

SOCIAL ORGANIZATION AND OLFATORY COMMUNICATION IN BROWN BEARS, EURASIAN BEAVERS, AND YELLOW-BELLIED MARMOTS

SOSIAL ORGANISERING OG LUKTKOMMUNIKASJON HOS BJØRN, BEVER OG MURMELDYR

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**Social organization and olfactory communication in
brown bears, Eurasian beavers, and yellow-bellied marmots**

Sosial organisering og luktkommunikasjon hos bjørn, bever og murmeldyr

Philosophiae Doctor (PhD) Thesis

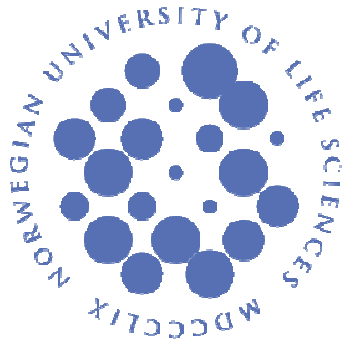
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Abstract

Communication systems in mammals are a key feature in the structure of social systems, and include a diversity of signals, sensory systems, and signaling behavior. In many mammals, scent marking is the dominant form of communication, putting the animal in contact with its environment, with conspecifics, and with other species. Scent marks may consist of glandular secretions, urine, and/or feces, and convey messages by way of volatile chemicals. Several factors contribute to the evolution of scent marking, but territoriality is the most prominent. Chemosignaling is a complex mode of communication critical to mediating social interaction in mammals. Behavioral experiments can reveal information perceived by a signal responder, and chemical analyses target the range of information potentially available within a chemosignal. Animals learn from their interactions with conspecifics, for example which provide benefits and which are to be avoided. Olfactory communication was investigated based on discrimination experiments in three mammals with different social systems: a) solitary, polygamous brown bears (*Ursus arctos*) with a dominance hierarchy, b) socially monogamous Eurasian beavers (*Castor fiber*) who are highly territorial, and c) harem polygynous yellow-bellied marmots (*Marmota flaviventris*), where males are primarily territorial and females form kin groups. Investigations were primarily of behavioral responses in field bioassays in which glandular secretions were “played back.” Some chemical analysis of secretions is included, but was not a primary focus of the project. Rather, it was used as a tool to aid our interpretation of classes of information (e.g., sex, age, or dominance) that could potentially be perceived behaviorally. In brown bear investigations, it was initially confirmed that they possess anal sacs and then hypothesized that the anal gland secretion (AGS) codes for sex. It

was also hypothesized that captive subadults could discriminate between unfamiliar adult male and female AGS. In Eurasian beavers, it was predicted that residents would find intruding subordinate sons of male territory owners a bigger threat than their fathers because they could potentially be in search of establishing a territory at a current owner's expense. AGS was also analyzed chemically for a status badge and for age. In yellow-bellied marmots, neighbor-stranger discrimination was investigated and the prediction was that female residents would display the dear enemy phenomenon toward adult females, whereas male residents would be indifferent. Whether female residents responded differently toward neighbors who were closely or distantly related was also assessed. It was confirmed that brown bears have anal sacs and that secretions may code for sex, and it was concluded that subadult brown bears can discriminate between male and female AGS. It was determined that resident Eurasian beavers can discriminate between AGS based on age and status badge codes. Finally, it was concluded that social bonds in yellow-bellied marmots, regardless of kinship, are likely important for neighbor-stranger discrimination of oral and cheek glands. The investigations contribute to the knowledge base of olfactory communication and increase our understanding of signals used in different social organizations of mammals.

Sammendrag

Kommunikasjonssystemene hos pattedyr er en nøkkelegenskap i sammensetningen av sosiale systemer, og består av et mangfold av signaler, sansesystemer og signaladferd. Hos mange pattedyr er luktmarkering den dominerende formen for kommunikasjon, og det setter dyrene i kontakt med sitt miljø, med sin egen art og med andre arter. Luktmarkeringen kan bestå av kjertelsekresjon, urin og/eller ekskrementer, og sender meldinger ved hjelp av flyktige kjemikalier. Det er flere faktorer som bidrar til utviklingen av luktmarkeringen, men territorialprinsippet er det mest fremtredende. Kjemisk signalisering er en komplisert kommunikasjonsmetode som er avgjørende for formidling av sosialt samspill hos pattedyr. Adferdseksperimenter kan avdekke informasjon som fornemmes av en signalmottaker, og kjemiske analyser rettes mot omfanget av informasjon som kan være tilgjengelig i et kjemisk signal. Dyrene lærer av samspillet med sin egen art, for eksempel hvem som er til nytte og hvem som bør unngås. Den olfaktoriske kommunikasjonen ble undersøkt på grunnlag av diskrimineringseksperimenter hos tre pattedyr med ulike sosiale systemer: a) enslige, polygyne brunbjørner (*Ursus arctos*) med et dominanshierarki, b) sosialt monogame, eurasiske bevere (*Castor fiber*) som er svært territorielle, og c) harempolygyne, gulmagede skogsmurmeldyr (*Marmota flaviventris*), der hannene primært er territoriale og hunnene danner familiegrupper. Undersøkelsene gikk primært på adferdsrespons på biologiske prøver ute i feltet, der kjertelsekret ble ”gjengitt”. Noen kjemiske sekretanalyser er inkludert, men det var ikke hovedfokus i prosjektet. De ble snarere brukt som et verktøy for å tolke grupper med informasjon (f.eks. kjønn, alder eller dominans) som muligens kunne fornemmes adferdsmessig. I undersøkelsene av

brunbjørn fikk vi i begynnelsen bekreftet at de har analsekker, og antok deretter at sekresjonen fra analkjertelen (AGS) inneholdt en kode for kjønn. Vi antok også at unge, voksne bjørner i fangenskap kunne skille mellom AGS fra ukjente, voksne hanner og hunner. Hos eurasisk bever kunne man forutse at de bofaste følte påtrengende, underordnede sønner av mannlige territorieiere som en større trussel enn fedrene, siden de potensielt kunne være ute etter å etablere et territorium på bekostning av nåværende eier. AGS ble også analysert kjemisk for å finne statusmerke og alder. Hos gulmaget skogsmurmeldyr ble forskjellsbehandling av naboer og fremmede undersøkt, og forutsigelsen var at bofaste hunner ga uttrykk for ”kjære fiende”-fenomenet mot voksne hunner, mens bofaste hanner var likegyldige. Vi vurderte også om bofaste hunner reagerte ulikt på naboer som var nært eller fjernt beslektet. Vi fikk bekreftet at brunbjørner har analsekker og at sekretet inneholdt koder for kjønn, og det ble konkludert med at unge, voksne brunbjørner kan skille mellom AGS fra hanner og hunner. Vi kunne fastslå at bofast, eurasisk bever kan skille mellom AGS basert på alder og statusmerkekoder. Til slutt ble det konkludert med at sosiale bånd hos gulmaget skogsmurmeldyr, uavhengig av slektskap, er viktige for skille mellom nabo og fremmed med kjertler i munn og kinn. Undersøkelsene bidrar til mer kunnskap om luktkommunikasjon, og øker vår forståelse for bruk av signaler hos ulike, sosiale grupper av pattedyr.

List of Papers

- I. Rosell, F., Jojola, S.M., Ingdal, K., Lassen, B.A., Swenson, J.E., Arnemo, J.M. and Zedrosser, A. 2011. Brown bears possess anal sacs and secretions may code for sex. *Journal of Zoology* 283:143-152.
- II. Jojola, S.M., Rosell, F., Warrington, I., Swenson, J.E. and Zedrosser, A. Captive subadult brown bears (*Ursus arctos*) discriminate between unfamiliar adult male and female anal gland secretion. *Submitted*
- III. Tinnesand, H.V., Jojola, S.M and Rosell, F. Does Eurasian beaver (*Castor fiber*) anal gland secretion signal territory ownership? *Manuscript*
- IV. Jojola, S.M., Cross, H., Blumstein, D.T. and Rosell, F. Do yellow-bellied marmots display a sex-bias in neighbor-stranger discrimination toward oral and cheek gland secretions? *Manuscript*

Synopsis

Olfactory communication in mammals

Olfactory communication

Communication systems in mammals are a key feature in the structure of social systems, and include a diversity of signals, sensory systems, and signaling behavior (Endler 1992). Multiple attempts have been made to define communication, but a single definition is not sufficient to encompass all aspects of animal behavior (see Dawkins 1995; Bradbury and Vehrencamp 1998). In a general sense, communication occurs when one animal's behavior can be shown to have an effect on the behavior of another, and in which 'signals' are the means by which these effects are achieved (Dawkins 1995). Signals are derived from movements, body parts, or molecules; such signals change in the course of evolution to enhance their function (Wyatt 2003).

Olfactory communication is defined as the process in which a chemical signal is generated by a presumptive sender and transmitted (generally through the air) to a presumptive receiver who by means of adequate receptors can identify, integrate, and respond (either behaviorally or physiologically) to the signal (Eisenberg and Kleiman 1972; Wyatt 2003; Müller-Schwarze 2006). It is assumed that the sender-receiver relationship is the result of natural selection such that signal production by the sender leads to an increased likelihood that the sender or the receiver will benefit from the transmission of the message (Eisenberg and Kleiman 1972; Wyatt 2003; Müller-Schwarze 2006).

Scent marks

In many mammalian species, scent marking is the dominant form of communication, putting the animal in contact with its environment, with conspecifics, and with other species (Müller-Schwarze 2006). Scent marks are a key source of information. The marks may consist of glandular secretions, urine, and/or feces (Johnson 1973; Gorman 1990; Müller-Schwarze 2006), and convey messages by way of volatile chemicals (Müller-Schwarze 2006). Gregarious species often integrate simultaneous visual, auditory, and behavioral displays into scent deposition (Candolin 2003; Partan and Marler 2005), whereas solitary species tend to use chemical signals as “bulletin boards” that relay messages in the absence of the signal sender (Alberts 1992). Olfactory communication enables solitary animals to leave messages that are relatively long lasting, can be ‘read’ later by conspecifics, and can also be used at night, underground, or in dense vegetation (Clapperton 1989; Alberts 1992; Hutchings and White 2000; Campbell-Palmer and Rosell 2011). Likewise, even in gregarious species, a visual or auditory component can at times be difficult to detect, especially in certain habitats (Gorman 1990), making reliance on olfactory signals more important.

Several factors contribute to the evolution of scent marking, but territoriality is the most prominent (Gorman 1990). Territorial animals defend areas to secure resources, such as food, shelter, and/or mating opportunities (Burt 1943; Grant 1993; Wolff 1997; Adams 2001). Scent marking is especially advantageous to territorial animals, because the scent persists after the animal has left (Gosling and Roberts 2001). Animals also use scent marks to attract a mate (Brown and Macdonald 1985), provide information on the female’s reproductive status or to initiate courtship (Eisenberg and Kleiman 1972; Jordan et al. 2007), influence sexual selection (Heymann 1998), warn

conspecifics, and assist in orienting or indicating population size (Hutchings et al. 2002). Scent marking can also be a reaction to novel objects or olfactory cues in the environment (Johnson 1973).

Many animals produce odors that are individually different and which may convey information on individual identity and serve a variety of functions (Halpin 1980, 1986). The ability to recognize individuals and predict their likely responses are important social skills that may have fitness benefits (Johnstone and Dugatkin 2000; Tibbetts and Dale 2007). Animals learn from their interactions with conspecifics, for example which provide benefits and which are to be avoided (Axelrod and Hamilton 1981; Mateo 2004). Open fights often only occur if the potential benefit outweighs the risk; making such decisions requires animals to assess their own strength and that of the opponent (Clutton-Brock & Albon 1979). Conflicts can be avoided if previously encountered scent marks are matched later in physical encounters (scent-matching; Gosling 1982). Recognition of individual identity (a perceptual process), for example, occurs when a predictable functional difference in behavior is shown toward the recognized individuals (Thom and Hurst 2004; Hurst et al. 2005). Discrimination, on the other hand, simply demonstrates a difference in behavior toward individuals without necessarily any meaning to the animals concerned (Thom and Hurst 2004, Lodé 2008).

Semiochemical messages

Chemosignaling is a complex mode of communication critical to mediating social interaction in mammals (Ralls 1971; Eisenberg and Kleiman 1972; Brown and Macdonald 1985). Semiochemicals, by definition, are chemical compounds or mixtures of compounds that fulfill the task of signaling (Müller-Schwarze 2006). Volatiles play

a key role in airborne transmission of information (Burger 2005; Müller-Schwarze 2006), and volatility is often related to the purpose of the signal. For example, scent cues for close-encounter contacts, e.g., mating, are less volatile than ones involved in long-distance interactions (Müller-Schwarze 2006). Behavioral discrimination experiments can reveal information perceived by a signal responder (e.g., Drea et al. 2002; Mateo 2003, 2006), and chemical analyses target the range of information potentially available within a chemosignal. Deciphering the chemical components contained in olfactory cues can provide important insight into the functional significance of mammalian scent marking (Albone 1984; Müller-Schwarze 2006). In recent years, there has been significant development of analytic techniques and increased efforts to identify compounds in mammalian semiochemicals, which have enhanced interdisciplinary studies (Albone and Shirley 1984; Johnston 2003; Brennan and Keverne 2004; Burger 2005; Novotny and Soini 2008). Examples of analytic techniques for analyzing semiochemicals include gas chromatography-mass spectrometry (GC-MS; e.g., Buesching et al. 2002a,b; Burger et al. 2008), gas chromatography-infrared spectrometry (e.g., Reiter et al. 2003, Lee et al. 2007), and near-infrared spectroscopy (Foley et al. 1998; Newey et al. 2008). Furthermore, several methods exist for sample preparation prior to using one of these techniques; which method to use depends upon the nature of the secretion and the question posed (e.g., Novakova and Vlckova 2009).

Aims of the Project

In this thesis, investigation of olfactory communication was based on discrimination experiments in three mammals with different social systems: a) solitary, polygamous brown bears (*Ursus arctos*) with a dominance hierarchy, b) socially monogamous Eurasian beavers (*Castor fiber*) that are highly territorial, and c) harem polygynous yellow-bellied marmots (*Marmota flaviventris*), where males are primarily territorial and females form kin groups. Investigations were primarily of behavioral responses in field bioassays in which glandular secretions were “played back”, or presented. Some chemical analysis of secretions is included, but it was not a primary focus. Rather, it was used as a tool to aid the interpretation of what classes of information (e.g., sex, age, or dominance) could potentially be perceived behaviorally. However, more effort was given to describe the chemical components of brown bear anal gland secretion (AGS), because it was confirmed only recently that they have anal sacs (**paper I**). In contrast, AGS of beavers has been investigated for longer (Rosell and Sunsdal 2001), and here, chemical analysis of beaver AGS investigated group patterns, rather than specific compounds. Yellow-bellied marmots have anal glands (Lassen and Ingdal 2008), but here, investigation was of behavioral responses to oral and cheek gland secretions.

Brown bears

Brown bears are solitary carnivores with a promiscuous, or polygamous, mating system (Schwartz et al. 2003; Bellemain et al. 2006; Steyaert et al. 2011). Males have larger home ranges than females, and home ranges overlap between sexes, among males, and among related females (e.g., Dahle and Swenson 2003; Støen et al. 2005). Species

having large home ranges to scent mark should produce marks that persist for long periods to be effective. Long-lasting, or delayed, communication enables an individual to save substantial energy associated with patrol (Yuan et al. 2004). Anal sacs in brown bears were only recently confirmed (**paper I**), hence, little is known about how AGS is used in communication. In another ursid, the giant panda (*Ailuropoda melanoleuca*), the anal glands are well-developed and have been studied for years. Giant panda AGS contains information about sex (Hagey and Macdonald 2003; Yuan et al. 2004; Zhang et al. 2008), age (Hagey and Macdonald 2003; Yuan et al. 2004), and individuality (Hagey and Macdonald 2003; Zhang et al. 2008). It is likely that brown bear AGS contains similar information. Functions of anal, anogenital, and subcaudal gland secretions have also been investigated in other carnivores (Asa et al. 1985; Macdonald 1985; Gorman and Trowbridge 1989; Buesching et al. 2002a; Begg et al. 2003). Specific messages include clan or group membership (Buesching et al. 2002a; Burgener et al. 2008), familiar and unfamiliar (e.g., Drea et al. 2002), sex (e.g., Jordan et al. 2011; Yuan et al. 2004; Zhang et al. 2003), age (e.g., Hagey and Macdonald 2003; Yuan et al. 2004), reproductive state (e.g., Yuan et al. 2004; Zhang et al. 2005, 2008), dominance status (e.g., Miller et al. 1998; Hayes et al. 2001), and individual identity (e.g., Buesching et al. 2002; Hagey and Macdonald 2003; Burgener et al. 2009).

Brown bear dispersal in Scandinavia occurs during the mating season (May-July); most males disperse as 2-yr-olds and most females disperse as 3-yr-olds (Støen et al. 2006). Subadult (1 and 2 yrs old) brown bears have a 15% chance of being killed by conspecifics in Scandinavia (Swenson et al. 2001) and most of these killings occur during the mating season (which is also the dispersal season). Thus, confrontations with adults have high risks for subadults, because they could be killed (Mattson et al. 1992;

McLellan et al. 1999; Swenson et al. 2001). Moreover, adult males kill subadults more often than do adult females (McLellan 1994; Swenson et al. 2001). Subadults, then, should benefit from the ability to assess potential threats on the basis of olfactory cues, enabling them to avoid risky encounters (Hurst 1990, 1993; Gosling et al. 1996a,b).

The aim of **paper I** was to confirm, for the first time, whether or not brown bears possess anal sacs, and to chemically investigate their AGS. We hypothesized that the AGS codes for sex, as in the giant panda (Yuan et al. 2004). A code for sex in AGS was investigated by digital (presence/absence) and analog (abundance) coding of compounds, the number of compounds, a comparison of color, and comparison of gas chromatogram profiles.

The primary aim of **paper II** was to determine whether captive subadult brown bears could discriminate between unfamiliar adult male and female AGS. We recorded side preference and frequency of side switches in their enclosure when AGS was present and absent. Additionally, we evaluated the frequency of visits to secretions, duration of visits, duration of time lapses between visits, and total time of visits. Finally, we characterized investigatory, or manipulatory, behaviour toward rocks containing the secretions, which has not been previously described.

Eurasian beavers

Eurasian beavers are territorial and socially monogamous rodents that rely highly on chemical communication (Schulte 1993; Rosell 2002; Campbell-Palmer and Rosell 2010). The basic family unit consists of a dominant pair living in a stable, long-term relationship within a defended “multipurpose/breeding” territory (Temeles 1994) together with their subordinate offspring, including young of the year (kits), yearlings,

and/or older offspring (Wilsson 1971; Rosell and Bjørkøyli 2002). Only the dominant couple reproduces, and subordinate members will normally not reach sexual maturity or be sexually active while living with a dominant animal of the same sex (Wilsson 1971; Svendsen 1980; Campbell et al. 2005). Offspring of both sexes usually disperse as 2-yr-olds (e.g., Hartman 1997; Sun et al. 2000), but delayed dispersal is common in high density populations, and yearlings can disperse when densities are low (Hartman 1997).

AGS of Eurasian beavers carries information about subspecies (Rosell and Steifetten 2004, but see Peterson et al. 2005) and sex (Grønneberg and Lie 1984; Rosell and Sundsdal 2001). In a similar species, the North American beaver (*C. canadensis*), which has been studied for longer, AGS carries information about sex and individuality (Sun 1996), kinship (Sun and Müller-Schwarze 1998a), and family membership (Sun and Müller-Schwarze 1998b). Age, body size, and dominance status are often correlated with fighting ability, or resource-holding potential (RHP), and frequently used to settle contests (Parker 1974; Archer 1987; Kemp and Wiklund 2004). On the other hand, asymmetries in fighting motivation can also affect contest behaviors and outcomes (Parker 1984), as a high motivation to fight might help in overcoming inferior RHP (e.g., Barnard and Brown 1984; Haley 1994). The latter could be the case in this social system, where subordinates do not breed while living with their parents, but must disperse from their natal site to find a territory and a mate.

In **paper III**, we investigated whether territory ownership (i.e., dominance) and age is coded in the AGS of male Eurasian beavers. We simulated a simultaneous intrusion into a resident's territory by a) an established dominant territory owner, and b) the subordinate son (i.e., has not yet dispersed and still lived with his father) of a). Subordinate sons (b) were either ≤ 1 or ≥ 2 -yrs-old. We predicted that residents would

show a stronger territorial response towards scent from the sons, despite their lower RHP, based on the fact that they, in contrast to their fathers, should be more motivated to acquire a territory.

Yellow-bellied marmots

Yellow-bellied marmots have been referred to as having polygynous harems with male dominance because the male maintains a territory where multiple females and her offspring live and the male is dominant to all other group members (Halpin 1985). However, in general, body size is an appropriate predictor for dominance among all marmots (Huang et al. 2011). Additionally, females establish kin groups by recruiting female offspring to build matriline (Armitage 1991, 2000). In the course of maintaining a territory, residents learn to discriminate neighbors from strangers (Ydenberg et al. 1988; Temeles 1994). Neighbor-stranger discrimination (NSD) can be influenced by genetic similarity (e.g., Rostain et al. 2004; Lodé 2008) and/or familiarity (habituation; e.g., Bates et al. 2008; Burgener et al. 2008). When a territory owner responds less aggressively toward an intruding neighbor than stranger, it is known as the dear enemy phenomenon (Fisher 1954). In a social system such as the yellow-bellied marmot's, adult male residents should attempt to mate with as many females as possible to maximize reproductive success (Trivers 1972), and hence should treat any intruding female cohesively (Downhower and Armitage 1971), because it could present a mating opportunity. Adult female residents, however, should display aggression toward intrusions by strange females, who may compete for resources, because reproductive success of the resident is increased by ensuring that their daughters survive and eventually reproduce.

In **paper IV**, we investigated NSD in adult marmots with both oral and cheek scents of adult females in separate experiments. We used scent from only adult females to specifically investigate a potential sex-bias in responses of residents. We predicted males would not respond differently toward neighbors and strangers because each presents a potential mating opportunity, whereas females would be more agonistic toward strangers, which pose potential threats. Additionally, we considered the effect of relatedness of neighbors on female resident responses.

Results

Brown bears

Paper I was the first to confirm that brown bears possess anal sacs and to investigate their secretions chemically. Many of the tentatively identified compounds were fatty acids, fatty acid esters, steroids, and hydrocarbons. Male and female secretions differed in analog coding, color, and in gas chromatogram profiles, suggesting a likely code for sex. A digital code was not detected for either male or female secretion, in which all males or females contained a single compound. Of the compounds with determined molecular weight, 68% were above 300, which is the upper limit for airborne pheromones (Wilson 1963; Bradbury and Vehrencamp 1998). Thus, their AGS is well-suited to persist for long periods and likely plays a role in delayed olfactory communication, as in the giant panda (Yuan et al. 2004).

In **paper II**, we concluded that subadult brown bears discriminated between unfamiliar adult male and female AGS based on the significant difference in duration of time to complete six visits to the secretion. Direct contact, in addition to the processing

time spent away from the AGS, characterized the discriminatory behavior. Subadults took longer to collect and process information from male secretion, and they waited longer to return. In the wild, collecting enough information from male AGS while maintaining caution would presumably increase the survival of subadults. We also concluded that female AGS was habituated to by a subadult's fourth visit, but that male AGS remained interesting through their sixth visit.

Eurasian beavers

Behavioral assays in **paper III** combined with the chemical analysis indicated there was a chemical difference between AGS from dominant territory owners and their subordinate sons, and that resident beavers can discriminate between the two categories. Residents showed a stronger territorial response towards AGS from subordinates, despite their lower RHP, when the subordinate scent donor was ≥ 2 years old, and only sniffed longer when the subordinate scent donor was a yearling. Thus, older sons were perceived as a greater threat than established territory owners, even when territory owners weighed significantly more than older sons, perhaps because older sons may be more motivated to engage in a physical conflict. Chemical analyses also indicated a difference in AGS between old and young sons, which indicates a code for age.

Yellow-bellied marmots

In **paper IV**, behavioral assays did not support the prediction that females would respond more agonistically toward strangers than neighbors, but the prediction that males would respond indifferently was supported. Additionally, females responded indifferently toward neighbors whether they were closely related or not. Our failure to

detect NSD in females was likely because neighbors were also perceived as strangers. In contrast to most other studies, our neighbor stimuli did not come from burrow-mates or the same social group. Rather, they came from adjacent social groups, where physical interaction was unlikely, but olfactory familiarity was likely. Thus, even though some neighbors were closely related, they were from a different social group and thus a social bond did not exist, which perhaps rendered them as strangers.

Discussion

Investigation of olfactory communication in mammals is a diverse and intriguing aspect of behavioral ecology. Behavioral and chemical information provides biologists with a better understanding of key elements that contribute to social organization of mammals, and the nature of their chemical senses that lead to their evolution.

The investigations in the thesis contribute to the knowledge base and emphasize the importance of olfactory communication in three different social systems. Even solitary animals, such as brown bears, must have an effective communication system to maintain their social organization and ensure reproductive success. Some studies indicate that solitary animals are more social than previously thought (Caro 1994; Waser et al. 1994, Sterling and Richard 1995; Kays and Gittleman 2001; Støen et al. 2005, 2006), which reinforces a continuum of sociality between strictly solitary and highly gregarious species. Being a predominantly solitary species does not preclude the evolution of complex social behaviors or group living (Bearder 1999), and understanding why the species are gregarious in some situations and solitary in others would contribute to our knowledge of why less flexible species have evolved a more

permanently gregarious social system, such as Eurasian beavers and yellow-bellied marmots. In addition, advances in technology such as GPS, and in analytic techniques, such as chromatography and genetics, allow for more refined and complex research questions that enable us to continue to understand the evolving signals in social organizations of species.

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Compilation of Papers

Paper I

Brown bears possess anal sacs and secretions may code for sex

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Keywords

analog coding; digital coding; gas chromatography–mass spectrometry; partial least squares regression; sex difference.

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Abstract

Olfactory communication occurs in carnivores and many scent-mark with anal gland secretions (AGS), which contain a variety of information including sex-related cues. Currently, there is disagreement about whether bear species, other than the giant panda *Ailuropoda melanoleuca*, possess anal glands or anal sacs. We documented anal sacs in brown bears *Ursus arctos* and analyzed AGS from 17 free-ranging, sexually mature individuals using gas chromatography–mass spectrometry. We hypothesized that brown bear AGS codes for sex, as it does in giant pandas, and predicted that AGS shows sex differences in gas chromatogram (GC) profiles, number of compounds, the digital and analog coding of chemical compounds, and color. We found 90 different compounds. Our results support the predictions that male and female AGS differs in GC, analog coding and possibly color. However, we found no significant difference between sexes in number of detected compounds or in the digital coding. Our results confirm that brown bears possess anal sacs, that secretions likely relay information about sex, and suggest other chemical information critical to the bears' social system is encoded in the AGS.

Introduction

Olfactory communication plays an important role in mammalian social and reproductive behavior, and many species use pheromones to send information about individual identity, reproductive status, territory boundaries and kin relations (Wyatt, 2003; Müller-Schwarze, 2006). Pheromones are often present in urine, various scent glands and feces and contain a mixture of chemical compounds with varying volatility (Brown & Macdonald, 1985; Wyatt, 2003).

Glandular tissues in the anal region of mammals are referred to collectively as 'anal glands' (Macdonald, 1985). Anal sacs are not anal glands (Scott, Miller Jr & Griffin, 2001), but secretion from the sacs is produced by glands in the anal region and is called anal gland secretion (AGS). There can be species differences in tissue structure, location and function as well as postural and behavioral deposits of AGS. In species of Hyanidae, Felidae and some Mustelidae, AGS is deposited separately from feces (see Macdonald, 1985). The use of AGS in olfactory communication has been documented in carnivores, as a code for sex in some mustelids (*Mustela* spp.) (e.g. Zhang *et al.*, 2002, 2003, 2005), for sex (Yuan *et al.*, 2004; Zhang *et al.*, 2008),

individuality (Hagey & Macdonald, 2003; Zhang *et al.*, 2008) and age (Hagey & Macdonald, 2003) in the giant panda *Ailuropoda melanoleuca*, and for social status and individual identity in the spotted hyena *Crocuta crocuta* (Burgener *et al.*, 2009). The AGS color differs between sexes in the Eurasian beaver *Castor fiber* (Rosell & Sun, 1999) and the subcaudal gland of European badgers *Meles meles* (Buesching, Newman & Macdonald, 2002a). In European badgers, there is a sex-related chemical difference in subcaudal glands (Buesching, Waterhouse & Macdonald, 2002b), but not anal sacs (Davies, Lachno & Roper, 1988).

Few studies have investigated AGS in large carnivores, such as bears (Ursidae). Giant pandas possess large anal glands that secrete a waxy substance (Schaller *et al.*, 1985). There is, however, virtually no information on AGS in the other seven bear species (Breiter, 2008) and there is disagreement about whether they even possess anal sacs. Pocock (1921) found greatly reduced anal sacs in the American black bear *Ursus americanus*, but did not find them in the brown bear *Ursus arctos*. Schaffer (1940) stated that the brown bear has anal glands, whereas Dyce, Sack & Wensing (1996) stated that bears do not.

One reason why little is known about olfactory communication in bears may be that they are elusive, have large home ranges (our study area: male, 1055 km²; female, 217 km²) (Dahle & Swenson, 2003), and are difficult to capture (Dahle & Swenson, 2003; Arnemo *et al.*, 2006). The brown bear is a solitary species with a polygamous mating system (Schwartz, Miller & Haroldson, 2003; Bellemain *et al.*, 2006). Males reach sexual maturity as early as 3 years of age (Zedrosser *et al.*, 2007), and primiparity varies from age 4 to 6 (McLellan, 1994; Zedrosser *et al.*, 2009). There is evidence that free-ranging brown bears can recognize and/or discriminate between individuals, because related females show more home-range overlap than unrelated females; suggesting that related females tolerate each other more than unrelated females (Støen *et al.*, 2005). Additionally, Zedrosser, Dahle & Swenson (2006) reported that young male brown bears disperse from their natal areas to avoid mate competition with older bears, implying that brown bears can discriminate between individuals and/or age classes (e.g. adults vs. subadults).

Thus, brown bears may use olfactory information for kin and status recognition, as in giant pandas (Swaisgood, Lindburg & Zhou, 1999), steppe polecats *Mustela eversmannii* (Zhang *et al.*, 2002), and coyotes *Canis latrans* (Tegt, 2004). Tschanz, Mayer-Holzappel & Bachmann (1970) observed that captive subadult bears withdrew in response to adult urine and feces at rubbing sites, suggesting a social communication function. The ability to discriminate between sexes benefits the scent donor by advertising its presence or attracting a potential mate. In turn, the receiver of the scent benefits by avoiding conflicts or recognizing individuals in later encounters (i.e. scent matching) (Gosling, 1982). American black bears rubbed marking trees more frequently in the mating season (Burst & Pelton, 1983), and giant pandas increased the use of AGS during this period (Schaller *et al.*, 1985).

Because brown bears have large home ranges, they have a vast area to scent mark. These marks should be durable to be an effective form of communication. Compounds of low volatility [i.e. high molecular weight (MW)] persist in the environment longer than those of high volatility, and are therefore more conducive to long-lasting or delayed communication because infrequent renewal could save substantial energy associated with patrol (Yuan *et al.*, 2004).

The aim of this study was to determine if free-ranging brown bears possess anal sacs, and, for the first time, chemically investigate their AGS. Although several codes potentially exist in brown bear AGS, we hypothesize that the AGS codes for sex, as in giant pandas (Yuan *et al.*, 2004), and predict that AGS shows sex differences in gas chromatogram (GC) profiles, number of compounds, digital (presence/absence) and analog (relative abundance) coding of chemical compounds and color.

Materials and methods

Study area and animals

The study was conducted in Dalarna and Gävleborg counties in south-central Sweden (61°N, 14°E), within the south-

ern part of the Scandinavian brown bear population (Sahlén *et al.*, 2007), and with a bear density at ~30 bears/1000 km² (Zedrosser *et al.*, 2006). The area is forest, dominated by scots pine *Pinus sylvestris*. Common tree species are Norway spruce *Picea abies*, birches (*Betula* spp.), aspen *Populus tremula* and lodgepole pine *Pinus contorta*. Timber management for clearcutting is intensive in the area, and together with roads, bogs and lakes, creates a patchy landscape.

As part of a long-term research project, we darted and immobilized free-ranging bears from a helicopter using a remote drug delivery system (Dan-Inject[®], Borkop, Denmark) with a combination of tiletamine/zolazepam and medetomidine (Arnemo *et al.*, 2006; Arnemo & Fahlman, 2008). We collected AGS samples during two periods before the mating season (18 April–18 May 2007, 6–30 April 2008; Table 1). Sex was determined by genital examination. Age of bears not captured as yearlings was determined based on cementum annuli in the root of the upper first premolar tooth (Matson *et al.*, 1993) analyzed at Matson's Laboratory (Milltown, MT, USA). Sexually immature bears (<3 years old) were excluded from analysis.

AGS collection

Anal sacs were located and identified by direct observation and manual palpation. We manually squeezed AGS from immobilized bears lying on their side or stomach by applying pressure on each anal sac separately. Samples were collected in 40 mL glass vials with teflon-lined caps (Lab Safety Supply[®], Janesville, WI, USA) and immediately put on ice. We used latex gloves during AGS collection to avoid contamination by human scent. All samples were frozen at –20 °C within 8 h of collection and kept frozen until analysis.

AGS color comparison

We compared the secretion colors with the natural color system (Scandinavian Colour Institute, Stockholm, Sweden) to evaluate sex differences in AGS color (Rosell & Sun, 1999). AGS colors were also ranked by eye into 12 categories from 1 = light to 12 = very dark (Buesching *et al.*, 2002a).

Chemical sample preparation

We performed a pilot study with different solvents and found that toluene–methanol 3:1 extracted most compounds from the AGS. We transferred 0.1 g of AGS into a glass test tube with a sterile needle. We added 1 mL of toluene–methanol 3:1 and vortexed the solution for 15 s. The compounds were extracted for 2 h at room temperature before centrifuging the sample for 3 min at 686 g. We covered the glass test tube with aluminum foil during extraction and centrifugation to avoid loss of volatile compounds. Next, we pipetted the particle-free solution into a GC-vial and used this solution in the analyses.

Chemical analysis

We used a Hewlett-Packard (HP, Oslo, Norway) 6890 Series II gas chromatograph equipped with a non-polar HP-5 MS 5% phenyl-methyl-siloxane column (30.0 m long \times 0.25 mm ID \times 0.25 μ m film thickness) connected to a HP 5973 Series mass spectrometer detector in the splitless mode. Helium gas was set to a constant flow of 1.0 mL min⁻¹ and the injection port temperature was 270 °C. The purge flow to split vent was 49.8 mL min⁻¹ at 1.00 min. The instrument was calibrated before analysis.

We injected 1 μ L of the particle-free AGS solution into the gas chromatograph-mass spectrometer (GC-MS) using an auto-injection system (Agilent 7683 Series Injector, Oslo, Norway). We used a HP single taper liner (4 mm inner diameter) with glass wool, and set the solvent delay to 5 min for every run to avoid damaging the detector. Initial oven temperature was set to 55 °C for 2 min then increased 6 °C/min to 310 °C, which was maintained for 5 min. Each run lasted 49.50 min; the first 8 min were eliminated from analysis because peaks in this interval stemmed from either solvent or the column. Control samples were run before, in the middle, and after all samples to control for changes in abundance or retention time. No major changes were observed.

We tentatively identified compounds by matching the retention time and mass spectra of the GC peaks with structures of 70 000 known compounds in the Wiley 275 Library, using a computer search. Structures of unidentified compounds were added to a new library and included in the search. The new compounds could then be recognized in different samples by comparing structures and retention times. The mass spectra from the GC peaks and the library were compared visually to determine if suggestions from the computer were reasonable. A positive identification of the compounds through known standards was not conducted because it was not the focus of this study.

The area of every peak was determined by computer-aided integration. We set the threshold to 17.0 to avoid integration of peaks that resulted from background noise. In order to quantify the relative abundance of each compound, we converted the single peak area into the percentage of the total peak area of the GC.

Olfactory information can also be examined by digital and analog coding of chemical compounds in the secretion (Sun & Müller-Schwarze, 1998a,b). Digital and analog coding is commonly used with GC-MS to reveal sex differences in composition of mammal AGS (e.g. Zhang *et al.*, 2003, 2005). We encoded the tentatively identified compounds by 0 (absent) and 1 (present) for all samples, and formed two digital matrixes of *X*-variables (detected compounds) and *Y*-variables (individuals). From the matrixes we examined the number of detected compounds by individual and sex.

Statistical analyses

We analyzed the data with partial least squares regression (PLSR) (Wold, Martens & Wold, 1983, 1984; Wold,

Sjöström & Eriksson, 2001). PLSR was used because it can analyze data with strongly correlated, noisy and numerous *X*-variables, and simultaneously model several response variables, *Y* (Wold *et al.*, 1983, 2001). PLS1, a type of PLSR, uses information in the *Y*-matrix to find the *Y*-relevant structure in the *X*-matrix (Esbensen, 2002) (for details see Rosell & Steifetten, 2004). As a basis for comparison, the abundance was measured for every time unit (165 time units min⁻¹) on the retention scale of the GC. The measured values formed a GC-matrix of *X*-variables (7012 time measurements) and *Y*-variables (sex of donors of 17 AGS samples). All values were scaled by mean normalization and standard normal variate to minimize the effect of variation in abundance between samples. Because of low sample size we used leverage correction to estimate the prediction residuals (Esbensen, 2002). The PLSR method extracts a small number of PLS components (PCs), which represent the relevant latent dimensions of the model. We used the values of validated R^2 and root mean square error of prediction (RMSEP) to evaluate the results. RMSEP is a measurement of the average difference between predicted and measured response values, with 0 showing least difference. We used the statistical software The UNSCRAMBLER 9.7 (Camo Software AS, Oslo, Norway).

We analyzed sex differences in the number of detected compounds with the Mann-Whitney *U*-test (Zar, 1998). A compound was defined to be sex specific if it was found in all males or females (Andersen & Vulpius, 1999). To check for sex difference in the digital composition of AGS, the digital matrixes were placed in a hierarchical cluster analysis with squared Euclidean distance (e.g. Yuan *et al.*, 2004). We used cluster analysis because the matrixes had more variables (compounds) than observations (individuals), and canonical discriminant analysis was therefore unsuitable (Johnson & Wichern, 1992). We checked for sex differences in analog coding of AGS using relative abundance of each compound in quantitative analyses. The Mann-Whitney *U*-test (SPSS for Windows, version 15.0, SPSS Inc., Chicago, IL, USA, 1999) was used to investigate the difference between the sexes in relative abundance and color. Significance levels were set to $P < 0.05$, and we defined a *P*-value of 0.05–0.1 as marginally significant.

Results

Anal sac description

The topography of the two anal glands is similar to that of the domestic dog *Canis familiaris*; the duct from each gland opens laterally at the cutaneous zone of the anal canal. All captured brown bears possessed paired anal sacs (Fig. 1). Males ranged in age from 3 to 17 years and females from 3 to 18 years (Table 1). The AGS had a clay-like substance with an unpleasant odor, but we detected no obvious sex difference in odor. AGS color varied from nearly black to light gray (Table 2). An overlap in color existed, but AGS of males was significantly darker than that of females ($U = 7.5$, $n_{\text{male}} = 5$, $n_{\text{female}} = 11$, $P = 0.02$).

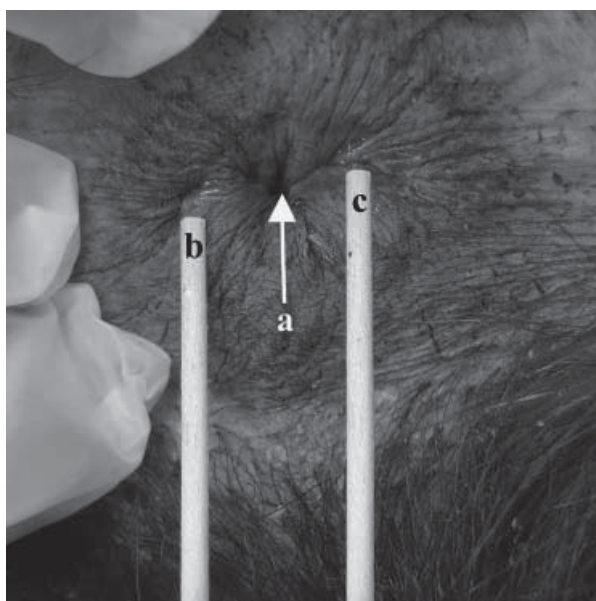


Figure 1 The anal region of a brown bear *Ursus arctos* showing the anus (a) and the location of the left (b) and right (c) opening of the anal sacs, indicated by the wooden sticks (photo: Andreas Zedrosser).

GC comparison

We found a difference in GCs between male ($n = 5$) and female ($n = 12$) AGS (Fig. 2). The PLS1 score plots tended to separate male and female AGS (Fig. 2). R^2 was 0.82, indicating a high predictive ability of the model. The RMSEP was 0.20, indicating a small average difference between predicted and measured response values. Of the total variation within the AGS GCs, PC1 explained 39% of the X -variance and 30% of the Y -variance, whereas PC2 explained 20% of the X -variance and 38% of the Y -variance.

Digital coding

We tentatively identified 90 compounds in the AGS that we were able to analyze ($n_{\text{male}} = 4$, $n_{\text{female}} = 7$; Tables 1 and 3). Unfortunately, some data were lost and no secretion remained to repeat GC-MS analysis, resulting in a lower sample size for total number of compounds, and digital and analog coding. The samples we analyzed are identified in Table 1. In general, these compounds were classified as fatty acids, hydrocarbons and steroids. Of the compounds with determined MW, 68% were above 300 MW (Table 3). We found a total of 74 (mean \pm SD = 38.00 ± 8.87) different compounds in males and 59 (29.14 ± 7.78) compounds in females (Table 3), but there was no sex difference ($U = 6.5$, $n_{\text{male}} = 4$, $n_{\text{female}} = 7$, $P = 0.12$) nor sex-specific compounds. Typical GCs of female and male AGS is shown in Fig. 3a and b. Hierarchical cluster analysis did not show any clear digital classification patterns in AGS between the sexes (Fig. 4).

Table 1 Individual number, sex and age of brown bear *Ursus arctos* donors of anal gland secretion

Number	Individuals	Date collected	Sex	Age
1	W9101 ^a	25 April 2008	F	18
2	W9301	18 April 2007	M	17
3	W0236 ^a	28 April 2008	F	15
6	W0624	21 April 2007	F	12–15 ^b
7	W9403 ^c	04 May 2007	F	14
8	W0004 ^a	24 May 2008	F	13
9	W9903 ^a	30 April 2008	F	11
10	W0424 ^a	30 April 2008	M	11
12	W0717	18 May 2007	M	> 10 ^b
13	W0803	14 April 2008	M	> 10 ^b
14	W0626	03 May 2007	F	8
18	W0217	03 May 2007	F	6
19	W0517	20 April 2007	F	6
23	W0416	22 April 2007	F	4
24	W0415	20 April 2007	F	4
26	W0508 ^a	29 April 2008	F	3
28	W0612	06 April 2008	M	3

^aThese individuals were not used in analysis of total number of compounds, digital or analog coding because some data were lost.

^bExact age of these individuals could not be determined.

^cThis individual was not used in the color comparison.

M = male, F = female.

Analog coding

We found differences between male and female AGS in the relative abundance of five of the shared compounds (Table 3). Females had a significantly higher abundance of four compounds than males: no. 57, 60, 73 and 80 (all steroids; $U = 2$, $P = 0.023$; $U = 3$, $P = 0.038$; $U = 1$, $P = 0.014$; $U = 0$, $P = 0.008$, respectively; Fig. 5), and males had a marginally significantly higher abundance of compound no. 76 (a steroid; $U = 4$, $P = 0.058$; Fig. 5).

Discussion

Our study is the first to confirm conclusively that brown bears possess anal sacs, and to investigate chemically their secretions. Our results supported our predictions that male and female AGS differ in GC, analog coding, and color, suggesting that AGS likely codes for sex in brown bears. However, our predictions of sex differences in number of detected compounds and digital coding of AGS were not supported.

Brown bear anal sacs were similar to those of domestic dogs, which are located between the external and internal sphincter muscles and help empty the contents of the intestine (Dyce *et al.*, 1996). It is unknown, but likely, that brown bears are capable of excreting AGS independently of feces, as observed in other carnivores with similar anal sacs, such as wolves *Canis lupus* (Asa *et al.*, 1985).

It is probable that brown bear AGS mediates information pertaining to mating. The amount of extractable AGS seemed to decrease as the mating season progressed

Table 2 Color of the anal gland secretions of brown bears (*Ursus arctos*)

Individual	Sex	Age	Color	Color rank ^a	NCS-code ^b
W9301	M	17	[Dark color swatch]	12	S8505-Y80R
W0424	M	11			
W0612	M	3	[Dark color swatch]	11	S8010-Y50R
W0508	F	3			
W0217	F	6	[Dark color swatch]	10	S8010-Y30R
W0004	F	13	[Dark color swatch]	9	S8005-Y50R
W0717	M	>10	[Dark color swatch]	8	S7020-Y30R
W0803	M	>10	[Dark color swatch]	7	S7020-Y20R
W0415	F	4			
W0624	F	12-15	[Dark color swatch]	6	S7010-Y30R
W0416	F	4	[Dark color swatch]	5	S6020-Y30R
W9101	F	18	[Dark color swatch]	4	S5020-Y20R
W9903	F	11			
W0626	F	8	[Dark color swatch]	3	S5010-Y30R
W0517	F	6	[Dark color swatch]	2	S5010-Y10R
W0236	F	15	[Dark color swatch]	1	S4040-Y20R

^aThe colors were ranked by eye in 12 categories (from 1 = light, to 12 = very dark).

^bNatural color system (NCS), Scandinavian Colour Institute AB.

(S. Brunberg, pers. comm.), but the production rate of AGS throughout the year is unknown. Less AGS later in the mating season implies that the normally solitary bears use AGS more frequently to communicate their presence when they are more likely to meet, as in giant pandas (Schaller *et al.*, 1985). On the other hand, a lower production of secretion after the mating season, as in the subcaudal gland of European badgers (Buesching *et al.*, 2002a), is also possible.

We documented a sex difference in AGS color; male secretion averaged darker than female secretion. In contrast to North American beavers (Schulte, Müller-Schwarze & Sun, 1995), aardwolves (Sliwa, 1996), Eurasian beavers (Rosell & Sun, 1999) and European badgers (Buesching *et al.*, 2002a), the color of male and female AGS overlapped in brown bears. Therefore, AGS color should not be used as the sole source for determining sex.

The sex differences in GCs and in analog coding imply that sex identification might be coded through a specific mix of several compounds (Albone, 1984), or by the relative abundance of some compounds. The total number of

compounds detected in brown bear AGS is relatively high compared with other scent-marking carnivores, like steppe polecat *Mustela eversmanni* (17 compounds) (Zhang *et al.*, 2003), Siberian weasel *Mustela sibirica* (14 compounds) (Zhang *et al.*, 2003) and domestic dog (13 compounds) (Preti *et al.*, 1976). However, the total number of compounds is similar to giant panda AGS (95 compounds) (Yuan *et al.*, 2004). Many of the compounds we identified in brown bear AGS were fatty acids, fatty acid esters, steroids and hydrocarbons, which are also found in wolves *C. lupus* (Raymer *et al.*, 1985), domestic dogs (Natynczuk, Bradshaw & Macdonald, 1989), giant pandas (Yuan *et al.*, 2004) and wolverines *Gulo gulo* (Wood, Terwillinger & Copeland, 2005). Similar to our results, Yuan *et al.* (2004) also found analog coding for nine compounds in giant panda AGS. The major differences in relative abundance of shared compounds in the giant panda were found in four steroids, but we cannot confirm whether these are the same four steroids as in brown bears.

We did not find sex-specific compounds in the AGS. Yuan *et al.* (2004) also concluded that information about

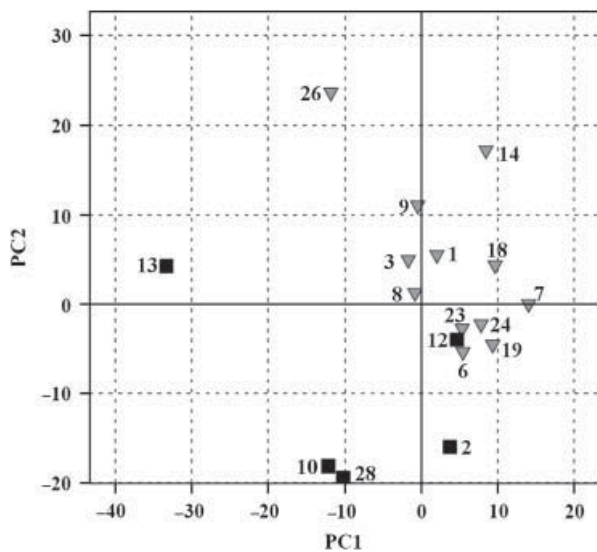


Figure 2 Partial least square regression score plot showing the position of each gas chromatogram of brown bear *Ursus arctos* anal gland secretions (■: male ($n=5$); ▼: female ($n=12$)) of the two first components, PLS component (PC) 1 and PC2. The numbers in the plots correspond with the number in Table 1.

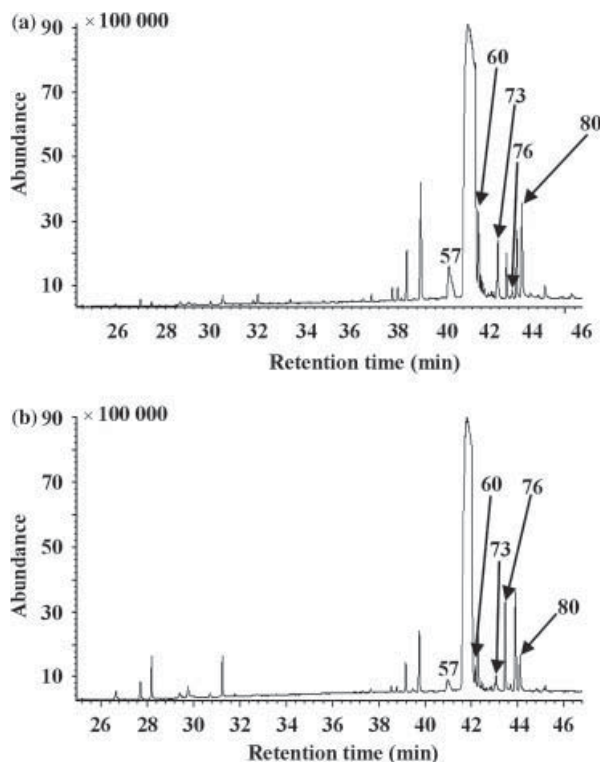


Figure 3 Typical gas chromatograms (GC) of the anal gland secretion from a female (a) and male (b) brown bear *Ursus arctos*. The numbers on the GC peaks correspond with compound numbers in Table 3. The x-axis is the retention time in minutes and the y-axis is the abundance.

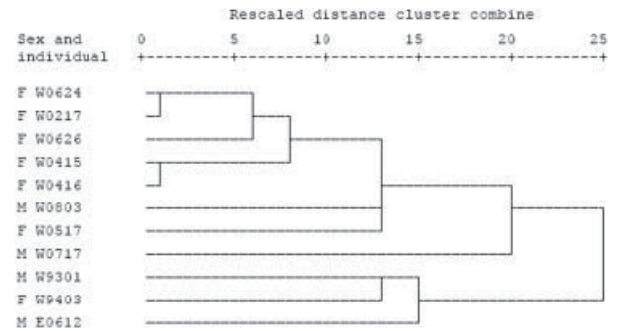


Figure 4 Dendrogram of hierarchical cluster analysis by using squared Euclidean distance for male and female brown bear *Ursus arctos* anal gland secretion. Labels indicate the bears' sex and individual number, and 'M' indicates male and 'F' indicates female.

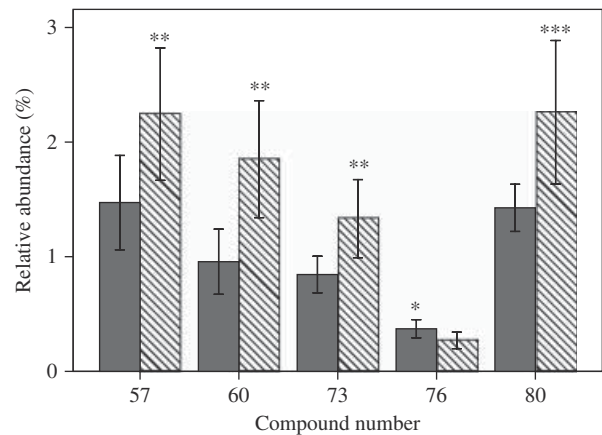


Figure 5 Sex-differences in relative abundance (mean \pm sd) of compounds found in the anal gland secretion of male (filled) ($n=4$) and female (hatched) ($n=7$), brown bears *Ursus arctos*. * $P=0.058$ (marginally significant), ** $P<0.05$, *** $P<0.01$. The numbers on the x-axis correspond with compound numbers in Table 3.

sex was not digitally coded in giant pandas. However, our result would have been different if we had used the same definition Zhang *et al.* (2003) used for the Siberian weasel. They concluded that (*Z*)-2-ethyl-3-methylthietane was a sex-specific compound because it was found in seven of 11 females and no males. According to this criterion, we would have defined compound no. 90 (unknown) as sex specific.

The hierarchical cluster analysis failed to show a clear grouping of sex in AGS. However, other information might be found in the digital composition. A similar cluster analysis of AGS from giant pandas revealed a clear grouping between adults and subadults rather than sex (Yuan *et al.*, 2004; Liu *et al.*, 2006). Differences between age groups in brown bears should be investigated in future studies.

Of the compounds detected in the AGS, 68% had a MW above 300. Compounds with MW >300 , the upper limit for airborne pheromones (Wilson, 1963; Bradbury & Vehrencamp, 1998), are well-suited for marking in large home

Table 3 Tentatively identified compounds in anal gland secretion of brown bear *Ursus arctos*

GC peak number	Retention time ^a (min)	Tentatively identified compounds	Molecular weight	Number of individuals		Relative abundance ^b	
				Male (n=4)	Female (n=7)	Male	Female
1	11.243	Piperidinone	–		3		0.174
2	21.686	Tetradecene (hydrocarbon)	196		1		0.105
3	23.511	Pentadecene (hydrocarbon)	210		1		0.565
4	25.249	Hexadecene (hydrocarbon)	224		1		0.443
5	26.650	Hexadecanoic acid (palmitic acid)	256	2	1	0.606	0.076
6	26.960	Unidentified nitrogen compound	299		1		0.115
7	27.689	<i>n</i> -phenyl benzensulfonamide	233	1		0.267	
8	27.702	Unknown	–	2	2	0.485	0.112
9	28.191	Unknown	–	2	1	1.721	0.104
10	28.627	10,13-octadecadienoic acid, methyl ester	294	1		0.056	
11	28.733	Hydrocarbon C21	296	1		0.042	
12	28.818	Unknown	236		1		0.078
13	29.408	Octadecenoic acid (oleic acid)	282	2	1	0.763	0.193
14	29.749	Octadecanoic acid (stearic acid)	284	2	1	0.915	0.094
15	29.809	Hydrocarbon	–	1	3	0.183	0.104
16	30.219	Hydrocarbon C22	310	1		0.066	
17	30.376	Hydrocarbon	–	1		0.074	
18	30.699	Unknown	–	2	2	0.175	0.089
19	31.156	Unknown	226	1		0.483	
20	31.247	Unknown	–	2	1	1.620	0.206
21	31.286	Unknown	236	2	6	0.139	0.084
22	31.411	Unknown	–		1		0.102
23	31.642	Unsaturated wax ester C24	366	1		0.121	
24	31.796	Unknown	–	1		0.055	
25	32.540	Unknown	–	1	4	0.147	0.113
26	32.551	Unknown	–	1		0.252	
27	32.751	Unknown	250	4	7	0.358	0.423
28	32.813	Unknown	–	1	1	0.123	0.086
29	32.900	Unknown	–		2		0.129
30	32.929	Hexanedioic acid, dioctyl ester	370	1		0.274	
31	33.011	Hydrocarbon C24	338	1		0.109	
32	33.220	Unknown	–		2		0.085
33	33.324	Phenol, 2,2'-methylenebis (6-(1,1-dimethylethyl)-4-methyl)	340	1		0.159	
34	33.533	Unknown	–	1		0.284	
35	34.006	Unknown	–	1		0.045	
36	34.152	Unknown	264	3	7	0.628	0.395
37	34.301	Unknown	294	2	3	0.097	0.073
38	35.300	Unknown	324	2		0.103	
39	35.535	Unknown	462	1	3	0.027	0.081
40	36.750	Unknown	292	2		0.083	
41	37.295	Unknown	320	3	3	0.108	0.110
42	37.644	A steroid	368	3	2	0.125	0.121
43	38.362	Squalene	410	1		0.112	
44	38.531	Unknown	334	4	7	0.237	0.299
45	38.771	A steroid	368	3	2	0.188	0.215
46	38.866	A steroid	366	1		0.061	
47	38.911	A steroid	366		1		0.076
48	39.144	A steroid	368	4	7	0.864	0.609
49	39.461	Hydrocarbon	364	3	4	0.231	0.092
50	39.527	Hydrocarbon	364	1		0.142	
51	39.749	A steroid	366	4	7	2.539	3.210
52	40.158	A steroid	–	2	2	0.109	0.094
53	40.555	Unknown	–	1		0.087	
54	40.655	Hydrocarbon	–		1		0.078

Table 3 Continued.

GC peak number	Retention time ^a (min)	Tentatively identified compounds	Molecular weight	Number of individuals		Relative abundance ^b	
				Male (n=4)	Female (n=7)	Male	Female
55	40.663	Unknown	–	2	2	0.178	0.165
56	40.837	Unknown	–	1	1	0.081	0.102
57	40.984	A steroid	380	4	7	1.472	2.244
58	41.808	Cholesterol	386	4	7	77.289	80.836
59	42.035	Unknown	394	2	4	3.118	3.512
60	42.177	A steroid	384	4	7	0.956	1.851
61	42.288	A steroid	386	3	6	1.181	0.477
62	42.323	A steroid	430	1		0.410	
63	42.409	Unknown	–	2	6	0.292	0.307
64	42.503	A steroid	414	2	2	0.358	0.201
65	42.519	A steroid	414	1		0.174	
66	42.660	Unknown	–		1		0.054
67	42.660	A steroid	–	1		0.103	
68	42.700	A steroid	382		1		0.156
69	42.735	Unknown	–	2	4	0.104	0.110
70	42.787	Ergost-5-en-ol (3 β)	400	4	7	0.343	0.396
71	42.908	A steroid	412	3	2	0.224	0.142
72	43.029	A steroid	–	1		0.211	
73	43.066	A steroid	408	4	7	0.845	1.332
74	43.447	A steroid	428	4	7	1.799	0.564
75	43.610	Unknown	–	1		0.090	
76	43.689	A steroid	414	4	7	0.371	0.269
77	43.813	A steroid	–		1		0.078
78	43.868	A steroid	426	1	3	0.275	0.336
79	43.898	Lanosta-8,24-diene-3-ol (3- β) (lanosterol)	426	3	4	2.989	1.216
80	44.115	A steroid	422	4	7	1.427	2.260
81	44.423	A steroid	404	2		0.332	
82	44.486	Unknown	–		3		0.213
83	44.828	Unknown	430	2	1	0.139	0.154
84	44.831	Unknown	–	1		0.117	
85	45.124	Unknown	436	4	7	0.183	0.308
86	45.200	A steroid	–	2		0.205	
87	45.817	Unknown	476	1		0.105	
88	46.191	A steroid	450	1		0.120	
89	46.206	Unknown	–	1		0.155	
90	46.278	Unknown	450		4		0.155

^aMean value of the retention time.

^bMean value for *n* possessing this compound.

GC, gas chromatogram.

ranges. This suggests that brown bears might use AGS for long-lasting or delayed olfactory communication, as in the giant panda (Yuan *et al.*, 2004). Interestingly, bears have been observed to frequently sit down in front of trees after rubbing and that, when coming to a tree, they often sniff the ground before rubbing (O. -J. Sørensen, pers. comm.).

Our study revealed that brown bear AGS likely codes for sex, and suggests that other vital information is probably encoded in their secretion, enabling the typically solitary bears to communicate indirectly. This increases our knowledge of the repertoire of communication modalities in ursids.

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Paper II

**Captive subadult brown bears (*Ursus arctos*) discriminate
between unfamiliar adult male and female anal gland
secretion**

Short title: Subadult brown bear discrimination of scent

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Summary

Olfactory cues have been investigated in several social carnivores and many use anal/anogenital gland secretion (AGS) for scent marking. Little is known about how solitary carnivores, such as ursids, use AGS in their communication, except for the giant panda (*Ailuropoda melanoleuca*). We hypothesized that subadult (12-36 mos old) brown bears (*Ursus arctos*) have the ability to discriminate between unfamiliar adult male and female AGS. Confrontations with adults carry high risks for dispersing subadults, so they benefit from the ability to assess potential threats based on olfactory cues, enabling them to avoid risky encounters. We presented AGS from free-ranging adult brown bears (male = 10, female = 10) to subadult brown bears (male = 13, female = 7) in outdoor zoo experiments from May to October 2010, and predicted that bears would avoid male more than female AGS. Male AGS was not avoided. Secretions appeared to be short-term enrichment for bears, but female AGS was relatively quickly habituated to and male AGS was investigated more intensively and not revisited too quickly. However, subadults took significantly longer time to complete six visits to male than female AGS, which indicated discrimination. We analyzed adult AGS samples by gas chromatography-mass spectrometry and used a partial least squares-discriminant analysis model to predict the sex based on chemical profiles. The predictability of the model was trustworthy, but would likely improve with increased sample size used in calibration for both sexes.

Keywords: anal gland secretion, gas chromatography-mass spectrometry, investigatory behavior, olfactory communication, partial least squares discriminant analysis, sex discrimination.

Introduction

Olfactory communication occurs commonly in mammals and our knowledge of the messages conveyed is increasing (Campbell-Palmer & Rosell, in press). Olfactory cues have been investigated in several social carnivores (e.g., Buesching et al., 2002a,b; Barja et al., 2005; Müller & Manser, 2007), and many use anal/anogenital gland secretion (AGS) for scent marking (e.g., Asa et al., 1985; Macdonald, 1985; Burgener et al., 2009). Variations in the composition of AGS among conspecific individuals allow carnivores to discriminate between males and females (e.g., Jordan et al., 2011), familiar and unfamiliar conspecifics (Drea et al., 2002), and kin and non-kin (Tegt, 2004).

Little is known about how solitary carnivores, such as ursids, use AGS in their communication, except for the giant panda (*Ailuropoda melanoleuca*). Chemical profiles of AGS of giant pandas contain information about sex (Hagey & Macdonald, 2003; Yuan et al., 2004; Zhang et al., 2008), age (Hagey & Macdonald, 2003; Yuan et al., 2004), and individuality (Hagey & Macdonald, 2003; Zhang et al., 2008). Both male and female giant pandas can discriminate between AGS from different individuals (Swaisgood et al., 1999), and subadult and adult giant pandas showed a significant investigative preference for adult versus subadult male AGS (White et al., 2003).

Brown bears (*Ursus arctos*) are solitary carnivores with a promiscuous, or polygamous, mating system (Schwartz et al., 2003; Bellemain et al., 2006; Steyaert et al., 2011). Males have larger home ranges than females, and home ranges can overlap between sexes, among males, and among related females (e.g., Dahle & Swenson, 2003; Støen et al., 2005). Females usually establish their breeding home ranges in, or adjacent

to, their natal areas, whereas males disperse long distances from the mother's home range (McLellan & Hovey, 2001; Støen et al., 2006). Bears are not considered to be territorial to the extent of defending specific areas (Stamps, 1994). However, some studies of both brown and American black bears (*U. americanus*) suggest they may be more territorial than previously thought because, among females, kin tolerate home range overlaps more than non-kin (Rogers, 1987; Støen et al., 2005). Thus, kinship and sex codes may be relayed in scent marking. Additionally, young male brown bears disperse from their natal areas to avoid mate competition with older bears (Zedrosser et al., 2007a), which suggests sex or age class (subadult versus adult) may be mediated chemically. Brown bears possess anal sacs and the secretion of bears ≥ 3 yrs old may contain a chemical code for sex (Rosell et al., 2011). Scent marking with AGS by brown bears, however, has not yet been documented. Tschanz et al. (1970) observed that captive brown bears avoided rubbing sites of unfamiliar adult males, which suggests a social communication function, such as age or dominance. However, there are no empirical data in the literature that characterize response behaviors of subadult brown bears to olfactory cues from unfamiliar adults and what can be inferred from these behaviors.

Sexually mature adult bears (≥ 3 yrs old) (McLellan, 1994; Zedrosser et al., 2007b, 2009) that scent mark benefit by advertising their presence within an area or by attracting a potential mate. The ability to discriminate between scent marks by sex and age, as an adult or subadult (< 3 yrs old; after weaning and before becoming sexually mature), benefits the receiver by avoiding conflicts or by recognizing individuals in later encounters (i.e., scent matching; Gosling, 1982; Gosling & McKay, 1990).

Brown bear dispersal in Scandinavia occurs during the mating season (May-July); most males disperse as 2-yr-olds and most females disperse as 3-yr-olds (Støen et al., 2006). Subadult brown bears have a 15% chance of being killed by conspecifics in Scandinavia (Swenson et al., 2001) and most of these killings occur during the mating season (which is also the dispersal season). Thus, confrontations with adults have higher risks for subadults than with other subadults, because they could be killed by adult bears (Mattson et al., 1992; McLellan et al., 1999; Swenson et al., 2001). Moreover, adult males kill subadults more often than do adult females (McLellan, 1994). Thus, subadults should benefit from the ability to assess potential threats on the basis of olfactory cues, enabling them to avoid risky encounters (Hurst, 1990, 1993; Gosling et al., 1996a,b).

We hypothesized that subadults could discriminate between unfamiliar adult male and female AGS. We predicted that the subadults would avoid male more than female AGS. Additionally, we intended to characterize the investigative, or manipulatory, behavior of subadult bears toward unfamiliar AGS within a familiar setting (their enclosure). Investigative behavior of brown bears toward novel, non-consumptive odors has not been previously described. We also revisited the investigation of a chemical code for sex in AGS by gas chromatography-mass spectrometry (GC-MS) analysis (Rosell et al., 2011), but this time we built a multivariate model to predict sex based on chemical profiles (i.e., chromatograms). This could be potentially advantageous in situations where AGS sample collection could occur without immobilizing a bear, such as near rubbing sites or at dens.

Material and methods

We used AGS from 20 (10 males, 10 females, ≥ 4 yrs old) free-ranging adult brown bears captured in a long-term study by the Scandinavian Brown Bear Research Project (Arnemo et al., 2011). Sex was determined by genital examination. Age of bears not captured as yearlings was determined based on cementum annuli in the root of the upper first premolar tooth (Matson et al., 1993) analyzed at Matson's Laboratory (Milltown, MT, USA). AGS was collected, by manually squeezing secretion from the anal sacs (see Rosell et al., 2011), during April-May 2008, April 2009, and April 2010, in Dalarna and Gävleborg counties in south-central Sweden (61°N, 14°E). AGS was collected in 40 ml glass vials with Teflon-lined caps and stored at -20 °C until taken to zoos.

Zoo experiments.

We conducted experiments in nine European zoos May-October 2010 (Table 1). Study subjects were male ($n = 13$) and female ($n = 7$) subadult bears (12-36 mos old; Table 1). Experiments were carried out in a familiar outdoor enclosure and each bear was exposed to three experiments at 45 min each: Control 1, Treatment, and Control 2. Treatment was the presentation of either male or female AGS of adult free-ranging brown bears into the enclosure, and Controls 1 (before the treatment) and 2 (after it) had no stimulus present. Study bears were housed with at least one bear their age ± 1 yr. Six bears were also housed with their mothers, four of which were also housed with their fathers. We considered the bears housed with their parents to be appropriate for

the study, because we investigated responses to unfamiliar AGS. Subadult bears were isolated from penmates during experiments, but whether the penmates remained visible depended upon the facility. We did not habituate bears to isolation prior to data collection. Rather, we held experiments at least 72 hrs apart to maintain the behavior associated with the novelty of isolation as much as possible. In other words, if a bear spent a majority of its time near access doors (den or enclosure doors) during Control 1, we expected them to allot a similar amount of time to these areas during Control 2. Experiments within subjects were held at the same time of day (\pm 1 hr).

Approximately 0.25 g of either male ($n = 10$) or female ($n = 10$) AGS was presented on a rock in the bear's enclosure. Rocks were gathered locally and were recently unearthed to avoid potential odors as a result of being exposed. We did not control for rock composition, because geology differed across zoos. We selected rocks with an approximate surface area of 30 cm x 20 cm and deposited AGS in the center. We handled AGS vials with latex gloves and rocks with leather gloves stored in a plastic bag to avoid contamination by human scent. Assignment of male and female AGS was balanced between sex of the study subjects, and then randomly assigned. Observers did not know which sex of AGS was being delivered. AGS was kept cool or frozen until use.

We video-recorded (Sony[®] digital video handycam, model no. DCR-SR35E, Komplet.no, Sandefjord, Norway) each experiment and observers were nearby, but not visible to the bears. All experiments were conducted before business hours, except in two zoos. In one of these zoos, visitors did not approach the bear exhibit during experiments; at the other zoo, bears did not appear to change their behavior when visitors were nearby. Even though visitors had minimal effect on the experiments, we

could not control for daily maintenance activities within zoos, such as construction, and human or vehicle traffic near, but not at, the enclosures.

Side preference and frequency of side switches.

After Control 1, the bear's activity was reviewed on video to document its time allotment and to assign a boundary line between a "preferred" and "non-preferred" side of the enclosure; the "preferred" side being that on which the bear spent > 1350 sec (i.e., 22.5 min, or half of 45 min). Because of the variability in each bear's activity and in zoo enclosures, we could not use a standard time allotment (e.g., "30 min") across all bears as the criterion for a preferred side. Each bear's boundary as determined in Control 1 remained constant for data collection during Treatment and Control 2. Based on Control 1 observations, the AGS was placed on the preferred side in a location frequented by the bear, in an attempt to shift the bear's preference across the boundary. We predicted a shift in side preference for bears assigned the male AGS (but not female AGS) during Treatment, and similar time allocation (no shift) by bears during Control 2 relative to Control 1.

We also recorded the number of times a bear switched sides during each 45-min experiment. The activity level of a bear may provide insight into its level of anxiety; the more a bear was active, the more anxious it probably was. We predicted more side switches during Treatment by bears assigned to male AGS, and no difference in side switches during Control 2 relative to Control 1 for either male or female AGS.

Frequency of visits, duration of visits, duration of time lapses between visits, and total time of visits.

We recorded the frequency of visits to the AGS during Treatment. A “visit” started when a bear’s nose came to within 30 cm of the AGS and ended when its forepaws stepped away from the rock. We predicted fewer visits to male AGS.

We also recorded the duration (in sec) of each visit, as defined above, to AGS. Each visit consisted of at least one sniff bout and potentially multiple investigative behaviors (described below). The duration of time that lapsed between visits to AGS, or a “time lapse”, was the time (in sec) a bear’s forepaws stepped away from the rock until its nose again came to within 30 cm of the AGS. Duration of time lapses can be an indication of how long it took bears to process gathered information, in addition to how soon more information was sought. “Total time of visits” was the sum of “duration of visits” and “duration of time lapses between visits”. We predicted shorter durations of visits, longer time lapses, and shorter duration of total time of visits for male AGS. We only analyzed data of visits during which $\geq 80\%$ of the study subjects participated.

Frequency of sniff bouts and investigative behaviors.

We recorded the frequency of sniff bouts for each visit to AGS. A “sniff bout” was where a bear’s nose was within 30 cm of the AGS to when it was 30 cm away from it. Multiple sniff bouts could occur within a visit. Additionally, we recorded frequencies of investigative, or manipulatory, behaviors of a) pawing or stepping on the rock containing AGS, b) nudging, pushing, or spinning the rock, and c) rolling the rock during each visit; multiple investigative behaviors could occur within a visit. We considered this order of behaviors to be an index of intensity levels of investigative

behavior by a bear. Frequencies of investigative behaviors were analyzed for visits where $\geq 80\%$ of the study subjects participated.

Statistical analysis.

We used a Mann-Whitney U test (Mann & Whitney, 1947; two-tailed) to examine significant differences between male ($n = 13$) and female ($n = 7$) subjects for each analysis. Of responses to AGS, we considered the three experiments (Control 1, Treatment, and Control 2) to be independent based on the 72-hr delay in between. We used one-tailed tests where predictions of our hypotheses were directional, e.g., less time spent at male AGS (Field, 2009). For only time spent on the preferred side, data were normal and had homogeneity of variance, so we used an ANOVA (e.g., Howell, 2007), where Control 1, Treatment, and Control 2 were within-subject factors, and sex of AGS was the between-subject factor. We used a Friedman test (Friedman, 1937) to evaluate frequency of side switches across Control 1, Treatment, and Control 2 by male and by female AGS, and a Mann-Whitney U test (one-tailed) to compare frequencies of side switches between male and female AGS for Control 1, for Treatment, and for Control 2. We used Mann-Whitney U tests (one-tailed) to compare male and female AGS differences in frequency of visits, duration of visits, duration of time that lapsed between visits, and total time to complete visits. We used a linear mixed model (LMM; alpha level was set to 0.05; e.g., Twisk, 2006) to examine frequencies of sniff bouts and investigative behaviors. Our model included fixed effects of Treatment, Visit, Behavior, Treatment*Behavior, and Visit*Behavior; study bear ID was fitted as a random effect. Planned pairwise comparisons were made on the estimated marginal

means. SPSS software, version 18 (PASW Statistics, IBM, Chicago, IL, USA) was used for all analyses.

Chemical analysis.

It was not possible to use every AGS sample that was used in the zoo experiments for chemical analysis, because minute amounts of AGS often were extracted from the males. Thus, we analyzed AGS of only 6 males, but all 10 females (Table 2).

Sample extraction.

Dichloromethane (400 μ l) was added to 0.10 g of AGS in a clean gas chromatograph (GC) vial (heated overnight in the GC oven at 150 °C) using clean utensils. The mixture was vortexed for 15 sec and left to stand at room temperature for at least 2 hrs, after which the mixture was again vortexed and then centrifuged for 4 min at 4500 rpm. The particle-free extract was pipetted into a glass vial insert in a new, clean GC vial for GC-MS analysis.

Analyses were carried out on a GC-MS system consisting of an Agilent 6890 Series II and an Agilent 5973 Series mass selective detector. The GC was fitted with a split/splitless injector with a single taper liner (4 mm inner diameter) containing a small plug of glass wool. The injector was operated at 230 °C in the splitless mode. Gas chromatographic separations were carried out using a DB-5 fused silica column (50 m x 0.25 mm) coated with non-polar phenyl-methyl-polysiloxane as stationary phase at a film thickness of 0.25 μ m. Helium was used as carrier gas at a constant flow of 1.0 ml/min. Using an Agilent 7683 Series auto-injector, samples of 2 μ l of particle-free

extract were injected for GC-MS analysis. Initial oven temperature was set to 40 °C (hold 5 min), then increased to 4 °C/min to 210 °C (hold 5 min), then 2 °C/min to 280 °C (hold 5 min), then 10 °C to 300 (hold 10 min) to condition the column. The total run lasted 104.5 min, which permitted ideal separation of peaks. Autotunes with perfluorotributylamine (PFTBA) were performed regularly.

A mixture of methyl esters (Mixture Me 63, Larodan Fine Chemicals, Malmö, Sweden) was used as a reference standard in the beginning, middle, and end of the run sequence to monitor a possible shift in retention time. Blank samples (dichloromethane) were run at the beginning, every fifth run, and at the end of the sequence to verify that the column was clear of interfering compounds.

Statistical analysis.

We used The Unscrambler[®] software (Generation X, version 10.0.1.0, CAMO, Oslo, Norway) to perform partial least squares-discriminant analysis (PLS-DA; Esbensen, 2002) to discriminate patterns in chemical profiles of male and female AGS. PLS-DA is the use of PLS regression (PLSR) for classification purposes. PLSR is a bilinear projection approach that extracts a small number of principle components (PCs) T from X, and each successive, orthogonal component explains a maximum covariance between X and y. These latent variables T are then used for bilinear modelling of both X and Y (Martens & Naes, 1996; Esbensen, 2002). AGS of males was assigned “1” and females “-1”, which represented Y. The total ion current (TIC) for each time unit on the retention scale of gas chromatograms was measured, which represented X (16,418 time units). PLSR uses information in the Y-matrix (16 bear AGS samples x dummy variable for sex) to find the Y-relevant structure in the X-matrix (16 samples x 16,418

time units). The purpose of the study was not to identify specific compounds in the chromatograms, but to characterize group differences of chemical profiles. Thus, we did not identify any compounds.

For PLS-DA, we first created a training data set for calibrating the model with AGS from bears (3 males, 5 females) that were used in a previous PLSR model (Rosell et al., 2011; Table 2). By calibrating with these samples, pseudoreplication in this analysis was avoided. We used full cross validation to create the PLS regression model, and had well-separated groups in the first two factors (Figure 1). Of the total variance, Factor 1 described 61% of the variation in X (chemical profiles) and 87% of the Y variance (sex of bear), and Factor 2 described 12% of the X variance and 7% of the Y variance. We used R-square and root mean square error of prediction (RMSEP) values to evaluate the results. Validated R-square represents the predictive ability of the model; the closer to 1, the better. RMSEP measures the average difference between predicted and measured response values at the prediction or validation stage; the closer to 0, the better. The R-square = 0.870 for calibration (where samples are put back in the same model they were a part of), and RMSE = 0.349. R-square for validation (from cross validation, which is more conservative because the samples were not part of the model when it was predicted) = 0.813, and RMSE = 0.479. “Prediction” here does not mean the model has been tested against new samples. Rather, it means the samples used to create the model were correctly classified for the cross validation.

Secondly, we used the new samples as the “test set” to see how well the model would predict (classify) the sex of the new samples (also, 3 males and 5 females; Table 2). To determine whether predictions can be trusted, the Inlier and Hotelling T^2 distances (Esbensen, 2002) were considered. The predicted sample must not be too far

from a calibration sample (Inlier distance), and projection of the sample in the model should not be too far from center (Hotelling T^2 distance). Samples falling outside the critical limit lines ($p = 0.05$) cannot be trusted (Esbensen, 2002).

We treated the data by applying a standard normal variate (SNV) transformation to remove scatter effects by centering and scaling each spectrum (i.e., to remove multiplicative interferences of scatter effects from the spectral data). Additionally, we applied a Detrending transformation (DT) to reduce multicollinearity, baseline shift, and curvature in the spectroscopic data. Detrending removes nonlinear trends by fitting a higher-order polynomial to each individual spectrum, then removing the estimated baseline curvature (Esbensen, 2002).

Results

Zoo experiments.

There were no significant differences between male and female subjects ($p \geq 0.100$) in side preference, frequency of side switches, frequency and duration of visits, duration of time lapses between visits, and total time of visits. Therefore, we pooled them to examine significant differences between male and female AGS.

Side preference and frequency of side switches.

Overall, within-subject effects indicated no change in the time spent on the preferred side across the three experiments (Control 1, Treatment, and Control 2) ($F(2, 36) = 0.268, p = 0.767$). Additionally, there was no change in time spent on the preferred side

relative to male or female AGS group assignment (i.e., no interaction effect) ($F(2, 36) = 0.066, p = 0.936$). Finally, between-subject effects (sex of AGS) revealed no significant difference, indicating that the time spent on the preferred side was the same for male and female AGS ($F(1, 18) = 0.100, p = 0.756$).

There was no significant difference in the number of times bears switched sides during Control 1, Treatment, and Control 2 in the male ($F = 1.590, p = 0.491$) or female AGS group ($F = 2.400, p = 0.368$), nor significant differences in side switches between male and female AGS during Control 1 ($U = 46.000, p = 0.391$), Treatment ($U = 49.000, p = 0.478$), or Control 2 ($U = 44.000, p = 0.334$).

Frequency of visits, duration of visits, duration of time lapses between visits, and total time of visits.

All 10 bears made at least 4 visits to male AGS, and 80% made 6 visits. All 10 bears made at least one visit to female AGS, 90% made 4 visits, and 80% made 6 visits. A maximum of 12 visits were made to male AGS, and a maximum of 20 to female AGS (but a single bear made visits 15-20). There was no significant difference in frequency of total visits between male and female AGS ($U = 39.500, p = 0.221$). The majority, $\geq 80\%$, of study subjects participated in up to 6 visits.

In general, for both male and female AGS, visit durations gradually decreased in subsequent visits. There was a significant difference between male and female AGS in duration of visit only for visit 3 ($U = 20.500, p = 0.026$; Figure 2a), where visits were longer at male AGS ($X_{\text{male}} = 23.400 \text{ sec} \pm 8.501 \text{ SE}$, $X_{\text{female}} = 6.888 \text{ sec} \pm 1.882 \text{ SE}$). We examined, post hoc, the decreasing trend in duration of visits 1–3 for female AGS. The duration of the first visit differed from the second visit (Wilcoxon signed-ranks test

(one-tailed); $Z = -1.838$, $p = 0.038$), and the second visit differed from the third ($Z = -1.838$, $p = 0.038$). For male AGS, we compared visits 4 and 5, which were not significantly different.

In general, for both male and female AGS, duration of time lapses gradually increased in subsequent visits. The time that lapsed between each visit 1-6 was not significant between male and female AGS (Figure 2b). Post hoc analysis of female AGS indicated the difference in time that lapsed between visits 3-4 was not significantly longer than the time that lapsed between visits 2-3 (Wilcoxon signed-ranks test (one-tailed); $Z = -1.364$, $p = 0.099$), but the time that lapsed between visits 4-5 was significantly longer than that for 3-4 ($Z = -1.820$, $p = 0.038$). For male AGS, the difference in time that lapsed between visits 4-5 and 5-6 was not significant, so there were no significant differences in time lapses between any of the immediately prior time lapses.

The total time to complete 6 visits (visit durations + time lapses) was significant between male and female AGS (Mann-Whitney U test (one-tailed); $U = 26.000$, $p = 0.037$). Bears took 8 minutes longer, on average, to complete 6 visits to male AGS ($X_{\text{male}} = 1311.200 \text{ sec} \pm 182.892 \text{ SE}$, $X_{\text{female}} = 811.900 \text{ sec} \pm 189.841 \text{ SE}$).

Frequencies of sniff bouts and investigative behaviors.

Based on head movements of the bears while investigating the AGS, we are confident that every bear at one point licked the AGS. This could not be reliably documented, however, because video cameras were often too far away to record this detail, or the bears faced away from the camera. For the same reasons just stated, in addition to bears walking out of the camera view, it was not possible to determine with confidence

whether bears urinated or defecated after visiting the AGS. In the few bears where defecation was observed on video (< 3), it would be difficult to conclude whether this was in direct response to the AGS. Thus, we did not quantify licks, urination, or defecation during Treatment.

Investigative behavior almost always occurred in the same order: directly sniffing the AGS and sniffing upwardly in the air, followed by pawing or stepping on the rock containing AGS, then nudging or pushing the rock, and in some cases eventually flipping or rolling the rock. We considered this order of behaviors to be an index of the level of intensity of investigation by a bear; sniffing being the least intense, rolling the most intense.

There were significant differences in frequency of sniff bouts between male and female subjects during only the first of 6 visits ($U = 17.500$, $p = 0.021$), where female subjects made more sniff bouts than male subjects ($X_{\text{males}} = 2.308 \pm 0.511$ SE, $X_{\text{females}} = 3.857 \pm 0.404$ SE); and in frequency of rock rolls for only the fourth of 6 visits ($U = 21.000$, $p = 0.044$), where male subjects rolled rocks more than female subjects ($X_{\text{males}} = 0.833 \pm 0.297$ SE, $X_{\text{females}} = 0$). Thus, female subadults sniffed more frequently than male subadults on their first encounter with unfamiliar AGS, and that by the fourth visit male subadults continued to intensively investigate (i.e., roll) AGS, whereas females did not. This significance may have been emphasized by the imbalance in sex of the study subjects. There were no significant differences between male and female subjects for paw/step or nudge/push.

Frequency of sniff bouts was not significant for Treatment ($F(16.787) = 0.362$, $p = 0.555$), Treatment*Behavior ($F(389.550) = 1.263$, $p = 0.287$), or Visit*Behavior ($F(389.550) = 0.747$, $p = 0.736$). Frequency of responses was significant for Visit

($F(394.029) = 6.310, p < 0.001$), which meant bears made one visit more frequently than six visits ($b = 1.515, t(392.405) = 2.369, p = 0.018$).

Frequency of responses was also significant for Behavior ($F(389.550) = 11.093, p < 0.001$). Pairwise comparisons indicated that sniff bouts occurred more frequently than nudge/push ($p = 0.010$; Table 3) and rolls ($p < 0.001$), and that paw/step also occurred more frequently than nudge/push ($p = 0.016$) and rolls ($p < 0.001$), but that sniff bouts and paw/step did not differ from each other. In turn, nudge/push occurred more frequently than rolls ($p = 0.016$), and rolls, then, occurred less frequently than other behaviors. Thus, the order of frequency for the index of intensity of investigatory behaviors was supported (i.e., sniff bouts were least intense and occurred more often; rolls were most intense and occurred less often).

Pairwise comparisons of Treatment*Behavior for male AGS indicated that only rolls occurred significantly less frequently than other behaviors (sniff bouts: $p = 0.001$; paws/step: $p = 0.012$; and nudge/push: $p = 0.031$; Table 4), hence the other behaviors, sniff bout through nudge/push, occurred in similar frequencies. For female AGS, rolls occurred less frequently than sniff bout ($p < 0.001$) and paw/step ($p = 0.003$), but not nudge/push ($p = 0.206$; Table 4). Likewise, nudge/push occurred less frequently than sniff bout ($p = 0.017$) and paw/step ($p = 0.003$). Thus, the male AGS elicited a higher intensity level of physical contact (up to nudge/push) than did female AGS (up to paw/step).

Pairwise comparisons of Visit*Behavior indicated that sniff bout, paw/step, and nudge/push occurred as frequently in each of the 6 visits, except in visit 6 where nudge/push occurred less frequently than only paw/step ($p = 0.016$; Table 5). Additionally, rolls occurred significantly less than all other behaviors in only the second

visit. Likewise, nudge/push occurred significantly more than rolls during only visit 2 ($p = 0.009$), which indicated that rolls, the highest-intensity investigatory behavior, were especially few during this visit. In the fourth and fifth visits, no behaviors differed. In the sixth visit, the more intense behaviors of nudge/push and rolls occurred significantly less frequently than paw/step ($p = 0.016$ and $p = 0.007$, respectively).

Chemical analysis.

The previously calibrated PLS model was used to classify the new samples. Female AGS should have a predicted value close to -1.0, and male AGS a value close to 1.0, with estimated deviations that do not include 0. The prediction value for female AGS was -0.8, and 0.3 for male AGS (Figure 3). The uncertainty (estimated deviation) of predicted values (horizontal lines) included zero for 130F, 418F, 263M, 429M, 422M, and 264F, which meant the model had difficulty assigning these samples to a group. The model was certain for 2 of 8 samples, which were correctly predicted as female (266F, 311F), and a third was borderline (264F). The model did not predict males with certainty, i.e., the predicted values were below zero and the estimated deviation included zero. Our samples were within the limit lines (Inlier distance = 1.28; Hotelling T^2 distance = 13.50) and, therefore, could be trusted based on the calibrated model.

Results were “projected” for visual comparison with the samples used in the calibration (Figure 4), where three factors best described the data. There is a cumulative effect for each Factor, so overall 88% of the variation in X and 66% of the variation in Y was accounted for: Factor 1 accounted for 61% of the variation in X (chemical profiles) and 30% of the variation in Y (bear sex), Factor 2 accounted for an additional

12% variation in X and 10% variation in Y, and Factor 3 accounted for an additional 15% variation in X and 26% variation in Y. The R-square (quality of prediction) for the predicted samples versus reference samples was 0.51, and the RMSEP (distance from the reference) was 0.68. Chemical profiles for females 418F and 130F had characteristics that classified them closer to the male group, and male 263M was classified closer to the female group.

Discussion

Zoo experiments.

Side preference and frequency of side switches.

We hypothesized that subadult brown bears could discriminate between unfamiliar adult male and female AGS. We predicted avoidance (less time spent on the preferred side) by bears in the presence of male AGS, which was not supported. Additionally, we predicted a higher frequency of side switches, an indication of anxiety, in the presence of male AGS, which was not supported. A likely possibility for no significant results may be that AGS served as an attraction in a captive setting. Captive animals retain a strong internally driven motivation to explore and learn about environmental contingencies (e.g., Inglis, 2000). Learning invariably occurs in situations that evoke exploration (Welker, 1961), and exploration can result in storage of data for future needs (Inglis, 1983; Toates, 1983). However, one of the first effects of captivity is to relieve the animal of the obligation to search for food and avoid predators (Hediger, 1955). Additionally, there is a high likelihood that a learning process occurs, in which

animals learn that objects placed within the enclosure are unlikely to be harmful and will usually be positively reinforcing (Hediger, 1955). This is partly because park managers are constantly challenged to develop enrichment for large mammals, such as bears, to encourage investigative and manipulatory foraging behaviors to prevent stereotypic behaviors (Carlstead et al., 1991, see also Montaudouin & Le Pape, 2004). Hence, the subadult bears in our study have likely learned that there is no reason to fear anything that is placed in their enclosure. Indeed, it was our hypothesis that avoidance would be detected by our method despite the security of captivity. But, especially without a paired physical threat, the subadults did not show avoidance to male AGS. Our failure to detect discrimination by the subadults based on their activity within their enclosures, however, does not imply an inability to discriminate between male and female AGS (e.g., Brown, 1979).

Frequency of visits, duration of visits, duration of time lapses between visits, and total time of visits.

We predicted that duration of visits would be shorter for male than female AGS. There were no significant differences for any visit, except visit 3, and the opposite outcome occurred: visit duration was longer for male AGS. We also predicted the duration of time lapses would be longer for male AGS than female AGS, which was not supported. Post hoc considerations of female AGS indicated a significant decrease in visit durations up to the third visit, which strongly suggested decreased interest in the AGS. And, the significant increase in time that lapsed between visits 3-4 and 4-5 indicated that subadults lost interest in female AGS after visit 4. For male AGS, there were no significant differences for duration of visits or duration of time lapses between any of

the immediately prior visits or time lapses, which suggested male AGS remained interesting through 6 visits. Thus, the subadults likely continued to acquire information from male AGS through visit 6, whereas they collected most of the important information from female AGS on the first visit and were habituated to it by visit 4.

We also predicted that the total time to complete visits would be shorter for male AGS, however, the subadults took significantly longer to complete six visits to male AGS. So, *each* visit (except visit 3) and time lapse was not statistically significant between male and female AGS, but the cumulative effect was. This meant that subadults took more time to process information of male AGS and did not return too soon. It's possible that male AGS took longer to process because it contained more information. Rosell et al. (2011) revealed no difference in the number of compounds in AGS between male and female adult brown bears, nor a difference in the presence or absence of particular compounds, but there was a significant difference in the abundance of compounds between the sexes. Hence, the subadult bears in our study likely discriminated male and female AGS based on the difference in abundance of the suite of chemicals present in AGS, which in turn supports that male AGS communicates different or additional messages than female AGS. The results of our PLSR model of GC profiles (given later), also suggested a code for sex. Moreover, by not returning to the male AGS too quickly, the subadults may have also been demonstrating cautious behavior; a behavior that would increase survival of free-ranging subadults. Scent marking with AGS by brown bears, however, has not been documented, but bears have been observed to sit at the base of a tree after rubbing, and to sniff the ground before rubbing (O.-J. Sørensen, pers. comm.). Based on the significant difference in time spent

collecting and processing information (i.e., cumulative time to complete 6 visits), we conclude that subadults were able to discriminate male from female AGS.

Direct contact, in addition to the processing time spent away from the AGS, characterized the discriminatory behavior of the subadults. Thus, our results were not directly comparable to many olfactory studies that define discrimination based on duration of contact. In general, however, our results were in accordance with many studies that strongly suggest that animals investigate odors longer if they are in some way more “important” to them, e.g., they play a larger role in competition or reproduction (e.g., Brown, 1979; Drickamer & Brown, 1998; Rasmussen & Schulte, 1998; Swaisgood et al., 2000, 2002), or survival, in the case of brown bear subadults.

Frequencies of sniff bouts and investigative behaviors.

Our prediction that male AGS would be avoided (i.e., fewer and less intensive investigative behaviors) was not supported. For female AGS, frequencies of sniff bouts and paw/steps were similar, and were statistically different from other investigative behaviors. For male AGS however, frequencies were similar for sniff bouts, paw/step, and nudge/push, which meant male AGS elicited a higher-intensity level of investigation (up to the nudge/push level, whereas female AGS was investigated mostly to the paw/step level).

Similar descriptions of the intensity levels of investigatory behaviors and their frequencies of occurrence have not been documented in other studies. White et al. (2003) recorded occurrences of flehmen, licking, and scent-rubbing, in addition to total duration of sniffing, in giant panda olfactory experiments. Not only did they record different behaviors than we, but they used fir boards (13 x 10 x 2 cm) to deliver stimuli,

thus different manipulatory behaviors toward flat boards and large rocks are imminent. A procedure has been developed to standardize experiments so that direct comparisons of investigatory behaviors can be made across experiments and species (Glickman & Sroges, 1966; Bacon, 1980), but the procedure quantifies responses to novel objects (which could be picked up or placed entirely in the mouth) rather than novel odors. The closest comparison to our study of manipulatory behaviors was of grizzly bears in zoos in response to objects that may or may not have contained food, and included pawing, swatting, picking up or tossing, licking, and chewing (McGowan et al., 2010). Regarding olfactory stimuli, Wells (2009) reviewed advances in olfactory enrichment in zoos, which included experiments with odors from prey, predators, and plant derived odors, but few investigated pheromones. Standardization of data collection methods, and materials used in delivery of stimuli, would simplify direct comparisons between studies and species, but may not be entirely plausible because the same stimulus (such as novel objects) may have differing significance for different species (Tinbergen, 1951). Likewise, the same object used in delivery of a stimulus (such as rocks or flat boards) may result in different manipulatory behaviors, because of the different phylogeny of different species (e.g., a bear species that digs may have the motor skills that are more conducive to flipping rocks than would a bear species that does not dig) (Nissen, 1951). Thus, even standardization of procedures may not necessarily produce directly comparable results.

Chemical analysis.

Based on our zoo experiments, chemical analysis, and on the previous chemical analysis by Rosell et al. (2011), there is good reason to believe a code for sex exists. The results of the PLS-DA model were trustworthy, and showed a trend toward discrimination by sex, but the model should be improved before it is used for prediction of unknown samples. The predictive ability of the calibrated model was moderate, and the predictions were weaker, but the target prediction was better for females than males. The weaker prediction ability for males may have been due to the fewer samples to build the model than for females. The predictability of the model would likely improve with increased samples for both sexes used in calibration. However, in the wild fewer adult male bears occur in a given area, which makes it difficult to acquire ample AGS samples to readily improve such a model. Moreover, AGS of male bears is often more difficult to obtain (less secretion available) than female AGS, possibly because they empty their anal sacs more often. Results of the current model should be used with caution until the Predictive versus Reference values are improved.

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Table 1. Subadult (1 and 2 yrs old) captive brown bears used in behavioral experiments to determine whether responses differed between adult male and female anal gland secretion. Experiments were conducted in May-October 2010.

No.	Sex	Age (years)	Location	Date of Experiments
1	M	2	Tbilisi Zoo- Tbilisi, Georgia	19-25 May
2	M	2	Tbilisi Zoo- Tbilisi, Georgia	19-25 May
3	M	2	Grönklitt Björnepark- Orsa, Sweden	4-10 June
4	M	2	Grönklitt Björnepark- Orsa, Sweden	4-10 June
5	M	1	Vassfaret Björnepark- Flå, Norway	17-23 June
6	M	1	Vassfaret Björnepark- Flå, Norway	18-24 June
7	M	1	Copenhagen Zoo- Copenhagen, Denmark	19-25 June
8	M	1	Copenhagen Zoo- Copenhagen, Denmark	20-26 June
9	F	2	Riga Zoo- Riga, Latvia	1-7 July
10	*M	2	Riga Zoo- Riga, Latvia	2-8 July
11	M	1	Polar Zoo- Bardu, Norway	4-10 July
12	F	1	Polar Zoo- Bardu, Norway	5-11 July
13	F	1	Ähtäri Zoo- Ähtäri, Finland	6-12 August
14	F	1	Ähtäri Zoo- Ähtäri, Finland	6-12 August
15	M	1	Ähtäri Zoo- Ähtäri, Finland	7-13 August
16	M	1	Borås Zoo- Borås, Sweden	6-12 September
17	M	1	Borås Zoo- Borås, Sweden	7-13 September
18	F	1	Grönklitt Björnepark- Orsa, Sweden	5-11 September
19	F	1	Grönklitt Björnepark- Orsa, Sweden	6-13 September
20	F	1	Karacabey Bear Sanctuary- Karacabey, Turkey	1-7 October

*Sterilized

Table 2. Samples of adult (≥ 4 yrs old) brown bear anal gland secretion used to characterize chemical profiles in partial least squares (PLS) regression.

	Sample ID	Sex	Age	AGS collected
1	130	F	4	2008
2	418	F	5	2010
3	126 ^a	F	9	2008
4	266	F	11	2008
5	124 ^a	F	14	2008
6	121 ^a	F	18	2008
7	125 ^a	F	11	2008
8	311	F	17	2009
9	264	F	8	2008
10	120 ^a	F	19	2008
11	263	M	5	2008
12	305 ^a	M	4	2009
13	129 ^a	M	19	2008
14	429	M	8	2010
15	422	M	> 4	2010
16	127 ^a	M	11	2008

^a Samples used to calibrate the PLS regression model.

Table 3. Planned pairwise comparisons of the estimated marginal means of a linear mixed model for frequencies of investigative behaviors of subadult (1 and 2 yrs old) brown bears toward anal gland secretion of adult male ($n = 10$) and female ($n = 10$) brown bears for their first six visits. Experiments were carried out in captivity trials in May-October 2010.

Behavior		Mean Diff	SE	df	P*
sniff bouts vs	paw/step	0.039	.259	389.55	0.879
	nudge/push	0.666	.259	389.55	0.010
	roll	1.290	.259	389.55	0.000
paw/step vs	nudge/push	0.627	.259	389.55	0.016
	roll	1.251	.259	389.55	0.000
nudge/push vs	roll	0.624	.259	389.55	0.016

* Bold indicates significance at the 0.05 level

Table 4. Planned pairwise comparisons of the estimated marginal means for Treatment*Behavior from a linear mixed model of frequencies of sniff bouts and investigative behaviors of subadult (1 and 2 yrs old) brown bears toward anal gland secretion of adult male ($n = 10$) and female ($n = 10$) brown bears carried out in captivity trials in May-October 2010.

Secretion	Behavior	Mean Diff	SE	df	P*
Male					
sniff bout vs	paw/step	0.305	.360	389.550	0.398
	nudge/push	0.441	.360	389.550	0.222
	roll	1.220	.360	389.550	0.001
paw/step vs	nudge/push	0.136	.360	389.550	0.706
	roll	0.915	.360	389.550	0.012
nudge/push vs	roll	0.779	.360	389.550	0.031
Female					
sniff bout vs	paw/step	-0.227	.370	389.550	0.540
	nudge/push	0.891	.370	389.550	0.017
	roll	1.360	.370	389.550	0.000
paw/step vs	nudge/push	1.118	.370	389.550	0.003
	roll	1.586	.370	389.550	0.000
nudge/push vs	roll	0.469	.370	389.550	0.206

* Bold indicates significance at the 0.05 level

Table 5. Planned pairwise comparisons of the estimated marginal means for Visit*Behavior from a linear mixed model of frequencies of sniff bouts and investigative behaviors of subadult (1 and 2 yrs old) brown bears toward anal gland secretion of adult male ($n = 10$) and female ($n = 10$) brown bears carried out in captivity trials in May-October 2010.

Visit	Behavior	Mean Diff	SE	df	P*
1					
	sniff bouts vs paw/steps	0.100	.601	389.550	0.868
	nudge/push	0.800	.601	389.550	0.184
	roll	1.900	.601	389.550	0.002
	paw/steps vs nudge/push	0.700	.601	389.550	0.245
	roll	1.800	.601	389.550	0.003
	nudge/push vs roll	1.100	.601	389.550	0.068
2					
	sniff bouts vs paw/steps	-0.382	.617	389.550	0.536
	nudge/push	-0.199	.617	389.550	0.748
	roll	1.425	.617	389.550	0.022
	paw/steps vs nudge/push	0.184	.617	389.550	0.766
	roll	1.807	.617	389.550	0.004
	nudge/push vs roll	1.623	.617	389.550	0.009
3					
	sniff bouts vs paw/steps	-0.225	.617	389.550	0.716
	nudge/push	0.643	.617	389.550	0.298
	roll	1.004	.617	389.550	0.105
	paw/steps vs nudge/push	0.868	.617	389.550	0.160
	roll	1.228	.617	389.550	0.047
	nudge/push vs roll	0.360	.617	389.550	0.560
4					
	sniff bouts vs paw/steps	0.618	.617	389.550	0.318
	nudge/push	1.064	.617	389.550	0.085
	roll	1.162	.617	389.550	0.061
	paw/steps vs nudge/push	0.447	.617	389.550	0.469
	roll	0.544	.617	389.550	0.379
	nudge/push vs roll	0.097	.617	389.550	0.875
5					
	sniff bouts vs paw/steps	0.813	.672	389.550	0.228
	nudge/push	0.750	.672	389.550	0.265

		roll	1.125	.672	389.550	0.095
	paw/steps vs	nudge/push	-0.062	.672	389.550	0.926
		roll	0.313	.672	389.550	0.642
	nudge/push vs	roll	0.375	.672	389.550	0.577
6	sniff bouts vs	paw/steps	-0.687	.672	389.550	0.307
		nudge/push	0.938	.672	389.550	0.164
		roll	1.125	.672	389.550	0.095
	paw/steps vs	nudge/push	1.625	.672	389.550	0.016
		roll	1.813	.672	389.550	0.007
	nudge/push vs	roll	0.188	.672	389.550	0.781

* Bold indicates significance at the 0.05 level

Figure legend

Figure 1. Partial least squares regression model showing a separation of groups in the Scores plot for the training data set of brown bear (*Ursus arctos*) anal gland secretion. Sex of a bear is denoted by F (female, $n = 5$) or M (male, $n = 3$) after each sample ID number.

Figure 2. a) Mean duration (sec) of the first 6 visits (\pm SE) by subadult brown bears ($N = 20$) (*Ursus arctos*), and b) mean duration of time (sec) that lapsed between the first 6 visits (\pm SE) by subadult brown bears toward male ($n = 10$) and female ($n = 10$) anal gland secretion of free-ranging adult brown bears. Error bars and means are calculated for only the bears participating in visits.

Figure 3. Predicted values (horizontal line) and estimated deviations (boxes around horizontal lines) for new brown bear (*Ursus arctos*) samples using the calibrated partial least squares regression model. Female (F) samples should be near -1 and males (M) should be near 1. The letter P above samples indicates those samples that were predicted (i.e., those not used to calibrate the model).

Figure 4. Projection of the training data set used in calibration (Figure 1) and the new predicted samples (test set) in a partial least squares regression model for adult male (M) and female (F) brown bear (*Ursus arctos*) anal gland secretion.

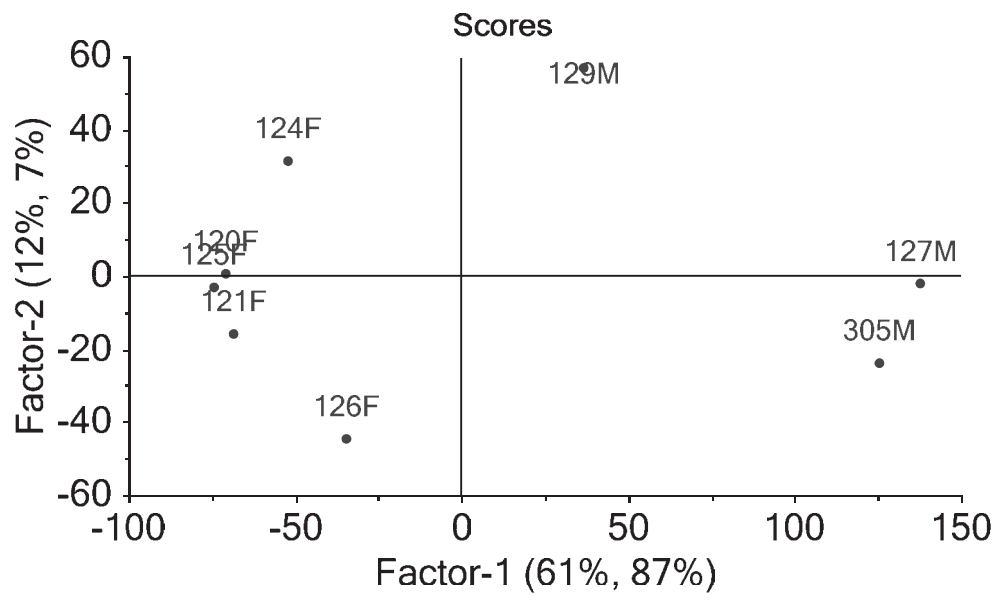


Figure 1

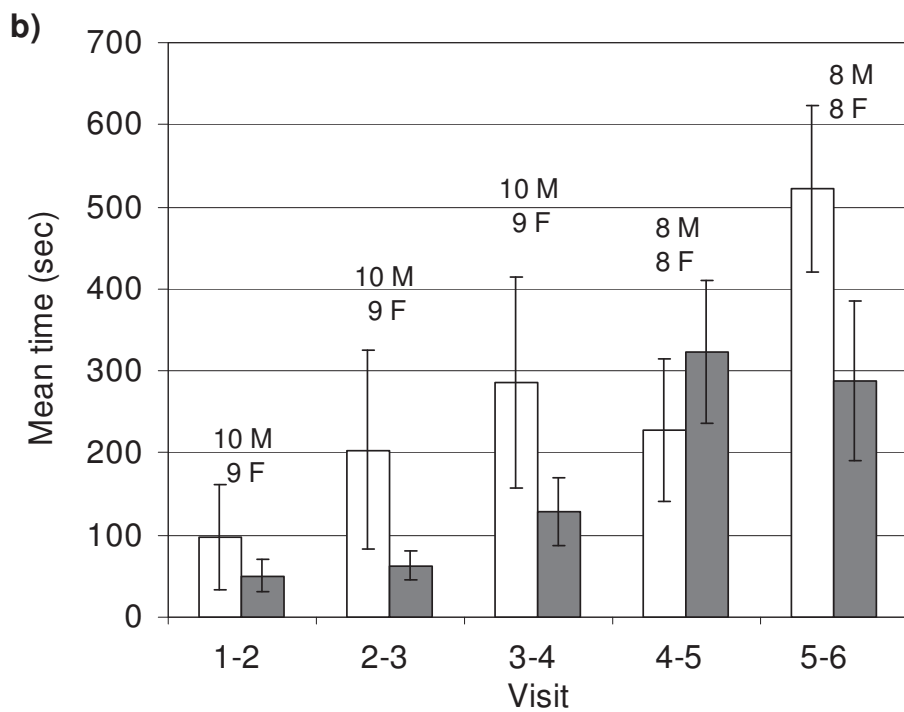
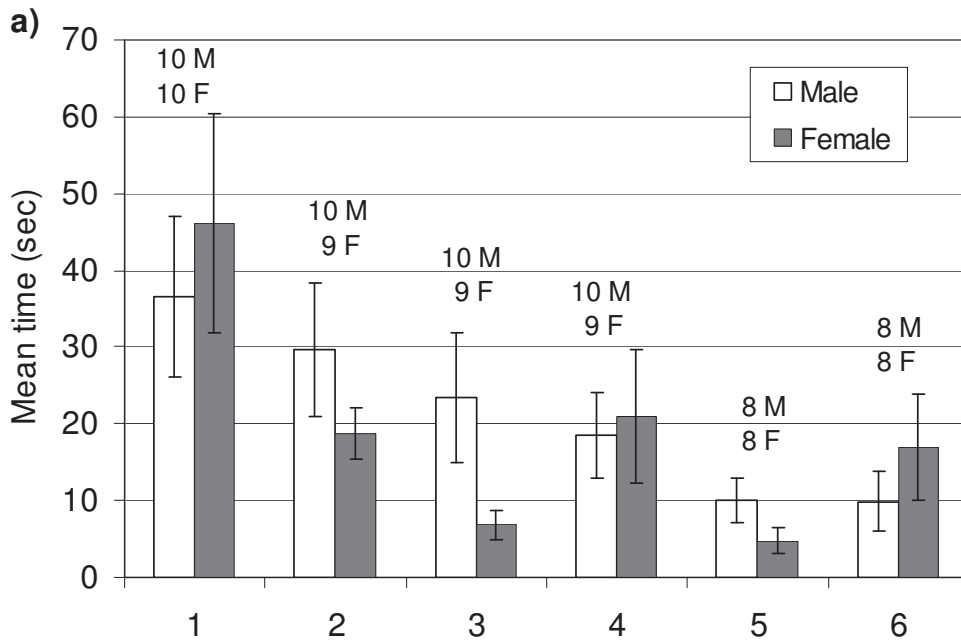


Figure 2

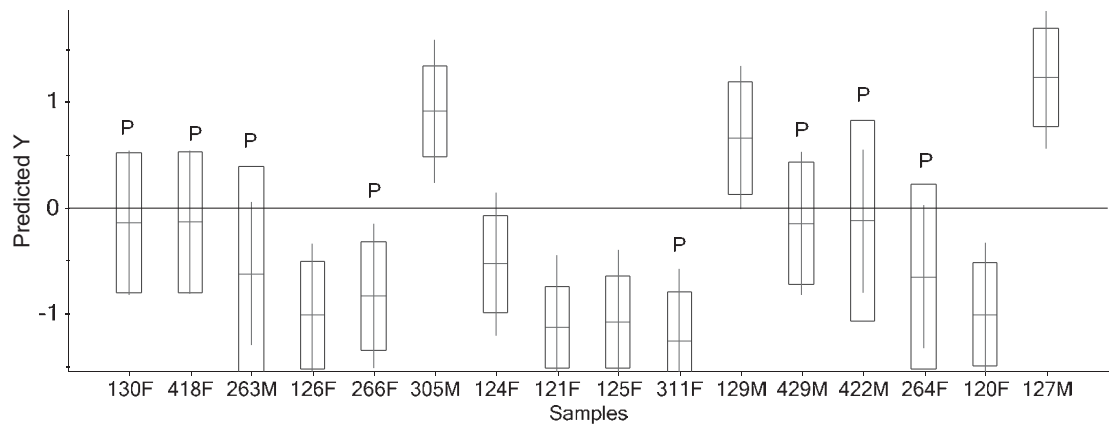


Figure 3

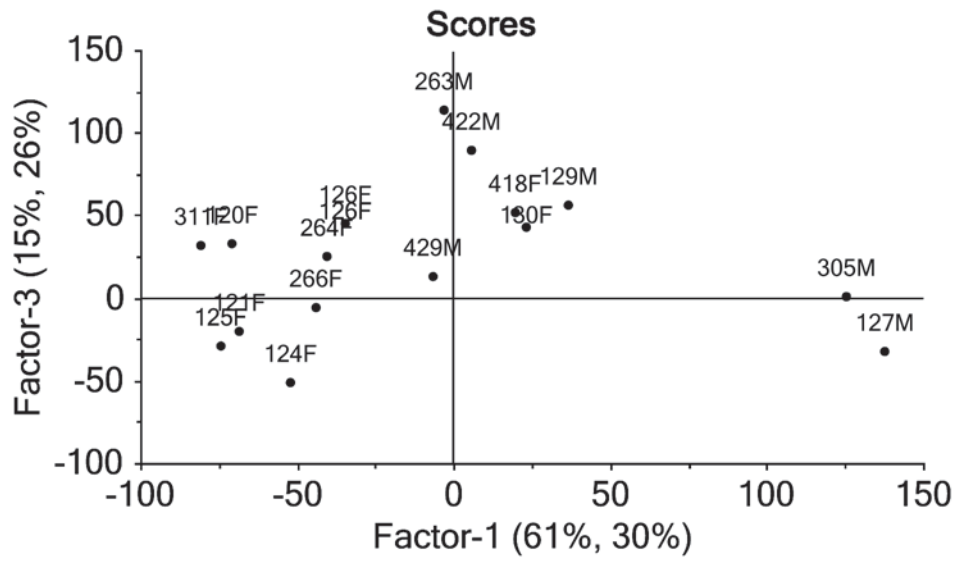


Figure 4

Paper III

Does Eurasian beaver (*Castor fiber*) anal gland secretion signal territory ownership?

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Abstract- Olfactory signals can contain information, such as age and social status, and play a vital role in competitor assessment. In many territorial species, subordinates must leave their natal colony to obtain their own territory and mate. These individuals could be aggressive opponents in agonistic encounters, as they will have little to lose (the desperado effect). In this study we tested the hypothesis that territory ownership is coded in the anal gland secretion (AGS) of the monogamous and highly territorial Eurasian beaver (*Castor fiber*). Territorial intrusions were simulated by presenting residents with a two-way choice test of AGS from an unknown male territory owner (i.e. dominant) and his son (i.e. subordinate; either one or \geq two years old). We predicted that subordinate sons would be considered a greater threat and elicit a stronger territorial response than their dominant fathers. Residents spent more time investigating AGS from subordinates, but only responded more aggressively when they were \geq two years old. Chemical analyses using gas chromatography-mass spectrometry supported our behavioral findings and revealed differences between chemical profiles of territory owners and subordinates, and also between the subordinates in different age groups. We suggest that our results are explained by differences in territory ownership, reproductive status, competitive ability (body weight), and that two year olds are more likely to disperse.

Key words- Anal gland secretion, *Castor fiber*, chemical communication, competitor assessment, competitive ability, desperado effect, dominant-subordinate discrimination, territory ownership

Introduction

Owning a territory often ensures exclusive access to food, mates, and nest sites, but there are several costs associated with territorial defense. The most important costs are time and energy spent patrolling and scent marking, chasing competitors, and injury or even death if an intruder does not back down (Bradbury and Vehrenkamp 1998). With the risk of a physical conflict, the ability to make the “right” decision can have a strong impact on fitness. For a resident, one way to avoid costly encounters is to use information conveyed by different signals to assess the threat level posed by an intruder (Temeles 1994, Müller and Manser 2007), and then scale the contest level by investing more aggression toward individuals that can inflict a greater loss (Parker 1974).

Olfactory signals are the basis of communication and social discrimination in many mammals (Ralls 1971, Wyatt 2003, Müller-Schwarze 2006), and scent marks have an important role in territorial behavior and competitor assessment (Gosling and Roberts 2001, Wyatt 2003, Müller-Schwarze 2006). Unique among social signals, scent marks can convey detailed information in the sender’s absence (Gosling and Roberts 2001), such as age (e.g. Buesching et al. 2002, White et al. 2003, Yuan et al. 2004, MacDonald et al. 2008) and dominance status (e.g. Hayes et al. 2001, Burgener et al. 2009). Age, body size and dominance status are often correlated with fighting ability, or resource-holding potential (RHP), and frequently used to settle contests (Parker 1974, Archer 1987, Kemp and Wiklund 2004). On the other hand, asymmetries in fighting motivation can also affect contest behaviors and outcomes (Parker 1984), as a high motivation to fight might help in overcoming inferior RHP (e.g. Barnard and Brown 1984, Haley 1994). One scenario where an intruder’s motivation could be expected to

be high is when territory ownership is a prerequisite for reproductive success and vacant territories are rare. If the intruder is not able to reproduce without gaining a territory, it could be expected to be an aggressive opponent, as it will have little to lose (the desperado effect, Grafen 1987). This could be the case in many territorial species where subordinates do not breed while living with their parents, but must disperse from their natal site to find a territory and a mate.

Beavers (*Castor* spp.) are territorial and socially monogamous rodents that rely highly on chemical communication (Schulte 1993, Rosell 2002, Campbell-Palmer and Rosell 2010). The basic family unit consists of a dominant pair living in a stable, long-term relationship within a defended “multipurpose/breeding” territory (Temeles 1994) together with their subordinate offspring; young of the year (kits), yearlings, and sometimes offspring from previous breeding seasons (Wilsson 1971, Rosell and Bjørkøyli 2002). Only the dominant couple reproduces, and subordinate members will normally not reach sexual maturity or be sexually active while living with a dominant animal of the same sex (Wilsson 1971, Svendsen 1980, Campbell et al. 2005). Offspring of both sexes usually disperse as two-year-olds (e.g. Hartman 1997, Sun et al. 2000), but delayed dispersal is common in high density populations, and yearlings can disperse when densities are low (Hartman 1997). The main dispersal period occurs in spring and summer (normally April-June) (Svendsen 1980, Rosell et al. 1998, Sun et al. 2000, DeStefano et al. 2006), which also includes the peak period in resident beavers’ territorial behavior (Svendsen 1980, Rosell et al. 1998). Aggressive encounters between conspecifics during this period are common (Piechocki 1977, Svendsen 1989, Nolet and Rosell 1994), and injuries inflicted by other beavers can be a major cause of death, especially in dispersing subadults (Piechocki 1977, Nolet et al. 1997).

Both the North American beaver (*Castor canadensis*) and the Eurasian beaver (*C. fiber*) have anal glands which they use when scent marking their territories (Rosell and Bergan 1998). All members in a family unit, except kits younger than five months, defend the territory with scent marking (Wilsson 1971, Buech 1995). The high number of compounds in AGS) of beavers (Sun 1996, Rosell and Sundsdal 2001) allows the potential coding of a wealth of information, and studies have shown that it contains information about species (Rosell and Sun 1999, Rosell 2002), subspecies (Rosell and Steifetten 2004, but see Peterson et al. 2005), gender (Grønneberg and Lie 1984, Sun 1996, Rosell and Sundsdal 2001), individuality (Sun 1996), kinship (Sun and Müller-Schwarze 1998a), and family membership (Sun and Müller-Schwarze 1998b).

The aim of this study was to investigate the hypothesis that territory ownership (i.e., dominance) is coded in the AGS of male Eurasian beavers. We simulated a simultaneous territorial intrusion by an established dominant territory owner and his subordinate son (i.e., son that had not yet dispersed and still lived with his father). We predicted that residents would show a stronger territorial response towards scent from sons, despite their lower RHP, based on the fact that they, in contrast to their fathers, should be more motivated to acquire a territory. We also hypothesized that there is a difference in the chemical profiles (i.e. chromatograms) of AGS from dominant territory owners and their subordinate sons. Thus, we performed chemical analyses with gas chromatography and mass spectrometry (GC-MS) of the AGS samples used in the bioassay to support any findings of discrimination.

Methods and materials

Study area and study animals. Field experiments were conducted in 2008 (10 April – 17 July) and 2009 (15 April – 20 July) using a population of free-ranging beavers in the rivers Straumen (59°29′ N, 09°153′ E), Gvarv (59°386′ N, 09°179′ E), and Saua (59°444′ N, 09°307′ E), in Telemark County, southern Norway. All three rivers form part of the catchment of Lake Nordsjø (Campbell et al. 2005). The study area has been inhabited by beavers since the 1920s (Olstad 1937), and hunting pressure is either low or nonexistent (Parker and Rosell 2003, Campbell 2010). The population in the study area appears to be at carrying capacity (Rosell and Hovde 2001, Parker et al. 2002). All territories in the study area are adjacent to each other with no unoccupied stretches of river in between, and territory overlap between neighbors is small to nonexistent (Herr and Rosell 2004).

The beavers in the study area have been monitored between March and November every year since 1998 through an extensive live-trapping program, using landing-nets from a motor boat (Campbell 2010). The trapping records, radio tracking, and observational data collected includes information about territorial borders, family composition, family member replacements, lengths of pair bonds, and breeding and dispersal events. Dominance status was determined by previous trapping and sighting history, body mass, and incidences of lactation in females (see Campbell 2010 for details). As beavers live in discrete family groups, individuals that were seen and/or trapped inside the territory of a known dominant pair on more than one occasion were assumed to be their subordinate offspring (Rosell and Hovde 2001, Campbell et al. 2005, Campbell 2010).

We immobilized captured individuals in cloth sacks while we collected measurements and samples. We assigned all trapped individuals to an age-class (1 years = yearling, 2 years = subadult and ≥ 3 years = adult) based on their body weight (Rosell et al. 2010) and trapping record, determined sex based on the color of their AGS (Rosell and Sun 1999), tagged them with a microchip (Avid[®] or Trovan[®]), and marked them with unique color combinations of plastic (Dalton) and metal (National Band and Tag Co.) ear-tags for individual recognition (Rosell and Hovde 2001, Campbell 2010). Before we obtained AGS samples, we lifted the beaver's tail (at the open end of the cloth sack) and evacuated the rectum. We rinsed the cloaca area with distilled water before we pushed out each of the two papillae of the anal gland separately and squeezed out the AGS (Rosell and Bjørkøyli 2002). All samples were placed in glass vials with Teflon-lined lids and frozen at -20 °C. The beavers were released after 20-30 min. All trapping and handling procedures were approved by the Norwegian Experimental Animal Board and the Norwegian Directorate for Nature Management and met the guidelines approved by the American Society of Mammalogists (Gannon and Sikes 2007).

Scent donors. We used AGS samples obtained during the main dispersal period (April – June) from 1999 – 2009 for field experiments. In the North American beaver, AGS codes for relatedness (Sun and Müller-Schwarze 1998a), and assuming that relatedness is also coded in Eurasian beavers, we used father-son pairs as scent donors to minimize the effects of genetic factors, and also habitat differences, on the chemical composition of AGS within a pair. Samples in a pair were taken the same year or one year apart, and all father-son pairs were living together at the time samples were obtained. As beavers

can already disperse at age one, both yearlings (born in May/June the previous year) and older were used as scent donors. Because yearlings are smaller in body size (see Rosell et al. 2010), we set up two different experiments. In experiment one, AGS was obtained from a territory owner (TO) and his old son (OS, age 2-7 years, N=12), and in experiment two, from a TO and his young son (YS, age 1 year, N=10; Table 1). To avoid pseudoreplication (Kroodsma et al. 2001), each scent donor was only used once in each experiment. Six dominant males and three subordinate sons were used in both experiments, but the two experiments were analyzed separately. When a scent donor was used in both experiments, he was not used two times in the same colony. Only one response from each resident was recorded in each experiment. If the same family unit was used in both experiments, the experiments were conducted at least one week apart to avoid carry-over effects. To minimize the probability that responding residents and scent donors had previous contact and/or were genetically related, we used AGS from scent donors caught > 15 km away and in a different watershed, which is greater than the average dispersal distance for males (e.g. Sun et al. 2000). The TO-OS experiment was conducted in 2008 (N=8) and 2009 (N=4), and the TO-YS experiment (N=10) was conducted in 2009.

Experimental design. We constructed two adjacent experimental scent mounds (ESMs) inside each territory and deposited AGS from a TO and his OS or YS. This simultaneous presentation controls for temporal variation in motivation of the resident and thus provides a more sensitive test of discriminatory abilities (e.g. Sun and Müller-Schwarze 1997, Rosell and Bjørkøyli 2002, White et al. 2003). A blank control was not included in the experimental design, because other studies have shown that beavers do

not respond to untreated mounds. Hence, it is not the actual sight of the ESM that elicits the response, but the scent itself (Schulte et al. 1995, Rosell et al. 2000).

We put 0.25 g of AGS into a white plastic bottle cap (2.5 cm top diameter, 1.5 cm high) to hold the sample and control the evaporation surface area (4.9 cm²) (Rosell and Bjørkøyli 2002, Rosell and Steifetten 2004), which has been shown to be enough to elicit a response in previous studies (e.g. Rosell and Bjørkøyli 2002, Rosell and Steifetten 2004). Bottle caps were pushed into the center of each ESM, so they were leveled with the surface of the mound.

We constructed ESMs in the center of the territory, defined as within 50 m from the active lodge (i.e., the lodge the residents used most frequently during the study period), in a location where the beavers could easily walk onto land. If the terrain on either side of the lodge was not suitable for construction of ESMs (e.g., too steep), a location on the opposite bank was used. We constructed ESMs by grabbing a handful of mud and debris from the bottom of the river. When handling the material we used clean latex gloves to avoid transmission of human odor. Each ESM was approximately 10 cm high and 15 cm wide, and made as identical as possible regarding size and the material used. The two ESMs in a pair were constructed 40 cm apart (between the centers of the bottle caps), and within 50 cm from the water's edge (Herr et al. 2006). The chosen distance between ESMs ensured that once a beaver responded to one of them, it could also respond to the other; hence, between-treatment effect could be compared (Sun and Müller-Schwarze 1997).

To control for side preferences, left and right positions were chosen at random, and the position in relation to the lodge was chosen according to the terrain. Scent was put out about 60 min before the beavers emerged from the lodge in the evening (1800-

2000 hours) (Rosell and Bjørkøyli 2002). Observations ended between 2100 and 2300 hrs because of insufficient daylight. After a beaver's response, we removed the ESMs. If no response from resident beavers was recorded during a trial, ESMs were removed, and we returned another evening. Responses were recorded with a tripod-mounted digital camera (Sony DCR-SR35E) set to record continuously. The camera was also handled using latex gloves to avoid human odor, and placed approximately 10 m from the ESMs, depending on the terrain.

Response measures. Video recordings were analyzed using Microsoft Windows Media Player (Microsoft®). The observer was blind to treatment assignments. We recorded the duration of two response patterns to ESMs in seconds: (1) sniffing (defined as when the beaver's nose was within 5 cm of the ESM), and (2) the physical response, (defined as the beaver standing with its hind feet on the ESM, or pawing and/or over-marking it with castoreum and/or AGS (Rosell and Bjørkøyli 2002). Duration of sniffing is used as a measure of the time a beaver needs to identify the scent, as well as an indication of the level of interest, whereas the duration of the physical response is used to describe how strong an agonistic behavior the scent on the ESM triggered; the longer the response, the more agonistic (Rosell and Bjørkøyli 2002, Rosell and Steifetten 2004, Rosell and Sanda 2006). Physical responses to scent on ESMs can be interpreted as agonistic, because beavers are highly territorial, and scent mounds containing odor from a stranger are typically destroyed by residents (e.g. Sun and Müller-Schwarze 1997, Rosell and Bjørkøyli 2002).

Only the first response from the first beaver was included in the analysis, because physical damage to ESMs might lead to carry-over biases in subsequent

responses (Sun and Müller-Schwarze 1997). Only responses from subadults and adults were included in the analyses (Rosell and Steifetten 2004). We attempted to identify all responders recorded during our live-trapping, based on body size, ear tags, and tail scars.

Chemical sample preparation. Chemical analyses were performed in December 2008. We used toluene-methanol 3:1 as the solvent to extract compounds from AGS (Rosell and Sundsdal 2001). Prior to extraction, we cleaned all laboratory materials in acetone and autoclaved them for 50 min. We transferred 0.10 g of AGS into a glass vial and added 2.5 ml of solvent. The solutions were left for two hours in room temperature, then filtrated through a filter paper (Schleicher and Schuell no. 595 ½) and transferred to a gas chromatography (GC) vial. To avoid loss of volatile compounds, samples were covered with aluminum foil during extraction.

Chemical analysis. Scent can communicate information through a mixed composition of chemicals that can be investigated by analyzing gas chromatograms. Use used an auto-injection system (Agilent 7683 Series Injector) to inject 1 µl of the AGS solution into a Hewlett-Packard (HP) 6890 Series II gas chromatograph. The gas chromatograph was equipped with a non-polar HP-5 MS 5% phenyl-methyl-siloxane column (30.0 m long × 0.25 mm ID × 0.25 µm film thickness) and connected to a HP 5973 Series mass spectrometer detector with a split/split-less inlet used in the split-less mode. Helium was used as the carrier gas at a constant flow of 0.7 ml/min. The initial oven temperature was set at 130°C and then increased 4°C/min to 310°C. The temperature was then kept

at 310°C for 15 min. A delay of 2 min was set for every run to prevent the solvent from damaging the detector.

Two sample pairs used in the TO-OS experiment were obtained in 2009, after the chemical analyses were conducted, thus they were not included in the chemical analyses. Therefore, in total, 40 samples were analyzed ($N_{TO} = 20$, $N_{OS} = 10$, $N_{YS} = 10$). Samples were analyzed in random order. After every nine samples, a blank sample was run to ensure that the column was clean.

Data analysis. The data did not fit the assumptions of normal distribution and homogeneity of variance for parametric analysis (Sokal and Rohlf 1995), so nonparametric tests were used (Siegel and Castellan 1988). Wilcoxon's matched-pairs signed-ranks test was used to evaluate differences in duration of sniffing and duration of physical response between ESMs, and Mann-Whitney U test to evaluate differences in the body weight of TO and OS. In the TO-OS experiment, data from the two years were combined, because there were no significant differences between years. All tests were two-tailed and the significance level was set to $P \leq 0.05$. The data analyses were performed with SPSS version 15.0 for Windows (SPSS Inc. 2006).

We used partial least squares regression (PLS-R) (Wold et al. 1983), as previously used by Rosell and Steifetten (2004), to investigate the chemical profiles of the dominant and subordinate groups. PLS-R is a particular type of multivariate analysis that uses the two-block predictive PLS-R model to model the relationship between two matrices, X and Y (Wold et al. 2001, Esbensen 2001) (for details, see Rosell and Steifetten 2004). PLS-R is especially suited to analyze GC data, because it can analyze data that are strongly collinear (correlated), noisy, and have numerous X-variables

(more than the number of samples), and also simultaneously models several response variables Y (Wold et al. 2001, Esbensen 2001).

To test whether the composition of the compounds detected by GCs differed among scent donor groups, the discriminating variable Y was defined as 1 for TO and 0 for OS or YS. We also compared OS and YS to evaluate age differences between the two groups, defining Y as 1 for OS and 0 for YS. As a basis for comparison, the total ion current (TIC) for each time unit on the retention scale (165 time units/min) was measured. The measured values formed a GC-matrix of X-variables (40 samples x 9561 time measurements) and the Y-variable. Due to considerable variation in TIC values among samples, all values were scaled by mean normalization and standard normal variate to minimize the effect of the considerable variation in abundance among the samples.

The PLS-R method extracts a small number of principal components (Factors) that represent the relevant latent dimensions of the model. We used the values of validated R-square and root mean square error of prediction (RMSEP) to evaluate the results. Validated R-square describes the predictive ability of the model, and should be close to 1. RMSEP is a measurement of the average difference between the predicted and measured response values, and should be close to 0. Full cross validation was used as the validation method. Analyses were performed using the statistical software The Unscrambler X 10.0 (CAMO Software AS 2010).

Results

Behavioural bioassay. In total, 22 responses were recorded, and we could identify the responder in 19 cases. Four of these responses were by subordinate offspring in the territory ($N_{\text{Male}} = 2$, $N_{\text{Female}} = 2$), and the remaining 15 were by dominant individuals ($N_{\text{Male}} = 8$, $N_{\text{Female}} = 7$). We found no consistent sex differences by resident responders. Responses from males and females were therefore pooled in the analysis. No yearlings were observed to respond, and all the identified subordinate responders were > 2 years old.

OS had a significantly lower body weight than TO (Mann-Whitney U test, $U = 20.5$, $N_{\text{OS}} = 12$, $N_{\text{TO}} = 12$, $P = 0.002$). Residents spent significantly more time sniffing AGS from OS than TO ($Z = -2.752$, $N = 12$, $P = 0.006$), and spent more time responding physically towards OS than TO ($Z = -2.668$, $N = 12$, $P = 0.008$) (Fig. 1a). Residents also spent significantly more time sniffing AGS from YS than TO ($Z = -2.191$, $N = 10$, $P = 0.028$), but did not show a stronger physical response towards YS than TO ($Z = -0.762$, $N = 7$, $P = 0.446$) (Fig. 1b).

GC comparisons. The GC comparison separated between TO and OS (Fig. 2a). Factor -1 explained 36 % of the X-variance and 24 % of the Y-variance, and Factor -2 explained 14 % of the X-variance and 37 % of the Y-variance (Validated R-square = 0.24, RMSEP = 0.47). When comparing TO and YS (Fig. 2b), Factor -1 explained 40 % of the X-variance and 34 % of the Y variance and Factor -2 explained 15 % of the X-variance and 42 % of the Y-variance (Validated R-square = 0.46, RMSEP = 0.39). OS and YS were also separated (Fig. 2c), with Factor -1 explaining 34 % of the X-variance

and 33 % of the Y variance and Factor -2 explaining 19 % of the X-variance and 38 % of the Y-variance (Validated R-square = 0.44, RMSEP = 0.39).

Discussion

The results from the behavioural bioassay combined with the chemical analysis supported our hypotheses that there is a chemical difference between AGS from dominant territory owners and their subordinate sons, and that other resident beavers can discriminate between the two categories. Our prediction that residents would show a stronger territorial response towards AGS from subordinates, despite their lower RHP, was supported when the subordinate scent donor was ≥ 2 years old, but only for the sniffing response when the subordinate scent donor was a yearling. The results from the chemical analyses showed that the GC profiles differed between dominant and subordinate beavers as well as between old and young sons.

Regardless of the subordinate age group, resident beavers spent significantly more time sniffing AGS from subordinates than territory owners. This suggests that beavers can discriminate between dominant and subordinate individuals based on the difference in chemical cues alone, which is consistent with results in other species, such as Iberian rock lizards (*Lacerta monticola*, Martín et al. 2007), house mice (*Mus domesticus*, Drickamer 1992) and bank voles (*Clethrionomys glareolus*, Rozenfeld and Rasmont 1991, Kruczek 1997). The difference in duration of sniffing between subordinates and territory owners also suggests that scent from subordinates is more interesting, but not necessarily more threatening than scent from territory owners, because we did not consider sniffing alone to be an agonistic response.

However, residents showed a significantly stronger aggressive response towards scent from older sons than from territory owners. This supports our prediction that older sons are perceived to constitute a greater threat than established territory owners, even if territory owners weighed significantly more (on average 3.7 kg) than older sons. The reason for this may be that older sons are more motivated to engage in a physical conflict. The effect of motivation on willingness to fight has been demonstrated in e.g. hermit crabs (*Pagurus longicarpus*), where individuals in lower quality shells were more willing to initiate and escalate fights than those with higher quality shells, as an attempt to overtake and occupy a higher quality shell (Gherardi 2006). The main dispersal period for beavers coincides with the peak in resident beavers' territorial behavior, and dispersers are often injured (personal observations) or killed by conspecifics during this period (Piechocki 1977). This suggests that dispersers do not always retreat without a fight, making it beneficial for residents to be more aggressive towards them. Several other studies have demonstrated that mammals adjust their level of aggression to the threat posed by intruders (Temeles 1994, Müller and Manser 2007), and that they respond more strongly toward scent marks from e.g. strangers than neighbors (Rosell and Bjørkøyli 2002, Palphramand and White 2007), non-relatives than relatives (Sun and Müller-Schwarze 1997, Tegt 2004, Rosell et al. 2008), same subspecies than different subspecies (Rosell and Steifetten 2004), and same species than different species (Rosell 2001).

Residents spent more time sniffing AGS from yearlings than their fathers, but there was no significant difference in the aggressive response. This suggests that yearlings were not considered to pose a greater territorial threat. It's likely that the lack of observable discrimination has a true biological basis. First, male beavers do not reach

sexual maturity until about 21 months (Wilsson 1971), thus the yearlings in this study were not yet sexually mature. The old son scent donors, on the other hand, are sexually suppressed but still sexually mature. It is likely that this difference would be reflected in their AGS profile. In territorial or dominance-based social systems, scent marks should also convey honest information about the signaler's competitive ability (Gosling and Roberts 2001). Yearlings are small compared to older individuals, and will not be able to compete physically with older conspecifics. It is often observed that when asymmetry in the body size of two competitors is very large, the individual with the relatively lower size (the probable loser) will usually retreat without escalating a fight in the first place (Just and Morris 2003). The signaling of competitive ability, whether superior or inferior, is probably beneficial, as it minimizes the potential costs of agonistic encounters, which could be the case with yearlings compared to older subordinates. Yearling beavers will probably also differ in dispersal behavior and motivation compared to older individuals, because of a less pronounced dispersal instinct (Hartman 1997). Wilsson (1971) observed that Eurasian beaver yearlings in captivity only showed some uneasiness in the dispersal season, while 2-year-olds became very restless and tried to leave their enclosures, indicating that 2-year-olds might be more "desperate" to leave their natal site than yearlings. The phenomenon of yearling dispersal is considered to be a result of the availability of vacant habitat and to be an exception rather than the rule (Hartman 1997). This supports the fact that yearlings are not able to compete for resources and therefore do not constitute a serious threat.

Also, the chemical analyses showed that there is a chemical difference due to age and social status. Because it was not the goal of this study to identify the compounds, but merely whether there was a difference between the chemical profiles,

we cannot identify the signal eliciting the aggressive response. One possibility is that dominant male beavers possess a status badge that can be perceived by conspecifics. An alternative explanation is that there is a signal in the AGS of the subordinates that triggers the aggressive response. Beavers are often observed to make exploratory trips outside the natal colony before they disperse successfully (Hartman 1997, Campbell et al. 2005, Herr et al. 2006). Such exploratory behavior can cause highly elevated levels of stress hormones, as shown in e.g. meerkats (*Suricata suricatta*, Young and Monfort 2009). The composition of glandular secretions are controlled by hormones (Ebling 1977), and there are often hormonal correlates with dispersal status (Holekamp and Smale 1998, Young and Montfort 2009). Thus, the stressors of either having to disperse to be able to reproduce, or to remain and be reproductively suppressed by the parents, could be reflected in the AGS of old sons and function as the signal that elicits a stronger territorial response from residents. The two explanations are not mutually exclusive, but further chemical studies should be conducted to provide better insight about the information coded in the AGS.

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Table 1. Mean (\pm SD) age* and weight of Eurasian beaver (*Castor fiber*) territory owners (TO), old sons (OS) and young sons (YS) used as scent donors in the two-sample choice tests.

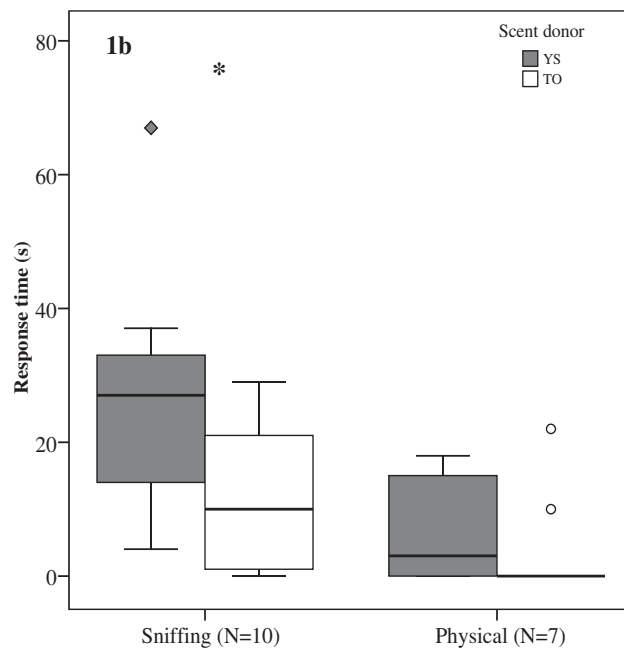
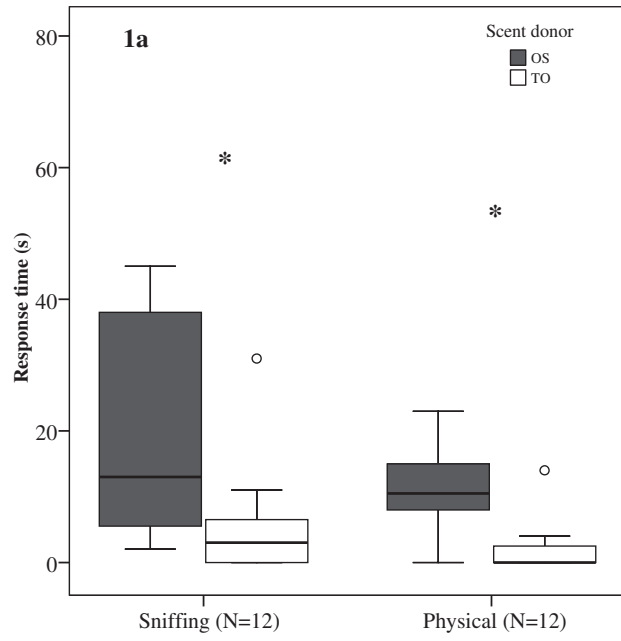
	TO-OS		TO-YS	
	TO (N=12)	OS (N=12)	TO (N=10)	YS (N=10)
Age* (years)	6.5 \pm 2.5 (range 4 - 11)	3.3 \pm 1.8 (range 2-7)	4.2 \pm 1.9 (range 3-9)	1
Weight (kg)	21.3 \pm 2.5 (range 18.0 - 25.0)	17.6 \pm 2.5 (range 14.8 - 22.5)	21.5 \pm 1.6 (range 19.5 - 24.0)	10.3 \pm 1.7 (range 7.5 - 12.2)

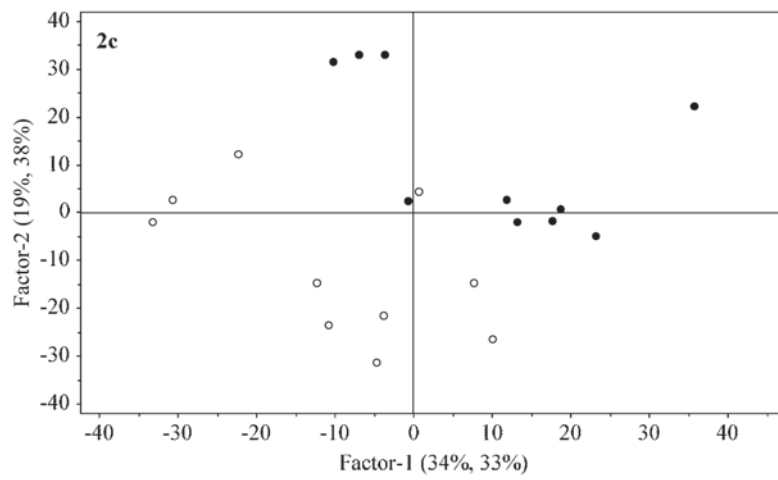
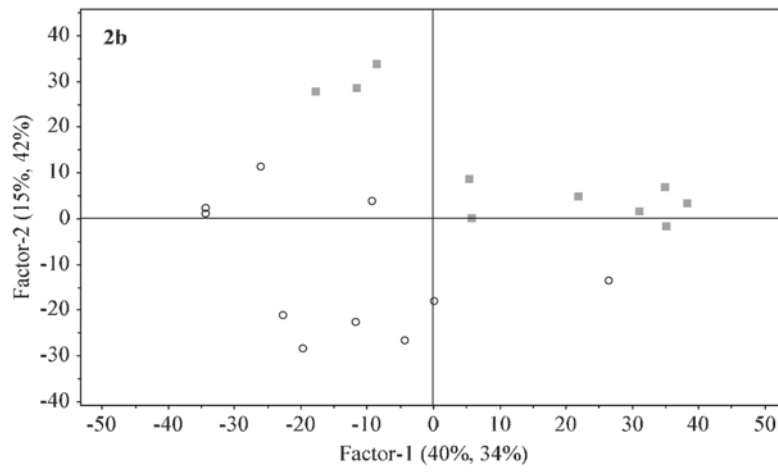
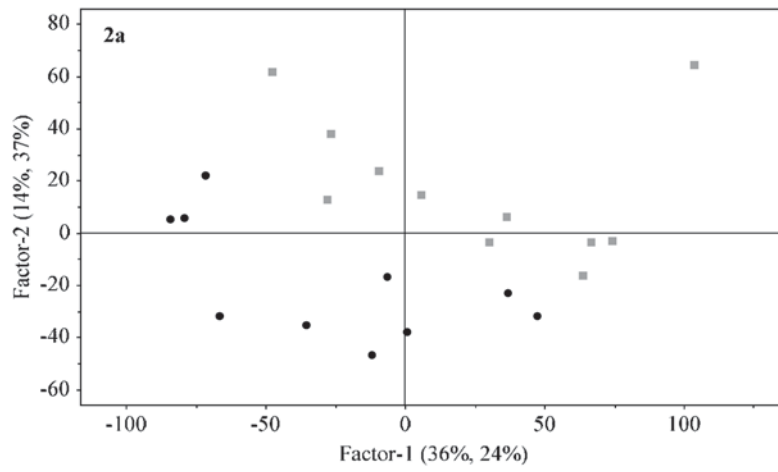
* For TO, age is the minimum age, as age is difficult to determine if beavers are adults the first time they are trapped.

Figure legend

Figure 1. Response time (in seconds) of Eurasian beavers to experimental scent mounds with anal gland secretion from (a) a territory owner (TO) and his old son (OS), and (b) a TO and his young son (YS). The line in the boxplot indicates the median; the lower and upper ends indicates the 25 % and 75 % values, respectively; and the whiskers indicate the distance from the end of the boxplot to the largest and smallest observed values that are less than 1.5 box lengths from either end of the box, \diamond outliers (1.5 – 3 box lengths from the end of the box); \circ extreme values (> 3 box lengths from the end of the box); N = number of responses, * P < 0.05.

Figure 2. Partial least square regression score plots showing the position of each gas chromatogram of Eurasian beaver anal gland secretion between (a) territory owner (TO) - old son (OS), (b) TO - young son (YS) and (c) OS - YS [\blacksquare : TO (N = 17), \bullet : OS (N = 16), \circ : YS (N = 12)] in the first two principal components, Factor 1 and Factor 2.





Paper IV

Do yellow-bellied marmots display a sex-bias in neighbor-stranger discrimination of oral and cheek gland secretions?

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Abstract- When a territory owner, or resident, responds more aggressively toward an intruding stranger than neighbor, it is known as the dear enemy phenomenon, and responses of the resident are influenced by the perceived threat. The social system of yellow-bellied marmots is referred to as polygynous harems with male dominance, because the male maintains a territory where multiple females and her offspring live, and he is dominant to all other group members. Females recruit offspring to build matriline. Male residents should respond cohesively to any intruding female to maximize his reproductive success, whereas female residents should display aggression toward intrusions by strange females because their reproductive success is increased by ensuring their daughters survive and eventually reproduce. We hypothesized that resident adult yellow-bellied marmots would display a sex-bias toward adult female oral and cheek gland secretions in neighbor-stranger discrimination experiments. We predicted no difference in responses of males, but that females would display the dear enemy phenomenon. Additionally, we considered the effect of relatedness of neighbors on female resident responses and predicted residents would be more agonistic to distantly related versus closely related kin. Results indicated that cheek glands were contacted longer than oral glands and that females contacted stranger scents longer than controls. Our prediction that males (n = 6) would respond indifferently toward neighbors and strangers was supported, whereas our prediction of the dear enemy effect in females (n = 12) was not. In addition, we did not detect a difference in duration of contact by female residents when neighbors were closely (n = 8) or distantly (n = 10) related. We conclude that the non-significant responses by females were the result of no perceived threat due to ample resources, including mates.

Key words- dear enemy phenomenon, kin discrimination, neighbor-stranger discrimination, *Marmota flaviventris*, olfactory communication, relatedness, yellow-bellied marmot.

Introduction

Olfactory communication plays an important role in the social behavior of many mammals (Eisenberg and Kleiman 1972; Wyatt 2003; Campbell-Palmer and Rosell 2011) and is also an important form of communication in many rodents, including ground-dwelling sciurids (Halpin 1984, Johnston 2003, Mateo 2006). Conspecific recognition, and more broadly, discrimination, based on olfactory cues are also important elements of social communication and have been demonstrated in several taxa, including amphibians (Waldman 1991), lizards (Mason 1992, Aragón et al. 2001), insects (Newey et al. 2010), fish (Olsén 1992), and mammals (Brown and MacDonald 1985, Halpin 1986, Swaisgood et al. 1999, Beauchamp and Yamazaki 2003). The ability to discriminate between conspecifics based on olfactory cues may be especially advantageous for territorial species because signals do not require the presence of the donor after a scent has been deposited (Eisenberg and Kleiman 1972; Campbell-Palmer and Rosell 2011). Moreover, deposition of scent by territory owners, or residents, likely reduces defense costs because it allows the resident to simultaneously identify itself to conspecifics and announce its presence to would-be intruders and/or neighbors (Bradbury and Vehrencamp 1998).

In the course of maintaining a territory, residents learn to discriminate neighbors from strangers (Ydenberg et al. 1988, Temeles 1994). Neighbor-stranger discrimination (NSD) can be influenced by genetic similarity (e.g., Rostain et al. 2004, Lodé 2008) and/or familiarity (habituation; e.g., Bates et al. 2008, Burgener et al. 2008). When a resident responds less aggressively toward an intruding neighbor than stranger, it is known as the dear enemy phenomenon (DEP; Fisher 1954), whereas more aggression toward an intruding neighbor is the nasty neighbor phenomenon (e.g., Temeles 1990, Müller and Manser 2007). Floaters (stranger intruders) sometimes establish a territory or acquire resources at the expense of a

current resident and can be a large potential threat. Neighbors with an established territory, on the other hand, pose less threat to a resident because their status is known and they have less to gain from a conflict (Parker 1974, Temeles 1994). The DEP has been documented in reptiles (López and Martin 2002), amphibians (Davis 1987), fish (Leiser 2003), ants (Dimarco et al. 2010) and mammals (Vestal and Hellack 1978, Rosell and Bjørkøyli 2002). Elicitors of aggression toward an intruder are influenced by the threat perceived and can be identified when genetic similarities and/or familiarity are controlled for experimentally. In response to these threats, a resident should escalate aggression when it already owns an area ('prior resident advantage'; Parker 1974), if they have a high resource holding potential (RHP), such as higher body mass (Archer 1987, Kokko et al. 2006), or if they occupy a more valuable territory (Krebs 1982, Kemp and Wiklund 2004).

Yellow-bellied marmots (*Marmota flaviventris*) have been referred to as having polygynous harems with male dominance because the male maintains a territory where multiple females and her offspring live, and because the male is dominant to all other group members (Halpin 1985). However, in general, body size is an appropriate predictor for dominance among all marmots (Huang et al. 2011). Additionally, females establish kin groups by recruiting female offspring to build matrilineal groups (Armitage 1991, 2000). In this social system, adult male residents should attempt to mate with as many females as possible to maximize reproductive success (Trivers 1972), and hence should treat females cohesively (Downhower and Armitage 1971), because any female intruder could present a mating opportunity. Adult female residents, however, should display aggression toward intrusions by strange females, because their reproductive success is increased by ensuring their daughters survive and eventually reproduce. And survival of daughters is in fact higher for recruits than dispersers (Armitage 1991). Additionally, access to foraging areas is strongly influenced by kinship; only closely related ($r \geq 0.25$) adult females share foraging patches (Frase and

Armitage 1984). Thus, adult female residents should display nepotism. Another reason female residents should exclude female intruders is because reproductive competition could occur between matrilineal lines in the form of infanticide (Armitage et al 1979, Brody and Melcher 1984).

Oral- and/or cheek-gland marking in the genus *Marmota* has been implicated as being important for defense of territories and burrows (Zimina 1978, Ouellet and Ferron 1988, Meier 1991), to communicate breeding status (Hébert and Prescott 1983), to signal dominance (Armitage 1976, Barash 1989, Hébert and Barrette 1989, Taulman 1990), to recognize individuals (Taulman 1990), and to self-reassure residents (Armitage 1976, Taulman 1990, Meier 1991). Based on choice tests with oral gland secretions, adult female yellow-bellied marmots discriminated between closely related group members and distantly related non-group members (Brady and Armitage 1999; Table 1), and the authors concluded it was supportive of a territoriality hypothesis. Additionally, adult male and female golden marmots (*M. caudata aurea*) discriminated between group members (males and females) and non-group members (males and females; Blumstein and Henderson 1996; Table 1), adult male woodchucks (*M. monax*) discriminated between oral gland secretion of neighbors and strangers (dear enemy effect), whereas adult females showed no difference between neighbors and strangers (Meier 1991; Table 1). Discrimination of unfamiliar versus familiar odors was not supported in male yellow-bellied marmots (oral gland; Olson and Blumstien 2010; Table 1), and alpine marmots (*M. marmota*) discriminated between non-group odors (Bel et al. 1995 [probably oral and cheek], 1999 [cheek]; Table 1) versus controls, but familiar versus unfamiliar odors were not investigated.

The above choice-tests in marmots could be generalized as familiar versus unfamiliar (i.e., group member or shared home range versus non-group member), with the exception of Bel et al. (1995, 1999). Our study is different because we played back odors of neighbors

from a different, but adjacent, social group that did not share home ranges. In other words, “familiar” odors in our study were from non-group members. Additionally, in a social system with philopatric females and recruitment of female offspring, it is expected that some neighbors are related. Hence, we considered the influence of kinship on resident responders.

Interpreting time at stimuli as an indication of an agonistic response by yellow-bellied marmots can be justified based on previous experimental and observational evidence. For example, females spent significantly more time investigating oral secretions of non-group members than group members, which in turn were investigated more than controls (although sex and age of the donors was not indicated; Brady and Armitage 1999). Furthermore, adult females engaged in more amicable interactions with other adult females who were familiar, and especially who were also close kin (Johns and Armitage 1979). Thus, a longer investigation time by females can be interpreted as an agonistic response. In male yellow-bellied marmots, the interpretation is not as straight forward. Adult male and adult female interactions were more agonistic whether they were burrow-mates or not (Johns and Armitage 1979). But interpretation of these specific interactions was heavily influenced by the female’s behavior (eg, avoidance) toward the male, rather than by the male’s behavior toward the female (attempt to copulate). If avoidance behaviors of female burrow-mates were ignored, there were more amicable interactions than agonistic. Either way, males should attract as many females as possible by treating them cohesively.

We investigated neighbor-stranger interactions in adult yellow-bellied marmots with both oral and cheek scents of adult females in separate experiments. We hypothesized there would be a sex-bias in responses by male and female residents toward neighbor and stranger females. It was predicted males would not respond differently toward neighbors and strangers because each presents a potential mating opportunity, whereas females would be more agonistic toward strangers (dear enemy effect), who pose potential threats. Additionally, we

considered the effect of relatedness of neighbors on female resident responses and predicted residents would be more agonistic to distantly related versus closely related kin.

Methods

Study area and sample collection. Yellow-bellied marmots were studied near the Rocky Mountain Biological Laboratory (RMBL; 38° 57' 29" N, 106° 59' 06" W) at 2,896 m elevation in the Upper East River Valley, Gothic, Colorado, USA, and are part of a long-term monitoring project. Marmot colony sites ranged in elevation from 2,867 to 3,008 m (Armitage 2003). Marmots have a patchy distribution in the valley and typically occupy sub-alpine meadows and forest openings. Habitat varies across sites but mainly consists of rolling grassy meadows to steep talus (Blumstein et al. 2006).

We trapped marmots in June and July 2009, from nine different colonies consisting of 2 to 6 social groups per colony. Scents collected from adult females in 10 social groups were used in experiments. Marmots were trapped with Tomahawk live-traps (Tomahawk, WI, USA) baited with Molene horse feed (Purina® Molene 100, Purina Mills, LLC, St. Louis, MO, USA), then transferred to a canvas handling bag for processing and sample collection. The sex of the animal was determined by the distance between the anus and the genitals, and age class (adult or yearling) was determined by weight (Armitage et al. 1976) and/or monitoring data. Marmots were marked with uniquely numbered metal ear tags and a unique mark on their dorsal pelage using Nyanzol fur dye (see Blumstein et al. 2008 for trapping and marking details). The fur around the cheek and oral glands was cleaned with isopropyl alcohol swabs prior to sample collection. Latex gloves were worn while handling all materials to prevent contamination by human scent. Samples were collected on cotton balls (Express Medical Supply, Inc., Fenton, MO, USA) using clean forceps, then placed directly

into 30 mL glass vials with Teflon-lined caps (Lab Safety Supply, Janesville, WI, USA), stored on ice in the field, and frozen at -20 °C within 3 hours.

Behavioral assays. Experiments were run 17 June to 28 July, 2009; after the spring breeding season. We ran two independent (at least 7 days apart), three-choice experiments with oral and cheek scents. The three treatments were: control (no odor stimulus), neighbor (female of an adjacent social group, or harem), and stranger (female from a social group >1.5 km away). Hair samples were taken to verify genetic relatedness which was determined based on 12 previously developed microsatellite loci (details in Blumstein et al. 2010), and the program Kingroup 2.0 (Konovalov et al. 2004) was used to calculate pairwise relatedness between individuals. Genetic similarity matrices were constructed from pairwise relatedness coefficients (higher r value = greater kinship).

Experiments were set up within 2 m of a burrow entrance of a targeted responder (adult male or female). Scents were each attached to 30.5 cm-long smooth shank spike nails (inserted 7.5 cm in the ground) with garden twine and arranged in an equilateral triangle, 50 cm apart. Nails were cleaned with isopropyl alcohol before each experiment. Latex gloves were worn during set-up to prevent cross-contamination or contamination by human scent. Treatments were randomly assigned to a nail beforehand, and observers were blind to treatments. We placed a small pile (50 g) of horse feed in the center of the triangle to encourage marmots to approach the scents. A video camera (Sony[®] digital video handycam, model no. DCR.SR35E, Komplett.no, Sandefjord, Norway) set up on a tripod nearby recorded approaches and responses. Cameras were placed at least 30 m away from scents, or camouflaged in nearby foliage to avoid distraction.

An observer watched the experiment through a spotting scope at a distance of 30-70 m, depending on terrain and habituation of marmots to humans. If a non-targeted marmot

approached the array, the observer would interrupt the marmot by rushing toward the setup until the marmot left. For each marmot's response, we recorded 1) time in seconds contacting the treatment (when a marmot's nose was within 5 cm of a treatment, and included sniffing, bites, and rubs), and 2) frequency of scent marking the treatment (bite or rub). Experiments were considered over after the targeted individual approached at least once, investigated at least one scent, and did not return after approximately 20 min.

Statistical analysis. We used a linear mixed model (LMM; alpha was set to 0.05; e.g., Twisk 2006) to analyze time in contact with each treatment by responders. Our model included fixed effects of the responder's sex, gland type (oral or cheek), relation (neighbor, stranger, or control), gland*relation, and sex*relation; the responder's ID was fitted as a random effect. We conducted planned pairwise comparisons on the estimated marginal means. Additionally, female responses to neighbors alone who were close kin ($r > 0.25$; $n = 8$) and distantly related kin ($r < 0.25$; $n = 10$) were compared (Mann-Whitney U test; two-tailed; Mann and Whitney 1947). We used a non-parametric test (Mann-Whitney U) to analyze frequencies of scent marking by males and females. All analyses were done using SPSS software version 18 (PASW Statistics, IBM, Chicago, IL, USA).

Results

Responses from 18 different adults were recorded; 12 females and 6 males. Nine of these individuals (6 females, 3 males) responded to both gland types for a total of 27 responses. The coefficient of relationship was < 0.094 for all strangers and responders, and ranged from 0.045 to 0.684 for neighbors and responders (Table 2).

Overall, there was no difference in responses by male and female responders ($F(1, 12.475) = 2.513, p = 0.138$), in responses toward treatments ($F(2, 53.191) = 1.216, p = 0.305$), or the interactions for gland*relation ($F(2, 53.191) = 3.100, p = 0.053$), and sex*relation ($F(2, 53.191) = 0.685, p = 0.509$). However, there was an overall effect for gland type ($F(1, 71.142) = 5.114, p = 0.027$), in which cheek glands were contacted significantly longer than oral glands ($b = 4.520, t(61.425) = 2.703, p = 0.009$; $X_{\text{oral}} = 3.077 \text{ sec} \pm 0.552 \text{ SE}$, $X_{\text{cheek}} = 5.619 \text{ sec} \pm 0.909 \text{ SE}$). Additionally, control scents in cheek gland experiments were contacted significantly less than stranger scents ($b = -5.446, t(53.191) = -2.371, p = 0.021$; Fig. 1a). Planned comparisons of sex*relation indicated that females responded significantly longer to stranger scents than to controls ($p = 0.023$; Fig. 1b), but responses to strangers did not differ from neighbors ($p = 0.240$; Fig. 1b), nor did responses differ between neighbors and controls ($p = 0.256$; Fig. 1b). And, as predicted, male responses did not significantly differ among any stimuli (C vs N: $p = 0.906$, C vs S: $p = 0.812$, N vs S: $p = 0.722$; Fig. 1b). Additionally, female residents did not respond differently toward neighbors relative to relatedness ($U = 30.000, p = 0.396$). Finally, the frequency of scent-marking (rub or bite) by marmots was very low (Table 3), thus we did not carry out a statistical test.

Discussion

We hypothesized that female marmots would display the dear enemy effect, whereas males would be indifferent. The prediction that females would respond more agonistically toward strangers than neighbors was not supported, but the prediction that males would respond indifferently was supported. However, male responses did not significantly differ either toward unfamiliar versus familiar male oral scents in a previous experiment (Olson and Blumstein 2010). In this social system, dominant males should defend their harems from

competitors. In another polygynous marmot, the woodchuck, males spent more time at strangers than neighbors, regardless of the sex of the scent donor (Meier 1991). Female woodchucks, however, did not respond differently to neighbors or strangers (regardless of sex), which was consistent with our results in females (even though we used only female scents). NSD has also been investigated in smaller rodents. In arena tests with the polygynous root vole (*Microtus oeconomus*), resident males or females were physically paired with a similar-sex neighbor or stranger, and males displayed the dear enemy phenomenon but females did not exhibit NSD, and thus a prior resident advantage (Rosell et al. 2008). The authors concluded that the absence of NSD by females could have been because female root voles are less territorial than males (see Andreassen et al. 1998), or because they may be more sensitive to intrusion by unfamiliar male root voles than by unfamiliar females. Both explanations are also plausible for the absence of NSD observed in female yellow-bellied marmots.

In a previous discrimination study (Brady and Armitage 1999), where group members were close kin to the responder, female yellow-bellied marmots responded longer to non-group (i.e., unrelated) individuals. However, we found that female responses toward neighbors alone did not differ whether they were closely related or not. Our failure to detect a difference in female responses could be because neighbors were also perceived as strangers. Despite some close relatedness of neighbors, they were from a different social group/harem and thus a social bond did not exist. Support for the importance of a social bond was given by Johns and Armitage (1979), where degree of association between adult females resulted in the following: Females were more amicable than agonistic toward burrow-mates, more agonistic than amicable toward non-burrow-mates but of the same social group/harem, and more agonistic than amicable toward females from different harems. Close and distant relatedness of neighboring marmots is expected in this type of social system, and our results suggested

that female residents may treat any female outside her social group/harem as a stranger, regardless of relatedness.

Infrequent scent marking of treatments by residents did not provide support for any of the implicated functions of oral and cheek glands, such as defense of territories and burrows (Zimina 1978, Ouellet and Ferron 1988, Meier 1991). However, Brady and Armitage (1999) suggested the stronger response by adult females toward non-group members was supportive of a territoriality hypothesis. In our experiments then, where neighbors were likely also perceived as strangers, similar response times may support a territoriality hypothesis. Yet, the absence of scent marking we observed does not support this. An alternative conclusion is that females responded equally passive toward strangers, rather than equally agonistic toward neighbors. In a recent study of the causes and consequences of dominance in harem-polygynous systems, Huang et al. (in press) found no effects of dominance rank on female yellow-bellied marmot reproductive success, and that dominance was unlikely to drive the reproductive suppression in the study population. Dominance, then, is probably less important among females because they do not compete for mates in this social system and are not subject to intense sexual selection (Armitage 1991). Furthermore, Wey and Blumstein (2010) documented affiliative networks formed between colonies (not social groups) related as little as 0.100, and that agonistic networks were formed between colonies related as much as 0.346. The agonistic networks pattern was in the opposite direction than they predicted, with more closely related kin being more likely to interact agonistically. They concluded that agonistic and amicable interactions are both important for building social bonds, so agonistic responses should not be interpreted as always “bad”.

Resident responses in NSD are influenced by perceived threat. Our non-significant results of male responders toward neighbors and strangers was expected, but non-significant responses by females suggested either a lack of perceived threat from each stimuli, or a

perceived threat from each. Infrequent scent marks by residents suggested the former. Temeles (1994) suggested that the DEP is not a permanent feature of all territory holders from the same species, but may vary depending upon conditions. Dominance in social groups is influenced by competition for resources, such as food and mates (Witting and Boesch 2003), but females in harem polygynous groups don't compete for mates, so a response to an intrusion by a stranger may not be necessarily perceived as a threat. Thus, in our experiments female responders likely did not discriminate between neighbors and strangers because there was no apparent shortage of resources and because competition for a mate is not necessary.

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Table 1. Social organization of four marmot species and a summary of choice-test experiments using oral or cheek gland secretions. Experiments investigated neighbor-stranger, familiar-unfamiliar, and/or close kin-distant kin discrimination by marmot resident responders.

Species, Social system & (study reference)	Gland Type (donor age)	Age & Sex of responders ^a (n)	Test & Stimuli ^b	Results by Responder: ^c	Author Definitions
woodchucks <i>Marmota monax</i> solitary; polygynous (Meier 1991)	oral (adult)	adult F (4) adult M (4)	3-choice: stranger F + neighbor F + C stranger M + neighbor M + C	M: stranger > neighbor M: M = F F: stranger = neighbor F: M = F	neighbor: occupy burrows immediately adjacent to or within the same home range as responder; stranger: from site >1 km away
golden marmots <i>M. caudata aurea</i> extended fam; monogamous (Blumstein & Henderson 1996)	cheek (adult)	adult F (6) adult M (7)	2-choice: non-group F + C; group F + C non-group M + C group M + C	M: non-group M > group M M: non-group F > group F F: non-group F > non-group M F: non-group F > group M	group member: same social group; non-group member: different social group
yellow-bellied marmots <i>M. flaviventris</i> female kin grp; harem polygynous (Brady & Armitage 1999)	oral (not specified)	adult F (7)	3-choice: group + non-group + C	F: non-group > group F: group > C	group member: same matriline; non-group member: of a distant or unrelated matriline (from a distant location)
(Olson & Blumstein 2011)	oral (adult)	adult M (23)	4-choice: familiar M + unfamiliar M + C + sheep C	all non-significant	familiar: from same social group; unfamiliar: from group >2.5 km away; sheep C: rubbed on domestic sheep
alpine marmot <i>M. marmota</i> extended fam; monogamous (Bel et al. 1995)	probably oral & cheek (not specified)	probably adults & juveniles	2-choice: non-group + C	non-group > C	non-group: another group
(Bel et al. 1999)	cheek (not specified)	not specified	2-choice: non-group + C	non-group > C	non-group: another family unit
(Bel et al. 1999)	cheek (not specified)	age unk F (6) age unk M (5)	2-choice: M + C; F + C	F: M = F M: M = F	

^aMale (M) or female (F) responders.

^bScents presented in choice trials consisting of male (M), female (F), or control (C) stimuli.

^c> read as, "was responded to significantly greater than"; = indicates no significant difference.

Table 2. Genetic relatedness (r) of adult female neighbor and stranger yellow-bellied marmots to adult responders used in oral and cheek olfactory experiments. Experiments investigated a sex-bias in neighbor-stranger discrimination and were carried out in June - July 2009.

Responder	Neighbor r^a	Stranger r
4655-4665	0.222	-0.103
4058-4720	0.275	-0.219
5285-5088	0.314	0.027
5236-5230	-0.043	0.063
5207-5212	-0.217	-0.012
5501-5503	0.069	-0.189
4901-5896	0.082	-0.022
4237-4329	0.262	0.094
4715-4716	0.572	-0.001
4948-4959	-0.173	0.006
4229-4217	-0.253	0.018
5395-5398	0.052	0.032
5472-5452	0.065	0.015
4830-4029	0.524	-0.025
4996-4107	0.140	0.022
5172-5850	0.045	-0.200
4447-4444	0.341	-0.118
4026-4050	0.684	-0.135

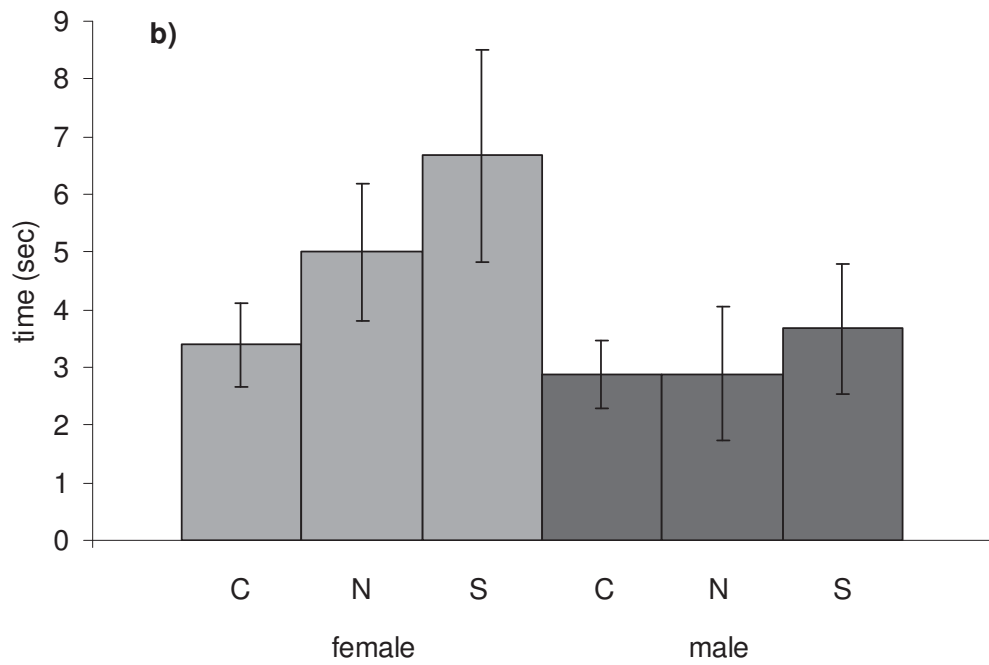
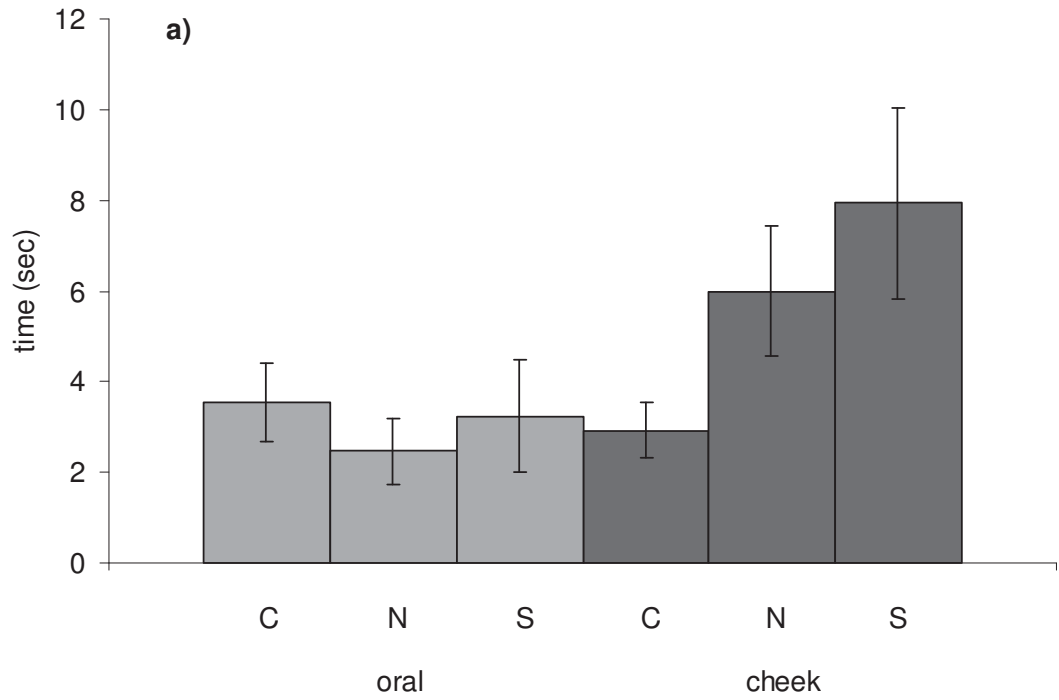
^aHigher r values indicate greater kinship.

Table 3. Frequency of scent marks by adult yellow-bellied marmots toward adult female oral (9 females, 4 males) and cheek (9 females, 5 males) gland secretions in olfactory experiments. Experiments investigated a sex-bias in neighbor-stranger discrimination and were carried out in June - July 2009.

Experiment	Rub or Bite Frequency	
	Female	Male
Oral		
Stranger	0	2
Neighbor	0	0
Control	1	1
Cheek		
Stranger	1	1
Neighbor	1	1
Control	0	0

Figure legend

Figure 1. Planned pairwise comparisons of the estimated marginal means of a linear mixed model for mean time in seconds (\pm SE) that adult yellow-bellied marmots (n = 18 females, n = 9 males) spent at neighbor (N), stranger (S), and control (C) stimuli by a) gland type, and by b) sex. Field experiments were carried out in June and July 2009.



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