

PUTATIVE PHEROMONES IN URINE OF RUTTING MALE MOOSE (*Alces alces*): EVOLUTION OF HONEST ADVERTISEMENT?

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Abstract—We tested hypotheses about how olfactory communication was related to mating behavior in Alaskan moose (*Alces alces gigas*). Males dig rutting pits where they deposit odiferous urine; females are strongly attracted to and often wallow in those pits. Moreover, mating and parturition are highly synchronized in moose. Consequently, male urine may play an important role in the mating system and in synchronizing reproduction in moose. Urine samples were collected from captive moose on the Kenai Peninsula, Alaska. Samples included those from the mating season and from the nonrutting period for two adult males, one yearling male, and one male and one female less than 1 year old. After pH adjustment, samples were extracted with methylene chloride to yield three fractions (acidic, neutral, and basic), which were analyzed by gas chromatography–mass spectrometry. Potential pheromones included unsaturated alcohols and homologs of tetrahydro-6-methyl pyranone and 2-nonen-4-one. We hypothesize that these compounds are related to hypophagia and catabolism of body reserves by rutting males, and thereby provide an honest advertisement of body condition by male moose during the mating season.

Key Words—*Alces alces gigas*, Alaskan moose, pheromones, urine, mating behavior, honest advertisement.

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INTRODUCTION

Olfactory communication plays a major role in the behavior and ecology of mammals (Ewer, 1968). Pheromones and other chemical signals are important in intra- and interspecific identification (Passanisi and MacDonald, 1990), and territorial marking (Gilbert, 1973; Kitchen, 1974; Gosling, 1987; Johanson et al., 1996; Massei and Bowyer, 1999), although many mammals that scent mark are not territorial (Ralls, 1971). Thus, the function of scent marking goes well beyond the delineation of territorial boundaries (Leuthold, 1977; Bowyer et al., 1998a). For instance, chemical signals are thought to play a fundamental role in alarm systems (Müller-Schwarze, 1969; Booth and Signoret, 1992), predator-prey relationships (Mattina et al., 1991; Young, 1993), maternal-infant bonds (Poindron et al., 1988; Poran et al., 1993a,b), social structure and organization (Passanisi and MacDonald, 1990), and sexual behavior (Alteri and Müller-Schwarze, 1980; Rasmussen et al., 1986, 1997; Rasmussen, 1988; Menzies et al., 1992). Ungulates exhibit a diverse array of scent-marking behaviors (Coblentz, 1976; Leuthold, 1977; Benner and Bowyer, 1988; Gosling, 1987; Bowyer et al., 1994; Oehler et al., 1995). Moreover, much evidence links olfaction and reproduction among ungulates (Watson and Radford, 1960; Fraser, 1968; Coblentz, 1976; Bakke and Figenschou, 1990; Miquelle, 1991; Booth and Signoret, 1992). Pheromones of domestic pigs and sheep have been identified and related to specific behavioral and physiological changes associated with reproduction (Booth and Signoret, 1992). Indeed, the role of chemical communication in the reproductive behavior of ungulates, especially the behaviors associated with scent marking by males, has been studied for many species (Coblentz, 1976).

During the mating season (rut), male urine provides an important chemical cue thought to relay information to conspecifics (McCullough, 1969; Altieri and Müller-Schwarze, 1980; Izard and Vandenbergh, 1982; Bakke and Figenschou, 1990; Miquelle, 1991). Scent urination by ungulates may serve at least two functions: mediating aggressive interactions between males and facilitating male-female interactions. Scent urination directed at other males in species such as Thomson's gazelles (*Gazella thomsoni*) (Estes, 1967), blackbuck antelope (*Antilope cervicapra*) (Schaller, 1967), and springbok (*Antidorcas marsupialis*) (David, 1973) may be used for territorial marking. Scent urination in bontebok (*Damaliscus dorcas dorcas*) (David, 1973) and wildebeest (*Connochaetes taurinus*) (Estes, 1969) is directed at other males as a part of a ritualized challenge. McCullough (1969) and Bowyer and Kitchen (1987) noted that scent urination in North American elk (*Cervus elaphus*) occurred primarily in dominance interactions between adult males and hypothesized that pheromones contained in urine might advertise the physical condition of males. Such an olfactory cue, however, also might be involved in male-female interactions (Coblentz, 1976; Bowyer and Kitchen, 1987). Scent urination by males directed at females in species such as

cattle (Izard and Vandenberg, 1982), sheep (Watson and Radford, 1960), feral goats (Coblentz, 1976), and pigs (Polikarpova, 1945) may serve to accelerate the onset of puberty or synchronize and actuate estrus and ovulation.

In cervids, scent urination during the mating season has been well documented. Male reindeer (*Rangifer tarandus*) scent mark by urinating on their hind feet and tramping the ground (Espmark, 1964; Mossing and Damber, 1981; Kojola, 1991). Female reindeer exhibit an interest in the male urine deposited by rubbing against the ground, a behavior that resembles the males rubbing against urine patches (Espmark, 1964). Mule deer (*Odocoileus hemionus*) also rub urinate (Bowyer, 1986), as do white-tailed deer (*Odocoileus virginianus*) (Marchinton and Hirth, 1984). Caribou (*Rangifer tarandus*) likewise scent urinate (Lent, 1965). Some cervids such as elk, moose (*Alces alces*), and fallow deer (*Dama dama*) dig rutting pits or wallows in which urine is deposited (McCullough, 1969; Bowyer and Kitchen, 1987; Miquelle, 1991; Massei and Bowyer, 1999).

Detailed descriptions of rutting pits and associated behaviors of moose have been provided previously (Miquelle, 1991; Van Ballenberghe and Miquelle, 1993). During the mating season, adult male moose paw a shallow depression (pit) in the ground, and urinate in the pit. Urine excreted during rut has a strong, pungent, and unique odor. These cervids impregnate their pelage with the scent by splashing urine and mud onto themselves using their front hooves, slapping urine with the underside of their antlers, and lying (wallowing) in the pit (Miquelle, 1991). Females also wallow in these pits and impregnate their pelage with urine deposited by adult males (Miquelle and Van Ballenberghe, 1985). Male moose attract females to their pits by scent marking with their urine or later in the rut by scent marking (rubbing) trees (Bowyer et al., 1994).

Scent urination in adult male moose is likely directed more towards females than males because that behavior is not temporally correlated with aggressive interactions between males, and female moose are strongly attracted to the urine of rutting males (Miquelle, 1991). Unlike many other cervids, female moose impregnate their pelage by wallowing in freshly made pits. Furthermore, females exhibit a high level of antagonism towards other females while attempting to obtain access to pits (Miquelle and Van Ballenberghe, 1985; Miquelle, 1991). Not only are female moose interested in the urine deposited in rutting pits, but so are subadult males that do not scent urinate, but presumably attempt to obtain the odor of dominants and gain the benefits of pits by wallowing in them (Miquelle and Van Ballenberghe, 1985; Miquelle, 1991). Those behaviors indicate that the urine of male moose may be a critical component in the reproductive biology of females.

Additional evidence exists that primer pheromones occur in the urine of ungulates (Watson and Radford, 1960; Verme and Ozoga, 1987). Synchronization of estrus and subsequent parturition would be of particular importance in ungulates because late-born young may experience higher rates of mortality than

those born earlier (Clutton-Brock et al., 1984; Fairbanks, 1993; Keech et al., 2000). Additionally, timing parturition to coincide with the resources necessary to meet the requirements of mother and young may be critical for successful reproduction (Bowyer, 1991; Rachlow and Bowyer, 1994; Bowyer et al., 1998b; Keech et al., 2000). Mating and parturition in moose are extremely synchronized (Schwartz and Hundertmark, 1993; Van Ballenberghe and Miquelle, 1993; Bowyer et al., 1998b).

Urinary constituents of some ungulates have been characterized. In red deer (*Cervus elaphus*), urine consisted mainly of carboxylic acids and their derivatives and some aromatic compounds (Bakke and Figenschou, 1990). Volatiles identified in the urine of white-tailed deer belong to the alcohol, aldehyde, furan, ketone, nitrile, alkene, alkane, thiol ester, disulfide, aromatic, ether, ketal, and amine classes of compounds (Miller et al., 1998). No studies have characterized urinary compounds and putative pheromones of moose.

We tested the hypothesis that there were differences in the chemical composition of urine from adult male moose during rut compared with the same nonrutting adult males, a subadult (yearling) male, and two younger individuals, and we characterized those substances. These data are a necessary first step in understanding the potential role of pheromones in the reproductive biology of moose.

METHODS AND MATERIALS

Urine samples (≥ 200 ml) were collected from captive moose at the Kenai Moose Research Center, a facility operated by the Alaska Department of Fish and Game, on the Kenai Peninsula, Alaska, USA (60°N, 150°W). Urine was collected by holding a 1-liter bottle affixed on a 1.8 m-pole while an animal urinated. Personnel of the Alaska Department of Fish and Game collected urine from five animals in August and September 1991. Five samples consisted of urine from the rut (September) and from the nonrutting period (August) for the same two adult males (≥ 8 years old), one yearling male (subadult), and one male and one female young both less than 1 year old. Samples were kept frozen at -20°C until analysis.

Frozen samples were thawed in a coldroom at 4°C . The pH of each sample was determined before adjustment of pH and extraction. We obtained three fractions based on pH (acidic pH 1, neutral pH 7, and basic pH 12). With the protocol of Menzies et al. (1992), we obtained organic compounds by eight successive extractions with equal volumes of methylene chloride (CH_2Cl_2). We did not, however, perform extractions with NaHCO_3 . The solvent was removed under reduced pressure by rotoevaporation at 40°C . The resultant extracts were weighed and diluted with chloroform (CHCl_3 100 $\mu\text{g}/\mu\text{l}$).

To assure that urine from an adult male moose contained substances that

TABLE 1. THIN-LAYER CHROMATOGRAPHY SOLVENT SYSTEMS USED TO ANALYZE EXTRACTED MOOSE URINE^a

Fraction	Solvent 1	Solvent 2
Acidic	CHCl ₃ -MeOH-H ₂ O (50:20:1)	CHCl ₃ -MeOH-H ₂ O (90:20:1)
Neutral	CHCl ₃ -MeOH-H ₂ O (90:20:1)	CHCl ₃
Basic	CHCl ₃ -MeOH-H ₂ O (40:10:1)	

^aPlates first were developed half way with a relatively strong eluting solvent (solvent 1), dried, and then developed completely with a weaker eluting solvent (solvent 2).

were of interest to an adult female, we performed a methylene chloride extraction on male urine collected during rut. We then offered the methylene chloride extract from male urine, the aqueous portion from that extraction, methylene chloride alone, and distilled water on small sponges to a female near the end of rut. The female responded strongly only to the sponge with the methylene chloride extract. Her behavior was similar to that observed when we offered urine from the adult male without performing the methylene chloride extraction. Social behavior of Alaskan moose has been well documented (Miquelle and Van Ballenberghe, 1985; Miquelle, 1990, 1991; Schwartz et al., 1990; Van Ballenberghe and Miquelle, 1993; Molvar and Bowyer, 1994), which facilitated our interpretation of those behaviors.

For preliminary analysis, we fractionated samples of urine by thin-layer chromatography (TLC) on silica gel plates by using a two-solvent system gradient. Plates first were developed half way with a relatively strong eluting solvent, dried, and then developed completely with a weaker eluting solvent. The basic fractions, however, were developed only with a single-solvent system (Table 1).

We identified volatiles and semivolatiles on a Hewlett Packard (HP) 5890 Series II gas chromatograph interfaced with a mass selective detector (HP 5972). We injected 1 μl of extract onto a HP 5% phenyl methyl siloxane capillary column (0.25 μm × 30 m) with a flow rate of 1 ml/min, and an injection temperature of 275°C. The initial oven temperature was maintained at 50°C for 3 min and then increased to 300°C at 4°C/min. Initial peak identification was made through the use of a library of standard mass spectra (Wiley 138), and subsequent identification was accomplished by matching retention times and mass spectra with authentic samples.

Volatiles and semivolatiles were quantified on a Hewlett Packard (HP) 6890 Series II gas chromatograph equipped with a flame-ionization detector. Samples of 1 μl were injected onto a HP 5% phenyl methyl siloxane capillary column (0.25 μm × 30 m) with a flow rate of 2.5 ml/min, and an injection temperature of 275°C. The initial oven temperature was maintained at 50°C for 3 min and then increased to 275°C at 5°C/min.

RESULTS

Urine collected in August (nonrut) from adult males lacked the characteristically pungent and musky odor, which was obvious to the human nose, of urine from rut. These qualitative differences in the urine of rutting and nonrutting adult moose were clearly established by TLC on silica gel. With the isolation techniques described here, we identified a total of 11 TLC bands unique to rutting males: 4 from the acidic fraction; 4 from the neutral fraction; and 3 from the basic fraction.

Five compounds from the urine of moose were identified from GC-MS analysis (Figures 1–3). Dimethyl sulfone was common to all samples except those from rutting males. Butyrolactone was present only in the samples from adult males. *p*-Cresol (i.e., 4-methyl phenol) occurred in the urine of all males. Although qualitative differences existed between the amount in rutting versus nonrutting urine, the amount of *p*-cresol in rutting samples was 5.5 times greater than for nonrutting males (Figure 1). Benzoic acid was identified in samples of all males except for adult rutting males. Lupanine, which was tentatively identified with mass spectral analysis, and sparteine only occurred in the sample from the young female.

Two of seven chromatographic peaks unique to rutting males have mass spectra indicating they are likely homologs of tetrahydro-6-methyl pyranone, and 2-nonen-4-one. Two other peaks have mass spectra similar to unsaturated alcohols.

DISCUSSION

Olfactory communication and associated scent-marking activities play a major role in the behavior and ecology of many species of mammals (Vandenberg, 1983; Bowyer et al., 1998a). During the mating season, scent marking with urine by male cervids is an important chemical cue imparting information to conspecifics (Bowyer and Kitchen, 1987; Miquelle, 1991). Specifically, adult male moose dig rutting pits in which they urinate, and females respond to the urine deposited in those pits in a characteristic manner (Miquelle, 1991; Van Ballenberghe and Miquelle, 1993). Because of the association and chronological order of events of moose courtship and copulation behavior (Van Ballenberghe and Miquelle, 1993), urine of rutting males likely is directed toward females and functions as a primer pheromone (Miquelle, 1991).

Four of the compounds identified in the chromatograph of moose urine are common constituents in the urine of many species. These compounds include butyrolactone, dimethyl sulfone, *p*-cresol, and benzoic acid (Figure 1). Butyrolactone occurred only in the urine of adult males. This compound also was

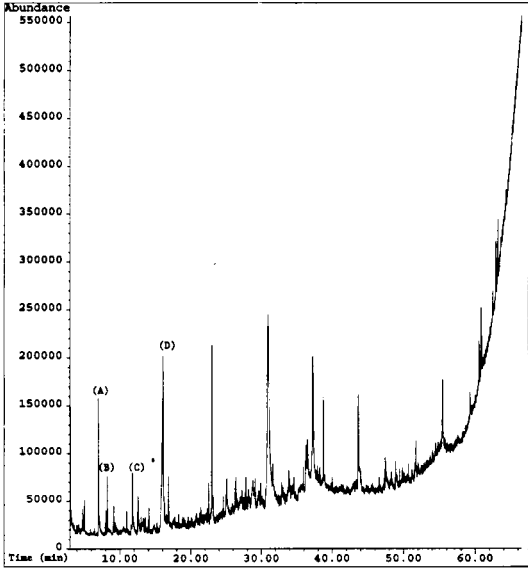
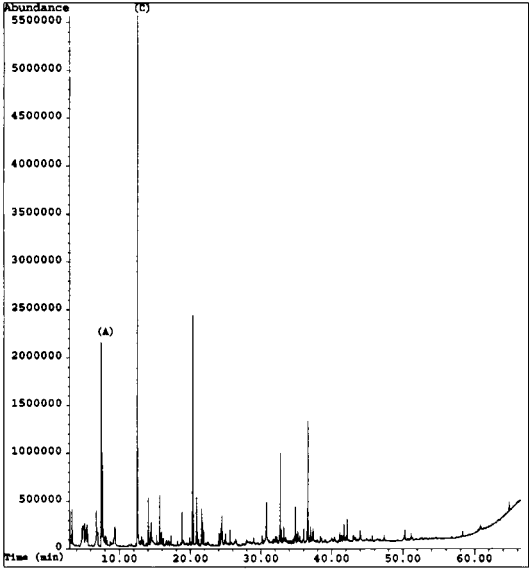


FIG. 1. GC-MS chromatograph of the acidic fraction of urine from an adult male moose during rut (above) and nonrut (below). Letters identify chromatographic peaks for butyrolactone (A), dimethyl sulfone (B), *p*-cresol (C), and benzoic acid (D).

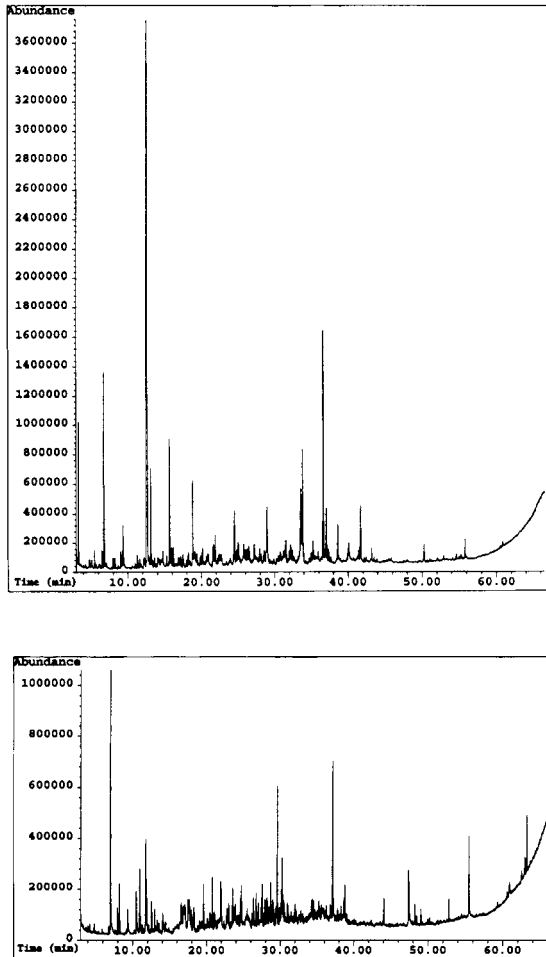


FIG. 2. GC-MS chromatograph of the neutral fraction of urine from an adult male moose during rut (above), and nonrut (below).

isolated from the sternal gland of the cockroach (*Nauphoeta cinerea*), but has not been identified as a pheromone (Sirugue et al., 1992).

Sparteine and lupanine occurred only in the urine of the young female. Those naturally occurring quinolizidine alkaloids are present in leguminous plants including the genus *Lupinus*. Although the moose in our study were given a commercial pelleted diet, there was an opportunity for them to feed on indigenous vegetation, including species of *Lupinus*. Sparteine appears to be unique

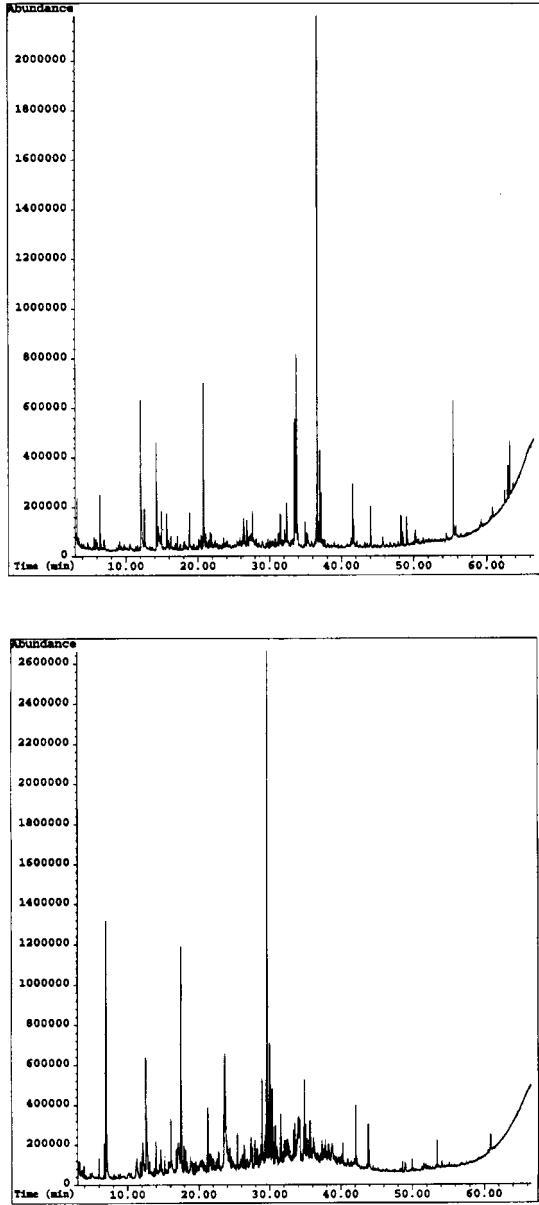


FIG. 3. GC-MS chromatograph of the basic fraction of urine from an adult male moose during rut (above), and nonrut (below).

to the urine of young female moose, but also has been reported in both genders of humans (Pfandl et al., 1992).

Two of seven chromatographic peaks unique to rutting males were tentatively identified by mass spectral analysis and were most likely homologs of tetrahydro-6-methyl pyranone and the ketone 2-nonen-4-one. Ketones, which are likely related to catabolism of body reserves, have been identified from the urine of rutting male white-tailed deer (Miller et al., 1998) and the Asian elephant (*Elephas maximus*) during musth (Rasmussen and Perrin, 1999). Two further peaks were characterized as unsaturated alcohols. That we identified these compounds only from rutting males may indicate that they are involved in pheromonal activity.

Ungulates excrete large quantities of aromatic metabolites in their urine primarily because of the ingestion of cellulose plant materials. These metabolites are the excretory products of dietary phenolic acids, alicyclic acids, and aromatic amino acids that are first fermented by rumen microbes and then further metabolized in body tissues after absorption (Martin, 1973, 1982). Martin (1970) noted that the quantity of various aromatic acid fractions excreted is directly proportional to the amount of food consumed. Typically, dominant male moose cease feeding (i.e., become hypophagic) and rely on the catabolism of endogenous reserves during rut to meet their metabolic needs (Miquelle, 1990). This hypophagic behavior may help explain why the chemical composition of urine from adult male moose during rut was markedly different when compared with nonrutting adults (Figures 1–3), a yearling male, male young, or female young. Indeed, studies investigating the role of malnutrition on the excretion of urinary compounds reported that the concentration of benzoic acid in urine was correlated positively with the amount of food eaten (Martin, 1969, 1970, 1973; Silanikove and Brosh, 1989). We isolated benzoic acid only from the urine of nonrutting males, although the concentration of that compound in urine from rut may have dropped below detection levels because of our analytical procedures. The absence of benzoic acid in rutting males, however, is consistent with their undergoing hypophagia during rut (Miquelle, 1990).

p-Cresol was identified from the urine of males, and a substantial difference occurred in the quantity of *p*-cresol in rutting versus nonrutting males (Figure 1). *p*-Cresol is an end product of anaerobic microbial degradation of tyrosine (Bakke, 1968; Bone et al., 1976; Spoelstra, 1978). Therefore, the quantitative differences of *p*-cresol also may result from the breakdown of endogenous reserves during rut when moose are hypophagic for about 18 days (Miquelle, 1990).

p-Cresol has been identified as a component of the pheromone in two species of hard tick (*Rhipicephalus appendicalatus* and *R. pulchellus*) (Wood et al., 1975) and cabbage looper (*Trichoplusia ni*) (Heath et al., 1992). Insect pheromones also may function as pheromones in mammals; Rasmussen et al. (1997) demonstrated that a lepidopteran-like pheromone occurred in the urine

of female Asian elephants, and that compound was identified as a female-to-male sex pheromone. Perhaps *p*-cresol also serves as a pheromone in moose.

Urinary compounds that we identified as unique to rutting male moose had molecular weights of <300 and had fewer than 20 carbon atoms. Airborne pheromones usually contain 5–20 carbon atoms and must be volatile to reach the receiver; pheromones typically have molecular weights near 300 (Bradbury and Vehrencamp, 1998). In white-tailed deer, several volatile compounds occur exclusively in the urine of dominant males during the mating season that had molecular weights <300 (Miller et al., 1998). Similarly, in red deer, volatile compounds possess molecular weight <300 (Bakke and Figenschou, 1990). Female Asian elephants release a female-to-male urinary pheromone with 13 carbons and a molecular weight near 300 (Rasmussen et al., 1997). The compounds we identified from rutting male moose have physical properties necessary for consideration as putative pheromones.

Bacterial interaction with exocrine secretions is responsible for the pungent axillary odor in humans (Leyden et al., 1981). Additionally, Stern and McClintock (1998) noted the importance for humans of female axillary secretions (pheromones) and their associated bacterial fauna (Leyden et al., 1981) in their possible role in synchronization of menstrual cycles. In moose, however, bacterial interaction is not likely important in the production of pungent-smelling urine during rut. The freshly collected urine of rutting males was already odoriferous, and a female was attracted to the smell of the freshly frozen and thawed urine from an adult male (personal observation). Additionally, females respond immediately to males urinating in rutting pits under natural conditions (Miquelle, 1991); there would not likely be sufficient time for bacterial interactions in that process.

Digging of rutting pits and deposition of pheromones in the pit should be considered a secondary sexual character related to mating (Möller, 1996). Expression of such sexual characters often shows evidence of being dependent on body condition (Andersson, 1986). For example, cervids usually grow larger antlers as a direct response to individual age and physical condition (Van Ballenberghe, 1982; Goss, 1983; Stewart et al., 2000). Such high-quality individuals with well-developed sexual characteristics can afford to invest relatively more in cost-reducing traits than can low-quality individuals (Zahavi, 1975; Zahavi and Zahavi, 1997).

Rutting activities not associated with scent marking are costly for adult male moose (Van Ballenberghe and Miquelle, 1993). A reduction in feeding, vigorous fights between males, and a concomitant loss of weight by rutting males occurs in many polygynous cervids (Taber and Dasmann, 1958; McCullough, 1969; Bowyer, 1981; Miquelle, 1990). During rut, adult male moose can lose from 12 to 19% of their prerut body mass (Franzmann et al., 1978; Schwartz et al., 1987). Additionally, rut occurs at the time of year when Alaskan moose will

be stressed physiologically by the subsequent demands associated with severe winters (Schwartz, 1998).

Adult males incur high energetic costs as a consequence of strenuous rutting activities. This cost handicap may be a self-imposed test upon the male. A male with a well-developed sexually selected character is an individual that has survived that test (Zahavi, 1975). A female moose that could discriminate a male of high quality has selected from among the best phenotypes, and presumably genotypes, and this trait likely will be passed on to her male offspring (Darwin, 1859; Zahavi and Zahavi, 1997). Thus, scent marking by male moose may be an honest advertisement of their physical condition and signal their quality as mates. More research is needed, however, on the putative pheromones we identified in the urine of male moose, and on their potential role in honest advertisement.

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