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Scent marking behavior as an odorant communication in mice

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Abstract

In rodents, where chemical signals play a particularly important role in determining intraspecies interactions including social dominance and intersexual relationships, various studies have shown that behavior is sensitive to conspecific odor cues. Mice use urinary scent marks for communication with individual conspecifics in many social contexts. Urinary scent involves genetic information about individuals such as species, sex, and individual identity as well as metabolic information such as social dominance, and reproductive and health status, which are mediated by chemical proteins in scent marks including the major histocompatibility complex and the major urinary proteins. The odor of the predator which can be considered to be a threatening signal for the prey also modulate mouse behavior in which scent marking is suppressed in response to the cat odor exposure in mice. These odorant chemicals are detected and recognized through two olfactory bulbs, the role of which in detection of chemosignals with biological relevant appears to be differential, but partly overlapped. Mice deposit scent marks toward conspecifics to maintain their social relationships, and inhibit scent marking in a context where natural predator, cat odor is contained. This suppression of scent marking is long-lasting (for at least 7 days) and context-dependent, while the odorant signaling to conspecifics tends to appear frequently (over 24 hrs but less than 7 days intervals) depending on the familiarity of each signal-recipient. It has been discussed that scent marking is a communicative behavior associated with territoriality toward conspecifics, indicating that the social signaling within species are sensitive to predator odor cues in terms of vulnerability to predation risk.

Keywords

Urine marking; predatory odor; predation risk; social memory; chemosignal; mice

1. Introduction

Animals exhibit a variety of adaptive behaviors for communicating with conspecifics and detecting and defending themselves from predators (Brown & MacDonald, 1985; Kats & Dill,

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1998). In a variety of mammals, odors may permit detection and recognition of individuals of the same and different species (Brown & MacDonald, 1985; Halpin, 1986; Staples et al., 2008). In addition to the use of chemosignals in the formation and maintenance of social relationships with conspecifics (Hurst, 1990a,b, 1993) prey may use predator odors to detect and subsequently avoid predation (Apfelbach et al., 2005; Blanchard et al., 1989a; Kats & Dill, 1998), whereas predators may use prey odors to detect and locate their meals (Koivula & Korpimäki, 2001; Rosell & Sanda, 2006). In the former case, mutual benefits may ensue, but in the latter cases, the odor-recipient is benefited by the interchange while the odor-donor is not.

Here, we briefly review social communication via odorants with a focus on scent marking behavior in mice. We also consider functional differences in response to interspecific and heterospecific (predator-prey) chemosignals; specifically the temporal characteristics of social memory formation and duration concerning conspecifics and predators.

2. Social odorant communication

Olfaction is a major modality through which animals may detect and possibly identify, other animals. As early mammals were likely nocturnal, olfaction, along with audition and tactile senses, were particularly important modalities of intraspecific communication as well as predator detection (Brown, 1979; Eisenberg, 1981). Auditory signals exchanged with conspecifics or alarm cries of conspecifics are particularly important for group-living animals (Warkentin et al., 2001; Litvin et al., 2007). Rapid onset – rapid off set auditory signals are particularly useful in acute emergencies, as olfactory signals typically have a delay between signal emission and reception (Eisenberg & Kleiman, 1972); however, the latter remain for longer durations. As a result, odors may provide information to a wider range of recipients, conspecific as well as allospecific, concerning locations of animals that are no longer present (Blanchard et al., 2003a; Brown & MacDonald, 1985; Hurst & Beynon, 2004). Most commonly used laboratory rodent species are macrosomatic, using odors as a major mode of social and nonsocial detection and potentially recognition. Odor-based interactions have recently come to occupy an increasingly important place in laboratory work on social, sexual, and antipredator behaviors.

2.1. Intraspecies communication

Mice and other rodents use odors in a number of social, agonistic, and defensive contexts. These odors may provide information about other animals including species, sex, and individual identity, using this information to adjust their behavior in subsequent interactions (Bowers & Alexander, 1967; Brown & MacDonald, 1985; Halpin, 1986). Scent marks deposited in the environment may communicate information on territory ownership when the owner is absent (Hurst, 1987, 1989), social status (Hurst, 1993; Jones & Nowell, 1973a, 1974); reproductive, health, and nutritional status (Barnard & Fitzsimmons, 1988; Brown & MacDonald, 1985; Brown & Schellinck, 1995; Kavaliers et al., 2005; Mossman & Drickamer, 1996); and enable recognition of individuals (Bowers & Alexander, 1967). The informational content of odors may be maintained for over 24 hours in the marker's absence (e.g., Hurst & Beynon, 2004) enabling a protracted interaction between individuals, compared to vocal or tactile communication (Hurst et al., 1998).

2.1.1. Male to male communication: Dominance relationships—Mice are territorial (Crowcroft & Rowe, 1963; Anderson & Hill, 1965), and urinary scent marking serves to indicate territorial boundaries (Humphries et al., 1999; Ralls, 1971). Scent marking and the counter-marking of the scent marks of other males are important components of dominance advertisement among male house mice and strongly influence their aggressive interactions (Hurst, 1993; Mugford & Nowell, 1970). In male mice, fresh scent marking propounds the

mouse's ability to dominate an area (Hurst & Rich, 1999) and to maintain the territory against male intruders (Gosling et al., 2000; Jones & Nowell, 1973b, 1989). Accordingly, dominant male mice make more urine marks, whereas subordinate males urinate in fewer locations (Desjardins et al., 1973; Hurst, 1990b), such that the number of scent marks can predict both aggression scores and social dominance status in mice (Drickamer, 2001). Subordinate males, in contrast, suppress such competitive signaling and avoid challenges (Desjardins et al., 1973), thereby reducing detection and attack.

The chemical signals in scent marks also modulate aggressive behavior in recipient mice (Lacey et al., 2007; Mucignat-Caretta et al., 2004). Resident male mice attack intact adult males, but not castrated male intruders or females (Mugford & Nowell, 1970). When the castrated male or the female (but not pups; Mucignat-Caretta et al., 2004) is sprayed with intact male urine, the resident male may attack them (Mugford & Nowell, 1970; Novotny et al., 1985). This suggests that chemicals present in male urine constitute an extremely important aggression-inducing signal (Mucignat-Caretta & Caretta, 1999).

Specificity of information available in scent marks is shown in studies indicating that dominant male mice countermark the scent marks from other males but show no such response to their own scent marks or to these from males genetically identical to themselves (Hurst, 1990b; Nevison et al., 2000). This countermarking response is an important part of male competitive advertisement to other males and to females (Hurst, 1993; Hurst & Rich, 1999). Although dominant males countermarked the urine marks of another when they could contact the mark, they failed to do this when contact was prevented by a sheet of nitrocellulose (Nevison et al., 2003), suggesting that non-volatile components of the scent mark are involved in this recognition (Hurst et al., 2005; Humphries et al., 1999).

Territorial urine marking is an androgen-dependent behavior influenced by dominance status (Desjardins et al., 1973; Kimura & Hagiwara, 1985, Ralls, 1971). Both testosterone propionate (TP) and estradiol benzoate (EB) were effective in restoring scent marking of castrated males (Kimura & Hagiwara, 1985). With some exceptions (CF-1 mice; Coquelin, 1992; Maruniak et al., 1975) intact female mice show low levels of marking that are further depressed by ovariectomy (Kimura & Hagiwara, 1985). Sexual experience and estrus cycle did not alter the frequency of scent marking in female mice (Maruniak et al., 1975), but TP and 5- α -dihydrotestosterone enhanced marking in ovariectomized females while EB restored the marking to the pre-ovariectomy level (Kimura & Hagiwara, 1985). Neonatally androgenized females showed much higher levels of scent marking than normal females (Kimura & Hagiwara, 1985). These findings suggest that sexual dimorphism in scent marking in mice is primarily determined by the hormonal (testosterone) environment during early postnatal age, with additional effects of hormones later in life.

2.1.2. Male to female communication: Reproductive relationships—Ultrasonic courtship vocalizations to females or female odors constitute another androgen-dependent reproductive behavior influenced by dominance status (Nyby et al., 1976, 1977). Dominant males vocalize more than subordinate males (Nyby et al., 1976) while castration decreases the frequency of vocalization to females as well as scent marking to males (Lumley et al., 1999; Nyby et al., 1992). TP replacement restores these behaviors to precastration level (Sipos & Nyby, 1998). However, courtship vocalizations and scent marking to females may be regulated by different mechanisms (Nyby et al., 1992). In castrated males, intracranial implants of testosterone into the medial preoptic hypothalamus, an important regulatory site for male sexual behavior (Meisel & Sachs, 1994), were sufficient to restore courtship vocalizations to female urine but only partly restore scent marking in response to female urine (Matochik et al., 1994; Sipos & Nyby, 1998). Repeated defeats suppress androgen levels in subordinate mice (Bronson & Desjardins, 1974). Following such defeats, DBA/2 males displayed prolonged

inhibition (over 4 weeks) of territorial scent marking to a matched pair male, but only a short-term decrement (less than 24 hrs) of courtship vocalizations to female odor (Lumley et al., 1999). These findings suggest that courtship vocalization and scent marking have different functions in social communication, although both behaviors are androgen-dependent (Lumley et al., 1999; Sipos & Nyby, 1998). While courtship vocalizations may be considered to be an urgent response to a potential mate regardless of the context, scent marking is likely to be context-dependent and perhaps to incur a greater risk of aggressive intention from other males in the vicinity, even when females are not present.

Odorant signals may also function as a primer that alters the endocrine state of the recipient animal (for review, Koyama, 2004). Male mice scent mark to adult females more than to juvenile males or females (Arakawa et al., 2007), regardless of estrous stage in the stimulus females (Coquelin, 1992; Brown, 1988). Male scent marks may induce estrus, accelerate the onset of puberty, and synchronize estrous cycles in females (Vandenbergh, 1969; Whitten, 1956, 1957, 1958), with odors of dominant males exerting a stronger influence on accelerating the onset of puberty in females than odors of subordinates (Lombardi & Vandenbergh, 1977). However, female odors produce a delay in the onset of puberty and suppression of estrus in females (Drickamer & Hoover, 1979; Drickamer, 1982, 1983; Lee & van der Boot, 1955, Vandenbergh, 1994). Luteinizing hormone (LH) and prolactin are two hormones important for regulating these effects (Dulac & Torello, 2003; Haplern, 1987), and female mice are likely to respond to males odor with an increase in LH and reduction in prolactin: Male mice display an increase in prolactin as a response to female odor (Keverne & de la Riva, 1982).

Unfamiliar male odors can interrupt the establishment of pregnancy in females (Bruce, 1959; Kumar & Dominic, 1993; Parkes & Bruce, 1961), providing a functional test of familiarity. Females appear to form an olfactory memory of the stud male in the accessory olfactory bulb shortly after mating (Brennan et al., 1990; Brennan & Peele, 2003). This is mediated by prolactin and LH, to stimulate neuronal production in the olfactory bulb and hippocampus, respectively (Mak et al., 2007). If exposed to the scent of an unfamiliar male from a different strain within five days of mating, prolactin release is disrupted and the embryos fail to implant. This test has been applied to assess the olfactory signature used to recognize the stud male (Peele et al., 2003; Yamazaki et al., 1983).

2.2. Interspecies odorant signaling: predator-prey interaction

Animals discriminate between chemosignals from their own species and those from other species (heterospecifics) (Meredith & Westberry, 2004; Wyatt, 2003). In general, the adaptive advantages of within-species signaling may be countered by disadvantages with reference to nonconspecifics. Odor-based and other signals to conspecifics can have a negative effect by advertising an individual's presence and location to predators (Koivula & Korpimäki, 2001; Roberts et al, 2001). Conversely, odors of predators may warn prey of their presence (Dickman, 1992; Merkens et al., 1991). Thus, rodents display aversion and avoidance responses to many of the odors of predators. This has been repeatedly demonstrated for the fur/skin odor of the domestic cat, which also supports rapid (1-trial) aversive conditioning, as an unconditioned stimulus (e.g., Blanchard et al., 1990a,b; Dielenberg & McGregor, 2001). Male and female mice also show a tendency to avoid cat odor (urinary and faecal odors) in a Y-maze choice situation (Kavaliers et al., 1994, 2001).

Predator odors may have major effects on within-species territorial scent marking (Roberts et al., 2001). High scent marking male laboratory mice approach competitors' scent marks more quickly and spend more time in countermarking than those with a low scent marking frequency. The urine of ferrets (*Mustela putorius furo*) reduced approaches to the competitor's marks, for high- but not low-marking males, suggesting that a high frequency of scent marking involves an inherent cost for predation risk. The odor of a non-predatory naked mole-rat

(*Heterocephalus glaber*) failed to elicit this response, indicating that it is a response to predator odor, not just to an unfamiliar odor. Similar discriminations have been shown in meadow voles (*Microtus pennsylvanicus*) in tests between odors of weasels (*Mustela erminea*) and guinea pigs (Parsons & Bondrup-Nielsen, 1995). Reductions of scent marking following predator odor may reduce the risk of predation but also the benefits associated with signaling (Belwood & Morris, 1997; Wolff, 2004). Roberts et al. (2001) have suggested that, from a resource competition perspective, predation risk may enable low-markers to increase their scent marking investment, for improved access to resources. In such cases, scent marking may have greater benefits in the form of territory, reproductive competition, or sexual advertisement, than the cost of increased predation risk (Wolff, 2004).

3. Chemical signals in urinary scent marks

Evolved odor signals involve some components that are genetically determined (Boyse et al., 1987) and not susceptible to disruption by metabolic and environmental influences. However, environmental factors, such as food type, bacterial gut flora, and social stress, (Schellinck et al., 1992; Yamazaki et al., 1999, 2002) as well as parasited status (Kavaliers et al., 2005; Zala et al., 2003) also induce changes in volatile odors.

3.1. Odor chemicals between conspecifics

To provide relatively unambiguous information on identity, odor signals need to be sufficiently polymorphic so that each individual has a low probability of shared signatures in the local population (Hurst & Beynon, 2004). Among inbred mice, there may be difficulties in discrimination between individuals, and in assessment of familiarity and/or kinship (Barnard et al., 1991; Nevison et al., 2000). Indeed, individuals of highly inbred strains are unable to discriminate between each others' volatile urinary odors when kept under identical conditions (Arakawa et al., 2008; Nevison et al., 2000; Yamaguchi et al., 1981).

3.1.1. The major histocompatibility complex associated chemosignal in scent marking—There are at least two chemical components of urinary scent that provide genetically-determined individual signatures in chemosignals (Brennan & Kendrick, 2006). The major histocompatibility complex (MHC) encodes highly polymorphic glycoproteins involved in self- non-self recognition in the immune system (Beauchamp & Yamazaki, 2003; Brown, 1995; Yamazaki et al., 1976). MHC-associated odors in mice are produced through a complex molecular mixture of volatile metabolites bound and released by urinary proteins (Singer et al., 1993, 1997). MHC associated urinary odors are used by mice in mate choice, discriminating kin from non-kin, and recognizing familiar individuals (e.g. Hurst, 1990a,b; Manning et al., 1992; Potts et al., 1994; Yamazaki et al., 1976, 1979). Yamazaki et al. (1976, 1988) demonstrated that female mice of congenic strains differing only in their MHC genotype tend to choose to mate with MHC type-different males (Jordan & Bruford, 1998). The maternal recognition of pups is also affected by MHC genotype (Brennan & Zufall, 2006; Yamazaki et al., 2000). Maternal mice preferred to retrieve pups of the same MHC types as themselves, while mouse pups were found to prefer nest odor of the maternal MHC type.

MHC genotype may play a significant role in the phenomenon of pregnancy block (PB). PB can be induced by scent from an unfamiliar strain that differs from the stud male only at the MHC (Yamazaki et al., 1983). Although many studies indicate that PB is a specific response to contact with androgen-dependent scents from an intact unfamiliar male (Bruce, 1960; Hoppe, 1975), Yamazaki et al., (1983) reported that urinary scent from unfamiliar females as well as males differing in MHC characteristics can induce PB (Yamazaki et al., 1983). Findings are inconsistent as to whether PB requires direct contact with urine odors, as opposed to airborne volatiles (Brennan & Peele, 2003; Dominic, 1966; Rajendren & Dominic, 1984; Yamazaki et al., 1983). Although there is no evidence that MHC proteins are able to bind

volatiles, MHC genotypes have been shown to influence the profile of volatile fatty acids in urine (Schaefer et al., 2002). Responsivity to airborne volatiles associated with mice of unfamiliar MHC genotypes may reflect a generalized stress response to unfamiliar mouse odors, rather than activation of specific pathways involved in individual recognition of the stud male.

3.1.2. The major urinary proteins—Major urinary proteins (MUPs) genes (Beynon & Hurst, 2003; Hurst et al., 2001) are largely expressed in the liver under stimulation by androgens and their products are released in the urine by filtration from blood serum, providing long-lasting nonvolatile odor (Bacchini et al., 1992; Brenan & Keverene, 2004). Urinary MUPs are expressed at high concentrations by adult mice of both sexes (Beynon et al., 2002; Payne et al., 2001), although males invest more than females in urine marks (Hurst, 1990c; Maruniak et al., 1975) and MUP production (Beynon et al., 2002; Stopka et al., 2007). Individual mice express many different MUP patterns (Hurst et al., 2001), which may be essential in allowing mice to distinguish another mouse's urine mark from their own (Hurst et al., 2001; Nevison et al., 2003).

Individuals of highly inbred strains have the same MUP patterns (Nevison et al., 2000; Yamaguchi et al., 1981). Male mice show countermarking when confronting urine odor from other males, but not their own, identical, odor (Hurst, 1993; Humphries et al., 1999; Nevison et al., 2000). Changing the MUP profile of the scent marks by the addition of artificially produced MUPs increased countermarking by the scent donor, indicating dependence on MUP profile rather than MHC genotype (Hurst et al., 2001, 2005).

MUPs bind small volatile urinary chemosignals that are slowly released in a testosterone-dependent fashion (Bacchini et al., 1992; Cavaggioni & Mucignat-Caretta, 2000), advertising the presence of a reproductively capable male (Novotny, 2003). Spraying of MUPs compounds on castrated males, or females, but not pups, can elicit aggressive behavior toward the stimulus by male mice (Mucignat-Caretta & Caretta, 1999; Mucignat-Caretta et al., 2004; Novotny et al., 1985). Female mice may also use MUPs to advertise reproductive state by varying the concentration of MUPs during their estrous cycle (Stopka et al., 2007). MUPs may regulate the reproductive state of females including accelerating puberty (Novotny et al., 1999) and inducing estrus cycles (Jemiolo et al., 1986).

Urinary chemosignals represent a complex compound that includes MHC-dependent volatile and non-volatile, and MUP-dependent non-volatile cues, each of which may be capable of signaling individual identity in different contexts. It is possible that these functions could be differentiated by behavioral test paradigms; MHC-based recognition has been described in the context of mate choice and parent-offspring interactions influenced by kin relationships, while MUP derived compounds in urinary scent have been reported to be associated with recognition between individual males and among male-female pairs, especially in scent countermarking situations.

3.2. Detection of odorant signaling

3.2.1. Two olfactory bulbs—Odorants may be registered by two distinct chemosensory systems originating in the nose: the main olfactory system, located in the dorsal posterior aspect of the nasal cavity, and the vomeronasal organ (VNO), located in the inferior aspect of the nasal cavity (Greer, 1991; Matsunami & Buck, 1997; Rodriguez et al., 2002).

Receptors of the main olfactory system, located in the olfactory epithelium, can detect thousands of different volatile odor molecules. These receptors encode olfactory signals to the main olfactory bulbs (MOB), while the VNO sends its axons to the accessory olfactory bulb (AOB) an anatomically independent region in the posterior part of the olfactory bulb. The AOB

and MOB, in turn, give rise to separate pathways that terminate in generally separate but sometimes overlapping areas of the basal telencephalon (Pro-Sistiaga et al, 2007). From the amygdala, vomeronasal pathways project to the medial preoptic area and the ventromedial nucleus of the hypothalamus (Rodriguez et al., 2002; Scalia & Winans, 1975).

3.2.2. Function of two olfactory organs—The roles of the two systems in detection of chemical signals with biological relevance appear to be partially overlapping (Restrepo et al., 2004). The MOB detects general odorants that provide information about the environment (Dulac & Torello, 2003; Lin et al, 2006). The VMO is particularly involved in the response to pheromones (Dulac & Torello, 2003; Halpern & Martínez-Morcós, 2003; Wysocki et al., 1982); and, likely, kairomones (Staples et al. 2007) mediating some particularly adaptive responses to the odors of other species such as predators; e.g., the vomeronasal receptor neurons are narrowly tuned, responding best to a more limited group of molecules.

Bilateral removal of the olfactory bulb, which abolishes input from both systems, eliminates both mating behavior (Bean, 1982; Keller et al., 2006) and agonistic behavior (Bean, 1982; Ropartz, 1968) in male mice. Nasal perfusion of zinc sulfate, disrupting the main olfactory bulb but leaving the accessory system functionally intact (Power & Winans, 1975), has only a minor effect on intermale aggression in mice (Bean, 1982; Connor, 1972), but completely disrupts mating behavior in male mice regardless of their sexual experience (Keller et al., 2006). Removal of the VNO alone does not affect mating behavior (Bean, 1982; Keller et al., 2006), but VNO removal markedly reduces scent marking responses and aggressive behavior to other male mice (Maruniak et al., 1986; Pankevich et al., 2004) as well as aggressive behavior of lactating female mice (Bean & Wysocki, 1989). After VNO removal, male mice are able to mate with females but do not show preference to estrous females (Pankevich et al., 2004). These findings suggest that normal male aggressive and scent marking behaviors toward other males are dependent on the presence of an intact vomeronasal system for their expression, while normal male-female sexual interactions require the presence of an intact main olfactory system. However, removal of the VNO does not appear to disrupt responsivity to general environmental stimuli, such as food buried under cage shavings (Wysocki et al., 1982).

Additional support from this view comes from studies of the TRP2 ion channel, a critical part of the vomeronasal system signal transduction pathway. Male mice with a genetic ablation of the *trp2* gene engage in sexual behavior with conspecifics of both sexes and fail to display male-male aggression (Leypold et al., 2002; Stowers et al., 2002). *Trp2* ^{-/-} males mount and emit ultrasounds to castrated male mice scented with intact male urine, a response that normally would be expected toward females. Since TRP2 is found exclusively in the vomeronasal epithelium especially in the vomeronasal V2 category of receptor neurons (Chamero et al., 2007), these results strongly support the idea that the vomeronasal system is critical for male-specific behavior in response to sensory cues for sex discrimination (Leypold et al., 2002; Stowers et al., 2002).

3.2.3. Detection of predatory odor—Several lines of evidence suggest that predator odors are mediated by the AOB rather than the MOB (McGregor et al., 2004; Apfelbach et al., 2005; Staples, 2008). Laboratory rats exposed to cat odor in a confined environment showed strong activation of the granular, glomerular and mitral cell layers of the AOB, but little activation in the MOB (McGregor et al., 2004). The AOB projects to the medial amygdala as well as the bed nucleus of the stria terminalis (Dielenberg et al., 2001; Pro-Sistiaga et al, 2007), providing input to a medial hypothalamic circuit that plays a key role in defensive responses to predators and their odors (Blanchard et al., 2003b; Canteras et al., 1997; Canteras, 2002). The synthetic fox feces-derived odor, trimethylthiazoline (TMT) (Vernet-Maury 1980; Vernet-Maury et al., 1984) does not appear to activate the AOB, instead producing activation of the MOB (Fendt et al., 2005). TMT is found to strongly activate the central nucleus

of the amygdala and paraventricular nucleus of the hypothalamus, while cat odor has no such effects (Day et al., 2004; Fendt et al., 2005). These findings suggest that cat fur/skin odor is a VNO-mediated kairomone (Apfelback et al., 2005), whereas TMT odor, although appearing to activate fear and aversion-related circuitry in the limbic system, is not (Day et al., 2004; Stables et al., 2007), a difference that may be influential in the patterns of defensive responses to these two predator odors when the two are tested under identical circumstances. Specifically, McGregor et al. (2002) suggested that TMT is a noxious, stimulus for rats and mice (as for humans!), a view that is compatible with findings (Endres & Fendt, 2007) that following 7 exposures, rats developed a conditioned place aversion for a chamber associated with TMT. In contrast, a single, brief, cat odor exposure reliably produces Pavlovian or associative conditioning to the exposure context, while TMT does not (Blanchard et al., 2003c; McGregor et al., 2002). These findings suggest that VNO involvement may be a factor in the specific and very rapid form of aversive conditioning seen with a cat fur/skin odor unconditioned stimulus.

4. Scent marking as communication with conspecifics

As noted earlier, mice deposit scent marks under circumstances suggesting that these function to communicate with a present or potentially present conspecifics. Male mice deposit more scent marks to adult females or their odors than toward juvenile females or juvenile males (Arakawa et al., 2007; Coquelin, 1992), suggesting male advertisement in a sexual context. Additionally, dominant but not subordinate males countermark when they find urine odor from genetically different males in their patrolling area or territory, but do not mark if they cannot detect any odor from conspecifics (Hurst, 1993; Hurst & Beynon, 2004; Rich & Hurst, 1999), or if the only odor is that of a familiar same-sex cagemate (Arakawa et al., 2007). These findings fit together very well in suggesting that in male mice scent marking can represent claims to ownership of a territory, along with the sexual opportunities that ownership may facilitate, with countermarking representing a challenge to the scent-mark mediated claims of another adult male; an interpretation congruent with findings that social defeat reduced both marking to a chamber, and ultrasonic vocalizations to a female mouse, albeit with different post-defeat durations of inhibition for the two behaviors (Lumley et al., 1999).

Habituation effects are consonant with these views: When repeatedly exposed to a chamber or a 15 conspecific, C57BL/6J males showed marked habituation to both, as expressed by decreased scent marking over trials (Arakawa et al., 2008). In terms of evolutionary adaptiveness of scent marking, these habituation effects may reflect reduced value of marking to familiar conspecifics, such that habituation may serve as an index of social memory in mice. In addition, however, persistent marking may incur an enhanced risk of predation to the marker (Robert et al., 2001; Rosell & Sanda, 2006), a risk that should be independently manipulable by cues suggesting predator presence.

To assess these relationships, we measured scent marking responses of male mice to a conspecific male, as influenced by cat odor, a cue suggesting the presence of a predator, to investigate whether odor from conspecifics and predators appear to mediate different functional mechanisms of odorant communication. Specifically, long-lasting effects of conspecific and predator odor exposure on scent marking responses were investigated in the context of social memory concerning conspecifics and predator odors.

4.1. Scent marking to conspecific and predator odors: Experiments

4.1.1. Materials and methods

Animals and rearing condition: Fifty nine male C57BL/6J (C57) mice (25–30 g), 16–18 weeks of age, were used as the subjects (32 males for experiment 1 and 27 males for experiment 2). They were bred from stock obtained from the Jackson Laboratory (Bar harbor, ME). All

subjects were weaned at 23–25 days of age, and then housed in groups of 2–3 same sex animals in standard polypropylene cages, 26.5 × 17 × 11.5 (height) cm, under 12L:12D cycle (lighting on 06:00) in a temperature- (22±2 °C) and humidity- (60 %) controlled room at the University of Hawaii Laboratory Animal Services. They were housed individually in the polypropylene cages at least for 1 week prior to testing, and then they were randomly assigned into one of four (experiment 1) or three (experiment 2) groups. Sixteen male CD-1 mice, 14 weeks of age, were obtained from Charles River Laboratories (Wilmington, MA), were used as the stimulus animals. They were housed individually in polypropylene cages at least for 1 week prior to the test.

All animals were allowed free access to food and water in their home cage. All protocols and animal handling and treatment were approved by the Institutional Animal Care and Use Committee at the University of Hawaii.

Apparatus: In both experiments, scent mark tests were conducted in a clean 46 × 24 × 21 cm Polycarbonate cage, placed upside-down on a rough paper (457 × 365 mm, Rough Newsprint paper, Bienfang) substrate. The cage was divided into two equal-sized compartments by a wire mesh screen that prevented direct physical contact between subject and stimulus, but allowed olfactory, visual, and auditory cues to be received.

In Experiment 2, a different cage was used to provide the novel context. This was a triangle-shaped opaque plastic cage, 47.4 × 36 × 18 (height) cm, with a clear Plexiglas top, one side of which was a wire mesh wall.

Test procedure: All test trials were conducted during the light phase of the light/dark cycle under dimly lit conditions. The subjects were moved from the holding room to the experiment room in their homecages 20 min before the beginning of the test. At the end of the each 20 min trial, the animals were returned to their home cage and moved back to the holding room. Between trials, the apparatus was cleaned with 15% alcohol, dried with paper towels, and given a fresh paper substrate. Scent marking was evaluated on each trial.

Experiment 1: The goal of this experiment was to investigate habituation of scent marking with repeated exposure to the same conspecific and to determine the duration of social memory as reflected in such habituation.

Subjects were divided into four groups; receiving the same or a different stimulus mouse on the test day, after an interval of 24 hr, or 7 days (N = 8/group). Each stimulus mouse was confronted with an initially novel CD-1 male in the test chamber, for 20 min, on each of four daily trials. On the fifth trial, animals in the SAME groups were exposed to the same CD-1 male following an interval of 24 hrs or 7 days, while animals in the DIFFERENT groups were confronted by a novel CD-1 male, after the same intervals.

Experiment 2: The aim of this experiment was to investigate the short- and long-term impact of exposure to cat fur/odor, in the homecage or in the test situation, on scent marking behavior in isolate C57 mice. In this experiment a HOMECAGE ODOR group was exposed to cat fur/skin odor in the home cage, whereas a TEST ODOR group encountered cat fur/skin odor in the test situation while CONTROL mice received no cat odor exposure. Tests 7 days later (D8) evaluated the durations of cat odor-induced changes in scent marking, and an additional test (D9) in a different chamber, determined the situational specificity of cat-odor effects on scent marking responses.

On D1, twenty minute before the beginning of the test, subjects were moved from the holding room to the experimental room in their homecages. Animals in the TEST ODOR and

CONTROL groups were left undisturbed for 20 min, while those in the HOMECAGE ODOR group had a cloth-wrapped plastic block (9 × 9 × 2 cm) placed on top of the wire mesh lid of their cage, for a similar duration. This block had been rubbed for 5 min against the fur of a laboratory-housed domestic cat and then stored in a Ziploc plastic bag until used as the cat-odor stimulus. During testing, each subject was placed in one compartment of the test chamber for 20 min, with a cloth block placed on the cage lid of the compartment on the other side of the wire mesh barrier. For the TEST ODOR group, this cloth had been rubbed with cat fur/skin, but was without odor for subjects in the HOMECAGE ODOR and CONTROL groups. At the end of the period, the cage lid was changed and all animals were returned to their holding rooms. Behaviors were recorded using an over-head video camera. On D2 and D8, each subject was placed in one compartment of the test chamber cage for 20 min. A no-odor cloth block was located in the other compartment. On D9, all subjects were evaluated in the novel, triangle-shaped test chamber.

Scoring scent marking: Mouse urine was fixed by Ninhydrin spray (LC-NIN-16, Criminal Research Products, LLC) (Fig. 1). After drying for 24 hrs, the number of scent marks was measured by placing a transparent grid sheet over the substrate paper and counting the total number of grids (each 10 × 10 mm) containing scent marks (maximum: 552 squares). Pools of urine larger than four square grids that formed a larger quadrant were not included in this count. Four squares in a row, however, were included. Numbers of urine pools and feces were also recorded.

Behavioral indices: In experiment 2, on D1 (first test exposure) subjects' behaviors were recorded by a DVD recorder, and subsequently analyzed. The floor of the test chamber was divided into four equal blocks (each 12 × 6 cm) by two perpendicular lines, and the number of entries into each block of the chamber area was counted as a measure of locomotion. To assess proximity to the scent block, durations in three locations were measured: "contact" was location in an area closer than 4 cm to the wire mesh barrier; "near" was in the remainder of the two blocks nearest the mesh, i.e. location in a 24 × 8 cm area just behind the "contact" area; "far" was location in the 24 × 12 area most distant from the mesh barrier. Durations of sniffing of the mesh barrier, climbing of the mesh barrier, and grooming were measured.

Statistical analysis: Data were analyzed by two-way analyses of variance (ANOVA) with a between-subject factor of groups (same or different for experiment 1, and HOMECAGE ODOR, TEST ODOR, or CONTROL for experiment 2) and a within-subject factor of trials (trials 1-5) for experiment 1 or day (D1, D2, D8 or D9) for experiment 2. In experiment 2, the number of crossings in each area of the chamber (locomotion) was analyzed by a within-subject factor of chamber area (near or far areas) and between-subject factor of group (HOMECAGE ODOR, TEST ODOR, or CONTROL). The duration of stay in each area and of each behavior (poking, climbing, and grooming) and risk assessment ratio were analyzed by a one-way ANOVA between groups. Post hoc comparisons used the Tukey's HSD test for between-subject factors and Bonferroni test for within-subject factors. A probability level of $p < .05$ was adopted as the level of statistical significance for all analyses.

4.1.2. Results

Experiment 1: Impact of repeated exposure and change of stimulus animal on scent marking response in C57 males: Fig. 2 depicts the number of squares with scent marks for C57 males toward an initially unfamiliar CD-1 male for the first 4 trials and then toward a same (SAME) or a novel (DIFFERENT) CD-1 male on trial 5. Two-way ANOVA on these scores found a significant main effect of trial, $F(4,56) = 15.788, p < .001$, but not of group, $F(1,14) = 0.217$. The interaction between trial and group was significant, $F(4,56) = 3.229, p < .019$ and subsequent analyses indicated that C57 males showed high levels of marking on the first trial

which decreased during 4 exposures to the same CD-1 male, with significantly reduced marking on trials 3 and 4 compared to trial 1. On trial 5 (Fig. 2a), C57 males exposed to the familiar CD-1 male showed less marking compared to those exposed to a novel CD-1 male ($p < .01$). Marking to the novel, but not the familiar, male on trial 5 was also significantly higher than to the habituated male on trial 4 ($p < .01$).

However, when a 7 day interval separated trials 4 and 5 (Fig. 2b), C57 males showed recovery of scent marking to the familiar stimulus mouse. This was confirmed by ANOVA, which found a significant main effect of trials, $F(4,56) = 16.226$, $p < .001$, but not of group, $F(1,14) = .107$. The interaction between trials and group was not significant, $F(4,56) = .441$.

There were no significant differences in the total number of squares that had urine pools, or in the number of fecal boli, for each group in either the 24 hrs or the 7 days retention tests.

Experiment 2: Immediate and long-term impact of cat odor on scent marking behavior:

Scent marking response: Fig. 3 indicates squares with scent marks for male C57 mice on D1–D9 for groups with or without cat fur/odor on D1. Two-way ANOVA conducted on these scores found significant main effects of group $F(2,24) = 13.567$, $p < .001$, but not of test days, $F(3,72) = 0.116$, n.s. The group \times test day interaction was significant, $F(6,72) = 2.275$, $p < .05$.

Subsequent analyses confirmed that mice exposed to cat odor in the test chamber (TEST ODOR) or in homecage prior to the test (HOMECAGE ODOR) showed significant reductions of scent marking compared to CONTROLS (D2, $p < .05$, and D1 and D8, $p < .01$, respectively). When the test chamber was changed to a different (triangle-shaped) cage on day 9, TEST ODOR mice showed similar levels of scent marking to CONTROLS, and both groups displayed significantly more scent marking than mice that had been exposed to cat odor in their homecage on day 1 ($p < .05$).

There were no significant differences in the number of urine pools [group, $F(2,24) = .715$, days, $F(3,72) = .082$, and group \times days interaction, $F(6,72) = 1.048$]. ANOVA on feces found a significant main effect of days, $F(3,72) = 2.775$, $p < .05$, but not of group, $F(2,24) = 1.464$, or of interaction between days and group $F(6,72) = 0.353$. For all groups combined, the number of feces was fewer on day 9 than on day 8 ($p < .05$).

These results indicate that mice exposed to cat odor in either the test chamber or their home cage prior to the test showed reductions of scent marking for at least 7 days. When the test chamber was changed to a differently shaped chamber on day 9, mice exposed to cat odor in the test chamber displayed recovery of scent marking but those exposed to cat odor in their home cages did not.

The impact of cat odor exposure on other defensive behaviors: With regard to other defensive behaviors on day 1 when animals were exposed to cat odor, location and locomotor activity in the test chamber were analyzed. The percentage of time that animals spent in each area (contact, near, and far areas) was calculated as shown in Fig. 4 (top panel). A one-way ANOVA for time in each area found a significant effect of condition, $F(2,24) = 6.311$, $p < .01$; mice exposed to cat odor in the test chamber and those exposed in their home cages showed significantly decreased time in contact with the wire mesh compared to mice not exposed to cat odor ($p < .05$ or less). Similarly, ANOVA for group was significant for location in the far side of the chamber $F(2,24) = 5.771$, $p < .01$, with mice exposed to cat odor in the chamber or in their home cages staying on the far side of the chamber longer than mice without cat odor ($p < .05$ or less). However, there was no significant group difference in the percentage of time spent on the near side of the chamber $F(2,24) = .835$.

The total number of entries into the blocks of the test chamber areas (2 near-side and 2 far-side blocks) is presented in Fig. 4 (middle panel). A two-way ANOVA conducted on these scores showed significant main effects of area, $F(1,24) = 92.962, p < .001$, and group, $F(2,24) = 5.853, p < .01$. The interaction between area and group was not significant, $F(2,24) = 2.765, p < .10$. Mice that were exposed to cat odor in the test chamber but not in their home cage, displayed decreased locomotion in the test chamber compared to mice exposed to the chamber without cat odor ($p < .05$).

Figure 4 depicts the total duration for each behavior; nose poking or sniffing at mesh barrier, climbing mesh barrier, and grooming, for each group of mice, as well as a risk assessment ratio (sniffing at barrier over total contact time). Group effects on sniffing at the barrier were significant $F(2,24) = 6.123, p < .01$, and mice exposed to cat odor in their home cages showed less sniffing compared to the other two groups ($p < .05$ or less). Group differences were not significant for the duration of climbing on the mesh $F(2,24) = 1.609$, but were significant for grooming $F(2,24) = 5.457, p < .05$: Mice exposed to cat odor in their home cage exhibited a longer duration of grooming in the test chamber than no-odor controls ($p < .05$). The risk assessment ratio was also significant for groups $F(2,24) = 7.364, p < .01$, with mice exposed to cat odor in the test chamber showing a higher risk assessment ratio compared to other two groups ($p < .05$).

These results indicate that mice exposed to cat odor in either the test chamber or their home cages prior to testing showed similar reductions in time near the wire mesh separating the subject compartment from the cat odor, whereas only those exposed to cat odor in the test chamber showed reduced locomotion, and an increased risk assessment ratio. However, mice exposed to cat odor in their home cages but not in the test situation showed reduced sniffing at the wire mesh and increased grooming. This may reflect that the stress of homecage odor exposure led to subsequent avoidance of highly salient novel stimuli (the wire mesh barrier) even in a novel situation.

4.2. Discussion: Expression and habituation of scent marking response to conspecific odor

The present experiments demonstrated that male C57 mice deposited substantial scent marks to an initially novel CD-1 male, with the number of marks decreasing over daily trials, indicating habituation to this CD-1 male. These C57 subjects showed a marked restoration of marking when confronted by a novel CD-1 male, indicating that they could differentiate animals on the basis of this previous exposure. However, when, following habituation to the same CD-1 male with consecutive 4 daily exposures, animals were exposed to the same CD-1 male 7 days later, they failed to show decrements in the deposition of scent marks.

There are two possible explanations for this finding. One is that social memory in this test paradigm cannot be maintained for 7 days due to limitations of mouse social memory systems. This view has some support in findings that rodent social memory evaluated as exploratory or aggressive behavior to a novel juvenile or ovariectomized female has a relatively short term duration (Winslow & Camacho, 1995; Sekiguchi et al., 1991; Thor & Holloway, 1981, 1982). However, one report using group-housed mice showed long-lasting, at least 7 days, social memory in this paradigm (Kogan et al., 2000).

Another possible explanation is that scent marking behavior is a communication tool for maintenance of social relationships that may be functional even if social memory for a specific individual is still remained. Male mice deposit scent marks in response to changes in the environment as well as opponents (Arakawa et al., 2007, 2008; Bronson & Desjardins, 1974), and tend to patrol and mark their territories frequently (Hurst, 1993). These factors reflect that scent marking represents the active emission of a signal, and not just an automatic response to the signal emitted by another mouse, and suggest that caution may be appropriate

in terms of the ability of such tests to evaluate the duration of mouse social memory. This view, urging consideration of functional aspects of behavior changes used to evaluate social memory, receives indirect support from the literature on pregnancy blocks in female mice, in response to the odor of a novel male (Brennan et al., 1990; Yamazaki et al., 1983). In this context social memory may be acquired in a single trial, and endure for 30 days (Brennan et al., 1990; Kaba et al., 1988).

4.3. Immediate and residual defensive responses of mice to predator odor

Experiment 2 demonstrated that exposure of mouse subjects to cat fur/skin odor, either in the home cage or in the scent-marking situation, produced strong, long-lasting (7 days) reductions in scent marking behaviors of male mice. When the test chamber was changed to a novel, different shaped chamber on day 9, mice exposed to cat odor in the initial test chamber showed recovery of scent marking, while home cage exposed mice did not, suggesting that the suppressive effects of cat odor exposure were conditioned to the test situation for the former, but not the latter, group. This is in agreement with a plethora of findings that exposure to a threatening stimulus results in rapid conditioning of aversive emotional responses to the situation in which exposure occurs (Blanchard & Blanchard, 1969; Blanchard et al., 2001, 2003d; Bouton et al., 2006; Fanselow, 2000; Rudy et al., 2004). The present findings clearly indicate that mice are able to maintain their memory of the context associated with cat fur/skin odor for at least 7 days.

Findings of long-lasting effects of cat fur/skin odor in the home cage on mouse behavior in the scent-marking test are amenable to two different interpretations. First, because the cat odor stimulus block was present in both the home cage and in the test situation, it is possible that these findings reflect a cue conditioning effect of the single home cage exposure. This view is compatible with earlier findings (Blanchard et al., 2001) of single trial conditioning of defensiveness to a cat odor block as indexed by enhanced defensiveness when the original odor context, but not the cue, was extinguished, in comparison to a group for which both cue and context were included in the extinction experience. The alternative view, that predator odor exposure can produce a lasting unconditioned increase in general defensiveness, is supported by findings (Hebb et al., 2003) that brief (2 to 10 min) exposure of mice to predator odor (rat-soiled bedding) can enhance acoustic startle several days later. This paradigm appears to have no obvious similarity between the exposure and test situations that might support conditioning. Adamec et al., 2006 also reported enhanced anxiety-like responses in an elevated plus maze for female, but not male, C57BL/6 mice following exposure to a room in which cats had previously confronted and come into contact with rodents. Thus, while one-trial cue conditioning to cat odor is certainly possible, it is not clear whether this provides the sole mechanism for the present home cage findings.

It might be noted that C57 mice may represent a particularly advantageous strain in terms of responsivity to cat odor, in that they, but not 129Sv mice, showed activity reductions in response to a cat fur/skin odor cloth (Raud et al., 2007). However, this specific difference may also reflect that the baseline activity levels of the 129Sv mice were also lower (Voilar et al., 2001), suggesting the possibility of a floor effect.

5. Conclusion

Odor discrimination, and urinary scent marks in unmarked situations or in response to the marks of a conspecific, have ethologically important roles in social communication among conspecifics in many rodent species (Brown, 1979; Eisenberg & Kleiman, 1972; Ralls, 1971). Social odor signals contain information about the individuals that deposit them, that may be of value to both the depositing individual and the conspecific recipients if that information (Hurst, 1993; Lacey & Hurst, 2005). Scent marking facilitates formation and

maintenance of a territory, reproductive competition, and sexual advertisement (Brown, 1979; Wolff, 2004). However, the advantages of scent marking may trade off with a potential cost in terms of predation risk. The present studies indicate that mice show suppression of scent marking in environments containing predator odor, compatible with the prediction that predator odor exposure produces rapid conditioning of defensiveness to the exposure situation. However, these data showed equally strong and durable inhibition of marking following cat odor exposure in the home cage just prior to placement in a situation that would normally elicit scent marking. The latter finding is in agreement with a growing literature indicating that exposure to cat fur/skin odor elicits a durable aversive emotional response that may be dependent on either the exposure context or on specific cues associated with the odor.

These findings, of the interactions of responsivity to predator and conspecific odors, are of interest in terms of the complex control of communicatory behaviors in rodents. Findings on habituation of scent marking to a familiar conspecific add to a body of findings (Arakawa et al, 2007, 2008) suggesting that scent marking in mice may provide a useful model for research on the physiology and pathology of communication in this species, complementary to mouse social recognition paradigms (Crawley et al., 1999; Crawley, 2004; Moy et al., 2004, 2007) that have been used for a variety of models associated with neurocognitive disorders including autism (Qiu et al., 2006; Tueting et al., 2006), Fragile X syndrome (Mineur et al., 2006; Spencer et al., 2005), and Rett syndrome (Moretti et al., 2005; Shahbazian et al., 2002) (cf. Ferkin & Li, 2005). Present findings that conditioned or unconditioned emotional responses to predator odors are capable of reducing initial scent-mark signaling (i.e. scent marking in the absence of conspecific scent) additionally suggest the potential use of this paradigm as a probe to assay motivations associated with scent marking behaviors.

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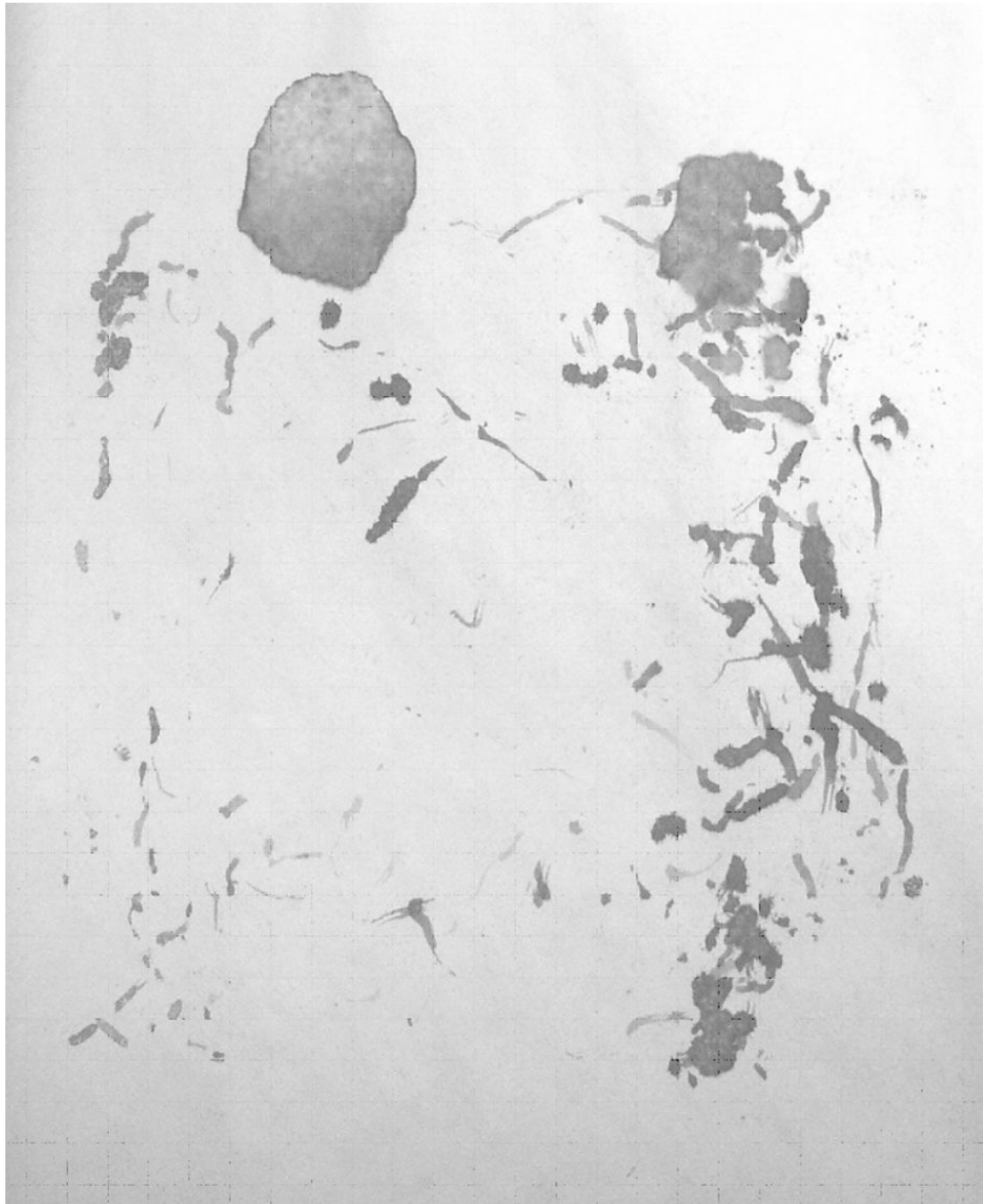


Fig. 1. Scent marks of C57 male mice fixed and colored by ninhydrin spray. A C57 male was introduced into a rectangular cage which was placed upside-down on a rough newsprint paper for 20 minute. Following removal of the mouse, the ethanol with ninhydrin was sprayed on the paper sheet and the sheet was left to be dried for 24 hours in room temperature. A large round shaped urine spot on an upper left portion of the sheet was counted as a urine pool.

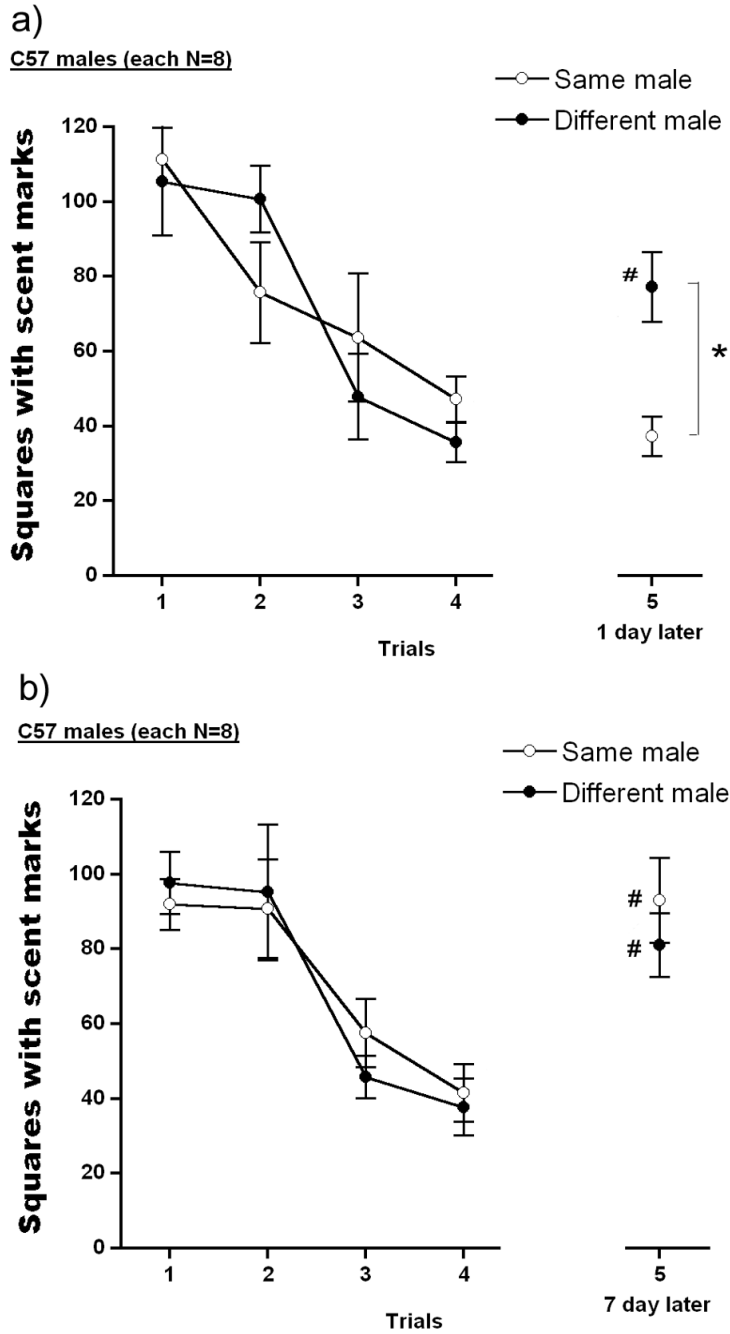


Fig. 2. Number of squares with scent marks of C57 males which were confronted with the same, initially novel, CD-1 male during the first 4 trials, and then on 5th trial, exposed to the same CD-1 male (same male) or a different, novel CD-1 male (different male). The inter-trial interval was 24 hrs on the first 4 trials, and on 5th trial 24 hrs (upper panel) or 7 days (bottom panel). Data are expressed as mean \pm S.E.M. Significant post hoc differences between groups; * p < .05, and between trials compared to trial 4, # p < .05..

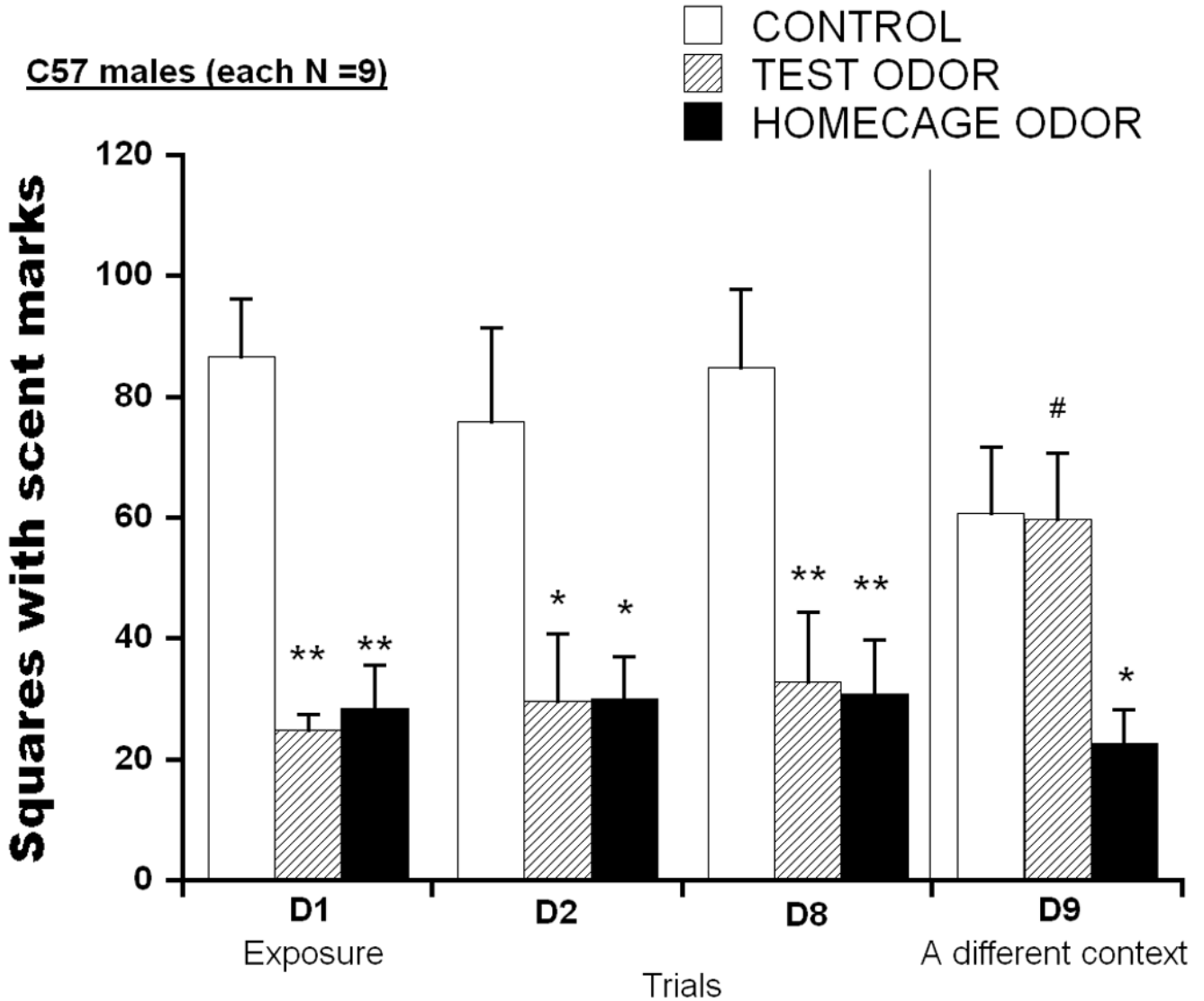


Fig. 3. Number of squares with scent marks of male C57 mice. On day 1, they were placed into the test chamber with a wire mesh partition, on the opposite side of which a towel block without odor (CONTROL), or with cat fur/skin odor (TEST ODOR) was placed. A third group was exposed to cat odor in their home cage prior to the test (HOMECAGE ODOR). Twenty-four hours (D2) and 7 days later (D8), they were replaced into the identical chamber and confronted with a towel block without cat odor. On day 9 they were placed into a differently shaped (triangular) chamber with a towel block without cat odor on the opposite side of a wire mesh partition. Data are expressed as mean \pm S.E.M. Significant post hoc differences between groups compared to CONTROL; * p <.05

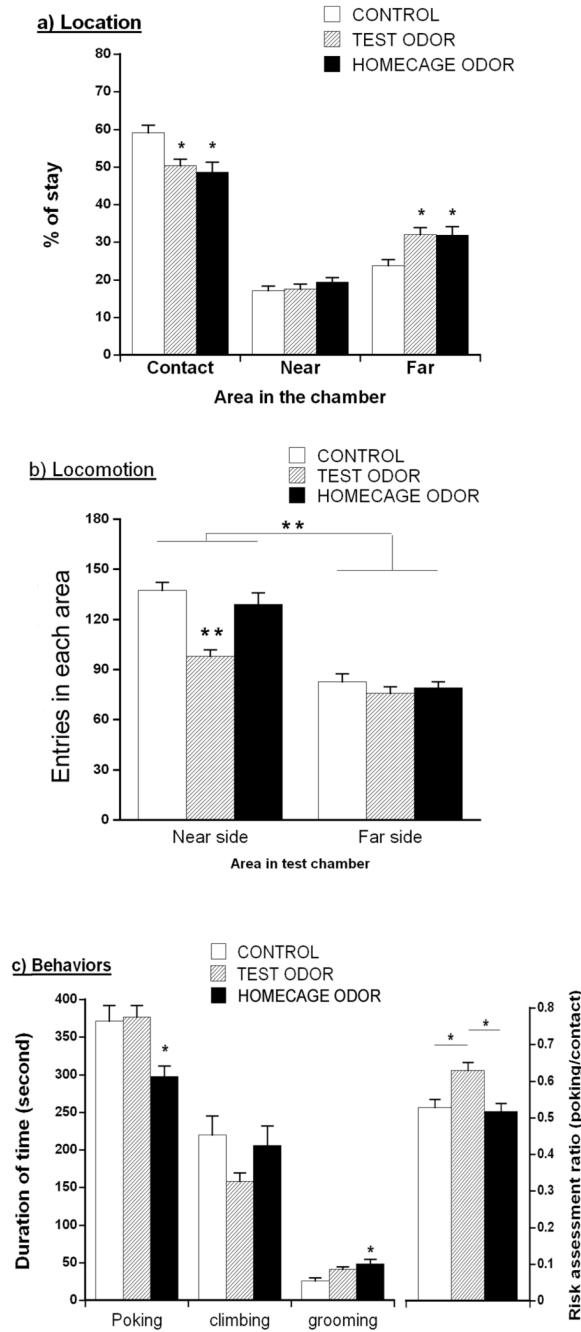


Fig. 4. Behaviors of C57 males in the test chamber on D1 when confronted with a towel block containing cat fur/skin odor (TEST ODOR) or no odor (CONTROL), or exposed to cat odor in their home cage prior to the test (HEMECAGE ODOR). a) Location: Time in each location (Contact; contacted with wire mesh; near; or far from the towel block side). b) Locomotion: Number of crossings of the lines on the chamber floor. c) Behaviors: Sniffing or poking at the wire mesh; climbing on the wire mesh; grooming; or risk assessment ratio -- duration of sniffing or nose poking per contact period with the wire mesh. Data are expressed as mean \pm S.E.M. Significant post hoc differences between groups compared to CONTROL; * $p < .05$.