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The Role of Pheromonal Responses in Rodent Behavior: Future Directions for the Development of Laboratory Protocols

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Pheromones—chemical signals that can elicit responses in a conspecific—are important in intraspecies communication. Information conveyed by pheromones includes the location of an animal, the presence of food or a threat, sexual attraction, courtship, and dam–pup interactions. These chemical messages remain intact and volatile even when animals, such as rodents, are housed in laboratories rather than their natural environment. Laboratory protocols, such as the cage cleaning and sanitation processes, as well as general housing conditions can alter a rodent's normal production of pheromones in both amount and type and thus may affect behavior. In addition, some procedures induce the release of alarm pheromones that subsequently alter the behavior of other rodents. To prevent pheromonal interference and stress-induced pheromonal release in their research subjects, experimenters should assess current laboratory protocols regarding cage cleaning processes, housing designs, and behavioral assays. Here we discuss how the most commonly used laboratory procedures can alter pheromonal signaling and cause confounding effects.

Abbreviation: VNO, vomeronasal organ.

A pheromone is a species-specific chemical signal that incites a response in another organism. Pheromones are involved in a wide variety of behaviors, including mate selection, food acquisition, alarm responses, territory marking, predation defenses, and other social behaviors indicating social status. In addition, pheromones contribute to a rodent's ability to court a potential mate; this attraction, partially mediated by pheromones, contributes to displays of female proceptivity—actions displayed to indicate interest to a male rodent.[4] Rodents, including mice and rats, are known to release and receive pheromonal signals both within and across sexes. Because mice and rats are commonly used as animal models in scientific research, an understanding of the role of pheromonal signaling in rodent behavior is essential. Specific laboratory protocols regarding cleaning and housing can affect rodent pheromone production and perception in ways that could confound experimental procedures. Laboratory procedures should both control for scents that will elicit differing physiologic and behavioral responses and consider that scent is an essential communication method in rodents. The extensive role of pheromonal signaling in a wide variety of rodent behaviors is indicative of a need to consider pheromonal signaling when designing lab protocols and experimental procedures.

Some mammals, including rodents, possess both a main olfactory epithelium and a vomeronasal organ (VNO); these organs provide similar but separate sensory functions. The main olfactory epithelium is triggered by airborne odorants, whereas the VNO is triggered by fluid-phase odorants that often act as pheromones.[23] Signals from the main olfactory epithelium travel to the main olfactory bulb and then to the olfactory cortex and other sensory centers. Alternatively, the signals of the VNO travel to the accessory olfactory bulb and then directly to the amygdala. From here, information is sent to the hypothalamus, resulting in changes in endocrine signaling. These alternate pathways therefore result in discrete behaviors. One group of investigators discovered a novel pheromone receptor gene family and hypothesized that differences in these pathways mediate their distinct functions.[12] Whereas the olfactory system uses higher-order brain regions, the pheromone system evades these regions and typically results in stereotyped behaviors.[12] Ultimately, the rodent's olfactory experience comprises 2 components, and many of their behaviors are regulated by these 2 systems.

Various aspects of lab protocols—for example, an experimenter's interaction with the rodents, the cage-cleaning process, and the housing conditions in their cages—can greatly affect a rodent's stress level, exploration behaviors, and social relationships. Pheromones play a large role in these factors, and they can all alter experimental results. Protocols and procedures should be refined so that effects on pheromonal signaling are minimized. For example, rodents may receive inadvertent exposure to pheromones (such as those present in urine) in transfer cages, temporary holding containers, and even experimental apparatus. All of these areas should be cleaned thoroughly after the introduction of each animal, which may diminish confounds in behavioral data. Here we outline current knowledge regarding pheromonal signaling in rodents and address how current laboratory protocols and procedures may induce pheromone mediated effects. In addition, we offer recommendations for further refinement of laboratory protocols and procedures through 3 major foci: cage cleaning and sanitation, housing, and the release of alarm pheromone.

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Cage Cleaning, Bedding, and Sanitation

In the United States, general guidelines mandate the procedures experimenters must follow when housing rodents for laboratory experiments. In addition to appropriate regulation of temperature, humidity, ventilation, and noise levels, part of the care process involves cleaning the cage that the rodents inhabit.[18]

Appropriate cage cleaning functions both to avoid substandard living conditions and to keep the cage fresh and pleasant for the housed rodents. According to the *Guide for the Care and Use of Laboratory Animals*,[18] cage cleaning and sanitation should occur approximately every other week. The process by which cages are considered sanitized and appropriate for rodent use includes cleaning out the cage, disinfecting, and lastly changing the soiled bedding.[18] Although cage cleaning must occur to keep the living environment from becoming uninhabitable, excessive cage cleaning can cause various repercussions.[28] The current standard for changing bedding indicates that it should be changed as often as necessary to prevent feces and ammonia from building up. However, experimenters need to remember that various factors, including pheromonal responses, can easily be altered through the interference associated with standard laboratory protocols.[9] The cage-cleaning protocol can affect a rodent's general health: excessive cage cleaning can increase the cannibalistic tendencies of rodents.[9] Although the current standard is to clean cages every other week, this frequency of cleaning may negatively affect a rodent's chemical signaling.

Measurement of stress responses in male rats that had their cages cleaned revealed that heart rate and arterial blood pressure were elevated for about 1 h in rats that were moved to new cages with fresh wood-chip bedding.[11] In addition, cage changing significantly altered the behavior of these rats, which reared and groomed more after the change compared with their baseline activity beforehand. Conversely, rats that only witnessed the cage changing of another rat did not show elevations in heart rate or blood pressure, and, behaviorally, these rats returned to baseline activity after approximately 15 min.[11] Experimenters should be aware that a rodent's behavior is altered for approximately 1 h after a cage change and should exercise caution in doing experiments during this time.

In a separate experiment, 1 cup of soiled bedding was placed in with the fresh bedding in the new cage, but this practice did not attenuate the increase of heart rate or blood pressure seen with the cage change.[11] In another study, routine cage cleaning resulted in elevations of heart rate and blood pressure that were similar to those after subcutaneous and tail-vein injections.[29] Another study found similar elevations in heart rate and blood pressure to cage changes in female rats.[30]

Increased rates of cage cleaning have the potential to cause cannibalistic behavior in rodents, due to the olfactory, physical, and auditory stresses that cage cleaning can induce.[9] Therefore, a rodent that becomes stressed due to the frequency of this process might respond by consuming one of its own pups.[9] However, if cages housing multiple rodents are not cleaned sufficiently frequently, the rodents will begin to display more aggressive behavior and, in turn, attempt to mask each other's scents with their own and fight for dominance. The mechanism underlying this behavior is thought to be that scents remain for longer periods in cages that are cleaned less frequently, causing animals to try to defend territory and thus be more dominant. Less aggression is expressed by a resident mouse toward an introduced mouse if urine from the resident is swabbed on the introduced mouse.[26]

Experimenters should use bedding that absorbs urine effectively; poor-quality bedding, rather than absorbing urine completely, will enable volatile pheromones in urine to disperse into other enclosures, perhaps induces behavioral changes in surrounding rodents, including increased vigilance and defensive behaviors (see also the section on alarm pheromones).[20] Monitoring the bedding used to see whether urine is seeping through to the floor of the cage would be beneficial. If such seepage occurs, the detection of pheromones also can induce responses including the Lee–Boot effect,[24,25] Whitten effect,[32] Bruce effect,[8] Vandenbergh effect,[31] and Hoover and Drickamer effect[16] all of which affect female rodents and can alter both their behavior and physiology[15] (Figure 1). To avoid airborne pheromones from diffusing among cages, soiled bedding should be replaced at a rate sufficient to keep the cage tidy but not so frequent as to eliminate the rodent's own pheromones in the environment and cause stress. Last, the bedding itself should not be scented before its introduction into cages, nor should the type of bedding be changed during the course of a behavioral experiment. Rodents are likely to habituate to the odor of their bedding, but a new type of bedding will represent an olfactory change in their environment. Following these suggestions will avoid exposing rats to novel scents, because these alone can alter a rodent's pheromonal responses. To this end, a lot of bedding is advertised as being scent-free.

Before bedding is replaced, cages must be sanitized. Caution is advised during the sanitation process due to the possibility of causing stress due to the introduction of a new scent.[10] Like scented bedding, scented sanitizers should be avoided, for the same reason: any new scent in the sanitizer will induce stress due to the fact that the rodent's own scent has been covered by a foreign scent.[18] Masking rodents' pheromones can be especially dangerous for female rodents, because their pheromones often signal care procedures for the pups. For example, pups release the pheromone dodecylproprionate to signal their mothers.[5] In rats, a mother's VNO is responsible for detecting dodecylproprionate, a pheromone released from the pup's preputial gland that stimulates maternal anogenital licking. This licking behavior stimulates pups to defecate. To determine whether dodecylproprionate was detected through the main olfactory system or the accessory olfactory system, investigators[6] removed the VNO or main olfactory system of rat dams. Dams that lacked VNO displayed disorganized anogenital licking: they did not lick individual pups for equal amounts of time and even ignored some pups completely. In addition, pup mortality increased and pup weight decreased in groups in which the dams lacked VNO.[6] The cited study demonstrates that the dam's ability to detect dodecylproprionate is dependent on the VNO and is crucial for effective rearing.

Investigators must be conscious of the frequency of cage cleaning because of its potential to alter rodents' established pheromones and affect the other rodents in the colony.

Housing

Exposure to scents of other mice can alter the behaviors of male mice in a laboratory setting.[22] Scent may play a role in organization of social status and competitive behavior in inbred male mice. Exposing mice to odors of different conspecifics during transfer, testing, or routine maintenance may alter their behavior.

One group of investigators exposed inbred mouse strains to bedding that contained the urine (odors) of either similar or different mouse strains.[22] Pairs of male mice were housed together in their homecage. A portion of their bedding was replaced with

Pheromone	Laboratory procedures	Effects on rodents	Species
Alarm pheromone[20]	Cage cleaning, experimental handling, stress-inducing behavioral assays	Increases acoustic startle response, increases stress-induced hyperthermia, increases locomotion	Mouse
Bruce effect[8]	Addition of a foreign male	Blocks pregnancy in females	Mouse
Diodecyl propriate[5]	Cage cleaning	Induces maternal licking of pup	Rat
Hoover–Drickamer effect[16]	Presence of urine from a foreign pregnant or lactating female	Prolongs estrus	Mouse
Lee–Boot effect[24]	Females are housed together and isolated from males	Suppresses or prolongs estrus; decreases luteinizing hormone; increases prolactin	Mouse
Vandenbergh effect[31]	Accidental exposure of prepubescent female mice to male urine	Accelerates female puberty	Mouse
Whitten effect[32]	Females exposed to male animal or urine	Induces estrus in a group of females	Mouse

Figure 1. Interactions between laboratory procedures and pheromone systems.

soiled bedding from other mice. The mice were removed from their homecage for behavioral testing in an arena and then replaced into their original homecage, which contained the newly introduced bedding and monitored for 10 min. The number of aggressive attacks made by both mice was quantified. Mice exposed in their homecage to soiled bedding from other mice, regardless of whether the soiled bedding was from mice of the same or a different strain, mounted more aggressive attacks than they did when exposed to the water control. Whether the soiled bedding was from the same mouse strain or a different strain did not differ: the number of attacks did not differ between these 2 groups. In light of these findings, researchers should be aware that transferring a mouse into a new but uncleaned cage may initiate aggression. In addition, researchers should avoid testing mice in an uncleaned testing apparatus that contains odors from previous subjects, because doing so may alter the behavior of the mouse to be tested. In addition, researchers should avoid exposing rodents to any soiled bedding or apparatus before, during, or after experimental manipulations.

To facilitate the behavioral scoring of identical mice, a group of researchers marked one of the mice in each pair with hair dye and unexpectedly found that the dyed male was significantly more likely to be the subordinate in the pair, whereas the unmarked male was more likely to be dominant.[22] Odor or visual cues (or both) likely helped establish this dominance hierarchy. The researchers suggest that because the male mice in each pair were selected randomly, the bias for the marked male to be the subordinate is not a confounding factor. However, in future experiments that require this type of marking, both mice in a pair should be marked, albeit with different patterns.

Housing conditions (such as individual and group housing) are believed to affect the physiology of male rats.[29] Rats housed with 1 or 3 cagemates of the same age, strain, and sex in a 930-cm^2 cage had lower average heart rates and mean arterial blood pressure than did singly housed rats. Decreases in heart rate and arterial blood pressure are indicative of a decrease in stress-like responses. Restraint and tail-vein injection resulted in greater heart rate and mean arterial blood pressure in rats housed 2-per-cage than in singly housed rats. These variations in stress levels of male rats in different housing conditions could affect pheromone production, which in turn might alter measured dependent variables in experimental tests. To eliminate this potential variable, housing conditions must be controlled for when results are compared within and across studies.

Some investigators[7] have suggested that male and female rats housed in identical housing conditions experience different biologic effects. Male rats in the crowded condition (housed 4 per cage; $32 \times 20 \times 18$ cm) have higher corticosterone than do female rats housed at the same density but in smaller ($27 \times 15 \times 13$ cm) cages. Male rats develop increased corticosterone levels under crowded conditions, whereas females show a significant decrease in corticosterone after crowding.[7] Because of the competitive nature of male rats, dominant male rats could increase the stress levels of the subordinate rats.

Although housing mice in groups can lead to physiologic changes, such as an increase in corticosterone, in subordinate animals, other studies show that group housing is not the direct cause of these changes but is a side factor that exacerbates the situation. When researchers used a T-maze and urine sampling to test the effects of group housing with subordinates and aggressive mice on learning and memory,[14] they found that although subordinate mice showed higher levels of corticosterone and deficits in learning and memory, the differences may not have been due to housing subordinates with more aggressive mice. Rather, the housing may have only contributed to the increased levels of stress hormone and subsequent deficits in cognition. The researchers came to this conclusion by measuring urine both before and after group housing; even after removing subordinates from group housing, they still showed elevated levels of corticosterone.[14] More importantly, even when these subordinates were housed alone again, they still showed deficits in the T-maze task. The authors suggested that subordinate mice had a preexisting proclivity toward having higher levels of corticosterone when exposed to the stress of group housing.[13] They extended their work by comparing corticosterone differences in subordinate and dominant males. Although subordinate mice had higher levels of corticosterone than did dominant mice, the subordinate mice did not perform poorly on the T-maze task.[13] This finding further supports the idea that group housing may not be the direct cause of physiologic and behavioral changes in mice; rather the hormone itself may, through the activation of the hypothalamic–pituitary–adrenal axis, induce cognitive deficits.[13]

Guidelines for housing conditions, bedding changes, and sanitation procedures have been developed for the welfare of the experimental animals and caretakers. However, these procedures affect the natural pheromonal environment of the rodents and can therefore affect their behavior both in their homecages

and in behavioral experiments. It is important to consider how these procedures might alter behavioral testing, not only within a given laboratory but also between laboratories that may have different housing and husbandry procedures.

Alarm Pheromones

In general, animals release alarm pheromones in response to stress or danger, as warning signals to conspecifics. In rodents, alarm pheromones most often result in defensive and vigilance behaviors. For example, mice responded with a significant increase in behaviors such as climbing, air sampling, and rearing when they were exposed to odors from stressed conspecifics.[33] The recipient mice in the cited experiment were exposed via chamber airflow to odors from either stressed or nonstressed conspecifics. Stressed conspecifics each received a footshock and an incision in the tail for blood sampling (a good source of alarm signal pheromones) and, 1 min after these procedures, were placed into a donor chamber in a separate room. A recipient mouse was placed immediately into a connecting chamber and therefore was exposed to odors from the stressed mouse: it had no other sensory experience of the stress other than the olfactory cue. Experimenters used the dual-chamber apparatus in a conditioned place-avoidance experiment; the time spent in the chamber with a stressed conspecific's odor compared with a nonstressed conspecific's odor was recorded, as well as the duration and frequency of behaviors including avoidance; freezing; licking of the body, head, or tail; sniffing of the air; urination; and defecation. Mice spent significantly more time in the nonodorized chamber when a stressed conspecific's odor was present than when a nonstressed conspecific's odor was applied. These differences diminished 30 min after exposure, indicating that the mice habituated to the odor.[33] Unlike pheromones that signal long-term or complex changes (for example, as sexual attractants, estrous mediators, social recognition signals), alarm pheromones result in immediate responses.[19] For this reason, researchers should consider the potential effects of alarm pheromones on the behavior of conspecifics during routine laboratory procedures, as they may confound behavioral testing data.

For example, researchers[2] demonstrated a possible role of alarm pheromones in rodent behavior in the Porsolt forced-swim paradigm. In an experiment designed to elucidate different interpretations of the behavioral despair aspect of the paradigm,[2] researchers compared the immobility times of male Long–Evans rats tested under multiple conditions. In one condition, subjects were tested in water that had been previously used during tests of other subjects. Immobility time differed significantly between subjects placed in clean water compared with used water; animals placed in clean water displayed significant immobility responses, whereas rats in contaminated water displayed little to no immobility. The authors hypothesized that alarm pheromone from the previous animals was present in the water.[2] To test this hypothesis, they assessed rats in water containing feces and urine from other animals. Under these conditions, rats behaved similarly to those tested in clean water. In addition, rats tested in water that had been used in a previous experiment 8 d prior displayed increased immobility, suggesting that alarm pheromone can remain biologically active for more than 1 wk.[1]

Alarm pheromone was investigated in more detail in another experiment.[17] Investigators found that the stress-induced alarm pheromone is water-soluble, is released from the perianal region, and aggravates stress-induced hyperthermia. This stress-induced pheromone also enhances the acoustic startle response. Other researchers[20] further characterized this response to alarm pheromone by demonstrating the presence of 2 different alarm pheromones in rodents, each released from a different area of the rodent and each with different functions. Odors released from the perianal region of donors that received electrical stimulation induced autonomic stress responses in recipient rodents, whereas odors released from the whisker region tended to induce behavioral changes. The whisker-area odors induced changes in resting behavior, rearing behavior, walking, and sniffing, all of which are dependent measures in many behavioral assays (Figure 2).[20]

One group of investigators[21] compared the behavior of intact rodents with that of rodents in which the VNO had been removed and thus demonstrated that pheromonal responses induce autonomic stress responses.[21] Three groups of male rodents (intact, vomeronasal organ-excised, and sham) were presented with alarm pheromones collected from donor animals after electrical stimulation to the neck or perianal region. Body temperature and behavior were recorded to determine the autonomic and behavioral responses to the pheromone presentation. Subjects with intact VNO showed significant autonomic responses (increases in stress-induced hyperthermia) when presented with pheromone, whereas subjects lacking intact VNO showed no such response.

Despite these known effects of alarm pheromones on rodent behavior, many current laboratory procedures and protocols do not take these effects into account. Laboratory stressors such as handling, injections, and tail-bleeds of a subject can affect the behavior of nearby but unmanipulated animals for as long as 30 min.[33] To avoid this confound, manipulated rodents could be separated from unmanipulated animals for 30 min after undergoing a stressor. This practice likely would dramatically reduce the alarm pheromone response. In addition, behavioral testing should not take place until approximately 30 min after a laboratory stressor to ensure that resulting data are not confounded by responses to alarm pheromone. However, immobility time in the Morris water maze did not change despite the number of retests,[1] suggesting that habituation may not occur. Additional research is needed to determine whether animals are able to habituate to this important signal over time.

Alarm pheromones[2] are also major concerns in behavioral testing. Any behavioral assay that uses water may not provide accurate data if the water is contaminated by alarm pheromone. Although difficult in practice, the ideal solution would be to change the water for every test subject. An alternative solution to this confound must be determined: the forced-swim test is used as a clinical model of depression,[27] and if one animal's behavior is dependent on that of the preceding animal, data from later tests must be considered to be influenced by responses to pheromones. In these cases, statistical analyses should be performed to look for an order effect in the animals tested during a given session. In addition, rodents should not be placed in short-term holding areas that previously held other rodents, because doing can cause stress in subjects even before the experiment has begun. Current research in alarm pheromone responses indicates that their effects on behavioral testing must be considered thoroughly during the design of lab protocols and procedures.

Conclusion

Although strict guidelines govern cage cleaning and sanitation procedures, data indicate that the standard frequency of cleaning may be excessive in light of its potential to eliminate elicited pheromones from the enclosures. The frequency of

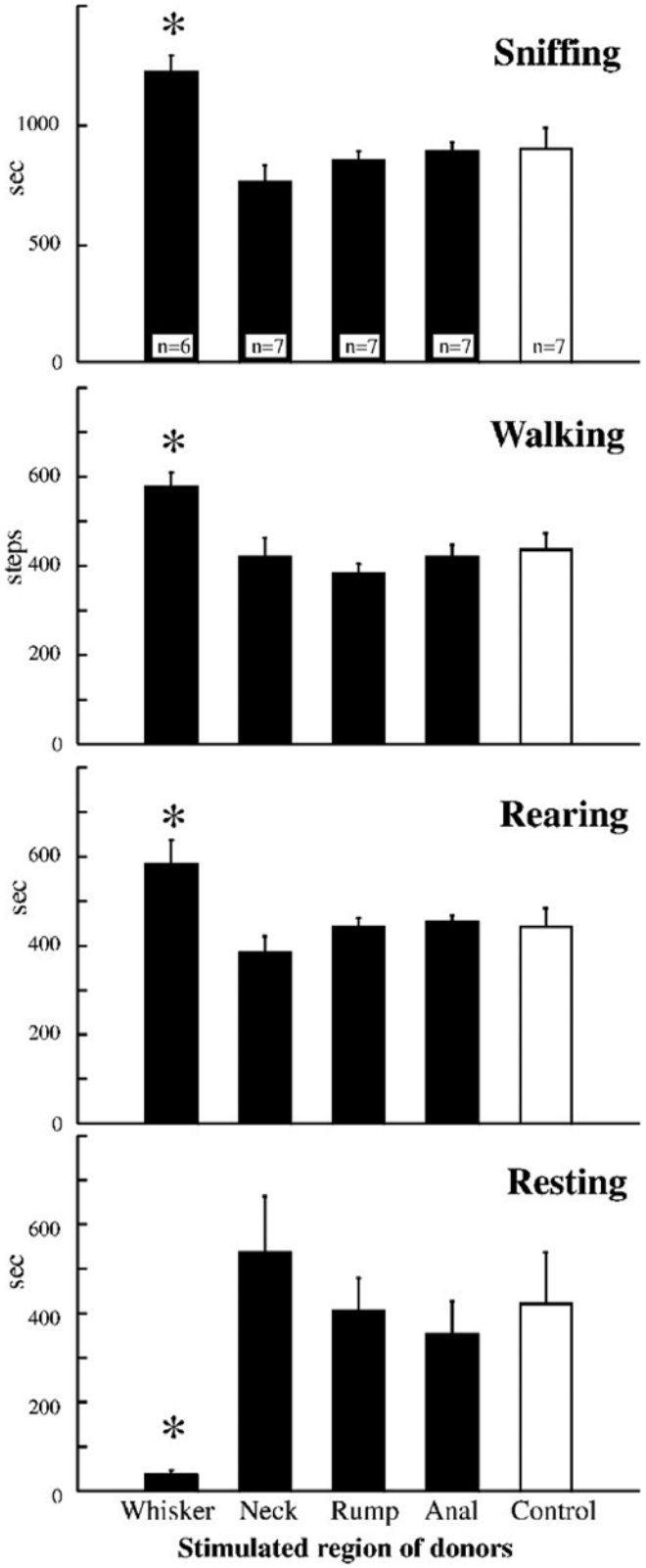

Figure 2. Stimulation of specific regions on the rodent leads to significant changes in behavior due to pheromone release. Pheromones released from the whisker area of the rat significantly reduced resting time but increased sniffing, walking, and rearing. Reprinted from reference 20 with permission from Oxford University Press.

cage-cleaning in vivaria typically varies from 3 to 14 d, depending on the colony size and housing conditions, with rodents housed in ventilated cages or in smaller colonies receiving less frequent bedding changes. Alternatively, frequent changes for rodents housed in static cages and those in larger colonies are necessary for the health of the colony. Clearly, laboratory protocols regarding bedding changes are focused on the health of animals, and this attention is paramount to potential consequences of these procedures on animal behavior. However, the effects of bedding changes on the natural pheromone-based environment of the rodents and even on various experimental results are largely unknown.

In particular, different housing conditions may be necessary to control stress in male and female rodents,[22] given that male animals are more competitive with each other than are females. Controlling exposure to other male scents may also be important in male rodents because of their competitive nature. Because of the demonstrated effects of alarm pheromonal signaling on conspecific behavior, continued research into the specific function and origin of the alarm pheromone is warranted. Current research in this field demonstrates that rats release alarm pheromones from various areas of rats, and these pheromones can induce specific stress behaviors that may confound common rodent behavioral tests. Research in pheromonal signaling should address the relationship between experimenter interaction with rodents and pheromone release. Additional studies might characterize the pheromones that mediate different responses to housing and cleaning conditions, including where and how the signals are released and their chemical composition. Finally, the effect of pheromonal signaling on behavioral responses in experiments should be addressed. Although the effect of alarm pheromones on performance in the Morris water maze and forced-swimming test has been demonstrated,[1-3] little research is available on pheromonal effects in other experimental paradigms.

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