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Aggression and plasma testosterone in male golden hamsters (*Mesocricetus auratus*) in response to encounters with receptive vs. nonreceptive females

Xin Zhao and Dingzhen Liu

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Abstract: Exposure to sexual stimuli can lead to increased aggression in male mammals, but it is unclear whether the aggression is related to the receptiveness of the females. Interactions with receptive females elicit testosterone (T) pulses that are important for sexual behaviors. We investigated the effects of male–female interactions on subsequent aggressive behaviors and T responses in the golden hamster (*Mesocricetus auratus* (Waterhouse, 1839)). Three groups (n = 18, 17, and 18) of males were exposed to receptive females, nonreceptive females, and blank (control), respectively. Then, we randomly chose eight animals from each group and measured their aggression toward an unfamiliar male conspecific; the remaining 29 males were used for a T assay (to avoid effects of aggression on T levels). The results show that interactions with females led to significantly higher male aggression and T levels than were found in control males. The increased aggression was not related to the receptiveness of the females, but receptive females elicited higher levels of T in males than nonreceptive females. Our findings suggest that the elevated aggression following the sexual encounter may serve to defend the female, by enhancing the ability of males to exclude other males from the vicinity of females, whereas the post-encounter T release may serve to assist mating behaviors and reproductive success.

Key words: aggression, golden hamster, *Mesocricetus auratus*, receptiveness, sexual encounter, testosterone.

Résumé : L'exposition à des stimuli sexuels peut mener à une plus grande agressivité chez les mammifères mâles, mais la présence d'un lien entre cette agressivité et la réceptivité des femelles n'est pas bien établie. Les interactions avec des femelles réceptives provoquent une production ponctuelle de testostérone (T) qui est importante pour les comportements sexuels. Nous avons examiné les effets d'interactions mâle–femelle sur les comportements agressifs subséquents et les réactions de la T chez le hamster doré (*Mesocricetus auratus* (Waterhouse, 1839)). Trois groupes (n = 18, 17, and 18) de mâles ont été exposés à des femelles réceptives, à des femelles non réceptives et à des blancs (témoins). Nous avons ensuite sélectionné aléatoirement huit individus de chaque groupe et mesuré leur agressivité envers un mâle conspécifique non familier; les autres 29 mâles ont été utilisés pour l'analyse de la T (pour éviter des effets de l'agressivité sur les concentrations de T). Les résultats montrent que les mâles ayant interagi avec les femelles présentent une agressivité et des concentrations de T significativement plus importantes que les mâles témoins. Cette agressivité accrue n'est pas reliée à la réceptivité des femelles, bien que les femelles réceptives aient élicité de plus fortes concentrations de T chez les mâles que les femelles non réceptives. Nos constatations portent à croire que l'agressivité élevée suivant un rapport sexuel pourrait servir à défendre la femelle en accroissant la capacité du mâle d'exclure d'autres mâles des environs de cette dernière, alors que la libération de T après le rapport pourrait servir à soutenir des comportements d'accouplement et le succès de reproduction. [Traduit par la Rédaction]

Mots-clés : agressivité, hamster doré, *Mesocricetus auratus*, réceptivité, rapport sexuel, testostérone.

Introduction

Sexual selection theory predicts that when females are a limited resource, males will increase aggression and challenge one another for access to females (Andersson 1994; Jirotkul 1999; Xu et al. 2012). From an evolutionary perspective, such increased boldness and aggression allows alpha males (dominant individuals) to monopolize access to females, which eventually would increase their reproductive fitness (Bernstein 1976; Wilson et al. 2010; Gilby et al. 2013). In seasonal breeding animals, male–male competition and aggression is closely associated with female reproductive state, e.g., estrous peak or receptiveness (Beach 1976). In contrast to seasonally breeding animals, female rodents typically have rapid estrous cycle times of 4–5 days (Dewsbury et al. 1977). These cycles continue unless the female becomes pregnant and these cycles are expressed as receptive and nonreceptive periods. Exposure to female golden hamsters (*Mesocricetus auratus* (Waterhouse, 1839)) at different stages of receptiveness can induce various copulatory responses and promote genital investigation behavior in male golden hamster (Macrides et al. 1984), yet male golden hamsters show similar preference for the odor of receptive to nonreceptive females, suggesting that nonreceptive females may also represent a potential reproductive resource (Johnston 1980). However, it is unclear whether males express differences in aggression after exposure to females that are either receptive or nonreceptive; if differences are expressed, then this would also further support the evidence that males can distinguish between receptive versus nonreceptive females.

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X. Zhao* and D. Liu. Ministry of Education, Key Laboratory of Biodiversity Science and Ecological Engineering, College of Life Sciences, Beijing Normal University, Beijing, People's Republic of China.

Corresponding author: Dingzhen Liu (email: dzliu@bnu.edu.cn).

*Present address: Department of Psychology, University of Wisconsin at Madison, Madison, WI 53706, USA.

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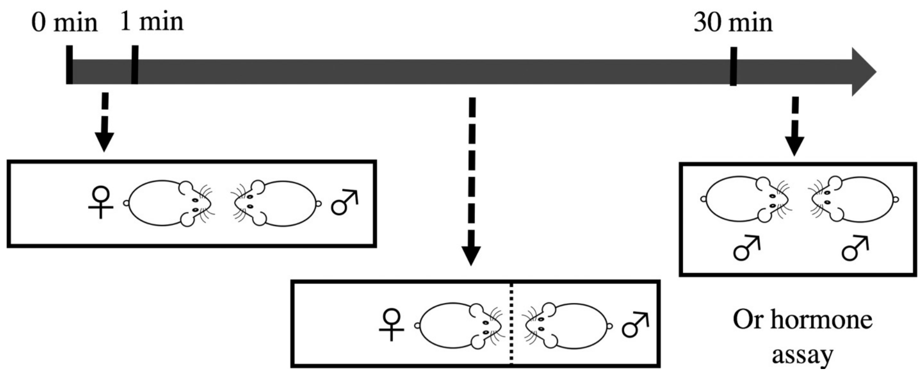
Fig. 1. A male–female encounter was conducted for 1 min during which the male golden hamster (*Mesocricetus auratus*) was allowed to investigate the female freely. We then inserted a wire mesh to separate the animals and left the apparatus untouched for 29 min. During this period, both the female and the focal male were able to interact but without physical contact. Immediately following the male–female encounter, males were randomly selected and experienced either a male–male agonistic encounter or blood sampling for future analysis of hormones.



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In addition to behavioral changes, golden hamsters exposed to a receptive female or its vaginal odor show elevated testosterone (T) levels in male golden hamsters (Macrides et al. 1974). However, the comparison between the T pulses after encountering receptive and nonreceptive females is still unavailable in this species. Testosterone pulses can be elicited both by male–male agonistic encounters and by male–female sexual encounters (for a review see Gleason et al. 2009). It is hypothesized that the T pulses may help males cope with the immediate situation (e.g., competition or a mating opportunity) that stimulates the release (Nyby 2008). Although nonreceptive females can represent a potential mating resources, only the receptive females show lordosis and are ready for mate; the T pulses may therefore be varied based on the receptivity of the females. Studies in rats and mice have demonstrated that encounters with receptive females elicit higher levels of T than encounters with nonreceptive females (Amstislavskaya and Popova 2004).

In this study, we used the laboratory golden hamster to investigate the aggressive behavior of males and the T pulses following short (30 min) interactions with females differing in receptiveness. The golden hamster is a solitary rodent species and is commonly used as an animal model for research on the hormonal mechanisms of aggression (Vandenbergh 1971; Payne 1973; Romeo et al. 2003). The females show a typical 4 day estrous period unless the female becomes pregnant (Deanesly 1938). Both male and female golden hamsters show aggressive behavior to conspecifics (Lai and Johnston 2002; Bath and Johnston 2007). We hypothesized that increased aggression serves to compete for access to mating resources and predicted that the levels of aggression does not rely on the receptivity of the females because the nonreceptive females also represent potential mating resources. We also hypothesized that the T release mainly serves to facilitate the mating behaviors and predicted that the levels of T would be higher in males exposed to receptive females than the males exposed to nonreceptive females.

Materials and methods

Subjects

The subjects were 77 male golden hamsters purchased (4–5 weeks old) from Weitong-Lihua Experimental Animal Technology Inc., Beijing, People's Republic of China. We used 16 female golden hamsters bred in our laboratory for male–female encounter (12–16 weeks old at the time of our experiments). All subjects were housed individually in polycarbonate cages (32 cm × 20 cm × 16 cm); food and water were provided ad libitum. The animal room was maintained on a reversed 14 h light : 10 h dark cycle (the lights came on at 1900), at a temperature of 23–27 °C, and a relative humidity of approximately 50%. All subjects were naïve and females had normal estrous cycles before the experiment. Males had no experience in fighting or sexual encounters and were kept individually for 6–7 weeks to allow them to adapt to their new environment and establish territoriality prior to the experiment. All experiments were completed within 3 h of commencement of the dark cycle; illumination was provided by a 25 W red light. Animal use and handling standards met the Animal Welfare Guidelines of the National Regulation on Laboratory Animal Research, Chinese Ministry of Science and Technology, People's Republic of China, and our protocols were approved by the Animal Research Ethics Board of Beijing Normal University.

Encounters between males and females

Fifty-three male golden hamsters (focal males) were randomly selected and divided into three groups that encountered receptive females (n = 18), encountered nonreceptive females (n = 17), or were placed in an empty arena (control; n = 18). Encounters took place in a rectangular arena (50 cm × 14 cm × 13 cm). A transparent polycarbonate cover was used to prevent animals from escaping. The arena contains two compartments divided by a wire mesh (0.6 cm grid): one compartment (35 cm × 14 cm × 13 cm) was for the female golden hamster and the other (15 cm × 14 cm × 13 cm) was for the male golden hamster (Fig. 1). A fan blew air from the female chamber to the male chamber.

We recorded the estrous cycle of the females based on a previous study in golden hamsters (Solomon et al. 2007). Briefly, we placed a cotton swab against the vaginal area. A thin, stringy vaginal discharge signified vaginal estrus. We defined a female as receptive if it displayed lordosis within 1 min of being placed with a sexually experienced male from a separate cohort (Johnston and Peng 2000). Briefly, we put a female on the top of the male cage. Both the male and female can sniff each other via the wire mesh of the male's cage without physical contact for 1 min. We then pressed the back of the female with a finger and tested if she showed lordosis or not. Once its receptiveness had been assessed, the receptive or nonreceptive female was placed in the arena with a focal male. The male was allowed to investigate the female freely for 1 min (by sniffing of snout and glands) so that he could detect both volatile and nonvolatile odor components from the female golden hamster. We then inserted a wire mesh to separate the animals, covered the arena, and left the apparatus untouched for 29 min. During this period, both female and focal male can see and sniff each other but have no physical contact. The arena was cleaned with 75% ethanol after each female exposure. Control males were allowed to investigate the whole cleaned and deodorized arena for 1 min and were then confined to the male compart-

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ment for 29 min (Fig. 1). Stimulus females were used up to three times, but never more than once in the same treatment group. Also, the use of the same female was counterbalanced across the animals in the behavioral and hormonal experiments.

Encounters between males and analysis of behavioral variables

Immediately following exposure to a stimulus female or control, eight focal males from each group were randomly selected and exposed to staged encounters with unfamiliar males. We assessed aggression using a neutral arena model in which two animals are placed in a novel neutral environment and aggressive behaviors are recorded. In a neutral arena, interactions between two male golden hamsters usually escalate into a fight until one male flees or shows an obvious submissive posture (Lai and Johnston 2002; Bath and Johnston 2007). The neutral arena in our experiment was a transparent plastic cage (36 cm × 23 cm × 21 cm) without a cover. Twenty-four male golden hamsters other than the focal male golden hamsters were selected to serve as opponents to the 24 focal males. Because it is unlikely that we would find a matched opponent with the same body mass, we consistently chose opponents that were 5–10 g heavier than the focal males to eliminate the advantage caused by size in golden hamsters (Petrulis et al. 2004); the additional benefit was that we refined the sensitivity with which experience-induced changes in aggression could be detected. In the arena, subjects were able to wander freely, sniff each other, or fight. The initiation of fighting was recorded as the time when one individual initiated an attack by jumping at or lying across the back of his opponent while biting its flank (Bath and Johnston 2007). The other male then either allowed itself to be placed on its back and remained there or initiated a counterattack by biting and kicking with its hind legs. If a counterattack occurred, then the fight developed into a rolling fight characterized by tussling. The end of a fight was defined when one individual (the loser) showed an obvious submissive posture such as freezing with rump upward or attempted to jump out of the arena. We used a field television system (Fuhrman Diversified Inc., Seabrook, Texas, USA) mounted 50 cm above the arena to record behaviors. We then recorded all behaviors that occurred, from the first sniff to the end of the fight, or for 8 min duration, which ever occurred first.

Video files were coded and analyzed by a person who was blind to the experimental design using Observer XT software (Noldus Information Technology, Wageningen, the Netherlands). The following variables were measured or calculated: mean frequency of attack (the number of attack divided by the sum of attack and submissive behavior) and latency to attack (the time from the first sniff to the first attack). In addition, to quantify the proportion of focal males winning the fight, we gave a score of one to the focal males that won the fight and elicited losing responses in the opponents (i.e., obvious submissive postures or attempts to jump out of the arena). We gave a score of zero to the focal males that lost the fight and a score of 0.5 to the focal males for which the fight ended in a draw after 8 min. These scores were used to calculate a mean proportion winning the fight for each group of focal males.

Hormonal assay

We collected blood samples from the remaining 29 focal males that did not engage in the aggressive encounters to measure hormone levels. The 29 male golden hamsters were divided into three groups that encountered receptive females (n = 10), nonreceptive females (n = 9), or the empty arena (control; n = 10), following the protocol described above. Previous research has shown that plasma T peaks 30–60 min after a male golden hamster encounters a female (Richardson et al. 2004). Thus, after spending 30 min in the arena with or without a female, males were immediately decapitated using a small animal guillotine (without anesthesia) and blood samples were collected based on the protocol of a previous study on golden hamsters (Romeo et al. 2003). Blood was centrifuged at 3000 rev/min for 10 min. Plasma was pipetted into a 1.5 mL centrifuge tube and stored at −80 °C. Samples were extracted with ethyl ether and steroids were separated using Celite chromatography. The samples were analyzed via radioimmunoassay kits (Aomei Biological Technology Inc., Beijing, People's Republic of China). The detectable range of the assay was 0–20 ng/mL and the sensitivity was higher than 0.02 ng/mL. The intra- and inter-assay coefficients of variation were 3.1%–6.1%.

Data analyses

We tested the continuous data for normality using the Kolmogorov–Smirnov test. For latency to attack, one outlier was removed from the control group based on the extreme Studentized deviate (ESD) outlier test. For each of the four variables, we compared the data for male golden hamsters exposed to receptive females, nonreceptive females, and no females (the control). We used the Kruskal–Wallis test followed by Dunn's multiple comparison test to analyze the data on latency to attack, which deviated from a normal distribution. We used one-way ANOVA followed by the post hoc least significant different (LSD) test to analyze the data on mean frequency of attack and plasma T. For the proportion winning the fight, we first used Fisher's exact test, then used the Cochran–Armitage trend test to analyze the trend between treatments and control and the probability of males winning the fight. We used SAS for all the statistical analyses with α = 0.05.

Results

Aggressive behavior in male golden hamsters and exposure to females

Prior interaction with females significantly affected the latency of attack in male golden hamsters (H = 11.73, p = 0.003). Dunn's multiple comparison test revealed that the latency to attack in males exposed to receptive and nonreceptive females was shorter than that in control males (both $p < 0.05$), but female receptiveness had no effect on the latency to attack in males (Fig. 2*a*).

Prior interaction with females also affected the mean frequency of attack in males ($F_{[2,23]}$ = 4.70, p = 0.02). The mean frequency of attack in males exposed to receptive and nonreceptive females was higher than that in control males (both $p < 0.05$), yet female receptiveness had no effect on the mean frequency of attack in males (Fig. 2*b*).

Fisher's exact test showed that prior interaction with females significantly affected the probability that male golden hamsters would win fights (p = 0.05). The Cochran–Armitage trend test showed that male golden hamsters that had been exposed to receptive females were more likely to win fights than males exposed to nonreceptive females or control males (p = 0.02; Fig. 2*c*).

Testosterone levels in male golden hamsters and exposure to females

Prior interaction with females significantly affected plasma T levels in male golden hamsters ($F_{[2,28]}$ = 15.44, p = 0.01). Plasma T levels were significantly higher in males that were exposed to receptive females than in males exposed to nonreceptive females (p = 0.04) and in control males ($p < 0.01$); T levels were also significantly higher in males exposed to nonreceptive females than in control males (p = 0.02; Fig. 2*d*).

Discussion

Our results revealed for the first time that the aggressiveness of male golden hamsters increased after a short (30 min) period of interaction with a female as a stimulus, and that this effect on aggression was not related to the receptiveness of females, but to the presence of females (reproductive resources). In comparison, the hormone assay revealed differences in T increases following the encounters with receptive versus nonreceptive females; the T

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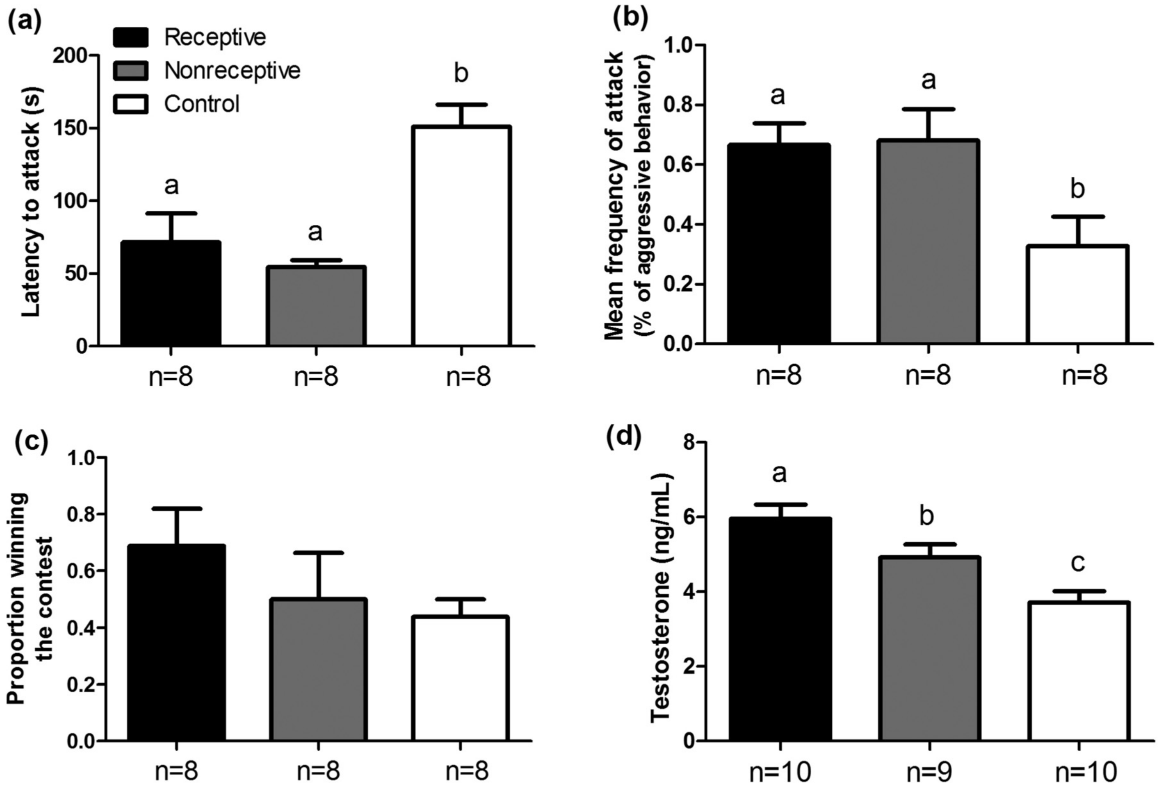
Fig. 2. Latency to attack (*a*), the mean frequency of attack (*b*), the proportion winning the fight (*c*), and plasma testosterone concentration (*d*) for male golden hamsters (*Mesocricetus auratus*) exposed to receptive females (black bars), nonreceptive females (gray bars), and controls (white bars). Data are mean + SE, with different letters above indicating significant differences ($p < 0.05$).



level in males exposed to receptive females was higher than in those exposed to nonreceptive females. This is consistent with previous studies showing that exposure to a receptive female increases the T levels in male golden hamsters (Macrides et al. 1974) and encounters with receptive female rats elicit higher levels of T in males than encounters with nonreceptive females (Amstislavskaya and Popova 2004); aggression was not, however, measured in these studies. Overall, our findings suggest that the increased aggression following the sexual encounter may facilitate the defense of mating resources (receptive and nonreceptive females), by enhancing the ability of males to exclude other males from the vicinity of females, while the T responses to the sexual encounters may facilitate the sexual behaviors, which supports the hypothesis that the T pulses may help males cope with the immediate situation (e.g., a mating opportunity) that stimulates the release (Nyby 2008).

At the behavioral level, irrespective of whether the females were receptive or nonreceptive, males that experienced prior contact with a female engaged in conflict with other males sooner and were more likely to initiate an attack than males in the control group that did not have contact with a female. In the control group, no males initiated any attack in the first 2 min of male contact, whereas more than half of the female-exposed males initiated a fight within 1 min. Although nonreceptive females do not elicit mounting behavior by males, they still represent a potential mating resource. Therefore, the level of aggression induced by female contact may be related to the perceived reproductive value of the female rather than to her receptiveness. This is consistent with a previous study, which showed that male golden hamsters have no preference for the odor of receptive or nonreceptive female conspecifics even though both males showed interests in the odors of both females (Johnston 1980). Female golden hamsters are continuously polyestrous and typically have rapid estrous cycle of 4–5 days (Dewsbury et al. 1977) throughout the year. Increased aggression in males exposed to females may therefore facilitate the attraction and protection of a potential mating resource, since dominance by aggressive male golden hamsters acts as a sexual display to bring about reproductively beneficial mating opportunities (Huck et al. 1986; Brown et al. 1988).

At the hormonal level, the pattern of the T surge of this study (Fig. 2*d*) was similar to that found in male golden hamsters that were exposed to a receptive female or its vaginal discharge (Macrides et al. 1974). Testosterone pulses in males can be elicited both by male–female sexual encounters and by male–male agonistic encounters (for a review see Gleason et al. 2009), but their functions may vary depending on the context. Goymann et al. (2007) suggested that T responses to male–female interactions should be considered to be distinct from T-level changes after male–male competition and from seasonal changes in plasma T. In the current study, we did not directly compare the T responses to male–female and male–male interactions, but our results may suggest that the T pulses following sexual encounters are more closely related to reproductive behaviors which relies on the receptivity of the females. Chemical studies of golden hamster vaginal discharge found that the concentration of the major proteins, which play a direct role in the facilitation of male golden hamster copulatory behavior, dropped by about 10-fold in transition from the estrous to the diestrous condition (Singer et al. 1984). Such difference in the chemosensory stimuli could alter the increases of T levels (Wood and Coolen 1997), which could help males to achieve mating, by suppressing distractions so that males are able to approach females and mate with them successfully (Aikey et al. 2002).

We found that nonreceptive females induced higher levels of T in males than occurred in control males exposed only to the

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empty arena, which is inconsistent with previous research showing that nonreceptive females did not elicit significantly increased levels of T in male mice and rats (Amstislavskaya and Popova 2004). The discrepancy may result from the distinct social environments experienced by group-living and solitary rodents. For solitary golden hamsters, guarding potential mating resources (e.g., nonreceptive females that are soon likely to become receptive) may be important for reproductive success, and the increased T pulses thus may be needed to facilitate aggressive behavior for guarding. In addition, T may reinforce learning associated with the site of a sexual encounter; at such a site, males may have a high probability of encountering a potential mate in future (Gleason et al. 2009). Previous studies in golden hamsters have demonstrated that the partner preference and copulatory behaviors are dependent on the actions of T (Powers et al. 1985; Ballard and Wood 2007). Also, the rewarding effects of T in producing a conditioned place preference have been demonstrated in golden hamsters (Wood et al. 2004), rats (Alexander et al. 1994) and mice (Arnedo et al. 2000; Zhao and Marler 2014, 2016).

Even though we speculate that the elevated T levels following the encounter with receptive females may primarily serve to facilitate mating behaviors, we cannot exclude the possibility that T influenced aggressive behavior in male golden hamsters. Aggression could be triggered when T exceeds a threshold and an increase above the threshold may not contribute to the enhancement of aggression. A T threshold for the maintenance of copulatory behaviors have been reported in male golden hamsters (Powers et al. 1985). Moreover, on the neurophysiological level, other factors in the T modulation pathway such as metabolic enzymes and receptor densities might affect the function of T. For example, the role of T in modulating aggression may vary with aromatase activity and estrogen receptor density in the central nervous system (Trainor and Marler 2002; Trainor et al. 2006), which are influenced by mating or mate-related cues (Hutchison et al. 1996; Gréco et al. 2003). In the male golden hamsters in our study, whether changes in T level, aromatase activity, and the density of estrogen receptors after contact with females collaboratively contribute to the increased aggressiveness needs further research.

Despite generating some potentially interesting findings, future studies could better tease apart what female stimuli influence male aggression and T levels. Firstly, in future studies, the sexual stimulus provided to the focal males could be extended by using females with other reproductive states, such as juvenile, pregnant, or lactating females, who will not come into estrus soon and so would have lower reproductive values. This would help to test the hypothesis that male aggression induced by encounters with females is related to the reproductive value of the female. Secondly, we could not demonstrate a causal relationship between hormone changes and increased aggressiveness in males because we used separate cohorts of male golden hamsters for the hormone assay and for the behavioral data. This could be ameliorated by collecting plasma samples from the same individuals that engaged in male–male encounters, by comparing the responses of intact and castrated males, or by comparing normal golden hamsters with those in which androgen receptors in the regions of the brain that regulate aggression had been blocked. Thirdly, in future studies, the effect of sexual experience on the relationship between female contact and male aggressiveness could be evaluated. All the male golden hamsters in the current study were sexually inexperienced, but the level of sexual experience may influence preferences and aggressive behavior (Bergvall and Hansen 1990).

In summary, male–male aggressive behavior and the proportion of winning the fight in golden hamsters were facilitated by encounters with females, regardless of the reproductive state of females (receptive or nonreceptive). The elevated aggression is likely to serve to defend females, by enhancing the ability of males to exclude other males from the vicinity of females. Plasma T levels in male golden hamsters were also increased after encounters with females, but the increase was greater when females were receptive than when they were nonreceptive. This suggests that the post-encounter T release may serve to assist male–female sexual encounters and reproductive success.

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