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THE ROLE OF THE GONADS AND INCEST AVOIDANCE IN THE REGULATION
OF SEXUAL BEHAVIOR IN DAMARALAND MOLE-RATS

by

Sara Nicole Carter

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ABSTRACT

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The Damaraland mole-rat (DMR; *Fukomys damarensis*) is one of a small number of mammals that are eusocial. In this mating system, only a single pair reproduce while the remaining colony members are non-reproductive. Non-breeders exhibit sexual behavior only when paired with unfamiliar opposite-sex individuals. The role of the gonads in the regulation of reproductive behaviors in non-breeders remains unknown; however, inbreeding avoidance has been hypothesized to account, in part, for the lack of reproductive behavior in non-breeding individuals. The mechanism that regulates incest avoidance also remains unknown. The aims of this study were twofold; first, to investigate the role of gonads in the expression of sexual behaviors and second, to identify the mechanism of incest avoidance. The results indicate 1) that the expression of sexual behavior is largely independent of the gonads and 2) that rather than incest avoidance *per se*, DMR avoid mating with familiar individuals irrespective of relatedness.

Table of Contents

Chapter	Page
1 Introduction	1
2 Social Cues Induce Sexual Behavior in Gonadectomized Male and Female Damaraland Mole-Rats	7
3 Incest Avoidance via Familiarity Rather than Genetic Relatedness	24
4 Conclusion	31
Appendix	40

List of Figures

Figure	Page
1. The probability of sexual behavior occurring between unfamiliar opposite-sex individuals. Both females and males were equally likely to engage in sexual behaviors when paired with an unfamiliar opposite-sex individual and gonadal status did not result in significant alterations in the probability of expression of sexual behavior in either sex (n = 8/group; $p > 0.07$ for each comparison)	40
2. (A) Duration of sexual behavior, (B) Latency to sexual behavior, and (C) Number of mounts expressed by GDX and gonadal-intact male DMR paired with unfamiliar females. Gonadectomy did not result in significant alterations in any measure ($p > 0.25$ for each comparison).	41
3. (A) Latency to female solicitation, (B) Number of bouts of female solicitation, Gonadectomy did not result in significant alterations in these measure ($p > 0.25$ for each comparison) (C) Latency to lordosis, (D) Number of lordoses, (E) Lordosis quotient; Gonadectomy did result in Significant alterations in these measures of sexual behaviors expressed by GDX and gonadal-intact female DMR paired with unfamiliar males (*; ANOVA; $p < 0.05$; n = 16).	42
4. Probability of sexual behavior mounting and thrusting for males, solicitation for females occurring between gonad intact familiar siblings and the same siblings after 5 weeks of separation. Overall, the probability of sexual behavior occurring between siblings increased following 5 weeks of separation (*; ANOVA; $p < 0.0001$; n = 16).	43
5. Duration of sexual behavior (A), Latency to sexual behavior (B), and Number of bouts of sexual behavior (C) between siblings before and after five weeks of separation. Rendering siblings unfamiliar after separation resulted in a significant increase in the duration of sexual behavior (ANOVA; * $p < 0.03$); a significant decrease in the latency to initiate sexual Behaviors (ANOVA; $p < 0.0001$), and an increase in the number of bouts of Sexual behavior (ANOVA; # $p < 0.037$).	44
6. Latency to sexual behavior (A), and Number of bouts of sexual behavior (B) between siblings before and after five weeks of separation. Rendering siblings unfamiliar after separation resulted in a significant	45

decrease in the latency to initiate female solicitation (ANOVA; $p = 0.0097$), and an increase in the number of bouts of female solicitation (ANOVA; $p < 0.0469$).

Chapter 1: Introduction

Two mammalian species meet the criteria to be considered eusocial, naked mole-rats (NMR; *Heterocephalus glaber*) and Damaraland mole-rats (DMR; *Fukomys damarensis*; Holmes, Goldman, Goldman, Seney, & Forger, 2009). Eusociality is a social system in which animals form colonies that consist of overlapping generations of adults in which only a single breeding pair within the colony engage in reproduction, while the remaining members are non-reproductive but support the reproductive efforts of the breeders. This rare strategy appears to have arisen independently multiple times in different species (Holmes et al., 2009). The selective advantages of eusociality in mammals remain speculative, although multiple non-mutually exclusive hypotheses exist to explain the evolution of eusociality. Two of these hypotheses that have received the most attention regarding the evolution of cooperative breeder, of which eusociality is an extreme form of, are the ecological constraints hypothesis and the life history hypothesis. The ecological constraints hypothesis posits that cooperative breeding occurs as a result of the lack of suitable territories capable of supporting individual breeding, coupled with a high mortality risk associated with dispersal, and low chances of finding a suitable mate. Thus, the odds of an individual successfully breeding are low (Hatchwell & Komdeur, 2000). The life history hypothesis emphasizes the role of life history traits such as clutch or litter size, dispersal, and longevity. This hypothesis suggests that in species with delayed maturity, high adult survival, and low reproductive and dispersal rates cooperative breeding is more likely to evolve (Hatchwell & Komdeur, 2000).

Within colonies of DMR, only a single female (often termed the “queen”) and her male partner mate and produce offspring. The remaining colony members help raise the

offspring and act as workers helping to forage, dig tunnels, and defend the colony (Jarvis & Bennett, 1993). Female and male non-breeders in the natal colony refrain from reproduction. The lack of reproductive effort in both sexes of non-breeders, may occur through different mechanisms. In other cooperatively breeding species, there are multiple levels at which reproduction can be interrupted; e.g., suppression of gamete development (Haigh, 1987), suppression of ovulation (Abbott, 1984), the reabsorption of embryos (Rood, 1980), or through lack of necessary stimuli from a potential mate (Schoech, Mumme, & Moore, 1991; Solomon, Brant, Callahan, & Steinly, 2001). In the wolf (*Canis lupus*), dominant females may suppress reproduction in subordinate females through interruption of sexual behavior (Moltano & Bennett, 2000). Similarly, in the NMR, it is thought that the breeding queen actively suppresses reproductive development in subordinate females through physical contact (Faulkes & Abbott, 1997). Both of these examples illustrate the “dominant control” model of active reproductive suppression. It is not known whether the lack of reproductive effort in non-breeding DMR is the result of active suppression by one of the breeders (i.e., dominant control), or due to the lack of the proper stimulatory cues in the natal colony (Burland, Bennett, Jarvis, & Faulkes, 2004; Clarke, Meithe, & Bennett, 2001; Faulkes & Bennett, 2001; Moltano & Bennett, 2000). Attempts to characterize the mechanism underlying female reproductive suppression in DMR have yielded conflicting results; Moltano and Bennett (2000) suggest that removal of a non-breeding female from the natal colony results in reproductive maturation. In contrast, other studies indicate that when a non-breeding female is allowed to interact with an unfamiliar male from a different colony, she will become reproductively competent even in the presence of the breeding queen (Cooney & Bennett, 2000; Snyman

et al., 2006). The latter result argues against the dominant control hypothesis and instead favors the hypothesis that in DMR, the lack of direct reproduction by non-breeders results from the lack of appropriate stimulatory cues. Remarkably, in DMR, there is also evidence that suggests that incest avoidance alone is sufficient to prevent sexual activity in non-breeding females (Cooney & Bennett, 2000). Introduction of a foreign male into a colony of DMR that had become reproductively quiescent following loss of the breeding female resulted in a renewal of sexual activity in formerly non-breeder females (Rickard & Bennett, 1997). Even more striking, Rickard and Bennett (1997) noted that introduction of a single unfamiliar male into a fully functional breeding colony of DMR resulted in the rapid appearance of soliciting behaviors among non-breeding females, directed in all cases toward the unfamiliar male. In five of 10 colonies so treated, one of the previously non-breeding females was the only female that continued to show sexual activity three weeks after introduction of the unfamiliar male (i.e., the status of breeding queen in these five colonies was usurped; Cooney & Bennett, 2000).

Non-breeding females do not exhibit reproductive behaviors when housed only with members of their natal colony, observations that indicate the importance of incest avoidance as a determinant of sexual behaviors in DMR (Jacobs, Reid, & Kuiper, 1998). Taken together, the above findings suggest that multiple mechanisms may act to keep females from breeding while in their natal colony. Regardless of the mechanism, female non-breeders, even after attaining a mature body size, fail to undergo puberty and exhibit underdeveloped uteri and ovaries (Holmes et al., 2009). These females exhibit lower concentrations of basal luteinizing hormone (LH) and progesterone when compared to breeding females. Non-breeding females also fail to ovulate, and do not exhibit sexual

behavior while living within their natal colony (Bennett, Jarvis, Faulkes, & Millar, 1993). The reproductive axes of non-breeding males, on the other hand, are virtually indistinguishable from those of breeding males. While non-breeding males exhibit smaller testes sizes compared to breeding males, they show similar number of sperm, and circulating concentrations of testosterone as compared to breeding males (Faulkes, Trowell, Jarvis, & Bennett, 1994; Holmes et al., 2009; but see Maswanganye, Bennett, Brinders, & Cooney, 1999). Therefore, it is possible that the mechanisms regulating reproductive behaviors differ between the sexes. Our primary objectives were to test the hypothesis that the expression of sexual behaviors in DMR is independent of the gonads in both males and females, and to determine the mechanism underlying inbreeding-avoidance. Thus, we determined whether the expression of sexual behaviors persisted following removal of the gonads, as well as the effect of separation on incest avoidance between previously familiar siblings.

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Chapter 2: Social Cues Induce Sexual Behavior in Gonadectomized Male and Female Damaraland Mole-Rats

Damaraland mole-rats (DMR) are eusocial mammals that live in colonies from two to approximately 40 individuals. Within these colonies only a single female and her male mate reproduce (Bennett et al., 1993). The remaining individuals are non-breeding workers that help raise the breeding pair's offspring. The non-breeders fail to exhibit reproductive behaviors while in their natal colony. Non-breeding female DMR fail to undergo puberty while in their natal colony, and exhibit underdeveloped ovaries and basal levels of ovarian steroid hormones (Holmes et al., 2009). Non-breeding males fail to exhibit sexual behaviors, even though they apparently undergo puberty while in the natal colony (Holmes et al., 2009). Models of eusociality hypothesize that the lack of reproductive effort by subordinates is necessary to maintain colony cohesiveness as non-breeders attempting sexual behavior are often met with aggression from the breeding female. If an unfamiliar male is introduced to a colony there is an increase in aggressive behaviors between the breeding female and high ranking non-breeding females. After a period of time, breeding becomes restricted to a single dominant female, although it is not always the original female (Burland, Bennett, Jarvis, & Faulkes, 2004). Reproductive efforts by subordinates are typically met with aggression. This may promote colony cohesion, because the cost of attempting to reproduce outweighs the benefits received via indirect fitness found within the colony (Jarvis, O'Riain, Bennett, & Sherman, 1994). Despite the presence of non-breeders resulting in advantages to individuals and the group, some non-breeding male and female DMR must be capable of becoming breeders, although the mechanism by which this occurs remains unknown. Interestingly, when non-breeders are paired with unfamiliar, opposite-sex conspecifics, they often exhibit mating

behavior (mounting, lordosis, etc.) within minutes (Cooney & Bennett, 2000; B. Goldman, pers. comm). It is remarkable that these prepubertal females mate so quickly following exposure to an unfamiliar male as female rodents typically require elevated levels of estrogen for at least 18-24 hours to permit the expression of mating behaviors (Pfaff & Schwartz-Giblin, 1988).

Given that non-breeding females are physiologically prepubescent, we hypothesized that gonadal hormones may not be necessary for the induction of sexual behavior, as it is unlikely that the hypothalamic-pituitary-gonadal (HPG) axis could be activated quickly enough to result in the expression of reproductive behavior within such a short time frame. The independence of sexual behavior from gonadal steroids is rare in vertebrates, especially females; occurring in relatively few species, including some birds, reptiles, and primates, including humans (Nelson, 2005). An integral role for ovarian hormones in the expression of sexual behavior stems from experiments in which ovariectomy resulted in the absence of estrous behavior, but the behaviors were restored following estrogen replacement (Ball, 1936; Young, 1961).

The lack of direct reproductive effort in female non-breeders may be due to differences in the regulation, or activity, of the HPG axis, as compared to breeding females. Within this axis, gonadotropin-releasing hormone (GnRH) regulates release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and is a necessary component of the vertebrate reproductive system (Hadley & Levine, 2007). There are no apparent differences in the number or size of GnRH neurons between breeding and non-breeding female DMR (Molteno, Kallo, Bennett, King, & Coen, 2004). Despite this, the response to GnRH at the level of pituitary could differ between breeding

and non-breeding females. Indeed, injections of exogenous GnRH, the GnRH “challenge” paradigm, resulted in greater LH secretion in breeding females than in non-breeding females (Bennett et al., 1993). However, with four repeated injections, that difference was no longer apparent (Holmes et al., 2009), indicating that the apparent difference in pituitary sensitivity may actually represent a downstream consequence rather than a cause of reproductive quiescence. These results indicate that the primary site of inhibition is upstream of GnRH neurons rather than at the pituitary (Faulkes, Abbott, Jarvis, & Sherriff, 1990a; Holmes et al., 2009).

Unlike females, male non-breeders do not differ in their reproductive physiology from male breeders). For example, plasma concentrations of LH, FSH and testosterone (T) are not different between reproductive and non-reproductive male DMR (Nice, Fleming, Bennett, Bateman, & Miller, 2010). Although testis weights are greater relative to body weight in breeding males as compared to non-breeders, there are no significant differences in numbers of spermatozoa (Faulkes et al., 1994; Holmes et al., 2009; but see Maswanganye, Bennett, Brinders, & Cooney, 1999). Thus, in males the lack of reproductive effort may be due to either inhibition of sexual behavior downstream of the HPG axis or to the lack of the proper social stimuli (Schoech, Mumme, & Wingfield, 1996; Maswanganye et al., 1999). This indicates that in non-breeding males regulation of sexual activity likely occurs at the behavioral, rather than physiological, level.

It is energetically costly to maintain an up-regulated reproductive system (Wingfield, Lynn, & Soma, 2001), and in a eusocial species it may be especially costly if it results in aggressive encounters between non-breeders and breeders. Thus, while in the natal colony, the absence of the expression of sexual behaviors in non-breeder males and

females may be a selected trait. The apparent sex difference in the pattern of reproductive regulation may be based on physiological differences in the time-course of gametogenesis between males and females; it can take up to two months for an azoospermic male to produce viable sperm (Johnson & Everitt, 1984). Therefore, it may benefit non-breeding males to have viable sperm already formed in case of copulatory opportunities. By comparison, the hormonal cascade that leads to ovulation in females may only require days to weeks (Nelson, 2005), thus, females remain reproductively suppressed until an opportunity to mate occurs. In the case of females, the act of mating may trigger reproductive maturation (Clark & Galef, 2001; Widowski, Ziegler, Elowson, & Snowdon, 1990). These observations indicate an obvious hurdle regarding the expression of sexual behavior in non-breeder female DMR; that is, she must be capable of exhibiting sexual behavior in the absence of fully functioning ovaries.

It is well established that for most mammals gonadal hormones are integral to the expression of sexual behavior. Testosterone is important for the stimulation of male sexual behaviors (Sachs & Meisel, 1988), whereas estrogen and progesterone are important for female sexual behaviors (Pfaff & Schwartz-Giblin, 1988). Thus, males and females of many species exhibit significantly reduced sexual behaviors when sex steroid hormones are removed via gonadectomy (GDX), and treatment with exogenous sex steroids often results in the return of sexual behaviors (Nelson, 2005). Thus, in most female mammals, ovariectomy eliminates sexual receptivity whereas treatment with estrogen, in some cases in combination with progesterone, restores it (Beach, 1976; Rissman & Bronson, 1987). In rodents, the lordosis reflex, a stereotyped female copulatory behavior involving the dorsiflexion of the vertebral column, is also dependent

on estrogen levels (Pfaff, Diakow, Zigmond, & Kow, 1974). Female lordosis is often presented as the lordosis quotient (LQ), which is calculated as the number of times a female exhibits lordosis divided by the number of mounts by the male within a test. LQ is heavily dependent on gonadal function. Thus, the LQ of ovariectomized female rats can approach zero, whereas intact females exhibit an LQ of ~0.95 (Hardy & DeBold, 1972). In males of some vertebrate species, castration leads to the elimination of all sexual behaviors and testosterone replacement restores sexual behavior (e.g., Japanese quail, *Coturnix coturnix japonica*; Adkins & Adler, 1972). However, to varying degrees, rats (*Rattus norvegicus*), guinea pigs (*Cavia porcellus*), golden hamsters (*Cricetus auratus*), dogs (*Canis lupus familiaris*), and cats (*Felis catus*) retain sexual behaviors post-castration (Beach, 1970), but this is often dependent on preoperative sexual experience (Costantini et al., 2007). Although there is interspecies variation in the importance of gonadal steroids for sexual activity, most male sexual behavior relies heavily on testosterone and its metabolites and after castration sex drive and the expression of sexual behaviors decline (Hull & Rodriguez-Manzo, 2009). A similar post-GDX persistence of sexual behaviors is rarely observed in non-primate females (Young, 1961). Restoration of the expression of sexual behaviors via hormone replacement requires weeks in males while only taking hours to days in females (Young, Goy, & Phoenix, 1961); thus, in mammals of both sexes, gonadal regulation of sexual behaviors is well-established.

Methods

Animals

Male and female DMR from colonies at the University of Memphis (animals originally provided by Dr. Bruce Goldman at the University of Connecticut), approximately two to three years of age were used in the behavior tests (these rodents can live up to 15 years [Holmes et al., 2009]). Their diet consists of ad libitum sweet potatoes (EasyWay Produce) with dry rodent pellets (Harlan 2019, 19% protein diet) provided as a supplement. Colonies were maintained in caging systems that consisted of two different sized (60 x 40 x 20 cm) and (48 x 25 x 20 cm) polypropylene tubs with PlexiglasTM lids and were connected by lengths of extruded polycarbonate tubing. Cages contained a 1:1 mixture of corncob and pine bedding. All experimental procedures and husbandry were approved by the University of Memphis Animals Care and Use Committee and comply with the criteria established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgeries

Sixteen adult mole-rats (eight of each sex) were anesthetized with an intraperitoneal injection of Ketamine/Xylazine/Acepromazine “cocktail” (0.0017 ml/g). Briefly, an abdominal incision was made in the males to remove their testes. Bilateral dorsal incisions were made to remove the ovaries from the females. Incisions were sutured with surgical thread following the removal of the gonads. Lidocaine cream (4%) was applied topically to the incision site to ameliorate pain and discomfort, and animals were subcutaneously administered Rimadyl (50 mg/ml).

Testing of Sexual Behavior

GDX animals were housed in isolation for five to seven days following surgery to allow for recovery. After the animals had recovered from surgery, testing began. Pairs of opposite sex unfamiliar (UNF) conspecifics were tested 12 times in sexual behavior tests.

Testing

Animals were housed individually except during behavioral tests. On two days each week, we tested pairs of unfamiliar partners by placing one GDX male and one GDX female in a large arena (60 x 40 x 20 cm) for 20 minutes. We recorded any instance of mating behavior, including female solicitation (backing the anogenital region toward the male's head) and lordosis (arching of the spine, deflection of the tail, and immobility), male mounting and thrusting, as well as the latency to initiation and number of each behavior. Animals were tested once with eight individuals, in the remaining four tests, pairs were repeated once. All tests were recorded using a JVC GZ-MG21U camcorder and scored from recordings.

Quantification of Sexual Behaviors

Behaviors were scored by a single blind observer. Only overt sexual acts were scored as sexual behaviors (mounting and thrusting, solicitation, and lordosis). The LQ was determined by dividing the number of times a female lordosed by the number of times a male mounted. For each individual, the mean values for each behavioral measure were calculated (e.g., the average number of mounts a male exhibited over the 12 tests with unfamiliar females) then analyzed using ANOVA or repeated-measures ANOVA. The proportion of individuals exhibiting sexual behavior was analyzed by Chi-square or

Fisher's exact tests. For all statistical analyses, differences were considered significant if $p < 0.05$.

We calculated the male's latency to express sexual behavior, duration of sexual behavior, and number of bouts. In females, the latency to the expression of solicitation behavior and lordosis were recorded as were the number of bouts of solicitation and lordosis. Means were obtained by averaging values for all tests for each condition (i.e., 12 trials paired with an unfamiliar individual). Additionally, to assess differences in the proportion of individuals in each group that expressed each behavior, we compared the number of individuals in which each behavior was expressed in at least one trial in each condition.

Results

Role of the Gonads in the Expression of Sexual Behaviors

Probability of Exhibiting Sexual Behavior. Gonadal status did not impact the proportion of either male or female DMR exhibiting sexual behavior, (mounting and thrusting for males and lordosis for females), when paired with unfamiliar opposite sex individuals. Thus 87.5 % (7/8) and 75% (6/8) of gonadal- intact and GDY males, respectively, exhibited mounting and thrusting behaviors in at least one trial, whereas 100% (8/8) and 87.5% (7/8) of gonadal-intact and GDY females, respectively, exhibited lordosis in at least one trial (Fisher's exact test: $p > 0.99$ for both males and females). Gonadal status also did not determine the probability of individuals engaging in sexual behavior when paired with unfamiliar opposite-sex individuals (i.e., the number of tests out of the 12 tests with unfamiliar individuals in which each behavior occurred; $p > 0.07$, Figure 1).

No sex difference existed in the proportion of DMR engaging in sexual behavior with an unfamiliar opposite-sex individual regardless of gonadal status (Fisher's exact: $p > 0.99$ for each comparison); nor in the probability of engaging in sexual behavior in these tests ($p > 0.5$, Figure 1).

Latency, Duration, and Number of Bouts of Sexual Behavior.

Males. Gonadal status did not significantly impact the average latency to mount, the duration of mount, or the average number of mounts exhibited by male DMR ($F_{1,14} = 1.34$; $p = 0.27$, Figure 2A, $F_{1,14} = 1.38$; $p = 0.26$, Figure 2B, $F_{1,14} = 0.92$; $p = 0.36$, Figure 2C, respectively).

Females. Gonadal status did not significantly impact the average latency to female solicitation ($F_{1,14} = 0.57$; $p = 0.47$ Figure 3A) or number of solicitations ($F_{1,14} = 1.33E-4$; $p = 0.99$, Figure 3B), but it did impact the average latency to lordose ($F_{1,14} = 10.00$; $p = 0.0069$, Figure 3C), the number of lordoses ($F_{1,14} = 7.23$; $p = 0.018$, Figure 3D), and LQ in female DMR ($F_{1,14} = 7.55$; $p = 0.016$, Figure 3E).

Discussion

The results of the current experiments are striking; indicating that the expression of sexual behavior in both male and female DMR occurs independently of the gonads. This is quite unusual, especially in rodents because gonadal steroid hormones have been closely linked to the expression of mating behaviors in most instances (Ball, 1936; Nelson, 2005; Young, 1961). Our results indicate that even months after GDX, the probability of DMR engaging in sexual behavior when paired with unfamiliar opposite-sex individuals is similar to that of intact individuals. Furthermore, in males, gonadal status failed to significantly alter measures of sexual motivation, including the latency to

mount, the total duration of sexual behavior expressed, or the number of mounts.

Whereas OVX failed to alter the proportion of females that exhibited lordosis, the results suggest that ovarian hormones play a modulatory role in the expression of lordosis. These results provide insights into this rare mammalian mating system and suggest possible mechanisms that mediating reproductive inactivity and subsequent activation in this eusocial species.

The role of gonadal signals in the expression of sexual behaviors has been appreciated for many years, at least since Berthold (1849) observed that castrated roosters (*Gallus gallus domesticus*) no longer displayed typical masculine sexual behaviors and Phoenix, Goy, Gerall, and Young (1959) demonstrated that gonadal hormones had direct effects on the sexual behaviors of guinea pigs. Similar findings have been reported in multiple rodent species (for review see Feder, 1984). Though the expression of sexual behavior typically depends on gonadal hormones, variation exists among species in the relationship between gonadal function and reproductive behaviors; thus, many animals, especially mammals, exhibit a so-called associated reproductive pattern. That is, during the breeding season, mating behavior coincides with maximal gonad size, gonadal steroid hormone concentrations, and gamete production (Crews & Moore, 1986). There are a few examples of dissociated reproductive systems, including male red-sided garter snakes (*Thamnophis sirtalis parietalis*) and the big brown bat (*Eptesicus fuscus*) (Crews & Moore, 1986; Mendonça, Chernetsky, Nester, & Gardner, 1996). Both species exhibit a temporal uncoupling of mating behavior from gonadal steroid hormone secretion; thus, the expression of sexual behavior precedes maximal gonadal activity and the appearance of mature gametes (Crews & Moore, 1986). Finally, the Asian musk shrew (*Suncus*

murinus) exhibits a mixed reproductive pattern, where the mechanisms regulating reproduction and mating behavior can differ between the sexes (Rissman, 1987 & Rissman & Bronson, 1987). In males, reproductive behavior coincides with testicular growth and depends on androgens, while females exhibit sexual receptivity independently of gonadal hormones (Crews & Moore, 1986). Despite the above exceptions, many experiments indicate that the gonadal hormones testosterone, estrogen, and progesterone, are critical for the expression of sexual behaviors across taxa (Nelson, 2005).

Importantly, the majority of experiments on the role of gonadal hormones on sexual behaviors have been carried out in rodent models that are solitary, or at the least, not highly social. The data presented herein provide an initial test of the role of gonadal hormones in the expression of sexual behavior within a very different mating system, namely, eusociality. Living within a eusocial mating system appears to require changes in the expression of sexual behaviors in non-breeding individuals (i.e., suppression of sexual behaviors). The present results show that the evolution of this mating system also resulted in modifications of the mechanisms underlying the expression of sexual behaviors. These results extend recent findings in both DMR and another eusocial mammal, the naked mole-rat (*Heterocephalus glaber*), in which sexual differentiation of the nervous system is either absent (in the NMR) or, in the case of DMR, greatly reduced (Anyan et al., 2011; Holmes et al., 2007), suggesting that both the traditional organizational and activational roles of gonadal hormones have been attenuated in eusocial mammals.

DMR are eusocial mammals in which social cues are thought to result in the suppression of reproduction in non-breeding colony members (Clarke, Meithe, &

Bennett, 2001). Results from the present and previous experiments also indicate that social cues are important in the activation of sexual behavior during the transition from non-breeding to breeding status (Clarke et al., 2001). Our results confirm those findings and indicate that both male and female non-breeding DMR exhibit rapid onset of sexual behavior when paired with unfamiliar opposite-sex individuals. Thus, it is important to consider social cues in addition to gonadal signals in regulating reproductive behavior in DMR. Social interactions have long been known to regulate mating behaviors in multiple vertebrate species. In the African teleost (*Haplochromis burtoni*) male reproduction is delayed in males that are reared in the presence of other adult males relative to those reared without adults present (Francis, Soma, & Fernald, 1993). Sexual maturation in the female house mouse (*Mus musculus*) is accelerated by exposure to unfamiliar male pheromones (Colby & Vandenberg, 1974), whereas olfactory cues from dominant female marmosets (*Callithrix jacchus*) play an important role in the suppression of ovulation in subordinate females in the group (Barrett, Abbott, & George, 1990). Many social influences on sexual behaviors lead to stimulation of the HPG axis. For example, parthenogenetic whiptail lizards (*Cnemidophorus uniparens*) rely on pseudosexual behaviors of same-sex conspecifics to facilitate reproduction (Crews & Moore, 1986). Another example of social regulation of sexual behavior is observed in the white-crowned sparrow (*Zonotrichia leucophrys*). The male does not exhibit mounting behavior until exposed to stimuli from a female (Moore, 1983). These behaviors are not mediated by gonadal steroids, because even long-term castrated, sexually inexperienced males, exposed to at least one period of long days, will mount receptive females (Moore, 1983). The results from the present experiment indicate that DMR depend heavily on social cues

to stimulate the expression of sexual behaviors. When housed within their natal colony, or when paired in an arena with a sibling, non-breeding DMR do not exhibit sexual behaviors, although when paired with an unfamiliar opposite-sex individual from a different colony, the expression of sexual behavior generally occurs within seconds to minutes. The present experiment tested the hypothesis that gonadal signals are a necessary component in the social induction of sexual behavior by pairing unfamiliar opposite-sex individuals that had been subjected to GDX. The results indicate that gonadal hormones are not necessary for the expression of socially-induced activation of sexual behavior in either male or female DMR. The results do demonstrate that gonadal hormones serve a modulatory role in the expression of lordosis in female DMR since intact females exhibited a higher LQ than OVX females. It should be noted that the LQ of ~0.3 exhibited by GDX female DMR greatly exceeds that normally observed in OVX female rodents; in most instances OVX results in a decline in LQ to zero (Hardy & DeBold, 1972; Yanase & Gorski, 1976).

The results of this experiment indicate that the evolution of eusociality in DMR may have resulted in alterations in the role of gonadal steroids in the expression of sexual behavior. Thus, the expression of sexual behaviors in non-breeding DMR appear to have escaped the strict regulation by gonadal steroids normally observed in rodents. DMR may have evolved more flexibility in the expression of sexual behaviors allowing them to balance the need to “suppress” sexual behavior while in their natal colony, and the requirement to rapidly initiate sexual behaviors given the opportunity to mate with an unfamiliar opposite-sex individual. These results are consistent with previous findings in

both the NMR and DMR indicating that the organizational effects of gonadal steroids on sexual differentiation of the nervous system are also attenuated (Anyan et al., 2011).

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Chapter 3 Incest Avoidance via Familiarity Rather than Genetic Relatedness

To avoid incest, organisms must be capable of identifying related individuals. Kin recognition enables incest avoidance and allows exposure to an unfamiliar conspecific to elicit sexual behavior. Damaraland mole-rats exhibit strict inbreeding avoidance (Burland, Bennett, Jarvis, & Faulkes, 2004). Kin recognition necessitates that an individual DMR be capable of identifying related versus unrelated individuals, which in DMR requires that individuals recognize fellow colony members. The mechanism by which DMR identify colony mates remains unclear. Several mechanisms can mediate kin recognition, including phenotype matching and prior association (or familiarity) (Mateo, 2003). Belding's ground squirrels (*Spermophilus beldingi*) employ phenotype matching, a system in which individuals identify related conspecifics based on comparing their own phenotypic cues, such as odor, with the cues of other individuals (Mateo, 2003). Prior association recognition can be based on familiarity: thus, individuals learn the phenotypes of siblings and parents during early development, and can later distinguish these familiar relatives from unfamiliar animals. Recognition by familiarity does not require that the individuals are genetically related, only that they were raised in close proximity. Kin recognition by prior association may be more likely in cases where relatives interact in environments that exclude non-kin, such as in exclusive burrow systems (Mateo, 2003). This experiment explored the mechanisms by which DMR practice incest-avoidance., Therefore the role of familiarity versus genetic relatedness was determined via the expression of sexual behaviors between siblings. Studies of agonistic behaviors directed toward test individuals revealed that DMR distinguish between familiar and unfamiliar conspecifics by use of individually distinct cues rather than kin-specific cues based on genetic relatedness or general colony odors. Thus, when members of a colony are

separated for an extended period of time (15-18 days; Jacobs & Kuiper, 2000; Jacobs, Reid, & Kuiper, 1998), they respond to each other with regard to colony defense and aggression the same way they would respond to an “unfamiliar” conspecific. Thus, males respond aggressively towards other males, even former colony mates, if they have been separated for more than 15 days (Jacobs & Kuiper, 2000). The present experiments were designed to determine whether separation would result in opposite-sex siblings responding to each other as unfamiliar with regard to the expression of sexual behaviors.

Methods

Animals

Male and female DMR from colonies at the University of Memphis (animals originally provided by Dr. Bruce Goldman at the University of Connecticut), approximately two to three years of age were used in the behavior tests. Their diet consists of ad libitum sweet potatoes (EasyWay Produce) and dry rodent pellets (Harlan 2019, 19% protein diet) were used as supplements. Colonies were maintained in several different sized (60 x 40 x 20 cm) and (48 x 25 x 20 cm) polypropylene tubs (with Plexiglas™ lids) containing a 1:1 mixture of corncob and pine bedding and connected by lengths of extruded polycarbonate tubing. All experimental procedures and husbandry were approved by the University of Memphis Animals Care and Use Committee and comply with the criteria established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Testing Sexual Behavior

Sixteen gonadal-intact mole-rats (eight per sex) were separated from their natal colony, individually housed, and subjected to the same testing protocol as the GD_X

animals described in Chapter 2. We exposed “familiar” animals (i.e., siblings from the same colony) to each other for 10-20 minutes on alternate days by placing them in a cage together, to maintain familiarity. Familiar animals (FAM) were defined as animals from the same natal colony that had not been isolated from each other for more than 5 days prior to testing. We placed 1 female and 1 male sibling in a large arena (60 x 40 x 20 cm) and observed behaviors for 20 minutes, recording mounting, thrusting, and solicitation. Animals were tested 12 times with FAM siblings and tested for the expression of sexual behaviors. Subsequently, sibling pairs were isolated from each other for 5 weeks. After 5 weeks of separation, the familiar siblings, now termed familiar post-separation (FAM PS), were again paired in sexual behavior tests.

This allowed us to discriminate between the phenotype matching and prior association hypotheses of kin recognition. We recorded any instance of mating behavior female solicitation (backing the anogenital region toward the male’s head) and male mounting and thrusting, as well as the latency to initiation and number of each behavior. All tests were recorded using a JVC GZ-MG21U camcorder and scored from recordings.

Quantification of Sexual Behaviors

Behaviors were scored by a single blind observer. Only overt sexual acts were scored as sexual behaviors (mounting and thrusting, and solicitation). For each individual, the mean values for each behavioral measure were calculated (e.g., the average number of mounts a male exhibited over the 12 tests with familiar females) then analyzed using ANOVA or repeated-measures ANOVA. The proportion of individuals exhibiting sexual behavior was analyzed by Chi-square or Fisher’s exact tests. For all statistical analyses, differences were considered significant if $p < 0.05$.

We calculated the male's mean latency to the expression of sexual behavior, mean duration of sexual behavior, and mean number of bouts. In females the mean latency to the expression of solicitation behavior was recorded as was the mean number of bouts of solicitation, again, averaged over all tests for each condition (i.e., 12 trials paired with a familiar individual, and six trials paired with a familiar individual post-five week separation). Additionally, to assess differences in the probability of the expression of each sexual behavior occurring, the proportion of individuals in which each behavior was expressed in at least one trial in each condition was compared among groups.

Note:

Female solicitation was used in lieu of LQ because equipment failure resulted in the loss of the majority of familiar post-separation videos prior to scoring for lordosis.

Results

Incest Avoidance

Probability of Expression of Sexual Behavior When Paired with a Sibling.

A significant difference was found in the probability of siblings expressing mounting and thrusting for males, and solicitation for females after they had been separated for 5 weeks (Males: $F_{1,7} = 21.64$, $p = 0.0023$; Females: $F_{1,7} = 15.007$, $p = 0.0061$). The results indicate that following five weeks of separation, sibling pairs behave no differently than DMR paired with unfamiliar individuals, thus, no difference was found in the probability of the expression of sexual behaviors between unfamiliar individuals and siblings post-separation (Males: $F_{1,7} = 0.034$, $p = 0.86$; Females: $F_{1,7} = 1.97$, $p = 0.20$). Further, no difference existed in the proportion of DMR engaging in sexual behavior with a sibling rendered unfamiliar as compared to pairings of unfamiliar animals ($p = 0.13$).

Latency, Duration, and Number of Bouts. Males exhibited a shorter latency to mounting, longer duration, and greater number of bouts with a sibling following five weeks of separation as compared to a familiar sibling ($F_{1,7} = 65.46$; $p < 0.0001$, Figure 5A, $F_{1,7} = 7.37$; $p = 0.03$, Figure 5B, $F_{1,7} = 6.37$; $p = 0.037$, Figure 5C, respectively). Females exhibited shorter latency to female solicitation and a greater number of solicitations with a sibling following five weeks of separation as compared to a familiar sibling ($F_{1,7} = 12.42$; $p = 0.0097$, Figure 6A, $F_{1,7} = 5.80$; $p = 0.046$ Figure 6B, respectively).

Discussion

The results suggest that incest-avoidance in DMR is accomplished by the failure of familiar individuals to engage in mating behavior, rather than the avoidance of mating between genetically related individuals, since separating siblings for five weeks resulted in a significant increase in mating behavior. In fact, the probability of mating, as well as measures of motivation (latency to initiate sexual behavior, duration of sexual behavior, and the number of bouts) did not differ when pairs of siblings rendered unfamiliar by five weeks of separation were compared to pairs of unfamiliar individuals. Thus, following five weeks of separation, DMR siblings treat each other as unfamiliar with regard to mating behavior. DMR exhibit incest avoidance (Burland et al., 2004), and results from the present experiments show that they recognize siblings via familiarity rather than genetic relatedness. Kin recognition involves three components of perception: (i) animals can be recognized indirectly from spatial cues, thus animals within the nest or burrow are treated as kin regardless of genetic relatedness; (ii) recognition can be based on familiarity via prior association, thus, animals learn the identity of individuals during

early development and later discriminate these familiar animals from unfamiliar animals; and (iii) animals learn their own phenotypes or those of their familiar kin, and later compare or match the phenotypes of unknown animals to this learned recognition template, a process called phenotype matching (Mateo, 2003). Our results suggest that DMR exhibit kin recognition based on prior experience rather than genetic relatedness. These findings are further supported considering there is no significant difference in the probability of the expression of sexual behaviors between unfamiliar DMR and siblings post-separation. These results are contrary to findings involving the social Ansell's mole-rat (*Fukomys anelli*, formerly *Cryptomys anelli*), in which Ansell's mole-rats exhibited a preference for genetically unrelated individuals over sibling that were rendered unfamiliar (Heth, Todrank, Begall, Wegner, & Burda, 2004). Thus, it appears that inbreeding avoidance may have evolved independently and by different mechanisms in DMR and Ansell's mole-rats, both of which differ from the eusocial naked mole-rat (*Heterocephalus glaber*) in which inbreeding is common (Holmes et al., 2009).

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Chapter 4 Conclusion

The DMR's mating system is not fully understood. While in the natal colony non-breeding individuals remain reproductively quiescent, but when exposed to the correct social stimuli, at least some individuals are capable of transitioning to the reproductive phenotype (Hazell, Bennett, Jarvis, & Griffin, 2000). Our results indicate that the expression of sexual behavior in DMR is almost completely dependent on social cues rather than on gonadal signals. The selective advantages of the dissociation of sexual behaviors from gonadal function in DMR remain speculative, although likely result from the need to suppress sexual behaviors while a member of the natal colony coupled with the need to rapidly respond to the rare opportunity to mate with an unfamiliar opposite-sex conspecific. The opportunity for out-breeding may occur only sporadically; therefore, an individual might benefit by having the capability to rapidly mate upon encountering a breeding partner to thereby begin a new colony. In the wild, non-breeding DMR have less than an 8 % chance of breeding in their lifetime (Clarke, Meithe, & Bennett, 2001) and the expression of mating or solicitation behavior by an unfamiliar female may be the only cue available to a dispersing male to initiate sexual behaviors. Copulation may initiate the transition from non-breeder to breeder status (Snyman, Jackson, & Bennett, 2006), allowing the formation of a nascent colony. Hazell et al. (2000) found that 81% of male and 40% of female dispersing DMR showed evidence of some reproductive development, although it is unclear whether this was the result of copulatory activity. The large percentage of reproductively mature males correlates with the findings that non-breeding males show little reproductive suppression even while within the natal colony.

One hypothesis to explain the persistence of sexual behaviors following gonadectomy poses that extra-gonadal sources of sex steroids drive the behavior. Several

researchers have speculated that the adrenal cortex produces androgens that maintain sexual behaviors (Asa, Goldfoot, Garcia, & Ginther, 1980; Everitt & Herbert, 1969; Gray & Gorzalka, 1980; Kindrick & Dixson, 1984; Rissman & Bronson, 1987). However, results failed to support a role for adrenal steroids in the maintenance of sexual behaviors in golden hamsters (Warren & Aronson, 1956) or cats (Rosenblatt & Aronson, 1958) and dogs (Schwartz & Beach, 1954). In many species, adult males with prior sexual experience often continue to express sexual behaviors after GDX (Costantini et al., 2007; Manning & Thompson, 1976; Rosenblatt & Aronson, 1958). For example, GDX cats with preoperative sexual experience retained a significant amount of sexual behavior as compared to GDX individuals with no prior experience (Rosenblatt & Aronson, 1958). Similarly, a significantly higher proportion of male Siberian hamsters (*Phodopus sungorus*) exhibited sexual behaviors post-castration if they had previous sexual experience; however, if the hamsters were castrated before puberty, none exhibited sexual behaviors (Costantini et al., 2007). Lastly, genetic factors may play a role in the retention of sexual behavior post-castration as certain strains of mice maintain copulatory behaviors for up to a year post-castration (Mendonça, Chernetsky, Nester, & Gardner, 1996; McGill & Hayes, 1973).

Biting, sniffing, and mounting by the female could all be considered proceptive behaviors. A female's lordosis behavior is the most obvious receptive behavior. In the female rat, proceptive behaviors are modulated by progesterone, while receptive behaviors are enhanced by progesterone in combination with estradiol (Frye, Bayon, Pursnani, & Purdy, 1998). In our study, the presence of the gonads had little effect on expression of sexual behaviors in either sex. This gonadal-independent expression of

sexual behavior places DMR among a small group of vertebrates in which mating behaviors are independent of gonadal hormones. It will be important to characterize the neuroendocrine pathways by which social cues are translated into sexual behavior in DMR. It is interesting to note that in both DMR and NMR, social status has a greater influence on brain morphology than do gonadal hormones (Anyan et al., 2011; Holmes et al., 2007, respectively). This finding is very unusual because most vertebrates exhibit sexual dimorphism in specific brain nuclei (Cooke, Hegstrom, Villeneuve, & Breedlove, 1998). The mechanism that mediates the effect of social status on brain morphology has yet to be characterized in either species. Taken together, the results from the present experiment and those noted above suggest that both the organizational and activational roles of gonadal steroids have been modified in eusocial mammals.

Since gonadal steroids are not required for the expression of sexual behaviors in DMR, neuropeptides or adrenal steroids may mediate the expression of these behaviors. Rissman and Bronson (1987) found that over 70% of adrenalectomized female musk shrews failed to exhibit sexual behavior when paired with a proven stud male. While this is the most dramatic effect of adrenalectomy on female sexual behavior, adrenalectomy also alters primate sexual behavior (Gray & Gorzalka, 1980). Oxytocin may also facilitate the expression of female sexual behavior, as rats treated with an oxytocin antagonist exhibited significantly reduced sexual behaviors despite having been primed with estradiol and progesterone (Pedersen & Boccia, 2002). Subsequently, Pedersen and Boccia (2006) found that arginine vasopressin (AVP) and oxytocin have opposite effects on female sexual behaviors in rats, as AVP inhibited the expression of sexual behaviors. Dopamine also appears to be stimulatory to the expression of male sexual behaviors and

motivation. Drugs that stimulate dopamine receptors and release increase the expression of mounts, intromissions, and ejaculations in male rats, while dopamine antagonists disrupt these behaviors (Pfaus & Phillips, 1991). Additional studies are needed to determine what role, if any, these neuropeptides and adrenal steroids have in the expression of sexual behaviors in DMR.

It is not clear at present whether non-breeding DMR are subjected to active reproductive suppression by the breeders, or if they remain reproductively quiescent due to incest avoidance coupled with the lack of proper social stimuli (e.g., an opposite-sex unfamiliar individual), or some combination of both. The NMR provides a classic example of the dominant control model of reproductive suppression through social contact with the dominant breeding female. Faulkes, Abbott, and Jarvis (1990b) found that non-breeding NMR females will become reproductively active when separated from the queen, even when exposed to other colony members and colony odors (Smith, Faulkes, & Abbott, 1997). These results indicate that non-breeding female NMR do not respond to olfactory cues, but are instead reproductively suppressed through physical contact with the queen. One hypothesis states that there are two types of behavioral suppression exhibited by the queen, overt and subtle (Faulkes & Abbott, 1997). Removal of the NMR queen results in social instability that is characterized by increased levels of aggression among the remaining high ranking females, and some high ranking males. It is thought that this initial aggression is sufficient to establish rank, after which only subtle aggression is required to maintain social control (Faulkes & Abbott, 1997). DMR appear to employ a different mechanism, as female non-breeders exhibit low levels of LH and underdeveloped gonads. Although speculative, this may be due to a combination of self-

restraint, absence of stimulation (i.e., avoiding reproduction until an unrelated male is available), and incest avoidance (Clarke et al., 2001). Whereas males appear to have a behavioral block on sexual behaviors that is likely a result of incest avoidance mechanisms or the lack of the appropriate social cue. The difference between NMR and DMR in the mechanisms that regulate reproductive inactivity could be due to the fact that NMR regularly inbreed and thus require a control mechanism to suppress reproduction in both sexes. In contrast, DMR are obligate out-breeders, in which case, incest avoidance alone might result in low intra-colony mating among siblings. The persistence of the prepubertal state in non-breeding female DMR may ensure that female non-breeders do not become pregnant in the event that a foreign male enters the colony (Burland et al., 2004). It is unlikely that reproductive control of non-breeding females is strictly due to behavioral dominance from the breeding female, because there is little intra-colony aggression in DMR colonies (Faulkes & Bennett, 2001). When the breeding male is removed from a colony and unrelated males are then introduced to the colony, aggression between the breeder female and her daughters increases dramatically and in 50% of these colonies a daughter usurps the queen (Faulkes & Bennett, 2001). This argues against a dominant control mechanism, since non-breeding females became reproductively active even in the queen's presence when provided social cues from an unfamiliar male. Thus, females may remain reproductively inactive in the natal colony because they lack the proper social stimuli, as opposed to active suppression.

The Damaraland mole-rat may prove to be a valuable model for understanding how mammalian sexual behavior can become dissociated from gonadal hormones. Although this happens to varying degrees for males in a number of species, it is very rare

for females. Humans, and probably some primates (e.g., bonobos), are among the very few mammals for which female sexual behavior does not depend on estrogen from the ovaries (for review see Feder, 1984). We suggest two possible explanations relating to our results indicating independence of sexual behavior from gonadal function in Damaraland mole-rats: 1) Steroids might be involved in some way in sexual behavior, even though not absolutely required. 2) It might be that activation of steroid receptors is required for support of sexual behaviors, but that activation is accomplished by non-steroidal ligands, perhaps a neurotransmitter. This second possibility is most intriguing and the activation of progesterone and estrogen receptors by non-steroidal ligands (dopamine, epidermal growth factor, GnRH) has been reported in laboratory rats (Blaustein, 2004, 2008; Mani, Allen, Clark, Blaustein, & O'Malley, 1994). However, because the overt effects were modest and most of the studies were conducted in vitro, whether these effects are of physiological significance remains to be determined.

Damaraland mole-rats almost certainly evolved from ancestors in which sexual behavior was steroid-dependent, as this is the case in the majority of today's species. Further, the activation of behavior in these species by steroid hormones by definition requires the presence of steroid receptors. We know that estrogen receptors can be activated by non-steroidal ligands, including dopamine (Power, Mani, Codina, Conneely, & O'Malley, 1991). Thus, it may be that direct activation of sexual behaviors by such non-steroidal ligands has evolved independently from gonadal steroids. It is clear that the characterization of the neural circuits and mechanisms by which social cues alter the expression of sexual behaviors independently of the gonads in DMR is the necessary next step to determine whether the above speculations have merit.

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Appendix

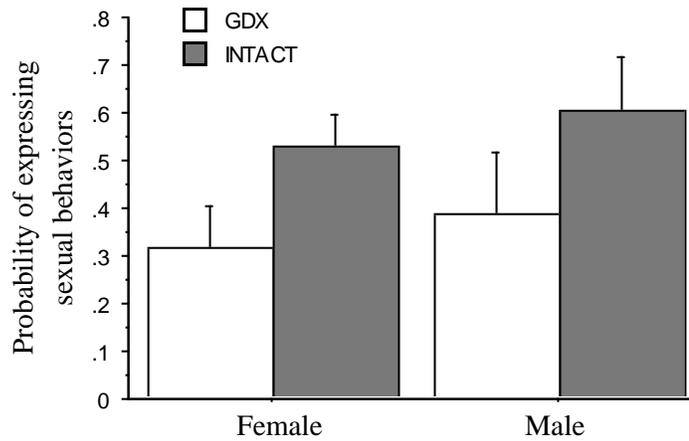


Figure 1. The probability (mean \pm s.e.) of sexual behavior (mounts for males and lordosis for females) occurring between unfamiliar opposite-sex individuals. Both females and males were equally likely to engage in sexual behaviors when paired with an unfamiliar opposite-sex individual and gonadal status did not result in significant alterations in the probability of expression of sexual behavior in either sex ($n = 8/\text{group}$; $p > 0.07$ for each comparison)

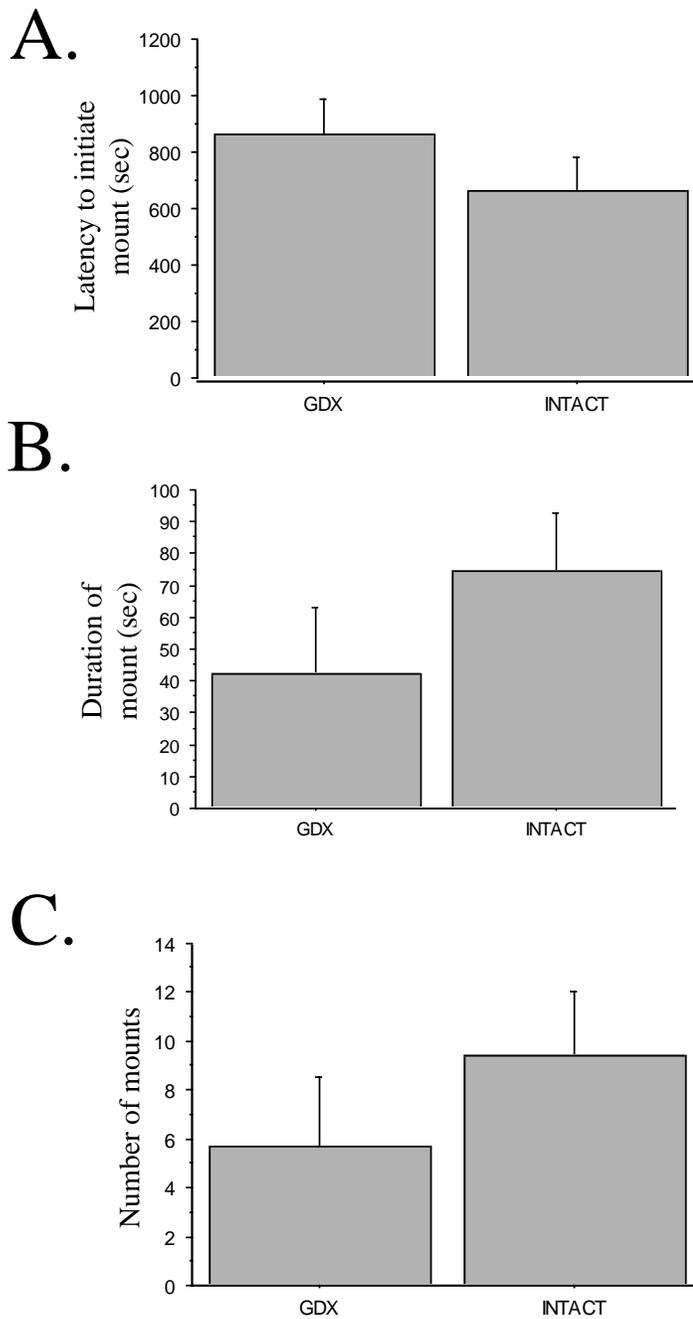


Figure 2. (A) Mean (\pm s.e.) latency to mount, (B) Duration of mount, and (C) Number of mounts expressed by GDX and gonadal-intact male DMR paired with unfamiliar females. Gonadectomy did not result in significant alterations in any measure ($n = 8$; $p > 0.25$ for each comparison).

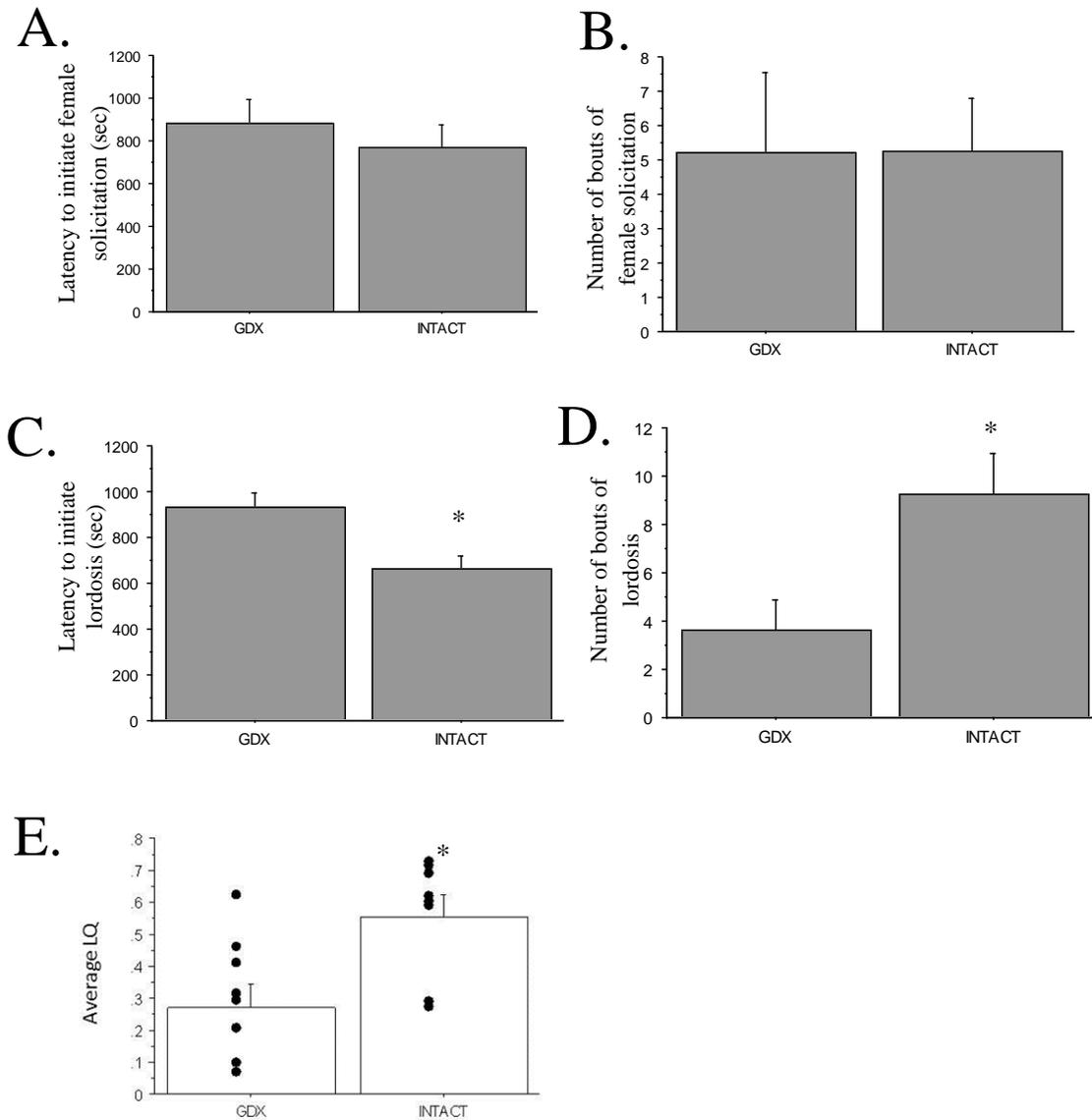


Figure 3. Mean (\pm s.e.) (A) Latency to female solicitation, (B) Number of bouts of female solicitation, Gonadectomy did not result in significant alterations in these measure ($p > 0.25$ for each comparison) (C) Latency to lordosis, (D) Number of lordoses, (E) Lordosis quotient expressed by GDX and gonadal-intact female DMR paired with unfamiliar males. In E each black dot represents an individual animal. Gonadal status significantly impacted measures of lordosis, but not solicitation. ($n = 8/\text{group}$; *; ANOVA; $p < 0.05$).

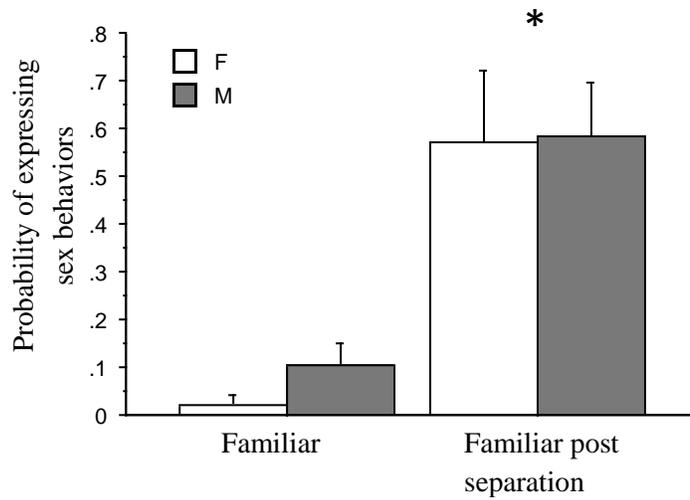


Figure 4. Probability (mean \pm s.e.) of sexual behavior (mounting and thrusting for males, solicitation for females) occurring between gonad-intact familiar siblings and the same siblings after 5 weeks of separation. The probability of sexual behavior occurring between siblings increased significantly following 5 weeks of separation ($n = 8/\text{group}$; *, ANOVA; $p < 0.0001$).

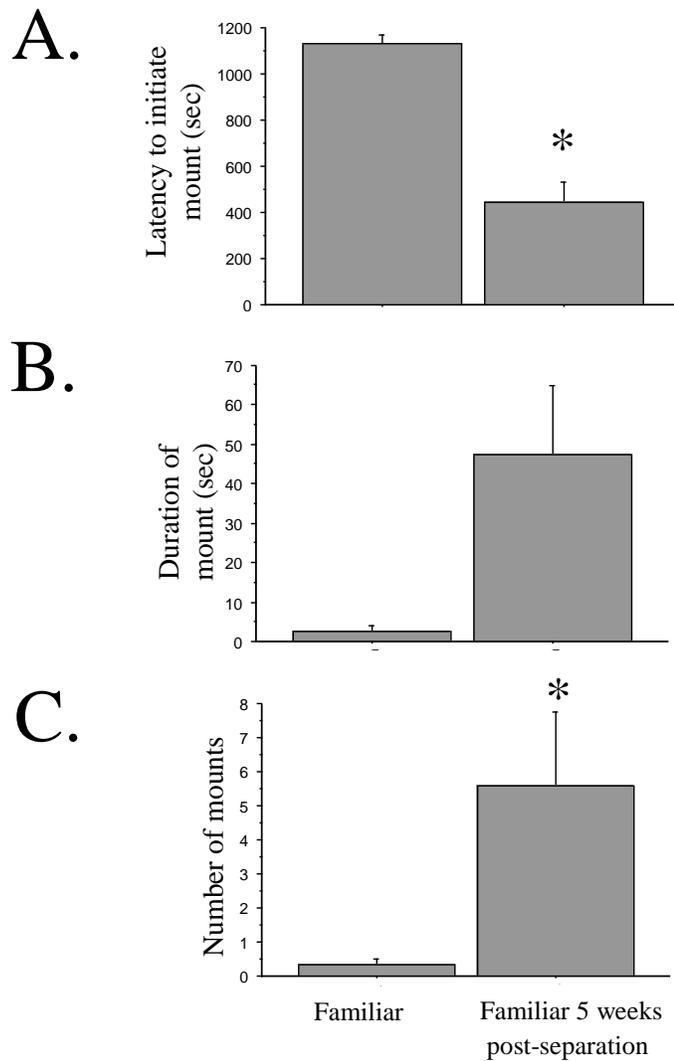


Figure 5. Mean (\pm s.e.) latency to mount (A), Duration of mounts (B), and Number of mounts (C) by males paired with female siblings before and after five weeks of separation. Rendering siblings unfamiliar after separation resulted in a significant decrease in the latency to initiate mounting behaviors ($n = 8$; ANOVA; $p < 0.0001$), an increase in the duration of male mounts (ANOVA; * $p < 0.04$); a significant increase in the number of bouts of mounts (ANOVA; * $p < 0.04$).

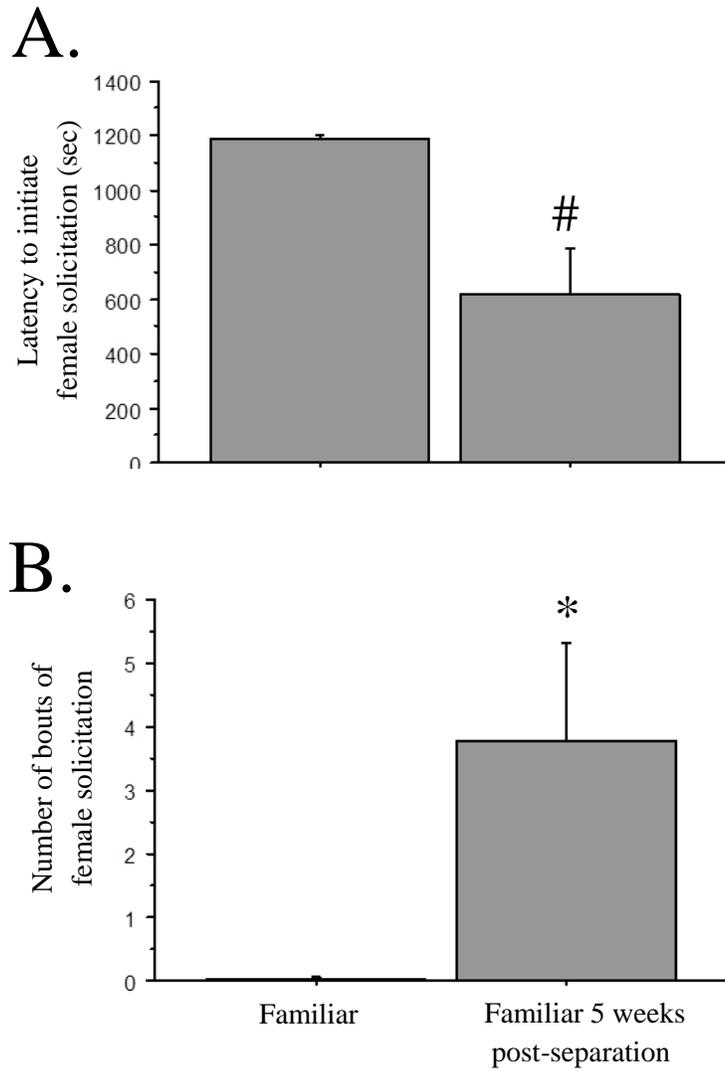


Figure 6. Latency (mean \pm s.e.) to initiate solicitation (A), and Number of bouts of solicitation (B) by females paired with a male siblings before and after five weeks of separation. Rendering siblings unfamiliar after separation resulted in a significant decrease in the latency to initiate female solicitation (n = 8; ANOVA; # $p = 0.0097$), and an increase in the number of bouts of female solicitation (ANOVA; * $p < 0.046$).



IACUC PROTOCOL ACTION FORM

To:	David Freeman
From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	9-26-11

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No.

0702(DMR sex and social behavior)

Your proposal is approved for the following period:

From: To:

Your protocol is not approved for the following reasons (see attached memo).

Your protocol is renewed without changes for the following period:

From: _____ To: _____

Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Revision Memorandum dated _____ for the following period:

From: _____ To: _____

Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Revision Memorandum dated _____

Prof. Guy Mittleman, Chair of the IACUC

Dr. Karyl Buddington, University Veterinarian
And Director of the Animal Care Facilities