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Hormones and Behavior 44 (2003) 209–221

Hormones  
and Behavior

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# Predatory aggression, but not maternal or intermale aggression, is associated with high voluntary wheel-running behavior in mice

Stephen C. Gammie,<sup>a,\*</sup> Nina S. Hasen,<sup>a</sup> Justin S. Rhodes,<sup>a</sup>  
Isabelle Girard,<sup>a</sup> and Theodore Garland Jr<sup>b</sup>

<sup>a</sup> Department of Zoology and Neuroscience Training Program, University of Wisconsin, Madison, WI 53706, USA

<sup>b</sup> Department of Biology, University of California, Riverside, CA 92521, USA

Received 3 June 2002; accepted 27 May 2003

## Abstract

Predatory (towards crickets), intermale, and maternal aggression were examined in four replicate lines of mice that had been selectively bred for high wheel-running (S) and in four random-bred control lines (C). In generation 18, individual differences in both predatory and intermale aggression were significantly consistent across four trial days, but predatory and intermale aggression were uncorrelated both at the individual level and among the eight line means. Latencies to attack crickets were significantly lower in S lines as a group. Intermale aggression, however, did not differ between S and C lines. S lines were significantly smaller in body mass, but did not differ in either testes mass or plasma testosterone. In generations 28 and 30, respectively, S and C lines did not differ in either maternal or intermale aggression. However, significant differences among the individual lines were found for maternal aggression, and one S line exhibited an extremely high mean time of aggression (>120 sec for a 5-min test). Maternal and intermale aggression were not correlated among the eight line means or at the level of individual variation. Overall, our results suggest: (1) predatory aggression and voluntary wheel-running are positively related at the genetic level; (2) predatory and intermale aggression are unrelated at a genetic level; and (3) maternal and intermale aggression are not tightly related at the genetic level. Possible relationships between predatory aggression, dopamine, and wheel-running behavior are discussed.

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**Keywords:** Maternal aggression; Intermale aggression; Predatory aggression; Mice; Wheel running; Exercise; Lactation; Dopamine; Artificial selection

Aggression occurs in many forms and can be an important adaptive behavior. Predatory aggression, for example, is critical for species that hunt other animals (Ramirez, 1998; Siegel et al., 1999). Maternal aggression, the expression of a fierce aggression against intruders by lactating mammals, plays an important role in protecting offspring (Agrell et al., 1998; Wolff, 1985; Wolff, 1993). Intermale aggression, although viewed as a social problem in humans, is adaptive for many species with respect to the creation and maintenance of territories, and hence for increasing access to resources, including food, shelter, and mates (Brain,

1981; Hurst, 1986; Parmigiani et al., 1998; vom Saal and Howard, 1982). Although all three behaviors are forms of aggression, to what extent they are similar in their neural and genetic bases, has been the subject of considerable debate.

One approach to examining genetic relationships is to conduct selective breeding for one type of aggression (e.g., high intermale aggression) and then to examine levels of other forms of aggression in subsequent generations. Two such studies found maternal aggression to be elevated in lines that had been selected for high intermale aggression (Hood and Cairns, 1988; Sandnabba, 1993), but another study did not (Benus, 2001).

Neuroendocrinology studies suggest critical differences in the control of maternal and intermale aggression. For example, intermale aggression in most rodents is facilitated

\* Corresponding author. 1117 W. Johnson St., Department of Zoology, University of Wisconsin, Madison, WI 53706, USA. Fax: +1-608-262-9083.

E-mail address: [scgammie@wisc.edu](mailto:scgammie@wisc.edu) (S.C. Gammie).

by testosterone, or its aromatization to estradiol (Compaan et al., 1994), whereas the expression of maternal aggression is facilitated by estradiol, progesterone, and prolactin released during pregnancy and lactation (Bridges, 1996; Mann et al., 1984; Stern and McDonald, 1989), as well as sensory input (including olfactory and tactile stimuli) to the lactating females from pups (Lonstein and Gammie, 2002; Stern and Kolunje, 1993; Svare et al., 1980). Additionally, certain neuromodulators have different effects on maternal and male aggression. For example, nitric oxide appears to inhibit intermale, but facilitate maternal aggression (Chiavegatto et al., 2001; Demas et al., 1999; Gammie and Nelson, 1999; Gammie et al., 2000b; Nelson et al., 1995). As described below, the same dose of serotonin can have an unequal action on the two forms of aggression in rodents.

The relationship between predatory and intermale aggression is controversial. It has been suggested that predation cannot be strictly accepted as aggression, because they are different in terms of both motivation and neural control (Ramirez, 1998). A relationship between predatory and intermale aggression was not found in several studies of house mice (Brain and Al-Maliki, 1978; Butler, 1973). However, other work on house mice has reported a positive relationship between intermale and predatory aggression (Sandnabba, 1995a). Injection of the same dose of a serotonin agonist into outbred mice significantly reduced intermale aggression, but did not affect predatory aggression (Ferrari et al., 1996). Likewise, the same dose of a serotonin agonist significantly reduces intermale aggression, but does not effect maternal aggression in outbred mice (Parmigiani et al., 1998). These findings suggests that at certain levels, serotonin has an unequal effect on the three forms of aggression. Although regulation of serotonin has been strongly implicated in the control of intermale aggression (Nelson and Chiavegatto, 2001) and other studies suggest a similar control of predatory aggression (Nikulina, 1991; Pucilowski and Kostowski, 1983) and maternal aggression (De Almeida and Lucion, 1994), it remains unclear exactly how serotonin interacts with the neuronal circuitry underlying different forms of aggression.

In some selection studies, intermale aggression has been associated with other behavioral traits. For example, one study found elevated intermale aggression in lines of mice that had been selected for high nest building, and also elevated nest building in lines that had been selected for high intermale aggression (Sluyter et al., 1995). Additionally, selection for high intermale aggression has been correlated with an increased rate of locomotion (Selander and Kvist, 1991), and, as noted by (Sandnabba, 1996) (p. 484), "Since aggressive behavior contains more elements of motor activity than does nonaggressive behavior, a positive correlation between aggression and the overall motor activity was expected."

Mice have successfully been selected for increased wheel-running activity in four replicate lines (S) compared to four control (C) lines (Swallow et al., 1998a), which has

resulted in various correlated responses (Garland, 2003). For example, compared to control lines, high-running lines were found to build smaller nests (Carter et al., 2000), to exhibit altered turning behavior in open-field tests (Bronikowski, et al., 2001), to have higher maximum oxygen consumption during forced treadmill exercise (Swallow et al., 1998b), lower body mass and greater food intake (Koteja et al., 1999), and reductions in hindlimb muscle mass (Garland et al., 2002). Most relevant to the study of aggression, tube-tests of male dominance against a standard opponent (from an inbred strain) revealed that mice from S lines were more likely to retreat, as compared with mice from C lines, during the second of two daily tests (Klomberg et al., 2002). The same study also found that S males tended to have larger testes, but did not differ in plasma testosterone levels.

In this study, we examined whether predatory, intermale, or maternal aggression had changed as a result of the selective breeding for high wheel running. Further, assuming there were differences in levels of aggression among the eight lines, we wanted to determine whether maternal and intermale aggression were positively, negatively, or not at all correlated. We also wished to explore the relationship between predatory and intermale aggression. Because of their possible relationships with aggression, at least intermale, we also measured body mass, size of testes, and plasma testosterone levels.

Voluntary wheel running is a widely studied behavior in laboratory rodents, yet the underlying cause of behavioral variation is not known (Sherwin, 1998). It has been proposed that wheel running is naturally rewarding and addictive (Belke and Belliveau, 2001; Nestler et al., 2001; Werme et al., 2002) and hence, may involve dopamine signaling. Recent work suggests altered dopamine signaling in the S lines with possible differences in the D1 receptor (Rhodes and Garland, 2003; Rhodes et al., 2001), which is consistent with idea that voluntary wheel running is naturally rewarding. However, the exact mechanisms of differences in dopamine signaling between S and C mice is still not known. Various studies have implicated dopamine in predatory aggression in rodents and carnivores (ferrets) (Baggio and Ferrari, 1980; Jimerson and Reis, 1973; Schmidt, 1979; Schmidt, 1983; Siegel et al., 1999). Given likely differences in dopamine signaling and recent work indicating higher levels of activation of the brain reward circuitry in S relative to C mice (Rhodes et al., 2003), we hypothesized that there would be significant differences in predatory aggression between S and C mice. The direction of possible differences in predatory aggression was not predicted because the differences in dopamine signaling between S and C mice are not fully understood. Inhibition of dopamine signaling has been linked to an elevation of maternal aggression in rats (Johns et al., 1998; Sorenson and Gordon, 1975) and, therefore, as with predatory aggression, we predicted differences (but not the direction of differences) in maternal aggression between S and C mice.

## Methods

### *Animals and housing*

We studied mice from of an artificial selection experiment for high voluntary wheel-running behavior. As described previously (Garland, 2003; Swallow et al., 1998a), the original progenitors were outbred, genetically variable laboratory house mice (*Mus domesticus*) of the Hsd:ICR strain (Harlan-Sprague-Dawley). After two generations of random mating, mice were randomly paired and assigned to 8 closed lines (10 pairs in each). In each subsequent generation, when the offspring of these pairs were 6–8 weeks old, they were housed individually with access to a running wheel for 6 days. Daily wheel-running activity was monitored by an automated system. In the 4 “selected” lines (S), the highest-running male and female from each family were selected as breeders to propagate the lines to the next generation. Wheel running was quantified as the total number of revolutions run on day five and day six of the 6-day test. In the four “control” lines (C), a male and a female were randomly chosen from each family. Within all lines, the chosen breeders were randomly paired except that sibling matings were not allowed. All cages for routine housing, breeding, and behavioral tests were clear plastic (27 × 17 × 12.5 cm) with metal or wire tops and wood shavings as bedding. Room temperature was controlled at approximately 22°C. Photoperiod was a constant 12:12, centered at 1400 hours (CST). Water and food were available ad libitum. Cages were cleaned weekly, and also 4 days prior to the first cricket trial. The numbers of animals used for each experiment are detailed below. An adherence to NIH standards and guidelines for treatment of animals was used for all experiments and all experiments were performed under the auspices of an approved animal care protocol. Predatory and intermale aggression and plasma testosterone levels were measured in males from generation 18. Maternal and intermale aggression were measured in mice from generations 28 and 30, respectively. All tests were conducted during the day. For each experiment, animals from any one line were always taken from different families (10 families were available per line) and thus, any possible effect of litter was avoided.

### *Predatory aggression*

Retired male breeders from generation 18 were used ( $N = 78$  total, 9–10 from each of the eight lines). The subjects had been weaned at 21 days of age. From weaning until breeding at approximately 10 weeks of age, males were housed four/cage. Males were left with females for 2.5 weeks and then housed individually until testing. Animals were assigned at random to one of two testing batches, with predatory aggression trials conducted during successive weeks. Mice had no prior experience with crickets.

Cricket-killing behavior has been employed as a model

for measuring predatory aggression in various rodents, including mice (Brain and Al-Maliki, 1978; Butler, 1973; Haug and Johnson, 1991). Our test protocol followed that employed by others (Sandnabba, 1995a; Sandnabba, 1995b). Mice were not food-deprived prior to testing. Tests began at 1300 hours on each of four consecutive days. Values for the four successive trial days were used to assess the consistency of individual differences and to determine whether the level of aggression changed with repeated testing.

In each test, a live cricket was dropped into the home cage on the side opposite where the mouse was at that instant. A sheet of ventilated Plexiglas was then placed on top of the cage, and behavior of the mouse was observed for 7 min. Observers were blind with respect to whether a mouse was from S or C line. Mice were weighed two days before and one day after the trial periods. Age at the first cricket trial averaged 115 days (range = 105–121).

Following protocols established by others (Sandnabba, 1995a; Sandnabba, 1995b), we emphasized latency to attack (attempt to grab) the cricket because this is the only variable that is directly comparable between predatory and intermale tests.

### *Maternal aggression*

Females from generation 28 were used ( $N = 64$  total, approximately eight from each of the eight lines). All subjects had been previously examined for wheel running, and had produced first litters as part of the routine selection protocol. Following weaning of their first litters, they were repaired with the same sire, and the males were removed after two weeks. The females remained individually housed for the remainder of the experiments. The day of birth was considered postpartum Day 0. On the 7th and 8th day postpartum, pups were removed from the home cage; after 2 min, a sexually naive intruder male was placed immediately into the cage for 5 min between 0800 and 1200 h. Intruder males were group-housed and were from the same Hsd:ICR outbred strain that had formed the base population for the selection experiment (Swallow et al., 1998a). In mice and hamsters, removal of the pups from a mother just before an aggressive test does not diminish the expression of maternal aggression (Siegel et al., 1983; Svare et al., 1981). Each test session was recorded on videotape and subsequently analyzed off-line to quantify aggressive behaviors by the female. All analysis of behavior was performed by individuals blind to experimental groups and expectations. The time to first attack, the duration of each attack, the total number of attacks, and the total duration of attacks were determined for each lactating female for each test day. All measures were recorded using pen and paper. In the case of time to first bite, if an animal was not aggressive, a time of 300 s was assigned (the maximum possible for the test). In the few cases where wounding occurred on the intruder male (8 out 128 tests), males were removed from the cage

immediately and replaced with a second intruder male. Wounds were treated with soapy water and bacitracin and wounded intruders were never used again for testing.

### *Intermale aggression*

Intermale aggression was tested in both generation 18 (retired breeders), to allow direct comparison with predatory aggression, and again in generation 30 (sexually naïve), for comparison with maternal aggression. In generation 18, males were housed individually beginning at an average of 88 days of age. The age at the first cricket trial averaged 115 days (range = 105–121) and intermale trials began 13 days after the first cricket trial. In generation 18, intermale tests consisted of mice used in batch one of the predatory aggression tests (see above) as the focal mice and batch two as the intruder mice. Hence, sample size was reduced by 50% for the intermale tests. Individuals used in batch one of the predatory aggression testing (intruder mice) were placed in the home cage of individuals used in week two (focal mice); thus, a total of 39 focal mice were observed. Behavior was recorded for 7 min. As above, a sheet of ventilated Plexiglas was used to cover the home cage. All trials were conducted by a single observer, who was blind with respect to mouse status. Testing commenced at 1300 hours on four consecutive days. On each day, the intruder mice were randomly assigned to focal mice and no two males were ever paired more than once. Mice were weighed on days directly before and after the 4-day trial period. Similar to the cricket tests, we emphasized the latency to first actual attack. This measure required that the focal male instigate contact (intruder males had been marked to allow identification).

In addition to latencies, we also recorded the number of occurrences each focal mouse spent in several behaviors: (1) Nosing/Sniff: olfactory investigation and nudging of intruder; (2) Wrestle: mice wrestle with one another, abstaining from biting/attacking each other (usually instigated by focal mouse, noted if otherwise); (3) Defensive Posture: mouse stands still on back feet with forelimbs in a “bracing” position; and (4) Tail Rattling. We then calculated a measure of total actions, which included these measures.

In generation 30, 31 S and 31 C mice (7–8 mice per line) were used. All mice were sexually naïve and were group housed with same-sex siblings until age 30 days, when they were individually housed. Following 40 days of isolation, males were tested for aggression for two consecutive days in their home cage. Intruder males were group-housed, sexually naïve, and of the Hsd:ICR strain. Aggression testing and analysis was conducted identically as for maternal aggression.

### *Blood sampling, testosterone assays, and mass of tests*

One week following intermale aggression testing of mice from generation 18, blood was drawn from the suborbital sinus (Klomberg et al., 2002) of the mice used as focal

subjects ( $N = 39$ ). Although testosterone levels are known to be elevated just following a successful encounter, work in birds suggests hormone levels would have returned to baseline one week following testing (Ramenofsky, 1984). Heparinized micro-hematocrit tubes were filled and produced a total blood volume of approximately 160  $\mu$ l. Blood sampling was done without anesthesia (under an approved animal-use protocol) as quickly as possible and times between initial restraint and the end of blood drawing ranged from 19 to 100 seconds with a mean of 47.6 seconds. Blood was centrifuged in a micro-hematocrit centrifuge for 8 min and the plasma was frozen until testosterone assays. Sampling occurred between 13:00 and 15:00 CST.

Plasma testosterone concentrations were analyzed by enzyme immunoassay as described previously (Klomberg et al., 2002). Assays were performed in facilities of the Wisconsin Regional Primate Research Center. Because of competitive binding of different steroids with the antibody, chromatographic separation of steroids was performed after extraction. Testosterone antibody (R156/7, raised in rabbits against testosterone-6-carboxymethyl oxime:BSA) was provided by C. Munro (University of California, Davis). The sensitivity of the assay at 90% binding was 0.6 pg. The inter- and intra-assay coefficients of variation of a mouse plasma pool assayed in duplicate were 9.4% and 2.7% respectively (9 assays).

Three days after drawing blood all animals were weighed, killed by CO<sub>2</sub>, and frozen. Four months later, they were defrosted and their testes dissected and weighed to the nearest 0.001 g.

### *Data analysis*

For predatory and intermale aggression tests in generation 18, we used Spearman rank correlations to test the consistency of individual differences across the four trial days. Paired *t*-tests (sometimes following transformation of values to improve normality) were used to determine whether average levels of aggression changed across the successive trials. Spearman rank correlations were used to test for correlations among the measures of predatory aggression, among the measures of intermale aggression, and among measures between predatory and intermale aggression. For these analyses, only day 2 values were analyzed, as both types of aggression showed substantial increase in average level from day 1 to 2, then relatively little change across days 2–4 (see Results).

Following previous studies of these lines, for all traits measured in all three generations, we used nested analysis of covariance (ANCOVA) to compare S and C lines, using Type III sums of squares in the SAS GLM procedure. Replicate line was nested within linetype (selected vs control), and the effect of linetype was tested over the effect of line (d.f. = 1,6). Body mass was used as a covariate in analyses of intermale and predatory aggression. Age and time of day were used as additional covariates in these same

analyses. For analyses of testosterone levels, the length of time between initial restraint and completion of blood drawing was also used. Interactions between covariates (e.g., body mass, age, time of day) and main effects (linetype and line), or between covariates, were not examined because they were not of interest here and because we had no a priori hypotheses concerning them. Procedure GLM calculated regression coefficients for covariates and least-squares adjusted means for selected and control lines, and for individual lines. Adjusted means were calculated using all covariates, regardless of significance levels. Traits were transformed as necessary to improve normality of residuals and linearity of relationships with covariates. For analysis of effect of specific line, not linetype, a one-way ANOVA was used. For analysis of specific differences between individual lines, a post hoc Tukey HSD test was used.

## Results

### Generation 18 predatory and intermale aggression

#### Individual variation

The rank order of individual differences in behavior was significantly consistent across trial days for both predatory and intermale tests. For latency to attack cricket, Spearman rank correlations for days 1–2, 2–3, and 3–4, respectively, were 0.73, 0.72, and 0.69 ( $N = 73$ – $78$ , all  $P \leq 0.001$ ). For latency to attack in the intruder male trial, Spearman correlations were 0.63, 0.72, and 0.58 ( $N = 36$ – $37$ , all  $P \leq 0.0002$ ). Mice became more aggressive across trials and latencies to attack crickets were significantly lower on day 2 than on day 1 (paired  $t$ -tests, for C mice,  $N = 36$ ,  $t = -4.74$ ,  $P < 0.0005$ ; for S mice,  $N = 42$ ,  $t = -6.77$ ,  $P < 0.0005$ ), but not from days 2–3 and from days 3–4 (data not shown).

For latency to attack an intruder male, paired  $t$ -tests also indicated a significant reduction in log latency to attack for day 1–2 (for C mice,  $N = 17$ ,  $t = -2.01$ ,  $P = 0.013$ ; for S mice,  $N = 19$ ,  $t = -3.00$ ,  $P = 0.008$ ). For days 2–3 and 3–4, the only significant further reduction was for S mice from day 2–3 ( $P = 0.038$ ).

The level of aggression for intermale aggression determined by latency to attack and total actions were highly correlated (results not shown). However, latency to attack for predatory aggression was not correlated with either of the two measures of intermale aggression (results not shown). Thus, at the phenotypic level, predatory and intermale aggression are independent aspects of behavior in these mice.

#### Difference between S and C mice

On test day 2, as compared with mice from C lines, those from S lines exhibited significantly shorter latencies to attack crickets (Fig. 1,  $p = 0.004$ ). In contrast, for intermale aggression, we found no significant differences in either

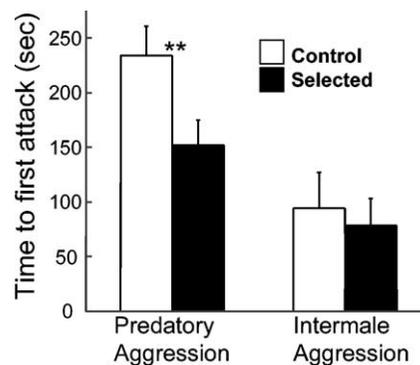


Fig. 1. Bar graphs indicating latencies to first attack for predatory and intermale aggression of mice from C and S lines in generation 18 on the second test day. Bars are means  $\pm$  SE. \*\* =  $p = 0.004$ , nested ANCOVA of log-transformed values indicating significant differences in predatory aggression between S and C mice.

attack latencies (Fig. 1,  $p = 0.493$ ) or total actions (data not shown,  $p = 0.250$ ). None of the behaviors showed significant differences among the replicate lines nested within linetype (data not shown; for day 2, all  $p > 0.5$ ).

We also tested whether latencies to attack on day two were related for predatory and intermale aggression, using adjusted line means as data points ( $N = 8$ ). Because S and C mice differed in predatory aggression, it would not be appropriate to compute a simple correlation of the eight line means, as they cannot be considered to come from the same underlying statistical distribution (i.e., the four S and four C lines constitute two separate populations in the statistical sense). Instead, we performed analysis of covariance on the adjusted line means from the nested ANOVAs, using a dummy variable to code for linetype. In this analysis, the effect of linetype was significant ( $P = 0.0014$ ) but the correlation between the two measures of aggression was not ( $P = 0.1184$ ).

### Generation 28 maternal aggression

There were no statistically significant differences between S and C lines in terms of time to first attack on either test day 1 ( $F(1,6) = 0.34$ ,  $P = 0.58$ ) or test day 2 ( $F(1,6) = 0.79$ ,  $P = 0.41$ ) (Fig. 2A) or mean time aggressive (Fig. 2B) for maternal aggression on either test day 1 ( $F(1,6) = 0.9$ ,  $P = 0.38$ ) or day 2 ( $F(1,6) = 1.14$ ,  $P = 0.33$ ). Also, in terms of total number of attacks, no differences between linetypes were detected on day 1 ( $12.2 \pm 1.9$  for C mice and  $15.6 \pm 1.9$  for S mice;  $P = 0.55$ ) or on day 2 ( $P = 0.43$ ).

In terms of total time spent in agonistic interactions, though, line S2 (lab designation is line 6) exhibited significantly higher levels of maternal aggression relative to all other individual lines on day 1 (Fig. 2C), and all lines except S4 on day 2 (data not shown). Line C3 exhibited the lowest levels of time aggressive, and these means were significantly different from line C2, S2, and

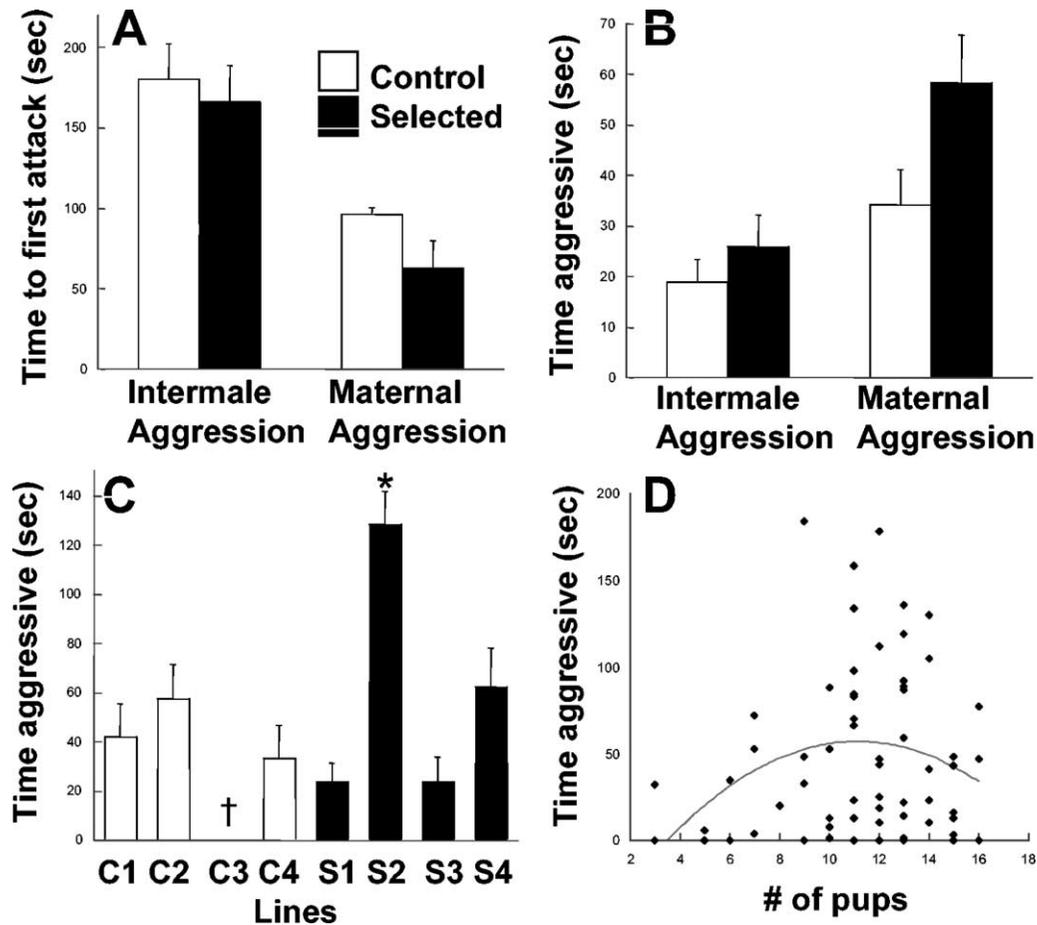


Fig. 2. Bar graphs indicating relative levels of aggression in generation 28 and 30, respectively, for intermale (test day 2) and maternal aggression (postpartum Day 7) between S and C mice in terms of (A) time to first attack and (B) time aggressive. (C) bar graphs of means levels of time aggressive for maternal aggression for each of the 8 lines on postpartum Day 7. (D) scatter plot of the relationship between mean time aggressive on postpartum Day 7 and the number of pups for S and C mice combined. \* =  $p < 0.05$  compared to all other lines; † =  $p < 0.05$  relative to lines C2, S2, and S4, Tukey HSD Post Hoc test.

S4 (on day 1) (Fig. 2C) and S4 (on day 2) (data not shown). As would be expected for line C3 that exhibited no aggression on day 1 and extremely aggression low aggression on day 2 ( $0.8 \pm 0.8$  sec), the latency to first attack ( $300.0 \pm 0.0$  sec on day 1,  $265.7 \pm 34.2$  on day 2) was significantly higher than for all other lines on both test days ( $P < 0.05$ ).

S lines had significantly more pups than did C lines ( $12.0 \pm 0.4$  compared with  $10.3 \pm 0.6$ ;  $F(1,6) = 6.40$ ,  $P = 0.04$ ). Therefore, we also compared maternal aggression between S and C mice after adjusting for number of pups by including number of pups and number of pups squared as covariates in the analyses. However, results remained the same, i.e., S and C lines did not differ for any measure of maternal aggression. Interestingly, we found a significant concave, polynomial relationship between time aggressive on day 1 and number of pups with C and S combined (1st and 2nd order polynomial regression coefficients were significant,  $F(1,54) = 5.12$ ,  $P = 0.03$ ,  $F(1,54) = 4.24$ ,  $P = 0.04$ , respectively; Fig. 2D).

#### *Generation 30 intermale aggression and comparisons with maternal aggression*

In Generation 30, in terms of time to first attack and mean time aggressive, S and C lines showed no significant differences on either test day 1 (data not shown) or day 2 (Figs. 2A and 2B). Also, in terms of number of attacks, no differences between S and C lines were detected (data not shown). Unlike for maternal aggression, individual lines showed no significant differences on any of these tests of intermale aggression on either test day (data not shown).

Maternal aggression was more intense than intermale aggression, as indicated by shorter latency to first attack, longer time aggressive, and greater number of attacks on both test days for maternal aggression (Fig. 3). Mean time of aggression increased significantly for intermale aggression from day 1 to day 2, but decreased significantly for maternal aggression, although this level was still higher than that for intermale aggression on day 2 (Fig. 3B). For intermale aggression, the latency to first attack decreased

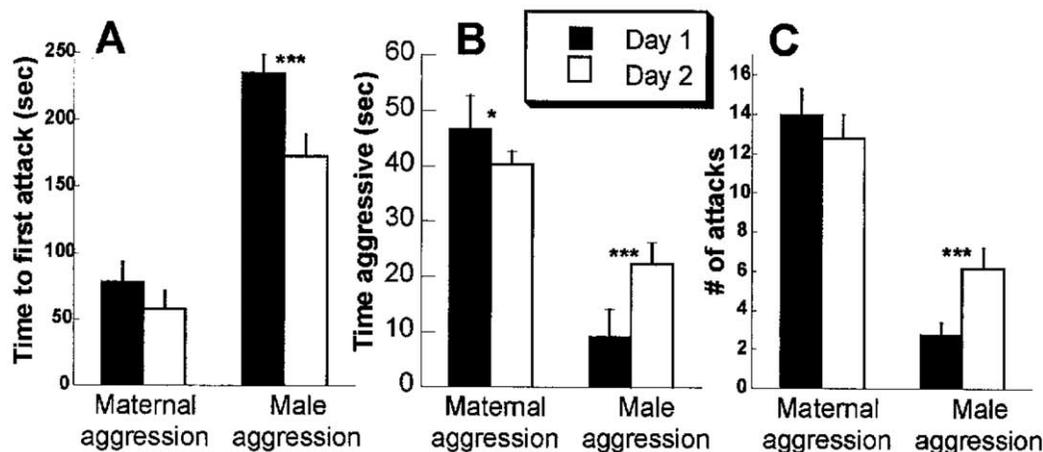


Fig. 3. Bar graphs indicating relative levels of aggression during maternal and intermale tests across the two trial days in terms of: (A) mean time to first attack; (B) mean time aggressive; and (C) mean number of attacks. Individuals from S and C lines were combined for this analysis because no significant differences between linetypes were found. For intermale aggression (generation 30), there was a significant decrease in time to first bite from day 1 to day 2, but a significant increase in mean time aggressive and mean number of bites. For maternal aggression (generation 28), there was a significant decrease in mean time from day 1 to day 2. For intermale aggression,  $N = 62$ ; for maternal aggression,  $N = 63$ . \* =  $P < 0.05$ ; \*\*\* =  $P < 0.0001$ ; paired  $t$ -test. Bars represent means  $\pm$  SE.

significantly from day 1 to day 2 (Fig. 3A), but for maternal aggression, this difference was not significant (Fig. 3A). Considering the higher of the two daily values, mean time aggressive for maternal (day 1) and male aggression (day 2) were not significantly correlated among the 8 line means ( $N = 8$ ; Pearson's  $r = 0.572$ ,  $P = 0.138$ ; Spearman rank correlation = 0.323,  $P = 0.434$ ).

#### Comparisons of generation 18 and generation 30 intermale aggression

Combining results for S and C mice for each generation, the mean latency to first attack on test day 2 for generation 18 was  $76.9 \pm 16.2$  sec and for generation 30 was  $173.0 \pm 15.9$  sec. These differences were significant using an unpaired  $t$ -test ( $T(99) = 4.0$ ,  $P < 0.001$ ). Because the maximum test for generation 30 was 5 min, but was 7 min for generation 18, a maximum latency of 300 sec was assigned to any generation 18 mice that took 5 min or longer to first attack.

#### Body mass, testes mass, and testosterone

As expected from previous studies (Girard et al., 2002; Swallow et al., 1999), generation 18 males from S lines were smaller (L.S. mean  $\pm$  SE of body mass =  $34.6 \pm 0.60$  grams) than those from C lines ( $38.9 \pm 0.65$ ). Testes mass (data not shown) did not differ between S and C lines ( $P = 0.467$ ), but mass-corrected testes mass did show highly significant differences among replicate lines ( $P = 0.001$ ), as was reported previously for mice from generation 10 (Klomberg et al., 2002). Plasma testosterone levels ( $0.519 \pm 0.064$  ng/ml for C lines,  $0.633 \pm 0.05$  for S lines) did not differ between S and C mice ( $P = 0.266$ ) when examined

one week after testing ceased. The lack of testosterone difference between S and C mice is consistent with a recent report of no differences in either baseline levels of testosterone or levels following a 10-min tube-test of social dominance (Klomberg et al., 2002).

#### Discussion

Results of this study indicate that voluntary wheel-running behavior is genetically related to predatory aggression, but not to either maternal aggression or intermale aggression. Further, the results suggest that predatory and intermale aggression are genetically unrelated, and likewise that maternal and intermale aggression are not closely related at the genetic level.

Studies of generation 18 indicate that predatory and intermale aggression are unrelated at both the genetic and phenotypic levels. Although predatory aggression was elevated in the S lines (discussed below), intermale aggression was not. Similarly, we observed no correlation at the level of line means (after adjusting for the linetype difference) or among individual mice. Our findings are consistent with work by others that also did not find an association between intermale and predatory aggression in mice (Brain and Al-Maliki, 1978; Butler, 1973; Popova et al., 1993). In one study that selectively bred mice for high intermale aggression, predatory aggression was significantly higher relative to a group selected for low intermale aggression, but not compared to a non-selected control group (Sandnabba, 1995a). As described below, low aggressive mice may reflect an elevation of pathologies or decreased physiological function, and a number of behavioral traits may also be indirectly impaired that are not directly associated with low

aggression. Thus, a lack of significant difference of predatory aggression between the non-selected control group relative to the high aggressive group (Sandnabba, 1995a), suggests a lack of association between predatory and intermale aggression.

Because injection of the same dose of a serotonin agonist into outbred mice significantly reduced intermale aggression, but did not affect predatory aggression (Ferrari et al., 1996), it is possible that, at certain levels, serotonin has an unequal effect on the two forms of aggression. However, increased levels of serotonin have been strongly implicated in the suppression of both intermale aggression (Nelson and Chiavegatto, 2001) and predatory aggression (Nikulina, 1991; Pucilowski and Kostowski, 1983). Thus, it is possible that serotonin acts similarly in the control of these two behaviors, but that intermale aggression is more sensitive to changes in serotonin levels. It could be expected, then, that some aspects of the control of different forms of aggression will have a common neural basis. Exactly how serotonin interacts with the neuronal circuitry underlying intermale and predatory aggression, especially across different doses, still needs to be determined.

The correlated response of increased predatory aggression in the S lines may result because both behaviors (wheel running and predatory aggression) share similar neuronal substrates. Recent work suggests that dopamine D1 receptor function is reduced in the S lines (Rhodes and Garland, 2003; Rhodes et al., 2001) and other studies have implicated dopamine in predatory aggression in rodents and carnivores (ferrets) (Baggio and Ferrari, 1980; Jimerson and Reis, 1973; Schmidt, 1979; Schmidt, 1983; Siegel et al., 1999). Dopamine facilitates predatory aggression, possibly via D2 receptors, in some studies (Shaikh et al., 1986; Siegel et al., 1999), yet dopamine receptor antagonists also facilitate this behavior in other studies (Baggio and Ferrari, 1980; Schmidt, 1979; Schmidt, 1983). These varying results suggest it will be critical to understand exactly where in the brain and at what levels dopamine is released (or inhibited from release) to determine how dopamine is involved in predatory aggression.

The lateral hypothalamus, which mediates predatory attack in cats and rats via brain stimulation (Siegel et al., 1999), is one of the few brain regions showing higher levels of neuronal activity in terms of cFOS expression in S versus C mice in association with increased motivation for wheel running (Rhodes et al., 2003). Therefore, it is possible that the same genetically altered neuronal function that causes increased voluntary wheel running (e.g., altered dopaminergic function or differential activation of the lateral hypothalamus) increases predatory aggression, and accounts for the correlated response between wheel running and predatory aggression. Given the correlation of high-wheel running behavior and predatory aggression, it is possible that an understanding of the alteration in dopamine signaling in S mice will help to understand the shared bases of these two behaviors. One possible line of speculation is that decreased

D1 receptor function in S mice causes an alteration of dopamine release and/or D2 expression that affects both running/reward behavior and predatory aggression. It is also possible, however, that neural structures involved in the correlated aggressive and wheel running behaviors could be related via pleiotropic effects of genes on separate neural pathways.

Intermale aggression, scored in resident-intruder tests at generations 18 and 30, did not differ between S and C mice. However, differences in latency to attack were noted between generation 18 and 30 when results from mice from S and C lines were combined. Because sexual experience has been linked to higher levels of intermale aggression (Goyens and Noirot, 1975; Parmigiani et al., 1998), the shorter attack latencies of generation 18 may reflect that these males were sexually experienced. Despite differences in the condition of resident and intruder mice in generations 18 and 30, in neither case were differences between S and C mice detected in terms of intermale aggression.

At generation 10, S mice were more likely to retreat in a tube-test of dominance against a standard (inbred strain) opponent (Klomborg et al., 2002). As reviewed briefly in that paper, the relationship between aggression and dominance is a complicated one, and those results, in combination with results of the present study, overall do not suggest that they are closely related in these lines of mice. Recent evidence suggests that the hypothalamic-pituitary-adrenal axis may be altered in the S lines (Girard and Garland, 2002). If so, then previous tube test of dominance may have reflected such a difference, rather than intermale aggression per se.

As described in the Introduction, the hormonal and neural control of maternal and intermale aggression differs, but it is still possible that they are genetically related. Although two previous studies of selected lines of mice reported a positive relationship between maternal and intermale aggression, both had shortcomings. In one, the mice used for the “control” group had been selected for low aggression and only one line for each group was used (Sandnabba, 1993). Thus, no replication of selection was made and because the control group had been subject to a different form of selection, no adequate non-selected control group existed for comparisons of either group. Further, with only one line used, it is more difficult to determine which behaviors have been fixed by random genetic drift or the selection itself (e.g., see discussions in Garland et al., 2002; Garland, 2003). However, in the Sandnabba study, maternal aggression was tested on Day 3 and we conducted the tests on postpartum Days 7 and 8, so it is possible that methodological differences account for differences in our results. The second study found a correlation between maternal aggression and high intermale aggression (Hood and Cairns, 1988), but the “intermediate aggression control mice” exhibited unusually low levels of intermale aggression for the generation tested, which leaves open the possibility that the behaviors exhibited by both males and females during that

generation were atypical. That study also lacked replication in the selection process and used males from the control group as intruders for all maternal aggression tests, which could have decreased levels of maternal aggression in the control group due to increased recognition (Hood and Cairns 1988). In other lines of mice selected for high intermale aggression, though, maternal aggression was not elevated (Benus, 2001) and our results are consistent with this finding.

The lack of a finding of correlation between male and female forms of aggression has precedence in other types of studies. For example, in two studies that selectively bred for high female-female (not maternal) aggression (Hyde and Ebert, 1976; Hyde and Sawyer, 1980) and in one study selecting for high intermale aggression (van Oortmerssen and Bakker, 1981), no correlation between intermale and female-female aggression was found. In another study, selection for high female-female aggression was found to be correlated to high maternal aggression (Hyde and Sawyer, 1979). Taken together, these studies provide an indirect line of evidence that maternal and intermale aggression are not tightly related at the genetic level.

Although our study examined mice selected for high wheel running, we were able to examine correlations of maternal and intermale aggression across eight different lines that have been separated for 28–30 generations. The differences in levels of aggression among the different individual lines may be attributable to simple genetic drift. The possibility of genetic drift highlights the value of having multiple lines in a selection study (see also Garland et al., 2002; Garland, 2003). If only two lines were used (for example, S2 and any one of the C lines), it might have been concluded (incorrectly) that high wheel-running behavior is associated with high maternal aggression. By analyzing each line as a separate population and finding a lack of correlation between maternal and intermale aggression, our results suggest that these two behaviors are not tightly linked at the genetic level in this population.

Another line of evidence of differences between maternal and intermale aggression is that although on the second test day the latency to first attack decreased for both forms of aggression, the mean time aggressive increased significantly for male aggression but decreased significantly for maternal aggression (Fig. 3B). Our finding of increases in male aggression from the first to second test is consistent with studies of multiple aggression testing of males that find peak aggression occurs during the first few tests before declining (Burrigh et al., 1988; Cairns 1983; Ryan and Wehmer, 1975). For maternal aggression in mice, peak aggression can occur around postpartum day 4 and remain high through day 10, but then decreases consistently until the pups have left the nest around 21 days postpartum (Svare, 1990). Maternal aggression levels were also higher and fiercer than intermale aggression (see Fig. 3: 5-fold higher on day 1, 2-fold higher on day 2), suggesting that there are differences in their etiology.

Despite the differences in expression and control of intermale and maternal aggression, it is still likely that common genetic and neural components contribute to both forms of aggression. The sex determining region of the Y chromosome (*sry*) has been implicated in the control of intermale aggression, but genetic and environmental background upon which these genes act greatly influences levels of aggression (Miczek et al., 2001). Females clearly lack the *sry* region, but it is likely that common neural circuitry is used for some aspects of intermale and maternal aggression. For example, the lateral septum, bed nucleus of the stria terminalis, medial amygdala, and periaqueductal gray are brain regions implicated in both maternal and intermale aggression (Gammie and Nelson, 2001; Halasz et al., 2002; Lonstein and Stern, 1998). Although we found a lack of tight correlation between maternal and intermale aggression, studies examining whether or how these two behaviors covary across populations can be a critical tool for determining the underlying genetic and neural basis of aggression.

One of the most striking findings from this study was the identification of extremely high maternal aggression in Line S2 (lab designation is line 6) with a mean time of over 120 sec aggressive for a 5-min test. As a general comparison to levels of maternal aggression in other rodents, for a 10 min test, the following rodents exhibit the following mean times aggressive: C57 mice from 20 sec to 35 sec (Gammie et al., 2000a; Gammie and Nelson, 1999), prairie voles ~40 sec (Gammie and Nelson, 2000), bank voles ~40 sec on day 3 of lactation and ~20 sec on day 8 (Koskela et al., 2000), outbred Hsd:ICR mice (the original strain used to generate mice in these studies) ~30 sec (Ferrari et al., 2000), dwarf hamsters ~60–300 sec (S.C. Gammie and R. J. Nelson, unpublished observations), rats ~30 sec (Flannelly et al., 1986), and wild mice (fourth generation in lab) ~150 sec (Ferrari et al., 2000). Although it is difficult to make comparisons of levels of maternal aggression across species and studies, the levels of maternal aggression in line S2 are at the high end of those reported in other studies. To determine whether the aggression exhibited by these mice was true maternal aggression, in pilot studies we tested the dams (postpartum day 5–8) for maternal aggression after removal of pups for 24 hours from the home cage and in each case aggression was absent. As expected from other studies in mice (Svare, 1973), reintroduction of the pups for only 30 min triggered the reexpression of maternal aggression in each S2 dam. Thus, the aggression in lactating females of line S2 appears to be true maternal aggression that can be manipulated by sensory input from the pups, as has been shown in other rodents (Svare and Gandelman, 1976; Svare et al., 1980). We are currently developing line S2 as a model system for the analysis of maternal aggression.

Prior to the onset of selection for high wheel running, all of the outbred mice used were randomly mated for two generations to ensure equal distribution of gene variation between what would become the S and C mice, and to allow

for acclimation to the new husbandry conditions (Swallow et al., 1998a). Some of the differences in levels of maternal aggression between lines is the likely result of random genetic drift (for another example in these lines, see Garland et al., 2002) which would be expected in relatively small populations (each line contains only 10 families). Line C3 exhibited extremely low levels of maternal aggression. We speculate that this characteristic also is the result of genetic drift. Because levels of maternal aggression can be influenced by overall health during reproduction, it is possible that deficiencies in maternal aggression may indirectly reflect general physiological problems that have accrued via genetic drift. Line C3 did show the lowest mean number of pups born, but this was not statistically different from any of the other lines. At this point, we are unable to determine whether the deficits in maternal aggression in line C3 are specific or reflect any general physiological problems associated with reproduction.

Because we examined a large number of lactating females with a large range of pup numbers, we were able to gain insights into how pup number correlates with levels of maternal aggression. As seen in Fig. 2D, our data suggest that either low (9 or fewer) or high (15 or more) pup numbers are associated with decreases in maternal aggression. In the case of high number of pups, it has been shown that a lactating dam defends its homeostasis and allows a larger number of pups to grow at a slower rate (Leon and Woodside, 1983). However, in dams nursing large litters, thermogenic activity is suppressed relative to smaller litters (Isler et al., 1984) and it is possible that there is an upper limit of pups that a dam can maintain without impacting her health. Previous work indicates that stressors can negatively impact maternal aggression (Maestripieri and D'Amato, 1991; Pardon et al., 2000), so it is possible that any physiological deficiencies due to high pup number could impact aggression. Another explanation for high pup number on decreases in maternal aggression could involve increases in levels of either prolactin or opioids in the dams. Placentophagia elevates both prolactin (Blank and Friesen, 1980) and opioids (Kristal, 1991) in the dams and an increased number of pups could provide additional placenta for consumption. An increased number of fetuses could elevate prolactin in the dams during pregnancy. Levels of prolactin are also elevated in dams as a result of suckling by pups (Amenomori et al., 1970) and an increased number of pups could further elevate prolactin levels in dams (Grigor et al., 1984). Peripherally administered prolactin (Wise and Pryor, 1977) and opioids (Haney and Miczek, 1989; Kinsley and Bridges, 1986) decrease maternal aggression in some studies in rodents, so differences in pup number that affect levels of these signaling molecules could affect aggression. However, why increased pup number corresponds to decreased maternal aggression still needs to be determined.

In the case of low numbers of pups, it is possible that decreased pup numbers reflect physiological deficits in the dams and the decrease in maternal aggression is a by-

product of this compromised state. Alternatively, low pup stimulus from a decreased number of pups could affect levels of maternal aggression. In bank voles, artificially increasing size of the litter from 5 to 7 increases maternal aggression (Koskela et al., 2000), so it is also possible that levels of maternal aggression reflect the level of parental investment up to a point where pups produce a strain on the dam.

Our finding of lower mean number of pups in the S lines differs from a recent report of no differences in pup numbers between the S and C lines (Girard et al., 2002). In that study, though, only first litters were examined and here only second litters were examined. Thus, it is possible that interesting changes (e.g., increased numbers in S lines or decreased numbers in C lines) occur from the first to second litter, but a separate study examining both first and second litters would be required to identify properly such changes.

In summary, our results suggest that maternal and intermale aggression are not closely related at the genetic level, and that neither trait is associated with voluntary wheel-running behavior. Further, one line, S2, exhibited extraordinarily high levels of maternal aggression, apparently the result of random genetic drift. Finally, intermale and predatory aggression appear to be disassociated at the genetic level, but predatory aggression is positively related to wheel running. The genetic and hence neural basis of an association between wheel running and predatory aggression has yet to be determined, but may involve alterations in dopamine function.

## Acknowledgments

We thank J. Blake, K. Blake, A. Dua, D. Haubenschild, M. Holland, C. Kober, H. Moss, A. Sorokin, L. Stenton, J. Swallow, M. Zawojski, and especially M. Manske for assistance with colony maintenance and/or behavioral observations. We thank K. Klomberg and the staff of the Wisconsin Regional Primate Research Center Assay Laboratories for conducting the testosterone assays. We also thank J. Alexander and K. Skogen for help with animal care. This research was supported by University of Wisconsin Graduate School start-up funds and Department of Zoology start-up funds to S.C.G., NINDS fellowship NS42872-02 to J.S.R., and NSF grants IBN-9728434 and IBN-0212567 to T.G.

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