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Testosterone response to courtship predicts future paternal behavior in the California mouse, *Peromyscus californicus*

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Abstract

In the monogamous and biparental California mouse (*Peromyscus californicus*), paternal care is critical for maximal offspring survival. Animals form pair bonds and do not engage in extrapair matings, and thus female evaluation of paternal quality during courtship is likely to be advantageous. We hypothesized that male endocrine or behavioral response to courtship interactions would be predictive of future paternal behavior. To test this hypothesis, we formed 20 pairs of California mice, and evaluated their behavior during the first hour of courtship interactions and again following the birth of young. We also collected blood from males at baseline, 1-hr after pairing, 3-weeks paired, and when young were four days old to measure testosterone (T). We found that male T-response to courtship interactions predicted future paternal behavior, specifically the amount of time he huddled over young when challenged by the temporary removal of his mate. Males that mounted T increases at courtship also approached pups more quickly during this challenge than males who had a significant decrease in T at courtship. Proximity of the male and female during courtship predicted paternal huddling during a 1-hr observation, and a multiple regression analysis revealed that courtship behavior was also predictive of birth latency. We speculate that male T-response to a female in *P. californicus* is an honest indicator of paternal quality, and if detectable by females could provide a basis for evaluation during mate choice.

Keywords

Monogamy; paternal care; testosterone; courtship; mate choice; *Peromyscus*

Courtship behavior has been described in a wide variety of taxa including birds, reptiles, insects, amphibians and fish (Adkins-Regan, 1981; Lindzey and Crews, 1986; Sturtevant, 1915; Wagner, 1989; Wootton, 1984). Courtship behaviors themselves are diverse, but it is generally accepted that courtship serves as a tool to assess a potential mate and determine whether to proceed with the act of mating (Adkins-Regan, 2005). Although courtship can provide basic information regarding sex and species of the signaler, it can also be used to evaluate a potential partner for desired qualities. An animal may select a mate on the basis of direct benefits such as nuptial gifts or access to resources, or indirect benefits including good genes or offspring defense (Andersson, 1994). Classically, females have been considered the “choosy sex”; due to the contribution of costly ova and in many cases larger parental

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investment, females are predicted to benefit from cautious evaluation of males and their ability to provide direct or indirect benefits that will ultimately produce surviving young (Trivers, 1985). For example, female barn swallows benefit indirectly from mating with males who possess the largest ornaments, given that ornament size is positively correlated with enhanced offspring viability (Moller, 1994). In a monogamous, biparental species we might predict that paternal care is an important benefit and that a male would be able to signal his competence as a future parent. Ostlund and Ahnesjo (1998) have shown that fifteen-spined stickleback females prefer males that shake their bodies more frequently during courtship, and the frequency of body shakes is in turn correlated with more frequent egg fanning bouts and increased egg hatching success.

Biparental care as a component of a breeding system can heighten the consequences of choosing an unsuitable mate. If a pair cannot coordinate its behavior to provide adequate resources to young, the associated reproductive consequences may be severe. Moreover, the opportunity to remate following the death of a partner may not exist (Thomas and Wolff, 2004). Although rare, monogamy and biparental care occur in approximately 3–5% of mammalian species, and may enhance offspring survival (Kleiman, 1977; Wright, 2006). The California mouse, *Peromyscus californicus*, is a prime example of a species whose behavioral ecology implicates the need for careful evaluation of potential mates during courtship. First, California mice are strictly monogamous. DNA fingerprinting studies have shown no evidence of extra-pair fertilizations in the field (Ribble, 1991), and the majority of animals do not mate with a novel individual when presented with the opportunity in a laboratory setting (Gubernick and Nordby, 1993). The presence of the father also appears to enhance offspring survival. In the field, only 26% of young born to father-absent families reach weaning-age, whereas 81% of father-present pups do (Gubernick and Teferi, 2000). Similarly, laboratory studies have found that male care enhances offspring survival in cold environmental conditions and when parents are required to wheel-run for food (Cantoni and Brown, 1997; Gubernick et al., 1993). Overall, these data present a strong case for careful evaluation of prospective partners in the California mouse, and suggest that quality of paternal behavior may be evaluated in the mate choice process.

Testosterone (T) promotes paternal care in *P. californicus*, and is one possible endocrine mechanism that could link courtship interactions with future paternal behavior (Trainor and Marler, 2001; Trainor and Marler, 2002). Testosterone supports courtship components of male sexual behavior in a variety of species, including ultrasonic courtship calling in rodents (Floody et al., 1979; Nyby et al., 1977; Pomerantz et al., 1983), and courtship singing in birds; in all avian species studied to date, T metabolites play a role in the expression of courtship behavior (Harding, Sheridan and Walters, 1983). As such, T may provide an endocrine link between behaviors and resources that are of interest to females. As an example, female grey partridges select males on the basis of calling and vigilance behavior, the latter of which is androgen dependent (Fusani et al., 1997). Thus, males that demonstrate high levels of vigilance are behaviorally signaling that they are in good physical condition and will be able to protect their mate and offspring from predators.

In the current study, we tested the hypothesis that endocrine and behavioral measures during courtship predict future paternal behavior in the California mouse. Given the strong selection pressure associated with paternal care in this species, courtship may be an important time period in which indicators of paternal behavior are assessed by potential mates. We hypothesized that these predictors could be either behavioral, biological (specifically via T measures) or a combination of both behavioral and endocrine courtship variables. Based on recent work with the dark-eyed junco showing that natural, individual variation in T responsiveness is associated with paternal care (McGlothlin et al., 2007), we were particularly interested in whether T responsiveness might also be related to paternal behavior in the California mouse. We paired sexually naive male and female California mice, and observed their behavior during the first

hour of pairing, once weekly until the birth of young, and during expression of paternal care in both challenge (female temporarily removed) and strictly observational paradigms. The use of a challenge paradigm is particularly important because paternal care may be more essential when the female is absent from the nest. Blood samples were drawn from males at baseline, following 1-hr of courtship, at 3-weeks paired and 4 days following the birth of young. Finally, we looked for associations between courtship variables and paternal behaviors.

Methods and Materials

Subjects

We used 20 male and 20 female reproductively inexperienced *P. californicus* reared in a laboratory colony at the University of Wisconsin, Madison. Animals were maintained in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*, and the University of Wisconsin-Madison IACUC approved all procedures. Mice were weaned at 30 days old and housed in same-sex groups of two to four, in 48.3 cm long $\times$ 26.7 wide $\times$ 15.6 cm high cages. Animals had free access to Purina 5015 mouse chow and water. The testing room was maintained at 25°C under a 14:10 light/dark cycle with lights on at 2200 hrs, and all behavioral observations were conducted at 1300 hrs under dim red light. Testing began at 6–8 months of age, when we randomly assigned males and females to pairs. No male siblings were used, and no male/female pair shared common ancestry for a minimum of two prior generations.

Pairing Procedure

One day prior to introduction, fur was shaved on both flanks or the lower back of each male and female for identification on videotape. The male was then placed in a 91 cm long $\times$ 46 cm wide $\times$ 43 cm high clear polycarbonate home arena equipped with a water bottle, mouse chow, a wooden nestbox (12.7 cm long $\times$ 11.4 cm wide $\times$ 8.3 cm high), a cotton nestlet, aspen bedding and a red transparent tube 15 cm long $\times$ 4.8 cm in diameter. During pilot studies we observed that using a smaller testing arena resulted in primarily aggressive interactions (E.D.G., personal observation), and thus we chose testing arenas approximately three times larger than a standard housing cage. We placed males in testing arenas for 24 hrs before the introduction of a female, allowing the males to establish territories (Bester-Meredith et al., 1999; Trainor and Marler, 2001); by introducing a pair within the male's territory, we sought to mimic the female-biased dispersal pattern of the California mouse (Ribble, 1991). At pairing the female was placed in the male's home cage, and 1-hr of videotape was recorded. Behavioral observations were recorded for 1-hr weekly until the pair delivered pups. An observer blind to pair identity scored the 1-hr courtship period for all behaviors that occurred; those that were consistently observed were selected for statistical analysis (Table 1). Although we intended to separate any pair displaying life-threatening aggression at pairing, no interventions were required. Courtship behaviors were subsequently scored by a reviewer blind to pair identity.

Paternal Behavior Assessment

We chose to assess paternal behavior using two separate observational paradigms, including a paternal "challenge", as well as an unmanipulated observation. On post-natal day 3 (PND3), we conducted a pup-displacement challenge (PDC) to measure paternal behavior in the absence of the female. An experimenter removed the mother and pups from the testing arena 90s before the start of the trial, returning pups to the location in the testing arena furthest from the nest (generally the opposite corner of the cage). At the conclusion of the 10-min trial, the mother was returned to the family. Latency to approach young, duration of huddling over young, duration of licking and grooming young, overall time spent in contact with young, and the number of retrievals were recorded. Huddling was defined as sitting crouched with an arched back over young, with minimal movement. Retrievals, although rare at this early stage in

development (Bester-Meredith et al., 1999), were defined as grasping the pup by the skin of its neck or back and carrying it to another location in the testing arena.

On PND4, we conducted a 1-hr observation of paternal behavior without manipulation to assess paternal behavior in the presence of the female. We recorded for each parent the duration of nest attendance, huddling over young, the number of times animals left the nest, proximity between mates, following, nose sniffs, grooming and nestbuilding. Neither pup retrievals nor aggressive behavior between mates was ever observed in the PND4 behavioral sample. For the 1-hr observation of paternal behavior, Z- scores were calculated for each of two correlated paternal huddling behaviors (huddling together with mate and pups, huddling alone with pups; $r=0.64$, $p=0.010$) and averaged, resulting in a composite “Paternal index” (PI) score in which each huddling behavior equally contributed to the overall composite score. All paternal observations were videotaped, and a reviewer blind to pair identity scored the tapes for paternal behavior.

Steroid Hormones and Courtship Behavior

To investigate the relationship between steroid hormones and behavior during courtship and paternal care, retroorbital blood samples (60–120 μ l) were collected from males under isoflurane anesthesia at four different stages in the study. Samples were always collected at 1400h to control for daily hormone fluctuations. Briefly, males were removed from their testing arenas, transported to a small induction chamber in the same room, and anesthetized. Each sample was collected using heparinized capillary tubes, and immediately transferred to a microcentrifuge tube placed on ice. Following collection, the researcher applied light pressure to the orbit using a gloved finger to ensure that bleeding had stopped. When an animal became fully ambulatory, as defined by the ability to walk and grip the experimenter’s handling glove, he was returned to his testing arena. The first sample, taken three days prior to pairing with a female, served as a baseline for hormone levels independent of sexual experience. After 1-hr of courtship, males were removed from the testing arena, sampled, and returned. Three weeks after pairing, another sample was collected to serve as a proxy for pair bonding, since 70% of females in our animal colony are pregnant within 3-weeks of pairing (compilation of colony data by E.D.G.). In monogamous mammals the physical act of mating is known to facilitate formation of the pair bond (Williams et al., 1992). Finally, we took a fourth sample on PND 4, following the 1-hr unmanipulated observation of paternal behavior. In all cases, blood samples were collected in less than 3 min from the time that the male was removed from his cage. Blood samples were centrifuged at 10,000 rpm for 10 min at 4°C (Eppendorf, model 5417R) after collection, and plasma was stored at –80°C. Analysis of samples was completed at the Wisconsin National Primate Research Center. Steroids were extracted twice with ethyl ether and separated using celite chromatography, and T was analyzed using enzyme immunoassay (T antibody R156, UC-Davis diluted to 1:35,000, validated for California mice by Bester-Meredith and Marler, 2001; Trainor and Marler, 2001; Davis and Marler, 2003). The intra- and inter-assay coefficients of variation for T were 7.5% and 15.4%, respectively.

Statistical Analysis

Data were analyzed using SPSS for Macintosh (version 16.0.1, SPSS Inc., Chicago, IL). Sample sizes for each steroid measure and behavior varied; three pairs did not produce young during the experiment and were thus excluded from paternal observations, and one pair had to be separated in the third week of testing due to injuries resulting from aggression that compromised the well being of the animals. Finally, technical difficulties removed additional data points in some cases. The smallest sample size used was 13 pairs, but more typically was 19 to 20 pairs. T-response to courtship interactions was evaluated using a change score, such that “T-response” was calculated by subtracting baseline T from T after 1-hr of courtship. For all steroid and behavioral data, normality was assessed using residual plots and the data were

transformed as needed; nose sniffs, latency of the male to approach the female, female jabs, male chases, wrestle latency and following behaviors were natural-log transformed.

For paternal observations, reviewers of the behavioral data were unable to visually distinguish the specific type of behavior in which a father was engaged (e.g., movement in nest versus grooming of pups) during the pup displacement challenge (PDC), and as such we chose to include only the time that a male spent huddled over his young (PDC huddling) and latency to approach pups in analyses for this sampling period. We noticed furthermore that male latency to approach and care for pups during the PDC followed a bimodal distribution, and animals were subsequently grouped into fast approach (FA < 27 seconds) and slow approach (SA > 50 seconds) fathers and compared using t-tests. Finally, we calculated birth latency by subtracting the average gestation length in our colony (31 days; E.D.G., unpublished data) from the number of days between pairing and the birth of a litter.

A repeated measures ANOVA was used to evaluate differences in mean T across each of the baseline, courtship, bonded and paternal phases of the study. Hypotheses regarding the predictive value of courtship endocrine and behavioral variables were tested using linear regression as well as Pearson correlations; for each family of correlations, the Benjamini-Hochberg procedure was implemented to control for Type I error (Benjamini and Hochberg, 1995). We conducted separate multiple regressions for two types of dependent variables that reflect pair success, paternal behavior and birth latency. Predictor variables, which included endocrine as well as behavioral measures, were entered into the model simultaneously. After testing our original hypotheses, we also chose to perform a follow-up multiple regression on T-response to further explore our findings.

Results

Hormones by Stage of Experiment

A repeated measures ANOVA revealed that mean T varied significantly by phase of experiment, $F_{3, 33} = 3.78$, $p = 0.02$. Tukey post-hoc tests indicated that paternal T was significantly lower than courtship T, $p < 0.05$, but no other group differences were detected (Fig. 1). The following results are presented in terms of paternal behavior and birth latency variables.

Paternal Behavior

Hormones: We began by asking whether courtship T was related to future paternal behavior. The change in male T during courtship, or T-response, was significantly correlated with huddling over young during the PDC ($r = 0.74$, $p = 0.006$) (Fig. 2), but not with PI score ($r = 0.003$, $p = 0.99$). Baseline T was not correlated with either PDC huddling ($r = -0.051$, $p = 0.87$) or PI score ($r = -0.11$, $p = 0.72$), whereas post-courtship T measures were correlated with PDC huddling ($r = 0.62$, $p = 0.025$), but not PI score ($r = 0.041$, $p = 0.89$). T-response was a stronger predictor of future PDC huddling than post-courtship T, accounting for 55% of variance in PDC huddling ($R^2 = 0.55$).

Intriguingly, of 17 males, 47% ($n = 8$) experienced an increase in T during courtship ($M = 0.99$, $SD = 0.84$), and 53% ($n = 9$) experienced a decrease in T during courtship ($M = -0.41$, $SD = 0.37$). Paired t-tests revealed that this change in T from baseline to courtship was significant for both the T-increase group ($t_7 = -5.18$, $p < 0.001$) and the T-decrease group ($t_8 = 3.33$, $p < 0.01$) (Fig. 3). T measures were statistically equivalent between T-increase and T-decrease males at baseline ($t_{14} = -1.06$, $p = 0.31$), but not at the end of courtship ($t_{14} = 2.68$, $p = 0.018$). Although there was no difference between T-increase and T-decrease males in the time it took for them to approach pups during the PDC ($t_{11} = -1.62$, $p = 0.13$), fathers that approached their pups

quickly had significantly higher courtship T than slow-approaching fathers ($t_{8,65}=2.63$, $p=0.028$) (Fig. 4). Thus, a rise in T at courtship was associated with faster latency to approach pups and longer duration of care for pups during the PDC, but not with PI score during a 1-hr observation with both parents present, demonstrating behavioral variation between paternal care paradigms.

With the knowledge that T-response to courtship interactions was an important predictor of future paternal behavior, we employed multiple regression to identify predictors of the T-response. Beginning with a full model that regressed T-response on the number of male siblings, all courtship behavior, and baseline T measures, we identified a reduced three-variable model that predicted T-response. Specifically, we found that female jabs, baseline T and number of male siblings accounted for 90 % of variance in courtship T ($R^2=0.90$, $F_{3,14}=33.99$, $p<0.001$). Regression coefficients indicated that baseline T was positively associated with the T-response, whereas female jabs and male siblings were negatively associated with T-response.

Courtship Behavior: With evidence that T-response predicted future paternal behavior, our next question was to address whether courtship behaviors (wrestle latency, number of wrestles, female jabs, following, nose sniffs and chases) could also predict future paternal behavior, as measured by PDC huddling or PI score. No courtship behavior was significantly correlated with either measure, with a maximum Pearson correlation coefficient of $r = -0.36$. However, proximity between animals during courtship was correlated with the amount of time the male spent huddling with young during the 1-hr paternal behavior observation ($r = 0.59$, $p = 0.019$) (Fig. 5). Therefore, there was variation between males in the amount of time spent huddling with pups during the undisturbed 1-hr observation, and proximity between the male and female during courtship predicted these variations in paternal behavior.

Birth Latency

Hormones: No endocrine measure from baseline or courtship was correlated with birth latency, with a maximum Pearson correlation coefficient of $r=-0.41$. There was no significant difference in birth latency between T-increase and T-decrease males ($t_{7,49}=-1.74$, $p=0.12$). Therefore, while T-response predicted future paternal behavior in the absence of the female, it did not predict birth latency.

Courtship Behavior: Behavior during courtship was naturally grouped into two categories of highly correlated variables that influenced birth latency. Amicable behaviors included following behavior and nose sniffs, which were positively correlated ($r = 0.73$, $p < 0.001$). Conversely, following was negatively correlated with number of wrestles, a measure of aggression ($r = -0.58$, $p = 0.011$). Proximity of animals during courtship was negatively correlated with birth latency, with pairs spending the greatest amount of time in the same cage quadrant during the courtship hour having the shortest birth latencies ($r = -0.61$, $p=0.010$) (Fig. 6). Relatedly, following behavior was also correlated with birth latency, such that pairs engaging in the longest duration of following had the shortest birth latencies ($r = -0.56$, $p=0.02$). Overall, proximity during courtship predicted both birth latency and future huddling behavior with pups when the mother was present.

Pairs typically engaged in both aggressive and amicable behaviors during the courtship period. As such, we were interested in how the combination of courtship behaviors might influence birth latency. Using multiple regression, birth latency was regressed on courtship behaviors (latency of the male to approach the female, following, nose sniffs, number of wrestling bouts, latency to wrestle, chasing by the male and defensive jabs by the female) to determine which of these behaviors influences birth latency. A reduced model including following by the male, chasing by the male and defensive female jabs was significant ($R^2=0.84$, $F_{3,16}=22.27$,

$p < 0.001$). Jabs were associated with a more extended birth latency, whereas chases and following were negatively associated with birth latency. Overall, both aggressive (female jabs) and amicable behaviors (following behavior) were found to be predictive of birth latency in our multiple regression analysis.

Discussion

The hypothesis that steroid hormones or behaviors during courtship would predict future paternal behavior was supported. We found that male T-response to courtship interactions positively predicted future paternal behavior, specifically the amount of time that a father huddled over his young when challenged by the temporary removal of his mate. Fathers that approached their young quickly (< 27 s) at the onset of this challenge also had higher courtship T on average than fathers who approached their young slowly (50 s $<$). The results of the multiple regression analyses furthered our understanding of this relationship, showing that male T-response to courtship interactions explained 55% of variance in future PDC huddling.

Although behavior measured by the paternal index (PI) on PND 4 was not related to T-response to courtship, we suggest that PDC huddling may be a more important and relevant behavioral measure. For conditions in which the entire California mouse family is present, such as our PND 4 behavioral observation, a male may not be obligated to care for young. When both parents are present, there is some indication that females will compensate for reduced paternal care in this species (Dudley, 1974; Trainor and Marler, 2001), and likewise when both parents are present, pups are rarely left unattended (Dudley, 1974). Previous research has shown that California mouse pups whose mother has been temporarily removed are unable to maintain their body temperatures without the presence of their father (Dudley, 1974), which may well translate into a higher rate of pup survival. Similarly, pup defense in the absence of the mother is likely to be extremely valuable (Wolff and Macdonald, 2004). Care by the father would also concurrently enable the mother to forage and replace calories lost via the demands of pregnancy and lactation. Thus, the duration of paternal care in the PDC might be a more decisive measure of paternal quality for California mice than the unmanipulated PI, and explain the absence of a link between T-response to courtship and PI. Whether a male will rise to the challenge of caring for young when the mother is absent might indicate a father that is highly responsive to his mate and pups, and who will be most successful in rearing offspring.

The ability of female stimuli to trigger a rapid rise in luteinizing hormone (LH) followed by T is well-documented in mammals (Johnston and Bronson, 1982; Maruniak and Bronson, 1976; Mendoza and Mason, 1989; Nyby, 2008; Roney et al., 2007). In house mice (*Mus musculus*), males show increased T 30–60 mins following an encounter with a female or her odor (Macrides et al., 1975). While the exact function of these rapid increases in T remains unclear, they are hypothesized to reflect the extent to which a male is aroused given that they occur in conjunction with courtship vocalizations (James et al., 2006; Nyby, 1983). Thus, while the observed increases in T following courtship interactions were not surprising, our finding that approximately half of the males in our study responded to courtship interactions with a statistically significant decrease in T was unexpected. While certain contextual information such as the odor of an adult male can block male T-response (Clancy et al., 1988), to our knowledge a statistically significant decrease in T in a sexual context has not been reported in a male mammal. If T-response does indeed reflect male arousal (James et al., 2006), our results suggest that an inhibition of sexual arousal, and potentially reproductive behavior, was warranted. Differences in social system is one likely reason for which the parameters of male arousal appear to differ in house mice and California mice; as a strictly monogamous and biparental species (Ribble, 1991; Ribble and Salvioni, 1990), sexual behavior in the California mouse is inextricably linked with pair formation, and ultimately the coordination of parental care.

It seems likely that male arousal will depend on a combination of factors including female attractiveness, male ability to mount a T-response, and the dynamics of pair interactions. As such, variability in T-response to courtship interactions could be attributed to a combination of internal and external factors. In the cooperatively breeding common marmoset, paired males caring for young do not mount T-responses to the ovulatory scent of a novel female, whereas single-housed males and those without offspring do (Ziegler et al., 2005). These data support an inhibitory role for certain social contexts on neuroendocrine response, or in shaping the exact cues to which the neuroendocrine system responds. We speculate that favorable signals from a female during courtship could trigger T-responses that shape the neuroendocrine system to support future parenting. In this view, variability in female attractiveness or pair dynamics could dictate T-response and future paternal behavior, with male ability to respond showing lower variability. While the concept of portioning parental investment based on mate quality is familiar (Velando et al., 2006), this explanation seems unlikely in a male of a genetically monogamous and biparental species. Once a pair has formed and infants are born, a large investment has already been made by both parents. Caring for young at this stage does not represent a tradeoff from care directed towards litters with other females, or from seeking additional mates.

While T-response may depend in part on a male's external social environment, it may also reflect natural variations in intrinsic male characteristics. For example, there are large individual differences in the ability to be paternal prior to sexual experience in the California mouse; a small but significant percentage of males (11%) are paternal prior to any sexual experience, whereas others become paternal only after their own young are born (Gubernick et al., 1994). While most male California mice (57%) eventually do become paternal, males that are paternal before pairing nearly always (97% of individuals) remain paternal, and a previously paternal male has never been observed to become infanticidal as a father (Gubernick et al., 1994). It is possible that T-response is a means by which females can identify the most paternal males, and avoid those that could potentially attack or ignore pups in the future. Recent work by McGlothlin and colleagues with dark-eyed juncos (*Junco hyemalis*) demonstrated that natural variations in paternal investment are reflected in T responsiveness. Specifically, individual T-responses to a gonadotropin-releasing hormone (GnRH) challenge were negatively correlated with parental behavior (McGlothlin et al., 2007). Moreover, a positive correlation between tail white, a plumage ornament used in mate choice, and T-responsiveness was also demonstrated (McGlothlin et al., 2008). Taken together, the data from these two papers suggest that tail white is an honest indicator of T-response, and raise the intriguing possibility that females are assessing future male behavior. In contrast to many paternal species, T is positively associated with the simultaneous expression of both aggressive and parenting behavior in the California mouse (Oyegbile and Marler, 2005; Trainor and Marler, 2001). For this reason, assessment of T responsiveness in courtship interactions could serve as an honest indicator of male paternal quality in California mice.

We speculate that the relationship between T-response at courtship and future paternal behavior in the California mouse may be a signal of male quality. It remains unclear how a rapid rise in T could quickly be translated into a perceptible signal, but several intriguing possibilities exist as androgens are increasingly being shown to exert rapid effects on behavior (Remage-Healy and Bass, 2006). Scent-marking behavior in mammals is androgen-dependent (Ulibarri and Yahr, 1996), and female rodents are capable of making distinctions about dominance status and male quality via urinary and chemosensory signals (Ferkin et al., 1994; Gottreich et al., 2000; Taylor et al., 1982). However, research has concentrated on circulating levels of hormones as opposed to rapid increases. Androgen-dependent ultrasonic courtship vocalizations are another means by which males could signal their endocrine responsiveness (Dizinho and Whitney, 1977; Nunez et al., 1978). In CF-1 mice, males begin to emit courtship vocalizations within minutes of encountering a female, and calls within the first minute are

predictive of T levels 30-min later (James et al., 2006). Courtship vocalizations are thus a potential conduit of early information regarding a male's ability to mount a T-response, prior to any actual change in T.

The strong positive correlation between T-response to courtship and future paternal behavior is very intriguing from the viewpoint of causality. It is possible that males who experience a rise in T at courtship already possess the correct neuroendocrine environment for responsiveness to pups during a challenge, but alternatively rises in T at courtship and any subsequent changes in T release could serve to prime the male for future parenting. The conversion of testosterone to estrogen (E2) by the aromatase enzyme is one potential endocrine mechanism that could link courtship and paternal care across time. Previous work in our laboratory by Trainor and Marler (2001; 2002) demonstrated that the conversion of T to E2 by aromatase is the critical step required for fathers to show normative amounts of pup huddling and grooming. Castrated fathers show reduced huddling and grooming, but administration of E2 can rescue the behaviors (Trainor and Marler, 2002). Prior to the birth of their own pups, male California mice have higher plasma T, but are not consistently paternal (Gubernick et al., 1994); this suggests a change in the underlying neuroendocrine system associated with the birth of young to prepare for fathering. Androgens are known to regulate the expression of aromatase in discrete brain areas of male mammals and birds (Roselli et al., 1997). In the California mouse, aromatase levels are increased in the medial preoptic area of California mouse fathers 2–3 weeks after the birth of pups when fathers are huddling, grooming and retrieving pups, as compared to males who have been paired with a female for 2 weeks (Trainor et al., 2003). While the timing of these studies is different, it is clear that the aromatase system changes to prepare a male for paternal care.

Testosterone increases may also impact paternal behavior via the AVP neurochemical system. Vasopressin has been implicated in a variety of social behaviors including aggression, pair bonding, and parenting. In the monogamous and biparental prairie vole, infusions of AVP into the lateral septum of sexually naive male prairie voles cause males to spend more time crouching over and in contact with young (Wang et al., 1994), and central infusion of AVP can trigger paternal behavior in facultatively parental meadow voles (Parker and Lee, 2001). In *Peromyscus*, comparisons indicate that species contributing paternal care show higher AVP immunoreactive (AVP-ir) staining in the bed nucleus of the stria terminalis (BNST), and a higher density of AVP V_{1a} receptor density in the lateral septum (Bester-Meredith et al., 1999). Specifically, males that spent more time engaged in huddling, grooming, and in the nest with their young had the highest levels of AVP-ir staining in the BNST (Bester-Meredith and Marler, 2003). Vasopressin pathways are androgen dependent both in development and throughout the lifespan of male rats, and respond to postcastration T treatment in a dose-dependent fashion (Magnusson and Meyerson, 1996). In male rats, castration causes vasopressin-ir cell bodies to disappear from the BNST and medial amygdala, but can be restored with T replacement (DeVries et al., 1985).

Courtship behavioral interactions predicted one element of future paternal behavior. Specifically, proximity between animals during courtship interactions was correlated with the total time that a male spent huddling over his young during the 1-hr observation period. In addition, courtship behaviors were associated with another measure of pair success, time to produce a litter. Following behavior and chases by the male during courtship were correlated with shorter birth latencies, whereas defensive jabs predicted longer birth latencies. Proximity during courtship may be an early indicator of compatibility of the pair, given that proximity could not be maintained if either the male or female was unwilling to interact with the other individual. Following behavior is represented within the proximity measure, and may be related to birth latency because a female nearing estrus is likely to be more tolerant of a male's persistence in sexual advances; in addition, an estrus female may also be more attractive to the

male. Likewise, male chasing may indicate sexual excitement due to behavioral receptivity of the female. It is worth noting that in a recent study with prairie voles, Ophir and colleagues (2007) found that female-directed aggression during choice tests, but not affiliation, was correlated with paternal behavior. It remains unclear whether species or methodological differences best explain these divergent patterns of results.

Interestingly, although several behavior variables were associated with shorter birth latency, in no case did we observe copulation within an hour of introducing animals. These findings are in agreement with previous work indicating that even with the use of a priming paradigm, California mice copulated on average 48 mins following initial pairing (Dewsbury, 1974). It does not seem surprising, then, that long mating and birth latencies were observed, particularly because we did not prime females in the present study. Female jabs were clearly a defensive behavior intended to deter males, and are consistent with pairs who had longer intervals between pairing and conception.

In sum, our findings are among the first evidence that endocrine responsivity and behavior during courtship interactions can predict future paternal behavior in a monogamous and biparental mammal. In terms of courtship behaviors, proximity of the pair predicted male huddling during a 1-hr paternal observation. In contrast to hormone measures before or after courtship, the direction and magnitude of T release in response to a female were linked to huddling behavior during a pup displacement challenge. In the California mouse, aggressive and paternal behaviors are expressed concurrently and have both been associated with T (Oyegbile and Marler, 2005; Trainor and Marler, 2001). We hypothesize that an honest indicator of male endocrine responsivity could be used during courtship to provide potential mates with information about a male's future paternal and territorial behavior, particularly in response to a challenge.

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Fig. 1. Mean male T (ng/mL) $\pm$ SEM by phase of study ($n=13$). Repeated measures ANOVA was significant $F_{3,33} = 3.78$, $p = 0.02$. Different letters over bars indicate a significant difference using Tukey HSD post-hoc comparisons ($p < 0.05$).

Fig. 2. Change in T (ng/mL) at courtship is significantly correlated with time spent (s) huddling over young during the pup-displacement challenge (PDC) ($n = 12$, $r = 0.74$, $p = 0.006$).

Fig. 3.

A significant increase in T at courtship was observed in some males (left panel, $n=8$, $t_7 = -5.18$, $p < 0.001$), whereas others experienced a significant decrease in T (right panel, $n=9$, $t_8 = 3.33$, $p < 0.01$, $p < 0.01$).

Fig. 4.

A bimodal distribution was observed in latency of fathers to approach pups during the pup-displacement challenge (PDC). All individuals who approached pups quickly (<27s) had experienced an increase in T at courtship, and all males who approached slowly (>50) had experienced a decrease. A significant difference in mean T change at courtship was observed ($p=0.028$).

Fig. 5.

Time animals spend in close proximity during the courtship period is positively correlated with male time spent huddling over young during the 1-hr paternal sampling period ($n=15$, $r=0.59$, $p=0.019$).

Fig. 6. Time animals spend in close proximity during the courtship period is negatively correlated with birth latency ($n=17$, $r=-0.61$, $p=0.010$).

Table 1

Description of behaviors consistently observed across pairs during the courtship period.

Behavior	Description
Proximity	The amount of time that animals are within the same quadrant of the testing arena.
Following	The female walks slowly around testing arena, and the male follows and investigates her anogenital region. The female may pause briefly, but continues to allow investigation by the male.
Nose sniff	The male and female simultaneously approach, touch noses and engage in mutual nose-to-nose investigation
Wrestle	Aggression developing into a tumble, in which animals attempt to pin one another
Chase	One animal pursues the other while running, attempting to make contact
Defensive jab	A fast, outward motion with the forepaw to deter an approaching animal, the result of which is typically a retreat by the offender. Only observed in females.