

Male fidelity expressed through rapid testosterone suppression of ultrasonic vocalizations to novel females in the monogamous California mouse

By: Joshua D. Pultorak, Matthew J. Fuxjager, [Matina C. Kalcounis-Rueppell](#), Catherine A. Marler

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Abstract:

The steroid hormone testosterone (T) is a well-known mediator of male sexual behavior in vertebrates. However, less is known about T's rapid effects on sexual behavior, particularly those involving ultrasonic vocalizations (USVs), a mode of communication that can influence mate acquisition in rodents. Using the monogamous California mouse, *Peromyscus californicus*, we tested whether T rapidly alters male USV production by giving T or saline injections to non-paired (sexually naïve) males and paired (paternally experienced and pair-bonded) males immediately prior to a brief exposure to an unrelated, novel female. Among non-paired males, no differences in the total number of USVs were observed; however, T increased the proportion of simple sweeps produced. Among paired males, T decreased the number of USVs produced, and this change was driven by a reduction in simple sweeps. These results suggest a differential rapid effect of T pulses between non-paired and paired males upon exposure to a novel female. Additionally, we observed a positive correlation in the production of USVs made between males and novel females, and this relationship was altered by T. Given the importance of USVs in sexual communication, our study supports an essential concept of monogamy in that mate fidelity is reinforced by decreased responsiveness to prospective mates outside of the pair bond. The central mechanism in pair bonded males that decreases their responsiveness to novel females

appears to be one that T can trigger. This is among the first studies to demonstrate that T can inhibit sexually related behaviors and do so rapidly.

Keywords: Monogamy | Mate fidelity | USVs | Ultrasonic vocalizations | Testosterone | California mouse | *Peromyscus californicus* | Infidelity | Rodent | Extra-pair

Article:

Introduction

Monogamy should be favored by evolution when multiple mating is costly or when environmental conditions demand high levels of paternal care (Emlen and Oring, 1977, Parker, 2006 and Trivers, 1972). However, species exhibiting strict sexual fidelity are scant in the animal kingdom, even among socially monogamous species. A broad review of socially monogamous birds, for example, indicates that extra-pair copulations occur in over 90% of species (Griffith et al., 2002), with some exhibiting up to 93% of nests showing extra-pair offspring (Mulder et al., 1994). Among mammals, social monogamy constitutes less than 5% of known species (Kleiman, 1977), and the regulation of extra-pair mating effort is less understood. Here we test for a potential mechanism underlying mate fidelity in the strictly monogamous (Ribble, 1991) and biparental (Cantoni and Brown, 1997) California mouse (*Peromyscus californicus*). California mice re-pair with a new mate only after the disappearance of the mate in the wild (Ribble, 1991). In the laboratory, pair-bonded males, but not non-paired (sexually naïve) males, show reduced sexual advertisement to novel females via scent marking (Becker et al., 2012), and most individuals do not copulate outside the pair bond when given an opportunity (Gubernick and Nordby, 1993). Thus, California mice make an excellent model system to explore potential mechanisms of mate fidelity. Processes promoting monogamy in mammals have been largely explored under the lens of neuropeptide regulation of social bonds (reviewed in Young et al., 2008); however, neurophysiological mechanisms underlying such examples of social bonding may not be identical to those regulating sexual fidelity. In the present study, we examined how the response to the sex steroid hormone testosterone (T) functions to modulate mating behavior in the form of ultrasonic vocalization production toward extra-pair females in a highly monogamous species.

Testosterone is a primary candidate for hormonal mediation of extra-pair mating effort since it has a well-established role in promoting suites of behaviors related to reproduction across a variety of taxa (e.g., Ball and Balthazart, 2004, Dixson and Anderson, 2004, Goymann, 2009, Muller and Wrangham, 2004 and Oliveira et al., 2002). Despite the wealth of research on androgenic regulation of mating effort, relatively few studies have directly examined T's influence on extra-pair mating effort in monogamous species, and the existing examples are primarily exclusive to avian models (Eikenaar et al., 2011, Peters et al., 2001, Raouf et al., 1997 and Van Roo, 2004). In mammals that pair bond, including humans, baseline circulating T levels are commonly reduced in response to bond establishment or the birth of offspring

(Burnham et al., 2003, Nunes et al., 2000, Reburn and Wynne-Edwards, 1999 and Van Anders and Watson, 2007), suggesting that lowered T facilitates increased paternal behavior and reduced mating effort outside the pair bond. However, a recent body of research suggests that the role of T in the transition to pairing and fatherhood is more nuanced, with fathers showing adaptive short term increases of T that positively contribute to paternal care (Juana et al., 2010, Luis et al., 2009, Neff and Knapp, 2009, Pradhan et al., 2014, Reburn and Wynne-Edwards, 1999, Stiver and Alonzo, 2009, Trainor and Marler, 2002 and Ziegler and Snowdon, 2000; reviewed by Hau, 2007 and Marler et al., 2003). Similarly, chronic baseline T levels may be insufficient to fully capture the role of T in mate fidelity.

Although most studies of T and mating effort have examined long-term changes in baseline T, recent attention has been given to the dynamic changes in T and other steroid hormones on sexual behavior on smaller time scales that can induce rapid changes in behavior, particularly in response to dynamic social conditions (Cross and Roselli, 1999, Mangiamele and Thompson, 2012, Remage-Healey and Bass, 2010 and Remage-Healey, 2012). In rodents, like other mammals, transient increases (henceforth “pulses”) of T have been widely observed among males in response to encounters with females (Batty, 1978, Coquelin and Bronson, 1980, James et al., 2006, Macrides et al., 1975 and Nyby, 2008; reviewed by Gleason et al., 2009), but the consequences of these T pulses are not well understood, and even less so regarding extra-pair mating in monogamous species. Thus, it is plausible that T pulses induce rapid changes in mating effort toward extra-pair females. Based on the classical emphasis on the positive association between baseline T and sexual behavior, one hypothesis is that T pulses stimulate pair bonded male mating effort toward extra-pair females. However, we propose an alternative hypothesis: T pulses may strengthen the pair bond by attracting a male to his partner and/or inhibiting extra-pair mating effort. This idea is supported in part by findings in *P. californicus* illustrating a positive association between high T levels in males and close proximity to their mates (Gleason and Marler, 2012). To extend the study of variation in behavioral response to rapid T effects, we also examined within-species variation by contrasting sexually naïve, non-paired individuals and paternally experienced, pair bonded individuals within the monogamous California mouse.

In the present study, we measured ultrasonic vocalizations (USVs) as a metric of sexual interest toward novel females. USVs are high frequency acoustic signals that are sensitive to changes in the social environment and are extensively implicated in mating behavior in rodents, being reliably produced by males upon exposure to females or female olfactory cues (Brudzynski and Pniak, 2002, Burgdorf et al., 2008, Chabout et al., 2012, Holy and Guo, 2005, Nyby, 1979, Sipos et al., 1993 and Whitney et al., 1973). Male house mice (*Mus musculus*) produce complex USVs in higher proportions toward cues of unfamiliar, unrelated females than to familiar, related females or cues of male conspecifics (Musolf et al., 2010) suggesting that USVs play a role in courtship. Furthermore, females prefer to spend time with vocal males over devocalized males (Pomerantz et al., 1983a) and prefer playbacks of unfamiliar males to related males or females

(Musolf et al., 2010). Finally, similar to vocal production in male songbirds, production of USVs is reduced in castrated polygynous male rodents and restored by T-treatment (Dizinno and Whitney, 1977, Pasch et al., 2011, Pomerantz et al., 1983b and Warburton et al., 1989).

Despite the positive associations among USVs, mating behavior and T, neither vocal behavior of monogamous male rodents in response to extra-pair females nor the sensitivity of this behavior to androgens are well studied. We speculated that behavioral responses to novel females in males of monogamous species such as California mice may differ from polygynous species given their differing life histories and mating strategies, at least for pair bonded males. Following this line of thought, we hypothesized that male mating status within a monogamous species will modulate the responsiveness to extra-pair females and/or hormones governing mating behavior such that non-paired males, as compared to paired males, would show behavioral responses more similar to those of males of polygynous species. In the present study, we tested the rapid effect of T on USV production toward novel females in two groups of male California mice: sexually inexperienced (non-paired) males and sexually experienced, paternally experienced, long-term pair bonded (paired) males. Additionally, we measured the USV response of the female, which is notable since it has recently been argued that female vocal behavior is frequently overlooked in studies of rodent ultrasonic vocalizations (Petric and Kalcounis-Rueppell, 2013 and Von Merten et al., 2014). Among non-paired males, we predicted that T injections would stimulate vocal behavior toward novel females, given the positive relationship of androgens and USVs in response to females in polygynous rodents (Dizinno and Whitney, 1977, Pasch et al., 2011, Pomerantz et al., 1983b and Warburton et al., 1989). Conversely, among paired males, we predicted that T pulses would promote mate-fidelity and thus inhibit vocal behavior toward novel females.

Methods and materials

Subjects

We used 45 adult male (26 paired males and 19 non-paired males) (age > 8 weeks) and 45 adult female California mice (age > 8 weeks) reared in our laboratory colony at the University of Wisconsin-Madison. Animals were maintained in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals*. The University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC) approved the animal treatment and research protocols. Animals were housed in standard cages (48.3 cm × 26.7 cm × 15.6 cm) with one to three same-sex conspecifics after weaning (post-natal day 30) and were given water and food (Purina 5015™ mouse chow) ad libitum. Colony rooms and testing rooms were maintained at 20-23 ° C under a 14:10 light/dark cycle. All behavioral testing was conducted under a red light within 3 h of the onset of the dark cycle. Animals used in dyads for trials did not share common ancestry for a minimum of two prior generations.

Experimental procedure

For a given trial, one male was removed from his cage mates (or, in the case of a paired male, removed from his female partner) and housed alone for 24 h before the trial to reduce the possible effects of cage mate behavior. We placed the male in one chamber of a two-chambered Plexiglas arena (see the Supplementary materials for a diagram) with a total size of 90.0 cm (length) by 30.0 cm (width) \times 30.0 cm (height), enriched with bedding, a wooden nestbox (12.7 cm \times 6.4 cm \times 6.4 cm) and cylindrical plastic tubing (15.2 cm in length, 3.8 cm diameter). The base of the Plexiglas dividing wall, which separated the arena into two equally sized chambers (45.0 cm \times 30.0 cm \times 30.0 cm), contained two symmetrically located circular openings (3.8 cm in diameter, center of opening 7 cm from the side wall) covered by a wire mesh. Centrally located on either distal end of the arena were the ultrasonic microphones (described below). One side of the divider was designated to the male, the other to the female. This setup provided opportunity for auditory and olfactory communication, but restricted physical contact between individuals. Before the addition of the female to the arena, the male was acclimated to this new environment for 30 min (Gleason and Marler, 2010). Immediately prior to the start of the trial, both paired and non-paired gonadally-intact males were randomly assigned to receive an intra-peritoneal injection of either T (36 μ g/kg cyclodextrin inclusion complex; n = 10 non-paired males, n = 13 paired males) or saline (n = 9 non-paired males, n = 13 paired males). Injections were performed quickly to minimize pain or discomfort for the animal. We used T-cyclodextrin inclusion complex since it rapidly increases plasma T levels and is quickly metabolized, resulting in a short-lived increase in T (Taylor et al., 1989). The injection concentration was selected because it was found to successfully produce a plasma T level that approximates naturally occurring physiologically high levels in this species (Gleason and Marler, 2010, Oyegbile and Marler, 2006 and Trainor et al., 2004). Thus, T injections were used to simulate this transient increase immediately prior to exposure to the stimulus female, as is typical of male mice (James et al., 2006 and Macrides et al., 1975). After the injection, the male was immediately returned to his assigned compartment in the arena, and an unrelated, sexually naïve, unfamiliar female was placed in the other compartment of the arena (each female was used exactly once). Females were not assessed for estrous, but were randomly assigned, thus making it unlikely that estrous females were distributed unequally across experimental conditions. Audio recording began immediately upon the addition of the female and ran continuously for 30 min (see below). An experimenter blind to the treatment of the male (T versus saline) performed all aspects of this procedure.

Ultrasonic vocalization (USV) recording and analysis

Ultrasonic vocalizations were collected using two Emkay/Knowles FG series microphones capable of detecting broadband sound (10-120 kHz). Microphones were placed centrally at the far ends of the arena (15 cm from the side walls, 45 cm from the dividing wall), one in the male chamber, and one in the female chamber, 20 cm above the arena floor. In order to minimize bias in the case of possible differential microphone sensitivity, the microphones were randomized across the trials between the male and female compartments of the arena. Microphone channels

were calibrated to equal gain (– 60 dB noise floor). We used RECORDER software (Avisoft Bioacoustics) to produce triggered WAV file recordings (each with a duration of 0.5 s) upon the onset of a sound event that surpassed a set threshold of 5% energy change (Kalcounis-Rueppell et al., 2010). Recordings were collected at a 250 kHz sampling rate with a 16 bit resolution. Spectrograms were produced with a 512 FFT (Fast Fourier Transform) using Avisoft-SASLab Pro sound analysis software (Avisoft Bioacoustics). All USVs were categorized and counted by visually inspecting spectrograms and listening to playbacks of the WAV files (sampling rate reduced to 11,025 kHz, corresponding to 4% of real-time playback speed). USVs were determined to originate from the male or female primarily based on visual assessment of amplitude comparisons across the two microphone channels that composed the stereo track. In rare cases of ambiguity, time of arrival of the start of the USV (accuracy up to 0.0001 s) was compared across the channels for origin determination. Instances in which both animals were close together at the center of the arena near the dividing wall accounted for a miniscule fraction of the trial time; this was verified through watching the video recording of a random subset of trials (n = 12), whereby individuals spent a maximum of 54 s (less than 3% of the total time) together near the center wall of the arena. Nonetheless, we also included a conservative “Dyad” measure (see the “Statistical analysis” section below) which summed the total vocalizations observed in the trial regardless of animal of origin in our analyses, similar to other studies of USVs and male-female interactions (Chabout et al., 2012, Hanson and Hurley, 2012 and Yang et al., 2013). An experienced observer blind to the condition analyzed all calls.

USV terminology and structure

Three categories of USVs, adopted and modified from previous studies focusing on *P. californicus* ultrasonic vocal communication, were used for mice of both sexes (Briggs and Kalcounis-Rueppell, 2011 and Kalcounis-Rueppell et al., 2010; Timonin, Marler, and Kalcounis-Rueppell, unpublished). These include simple sweeps, complex sweeps, and 1-, 2-, 3-, 4-, and 5-syllable vocalizations (SVs; Fig. 1). SVs are low bandwidth calls with a low modulation and a peak frequency of around 20 kHz and duration of approximately 100-500 ms. SVs exhibit strong upper harmonics and can vary in number of syllables within a given bout in the wild and in the laboratory (Briggs and Kalcounis-Rueppell, 2011 and Kalcounis-Rueppell et al., 2010). Because we were interested in changes in production rate rather than call structure (see Brudzynski and Pniak, 2002), we counted each syllable as a unique “SV”. Simple sweeps are single syllable, modulated (“down-sweep”) short calls (< 50 ms) with a peak frequency of around 40 kHz. Complex sweeps are also modulated but exhibit a much higher peak frequency (around 100 kHz), typically contain multiple inflection points, and are longer in duration (< 100 ms).

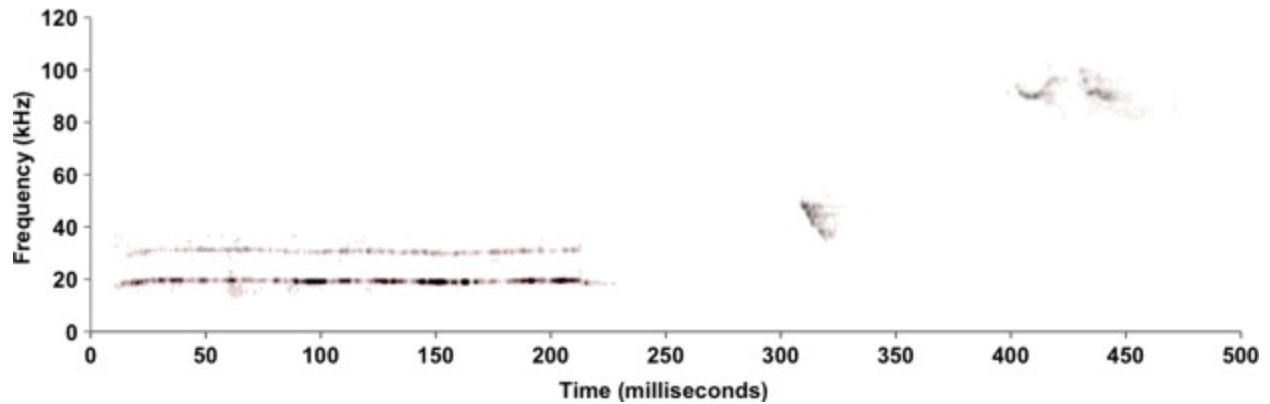


Fig. 1. Types of ultrasonic vocalizations observed in *Peromyscus californicus* males and females. SV (left), simple sweep (middle), and complex sweep (right).

Statistical analysis

Data were analyzed using SPSS (Version 16.0.1, SPSS Inc., Chicago, IL). Consistent with other studies examining changes in vocal behavior in rodents, we analyzed group differences in the total number of each type of vocalization produced (Brudzynski and Pniak, 2002) as well as the proportion of each type produced relative to the total amount of vocalizations produced (Musolf et al., 2010). To capture possible synergistic effects of T-treatment on vocal production that may be missed in analyses of individual sexes, we also included a conservative “Dyad” (both sexes combined) measure for each vocalization type in our analysis, in which we totaled all USVs of each type observed during each trial (i.e., a summation of vocalizations of the male and female). Due to ubiquitous non-normality based on visual inspection of normality probability plots, USV totals of all types were treated with natural log transformations to restore normality. Levene's test was used to assess equality of variance. The effect of T-treatment was assessed separately within paired and non-paired males, using one-way ANOVA or ANCOVA (see below). Bivariate relationships were assessed with Pearson's correlation. Partial eta-squared (η_p^2) effect size estimates (Cohen, 1973 and Levine and Hullett, 2002) are reported for each significant result. In order to reduce the possibility of inflated Type 1 error due to multiple comparisons, p-values pertaining to group comparisons for all USV types were adjusted using the Benjamini-Hochberg False Discovery Rate (FDR) procedure ($Q = 0.10$; Benjamini and Hochberg, 1995 and Thissen et al., 2002).

Separate analyses for paired and non-paired males

Our question addressed the effect of T across two dissimilar groups of males. At the time of the experiment, non-paired males were same-sex housed and sexually inexperienced, whereas paired males were obtained from established breeding pairs; each paired with one exclusive mate and having fathered more than three litters with their mate. We assessed the effect of T within each group separately because the groups significantly differed in age (paired male mean = 890 days, non-paired male mean = 263 days, $t = 10.06$, $p < 0.001$), which was found to contribute to vocal

behavior (see below). Nonetheless, we briefly present the results of analyses that include both groups in the same factorial model to corroborate our significant findings. Specifically, we used two-way ANCOVAs with male mating status (non-paired versus paired) and hormone treatment (T versus saline) as factors, and male age as a covariate. While the results of the two statistical approaches are similar, our emphasis is on the conservative approach of analyzing each group independently of the other.

Results

Age effect and covariance analyses

Male age was significantly negatively correlated with total male vocalizations produced among paired males ($R^2 = 0.38$, $p = 0.001$, $n = 26$), but not among non-paired males ($R^2 = 0.004$, $p = 0.80$, $n = 19$). Furthermore, paired male age was significantly negatively correlated with totals for each USV type ($p \leq 0.01$, $n = 26$). We therefore statistically controlled for the effect of paired male age by including the variable as a covariate in comparisons of hormone treatment groups. In addition to age, we also statistically assessed the impact of measures of pairing experience with the mate (pairing duration) and paternal experience (number of litters produced with mate), as well as pregnancy state of the male's mate (days from most recent weaning to trial and days from trial to subsequent birth of a litter). Measures of pregnancy state were not correlated with male total vocalizations ($p \geq 0.18$). Pairing duration was significantly negatively correlated with male total vocalizations ($R^2 = 0.34$, $p = 0.002$, $n = 26$), as was the number of litters produced ($R^2 = 0.54$, $p = 0.02$, $n = 26$). Since the three measures (age, pairing duration, and number of litters) were highly correlated ($p \leq 0.001$), we ran a general linear model to assess the contribution of each measure to the variance in male vocalization production. The model, which included hormone treatment as a fixed factor, male total vocalizations as the dependent variable and age, pairing duration and number of litters as covariates, revealed that age was a significant factor ($F = 5.24$, $p = 0.03$, $\eta^2 = 0.20$), whereas pairing duration and number of litters were not ($p \geq 0.22$). Thus, we retained male age as the sole covariate for all analyses of group comparisons. Finally, within each group of males (paired and non-paired males), it was confirmed that age did not statistically differ between hormone treatment groups (p 's > 0.50), as expected since T-treatment was randomized.

Effect of T on paired male and corresponding female production of USVs

Among paired males, T-treatment significantly decreased the number of total USVs ($F_{1, 24} = 8.20$, $p = 0.009$, $\eta^2 = 0.26$, $n_T = 13$, $n_{\text{saline}} = 13$) and simple sweeps ($F_{1, 24} = 8.57$, $p = 0.02$, $\eta^2 = 0.27$) produced upon exposure to a novel female (Fig. 2A, Table 1), but did not change the number of complex sweeps ($F_{1, 24} = 2.45$, $p = 0.13$), SVs ($F_{1, 24} = 3.74$, $p = 0.10$) produced or relative proportion of each USV type ($p \geq 0.26$; Table 2). Testosterone treatment also did not affect the variance of simple sweep production ($F_{1, 24} = 0.28$, $p = 0.60$). Among females

exposed to paired males, male T-treatment did not affect the number of total USVs ($F_{1,24} = 2.83$, $p = 0.11$, $n_T = 13$, $n_{\text{saline}} = 13$), complex sweeps ($F_{1,24} = 1.07$, $p = 0.47$), SVs, ($F_{1,24} = 0.02$, $p = 0.89$) or relative proportion of USV types ($p \geq 0.50$). Testosterone treatment produced a non-significant trend of decreased number of female simple sweeps ($F_{1,24} = 5.04$, $p = 0.09$, $\eta_p^2 = 0.17$) (Fig. 2B). Dyads containing a T-treated paired male produced significantly fewer total USVs ($F_{1,24} = 4.80$, $p < 0.001$, $\eta_p^2 = 0.17$) and simple sweeps ($F_{1,24} = 9.74$, $p = 0.005$, $\eta_p^2 = 0.29$) than dyads containing a saline-treated paired male, but did not differ in the production of complex sweeps or SVs ($p \geq 0.37$; Fig. 2C).

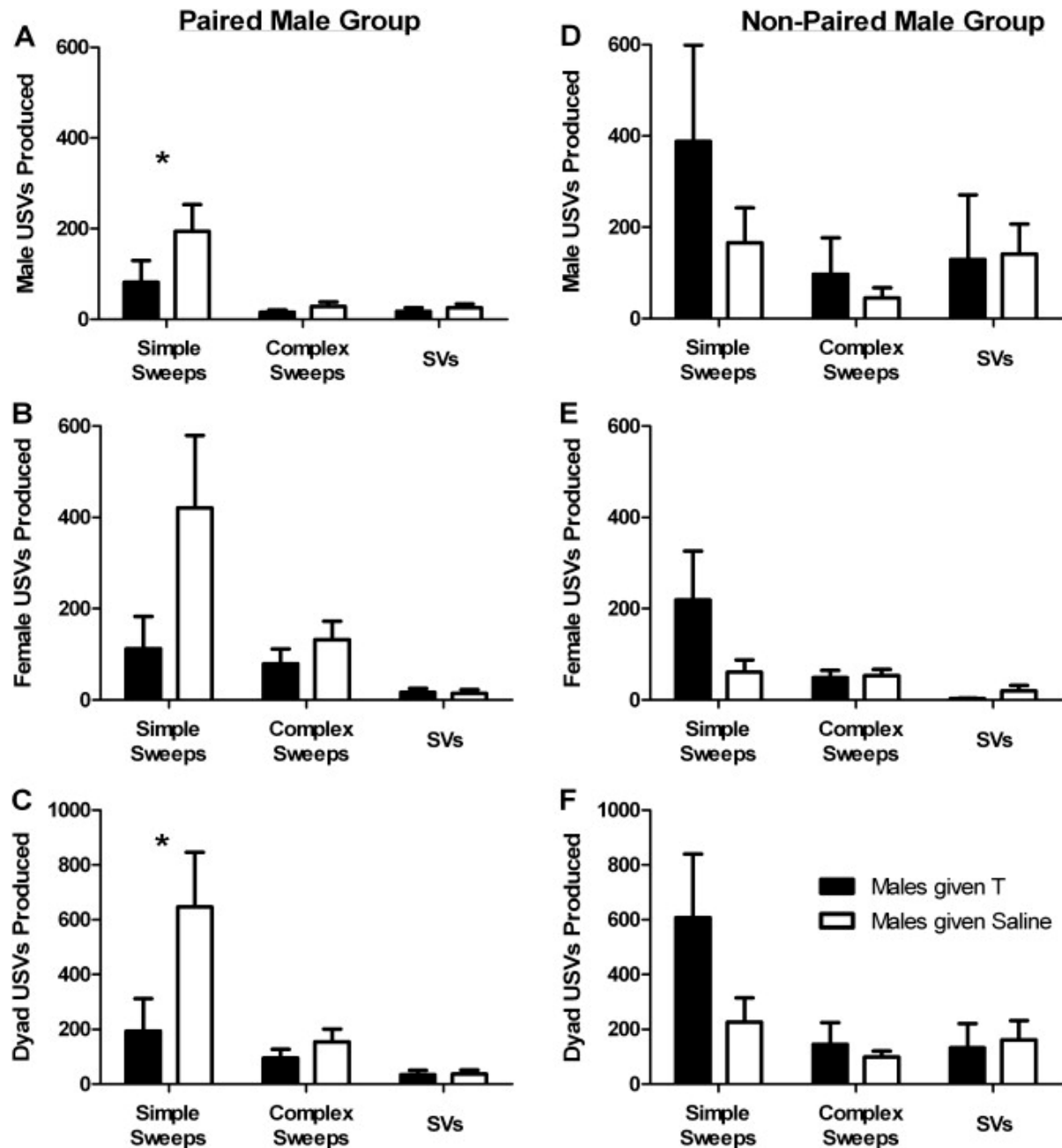


Fig. 2. Ultrasonic vocalizations produced during the 30 min dyadic male-female restricted interaction encounter. Trials involving novel female exposure to paired males on the left (N = 26), trials involving novel female exposure to sexually naïve non-paired males on the right (N = 19). Mean \pm SEM of male USVs (A and D), female USVs (B and E), and dyad (male + female) USVs (C and F). * indicates $p < 0.05$.

Table 1. Ultrasonic vocalizations (by type) produced by paired males and novel females during the 30 min restricted interaction encounter. Raw averages with associated p-values and effect size estimates derived from natural log transformed data are presented.

USV type totals	Paired males				Novel females			
	T	Saline	p	η^2_{ρ}	T male	Saline male	p	η^2_{ρ}
Simple sweeps	81.8 \pm 48.1	194.4 \pm 58.8	0.02	0.27	112.3 \pm 70.6	420.8 \pm 159.2	0.09	0.17
Complex sweeps	15.5 \pm 5.4	28.2 \pm 10.2	0.13	0.10	79.7 \pm 32.0	131.8 \pm 41.1	0.47	.04
SVs	17.2 \pm 7.9	25.1 \pm 8.9	0.10	0.14	16.69 \pm 8.7	14.23 \pm 8.6	0.89	< .01

Table 2. Proportion (by type) of ultrasonic vocalizations produced by paired males and novel females during the 30 min restricted interaction encounter. Raw averages with associated p-values and effect size estimates are presented.

USV type proportion	Paired males				Novel females			
	T	Saline	p	η^2_{ρ}	T male	Saline male	p	η^2_{ρ}
Simple sweeps	0.727 \pm 0.058	0.74 \pm 0.054	0.90	< .01	0.479 \pm 0.075	0.633 \pm 0.075	0.51	0.07
Complex sweeps	0.172 \pm 0.044	0.108 \pm 0.030	0.26	0.06	0.434 \pm 0.086	0.322 \pm 0.070	0.51	0.04
SVs	0.101 \pm 0.424	0.149 \pm 0.044	0.39	0.03	0.087 \pm 0.040	0.046 \pm 0.025	0.50	0.02

Effect of T on non-paired male and corresponding female production of USVs

Among non-paired males, T-treatment did not affect the number of total USVs ($F_{1,17} = 0.01$, $p = 0.94$, $n_T = 10$, $n_{\text{saline}} = 9$) or of any particular USV type ($p \geq 0.36$) produced upon exposure to a novel female (Fig. 2D, Table 3). However, T-treated males produced a higher proportion of simple sweeps than saline-treated males ($F_{1,17} = 6.87$, $p = 0.05$, unadjusted $p = 0.018$, $\eta^2_{\rho} = 0.31$; Table 4), but not complex sweeps or SVs ($p \geq 0.11$). Similar to paired males, T

treatment did not affect the variance of simple sweep production ($F_{1, 17} = 0.39$, $p = 0.54$). In females, male T-treatment did not affect the number of total USVs ($F_{1, 16} = 0.95$, $p = 0.34$, $n_T = 10$, $n_{\text{saline}} = 9$), or number of any particular USV type ($p \geq 0.14$) produced (Fig. 2E). Similarly, male T-treatment did not affect relative proportion of any USV type among females ($p \geq 0.20$). Among dyads containing non-paired males, male T-treatment did not affect the number of total USVs ($F_{1, 17} = 0.12$, $p = 0.74$), or of any particular USV type produced ($p \geq 0.56$; Fig. 2F).

Table 3. Ultrasonic vocalizations (by type) produced by non-paired males and novel females during the 30 min restricted interaction encounter. Raw averages with associated p-values and effect size estimates derived from natural log transformed data are presented.

USV type totals	Non-paired males				Novel females			
	T	Saline	p	η^2_{ρ}	T male	Saline male	p	η^2_{ρ}
Simple sweeps	388.1 ± 211.1	165.6 ± 76.7	0.36	0.04	218.7 ± 107.6	60.6 ± 27.0	0.65	0.04
Complex sweeps	96.6 ± 79.97	45.3 ± 21.7	0.56	0.03	48.7 ± 15.9	53.1 ± 13.5	0.70	< .01
SVs	129.2 ± 141.3	141.3 ± 65.5	0.92	0.06	2.5 ± 1.21	19.9 ± 12.0	0.14	0.21

Table 4. Proportion (by type) of ultrasonic vocalizations produced by non-paired males and novel females during the 30 min restricted interaction encounter. Raw averages with associated p-values and effect size estimates are presented.

USV type proportion	Non-paired males				Novel females			
	T	Saline	p	η^2_{ρ}	T male	Saline male	p	η^2_{ρ}
Simple sweeps	0.709 ± 0.07 9	0.417 ± 0.07 8	0.05	0.3 1	0.552 ± 0.11 8	0.371 ± 0.06 8	0.3 8	0.0 8
Complex sweeps	0.143 ± 0.05 0	0.304 ± 0.11 6	0.11	0.1 9	0.407 ± 0.11 0	0.515 ± 0.08 4	0.5 2	0.0 3
SVs	0.148 ± 0.07 4	0.280 ± 0.08 7	0.31	0.0 6	0.042 ± 0.02 6	0.114 ± 0.03 0	0.2 0	0.2 0

Corroboration of full model incorporating non-paired and paired males

Analyses of covariance that combined mating status (paired versus non-paired) and hormone treatment (T versus saline) into the same model using male age as a covariate corroborated our significant findings above from independent comparisons within paired and non-paired male

groups; additionally we were able to statistically compare USV production between non-paired and paired male groups. There was a significant main effect of mating status on total male USVs ($F_{1,41} = 4.71$, $p = 0.036$, $\eta^2_{\rho} = 0.11$, $N = 45$) and male simple sweeps ($F_{1,41} = 5.29$, $p = 0.027$, $\eta^2_{\rho} = 0.12$, $N = 45$), whereby paired males vocalized less than non-paired males in response to novel females. There was not a significant main effect of hormone treatment on total male USVs ($F_{1,41} = 2.55$, $p = 0.12$, $N = 45$) or male simple sweeps ($F = 0.45$, $p = 0.51$, $N = 45$), however there was a significant interaction of mating status and hormone treatment for male simple sweeps produced ($F_{1,41} = 4.42$, $p = 0.042$, $\eta^2_{\rho} = 0.10$, $N = 45$), whereby T decreased the number of simple sweeps among paired males but not non-paired males. Similarly, there was significant interaction of mating status and hormone treatment for relative proportion of male simple sweeps produced ($F_{1,41} = 5.02$, $p = 0.031$, $\eta^2_{\rho} = 0.11$, $N = 45$), whereby T increased the proportion of simple sweeps among non-paired males, but not paired males. Among females, there was a main effect of male mating status ($F_{1,41} = 7.11$, $p = 0.011$, $\eta^2_{\rho} = 0.15$, $N = 45$) but not male hormone treatment ($F_{1,41} = 0.58$, $p = 0.45$, $N = 45$) on total USVs produced, whereby females vocalized less in response to paired males. There was a significant interaction of mating status and hormone treatment for dyadic total USVs produced ($F_{1,41} = 5.06$, $p = 0.03$, $\eta^2_{\rho} = 0.11$, $N = 45$) and dyadic simple sweeps produced ($F_{1,41} = 8.54$, $p = 0.006$, $\eta^2_{\rho} = 0.18$, $N = 45$), whereby T lowered vocal production for trials containing paired males but not non-paired males.

Correlations between male and corresponding female USV production

In dyads containing paired males across both treatments (T and saline collapsed), male total USVs and female total USVs were significantly positively correlated ($R^2 = 0.22$, $p < 0.017$, $N = 26$), as were male and female simple sweeps ($R^2 = 0.29$, $p = 0.004$, Fig. 3A), complex sweeps ($R^2 = 0.23$, $p = 0.01$) and SVs ($R^2 = 0.31$, $p = 0.003$). Similar to dyads containing paired males, dyads containing non-paired males and females showed a significant positive correlation of simple sweeps ($R^2 = 0.37$, $p = 0.006$, $N = 19$, Fig. 3B) and SVs ($R^2 = 0.34$, $p = 0.01$). By contrast, there was no significant correlation between non-paired male and female total USVs ($R^2 = 0.09$, $p = 0.22$) or complex sweeps ($R^2 < 0.001$, $p = 0.94$). Further analysis of these relationships within each hormone treatment condition revealed significant positive correlations between saline-treated non-paired males and females for simple sweeps ($R^2 = 0.53$, $p = 0.02$, $n = 9$) but not complex sweeps or SVs ($p \geq 0.10$), as well as saline-treated paired males and females for complex sweeps ($R^2 = 0.38$, $p = 0.02$, $n = 13$) but not simple sweeps or SVs ($p \geq 0.30$). Testosterone-treated non-paired males showed no significant correlations with females for any call type ($p \geq 0.13$, $n = 10$), whereas testosterone-treated paired males and females demonstrated a positive correlation for simple sweeps ($R^2 = 0.47$, $p = 0.01$, $n = 13$) and SVs ($R^2 = 0.77$, $p < 0.001$, $n = 13$), but not complex sweeps ($p = 0.32$).

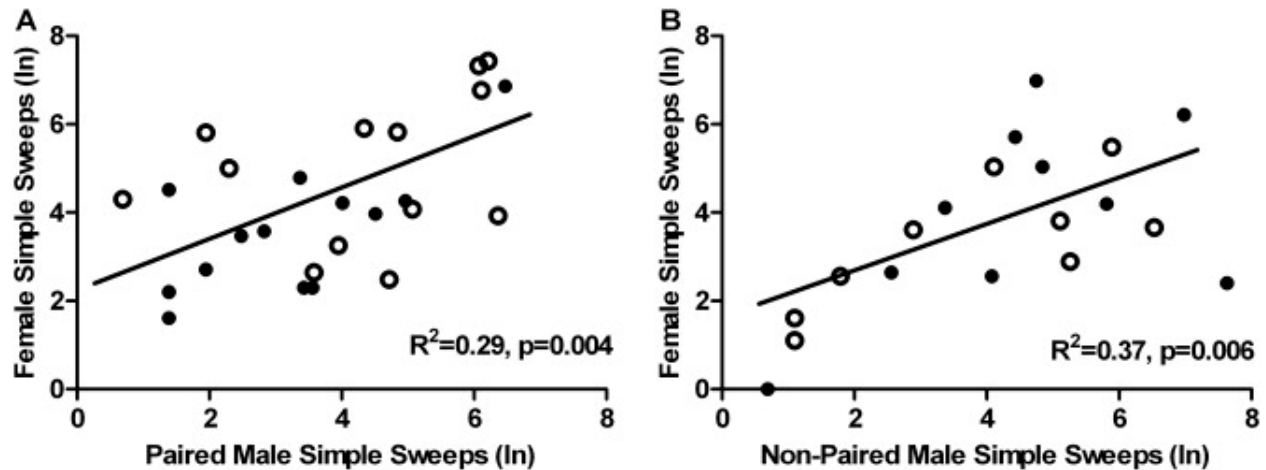


Fig. 3. Relationship of male and female vocal behaviors during the 30 min dyadic male-female restricted interaction encounter. Total number (natural log transformed) of simple sweep USVs produced by (A) paired males with novel females (N = 26), and (B) non-paired males with novel females (N = 19). Filled circles indicate males given T and open circles indicate males given saline (see text for correlations within T and saline groups).

Discussion

The present study reveals intriguing and novel effects of T on male USV production toward unfamiliar females in a monogamous mammal. We found within-species variation based on mating status in that T differentially affected pair bonded and non-paired male vocal behavior toward a novel female; T inhibited calling behavior in paired but not non-paired males, suggesting that T can promote mate fidelity. Our results further suggest that males from a monogamous species can respond differently than those from polygynous species to the modifying effect of T when presented with novel females in which T increases extra-pair advertisement (see below). To our knowledge, our study is the first demonstration of a novel mechanism incorporating brief T pulses that may play a role in supporting monogamy via reduced extra-pair advertisement.

We examined predictions arising from two separate hypotheses about the role of rapid T regulation of mate fidelity in this monogamous species: (1) T stimulates pair bonded males to vocally advertise to extra-pair females as classically expected, or (2) T acts to inhibit vocal advertisement to extra-pair females. The present results are consistent with the latter hypothesis since T reduced the total number of USVs produced by pair bonded males, primarily driven by a reduction in the number of simple sweeps. This hypothesis is further supported by previous studies in our model species, specifically that high T in pair bonded males is associated with increased proximity to the mate (Gleason and Marler, 2012). Furthermore, previous studies in the California mouse indicate that pair bonded males show reduced advertisement, as compared to non-paired males, to novel females via scent marking (Becker et al., 2012). While the role of T in the regulation of scent marking behavior has not yet been explored in this species, in a closely

related, but polygynous species, the white-footed mouse (*Peromyscus leucopus*), T can rapidly inhibit scent marking behavior in subordinate but not dominant males, suggesting plasticity in the sensitivity to T based on both mating status and social status, as well as in response to development of the winner effect in which changes in androgen receptors were identified (Fuxjager et al., 2010 and Fuxjager et al., in press).

The transient nature of the T administration mimicked natural-occurring pulses seen in intersexual rodent social encounters (James et al., 2006 and Macrides et al., 1975) and in the current study, this pulse inhibited acoustic communication with a novel female within 30 min, which is considered rapid for steroid hormones (Cornil et al., 2012, Ramage-Healey, 2012 and Wu et al., 2001). The precise mechanism through which T is acting cannot be determined by the present study, but recent research has identified intriguing rapid effects of steroid hormones achieved by non-genomic mechanisms (Cornil et al., 2013 and Mangiamele and Thompson, 2012), including effects on acoustic communication (Ramage-Healey and Bass, 2010 and Ramage-Healey and Joshi, 2012). Aromatase conversion of the precursor T to estradiol has been identified as a key process in rapid modulation of vocal behavior (Schlinger et al., 1999), which is interesting considering that aromatase expression in the medial preoptic area (mPOA), a nucleus with known involvement in both sexual and parental behavior (Dominguez and Hull, 2005 and Kentner et al., 2010), increases in response to fatherhood in male California mice (Trainor et al., 2003). This may contribute to the present findings that T had non-parallel effects between paired paternal males and non-paired males.

Additionally, changes in endogenous circulating T levels may play a role in responsiveness to novel females, similar to another monogamous mammal, the common marmoset, in which paternal males do not mount a T response to novel female scents, whereas non-paired males do (Ziegler et al., 2005). In the California mouse, baseline T levels are higher in non-paired males exposed to a novel female but drop as the pair bond forms (Gleason and Marler, 2010) but the nature of pair bonded, paternal male T-release response to novel females is unknown. It is possible that male T-release or vocal response could vary based on the estrous state of the novel female. Because it was not measured in our design, it is conceivable that estrous variation was non-uniform across T and saline treatment conditions. However, variability in production of male simple sweeps, which might be expected to covary with female estrous variability, did not significantly differ across conditions, within both paired and non-paired male groups. It is also possible that female estrous does not influence male calling behavior in this species; recent experimental evidence indicates that estrous state does not predict any behavioral measure of male California mouse sexual interest, whereas it is highly predictive in a closely related promiscuous species of *Peromyscus* (Karelina et al., 2010). Moreover, paired female scent marking behavior, in the form of frequency, distribution or volume, does not differ based on her estrous state, suggesting that females are not widely advertising estrous state (Petruno, Fuxjager and Marler, unpublished). Indeed, unlike many polygynous rodent species, California mice rarely copulate upon initial introduction (Dewsbury, 1974), with birth latencies suggesting that

copulatory behavior is not common until days or even weeks after pairing (Gleason and Marler, 2010; Pultorak and Marler, unpublished data) suggesting a reduced salience of estrous during courtship. We also acknowledge the possibility that endogenous T levels may have been altered among paired males due to stress resultant from mate separation (Martin et al., 2006), although a recent study in this species suggests that corticosterone changes are not evident until several days after separation, if at all (Harris et al., 2013), whereas our design employed only a 24 h separation period. Regardless of possible endogenous hormonal changes, the present study indicates that pair bonded males presented with novel females are sensitive to inhibitory actions of exogenous T.

We also tested the prediction that a pulse of T would rapidly promote vocal production in non-paired male California mice. We suspected that rapid effects of T would parallel previous findings using long-term changes in T that demonstrated a positive association between T and vocal output in polygynous house mice (Pomerantz et al., 1983a, Pomerantz et al., 1983b and Warburton et al., 1989) and singing mice (*Scotinomys teguina*; Pasch et al., 2011). Our predictions were not directly supported since, unlike in paired males, T did not affect the total number of USVs produced in non-paired males. This could be explained by the differences in T administration: short-term versus long term manipulations using castration and implants. We are exploring the effects of the different modes of administration. Other evidence within California mice suggests that T injections in intact males are sufficient to induce behavioral changes related to aggression and winning ability in male-male encounters (reviewed in Gleason et al., 2009).

Despite the lack of effect of T on total vocal production among non-paired males, we found that T altered the distribution of USV types produced, in that simple sweeps were proportionally more common. This redistribution is likely socially relevant since previous studies in house mice show that males modify the relative proportion of specific call types based on olfactory cues of kin versus non-kin conspecifics, and same-sex versus opposite-sex conspecifics (Musolf et al., 2010). Although USVs have been implicated in courtship behavior in related rodent species (Brudzynski and Pniak, 2002, Musolf et al., 2010, Nyby, 1979, Sipos et al., 1993 and Whitney et al., 1973), the functional roles of USVs in the California mouse are hitherto less well studied (but see Briggs and Kalcounis-Rueppell, 2011). USVs may communicate a variety of signals, including non-sexual information, and functions likely differ based on call type. Recent indirect evidence in the laboratory (Pultorak and Marler, unpublished data) and behavioral context data from the field (Timonin et al., unpublished data) suggests that SV vocalizations may play a role in pair bond maintenance. Based on our findings in the present study of fewer simple sweeps among paired males (as compared to non-paired males) and a proportional increase of simple sweeps among T-injected non-paired males, we speculate that simple sweeps may underlie courtship behavior and indicate socio-sexual interest in this species. Intriguingly, T-mediated changes in vocal behavior, specifically the proportion of sweeps, may provide cues during courtship that allow for female assessment of male quality; recently it was found that a male's T

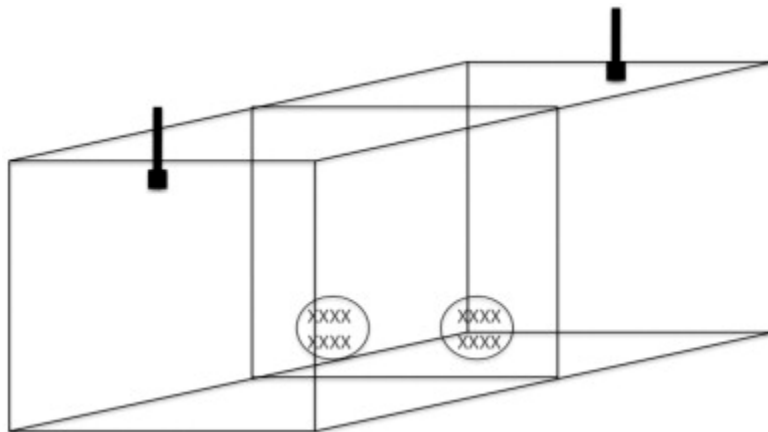
level during courtship is positively predictive of his paternal quality in this biparental species (Gleason and Marler, 2010).

We speculate that one measure of female assessment of male quality is female vocal production in relation to a male's vocal production. In the present study, we found evidence of vocal responsiveness between males and females, specifically involving simple sweeps. Analysis of the relationship between male and female production of simple sweeps reveals a significant positive correlation among dyads involving either non-paired males or paired males (Fig. 3). Interestingly, this relationship may be driven by females since the T-induced reduction of simple sweeps among paired males was mirrored by a non-significant (due to multiplicity correction) reduction of simple sweeps in females. This positive relationship in vocal production between males and females is intriguing because it suggests that some degree of vocal coordination or responsiveness may be occurring, possibly as a means of compatibility assessment, which is known to influence reproductive success in this species (Gleason et al., 2012). Interestingly, T administration altered the relationship of vocal responsiveness, but not in a classically expected direction, in that female and male vocal output was uncorrelated among T-treated, non-paired males. Ultrasonic vocal duetting in monogamous rodents has not been investigated to our knowledge, making this an exciting avenue for future research.

Mechanisms involved in pair bonding have been examined extensively with a focus on vasopressin and oxytocin, and several studies in prairie voles have elegantly demonstrated associations of pair bonding with these peptide hormones (reviewed in Lim and Young, 2006 and Young et al., 2011) and other proximate mechanisms (DeVries et al., 1995 and Hostetler et al., 2012). What has remained an enigma is how high levels of pair bonding in a species can also be associated with a high level of extra-pair copulations. The “Challenge Hypothesis” holds that T positively influences mating effort and negatively influences paternal care (Ketterson et al., 1991 and Wingfield et al., 1990), but recent evidence supports alternative mechanisms that circumvent the supposed trade-off between mating effort and paternal effort (reviewed by Stiver and Alonzo, 2009; meta-analysis by Hirschenhauser and Oliveira, 2006) and further work is needed to elucidate context-specific effects of T such as opportunistic extra pair-mating effort in monogamous species. A framework has been built, primarily via studies in humans, that illustrates the complex interactions of androgens and peptide hormones across social contexts, including competitive versus nurturant contexts, and single versus multiple mating contexts (Van Anders et al., 2011 and Van Anders, 2013). Indeed, low circulating T is associated with long term pairing whereas high circulating T is associated with multiple mating in humans (Burnham et al., 2003; Gray et al., 2004 and McIntyre et al., 2006; Van Anders et al., 2007 and Van Anders and Goldey, 2010). However, T may act to regulate opportunistic mating behavior through mechanisms other than long-term changes in baseline levels, such as rapid behavioral effects resultant of brief pulses of T, or altered sensitivity to these pulses, especially in species for which periods demanding high paternal effort overlap with periods requiring high levels of aggression or high mating effort (Onyango,

2013; Ostner et al., 2008, Teichroeb and Sicotte, 2008 and Ziegler and Snowden, 2000). California mouse females exhibit a post-partum estrous in the few days following birth (discussed in Marler et al., 2003) and interestingly, paternal behavior is actually promoted, not inhibited, by T (Trainor and Marler, 2002). Thus, it may be adaptive for a co-opted mechanism that is plastic enough to allow closer temporal association between paternal behavior and mate-specific mating behavior to be in place. As such, classical predictions of the function of T may need re-evaluation with newfound knowledge of the plasticity evident in many animal model systems. California mice represent an important model given the evidence of such a high level of genetic monogamy. The present study suggests evidence of the co-opted evolution of T pulses to rapidly inhibit vocal advertisement and perhaps thereby decrease the probability of extra-pair copulations, thus acting as a novel mechanism promoting mate fidelity in a strictly monogamous species.

The following are the supplementary data related to this article.



Supplemental Figure. Diagram of the two-chambered experimental arena. The chambers are separated by a wall with two mesh openings to allow for acoustic, visual and olfactory communication, but limited physical interaction between the male and female.

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