Testosterone pulses at the nest site modify ultrasonic vocalization types in a monogamous and territorial mouse

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Abstract:

Modulation of baseline testosterone (T) via long-lasting T implants alters territorial, sexual, and social behavior of animals in the field. Transient T increases occur in numerous species after social interactions, but these transient increases in T have not been manipulated in the field. In the laboratory, these T increases can influence future behavior for days, causing changes in social behavior and inducing preferences for specific locations. We manipulated transient increases in T in the field at the nests of the monogamous and territorial California mouse (*Peromyscus californicus*) to examine long-term (>24 hr) changes in ultrasonic vocalizations (USVs). Males of bonded male–female dyads (=pair) were administered a T injection (vs. saline) three times over seven days and USVs of the male–female dyad were measured for three days after the last injection. At T nests, the male–female dyad produced significantly more 1SV (one call SV: an SV is a sustained vocalization that is long in duration and low in modulation) and 4SV (four call SV) type USVs than controls, but no significant changes in aggressive barks. Overall, male–female dyad mice at T nests produced a greater diversity in call types such that 1SV, 4SV, 5SV, and a complex sweep were produced at T nests but not control nests. There were significantly more USVs produced at T nests on night 2 after the final injection. There were no differences in spectral characteristics of SV calls or aggressive barks between T and control nests. The function of the changes that occurred is unknown, but is consistent with increased long-term changes in behavioral interactions with nest mates and may reflect T-induced conditioned place preferences to the nest site. Significantly, transient increases in T influence future acoustic communication under field conditions with competing biotic and abiotic stimuli.

Keywords: androgens | conditioned place preference | field | male-female dyad | pair bond | Peromyscus californicus | social behavior | vocal communication

Articles:

1 INTRODUCTION

The effect of testosterone (T) on vocalizations associated with reproduction is typically examined using the classical behavioral endocrinology method of removing the gland, in this case the testes, and subsequently replacing the hormone (T) with a long-lasting implant that maintains a constant level of T (Adkins-Regan, **2005**). The classic behavioral endocrinology approach has nicely illustrated that, compared to vocal production in male songbirds, production of ultrasonic (high frequency) vocalizations (USVs) is reduced in castrated male rodents and restored by T implants (Dizinno & Whitney, **1977**; Pasch, George, Hamlin, Guillette, & Phelps, **2011**; Warburton, Stoughton, Demaine, Sales, & Milligan, **1988**). While T implants can address functions of baseline or seasonal changes in T levels, the advantage of examining socially induced transient increases in T (for an example in birds see Wingfield & Wada, **1989**) is that these mimic the naturally occurring transient increases in T (surges) above the breeding baseline. T release can occur after both male–male encounters (challenge effect) as well as male–female encounters. Both baseline and socially induced transient T release are composed of smaller pulses of T release that result in larger secretory episodes of T (review by Velduis, Keenan, & Pincus, **2008**; Nelson & Kriegsfeld, **2018**). Testosterone that is released after a fight or competition between males may influence the ongoing encounter or future encounters (referred to as the "Challenge Effect"; Wingfield, Hegner, Dufty, & Ball, **1990**; reviewed in Fuxjager, Trainor, & Marler, **2017**; Hirschenhauser & Oliveira, **2006**).

Testosterone is also released in males in response to females and female odors (review by Gleason, Fuxjager, Oyegbile, & Marler, **2009**; California mice: X Zhao & CA Marler, unpublished), which may provide a link between transient increases in T and male reproductive behaviors including mate seeking, courtship, and mate guarding. Manipulations of such transient T increases using T injections have not been conducted under field conditions, in part, because of the challenges of field research. Here we begin a series of studies in which we manipulate transient T increases in a monogamous rodent using T injections and focus on vocalizations at an established field site at which USVs have previously been recorded (Briggs & Kalcounis-Rueppell, **2011**; Kalcounis-Rueppell & Millar, **2002**; Kalcounis-Rueppell et al., **2010**).

There has been uncertainty as to how these individual transient increases in T (as compared to long-term changes via implants) can influence behavior (Gleason et al., **2009**; Marler, Oyegbile, Plavicki, & Trainor, **2005**). For example, male California mice produce an increase in T over baseline levels 45 min after both male–male encounters (Marler et al., **2005**) and male–female encounters (X Zhao & CA Marler unpublished data). Transient increases in T likely occur in California mice in natural settings in the field, as elegantly described by Wingfield and others for avian species (Wingfield et al., **1990**). Strong evidence, however, in the monogamous and territorial California mouse (*Peromyscus californicus*) supports the robust behavioral effects of one to three transient increases in T via injections with effects ranging from rapid effects (20 min) (Pultorak, Fuxjager, Kalcounis-Rueppell, & Marler, **2015**) to more long term, and even cumulative effects, up to at least a week (Fuxjager, Oyegbile, & Marler, **2011**; Pultorak et al., **2015**; Trainor, Bird, & Marler, **2004**; Zhao & Marler, **2014, 2016**). After a male–male encounter, a transient increase in T is released in males that helps to drive development of the winner effect (increased ability to win based on previous wins) (Fuxjager, Montgomery, &

Marler, **2011**; Fuxjager, Oyegbile, et al., **2011**; Oyegbile & Marler, **2005**; Trainor et al., **2004**). A single transient increase in T of the same magnitude can rapidly inhibit USVs in pair-bonded (but not unpaired) male California mice when exposed to an unfamiliar female of reproductive age (Pultorak et al., **2015**). Moreover, three transient increases in T over a week can result in the development of a preference for a specific location (conditioned place preference; Zhao & Marler, **2014, 2016**) in addition to the previously mentioned winner effect. However, the effects of T in the form of either pulses or long-term implants in this monogamous species have not been studied in the field, or in the context of the pair bond itself, and its associated social complexities.

We chose to work with California mice, *P. californicus*, for these studies of hormones and behavior from an ecological and physiological perspective because they have been well studied in both the field and laboratory. In addition to information described above, this is a strictly monogamous and territorial (Ribble, **1991**; Ribble & Salvioni, **1990**) species in which both sexes display high levels of aggression (Bester-Meredith & Marler, **2007**; Bester-Meredith, Young, & Marler, **1999**; Davis et al., **2004**; Fuxjager, Mast, Becker, & Marler, **2009**; Ribble, **1992a**; Ribble & Salvioni, **1990**; Trainor and Marler, **2001, 2002**), and male–female dyads display remarkable similarities in behaviors in the laboratory and field. For example, USVs are similar between both laboratory and field settings (e.g., Kalcounis-Rueppell et al., **2010**). In the current study, we examined the enduring effects of three injections of either T or saline over seven days, with a minimum of one day between injections, on USV production (as part of a larger ongoing field study) in California mice. All injections were conducted at the nest site of the male–female dyad, thus potentially conditioning them to the nest site (Zhao & Marler, **2014, 2016**). We used a similar pattern of T administration that mimics natural changes as previously used in the laboratory to examine other changes in behavior (e.g., Fuxjager, Oyegbile, et al., **2011**; Fuxjager, Montgomery, et al., **2011**; Trainor & Marler, **2001**; Trainor et al., **2004**; Gleason et al., **2009**; Zhao & Marler, **2014, 2016**). We combined this with previously used techniques for studying USVs under field conditions in which both sexes have been discovered to produce USVs that can be recorded remotely and used as a measure of mouse social behavior (Briggs & Kalcounis-Rueppell, **2011**). During the three days following the final injection, we recorded USVs produced by the male-female dyad (=pair) at their nest.

2 METHODS

Fieldwork was carried out at the Hastings Natural History Reservation, Carmel Valley, California, USA, from September 2012 to February 2013. Acoustic recordings were conducted between September 26–December 03, 2012 and January 11–February 26, 2013. Our study was conducted exclusively on the Lower Robertson Creek (LRC) trapping grid, one of three historic trapping grids used in previous field studies with *P. californicus* (Briggs & Kalcounis-Rueppell, **2011**; Gubernick & Teferi, **2000**; Kalcounis-Rueppell & Millar, **2002**; Kalcounis-Rueppell et al., **2010**; Ribble, **1992a, 1992b**). The LRC trapping grid consists of approximately 136 trap stations arranged in a 4 × 34 array with 10 m spacing between each station. The LRC grid runs parallel to Robertson Creek, through a canyon bottom with an overstory dominated by live oak (*Quercus agrifolia*), California bay-laurel (*Umbellularia californica*) and California buckeye (*Aesculus californica* (Griffin, **1977**; Kalcounis-Rueppell & Millar, **2002**; Ribble, **1992a**; Ribble & Salvioni, **1990**). We first identified breeding male–female dyads

through our live-trapping program and used handheld radio-telemetry to locate the male–female dyad's nest site. Males from established male–female dyads were then captured near the nest site and injected with either T or saline and immediately released near the nest site. We then recorded USVs at the nest site for three nights following administration of the last injection. As these mice are monogamous and territorial, we assumed that USVs being recorded at the nest site were from the male–female dyad (=pair) that nested at the nest site. Different calls were identified as described below and measured by number and proportion of total calls.

2.1 Care and use of animals

All applicable guidelines for the care and use of animals were followed, including the US National Research Council's *Guide for the Care and Use of Laboratory Animals* and the US Public Health Service's *Policy on Humane Care and Use of Laboratory Animals,* the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences, and ASAB/ABS Guidelines for the care and use of animals. Research protocols were approved by both the University of North Carolina at Greensboro and University of Wisconsin, Madison College of Letters and Sciences Institutional Animal Care and Use Committee (IACUC); UNCG: 11-12, and 12-04 and UWM: L005047-A01). In addition, we had UC Davis approval (UNCG IACUC protocols 11-12 and 12-04) and were authorized by California Department of Fish and Wildlife under Scientific Collection Permits (SC-001358, SC-12294, SC-12295, and SC-12308).

2.2 Live trapping

To establish the identity of resident adult California mouse male–female dyads (=pairs), we trapped sections of the LRC trapping grid throughout the field season, using standard live-trapping methods (Briggs & Kalcounis-Rueppell, **2011**; Kalcounis-Rueppell & Millar, **2002**; Petric & Kalcounis-Rueppell, **2013**). We divided the grid into three sections (lower, middle, and upper), each with approximately 45 trap stations per section. To capture mice, we opened two Sherman traps provisioned with a mixture of crimped oats and sunflower seeds at each station. A trapping session occurred when all traps were open at all of the stations in at least one section of the grid for three consecutive nights, with traps set at dusk, and checked and closed prior to sunrise. Throughout the season, the lower section was trapped for nine sessions (27 trap nights), the middle section for eight sessions (25 trap nights), and the upper section for seven sessions (21 trap nights).

For individual identification, all mice were given a single ear tag (Monel Numeric, National Band and Tag Co., Newport, KY, USA) with a unique number when they were first captured. We also took standard measures including mass, sex, age, and reproductive status every time an individual was captured (as in Briggs & Kalcounis-Rueppell, **2011**; Petric & Kalcounis-Rueppell, **2013**). All mice were released at the site of capture. Adults were considered to be residents if they were captured at least three times between two separate trapping sessions, and within trapping stations that were no more than two trap stations away from initial trap station of capture. Information we gathered from live trapping was used to determine putative male–female dyads, reproductive status of males and females, and maternal status of breeding females. An adult male resident and female resident that were consistently captured at the same sites were

assumed to be pair-bonded and we confirmed this through handheld radio-telemetry to show the male–female dyad shared a nest.

2.3 Nest sites

From the identified resident adults, we selected males for our study that were captured regularly (i.e., at least once during each consecutive trapping session), and frequented trap stations in an area of the grid that was suitable for recording equipment. We then determined the female member of the dyad using trapping records for resident females captured at the same subset of trap stations as the male.

After identification of a putative male–female dyad, we opened the subset of traps typically frequented by the individuals of interest (typically 3–4 trap stations). Upon capture, both the male and female were outfitted with mouse-sized radio-collars that emitted at a unique frequency (MD-2T Advanced Telemetry Systems, Isanti, MN, USA) and then released. The following day, the male and female were located using a handheld radio-telemetry receiver (R4000 or R4500S, ATS) and a 4-element Yagi antenna to determine the location of the nest site, by simply localizing the signal by hand, and to confirm that the animals were indeed a dyad and sharing the same nest. When a suitable dyad and their nest site were identified, we placed additional traps directly around the nest to capture the male to administer injections. In some instances, we only tracked the male to the nest if we were unsure of his mate and then used the additional traps around the nest to determine his mate if she had not already received a radio-collar.

2.4 Male treatment

Males of the male–female dyad were randomly assigned to receive testosterone (T; experimental group) or saline (control group) injections with the constraint that we wanted half of the males to be treated with T. The researcher responsible for administering injections (MET) was blind to treatment type. Males received three 0.1 ml subcutaneous injections over a period of seven days, with a minimum of one day and a maximum of six days between injections. One control mouse received only two injections of saline. The exact injection day depended on our ability to trap the focal male on a particular night. On average, males had 1.7 ± 0.4 days (range 0–6) between consecutive injections. The T injectate was prepared by dissolving cyclodextrin-conjugated testosterone inclusion complex in saline to a final concentration of 1.44 μg/ml, for an approximate dosage of 36 μg/kg (Trainor & Marler, **2001**) (average dosage: 36.0 ± 0.8 μg/kg, range: 33.2–41.7 μg/kg). This dose produces an increase in T levels 3–5 times higher than baseline, reaching a maximum of 4–5 ng/ml and lasts for approximately 10 min (Trainor et al., **2004**). Moreover, it mimics natural changes in T found in intact California mice after winning a male–male encounter (Fuxjager et al., **2009**; Marler et al., **2005**; Oyegbile & Marler, **2005**) and in male–female encounters (X Zhao & CA Marler, unpublished). On a behavioral level, this dose increases future ability to win in male–male encounters (i.e., Fuxjager, Montgomery, et al., **2011**; Fuxjager, Oyegbile, et al., **2011**; Trainor et al., **2004**), induces conditioned place preferences (Zhao & Marler, **2014, 2016**), and alters USVs in the laboratory (Pultorak et al., **2015**).

2.5 Recording USVs

We set up two microphones (Emkay FG Series; Avisoft Bioacoustics, Glienicke, Germany) adjacent to the nest at an identifiable entrance (Kalcounis-Rueppell & Millar, **2002**) to record mouse vocal behavior (full spectrum sound: sonic, ultrasonic, harmonics; 10–250 kHz). Two microphones were set up to maximize the chances of recording (through the same channel) clear USVs at nest sites where there were multiple openings, whereas one microphone was used if the nest was shallow and/or had only one opening and USVs were easily detected.

Microphones, which sampled at 250 kHz with a 16-bit resolution, were wired to a 1216H UltraSoundGate system (Avisoft Bioacoustics, Glienicke, Germany) attached via a 2.0 USB interface to a laptop (Dell Latitude D410, Dell, Inc., Round Rock, TX, USA). Vocalizations were recorded using Avisoft Bioacoustics Recorder software (Avisoft Bioacoustics, Glienicke, Germany). Inverters and 12 V 33 AMP batteries powered equipment. Recording equipment was manually turned on at dusk each evening, and operated until after sunrise. USVs recorded at an amplitude of greater than or equal to −30 db were assumed to be generated at the nest site (see Data **S1**) and were used in the analysis.

2.6 USV characterization and analysis

The first author examined every recording we made to ensure that every USV we recorded was detected. When a USV was detected, we classified the USVs by type; California mouse vocalizations recorded in the wild typically fall into two USV types previously described by Briggs and Kalcounis-Rueppell (**2011**) and Kalcounis-Rueppell et al. (**2010**) and summarized in (Kalcounis-Rueppell, Pultorak, & Marler, **2018**). The first and most common vocalization in the field recordings is a sustained vocalization (SV) that can have from 1 to 5 or more calls in a bout (i.e., 1SV, 2SV, 3SV, 4SV, and 5SV). The SV call is relatively long in duration and low in modulation. In bouts of SV calls, each call is approximately 50–200 or more milliseconds in duration, with similar duration between calls in a bout. In contrast, time between bouts of SV calls occurs on the order of seconds, minutes, or hours. The behavioral context of SVs in the field was studied by Briggs and Kalcounis-Rueppell (**2011**), and SVs were used both when mice were alone and together in the presence of mates and non-mates, suggesting relevance for both territory and pair-bond maintenance. The second USV type is the bark call, which is an inverted chevron shaped call. Both SV and barks are produced by *Peromyscus* mice at relatively low fundamental frequencies that pass through approximately 20–30 kHz. California mice also produce complex and simple sweeps as characterized by Pultorak et al. (**2015**) that are produced at relatively high frequencies. Simple and complex sweeps, when compared to SVs and barks, are not easily recorded in the field because of their relatively low amplitude and use by mice in close-contact interactions (Pultorak et al., **2015**).

We used the automated measurement feature in SASLab Pro (Avisoft Bioacoustics, Glienicke, Germany) to analyze spectral and temporal characteristics of all SVs and barks (FFT length 512; Hamming window; 100% frame size; 50% resolution overlap). Consistent with our previous study (Pultorak et al., **2015**) and other studies examining rodent vocalizations, we measured both the number of calls of each type (Brudzynski & Pniak, **2002**) and proportion of calls of each type relative to total calls of USVs within specific types. We measured the following parameters: bout duration, call duration, minimum and maximum frequency of each call, peak frequency at the

start and end of each call, and frequency at the time point of maximum amplitude of each call. Here, a call is defined as an uninterrupted sound, consistent with definitions in Kalcounis-Rueppell et al. (**2018**). Additionally, we calculated the internal modulation and bandwidth of each call (for details and an annotated spectrograph of measurements see Kalcounis-Rueppell et al. **2010**).

We examined the mean number and total number of USV bouts and calls recorded at T and control nest sites on Night 1, Night 2, and Night 3 after the final injection. To determine whether male–female dyads at the T and control nest sites produced the same USV types, we calculated proportions of calls of each USV type per night per nest site. Because of a large number of zeros in our data set, data were not suitable for parametric statistics and we examined differences between T and control nests using Wilcoxon one-way tests. Because we had repeated measures across nights and we needed to use a parametric repeated measures ANOVA, we examined treatment effects on rank-transformed data (Conover & Iman, **1981**). We also examined treatment effects on particular types of calls using Wilcoxon one-way tests. We used nonparametric approaches here, as opposed to full factorial GLMM (with individuals as random effects nested within treatment and nights as replicates), because of our small sample size and the scale differences in our call response for T males (two T males responded to treatment at an order of magnitude higher than the other T males and control males; see Figure **1**); this resulted in non-normal response variables and unstable GLMM models that never converged (not shown). All analyses were conducted in JMP Pro 13 (SAS Institute Inc., Cary, NC). We used a rejection criterion of $p = 0.05$.

	Total USVs	**ISV**	**2SV**	**3SV**	**4SV**	**5SV**	**Barks**	**Complex Sweeps**
Control	29 (74)	0	8 (16)	6 (18)	0	0	15 (40)	0
Testosterone	220 (512)	39 (39)	58 (116)	30 (90)	10 (40)	4 (20)	78 (206)	1 (1)

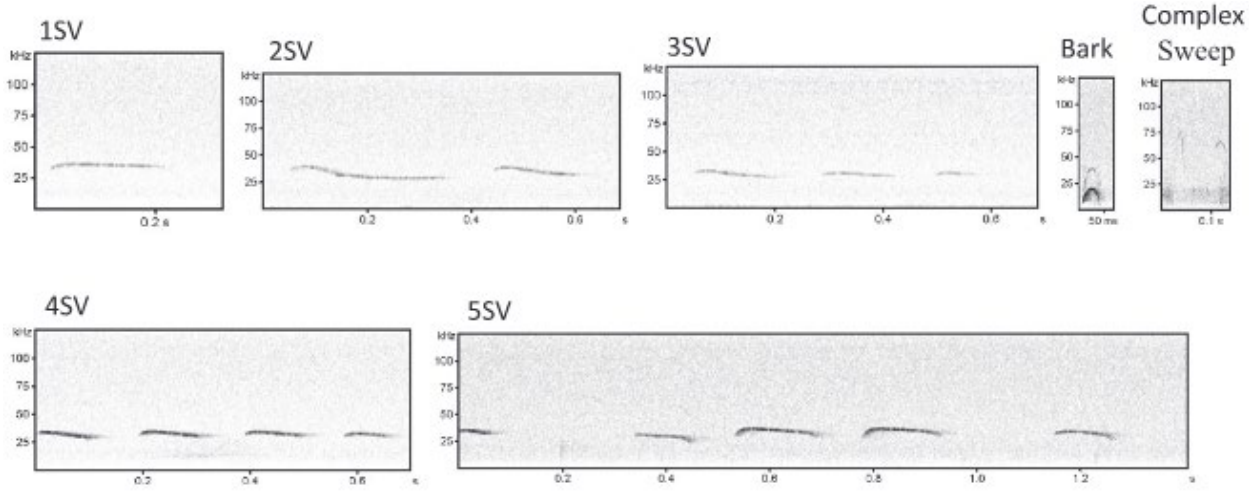

Figure 1. Median and quantiles of USV bouts (left) and calls (right) by treatment recorded from nest sites where the male received a series of three injections of either testosterone ($n = 6$) or saline (control; $n = 5$). Recordings were made on the three nights following the final injection in the series. Each dot represents the mean USVs for a pair across the three nights post-injection

2.7 Sample sizes

We captured 183 different individual California mice 344 times from September 2012 to February 2013. Of those 183 mice, 109 were classified as residents (as described above) and 57 residents were outfitted with radio-collars. Eleven males received either 3 T (T nests; $n = 6$) or three saline (control nests; $n = 5$) injections with one exception; for one control male, we were only able to give two injections instead of three, but in this case, we still recorded the response for the three nights after the last injection. We were able to follow nine of the pair-bonded mates of the 11 males (5/5 mates of the control males and 4/6 mates of the T males). As we were only able to identify the female of the male-female pair for 4 of 6 T males, this leaves open the small possibility that there were no females paired with males with 2 of the 6 T males. The more likely scenario based on our trapping data (not shown) is that these males were paired but it was difficult for us to determine who the female was because there was a high density of female mice around their nest sites in the 2012/2013 field season. In any case, to determine whether the inclusion of these individuals influenced our results, we analyzed our datasets with and without these two T nests. Conclusions were consistent with and without these two T nests (data not shown), and we therefore include them in our analyses.

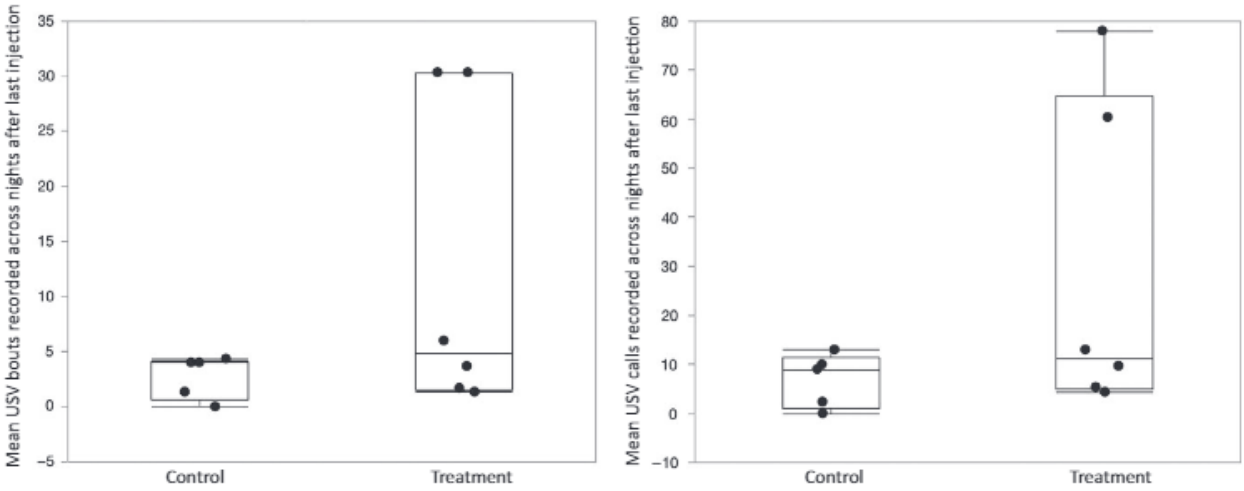

Figure 2. The total number of ultrasonic vocalizations (USVs) bouts (calls) recorded at the nest sites of males receiving testosterone or saline control injections. Representative spectrographs of types from each male–female dyad at the nest are shown. Recordings were made on the three nights following administration of the last injection in a series of three injections in *Peromyscus californicus* males at the HNHR during the 2012–2013 breeding season

Ultrasound recording equipment was set up at 11 nests for three nights in a row ($n = 33$ nest recording nights), following the last injection, from dusk until sunrise. However, due to technical problems, equipment failed to record on three of the 33 nights at two control nest sites (night 3 at one control nest and night 2 and night 3 at another control nest). We recorded 249 USV bouts at nest sites with 220 bouts from nests where the male was T-injected and 29 bouts from nests where the male was a saline-injected control (Figure **2**). These bouts consisted of 586 USV calls at nest sites with 512 calls from nests where the male was T-injected and 74 calls from nests where the male was a saline-injected control (Figure **2**). We analyzed the response to T treatment at the level of the male–female dyad at the nest as opposed to the individual because we could not assign the USV to the male or female of the dyad. In addition, we did not examine our results in the context of reproductive condition or pup presence because this was an unusual year in that few mice were in reproductive condition, and only one nest had pups during the experiment (this

individual male was a control male and anecdotally did not stand out as being different from other individuals sampled in the study).

3 RESULTS

We recorded seven different USV types, ranging from SVs with a single call (1SV) to those with five calls (5SV), as well as barks and complex sweeps (Figure **1**). At T nests, but not controls, we recorded 1SV, 4SV, 5SV, and one complex sweep (Figure **1**), suggesting that either the male–female dyad at T nest sites called more and produced a greater variety of calls as a by-product, or that T is inducing a greater diversity in USV types.

Table 1. Descriptive statistics for mean number of USV bouts and calls recorded around the nest sites of males receiving testosterone (T; $n = 6$ nests) or saline (Control; $n = 5$ nests) injections following administration of the last injection in a series of three injections in *Peromyscus californicus* males. Means were calculated on the number of USV bouts and calls recorded across all three nights

	Mean Male–Female Dyad USV bouts		**Mean Male–Female Dyad USV calls**	
	Control	**T**	**Control**	**T**
Median	4.00	4.84	9.00	11.33
Min	0.00	1.33	0.00	4.33
Max	4.33	30.33	13.00	78.00

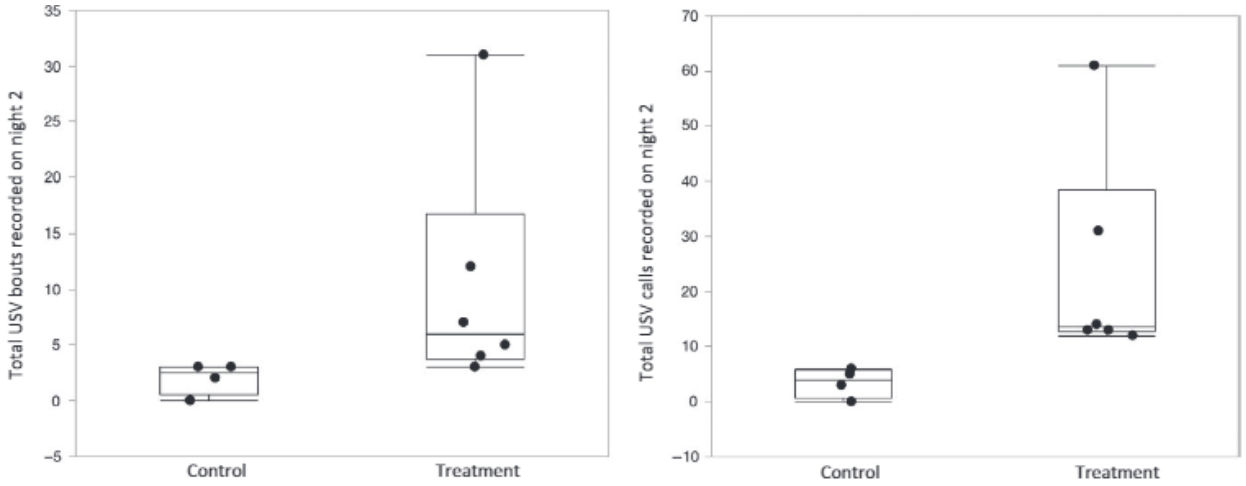

Figure 3. Median and quantiles of USV bouts (left) and calls (right) by treatment recorded from night 2 post last injection at nest sites where the male received a series of three injections of either testosterone ($n = 6$) or saline (control; $n = 4$). Each dot represents the total USVs for a pair recorded during night 2

The mean number of male–female dyad bouts (chi-square = 1.02, $df = 1$, $p = 0.31$, $n = 5$ control dyads and 6 T dyads) and calls (chi-square = 1.41, $df = 1$, $p = 0.23$, $n = 5$ control dyads and $n = 6$ T dyads) recorded at T and control nests across three nights post-injection were not significantly different, although there was a trend for the mean number of USVs bouts and calls to be higher at T nests (Figure **2**; Table **1**; see below for significant patterns in specific USV types and the effect of night). No treatment effect was found when we considered total number of bouts (chi-square = 0.42, $df = 1$, $p = 0.52$, $n = 5$ control dyads and 6 T dyads) or calls (chi-square = 0.68, $df = 1$, $p = 0.41$, $n = 5$ control dyads and 6 T dyads) on night 1. There was a treatment effect for total number of bouts (chi-square = 5.64, $df = 1$, $p = 0.02$, $n = 4$ control dyads and 6 T dyads) and calls (chi-square = 6.58, $df = 1$, $p = 0.01$, $n = 4$ control dyads and 6 T dyads)

on night 2 with more USVs produced by T dyads (Figure **3**). No treatment effect was found when we considered total number of bouts (chi-square = 0.84, $df = 1$, $p = 0.36$, $n = 3$ control dyads and 6 T dyads) or calls (chi-square = 0.15, $df = 1$, $p = 0.70$, $n = 3$ control dyads and 6 T dyads) on night 3.

As an additional control analysis, we examined the effect of night on treatment using repeated measures on both raw (Table **2**) and rank transformed data. There was no effect of treatment or night, and no treatment by night interaction on raw data for total bouts (between subjects treatment: $F_{1,7} = 1.48$, $p = 0.26$; within subjects night: $F_{2,6} = 2.09$, $p = 0.20$; within subjects treatment*night: $F_{2,6} = 3.10$, $p = 0.12$) or total calls bouts (between subjects treatment: $F_{1,7} = 1.45$, $p = 0.27$; within subjects night: $F_{2,6} = 0.18$, $p = 0.84$; within subjects treatment*night: $F_{2,6} = 3.75$, $p = 0.09$). There was no effect of treatment or night, and no treatment by night interaction on rank transformed data for total bouts (between subjects treatment: $F_{1,7} = 3.71$, $p = 0.10$; within subjects night: $F_{2,6} = 0.71$, $p = 0.53$; within subjects treatment*night: $F_{2,6} = 3.25$, $p = 0.11$). There was no effect of treatment or night on rank transformed data for total calls (between subjects treatment: $F_{1,7} = 3.50$, $p = 0.10$; within subjects night: $F_{2,6} = 0.10$, $p = 0.91$). Importantly, there was a treatment by night interaction (within subjects treatment*night: $F_{2,6} = 6.40$, $p = 0.03$). The effect of the T injection was different on different nights and most evident on night 2, consistent with our nonparametric univariate results (Figure **4**).

Table 2. Descriptive statistics of total number of USV bouts and calls recorded at the nest sites of males receiving testosterone (T) or saline (Control) injections each night following administration of the last injection in a series of three injections in *Peromyscus californicus* males. Sample sizes are listed in the table for each night and on some nights are lower than 5 (Control) or 6 (T) due to equipment failure at two control nests (see text)

	Night 1		**Night 2**		**Night 3**	
	Control	**T**	**Control**	**T**	**Control**	**T**
USV Bouts						
n	5.00	6.00	4.00	6.00	3.00	6.00
Mean	3.00	19.33	2.00	10.33	2.00	7.00
SE	1.05	11.68	0.71	4.33	1.53	3.79
Median	4.00	4.50	2.50	6.00	1.00	4.00
Min	0.00	0.00	0.00	3.00	0.00	0.00
Max	5.00	71.00	3.00	31.00	5.00	25.00
USV Calls						
n	5.00	6.00	4.00	6.00	3.00	6.00
Mean	6.2	46.5	3.5	24.00	9.67	14.83
SE	2.42	29.87	1.32	7.99	8.21	7.70
Median	8.00	11.00	4.0	13.5	3.00	9.50
Min	0.00	0.00	0.00	12.00	0.00	0.00
Max	12.00	186.00	6.00	61.00	26.00	51.00

We also examined how each USV call type responded to treatment by determining the number and proportion of each call and bout type recorded at control and T nest sites (Table **3**). Results were consistent between bouts and calls: There were treatment effects for 1SVs and 4SVs with both median number and proportion of these types of USVs being higher at T nests (Table **3**).

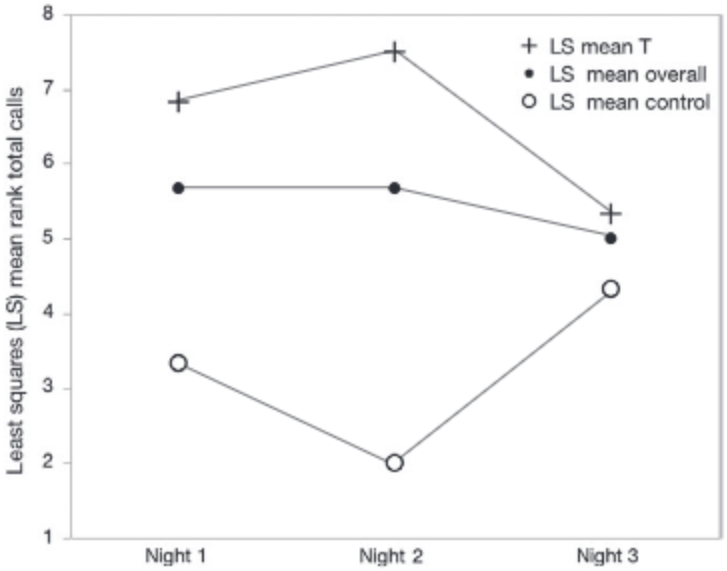

Figure 4. Least squares means calculated from the repeated measures analysis of rank total calls per night across each of 3 nights post last injection at nest sites where the male received a series of three injections of either testosterone (T; $n = 6$) or saline (control; night 1 $n = 5$, night 2 $n = 4$, night 3, $n = 3$)

Table 3. The effect of treatment on number and proportion (prop) of each type of USV bout and call produced at nest sites where the male received a series of three injections of either testosterone (T) ($n = 6$) or saline (control; $n = 5$). Recordings were made over three nights following the final injection. Because of small sample sizes, analyses were not performed for 5SVs or sweep USVs **bold =** $p < 0.05$

USV Type	Measure	Median Control	Median T	Chi-square	*df*	*p*
1SV	Number bouts	**0.00**	**2.00**	**6.23**	**1**	**0.01**
	Prop bouts	**0.00**	**0.13**	**5.17**	**1**	**0.02**
	Number calls	**0.00**	**2.00**	**6.22**	**1**	**0.01**
	Prop calls	**0.00**	**0.06**	**5.21**	**1**	**0.02**
2SV	Number bouts	1.00	3.50	1.23	1	0.27
	Prop bouts	0.38	0.18	0.73	1	0.39
	Number calls	2.00	7.00	1.23	1	0.27
	Prop calls	0.34	0.16	0.73	1	0.39
3SV	Number bouts	1.00	2.00	1.05	1	0.31
	Prop bouts	0.23	0.13	0.29	1	0.59
	Number calls	3.00	6.00	1.05	1	0.31
	Prop calls	0.29	0.17	0.29	1	0.59
4SV	Number bouts	**0.00**	**1.00**	**6.33**	**1**	**0.01**
	Prop bouts	**0.00**	**0.07**	**5.17**	**1**	**0.02**
	Number calls	**0.00**	**4.00**	**6.33**	**1**	**0.01**
	Prop calls	**0.00**	**0.12**	**5.17**	**1**	**0.02**
Bark	Number bouts	1.00	3.00	0.04	1	0.85
	Prop bouts	0.44	0.17	0.60	1	0.44
	Number calls	1.00	6.50	0.04	1	0.85
	Prop calls	0.38	0.17	0.60	1	0.44

We found no differences in spectral characteristics (duration of call, peak frequency at maximum amplitude of call, and modulation of call) of USVs produced by the male–female dyad T and

control nests (Table **4**). Only 2SV, 3SV, and barks were included in the spectral and temporal analyses because no 1SV, 4SV, 5SV, or complex sweeps were recorded at control nests (Figure **2**). Lastly, we found no difference in the number of calls present in bouts of barks produced at T and control nests (chi-square = 1.21, *df* = 1, *p* = 0.27; mean ±1 SD: T = 2.52 ± 0.30 calls/bark USV bout, control = 1.70 ± 1.21 calls/bark USV bout).

Table 4. Comparison of spectral and temporal characteristics of 2SV, 3SV, and bark bout types among nest sites where the male received a series of three injections of either T or saline (control). Recordings were made on the three nights following the final injection. For 2SV and 3SV bout types, we did a separate comparison for each call within the bout type. For 2SV bouts, there were 243 bouts that contributed to the analysis from four control nests (number of bouts = 9) and five T nests (number of bouts = 234). For 3SV bouts, there were 114 bouts that contributed to the analysis from three control nests (number of bouts = 6) and six T nests (number of bouts = 108). For barks, there were 92 bouts with a total of 108 calls that contributed to the analysis from three control nests (number of calls = 22) and six T nests (number of calls = 86). Because of the shape of bark calls, we did not calculate modulation

USV Type	**Call duration (ms)**					**Call modulation (kHz/ms)**					**Call frequency at maximum amplitude (kHz)**				
	Median Control	**Median T**	**Chi-square**	***df***	***p***	**Median Control**	**Median T**	**Chi-square**	***df***	***p***	**Median Control**	**Median T**	**Chi-square**	***df***	***p***
2SV															
First call	0.17	0.15	0.96	1	0.33	86.00	100.26	0.24	1	0.62 200.20	30.00	29.10	0.54	1	0.46
Second call	0.14	0.14	0.24	1	0.62	200.20	360.73	1.50	1	0.22	33.11	32.64	0.54	1	0.46
3SV															
First call	0.14	0.14	0.07	1	0.8	32.19	85.10	1.07	1	0.3	27.30	22.83	1.07	1	0.3
Second call	0.17	0.17	0.00	1	1	128.33	129.00	0.07	1	0.8	34.15	28.66	2.40	1	0.12
Third call	0.11	0.11	1.67	1	0.2	202.32	244.69	1.07	0.3	0.3	34.13	32.97	1.07	1	0.3
Bark															
Number Bouts	0.09	0.04	1.19	1	0.28	n/a					12.60	10.31	2.33	1	0.13

4 DISCUSSION

The challenge effect is distinct from the pulsatile nature of endogenous T that contributes to stable but heightened levels of T needed for the breeding season (Nelson & Kriegsfeld, **2018**). As described by Wingfield et al. (**2000**), there is a complex pattern of T release above the baseline levels needed for the breeding season. This complex pattern includes fluctuations in response to changing social conditions. Here, we artificially induce three individual transient increases via injections that mimic natural changes in T found in intact California mice both after winning a male–male encounter (Fuxjager et al., **2009**; Marler et al., **2005**; Oyegbile & Marler, **2005**) and also after male–female encounters (X Zhao & CA Marler, unpublished).

Our T manipulations induced an effect on future USVs in the monogamous California mouse. These changes occurred despite the small sample sizes and despite conducting the study within the complexity of a field setting. Specifically, three transient increases in T via injections

administered to males of mated male–female dyads over seven days influenced USVs at the nest during the three days following the last injection. Male–female dyads at T nests produced a greater diversity of call types such that 1SV, 4SV, 5SV, and a complex sweep were produced at T nests but not control nests. When we statistically compared individual call types, we observed that transient increases in T induced a significantly greater number and proportion of 1SV and 4SV vocalizations, but not 2SV, 3SV, 5SV, complex sweeps, or barks at the T nests, indicating that T has selective effects on specific call types calls. At the level of overall number of calls, at T nests there was also an effect on future calls produced but only on night 2 and not when all three nights after the last T injection were combined. An examination of the number of calls and call bouts across the three nights (Figure **1**) reveals a greater variance at the T nests that was not found at the control nests; results were driven by two males displaying a very strong response to the T pulse exposure (such variation was not observed in the controls). Future studies will investigate whether variation in the effects of T are induced by environmental factors such as the social context.

We previously demonstrated that three injections over the span of seven days have a future effect on both ability to win a male-male encounter (Fuxjager, Oyegbile, et al., **2011**; Trainor et al., **2004**) and an increased preference for a location (Zhao & Marler, **2014, 2016**; via conditioned place preferences). Here we demonstrate future changes in USVs in response to these same transient increases in T. Our results support the concept that social behavior in the field is causally influenced by more than the long-term changes in T such as those associated with the breeding season, as previously found via implants in other species (e.g., Ketterson, Nolan, Wolf, & Ziegenfus, **1992**; Marler & Moore, **1988**), but can also be altered significantly by the transient increases in T that normally occur in response to male–female and male–male ("challenge effect") encounters in California mice (Marler et al., **2005**; Oyegbile & Marler, **2005**; X Zhao & CA Marler, unpublished) and other rodents (reviewed in Gleason et al., **2009**). The isolated changes observed here from three injections over seven days may contribute to the longer lasting patterns of T change above baseline (e.g., Wingfield et al. **2000**) if individuals experience a high sustained rate of social interactions.

More generally, transient post-encounter increases in androgens have been documented for male–male interactions across a wide variety of species and taxa (reviews by Hirschenhauser & Oliveira, **2006**; Fuxjager et al., **2017**); the effect of transient T surges on communication therefore may be relevant for a wide range of species, but remains to be tested. Within rodents, including male California mice (X Zhao & CA Marler, unpublished data), it has been widely documented that male encounters with a female can induce transient increases in T (review by Gleason et al., **2009**). We previously documented a rapid effect of transient increases in T on male USVs (within at least 20 min) in the laboratory when exposed to unfamiliar/strange females (Pultorak et al., **2015**); these past results combined with the current study suggest that transient increases in T can have both rapid effects acting within minutes and long-term effects that are lasting days. We suggest that the short duration of the hormonal signal therefore does not preclude an important effect on behavior, such as vocal communication. It is of significance, however, that these changes in USVs are measures of the male–female dyad and may be occurring in the males receiving the T injections and/or changes in other male behaviors that are inducing changes in vocalizations of the female partner. Thus, our results should be viewed in the context of male–female dyad interactions within a monogamous species.

In general, our results support a positive association between T and USV production that is consistent with T effects seen in males of other species following castration and T replacement via long-lasting T implants (Dizinno & Whitney, **1977**; Warburton et al., **1988**), such as the positive association found between T and the trilled songs produced by male Alston's singing mice (*Scotinomys teguina*; Pasch, George, Hamlin, et al., **2011**). We again suggest, however, that the pulses used in the current study, in contrast to baseline changes in T, allow a more rapid change in response to fluctuating social conditions and may provide an internal messaging system representing a cumulative sampling of the environment.

We can only speculate on the function of changes in USVs in response to T in the field because other behavioral data were not available. One possibility is that T is increasing male sensitivity to environmental stimuli. For example, the increase in both 1SVs and 4SVs and a lack of an increase in barks could reflect an increase in affiliation and/or sexual attractiveness to females at the T nests as suggested by a negative association between male T and distance between the male and his mate (Gleason & Marler **2012**). Laboratory studies reveal a positive association between the production of SV calls, SV bout size, and SV call duration of the male/female dyad with male affiliative behavior, such as when a male follows the female mate (Pultorak, Matusinec, Miller, & Marler, **2017**). In addition, after a male and female are introduced and the pair bond forms, there is an increase in dyad SV calls (Pultorak, Alger, Loria, Johnson, & Marler, **2018**).

In the field, SVs are the most common call type detected, but occur both when individuals are alone or during social interactions (Briggs & Kalcounis-Rueppell, **2011**). While SVs occur in aggressive contexts, as recorded from same-sex dyadic interactions, the calls are typically short and co-occur with vocalizations referred to as barks that represent defensive aggression (Rieger & Marler, **2018**). In the current context, the increase in SVs and absence of a change in barks suggests that the T-induced social interactions in the context of the nest site may have been affiliative. Because T injections produce conditioned place preferences in male California mice in the laboratory using the same three T injections as in the current study (Zhao & Marler, **2014, 2016**), an increase in social interactions may have been induced by an increase in time males spent at the nest. In the laboratory, the rewarding effects of T have been demonstrated both through self-administration studies (Wood, **2002**; Wood, Johnson, Chu, Schad, & Self, **2004**) and through the development of a conditioned place preference (CPP) for a location where multiple transient increases in T have been administered (e.g., Zhao & Marler, **2014, 2016**). Future studies will examine whether the T injections are producing responses more consistent with affiliation or aggression in the context of a pair bond and whether there is an interaction between conditioned place preferences and social contact.

In summary, the effect of multiple transient increases in T on USVs in the field had a fairly dramatic effect on vocalizations considering the myriad of competing biotic and abiotic stimuli (Hurley and Kalcounis-Rueppell, in press). The primary change was documented in number of and proportion of SVs that can be used in aggressive, sexual, or pair bonding interactions. We also cannot rule out the potential importance of context in determining the function of SVs in California mice (Hurley and Kalcounis-Rueppell, in press; Kalcounis-Rueppell et al., **2018**), as the songs produced by male Alston's singing mice, for example, also appear to be important in both aggressive and courtship behaviors (Pasch, George, Campbell, & Phelps, **2011**; Pasch,

George, Hamlin, et al., **2011**). Our findings support and add to the growing evidence for the functionality of transient increases in T on not only behavior in the laboratory (as described in the introduction), but also on USVs. It will also be important to eventually expand such studies to understand how animals sample their social environment and when translated into transient increase in T, how this contributes to animals' ability to integrated multiple social interactions at the level of the central nervous system and influence future behavior.

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