PETRIC, RADMILA, Ph.D. Do Physiological and Environmental Factors Influence Vocal Communication and Associated Behaviors in *Peromyscus*? (2020)
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Vocal communication is an integral component of animal behavior and individuals rely on vocal signals to mediate a myriad of daily activities. Despite valuable work from the laboratory, we do not understand how physiological and environmental factors alter vocal communication and activities in complex field settings where multiple competing stimuli occur simultaneously. The goal of my research is to understand how transient testosterone (T) pulses, a physiological factor, and anthropogenic noise, an environmental factor, alters the allocation of time and energy to influence vocal output, signal structure, and other reproductively related behaviors in free-living animals.

Physiological Factor: T-pulses naturally occur after social interactions in a variety of species and can modulate call production and alter animal preferences for the physical location at which the T-pulse occurred (conditioned place preference; CPP). Manipulation of T-pulses has been conducted under controlled laboratory conditions; here, I ask how multiple T-pulses alter time allocation in complex field setting and influence future vocal behavior. **H_1:** *T-pulses reinforce behaviors in the area where the experience occurred in the form of conditioned placed preference (CPP) that in turn alter call production and the allocation of time and energy spent towards specific social interactions.* To determine whether T-pulses induce CPPs and alter call production in the wild, I used a monogamous, territorial, and vocal rodent, the California mouse (*Peromyscus californicus*). California mice are well studied both in the laboratory and the wild, and in this species, males must balance mate attendance, offspring care, and

territory defense with T being an important mediator of these social behaviors. I assessed the effects of three exogenously administered T-pulses or saline (control; C) on the following: 1) spatial preference 2) number of calls produced, and 3) spectral and temporal characteristics of calls (frequency, amplitude, and duration). I found that in the field, environmental location dictates the effects of T injections, suggesting that T-pulses are highly context dependent. At the nest, T-males spend more time at the nest and their non-injected mates spent less time at the nest. At the territory boundary, T-males and their non-injected mates spent less time at the boundary, but T-males traveled further outside their original territory than C-males. At the nest, T-mice produced more calls with a lower mean bandwidth whereas at the territory boundary T-males produced more short duration calls than C-males. In free-living and pair-bonded males, T-pulse induction of CPPs is based on the physical environment and the interactions that occur in that space. Together, these results suggest there is behavioral plasticity in inducing CPPs and that it is context dependent. Lastly, I found that independent of treatment type, the acoustic properties within a pair were more similar than among pairs, providing evidence for vocal convergence in pair-bonded California mice.

Environmental Factor: Anthropogenic noise is a global pollutant that alters the natural soundscape which animals rely on for communication, foraging, navigation, exploring, and predator avoidance. Anthropogenic noise is pervasive in the audible range during the day, but it also extends into the ultrasonic range and into the night. Here, I ask how broadband (audible and ultrasonic) anthropogenic noise influences behaviors of free-living and nocturnal mammals. **H_2**: *Broadband*

anthropogenic noise alters the allocation of time and energy to influence activity, foraging, and vocal communication. To test my hypothesis, I broadcasted anthropogenic or familiar noise to examine 1) activity, 2) foraging and 3) call production of the deer mouse (*Peromyscus maniculatus*) and woodland jumping mouse (*Napaeozapus insignis*). I found that deer mice and woodland jumping mice spent less time at sites with anthropogenic noise compared to familiar noise. I also found that deer mice were less likely to approach food than woodland jumping mice during broadcasts of anthropogenic noise, however, both species spent less time foraging and vocalizing in the presence of anthropogenic noise. My results show species-specific responses to noise in nocturnal rodents that vocalize in the ultrasonic range. Overall, my data are consistent with previous research from other taxonomic groups, which demonstrate that anthropogenic reduces activity, foraging and vocalization production of animals.

DO PHYSIOLOGICAL AND ENVIRONMENTAL FACTORS INFLUENCE VOCAL COMMUNICATION AND ASSOCIATED BEHAVIORS IN *PEROMYSCUS*?

by

Radmila Petric

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Approved by

_____
Committee Chair

APPROVAL PAGE

This dissertation written by RADMILA PETRIC has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

_____

_____

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

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CHAPTER I

INTRODUCTION

Vocal communication is an integral part of animal behavior. Vocal signals contain information and elicit predictable behavioral responses by the receivers (Wilkins et al. 2013). Animals rely on vocal signals to mediate a myriad of daily activities (Panyutina et al. 2016). In various taxonomic groups both males and females vocalize to convey information about species (Höbel and Gerhardt 2003), sex (Aubin et al. 2007; Smith et al. 2009), reproductive state (Klappert and Reinhold 2003), and sender and receiver identity (Janik 2009; Prat et al. 2016). Vocal recognition is documented based on spectral and temporal characteristics, such as frequency, duration, amplitude, redundancy, bandwidth, and timing (Bee et al. 2001; Soltis et al. 2005; Merten et al. 2014).

Call production and other social behaviors are a result of information transfer that begins with a stimulus and ends in a behavioral response (Fujii et al. 2016). To coordinate internal with external signals, environmental sensors are connected to the endocrine system (Barclay et al. 2016). Sensory systems collect information from the environment and forward the message to the central nervous system (CNS) for processing (Barclay et al. 2016). The CNS processes the environmental stimulus which triggers physiological stimuli that alter cellular processes to initiate an appropriate behavioral response (Eraslan et al. 2015; Arthur and Cooley 2012).

Testosterone, the Physiological Factor

Behaviors, including vocal communication, are modulated by hormones, a physiological factor (Arch and Narins 2009). Fluctuation in hormone levels is correlated with changes in multiple aspects of vocal communication (Fernandez Peters 2016) such as call production (Burmeister and Wilczynski 2001), spectral characteristics of vocalizations (Klomberg and Marler 2000), and interpretation and behavioral response by the receiver (Caras 2013). One of the main regulators of vocal communication are gonadal steroids (Wilczynski et al. 2005). In both males and females, gonadal steroids (androgens and estrogens) initiate vocal communication and other sexual behaviors (Pasch et al. 2011). Gonadal steroids coordinate changes between the CNS for initiating the vocal behavior and the peripheral system responsible for generating the actual signal (Harding 2004). In various animal species, the androgen hormone testosterone modulates vocal communication (Harding and McGinnis 2003; Pasch et al. 2011). In rodents, behavioral endocrinology studies have demonstrated that castration of males decreases or eliminates vocal communication, and exogenous administration of long-lasting T implant restores vocalization production (Pasch et al. 2011; Kerchner 2004).

Variation in vocal signals influences mate selection, as vocal communication is an honest signal that provides accurate information about the quality of a potential mate (Spencer et al. 2003; Drăgănoiu et al. 2002; Pasch et al. 2011). Increased testosterone levels and its metabolites can influence vocalization features (Bass and Remage-Healey 2008). Males with elevated androgens can exhibit greater modulation of spectral and temporal characteristics of vocalizations which females tend to prefer (Pasch et al. 2011).

Testosterone can generate a reward by indirectly activating the dopamine receptors (Bell and Sisk 2013; Robichaud and Debonnel 2005). The rewarding properties of testosterone can condition animals via conditioned placed preferences (CPPs) to a location in the environment at which the hormone release occurred (Arnedo et al. 2000; Frye et al. 2001; Zhao and Marler 2014; 2016). Winners of aggressive encounters experience an increase in testosterone and the winning male can altered spatial preferences by forming CPPs for the encounter location at which the encounter occurred (Martínez et al. 1995).

Studies that have examined the relationship between behavior and testosterone have mainly focused on long-term changes in baseline T levels; however, rapid and transient increases in testosterone (T-pulses) can also induce behavioral changes. In male mice, T-pulses occur after social interactions (review by Gleason et al. 2009). For example, male California mice experience a T spike 45 minutes after a social encounter (Gleason et al. 2009). The proposed function of T-pulses is to rapidly modulate male reproductive behaviors (Mangiamele and Thompson 2012). Interestingly, a single T-pulse can rapidly (<1 hour) alter male vocal behavior (Pultorak et al. 2015; Remage-Healey and Bass 2004). In addition to the altered vocal response, a T-pulse can also induce an acute sexual response by rapidly increasing sperm volume and cell density (Mangiamele and Thompson 2012). Furthermore, multiple T-pulses can have long term (> 24hours) effects on vocalization production (Timonin et al. 2018).

The classic mechanism through which steroid hormones alter behavior is via gene transcription, however, T-pulses most likely induce behavioral changes through a non-

transcriptional mechanism (Mangiamele and Thompson 2012; Cornil et al. 2013). Studies have demonstrated that T-pulses can alter male vocal and sexual behaviors in less than an hour, which for a steroid hormone is considered fast (Remage-Healey and Bass 2006; Cornil et al. 2013; Pultorak et al. 2015). These rapid behavioral changes indicate a non-transcription mechanism, as genomic changes take more time (Mangiamele and Thompson 2012). A potential non-transcriptional mechanism for the rapid effects of T-pulses on reproductive behaviors is the conversion of testosterone to estradiol via aromatase (Cornil et al. 2013). Aromatase is expressed in the medial preoptic area (mPOA) of the hypothalamus, area of the brain that controls vocal, sexual, and parental behavior (Kentner et al. 2010; Dominguez and Hull 2005). Within seconds to minutes, estradiol binds to estrogen receptors and rapidly modulates vocal and sexual behavior (Mangiamele and Thompson 2012; Cross and Roselli 1999; Cornil et al. 2006). Estradiol can have acute effects by directly acting on the vocal communication circuits in the CNS and on the motor neurons (Remage-Healey 2012). Strong synapses are important in producing fast, and loud vocalizations and estrogen can alter the synaptic strength at the laryngeal neuromuscular junction to alters muscle contractions (Remage-Healey 2012). Testosterone and its metabolites can modify vocalizations by altering neuromuscular control to allow for greater modulation of spectral and temporal characteristics (Harding 2004). Specifically, testosterone can lead to modification of duration, frequency, bandwidth, and amplitude of vocalizations (Pasch et al. 2011; Pasch et al. 2011; Fusani, et al. 1994).

The modification of spectral or temporal characteristics between members of a pair or group to show similarities in call structure is known as vocal convergence (Tyack 2008). Vocal convergence occurs in animals are form-long term social bonds (Tyack 2008). In order for vocal convergence to occur, individuals must have control of the vocal organ and the neural circuitry to coordinate the fine-tuning of the vocalizations (Knörnschild 2014).

Anthropogenic Noise, the Environmental Factor

Anthropogenic (human-made) noise is a global pollutant that emanates from human infrastructure and activities and changes the natural soundscape (Barber et al. 2010). Studies on novel environmental sounds suggest that to some degree all anthropogenic sounds negatively affect vocal communication in a variety of species (Bee and Swanson 2007). Anthropogenic noise can have negative population effects by altering both senders and receiver behaviors (Slabbekoorn and Ripmeester 2008). We know that vocal communication is a crucial component of animal behavior (mate attraction, territory defense, pair-bonding, parent-offspring communication, and predator avoidance) and anthropogenic noise can have a detrimental effect on vocal signals (Slabbekoorn and Ripmeester 2008). Signal impairment in any context could lead to decreased individual fitness which leads to a decline in population size and altered species composition (McLaughlin and Kunc 2013). Anthropogenic noise can affect vocal communication by directly and indirectly influencing the production and propagation of signals, which alters animal behavior, physiology, and survival (Babisch 2003; Jarup et al. 2008; Radford et al. 2014; Slabbekoorn and Ripmeester 2008). In general, noise

negatively affects vocal communication in two ways: 1) by masking vocal signals which prevent successful propagation, detection, and interpretation of vocalizations and 2) by eliciting energetically costly anti-predator behaviors which shift energy away from vocal communication, as well as, spatial behavior, and foraging (Frid and Dill 2002).

Anthropogenic noise can have numerous physiological effects that can lead to altered performance and decision making (Torre and Snowdon 2002). These effects can be mild, short-term and reversible or they can be permanent and irreversible (Torre and Snowdon 2002). Noise can disrupt normal functions of the endocrine system and lead to elevated glucocorticoid production (Barber et al. 2010; Babisch 2003). The increase in circulating glucocorticoids has various physiological effects including inhibition of glucose uptake by muscle cells and breakdown of amino acids. Furthermore, chronic stress and prolonged secretion of glucocorticoids can stifle the immune response which makes the individual vulnerable to infection (Torre and Snowdon 2002). Glucocorticoids increase leads to signal disruption of the hypothalamus – pituitary – gonadal axis which in turn inhibits sex-hormone production. Specifically, in males, glucocorticoids inhibit testosterone production by suppressing the secretion of gonadotrophins, enzyme activity necessary for the synthesis of testosterone (Orr et al. 1994), and by inducing apoptosis of the Leydig cells (Hardy et al. 2005).

There is a knowledge gap in understanding the long-term (>24 hours) effects of transient T-pulses (physiological factor) on vocalization production and social behaviors in pair-bonded monogamous species living under natural conditions. For my dissertation, I investigated the effects of T-pulses on time allocation at the site of injection (nest site or

territory boundary) as well as the number and type of calls produced and spectral characteristics of those calls. Furthermore, our understanding of anthropogenic noise effects on animals is biased toward the impacts of audible noise on diurnal bird behavior (Slabbekoorn and Ripmeester 2008). Noise is pervasive in the audible range, but it also extends into the ultrasonic range, and we do not understand how nocturnal mammals perceive and respond to broadband anthropogenic noise in nature. As part of my dissertation, I also investigated the vocal, spatial and foraging response to the presence of anthropogenic noise.

H_1: I hypothesized that, in the field, T-pulses would reinforce behaviors in the area where the social experiences induced T-pulses through the formation of CPPs that would, in turn, alter associated social behavior. **H_2**: I hypothesized that broadband anthropogenic noise would alter the allocation of time and energy spent toward vocalization production, activity, and foraging.

My hypotheses were successfully tested using *Peromyscus* mice. The genus *Peromyscus* contains over 50 known species, are abundant, widely distributed, and occupy almost every terrestrial habitat in North America (Bedford and Hoekstra 2015). In the wild, *Peromyscus* produce a wide array of vocalizations, most of which are high-frequency signals in the ultrasonic range (Briggs and Kalcounis-Rueppell 2011; Petric and Kalcounis-Rueppell 2013; Kalcounis-Rueppell, et al. 2006; Pomerantz and Clemens 1981; Kalcounis-Rueppel et al. 2018).

To address **H_1**, I studied the California mouse (*Peromyscus californicus*) because it forms lifelong pair-bonds and is a model species for monogamy, territoriality, and

paternal care. Male California mice must balance territory defense, paternal care, and mating all at the same time and T is an important mediator of these social behaviors. Furthermore, I tested $\mathbf{H_2}$ using the deer mouse (*Peromyscus maniculatus*) because it is the most abundant and widely distributed native rodent, making my anthropogenic noise results relevant to a wide geographic range. To my knowledge, this is the very first study to examine the effects of both environmental and physiological stimuli on the behavior of free-living individuals in a complex field setting. I had four specific aims:

Specific Aim 1 (Chapter II): Assessed whether T-pulses or saline at the nest site have lasting effects on the number and type of vocalizations produced and spatial preference for the nest site.

Specific Aim 2 (Chapter III): Assessed whether T-pulses or saline at the territory boundary site have lasting effects on the number and type of vocalizations produced and on spatial preference at the territory boundary.

Specific Aim 3 (Chapter IV): Examined vocal convergence in wild California mouse pairs based on similarities within and among pairs in spectral and temporal characteristics of vocalizations.

Specific Aim 4 (Chapter V): Examined the effects of broadband anthropogenic noise or familiar noise on vocalization production, spatial behaviors and foraging effort.

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CHAPTER II

TESTOSTERONE INDUCES A CONDITIONED PLACE PREFERENCE TO THE NEST OF A MONOGAMOUS MOUSE UNDER FIELD CONDITIONS

This Chapter is coauthored by Radmila Petric, Matina Kalcounis-Rueppell and Catherine Marler

Abstract

Rapid transient changes in testosterone (T-pulses) in males occur after social interactions with males and females. One underexplored function of T-pulses is its rewarding nature which raises the question of whether behavioral changes occur other than aggressive or sexual behavior. In the laboratory, T-pulses can induce a preference for a location through conditioned place preference (CPP), as found in the California mouse (*Peromyscus californicus*), a monogamous, biparental and territorial species. This prompted us to ask, what is the adaptive value of these T-pulses under complex field conditions. We hypothesized that the T-pulses center the male around an area of reproductive importance via the ability of T to induce a preference for the location in which the pulse was experienced (condition placed preference; CPP) and indirectly reinforce location-associated behaviors. Wild California mouse males that were administered three T-pulses at the nest site spent more time at the nest than controls regardless of whether they had pups or not. T mice produced more calls, and these calls have a lower bandwidth that can travel further in the environment, potentially to

communicate with the mate across a distance. Females adjusted for male behavioral changes by spending less time in the nest. These results suggest that T-pulses can induce a male to alter allocation of time to a specific location of focused activity, in this case, the nest, as opposed to behaviors such as mate guarding, courting, pair-bonding or aggressively pursuing other males.

Introduction

Animals must frequently adjust their allocation of time as they move through various life-history stages and meet different social challenges. One mechanism that can adjust the approach to a stimulus is through rewarding or reinforcing neural processes that increase the probability that an individual will approach a stimulus (Glickman and Schiff 1967) through the repeated linkage between testosterone (T) release and the presence of a stimulus. Testosterone pulses can act as an internal reward (Gleason et al. 2009) or reinforcing stimulus that when released naturally or through an injection can increase approach to different stimuli such as the physical location in which the T-pulse was experienced, at least under laboratory conditions in rodents (e.g. Zhao and Marler 2014). Because male T-pulses are released in response to social conditions such as a male-male challenge or a male-female interaction across a variety of species including humans (Gleason et al. 2009), we speculate that reward systems which allow reinforcement mechanisms to adjust to changing social challenges can be linked to location (Zhao and Marler 2016), such as different areas within a territory. In the case of a biparental species, T release near the nest may provide a mechanism for increasing a male's attendance at the nest, as suggested by the results of a laboratory study (Zhao and

Marler 2014). Reinforcing effects can alter the probability for the successful acquisition of essential resources necessary for survival and reproduction (Tinbergen 1957). One paradigm for testing reinforcing effects is to assess changes in behavioral preference for a location at which the stimulus (i.e. T-pulse in response to a social stimulus; Gleason et al. 2009) occurred in the form of a conditioned placed preference (CPP) (Arnedo et al. 2000; Frye et al. 2001). The reinforcing effects occur via activation of the internal reward system (Bell and Sisk 2013). We explore the hormone testosterone as a naturally occurring stimulus that has rewarding/reinforcing effects (Arnedo et al. 2000; Frye et al. 2001; Zhao and Marler 2014; 2016; Zhao et al. 2019; 2020).

The "Challenge Hypothesis" proposed by Wingfield and colleagues states that there are temporal changes in circulating testosterone levels determined by a trade-off in paternal care that requires a decrease in T and male-male competition that requires an increase in T (Wingfield et al. 1990). In California mice (*Peromyscus californicus*), the Challenge effect is activated with T-pulse release after male-male aggressive encounters which influence future behavior under laboratory conditions (Fuxjager et al. 2016). Males that previously won a dispute can form CPPs for the encounter location (Gleason et al. 2009). In monogamous species, the formation of CPPs can be dependent on the familiarity of the environment and the pair-bond status (Zhao and Marler 2014; 2016). For example, in pair-bonded California mice, T-pulses induce CPPs in familiar but not unfamiliar environments (Zhao and Marler 2014; 2016). Interestingly, the opposite was true for sexually naïve males, in which T-pulses induced CPPs in unfamiliar but not in

familiar environments (Zhao and Marler 2014; 2016). Therefore, these T-pulses can influence both social interactions and location preferences.

T-pulses modulate other behaviors such as vocalizations (Remage-Healey and Bass 2006; Pultorak et al. 2015), which can affect aspects of sexual selection. In Gulf toadfish (*Opsanus beta*) and plainfin midshipman fish (*Porichthys notatus*), within minutes of a T-pulse, males increase call rate and duration of calls which females tend to prefer (Remage-Healey and Bass 2004; 2006). Male California mice administered a single T-pulse and placed in the presence of a novel female decrease production of calls associated with courtship in pair-bonded but not unpaired mouse males in the laboratory (Pultorak et al. 2015). This finding indicates that in this species, T-pulses may reduce extra-pair mating effort by inhibiting the production of courtship calls to unfamiliar females and act as a fidelity mechanism (Pultorak et al. 2015). T-pulses also have long-term effects on the call production in California mice, such that days after multiple T-pulse administration in the field, males produced more call types and a trend to produce more ultrasonic vocalizations (USVs) that are above 20 kHz in frequency (Timonin et al. 2018).

While single and multiple T-pulses can alter behavior, the effect of T-pulses on location preferences have only been examined in controlled and simplified laboratory conditions with few competing behavioral choices available, as would occur in the field. There is also a knowledge gap regarding long-term effects (greater than 24 hours) of multiple T-pulses, especially how T-pulses alter time allocation and vocal behavior in complex field settings characterized by multiple and competing biotic and abiotic cues.

We hypothesized that, in the field, T-pulses would reinforce behaviors in the area where the social experiences induced T-pulses through the formation of CPPs that would, in turn, alter associated social behavior. Here we tested three predictions: 1) pair-bonded males receiving T injections at the nest, would spend more time at the nest; 2) females would adjust for the increased time that her T-injected mate spent at the nest by decreasing her time at the nest and allocating more time to activities away from the nest (based on Trainor et al. 2011); 3) T-pulses would induce changes in type and number of USVs produced as part of both the direct effects of T on behavior and the indirect effects on the pairs' social adjustment to the altered time allocation to a specific location (Timonin et al. 2018).

We tested our hypothesis in the well-studied monogamous and territorial California mouse. In this species, males balance their time between mate attendance, offspring care, and territory defense, all behaviors that can be influenced by T. In the laboratory and the wild, California mouse adults frequently produce USVs. In the field, sustained vocalizations (SVs) and barks are frequently recorded (Kalcounis-Rueppell et al. 2006; Briggs and Kalcounis-Rueppell 2011; Timonin et al. 2018), but in the lab, SVs, barks along with simple sweeps and complex sweeps are recorded (Pultorak et al. 2015; 2017; 2018; Rieger and Marler 2018; USVs are described in more detail in the methods). SVs are the most common call type recorded in the field outside of the nest (Briggs and Kalcounis-Rueppell 2011; Kalcounis-Rueppell et al. 2006; 2010; Timonin et al. 2018). The SVs often occur when a member of a pair is greater than 1m away and under these conditions may serve as long-distance contact vocalizations, possibly for maintenance of

the pair-bond (Briggs and Kalcounis-Rueppell 2011). Free-living California mice maintain strict territories and generally do not overlap with other California mice (Ribble and Salvioni 1990), therefore, social interactions at the nest occur primarily between pair members and these interactions are often accompanied by the production of SVs. This is consistent with the common production of SVs between pairs in the laboratory (Pultorak et al. 2018). Thus, the monogamous reproductive system of California mouse and their known time management and vocalization behaviors contribute to a compelling system for assessing behavioral responses to T-pulses and the establishment of male T-induced CPP in the field to alter the amount of time that males spend at the nest.

Methods

Field work was conducted at the Hastings Natural History Reservation (HNHR), Carmel Valley, California, USA, from January to June 2015 (season one) and from September to December 2015 (season two) on trapping grids that this species was previously studied (Timonin et al. 2018; Briggs and Kalcounis-Rueppell 2011; Kalcounis-Rüppell and Millar 2002; Kalcounis-Rueppell et al. 2006). We tagged 323 mice, of those we identified 33 reproductively active mated pairs (males with enlarged testis and females were pregnant and/or lactating). Once putative pairs were identified, we trapped the target and both the male and the female in the pair were outfitted with a 0.55g M1450 mouse style transmitter (Advanced Telemetry System [ATS], Isanti, MN, USA), adjusted for California mice (Briggs and Kalcounis-Rueppell 2011). We tested each transmitter to ensure proper functioning, attached the transmitters (Briggs and Kalcounis-Rueppell 2011), and released all mice at the site of capture. Using an R4500S

DCC receiver/datalogger and a Yagi antenna (ATS), we located the pair the following day at the nest (described below). All 33 putative pairs were confirmed as pairs when the signals from both the male and female transmitters were emitted from the same nest. We ensured the tracked nest location was the primary nest and not one of the satellite locations by monitoring nest occupancy for up to three days. A total of 28 pairs did not relocate and the nest was in a suitable location for setting-up our remote sensing equipment (described below), we place 15-20 Longworth traps (14 x 6.5 x 8.5cm, NHBS, Totnes, Devon, UK) within a 2m radius surrounding the nest to trap the male and administer the injections (described below).

Treatment

We randomly assigned 28 males to receive either testosterone (T, n=15) or saline (control, C, n=13) injections. Based on prior laboratory research, the dose of T injection was approximately 36ug/kg (T-cyclodextrin dissolved in saline; Oyegbile and Marler 2005) which mimics natural T-pulses (Trainor et al. 2004). All animals were injected subcutaneously, and the researcher was blind to the treatment type. Each focal male received three injections of 0.1 ml of the injectate regardless of body mass. We, therefore, included body mass as an independent variable in our statistical analysis. Our requirement was that all three injections were administered within five days, with only one injection per day. One male was excluded because it did not receive all three injections within five days. We refer to females whose mate received T as "T-females" and the nests as T-nests while females whose mate received saline are referred to as "C-females" and the nests as C-nests. We also recorded "injection night" which represents

the total number of nights needed to administer all three injections (three or four nights). Therefore, we included injection night as an independent variable in our statistical analysis.

After the third and last injection, we deployed the remote sensing equipment (automated radio telemetry, audio recording, and thermal imaging; described below) to record for three consecutive nights ("recordings nights" 1-3). We treated data collected by the remote sensing equipment over one night as a sample unit and included recording night in our analyses. For each recording session, all equipment was set-up to record from sunset to sunrise. T and C solutions were issued by Dr. Brian Trainor from the Department of Psychology at the University of California Davis (IACUC Protocol number 19849).

Automated Radio Telemetry

We used two R4500S DCC receiver/dataloggers (Advanced Telemetry System [ATS], Isanti, MN, USA) to monitor the number of minutes radio-collared mice spent at the nest each night and the amount of time the male and female were together and apart. We, therefore, monitored both male behavioral changes in response to treatment type and the female response to male behavioral changes. The time at the nest was standardized by counting and totaling the number of minutes the mouse spent in the nest area from sunset to sunrise divided by the total number of minutes in the night to obtain a proportion of time spent in the nest area ("time at the nest"). Using proportions allowed us to account for differences in length of recordings. Each datalogger was connected to an antenna and programmed to detect one unique transmitter frequency (one for each pair member).

Antennas were placed either on top of or next to the nest. When the collared mouse was detected by the receiver, signal strength was stored in the datalogger. Each day we conducted manual telemetry on the collared pair and found the nest location with the strongest signal strength. For each individual, we assessed a reference signal (range 130 – 155dB signal strength) during the day when we knew the mouse was in the nest. To assess how long a mouse spent in the nest per night, we only counted the number of minutes during which the signal fell within the reference range. Each morning, the dataloggers were removed from the field and data were downloaded. The telemetry equipment was set-up at 27 nest sites. Due to equipment failure, we did not record male time at the nest for five T-nests and one C-nest and we did not record female time at the nest for one T-nest and three C-nests. Our final dataset consisted of 63 recording nights from 21 nest sites (T=10, C=11) for males and 69 recording nights from 23 nest sites (T=14, C=9) for females. We did not have matching pair time at the nest for five T-nests and four C-nests. Our final matching pair dataset consisted of 54 recording nights from 18 nest sites (T=10, C=8) and we used night as a sample unit in our analysis.

To validate our automated telemetry system, we also conducted manual telemetry. For at least six hours per night, starting at sunset, we manually tracked (4 MHz R4000 receiver from ATS) the signal of the male. To determine percent agreement between the two methods, we randomly selected and examined time at the nest over 24 nights from 12 nest sites during which both manual and automated telemetry data were recorded. We compared the two methods using Cohan's Kappa test of agreement for each of the 24 nights. According to Cohan's Kappa Index, any value that falls between 0.81 and 0.99 is

considered a very high level of agreement. Our values for each of the 24 days fell between 0.88 to 0.97, therefore we use automated telemetry in our final analysis.

Audio Recording

Our goal was to record a variety of USVs as described here. The SVs have a peak frequency around 20kHz, and are approximately 50 – 1000ms in length, low modulation calls that can be emitted as a single or bout of multiple calls that can be categorized based on the number of calls in a bout (1SV, 2SV, 3SV, 4SV, etc.; Kalcounis-Rueppell et al. 2018). Bark calls are shorter in duration (50ms or less), resemble an upside-down U with the beginning and the end of the call dips into audible range at approximately 12kHz with a peak frequency around 20kHz and tend to be "noisy" vocalizations (Pultorak et al. 2018). Complex sweeps are short duration (100ms or less), that pass through multiple high to low and low to high frequencies with a peak frequency of around 100kHz (Pultorak et al. 2015). Similar to the SVs, the barks, simple sweeps, and complex sweeps occur as a single call or bout of calls.

We used ultrasonic microphones (Emkay FG Series from Avisoft Bioacoustics, Berlin, Germany) to assess the number and type of USVs produced at the nest. We set-up two microphones; one next to the nest entrance and a second 2m away directly from the nest entrance. Microphones were tested prior to each recording session by blowing a dog whistle and clapping our hands. Microphones were connected to an UltraSoundGate system 1216H (Avisoft Bioacoustics, Berlin, Germany) that was connected to a small laptop (DELL Latitude E6230). Using Avisoft RECORDER Software, the system recorded when sonic and ultrasonic sounds were detected by the microphone(s). Each

morning, files were downloaded. All files were examined using Avisoft SAS Lab Pro (Avisoft Bioacoustics) and files with mouse USVs were transferred to a different folder for additional analyses. When possible, we assigned USVs to individuals by matching the radio telemetry data with the time of the mouse USV. By examining telemetry data within one minute of USV production and based on the transmitter signal strength (Briggs and Kalcounis-Rueppell 2011), we determined if the male or the female produced the USV. We were not able to assign 51% of the USVs to one individual because both the male and the female were at the nest with strong transmitter signal strengths and therefore, we only used the assigned data to test the treatment effect on the spectral and temporal characteristics of USVs. The acoustic recording system was set-up at 27 nest sites (T=15, C=12). Due to equipment failure, we did not record data at one T-nest. Our final dataset consisted of 78 recording nights from 26 nest sites (T=14, C=12). Mouse USVs were counted and classified into one of the following types: 1SV, 2SV, 3SV, 4SV, 5SV, 6SVs or barks (Kalcounis-Rueppell et al. 2018). We counted USV numbers recorded from sunset to sunrise and refer to the value as "total USVs". Lastly, we determined if the proportion of a specific type of USV (1-, 2-, 3-, 4-, 5-, 6SVs and barks) differed between treatments by totaling each USV type per nest site and dividing by the total number of USVs produced at that nest.

Using SAS Lab Pro, we extracted spectral and temporal characteristics (duration, bandwidth, and five frequency variables [peak, minimum, maximum, start, and end]) from USVs recorded at the nest. Each spectrogram was generated with a 512 FFT (Fast Fourier Transform), and a 100-frame size with Hamming window. For each call, we

measured duration, bandwidth, and five frequency parameters (start, end, minimum, maximum, and frequency at maximum amplitude).

Thermal Imaging

We used a thermal imaging lens (Photon 320 14.25 mm; Flir/Core By Indigo) to assign social context to USVs. The thermal imaging lens was suspended to capture the full view of the nest and a circular area with a 2m radius surrounding the nest. The lens was connected to a JVC Everio HDD camcorder which recorded continuously throughout the night. We watched the video footage in three-minute increments, (a minute before, minute during and a minute after call production) to determine behavior and number of mice on the screen. If there was only one member of a pair present at a time, the behavior was assigned as "alone". If both mates were present, we determined the proximity of mice to each other by using a 1m scale that was overlaid in the video for each site. If mice were less than 1m apart, we assigned them as "<1m", and if the mice were more than 1m apart, we marked them as ">1m". We assessed the types of USVs (1-, 2-, 3-, 4-, 5-, 6SVs and barks) produced by context (alone, <1m or >1m) and treatment type.

Statistical Analyses

Time at the nest for both the male and the female was normally distributed and therefore we fitted Gaussian distribution. Pair time at the nest and total USVs were in violation of normality and variances and could not be normalized and therefore we used either Quasibinomial or Poisson distribution. We used General Linear Models (GLM) with time at the nest, pair time at the nest and total USVs as the dependent variables and we included individual identification code independent of treatment type to account for

individual differences. Using the package lem4 (Bates et al. 2015) we fitted Generalized Linear Mixed Models (GLMM) with the individual identification code as a random term and treatment as the fixed term.

In addition to treatment type, we also considered the following covariates: presence of pups at the nest, season, male and female body mass, injection night, and recording night. Due to our small sample size, when modeling covariates we included a maximum of two fixed terms in one GLMM model (treatment type and one covariate). We first modeled the interaction term between treatment type and the one covariate. If the interaction term was not significant, the term was dropped. We also used the non-parametric Wilcoxon Rank Sum test for our comparison of USV types. We compared the median of the proportion of each USV type (1SV, 2SV, 3SV, 4SV, 5SV, 6SV, and barks) by treatment. We used a GLM to examine the relationship between USV types by context and treatment.

For the analysis of the spectral and temporal characteristics, we used factor analysis to extract principal component (PC) scores for the frequency variables (peak, minimum, maximum, start, and end). We only analyzed calls assigned to an individual male or female and the calls were analyzed separately. Following a previous protocol (Kalcounis-Rueppell et al. 2010) we generated a single PC score that represented the frequency variables using the first call in the 1-, 2-, 3- and 4SVs sequence. We dropped the 5SVs, 6SVs, and barks due to a small sample size (<4). The PC1 score accounted for 67% of the variation in acoustic variables for the male and 71% variation for the female. We fitted GLMM with ID as a random term and USV type and treatment as the fixed

terms. For both the male and female data, duration and bandwidth variables were in violation of normality and variances. We, therefore, fitted our models using Poisson family distribution. PC scores were normally distributed, and we used Gaussian distribution in our models. All data are represented using box plots. We used an alpha level of $p<0.05$ for the rejection criterion. All data were analyzed using R software (Version 3.2.2.)

Results

Time at the Nest

Overall, T-males spent 14% more time at the nest than C-males (GLMM Estimate 0.14±0.05, p=0.02; Figure 2.1). The T conditioning was amplified in the response to pups where T-males with pups spent 23% more time at the nest than T-males without pups (GLMM Estimate 0.21±0.04, $p<0.01$; Figure 2.1; Table 2.1). Male time at the nest was not statistically influenced by season (GLMM Estimate -0.09±0.06, p=0.17), body mass (GLMM Estimate -0.01±0.01, p=0.51), injection night (GLMM Estimate -0.09±0.08, p=0.26) or recording night (night two GLMM Estimate 0.04±0.04, p=0.39; night three GLMM Estimate 0.07±0.04, p=0.11; nights two and three are compared to night one after final injection for this type of analysis).

Females were not subjected to T-injections, but we examined their responses to their T-injected mates. T-females spent 15% less time at the nest than C-females (GLMM Estimate -0.16±0.06, p=0.02; Figure 2.1C). Females with pups in the nest spent more time at the nest than females without pups (pups GLMM Estimate 0.19±0.06, $p<0.01$; Figure 2.1D; Table 2.2). T-females without pups spent 19.4% less time at the nest than T-

females with pups, whereas C-females without pups spent 11.6% less time at the nest than C-females with pups (Table 2.2). T- and C-females spent more time at the nest on night three of recording compared to night one of recording (night three GLMM Estimate 0.10±0.04, $p<0.02$; Table 2.2). T-females spent 13% more time on night three than night one and control females spent 6% more time on night three than night one (Table 2.2). Females spent less time in the nest during season one than season two, independent of treatment (season one GLMM Estimate -0.15±0.06, $p=0.02$; Table 2.2). T-females spent 15.6% less time at nest than during season one than season two (Table 2.2). C- females spent 10.3% less time at the nest during season one than season two (Table 2.2). Female time at the nest was not influenced by body mass (GLMM Estimate 0.01±0.01, $p=0.24$) or mass difference between the female and the male (GLMM Estimate 0.01±0.01, $p=0.17$). We also examined within pair comparisons and found a significant negative effect of T and male time at the nest on female time at the nest (T GLMM Estimate -0.15±0.07, $p=0.04$, Time at the Nest GLMM Estimate 0.36±0.17, $p=0.04$; Table 2.3). T-females spent 5% less time at the nest than their mates, whereas, C-females spent 18% more time at the nest than their mates (Table 2.3).

USVs at the Nest

We recorded a total of 549 USVs across the 26 nest sites (T USVs=368, C USVs=181). We assigned context to 385 USVs from video; 157 USVs were produced when a mouse was alone (T USVs=101, C USVs=56), 119 USVs were produced when the mouse was <1m away from another mouse (T USVs=94, C USVs=25), and 109

USVs were produced when the mouse was >1m away from another mouse (T USVs=76, C USVs=33).

T-pairs produced twice as many total USVs at the nest than C-pairs (GLMM Estimate 0.87±0.40, p=0.04; Figure 2.2A). Across treatments, pairs also produced more USVs on night one than night three after the last injection, (night three GLMM Estimate -0.76±0.26, p=0.01; Figure 2.2B; night two GLMM Estimate -0.33±0.26, p=0.15). Both C- and T-pairs produced twice as many total USVs on recording night one than on recording night three. Total USVs recorded was not influenced by pups (GLMM Estimate -0.48±0.40, p=0.25), season (GLMM Estimate -0.68±0.40, p = 0.10), body mass (GLMM Estimate 0.01±0.06, p=0.92) or injection night (GLMM Estimate -0.85±0.64, p=0.20).

T-pairs produced proportionately more 4SVs at the nest than control pairs (W=43, p=0.03). All call types (1-, 2-, 3-, 4-, 5-, 6SV, and barks) were recorded for the male and the female at both C- and T-nests. There was no significant difference between treatments in the proportion of any other call type produced (1-, 2-, 3-, 5-, or 6SV; p>0.137). Mice were more likely to call when the mate (or any other individuals besides the potential presence of pups) was not at the nest (GLM Estimate -0.80±0.34, p=0.02), independent of treatment type (GLM Estimate -0.37±0.30, p=0.22). When alone (regardless of pup presence), T-pairs were more likely to produce 1-, 2-, and 4SVs ($1SV\chi2=9.95$, df=2, p<0.01; $2SV\chi2 = 9.59$, df=2, p<0.01; $4SV\chi2 = 9.48$, df=2, p<0.01).

T-males produced calls with a smaller mean bandwidth than C-males (GLM Estimate -0.11±0.04, p = 0.02). There was no significant difference between treatment

types in call duration (GLM Estimate -0.09±0.12, p=0.46; Table 2.6) or PC1 score (GLM Estimate 0.77±1.07, p=0.48; Table 2.6). For females, there was no significant difference between treatment types and any call characteristics, duration (GLM Estimate -0.09±0.21, p=0.68), bandwidth (GLM Estimate -0.11±0.07, p=0.88) or PC1 score (GLM Estimate 0.51±1.02, p=0.63). There was a negative correlation between the number of USVs produced and female time at the nest (t=-1.96, df=64, p=0.05).

Discussion

Under natural settings, we revealed that T-pulses administered to a male at the nest increased time allocation to that site in the male's territory, likely through the development of CPPs. We used T-injections in male California mice to mimic T-pulses that naturally occur after male-female or male-male encounters (Gleason et al. 2009), and in the laboratory can alter both time in a location (Zhao and Marler 2014; 2016) and social behaviors (Zhao et al. 2019; 2020; Pultorak et al. 2015; 2018). Using a classic laboratory paradigm for examining drug reinforcing effects, we showed that T-pulses induced that same effect in a field setting at the nest site. Male California mice experiencing three T-injections over three days spent more time at the nest and the conditioning was amplified at the location in the presence of offspring. It is unlikely that the increased time at the nest was caused by behavioral changes other than the T-induced CPPs found in the laboratory for several reasons discussed below. This effect may be unique to T-pulses as compared to manipulations of T-implants used to induce long term behavioral changes in the field (Ketterson et al. 1992; Marler and Moore 1989). T-pulse release in response to social interactions occurs in variety of different species, including

humans (Gleason et al. 2009; Fuxjager et al. 2017; Marler and Trainor 2019) and our results are consistent with laboratory observations in mice, rats, and hamsters showing that T-pulses have reinforcing/rewarding effects as described in the introduction (Zhao and Marler 2016; Arnedo et al. 2000; Zhao and Marler 2014; Wood 2004; Alexander et al. 1994).

T-pulses in response to male-male social challenges is a defining hallmark of Wingfield's Challenge hypothesis but also occur in males after male-female sexual interactions (Gleason et al. 2009). Male mice and rats exposed to an estrous female or her olfactory cues show a preference for the location at which the sexual encounter occurred (Camacho et al. 2004; Hughes et al. 1990; Mehrara and Baum 1990; Frye et al. 2001). This likely serves a reproductive function as the male may use previous experiences to increase the likelihood of encounters with an estrous female and potential mating opportunity (Gleason et al. 2009). Based on the knowledge of functions of T, one might predict that increased T causes males to allocate more time toward mate guarding, courting, or aggressively pursuing other males. In the current study, the change in spatial preference was most likely not a result of behavioral changes other than the T-induced CPPs. We found no evidence for increased mate guarding behavior since females spent more time away from the nest when males spent more time at the nest. Males were not increasing their sexual behavior by pursuing their mate or other females. We found that T-pairs spend more time apart at the nest than C-pairs suggesting that males were not pursuing their mates. Additionally, T-males did not increase vocalizations associated with courtship (sweeps) that unpaired males express at high levels towards unfamiliar females

(sweeps; Pultorak et al. 2015) that would be expected from courting an unfamiliar female. This lack of increased sexual behavior to unfamiliar females is also consistent with the finding that the administration of a single T-pulse caused unpaired but not paired male California mice to decrease sweep USVs to unfamiliar females in the lab (Pultorak et al. 2015), suggesting an internal fidelity mechanism. In the context of the nest site, there was no evidence that T increased aggression, as evidenced by a lack of increase in aggressive barks (Pultorak et al. 2018). We cannot, however, rule out that males may have been actively pushing females out of the nest as has been anecdotally observed in laboratory situations by either sex (Rieger & Marler, unpublished data). What then were males doing at the nest? In this case, the most likely explanation is increased paternal behavior in the form of increased nest defense or paternal care of pups based on evidence, described below, that T can increase paternal care in California mice in the laboratory. We suggest that T increases the focus on the reproductive or aggressive behaviors most relevant at that time depending on social and physical context for that specific species. This is consistent with previous findings that the ability to create T-induced conditioned location preferences is plastic and varies with social experience and current social and physical (familiar versus unfamiliar locations) contexts. It would be valuable in the future to examine the natural expression of T-pulses in males in response to social stimuli in the field. We can not rule out the alternative that males simply spend more time at the nest without altering paternal or direct pup defense behaviors.

In nature, T-pulse release following a sexual encounter most likely occurs at the nest site when females first approach a male that has established a territory, further T-

pulses likely occur when the female is in postpartum estrous (Gubernick and Nelson 1989) and T-induced CPPs could be the potential mechanism for increasing paternal care through increased preference for spending time at the nest. Therefore, T-induced CPPs at the nest could drive the male to spend more time at the nest where future sexual encounters are more likely to take place, which could also be a mechanism for maintaining paternal care. In addition, in California mice and other species, T promotes paternal care in males (Trainor and Marler 2002; Juana et al. 2009). If young are altricial, the offspring demand extensive paternal investment, especially if the young are considered exothermic and depend on adult presence to maintain their body temperature (Gubernick and Alberts 1987). In the California mouse, the presence of the father has a significant positive effect on offspring survival when temperatures are low and the parents have to forage, but there is no effect of father's presence in warm temperatures (Gubernick et al. 1993). The main limiting factor in California mouse reproduction is water availability (Nelson et al. 1995) and therefore, in nature, California mice breed during the cold rainy season and cease breeding during the dry summer months (Nelson et al. 1995). When reproduction occurs during harsh environmental conditions and offspring require constant care, there must be a balance in the time invested towards offspring maintenance and time spent towards foraging and resource defense. To achieve balance, biparental care is essential for facilitating offspring survival and maximizing reproductive success. We, therefore, propose that in some biparental species, T-induced CPPs could be a mechanism for keeping the male at the nest to care for the young while the female forages. Another selection pressure for T-induced paternal behavior may be

increased protectiveness of pups to prevent the high levels of conspecific infanticide found in rodents (Agrell et al. 1998). Van Anders et al. (2012) speculate that infant protection may be positively associated with T and more nurturing behaviors negatively associated with T. The reinforcing effects of T-pulses may function to allocate more time in the familiar environment and display behaviors that have direct fitness benefits.

Interestingly, changes in male allocation to tasks closer to the nest resulted in females spending more time away from the nest. This could occur through female preference/choice or because males aggressively pushed females out of the nest. We again do not think that there was an increase in aggression between the mated pair because of the absence of increased barks at the nest as has been shown in mated pairs that are stressed (such as through a fidelity challenge; Pultorak et al. 2018). An alternative is that females change their spatial preference to be away from the nest to compensate for the T-induced changes in male spatial preferences. In species that form pair-bonds where both members of a pair are engaged in offspring care and territory defense, the delegation of tasks is beneficial. In a wider variety of taxonomic groups, including insects, birds, fish, and mammals that engage in cooperative breeding, members of a pair or group often distribute tasks (Arnold et al. 2005; Ahern, et al. 2011; Rieger et al. 2019; Mathews 2002; Quinard and Cézilly 2012; Page et al. 2006; Rogers 1988). In the laboratory, when challenged with a potential intruder, California mouse pairs either coordinate their behavior in joint defense or employ labor division strategies, with the latter strategy potentially more likely to occur after pups are born (Rieger et al. 2019). In the California mouse, when the male is present but decreases paternal care due

to castration, the female will compensate for the mate's behavior by increasing the huddling of her pups (Trainor and Marler 2001). Species in which both members provide offspring care, such as in Midas cichlid, great tits, and voles, the presence of offspring increases the pairs' use of division of labor (Ahern et al. 2011; Rogers et al. 2018; Rogers 1988; Boucaud et al. 2016). This division of labor can have important long-term benefits for the persistence and survival of a social group (Arnold et al. 2005). In the case of California mice, if the male is spending more time in one location, such as the nest to care for offspring, then the female is adjusting her space use by allocating more time to other parts of the territory, such as foraging and/or defending the territory against potential intruders. Interestingly, T-induced CPPs regulate behaviors of other animals by influencing space use which in turn alters social interactions.

We also found that the same transient increases in T which induce CPPs also had long-term effects (>24 hours) on vocal communication by increasing the number of USVs produced and altering both the proportion of specific call types and call bandwidth. Our results are consistent with Timonin et al (2018) work that examined the effect of T on USVs in this species in the wild; T-pairs from both studies produced more total calls across the three nights with fewer USVs produced on night three. One difference between the studies is that Timonin et al (2018) found that T-pairs produced proportionately more 1-, 4- and 5SVs, whereas we only found a difference in 4SVs, however, we did find that T-pairs were more likely to produce 1-, 2-, and 4SVs when alone. The difference between the studies could be attributed to year, population densities, or sample size difference between the two studies. Anecdotally, densities were lower in the current study which

could alter social interactions. Contrary to our findings that T-pulses decreased bandwidth in SVs, in golden hamsters T-pulses increased bandwidth of the one and multi-note sudden frequency change calls that are produced in a sexual context (Fernández-Vargas 2017). Furthermore, males in other species that are administered T-implants produced produce frequency modulated trills (mating calls) with increased bandwidth, which females tended to prefer (Pasch et al. 2011; Pasch et al. 2011). However, it is important to consider the function of the call. While narrow bandwidth calls are less attractive to novel females, these calls can also transmit more effectively in the soundscape (Slabbekoorn 2013). This further supports the concept that in California mice, call production at the nest is most likely directed toward a member of a pair (Briggs and Kalcounis-Rueppell 2011) and not toward pups because in the current study offspring presence did not influence call production. In the wild, California mouse SVs travel an average of 3.12m and a maximum of 7m (Timonin et al. 2018). If the male is altering spectral characteristics of SV calls, it is unlikely for the function of attracting a new mate, but instead to get the attention of his own mate and attract her back to the nest. It is also possible, that the calls serve a dual function, for mate attraction and as territorial advertisement. We argue that territorial advertisement is unlikely the primary function of the calls because members of a pair vocalize more when alone than in the presence of mate. The production of narrow bandwidth calls more often increases the likelihood of signal transmission of the vocal signal across a large area (Barber et al. 2010). The increased calling behavior when the pair is apart but decreased calling behavior when the pair is together could be the mechanism for reducing extra-pair copulation and

maintaining sexual fidelity. We found that mice produce twice as many USVs on night one than night three. Our data show that call production at the nest negatively corresponds to the time allocation by the female at the nest. Additionally, increased calling behavior when the pair is apart and decreased calling behavior when the pair is together could simply be a way to keep contact between members of a pair (Briggs and Kalcounis-Rueppell 2011).

In summary, this is the first field study that has shown a natural function of transient T-pulses for CPPs. T-pulses naturally occur in a variety of different species, including humans, and our results are consistent with other research in which T-pulses have rewarding properties and can condition animals to the physical location in which the hormone release occurred (Arnedo et al. 2000; Frye et al. 2001). This change in the allocation of time spent in the physical environments also leads to changes in social interactions and call productions during those interactions. Here, we provide evidence that in the wild, T-pulses may be an important internal stimulus that can control spatial preference and vocalization plasticity of the male and his mate. T-pulses can have important long-lasting effects on paternal and partner behavior which influence family dynamics. Overall, T-pulses induced long-term spatial and vocal changes that impacted the time management and potentially reproductive fitness of individuals. There could be an adaptive significance for a co-option mechanism that allows such a close association between mating and paternal behavior. In California mice, T related behavioral response is context dependent, occurs in both sexes, and further suggests that this species is a good model for testing hypotheses related to monogamy and family dynamics.

Ethical Statement

All animal care and use guidelines were followed and research protocols for this study were approved by the University of North Carolina at Greensboro and University of Wisconsin-Madison Institutional Animal Care and Use Committees (IACUC; UNCG 12-004 and UWM L005047-A01) and by California Department of Fish and Wildlife under Scientific Collection Permits (SC-9663 and SC-13190).

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Figure 2.1. California Mouse (*Peromyscus califronicus*) Time at the Nest by Treatment Type. **A)** Male time at the nest by treatment type (T = 10 and C=11). T injected males spent 14% more than C-males (GLMM Estimate 0.14±0.05, p = 0.02). **B)** Male time at the nest by treatment type and pups (T with pups, n=6; T without pups, n=4; C with pups, n=6; C without pups, n=5). T-males with pups spent 15% more time at the nest than C-male with pups, and T-males without pups spent 12% more time at the nest than C-males without pups (treatment GLMM Estimate 0.13±0.03, p >0.01; pups GLMM Estimate 0.21±0.03, p >0.01). **C)** Female time at the nest by treatment type (Testosterone = 14 and Control=9). T-females spent 15.8% less time at the nest C-females (GLMM Estimate -0.16±0.06, p = 0.02). **D)** Female time at the nest by treatment type and pups (T with pups, n=6; T without pups, n=8; C with pups, n=6; C without pups, n=3). There was a significant effect of pups on female time at the nest (pups GLMM Estimate 0.54±0.24, p >0.04), but there was no treatment effect (treatment GLMM Estimate -0.05±0.25, p >0.84). Control females with pups spent 11.6% more time at the nest than female without pups. Testosterone females with pups spent 19.4% more time at the next than females without pups. Black dots represent outliers.

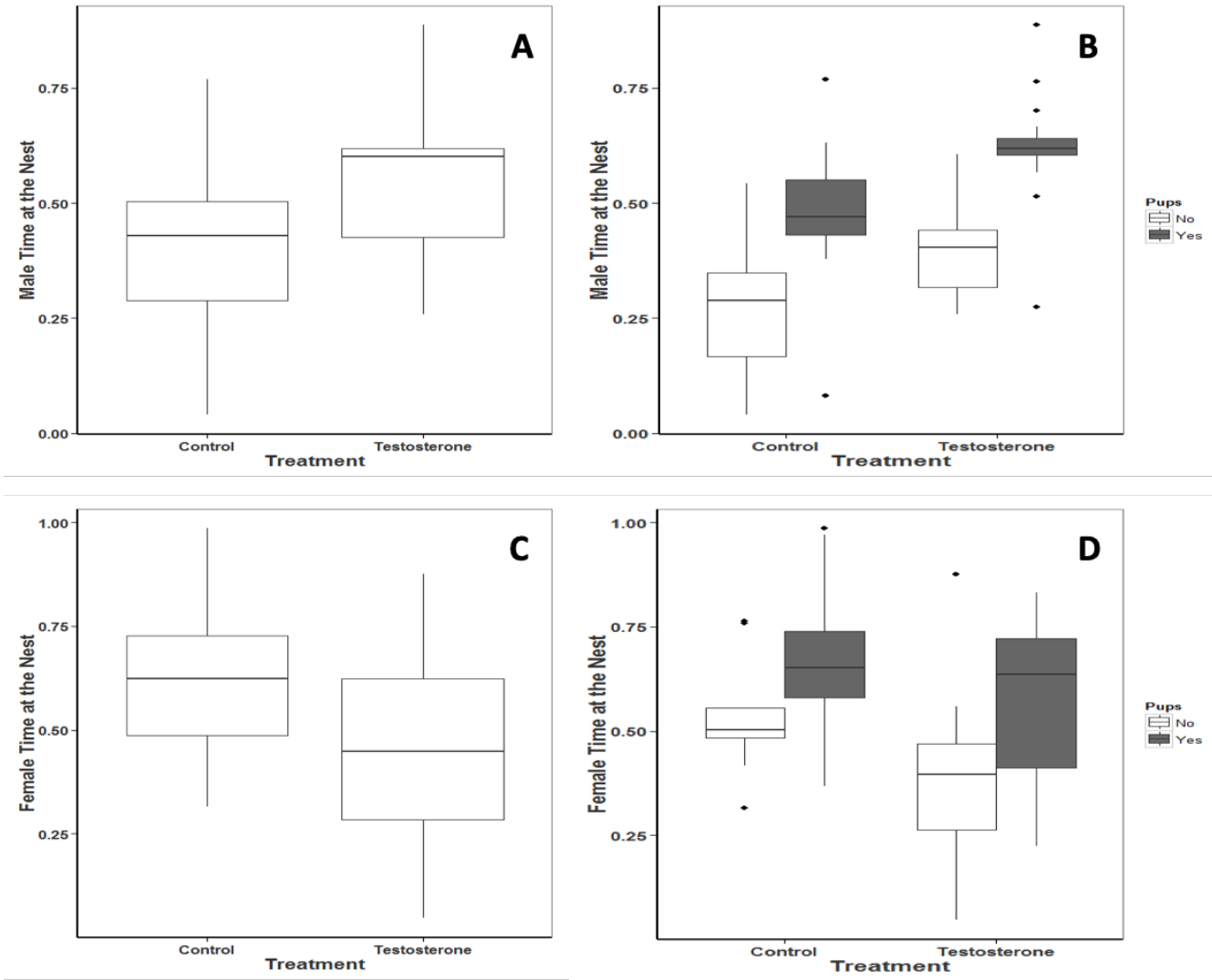
A
B
C
D
Male Time at the Nest
Female Time at the Nest
0.00
0.25
0.50
0.75
1.00
Control
Testosterone
Treatment
Pups
No
Yes

Figure 2.2 Total USVs Produced at the California Mouse (*Peromyscus califronicus*) Nest. A) across the three nights and B) by night (Testosterone=14 and Control=12). Male-female dyads produced more total USVs at testosterone nests than control nest (GLMM Estimate 0.87±0.40, p=0.04). Both treatment type and night three post injection significantly influenced total USVs produced at the nest (treatment GLMM Estimate 0.91±0.42, p=0.04; night three GLMM Estimate -0.76±0.26, p <0.01). Black dots represent outliers.

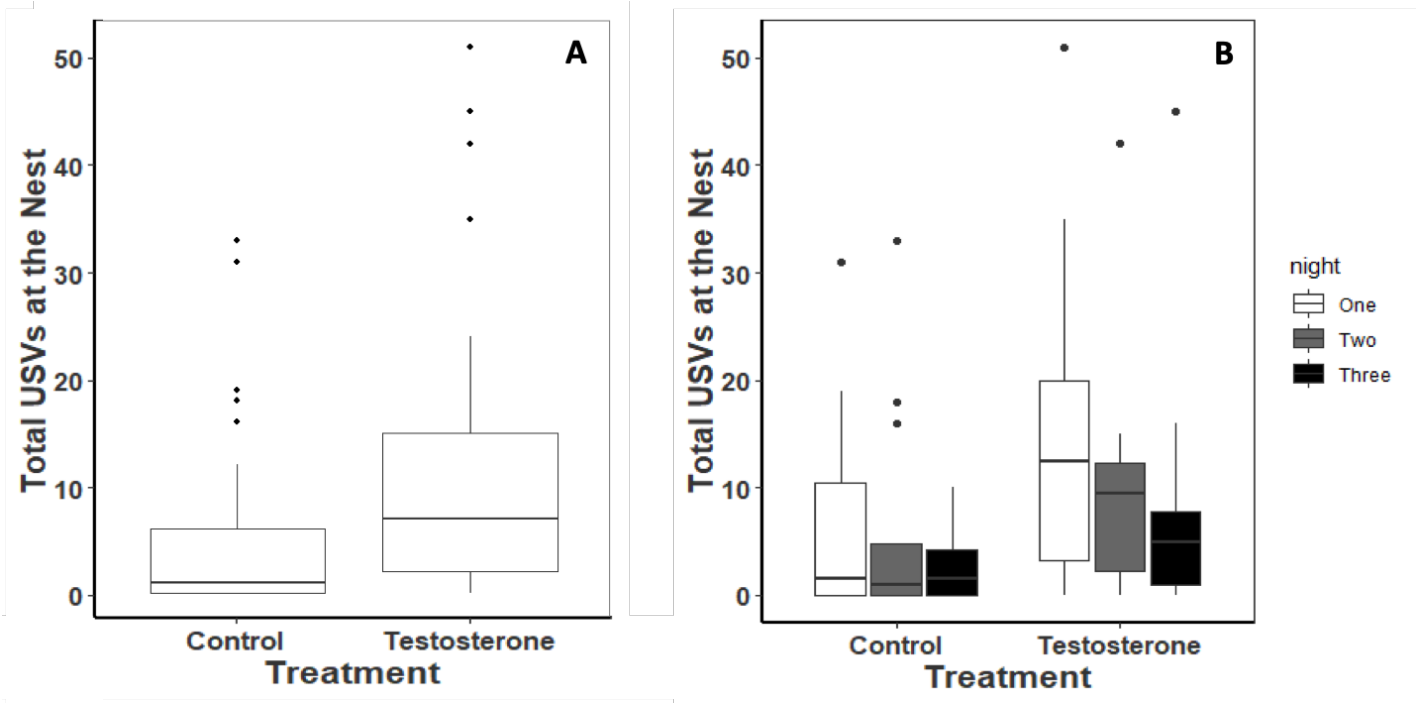

Table 2.1. Descriptive Statistics on California Mouse (*Peromyscus californicus*) Male Time at the Nest, Presence of Pups at the Nest, Season, Body Mass, Number of Nights Required to Administer Three Injections, and Recording Night After the Last Injection by Treatment Type. Each *Peromyscus californicus* male received three testosterone (T=10) or saline (C=11) injection at the nest, after the final inject, we recorded time spent at the nest for three consecutive nights.

		n	Mean	SE	Median	Min	Max
Time at the Nest	C	33	0.39	0.03	0.43	0.04	0.77
	T	30	0.53	0.03	0.6	0.26	0.89
Time at the Nest and Pups	C with Pups	18	0.48	0.03	0.47	0.08	0.77
	T with Pups	18	0.63	0.03	0.62	0.27	0.89
	C without Pups	15	0.28	0.04	0.29	0.04	0.54
	T without Pups	12	0.4	0.03	0.4	0.26	0.61
Time at the Nest and Season	C Season One	21	0.344	0.04	0.313	0.041	0.769
	T Season One	12	0.549	0.054	0.587	0.258	0.886
	C Season Two	12	0.476	0.034	0.491	0.178	0.631
	T Season Two	18	0.523	0.031	0.601	0.281	0.7
Body Mass	C	33	39.05	0.707	40	30.5	43
	T	30	38.7	0.37	38.75	36	42
Injection Administration	C	11	3.273	0.141	3	3	4
	T	10	3.1	0.1	3	3	4
Time at the Nest and Recording Night	C - Night One	11	0.366	0.058	0.414	0.134	0.631
	T - Night One	10	0.49	0.052	0.569	0.258	0.666
	C - Night Two	11	0.375	0.045	0.429	0.081	0.552
	T - Night Two	10	0.555	0.044	0.604	0.317	0.763
	C - Night Three	11	0.435	0.056	0.435	0.041	0.769
	T - Night Three	10	0.554	0.053	0.564	0.317	0.886

Table 2.2. Descriptive Statistics on California Mouse (*Peromyscus californicus*) Time at the Nest, Presence of Pups at the Nest, Season, Body Mass, Number of Nights Required to Administer Three Injections, and Recording Night After the Last Injection by Treatment Type. Each female was paired with a male who received three testosterone (T=14) or saline (C=9) injection at the nest, after the final inject, we recorded time spent at the nest for three consecutive nights.

		n	Mean	SE	Median	Min	Max
Time at the Nest	C	27	0.614	0.032	0.624	0.316	0.985
	T	42	0.456	0.033	0.448	0.048	0.875
Time at the Nest and Pups	C with Pups	18	0.653	0.039	0.651	0.369	0.985
	T with Pups	18	0.567	0.048	0.635	0.225	0.831
	C without Pups	9	0.537	0.049	0.502	0.316	0.763
	T without Pups	24	0.373	0.037	0.397	0.048	0.875
Time at the Nest and Season	C Season One	12	0.557	0.034	0.526	0.417	0.771
	T Season One	24	0.39	0.043	0.338	0.048	0.875
	C Season Two	15	0.66	0.048	0.679	0.316	0.985
	T Season Two	18	0.546	0.044	0.501	0.108	0.832
Body Mass	C	27	43.06	0.85	43	34.5	51
	T	42	41.93	0.752	41	35	51
Time at the Nest and Recording Night	C - Night One	9	0.566	0.057	0.555	0.316	0.771
	T - Night One	14	0.409	0.065	0.382	0.048	0.832
	C - Night Two	9	0.649	0.054	0.625	0.452	0.971
	T - Night Two	14	0.425	0.051	0.42	0.166	0.875
	C - Night Three	9	0.628	0.057	0.624	0.417	0.985
	T - Night Three	14	0.536	0.051	0.509	0.225	0.801

Table 2.3. Descriptive Statistics on California Mouse (*Peromyscus californicus*) Pair Time at the Nest. Each female was paired with a male who received three testosterone (T=10) or saline (C=8) injection at the nest, after the final inject, we recorded time spent at the nest for three consecutive nights.

		n	Mean	SE	Median	Min	Max
Pair Time at the Nest	C Female	24	0.610	0.036	0.580	0.316	0.985
	T Female	30	0.495	0.036	0.470	0.108	0.832
	C Male	24	0.435	0.025	0.440	0.147	0.631
	T Male	30	0.533	0.028	0.601	0.258	0.886

Table 2.4. Descriptive Statistics on California mouse (*Peromyscus californicus*) USVs Produced Per Minute and Per Night at the Nest, Presence of Pups at the Nest, Season, Body Mass, Number of Nights Required to Administer Three Injections, and Recording Night After the Last Injection by Treatment Type. Each male received three testosterone (T=14) or saline (C=12) injection at the nest, after the final inject, we recorded USVs at the nest for three consecutive nights.

USVs at the Nest		n	Mean	SE	Median	Min	Max
USVs	C	36	5.083	1.421	1	0	33
	T	42	10.81	1.942	7	0	51
USVs and Pups	C with Pups	21	3.381	1.096	1	0	18
	T with Pups	15	10.6	4.154	3	0	51
	C without Pups	15	7.467	3.003	1	0	33
	T without Pups	27	10.926	2.023	8	0	42
USVs and Season	C Spring	21	6.643	2.189	1	0	33
	T Spring	27	13.296	2.748	8	0	51
	C Fall	15	3.2	1.448	0	0	18
	T Fall	15	6.333	1.866	5	0	24
Body Mass	C	36	39.21	0.653	40	30.5	43
	T	42	38.5	0.432	38	34	45
Injection Administration	C	36	3.333	0.08	3	3	4
	T	42	3	0	3	3	3
USVs and Recording Night	C - Night One	12	6.75	2.834	1.5	0	31
	T - Night One	12	15.071	3.081	12	0	51
	C - Night Two	12	5.916	0.933	1	0	33
	T - Night Two	14	9.857	3.959	9.5	0	42
	C - Night Three	14	2.583	2.84	1.5	0	10
	T - Night Three	14	7.5	3.108	5	0	45

Table 2.5. Descriptive Statistics and Results from the Wilcoxon Rank Sum Test for the Comparison of USV Proportion by Type and Treatment Produced at the Nest. Each *Peromyscus californicus* male received three T (n=14) or C (n=12) injections at the nest, after the final inject, we recorded USVs at the nest for three consecutive nights. Alpha values of p <0.05 are in **bold**.

	USV Proportions at the Nest															
	C							T								
Call Type	**n**	**Mean**	**SE**	**sd**	**Median**	**Min**	**Max**	**n**	**Mean**	**SE**	**sd**	**Median**	**Min**	**Max**	**W**	**p**
1SV	12	0.205	0.086	0.299	0.078	0	1	14	0.287	0.078	0.293	0.202	0.029	1	59.5	0.215
2SV	12	0.149	0.064	0.221	0.019	0	0.667	14	0.215	0.037	0.138	0.225	0	0.467	55	0.137
3SV	12	0.251	0.094	0.326	0.158	0	1	14	0.214	0.045	0.168	0.2	0	0.458	73.5	0.602
4SV	12	0.058	0.033	0.114	0	0	0.385	14	0.127	0.024	0.089	0.137	0	0.257	43	**0.03**
5SV	12	0.029	0.019	0.065	0	0	0.194	14	0.014	0.005	0.018	0	0	0.047	73	0.525
6SV	12	0.003	0.003	0.009	0	0	0.032	14	0.005	0.004	0.014	0	0	0.043	79	0.407
BARK	12	0.055	0.052	0.18	0	0	0.625	14	0.139	0.072	0.268	0	0	0.926	61	0.157

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CHAPTER III

T-PULSES ALTER THE SPATIAL PREFERENCE AND CALL DURATION AT THE TERRITORY BOUNDARY OF THE FREE-LIVING AND MONOGAMOUS CALIFORNIA MOUSE (*PEROMYSCUS CALIFORNICUS*)

This Chapter is coauthored by Radmila Petric, Matina Kalcounis-Rueppell and Catherine Marler

Abstract

Individuals must adjust the allocation of time and energy in response to different social challenges. Transient testosterone increases (T-pulse) occur after social interactions and rapidly modulate male behaviors. In nature, at the nest site, T-pulses alter spatial preference, social interactions and call production but how do T-pulses alter behavior in other context such as the territory boundary. In the context of social interactions at the territory boundary, T-pulses may reinforce learning associated with aggression in the form of conditioned placed preference (CPP). We hypothesized that under complex natural conditions, the reinforcing effects of T-pulses will induce CPPs at the territory boundary and promote territory defense and establishment with vocalizations associated with aggression. We tested our hypothesis in the field using the monogamous and territorial California mouse (*Peromyscus californicus*). We found that three exogenously administered T-pulses over five days alter the spatial preference of the male by decreasing time allocation at the original territory boundary and promoting

territory/home range expansion, which in turn decreases time allocation at the boundary by the female in the pair. We also found that T males produce shorter duration calls and calls were more likely to be produced when two mice are less than 1 meter apart. Shorter calls have previously been associated with greater levels of aggression and inversely associated with affiliative behavior with the pair mate. Overall, in the complex field setting, CPP induction on California mouse is context dependent. The variation of T in inducting CPPs at the nest but not at the territory boundary suggests there is plasticity in the sensitivity to T based on the physical location. Environmental location dictates the effects of T-injections suggesting the effects of T-pulses are highly context dependent. We suggest that these T-pulses, therefore, act differently from long term testosterone implants that induce long term stable changes.

Introduction

The androgen hormone testosterone is responsible for the activation of reproductively related behaviors including territoriality (Wingfield 2005). In nature, male-male agonistic encounters most often occur near territory boundaries which are followed by a hormonal response that results in a transient testosterone increase (T-pulse) (Gleason et al. 2009). While seasonal changes in circulating T levels increase aggression, T-pulses influence social behaviors (Gleason et al. 2009). The proposed function of T-pulses is to rapidly modulate male behaviors and in the context of social interactions at the territory boundary, T-pulses may reinforce learning associated with aggression (Marler et al. 2005). The challenge hypothesis states that when the status of the individual is threatened during social interaction, testosterone is elevated to facilitate approach and

dominance seeking behaviors (Gleason et al. 2009). T-pulse release is the proposed mechanism for rapidly initiating the behavioral response during challenging social interactions.

T-pulse release after winning a male-male aggressive encounter can influence future behavior at the location at which the aggressive interactions occurred (Booth et al. 1989; Farrell and Wilczynski 2006). Testosterone along with previous experience and physical environment play an important role in the final formation of the winner effect (Martínez et al. 1995). Winning aggressive encounters in a specific location leads to the ability of an individual to win future aggressive encounters in that location following previous victories (Dugatkin 2001). The winner effect is observed in a variety of taxonomic groups including birds (Drummond and Canales 1998), reptiles (Schuett 1997), fish (Chase, Bartolomeo, and Dugatkin 1994), and mammals (Oyegbile and Marler 2005) and the proposed function is to contribute to the establishment and adjustment of a territory.

T-pulses have rewarding properties and lead to the development of a location preference at which the hormone release occurred in the form of conditioned placed preferences (CPPs)(Arnedo et al. 2000; Frye et al. 2001). Males that previously won a dispute can form CPPs for the encounter location (Martínez et al. 1995). A series of laboratory studies in the California mouse (*Peromyscus californicus*), found that CPP induced by T are context dependent. In pair-ponded laboratory males, T-pulses induce CPPs only for the familiar environment containing the nest site associated with the female mate (Zhao and Marler 2014; 2016). In unpaired laboratory males, T-pulses

induce the formation of a CPP for the unfamiliar environment which most likely promotes territory formation (Zhao and Marler 2014; 2016). Furthermore, in free-living and pair-bonded males, T-pulses induce CPPs for the nest location and alter social interactions at the nest between members of a pair (Petric et al. unpublished). Furthermore, a single T-pulse alters social preference from a receptive female to an interaction with a potential competitor in unpaired males (Zhao et al. 2019). Laboratory studies suggest that CPP formation for the territory boundary may not occur but instead induce males to move farther and seek challenges with other males (Zhao et al. 2019). Context can be critical for hormonal effects on behavior. Such plasticity was foreshadowed by the finding that male T release can occur in response to both male-male and male-female encounters. The variation in the effects of the T pulses on location preferences is intriguing because it strongly supports the perspective that rewarding/reinforcing properties of a compound can vary among individuals depending on experience and context.

T-pulses can regulate vocalization output both directly and indirectly. In various vertebrate species, T-pulses can influence vocal behavior directly through multiple neural network pathways associated with vocalization production (Remage-Healey and Bass 2004). Within minutes of a T-pulse, males increase the duration of individual calls and call rate (Remage-Healey and Bass 2004; 2006). Interestingly, context is also important when considering T-pulse effect on call production. In the California mouse, a single T-pulse decreases courtship call production of pair-bonded but not unpaired males in response to a novel female (Pultorak et al. 2015). This suggests that T-pulses are

potentially not inducting paired males to seek other females for extrapair copulation. Furthermore, T-pulses can also alter call production indirectly. The development of location based CPPs changes social interactions and therefore alters vocalization production associated with the social interactions. For example, in the wild a pair-bonded California mouse male treated with T spent more time at the nest which causes his female mate to spend significantly less time at the nest (Petric et al. unpublished). In response to the female absence, more vocalizations are produced, and a specific type of vocalization is produced when the mouse is alone (Petric et al. unpublished).

In nature with multiple competing abiotic and biotic stimuli, an individual must adjust the allocation of time and energy in response to different social challenges (Hurley and Kalcounis-Rueppell 2018). At the nest site of a free-living and paired California mouse, T-pulses alter spatial preference, social interactions and call production (Petric unpublished). However, there is a lack of knowledge on the effect of T-pulses on behavior in other context such as the territory boundary. Here, we investigated if rapid changes in T at the territory boundary will induce behavioral changes by the development of a learning associated location preference. We hypothesized that under complex natural conditions, the reinforcing effects of T-pulses will induce CPPs at the territory boundary and promote territory defense and establishment with vocalizations associated with aggression. We predict that if pair-bonded males receive T injections at the territory boundary, then T males will alter spatial preferences and spend more time at the boundary. Furthermore, if a female responds to the altered behavior of her mate, then the female within the pair will alter her spatial behavior. Finally, if T injected males spend

more time at the territory boundary, then the change in spatial behavior will alter social interactions toward more aggression accompanied by the production of associated calls.

We tested our hypothesis using the monogamous and territorial California mouse (*Peromyscus californicus*). In this species, males balance mate attendance, offspring care, and territory defense all at the same time (Gleason et al. 2009). The California mouse produces ultrasonic vocalizations (USVs) in various social setting both in the laboratory and the wild (Briggs and Kalcounis-Rueppell 2011; Kalcounis-Rueppell et al. 2010; Kalcounis-Rueppell et al. 2006; Kalcounis-Rueppell et al. 2018; Pultorak et al. 2015; Pultorak et al. 2017; 2018). The sustained vocalizations (SVs) are the most common type of USV produced (Briggs and Kalcounis-Rueppell 2011; Kalcounis-Rueppell et al. 2018; Pultorak et al. 2015) and the proposed function of the SVs is to maintain the pair-bond between members of a pair (Briggs and Kalcounis-Rueppell 2011). Agonistic interactions are often associated with a specific type of USV know as a bark (Kalcounis-Rueppell et al. 2018; Pultorak et al. 2018) as well as short-duration SV calls (Rieger and Marler 2018).

Methods

Our field work was conducted at the Hastings Natural History Reservation (HNHR), Carmel Valley, California, USA from October 2017 to June 2018. The experiment took place at a study site at which California mice were previously studies (Kalcounis-Rüppell and Millar 2002; Kalcounis-Rueppell, et al. 2006; Briggs and Kalcounis-Rueppell 2011; Kalcounis-Rueppell and Petric 2013). Using previously established live-trapping techniques and pair assignment techniques (Timonin et al. 2018)

we only selected paired mice for our study. To track the individuals to their nest and territory boundary, each mouse within the pair was collared with a 0.55g M1450 transmitter (Advanced Telemetry System [ATS], Isanti, MN, USA) (Timonin et al. 2018).

For each male, we determined the territory boundaries by generating home range estimates of individual mice based on trapping data. Home ranges were manually estimated using study grid maps and individual mouse recapture information. The estimates were based on at least three trapping events (range three to ten recaptures). For each mouse we selected a seven-meter section of the territory boundary to use as the study site based on the following criteria 1) the site was a minimum of ten meters away from the nest, 2) there was at least one pair-bonded neighbor in the adjacent territory boundary, and 3) feasibility of assembling the remote sensing equipment (automated radio telemetry, audio recording and thermal imaging; described below). We also used R4500S DCC receiver/datalogger and a Yagi antenna for stationary and manual tracking of the collared mouse at the specific section of interest in the territory. We did this to ensure that our home range estimates accurately represented the territory range of the target mouse.

We tagged a total of 331 *P. californicus* that were recaptured approximately 3,531 times from October 2017 to May 2018. We radio-collared 255 resident mice and identified 72 potential pairs for our experiment. Of these 72 mated pairs, we injected and followed 30 mated males at the territory [15 testosterone (T), and 15 control (C)].

Treatment

Males were randomly assigned to either testosterone (T; T-males) or saline (control, C; C-males) group. At the selected territory boundary, we placed 16 numbered Longworth traps in 2x8 configurations one meter apart that covered the target pairs territory but could have overlapped with an adjacent territory. Upon capture in one of the 16 Longworth traps, the male received the assigned injection and was returned to the same trap of capture for one hour, and we refer to this trap as the target trap. We used the target trap for the conditioning of the mouse because the male chose and willingly entered that specific trap. The one-hour time period was selected to allow for treatment to take effect at the site of injection. After the hour, the male was released from the trap. For each subsequent injection, the male was returned and held in the target trap of capture from the first night. We also measure the distance between the target trap to that of his nest and refer to the variable as the distance to nest. The T injection dose was approximately 36ug/kg, a dose that is three to five times higher from the baseline T levels, therefore mimicking a transient increase in T levels (Trainor et al. 2004). Each male received 0.1 ml of the injectate which produces a CPP in California mouse males. Regardless of body mass, each male received the same dose therefore we included body mass as an independent variable in our statistical analysis. Males received three subcutaneous injections over a period of five days with a maximum of two days between injections. We recorded injection night which represents the number of nights required to administer the three injections (three, four, or five nights) and included the variable in our statistical analysis.

Automated Radio Telemetry

We monitored the amount of time radio-collared mice spent at the territory boundary using two R4500S DCC receiver/dataloggers (Advanced Telemetry System [ATS], Isanti, MN, USA). Dataloggers were connected to an antenna and programmed to detect one transmitter frequency, one for the male and the other for the female. The antennas were placed at the target trap. To account for differences in night length over the recording period, we totaled the number of minutes the mouse spent in the recording area from sunset to sunrise divided by the total number of minutes in the night to get a proportion of time spent in the recording area. We used proportions for our spatial analysis and refer to the variable as the time at the boundary.

Audio Recording

To assess the number and type of USVs produced, we used five ultrasonic microphones (Emkay FG Series from Avisoft Bioacoustics, Berlin, Germany). One microphone was placed next to the target trap at which the T injections were administered, and the other four microphones were placed two meters away from the target trap, 90 degrees apart from each other. The microphones were triggered, and files were recorded when sonic and ultrasonic sounds were detected. All files recorded were examined using Avisoft SAS Lab Pro (Avisoft Bioacoustics). Files with mouse USVs were assigned to individuals by matching the time of the vocalization to the transmitter signal strength to determined which of the radio-collared mice produced the vocalization (as described in Briggs and Kalcounis-Rueppell 2011). All USVs were counted and classified as 1SV, 2SV, 3SV, 4SV, 5SV, 6SVs or Bark. We counted the number of USVs

recorded per night and refer to the variable as total USVs. Lastly, we assessed the proportion of USVs types (1-, 2-, 3-, 4-, 5-, 6SVs and Bark) produced by treatment type. We totaled each USV type per site and divided it by the total number of USVs produced at territory boundary. This allowed us to determine if the proportion of a specific type of USV differed between treatments. We also measured duration, bandwidth, and frequency at five points (start, end, minimum, maximum, and frequency at maximum amplitude) of the call. For each USV we generated a spectrogram with a 512 FFT (Fast Fourier Transform), and a 100-frame size with Hamming window using SAS Lab Pro. The acoustic recording system was set-up at 30 territory sites (T=15, C=15) and we record USVs at all sites. Our final dataset consists of 90 recording nights from 30 territory sites.

Thermal Imaging

We assigned context to USVs by using a thermal imaging lens (Photon 320 14.25 mm; Flir/Core By Indigo) at the territory boundary to capture the full view of the target trap with a two-meter radius surrounding the trap. The lens was connected to a JVC Everio HDD camcorder, which recorded continuously throughout the night. We watched video footage in three-minute increments surrounding each call (a minute before the call, during the call, and the a minute after the call) to determine the number of mice on the screen and behaviors of those mice. For each study site, we measured and marked a two-meter diameter surrounding the target trap. The two-meter ruler was visible on the thermal imaging screen which we used to assign context to each USV. Context was classified as alone if there was only one mouse present, close if mice were <1 meter apart, or far if mice were >1 meter apart. We assessed the types of USVs (1-, 2-, 3-, 4-,

5-, 6SVs and Bark) produced by context (alone, closer or far) and treatment type. We totaled each USV type by context and treatment.

The automated radio telemetry, audio recording, and thermal imaging were set to record for three consecutive nights from sunset to sunrise. In our statistical analysis, we included night post-injection (night one, two, and three) and refer to the variable as recording night. We treated data collected over one night as a sample unit. The telemetry equipment was set-up at 30 territory sites. Due to equipment failure, we did not record data at one control site. Our final telemetry dataset consisted of 87 recording nights from 29 territory sites (T=15, C=14). Females within the pair were not injected, however, we refer to females whose mate received the treatment injections as either testosterone (T-females) or control females (C-females). Due to equipment failure, we did not record female time at the boundary for two control sites. Our final dataset consists of 84 recording nights from 28 territory sites (T=15, C=13). We did not have matching pair time at the boundary for three control pairs. Our final dataset consists of 81 recording nights from 27 territory sites (T=15; T-pairs, C=12; C-pairs).

Boundary Expansion

We used the live trapping data to measure the distance injected males traveled outside their territory. This travel distance allowed us to assess if and how far territory injected males were moving from their territory. We measured the following four variables 1) distance - distance in meters between the territory injection site to the first trapping location after the 3rd injection; 2) capture night - the number of nights required to capture the male after the 3rd injection; 3) new station - was the mouse captured in a

new location post-treatment; and 4) travel distance post-treatment - distance in meters between capture stations pre-treatment to capture stations post-treatment. For the male to be considered, the male had to be captured at least three times pre-treatment (not accounting the trapping captures for injection administration) as well as three times post --treatment. If the male was not captured in any other station post-treatment when compared to the pre-treatment stations, the travel distance was assigned as "0". If the male was captured at a new station post-treatment, we measured the distance from the new station to the closest station pre-treatment. The live-trapping dataset for determining male travel distance outside the original territory was assessed for 28 males (T=13 and C=15). Two testosterone males were excluded from the analysis because the minimum capture data post-treatment was not available for them.

Statistical Analyses

Data were checked for normality and equality of variances. Female time at the boundary was normally distributed and therefore we fitted Gaussian family distribution. Male time at the boundary and total USVs was in violation of normality and transformations could not normalize the data. Male time data ranged between 0 and 1 thus we fit our models using Quasibinomial family distribution and for total USVs we fit our models using Poisson family distribution. We used a General Linear Model (GLM) with time at the boundary and total USVs as the dependent variables and individual identification code (ID) as the independent variable to determine if there were individual differences. We found that some individuals spent more time or produced more USVs at the territory site than others independent of treatment type. Therefore, for the rest of the

analysis, we included ID to account for individual differences. Using the package lem4 (Bates et al. 2015) we fitted Generalized Linear Mixed Models (GLMM) with ID as a random term and treatment as the fixed term. We also considered the following covariates: presence of pups at the nest, season, body mass, injection administration, recording night and distance to nest. When modeling covariates, due to our sample size, we included a maximum of two fixed terms in one GLMM model (treatment type and one covariate). We first modeled the interaction term between treatment type and the one covariate. If the interaction term was not significant, we would drop the interaction term.

We used factor analysis to extract principal component (PC) scores for call characteristics. The male and female calls were analyzed separately, and we only analyzed 1-, 2-, 3- and 4SVs. The sample size for 6- to 9SVs and the Barks were less than three and could not be included in the analysis. We generated a single PC score (frequency variable) from the first call in the sequence of analyzed calls. The PC1 score accounted for 72% of the acoustic variation for the male and 74% variation for the female. We used the PC score and the original duration and bandwidth variables in the final analysis. We fitted GLMM with ID as a random term and USV type and treatment as the fixed terms. We fitted a Quasibinomial distribution because the data were in violation of normality and variances. We used Gaussian distribution for PC scores because data were normal.

We also used the non-parametric Wilcoxon Rank Sum test for our comparison of USV types. We compared the median proportion of each UVS type (1SV, 2SV, 3SV, 4SV, 5SV, 6SV, and Barks) by treatment. Finally, we performed Chi-Squared Test of

Independence to determine if there was a significant relationship between USV types and context by treatment. All data are represented using box plots to show skewness and outliers in the data. We used an alpha level for the rejection criterion of $p<0.05$. All data were analyzed using R software version 3.2.2.

Results

Time at the Boundary

T-males spent 7% less time at the boundary than C-males (GLMM Estimate -0.81±0.33, p=0.02; Table 3.1; Figure 3.1). T-males spent 2.4% less time at the boundary on night three than night one and control males spend 1.8% more time on night three than night one (Testosterone: Recording night three GLMM Estimate -0.58±0.23, p=0.02; Table 2.1; Figure 3.3). Male time at the boundary was not influenced by pups (GLMM Estimate -0.33±0.37, p=0.42; Table 3.1), season (GLMM Estimate -0.35±0.37, p=0.36; Table 3.1), body mass (GLMM Estimate -0.05±0.05, p=0.34; Table 3.1), injection administration (GLMM Estimate 0.11±0.29, p=0.72; Table 3.1) or distance to nest (GLMM Estimate -0.02±0.02, p=0.29; Table 3.1).

Travel distance post injection was different between control and testosterone injected males (W=4.51, df=1, p=0.03). Post-treatment T-males were captured at stations that were on average 13.4 meters further from the pre-treatment stations than C-males (Figure 3.2). There was no treatment effect on distance (W=0.85, df =1, p=0.36), capture night (W=0.09, df=1, p=0.77) and new station (W=1.69, df=1, p=0.19).

T-females spent 5% less time at the boundary than control females (GLMM Estimate -0.55±0.27, p=0.05; Table 3.2; Figure 3.4). Independent of treatment type,

females with pups in the nest spent less time at the boundary than females without pups (pups GLMM Estimate -0.55±0.24, p=0.03; Table 3.2; Figure 3.5). T-females with pups spent 4.4% less time at the boundary than C-females with pups (Table 3.2). T-females without pups spent 6.4% less time at the boundary than C-females without pups (Table 3.2). Females spent more time at the boundary during the first season than the second season (pups GLMM Estimate -0.55±0.24, p=0.03; Table 3.2). In the first season, T-females spent 3.7% more time at the boundary than during the second season. C-females spent 7.7% more time during the first season than the second season. T- and C- females spent more time at the boundary on night one than night three (night three GLMM Estimate -0.35±0.14, p =0.01; Table 3.2; Figure 3.6). T-females spent 4.2% more time on night one than night three and C-females spent 1.8% more time on night one than night three (Table 3.2). There was no body mass (GLMM Estimate -0.05±0.05, p = 0.34; Table 3.2) effect on time spent at the territory.

There was a treatment and mate time effect on the female time at the boundary (testosterone GLMM Estimate -0.87±0.34, p = 0.02, mate time GLMM Estimate 2.07±0.99, p = 0.04). C-females spent 2.6% less time at the boundary than their mates. T-females spent 2.2% more time at the boundary than their mates (Table 3.3).

USVs at the Territory

We recorder a total of 1028 USVs across the 30 territory sites (T USVs=469, C USVs=559). Of the 1028 USVs, 373 were produced by the female in the pair, 227 were produced by the male in the pair, and 428 USVs we could only assign the vocalization to the pair because both the male and the female were there at the same time. Of the 1028

USVs recorded, we assigned context to 854 USVs using our thermal imaging video. We could not assign context for 174 USVs, for 27 USVs we did not have matching video footage (equipment failure or outside the recording video time), and for the remaining 147 USVs mice were not visible on the video (mice were most likely under thick brush). Of the 854 USVs which we assigned context from thermal video, 359 USVs were produced when a mouse was alone (T USVs=160, C USVs=199), 122 USVs were produced when the mouse was <1 meter away from another mouse (T USVs=52, C USVs=70), and 373 USVs were produced when the mouse was >1 meter away from another mouse (T USVs=157, C USVs=216).

There was no treatment effect on the number of USVs produced (total USVs GLMM Estimate 0.27±.42, p = 0.53). There was a positive interaction between treatment and number of USVs produced across the three nights (Testosterone: Recording Night Two GLMM Estimate 1.40±0.64, p =0.03; Testosterone: Recording Night Three GLMM Estimate 1.74±0.62, p =0.01; Table 3.4; Figure 3.7). C-pairs produced eight more USVs on night one than nights two and three. T-pairs produce seven more USVs on night two than night one and 14 more USVs on night three than night one. Total USVs produced were not influenced by pups (GLMM Estimate 0.23±0.51, p = 0.65; Table 3.4), season (GLMM Estimate 0.35±0.42, p = 0.42; Table 3.4), body mass (GLMM, Estimate -0.01±0.06, p = 0.91; Table 3.4), injection administration (GLMM Estimate 0.13±0.33, p = 0.70; Table 3.4), or distance to nest (GLMM Estimate 0.03±0.03, p = 0.24; Table 3.4).

We recorded all call types for both the male and the female at the territory boundary. T-pairs produced proportionately fewer 3SVs at the territory than control pairs

(W=153, p=0.03; Table 3.5; Figure 3.8). There was no difference between treatments in the proportion of any other call type produced (1-, 2-, 4-, 5-, or 6SV). There was a treatment effect when we considered USVs produced by a mouse in close proximity to another mouse at the territory ($\chi 2 = 18.07$, $df = 1$, $p < 0.01$). There was no treatment effect when we considered USVs produced by a mouse alone ($\chi 2 = 5.88$, $df = 1$, $p = 0.32$) or when mice were far apart ($\chi 2 = 7.68$, $df = 1$, $p = 0.10$).

T-males produced calls with a shorter mean duration than control males (GLM Estimate -0.03±0.01, p = 0.04; Figure 3.9). There was no difference between treatment types in call bandwidth (GLM Estimate 76.49±101.86, p = 0.45) or PC1 score (GLM Estimate 0.32±0.32, p = 0.32). For females, there was no difference between treatment types and any call characteristics, duration (GLM Estimate 0.01±0.01, p = 0.58; Table 2.7), bandwidth (GLM Estimate 142.72±100.00, p = 0.15; Table 2.7) or PC1 score (GLM Estimate 0.31±0.25, p = 0.21; Table 3.7).

Discussion

Environmental location dictates the effects of T-injections suggesting the effects of T pulses are highly context-dependent. Our present study found that T-pulse manipulations alter future spatial behavior, and spatial preference is modulated by the physical environment. Three artificially induced T-pulses over a five-day period altered the behavioral response of the free-living dyads at the territory during the three days following the last T-administration. Our study is the first to demonstrate that 1) T-pulses alter the spatial preference of the male by decreasing time allocation at the territory boundary and promoting territory expansion, 2) T-males time allocation at the territory

boundary regulates female spatial preference, and 3) T-pulses alter spectral characteristics of calls in response to different social interaction.

Males injected with T at the territory boundary spent less time at the boundary than control males. However, T-males traveled further outside their territory than control males. Night post-injection was important in explaining the variation in the amount of time the male spent at the territory boundary. T-males spent more time in the boundary on night one than night three, where the opposite was true for control males. We did not find that any other variable tested (pups, season, body mass, injection administration, or distance to nest) contributed to the variation in the amount of time the male spent at the territory boundary. Our results suggest that T-pulses do not have the same rewarding effects to elicit a location preference for the specific section of the territory boundary we tested, as they do at the nest site (Petric et al. unpublished). The lack of conditioning by pair-bonded males at the territory boundary was unexpected because laboratory experiments on this species found that T-pulses induce CPPs in pair-bonded males in familiar but not in unfamiliar environments (Zhao and Marler 2014; 2016). California mice maintain strict territories and neighboring grounds can be considered an unfamiliar environment (Ribble and Salvioni 1990). Interestingly, the opposite was true for sexually naïve males, in which T-pulses induced CPPs in unfamiliar but not familiar environments (Zhao and Marler 2014; 2016). In free-living mice, T-pulses at the territory boundary most likely induce a behavioral change that promotes active territory expansion to secure additional resources instead of defending the currently controlled boundary. We speculate that based on travel distance outside the original boundary, T-males are most likely

challenging other males and staking out a larger territory to acquire novel resources instead of going back to the nest. In birds, T increases influence territory acquisition and expansion (Wingfield et al. 1987) and T can induce polygyny in a monogamous species (Wingfield 1984). The ability to approach other males and secure resources are essential for survival and reproduction (Maher and Lott 1995). T-pulses at the territory may enforce the drive for approaching and challenging heterospecifics in the adjacent territory which could be tested using playback studies. This could also explain why we see a continuous decrease in time spent at the territory boundary from night one to night three for T-males but not C-males. In nature, the number of aggressive encounters increases in response to the transfer of space as the male is challenged by surrounding males who are defending their resources (Potts and Lewis 2014, Hegner and Wingfield, 1987). Males will show stronger territory defense tendency for areas that were newly acquired than for those already controlled (Mayer et al. 2017; Chandler et al.1994). It is also possible and not mutually exclusive that males, while involved in territory conflicts, are also seeking to mate with additional females. In banded mongooses (*Mungos mungo*), aggressive encounters between neighbors lead to mating between the invading males and the opposing females (Cant et al. 2002). If T-injected California mouse males are spending less time at the original territory boundary, then it is possible the males are spending more time challenging neighboring males and patrolling their territory, but this remains to be tested.

We found that testosterone altered the spatial behavior of the female. Females whose mate received T spent less time at the territory boundary than females whose mate

received saline control. In California mice, pair-bonded mice form a partnership and both members of a pair are engaged in offspring care and territory defense (Gubernick and Alberts 1987). If T-females are spending less time at the boundary than the females could spend more time in three potential places 1) the nest to take care of her young, 2) patrolling and defending other parts of the already established territory, and/or 3) engaging in more cooperative behavior with her mate to encroach on other territories. In cooperatively breeding banded mongooses (*Mungos mungo*), both male and female presence is essential for a successful territory overtake (Cant et al. 2002). Furthermore, in the Arabian babbler (*Turdoides squamiceps*), researchers found that coalition territory overtake in this avian species is more successful and energetically effective than an overtake attempt by a single individual (Ridley 2012). It remains to be tested if California mice are dividing tasks or invading together and the answer is most likely context-dependent (Hurley and Kalcounis-Rueppell 2018).

We found that pup presence and season influenced the amount of time the female spent at the territory. Overall, females with pups and during the second season spent less time at the territory boundary. T-females with pups spent the least amount of time at the boundary. As discussed earlier, in the California mouse, both sexes engage in paternal care and females with pups most likely allocate more time at the nest to offspring care. This is especially true during the breeding season. Season two corresponds to the breeding times when nights are cooler, and pups require more care. In the California mouse, pups are considered exothermic until the age of 15 days and young depend on adult presence to maintain their body temperature (Gubernick and Alberts 1987).

Therefore, females are most likely going back to the nest to care for their young. There was a significant night effect with T-females spending more time at the boundary on night one than night three. The spatial territory boundary trend across the three recording nights is similar between the male and female, suggesting the female can adjust her behavioral response to that of her mate.

Together, our results suggest there is behavioral plasticity in inducing CPPs and that it is context-dependent. In free-living and pair-bonded males, T-pulse induction of CPPs is based on the physical environment and the interactions that occur in that space. T-pulse induced CPPs only at the nest site (Petric, Kalcounis-Rueppell, and Marler unpublished) whereas, at the territory boundary, T-pulses are possibly inducing novelty-seeking behavior or allocation of time in other parts of the established territory and interacting with known neighbors.

Our study also found that in response to the same transient increases in T, the California mouse alters future vocalization production at the territory boundary. The territory USV results correspond to the spatial preference of males and females at the territory boundary. Control males and females spend more time and produce more USVs at the territory boundary on night one than night three. Where we see the opposite trend for the T-pairs. The USV results for T-pairs are possibly driven by a single pair that exhibited a strong response to T on night three. In the laboratory, there is a positive relationship between SV calls and male affiliative behavior in the California mouse (Pultorak et al. 2017). Furthermore, in the lab, California mice pairs increased SV production after the introduction of a male and female and the formation of a pair bond

(Pultorak et al. 2018). We found that C-males produced proportionately more 3SVs and there are several potential hypotheses related to the function of the 3SV at the territory boundary. The 3SVs could be aggressive calls aimed at neighbors. More likely the 3SVs serve an affiliative function between members of a pair, or to coordinate territory defense behaviors between the male and the female in the pair. In many species of tropical birds, both sexes coordinate their songs to defend their territory (Logue 2005). Pair cooperation deters other individuals from overtaking the territory (Wickler 1976). When the individual is alone, the SVs may serve as territory defense vocalizations and/or an attraction vocalization between members of a pair. Males but not females produce shorter SVs during none-mate interactions is consistent with laboratory results. Rieger et al. (2018) found that during same-sex interactions, resident males shorten SVs both before and during the encounter. These results are consistent with other studies that examined call characteristics and aggression. In squirrel monkeys and big brown bats, call duration decreases as the level of aggression increase (Weerts and Miczek 1996; Gadziola et al. 2012). Fitch and Hauser (1995) suggest that during aggressive encounters individuals perceive the situation as urgent and tense, which triggers a physiological response that results in the release of higher air pressure in the vocal organ and leads to the production of shorter duration calls. In order to tease apart the function of specific SVs, playback studies in various social contexts are necessary.

Overall, rapid and transient increases in androgen hormone testosterone influence both spatial and vocal behavior of the male and the female. In the complex field setting, CPP induction on California mouse is context-dependent (Zhao and Marler 2014; 2016).

This change in the allocation of time spent in the physical environments also leads to changes in social interactions and USV productions during those interactions. Here, we provide evidence that in the wild where multiple biotic and abiotic competing stimuli occur simultaneously, testosterone is an important factor that can control spatial preference and vocalization plasticity of the male and his mate. This research contributes to the growing body of literature for elucidating the functional significance of T-pulses on animal behavior. The variation of T in inducting CPPs at the nest but not at the territory boundary suggests there is plasticity in the sensitivity to T based on the physical location.

Ethical Statement

Research protocols for this study were approved by the University of North Carolina at Greensboro and University of Wisconsin-Madison Institutional Animal Care and Use Committees (IACUC; UNCG 16-002 and UWM L005047-A01) and by California Department of Fish and Wildlife under Scientific Collection Permits (SC-9663 and SC-13190).

Acknowledgments

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Figure 3.1. Proportion of Time Male California Mouse (*Peromyscus californicus*) Spend at the Territory Boundary by Treatment Type. Proportion of time spent at the territory boundary was recorded for three consecutive nights after the administration of the third injection (Testosterone = 15 and Control=14). T injected males spent 7% less time at the territory boundary than C males (GLMM Estimate -0.81±0.33, p = 0.02).

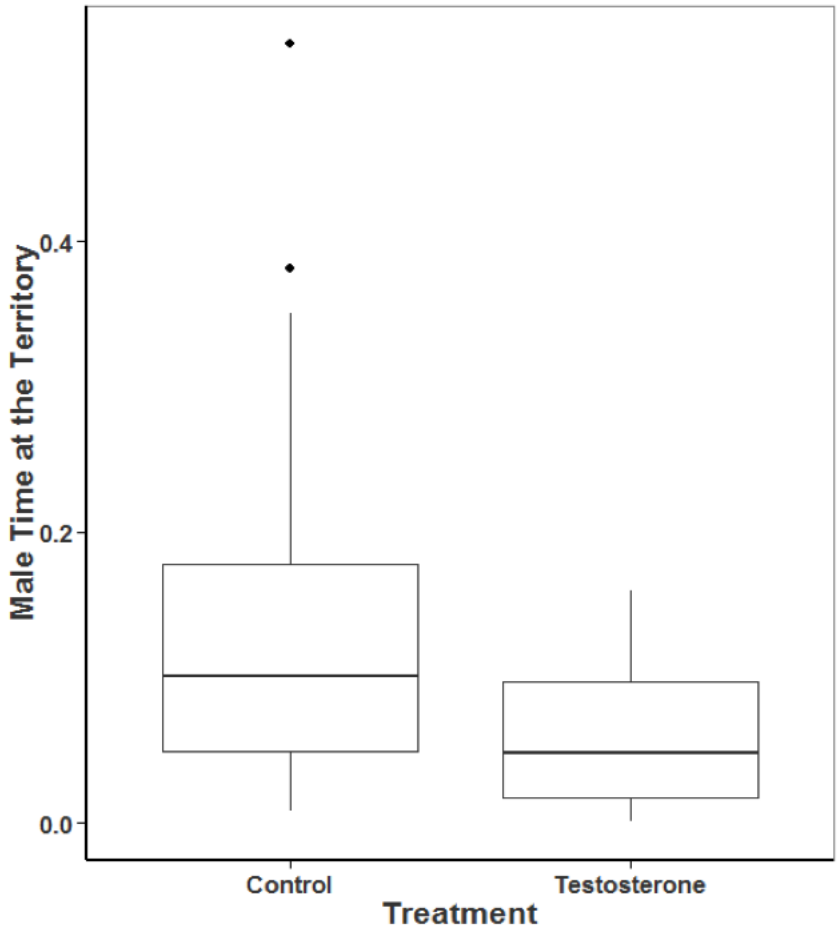

Figure 3.2. Proportion of Time Male California Mouse (*Peromyscus californicus*) Traveled Based on Live-Trapping Data When Comparing Capture Stations Pre-Treatment to Capture Stations Post-Treatment by Injection Type. Testosterone injected males (Testosterone = 13 and Control=15) traveled an average of 13.4 meters further from the pre-treatment stations than control males (W=4.51, df=1, p=0.03).

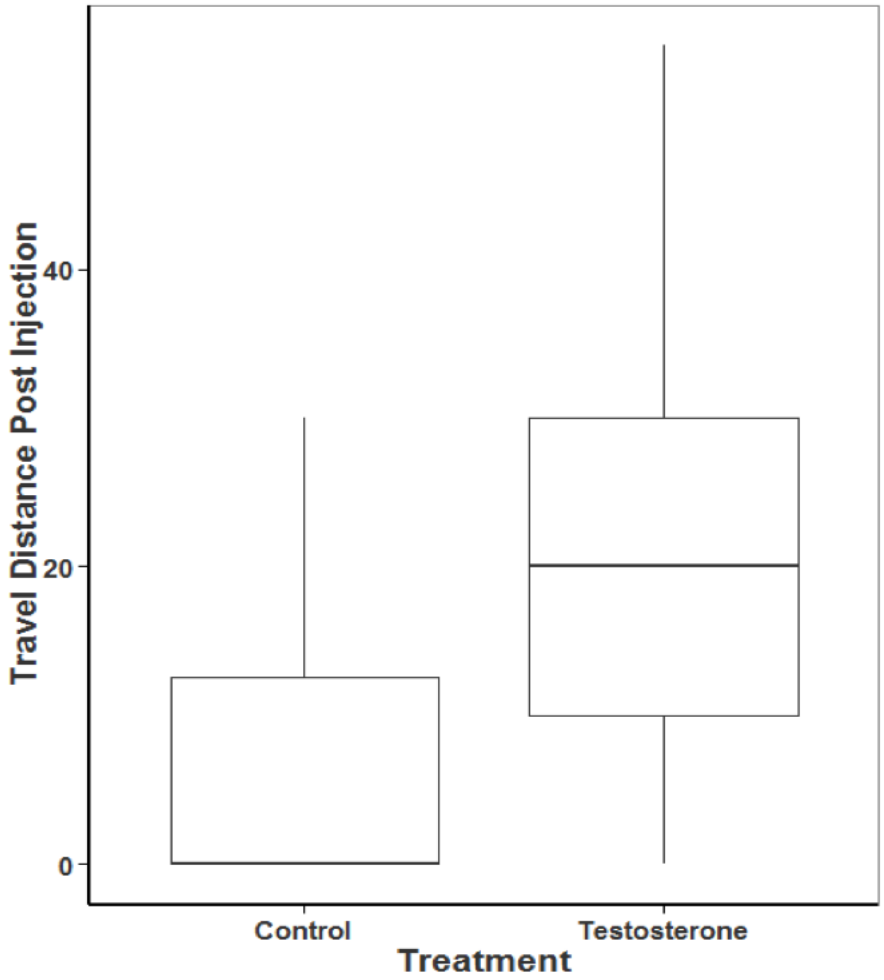

Figure 3.3. Proportion of Time Male California Mouse (*Peromyscus californicus*) Spent at the Territory Boundary by Treatment Type and Night. Time at the territory boundary was recorded for three consecutive nights after the administration of the third injection (Testosterone nights one, two and three each = 15 and Control night one, two, and three each =14). Control males spend 2% more time at the territory boundary on night three than night one, whereas, testosterone males have the opposite effect and spend 2% less time on night three than night one. There was a negative interaction between treatment and recording night three (Testosterone: Recording Night Three GLMM Estimate -0.58±0.23, p =0.02). Black dots represent outliers.

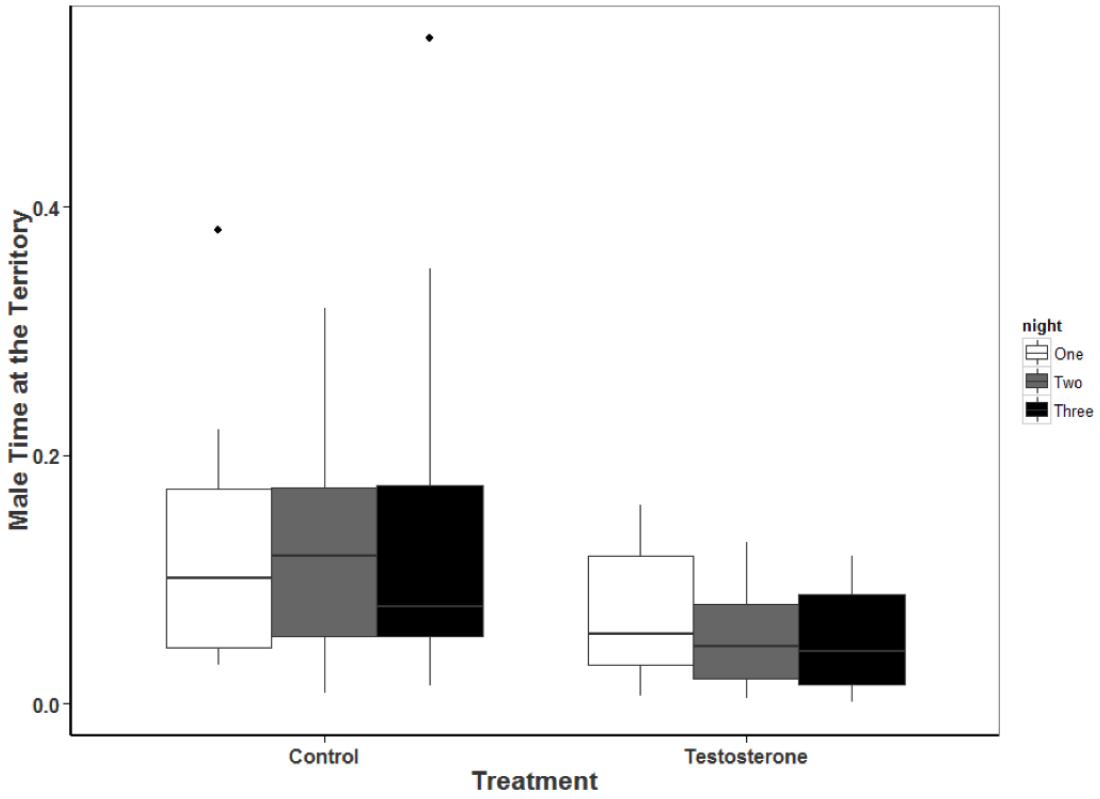

Figure 3.4. Proportion of Time Female California Mouse (*Peromyscus californicus*) Spend at the Territory Boundary by Treatment Type. Proportion of time spent at the territory boundary was recorded for three consecutive nights after the administration of the third injection (Testosterone = 15 and Control=13). T females spent 7% less time at the territory boundary than C females (GLMM Estimate -0.55±0.27, p = 0.048).

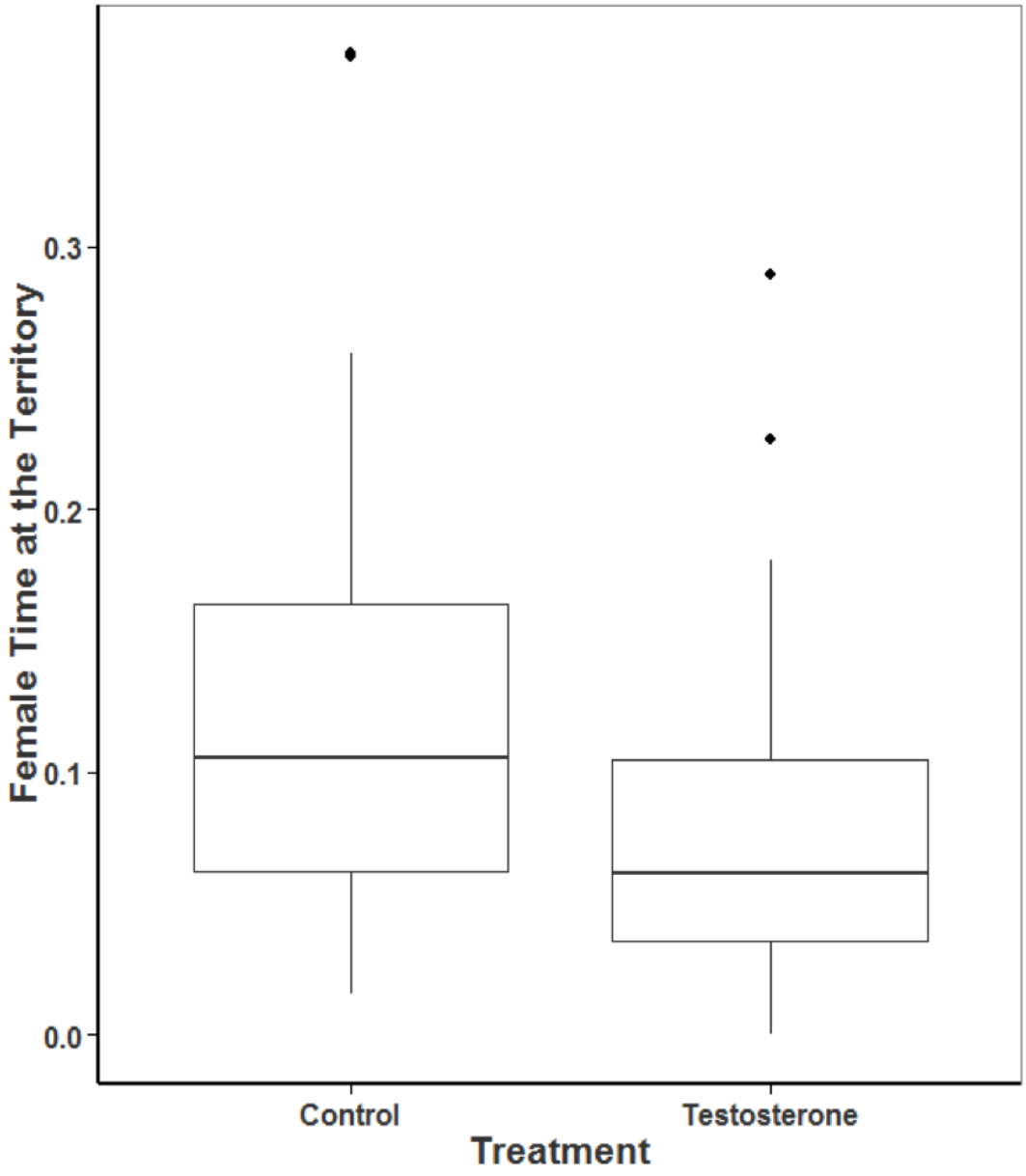

Figure 3.5. Proportion of Time Female California Mouse (*Peromyscus californicus*) With and Without Pups Spend at the Territory Boundary by Treatment Type. Proportion of time spent and the territory boundary was recorder for three consecutive nights after the administration of the third injection (T with pups, n=7; T without pups, n=8) or saline (C with pups, n=8; C without pups, n=5). T females with pups spent 4.4% less time at the territory boundary than C female with pups, and T females without pups spent 6.4% less time at the territory boundary than C females without pups (treatment GLMM Estimate -0.59±0.24, p =0.02; pups GLMM Estimate -0.55±0.24, p =0.03).

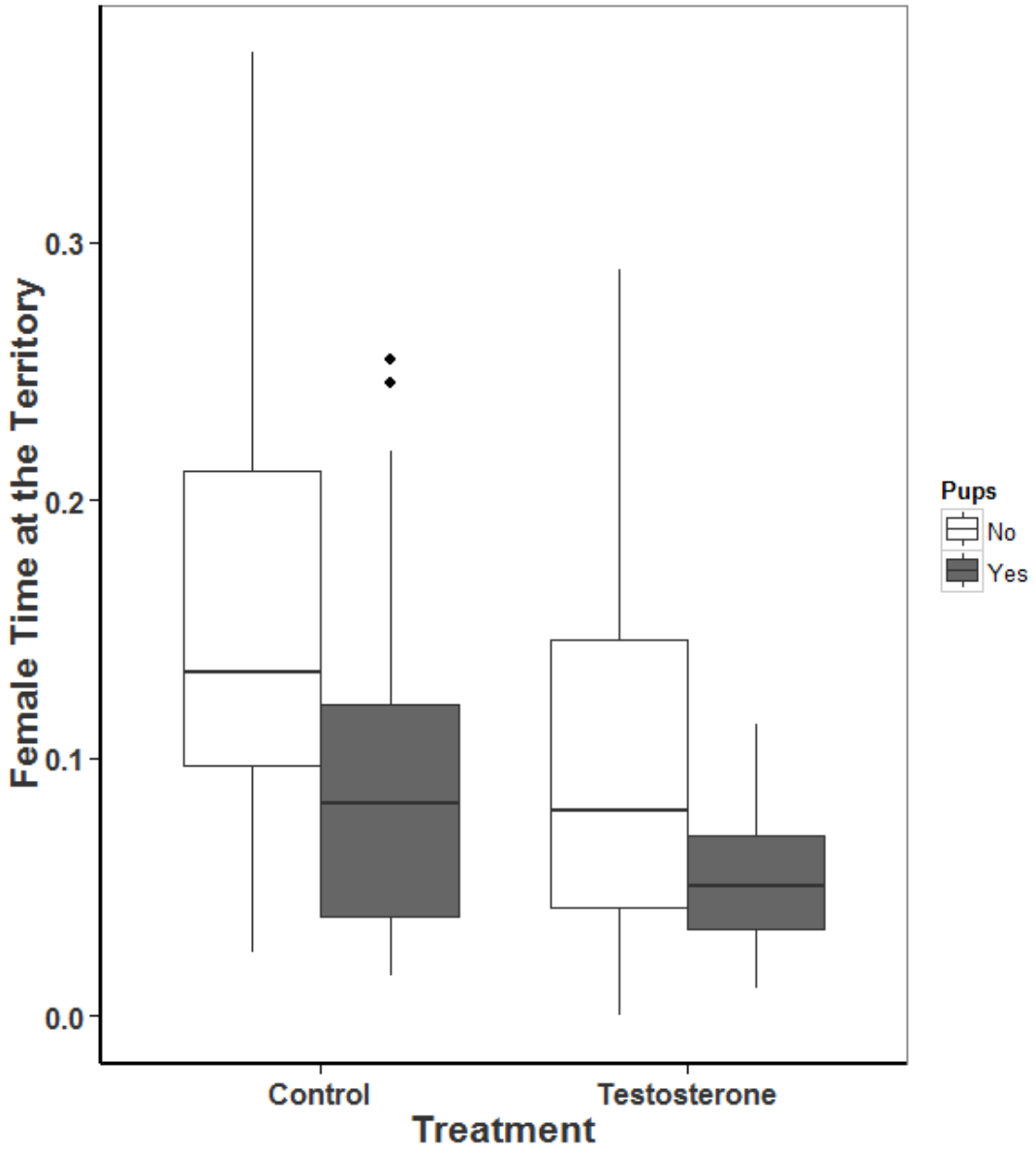

Figure 3.6. Proportion of Time Female California Mouse (*Peromyscus californicus*) spent at the Territory Boundary by Treatment Type and Night. Black dots represent outliers. Time at the territory boundary was recorder for three consecutive nights after the administration of the third injection (Testosterone nights one, two and three each = 15 and Control night one, two, and three each =14). Control females spend 2% more time at the territory boundary on night one than night three, whereas, testosterone females have the opposite effect and spend 2% less time on night one than night three. There was a negative interaction between treatment and recording night three (Testosterone: Recording Night Three GLMM Estimate -0.35±0.14, p =0.01).

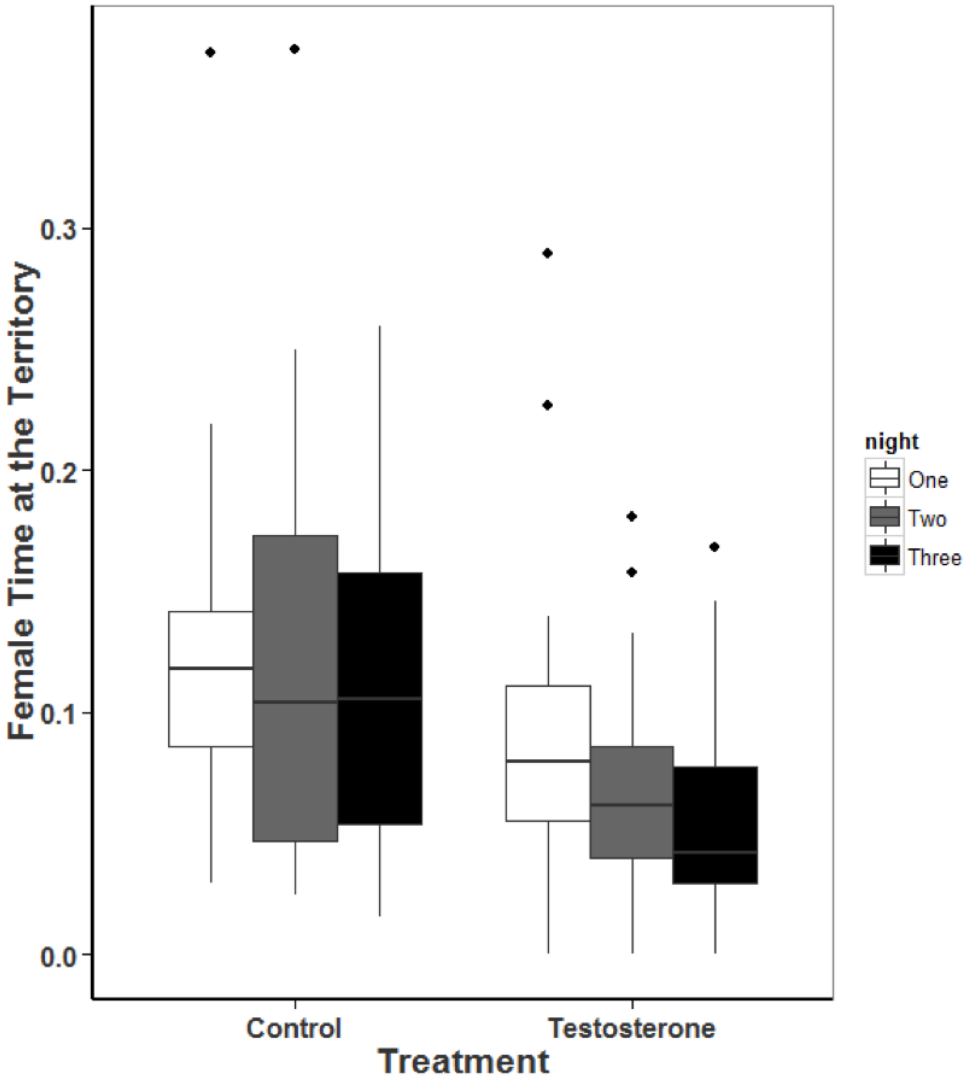

Figure 3.7. California Mouse (*Peromyscus californicus*) USV Produced at the Territory Boundary by Treatment Type and Night. USVs at the territory boundary by treatment type and night post injection (Testosterone nights one, two and three each = 15 and Control night one, two, and three each =14). Black dots represent outliers. Time at the territory boundary was recorder for three consecutive nights after the administration of the third injection. There was a negative interaction between treatment and recording night (Testosterone: Recording Night Two GLMM Estimate 1.40±0.64, p =0.03; Testosterone: Recording Night Three GLMM Estimate 1.74±0.62, p =0.01). Control dyad produce more USVs on night one and decrease USV production on nights two and three, whereas, we see the opposite effect for testosterone dyad with fewer USVs produced on night one and increase USV production for nights two and three.

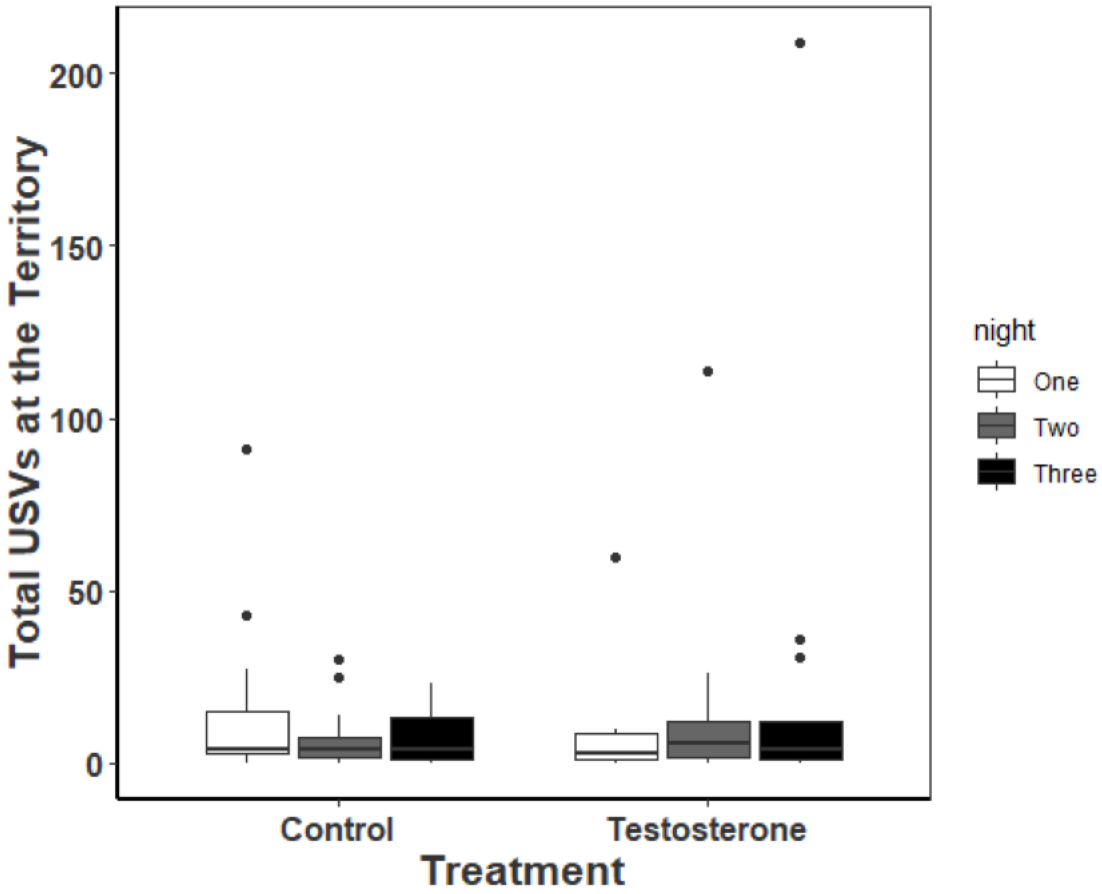

Table 3.1. Descriptive Statistics on Male California Mouse (*Peromyscus californicus*) Time at the Territory Boundary, Presence of Pups at the Nest, Season, Body Mass, Number of Nights Required to Administer Three Injections, and Recording Night After the Last Injection. Each *Peromyscus californicus* male received three testosterone (T=15) or saline (C=14) injection at the territory boundary, after the final inject, we recorded time spent at the territory boundary for three consecutive nights.

		n	Mean	SE	Median	Min	Max
Time at the Boundary	C	42	0.130	0.017	0.101	0.009	0.535
	T	45	0.060	0.007	0.048	0.001	0.159
Time at the Boundary and Pups	C with Pups	24	0.149	0.028	0.127	0.009	0.535
	T with Pups	24	0.040	0.007	0.031	0.001	0.136
	C without Pups	18	0.106	0.015	0.084	0.015	0.263
	T without Pups	21	0.082	0.010	0.097	0.012	0.159
Time at the Boundary and Season	C Season One	15	0.158	0.027	0.142	0.009	0.351
	T Season One	18	0.064	0.009	0.053	0.012	0.139
	C Season Two	27	0.115	0.022	0.068	0.012	0.535
	T Season Two	27	0.056	0.010	0.034	0.001	0.159
Body Mass	C	42	38.930	0.561	39.000	32.000	45.000
	T	45	38.370	0.545	38.000	32.000	47.000
Injection Administration	C	42	3.357	0.075	3.000	3.000	4.000
	T	45	3.600	0.107	3.000	3.000	5.000
Time at the Boundary and Recording Night	C - Night One	14	0.124	0.026	0.101	0.031	0.381
	T - Night One	15	0.074	0.014	0.056	0.006	0.159
	C - Night Two	14	0.126	0.025	0.119	0.009	0.318
	T - Night Two	15	0.054	0.011	0.046	0.004	0.130
	C - Night Three	14	0.142	0.039	0.078	0.015	0.535

	T - Night Three	15	0.051	0.010	0.042	0.001	0.119

Table 3.2. Descriptive Statistics on Female California Mouse (*Peromyscus californicus*) Time at the Territory Boundary, Presence of Pups at the Nest, Season, Body Mass, Number of Nights Required to Administer Three Injections, and Recording Night After the Last Injection. Each *Peromyscus californicus* female was paired with a male who received three testosterone (T=15) or saline (C=13) injection at the territory boundary, after the final inject, we recorded time spent at the territory boundary for three consecutive nights.

		n	Mean	SE	Median	Min	Max
Time at the Boundary	C	39	0.125	0.014	0.106	0.016	0.374
	T	45	0.076	0.009	0.061	0.000	0.289
Time at the Boundary and Pups	C with Pups	24	0.100	0.014	0.083	0.016	0.255
	T with Pups	21	0.056	0.006	0.050	0.011	0.113
	C without Pups	15	0.164	0.027	0.133	0.024	0.374
	T without Pups	24	0.100	0.017	0.080	0.000	0.289
Time at the Boundary and Season	C Season One	14	0.175	0.031	0.159	0.035	0.374
	T Season One	18	0.098	0.016	0.095	0.017	0.289
	C Season Two	25	0.096	0.011	0.087	0.016	0.025
	T Season Two	27	0.061	0.010	0.049	0.000	0.226
Body Mass	C	39	45.120	0.903	45.000	35.000	55.500
	T	45	43.330	0.832	42.000	37.000	59.000
Time at the Boundary and Recording Night	C - Night One	13	0.132	0.025	0.118	0.029	0.372
	T - Night One	15	0.099	0.019	0.080	0.000	0.289
	C - Night Two	13	0.128	0.029	0.104	0.024	0.373
	T - Night Two	15	0.070	0.013	0.061	0.000	0.180
	C - Night Three	13	0.114	0.022	0.106	0.016	0.260
	T - Night Three	15	0.059	0.013	0.042	0.000	0.168

Table 3.3. Descriptive Statistics on California Mouse (*Peromyscus californicus*) Pair Time at the Territory Boundary. Each *Peromyscus californicus* female was paired with a male who received three testosterone (T=15) or saline (C=13) injection at the territory boundary, after the final inject, we recorded time spent at the territory boundary for three consecutive nights.

		n	Mean	SE	Median	Min	Max
Pair Time at the Boundary	C Female	36	0.122	0.015	0.105	0.016	0.373
	T Female	45	0.082	0.010	0.061	0.000	0.254
	C Male	36	0.148	0.194	0.119	0.011	0.535
	T Male	45	0.060	0.007	0.048	0.001	0.159

Table 3.4. Descriptive Statistics on USV Production by California Mouse (*Peromyscus californicus*) at the Territory Boundary, Presence of Pups at the Nest, Season, Body Mass, Number of Nights Required to Administer Three Injections, and Recording Night After the Last Injection. Each male received three testosterone (T=15) or saline (C=14) injection at the territory boundary, after the final inject, we recorded USVs at the territory boundary for three consecutive nights.

USVs at the Territory		n	Mean	SE	Median	Min	Max
USVs	C	45	9.867	2.336	4.000	0.000	91.000
	T	45	14.490	5.289	4.000	0.000	209.000
USVs and Pups	C with Pups	27	13.778	3.694	7.000	0.000	91.000
	T with Pups	24	12.958	4.818	5.500	0.000	114.000
	C without Pups	18	4.000	0.784	3.500	0.000	12.000
	T without Pups	21	16.238	10.054	2.000	0.000	209.000
USVs and Season	C Spring	18	7.167	2.443	3.500	0.000	43.000
	T Spring	18	25.222	12.832	2.000	0.000	209.000
	C Fall	27	11.667	3.532	4.000	0.000	91.000
	T Fall	27	7.333	1.484	5.000	0.000	31.000
Body Mass	C	45	38.867	0.525	39.000	32.000	45.000
	T	45	38.367	0.545	38.000	32.000	47.000
Injection Administration	C	45	3.333	0.071	3.000	3.000	4.000
	T	45	3.600	0.107	3.000	3.000	5.000
USVs and Recording Night	C - Night One	15	15.067	6.326	4.000	0.000	91.000
	T - Night One	15	7.600	3.863	3.000	0.000	60.000
	C - Night Two	15	7.133	2.347	4.000	0.000	30.000
	T - Night Two	15	14.533	7.354	6.000	0.000	114.000
	C - Night Three	15	7.400	2.093	4.000	0.000	23.000
	T - Night Three	15	21.333	13.712	4.000	0.000	209.000

Table 3.5. Descriptive Statistics and Results from the Wilcoxon Rank Sum Test for the Comparison of USV Proportion by Type and Treatment Produced at the Territory Boundary. Each *Peromyscus californicus* male received three T (n=15) or C (n=14) injections at the territory boundary, after the final inject, we recorded USVs at the territory boundary for three consecutive nights. Alpha values of p <0.05 are in **bold**.

	USV Proportions at the Territory															
	C							T								
Call Type	**n**	**Mean**	**SE**	**sd**	**Median**	**Min**	**Max**	**n**	**Mean**	**SE**	**sd**	**Median**	**Min**	**Max**	**W**	**p**
1SV	14	0.164	0.044	0.166	0.097	0.000	0.600	15	0.198	0.056	0.218	0.136	0.000	0.667	106.0	0.983
2SV	14	0.099	0.026	0.097	0.077	0.000	0.296	15	0.065	0.024	0.094	0.031	0.000	0.333	127.5	0.327
3SV	14	0.359	0.052	0.195	0.389	0.000	0.611	15	0.206	0.045	0.174	0.161	0.000	0.438	153.0	**0.038**
4SV	14	0.180	0.042	0.157	0.148	0.000	0.500	15	0.156	0.040	0.156	0.100	0.000	0.500	119.0	0.554

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CHAPTER IV

CALL CONVERGENCE WITHIN PAIRS OF THE MONOGAMOUS CALIFORNIA MOUSE (*PEROMYSCUS CALIFORNICUS*)

This Chapter is coauthored by Radmila Petric and Matina Kalcounis-Rueppell

Abstract

Animals that form long-term social bonds modify vocal characteristics to show similarities in calls. Vocal convergence has been observed in birds and several mammal groups but remains largely unexplored in rodents. Here, we examine the monogamous and free-living California mouse (*Peromyscus californicus*), a rodent that forms lifelong pair-bonds and produces ultrasonic vocalizations (USVs). One type of call, the sustained vocalization (SV) is produced when a member of a pair is separated and most likely serves as a type of contact call. We examined frequency, duration and bandwidth of SV calls among pairs, as well as the difference within pairs compared to differences among individuals in the population, to determine if there are pair similarities greater than would be expected by chance. We found frequency, duration, and bandwidth differences among pairs. Furthermore, call characteristics between members of a pair were more similar than those between other individuals in the population. Our results suggest that vocal convergence occurs in pair members of the California mouse. In a monogamous rodent, the shared acoustic properties most likely have the adaptive significance of strengthening social bonds between members of a pair by serving as pair distinctive identifiers.

Introduction

Animals that form long-term social bonds tend to modify vocal characteristics to show similarities in contact vocalizations (Tyack 2008). These similarities are the result of learning by modification of spectral and temporal characteristics to match individual or group vocalizations, a concept known as vocal convergence (Balsby and Scarl 2008). In the laboratory, vocal convergence is often observed in animals that are housed together (Tyack 2008). Socially bonded individuals tend to modify vocal characteristics into pair distinctive vocalizations (Hile et al. 2000). Vocal convergence has been observed in birds, primates, cetaceans, bats, elephants, and pinnipeds (Janki 1997) but underexplored in other groups that form social bonds, like rodents.

Vocal learning is essential for the development of a language. The modification of vocalizations based on auditory feedback is known as vocal production learning (Janki 1997). In order for vocal production learning to occur, individuals must have control of the vocal organ as well as the neural circuitry to coordinate the fine-tuning of the vocalizations (Knörnschild 2014). There are two types of vocal production learning, learned acquisition and social modification. Learned acquisition is the production of new vocalizations, whereas social modification is the adjustment of already existing vocalizations (Knörnschild 2014). In songbirds, vocal learning is controlled by a specialized vocal center, and the volume of this neural circuit is directly related to testosterone levels.

The basic functional significance for the evolution of vocal convergence is to diminish the uncertainty of the callers' group affiliation (Tyack 2008). In nature, socially

living pairs or groups often separate to forage and/or defend essential resources. These pairs or groups rely on vocal communication to identify members from a distance or in noisy environments. Group-specific vocalizations play an important role in rapidly identifying a member of a pair or group, coordinating the defense, and strengthening the social bond (Tyack 2008). In wild chimpanzees (*Pan troglodytes verus*), as individuals in a group form social bonds the vocal features of calls are modified to match the group, and the variation in the vocal convergence among groups has no genetic influence (Crockford et al. 2004). Furthermore, from the same study, the authors found that call characteristic were more similar between neighboring communities than a distant community (Crockford et al. 2004). Different mechanisms control different spectral characteristics of calls. Exhalation can control the duration of the call and air pressure can control the amplitude of the call (Janki 1997). Testosterone and its metabolites can modify vocalizations by altering neuromuscular control to allow for greater modulation of spectral and temporal characteristics (Harding 2004). Specifically, testosterone can lead to modification of duration, frequency, bandwidth, and amplitude of vocalizations (Pasch et al. 2011a). For example, in Alston's singing mouse (*Scotinomys teguina*) and the grey partridge bird (*Perdix perdix*), testosterone-treated males produced longer duration vocalizations that spanned across a greater number of frequencies with a lower mean fundamental frequency (Pasch et al. 2011b; Pasch et al. 2011; Fusani et al. 1994). Furthermore, in barn swallows (*Hirundo rustica*), testosterone levels were correlated with song complexity and the ability to modify vocal characteristics to match songs of neighboring males (Galeotti et al. 1997).

Generally, call production by rodents is considered to be innate as mice deafened at birth still produce vocalizations (Arriaga and Jarvis 2013). However, mouse calls may not be fixed at birth but instead undergo structural changes in response to various stimuli (Arriaga and Jarvis 2013). For example, vocalizations between deaf and control male mice were significantly different, as mice rely on auditory feedback to modify vocalization characteristics (Gustavo et al. 2012), suggesting that mice must compare their vocal output with auditory input to match call characteristics from memory. The mouse brain region for call production has some characteristics of the vocal center found in humans and vocal-learning birds, and male mice have the necessary neuromuscular features for vocal learning (Gustavo et al. 2012). Despite our knowledge about vocal learning, relatively little is known about learning by modification of spectral and temporal characteristics that result in vocal convergence in rodents.

The California mouse (*Peromyscus californicus*) is a vocal rodent, that forms lifelong pair-bonds (Ribble 1991). Calls are an important part of the California mouse behavior. In the wild and the laboratory, California mice produce the same types of USVs, however, there is greater variability in duration and frequency of USVs produced by wild than the laboratory mice (Kalcounis-Rueppell et al. 2010; Kalcounis-Rueppell et al. 2018). In the wild, the most common call type recorded by California mice are sustained vocalizations (SVs)(Briggs and Kalcounis-Rueppell 2011; Kalcounis-Rueppell et al. 2006; Kalcounis-Rueppell et al. 2010; Timonin et al. 2018; Kalcounis-Rueppell et al. 2018). Both sexes produce SVs in various social contexts and they can range in bouts from 1-11 calls (Briggs and Kalcounis-Rueppell 2011; Kalcounis-Rueppell et al. 2018).

The SVs are often produced when a member of a pair is greater than 1m away and in this context, SVs most likely serve as contact vocalizations, possibly for maintenance of the pair-bond (Briggs and Kalcounis-Rueppell 2011; Kalcounis-Rueppell et al. 2018), which is consistent with the regular production of SVs between pairs in the laboratory (Pultorak et al. 2018).

Free-living California mice maintain strict territories and generally do not overlap with other California mice (Ribble and Salvioni 1990). Therefore, social interactions within the core of the territory are primarily between pair members and these interactions are often accompanied by the production of SV calls (Briggs and Kalcounis-Rueppell 2011; Kalcounis-Rueppell et al. 2018). Thus, the monogamous and vocal California mouse is a compelling species for assessing vocal similarities between members of a pair.

We hypothesized that in the California mouse, there will be vocal convergence between members of a pair. We predicted that 1) call characteristics among pairs will differ; 2) call characteristics between members of a pair will be more similar than between other individuals in the population; 3) similarities among pairs would differ based on the geographic distance between pairs, with pairs that are closer to one another having more similar call characteristics than pairs that are further apart from one another.

Methods

As part of another study (see Dissertation Chapter II) we recorded vocalizations from known California mouse individuals and known pairs, and this allowed us to examine call convergence within and among pairs. We were able to assign 98 USVs (1SVs, 2SV, 3SV) to 12 known and paired individuals belonging to six different pairs. As

part of the same study (see Dissertation Chapter II), males within a pair were randomly assigned to receive three injections of either testosterone (T; n=3) or saline control (C; n=3).

Acoustic Analysis

For each call, we used an automatic feature to measure duration, bandwidth and five frequency variables (start, end, maximum, minimum, and frequency at maximum amplitude) using Avisoft SAS Lab Pro (Avisoft Bioacoustics). For each call a spectrogram was generated with a 512 FFT (Fast Fourier Transform), and a 100-frame size with Hamming window in Avisoft SAS Lab Pro (Avisoft Bioacoustics). To reduce the number of frequency variables in our analysis, we used a Principal Component analysis to yield a single PC score. For among-pair comparisons, we treated the male and female calls as belonging to the same pair and generated an average PC score for the pair. For the within-pair comparison, we did not pool the male and female calls and generated average PC scores for individuals. The PC scores accounted for 89.8 to 95.3% of the variation in frequency variables (Supplemental Table 4.1). For our statistical analyses, we used three dependent acoustic variables (duration, bandwidth and PC1) to examine pair differences. We examined all SV call types (1-, 2-, and 3SV) together and each call type separately.

For among-pair comparisons, we treated the male and female calls as belonging to the same pair. To determine if the difference between a male and a female within pairs is smaller than differences that would be expected from any pairing within the population, we measured 1) the difference in mean call characteristics between a male and a female

within a pair (this represents within pair difference) and 2) difference in mean call characteristics between any two possible mouse combinations outside the original pair but from within the individuals representing the other 5 pairs (this represents within the population difference). To determine if differences between pairs scales with geographic distance between pairs, we measured the distance in meters between the nest sites of all pairs. Our study sites were set-up in a grid formation with 10-m spacing, and all the pair nest sites were located within the girds (see Dissertation Chapter II). Therefore, each cell within the grid represented a distance of 10-meters, we counted the number of cells from the shortest distance between each nest site to get the approximate distance.

Statistical Analyses

All data were checked for normality using a Shapiro-Wilk test. We used a Mann-Whitney U test to examine sex and treatment (testosterone and control) effects on pair call characteristics. To examine differences among pairs, we used a Kruskal Wallis Test. To determine if the difference between a male and a female within pairs is smaller than differences that would be expected from any pairing within the population, we used a T-test. To examine whether differences between pairs scale with the geographic distance we used regression analysis for each call characteristic (duration, bandwidth, and for PC1 we used Frequency at Maximum Amplitude because it had the largest loading score (Supplemental Material S4.1) with distance. We used a Bonferroni correction rejection criterion of $p<0.01$ to account for multiple tests. All data were analyzed in R (Version 3.6.0).

Results

We did not find a treatment effect between testosterone and control pairs for any vocal characteristics in any call types ($p>0.05$). We also did not find any sex differences between the males and the females for any call characteristics in any call types ($p>0.05$). Therefore, we did not use treatment or sex as factors in our analysis of call convergence.

Among Pair Differences

We found a difference among pairs in duration ($\chi2=33.07$, $df=5$, $p<0.01$; Figure 4.1A; Table 4.1), PC1 ($\chi2=44.03$, $df=5$, $p<0.01$; Figure 4.1B; Table 4.1), and bandwidth ($\chi2=33.13$, $df=5$, $p<0.01$; Figure 4.1C; Table 4.1). When we examine each call type separately, we found a difference among pairs in the PC1 for the 1SV and calls two and three of the 3SV (1SV$\chi2=13.27$, $df=3$, $p<0.01$; 3SV-2$\chi2=14.80$, $df=3$, $p<0.01$; 3SV-3$\chi2=20.11$, $df=3$, $p<0.01$). We also found a difference among pairs in the duration for the first call in the 2SV (2SV-1$\chi2=12.12$, $df=3$, $p<0.01$) and bandwidth for the second call in the 2SV (2SV-2$\chi2=11.73$, $df=3$, $p=0.01$).

Within Pair Compared to Within the Population Differences

We found a trend for smaller within pair difference than within the population difference in PC1 ($t=2.37$ $df=8.69$, $p=0.04$) and bandwidth ($t=2.40$ $df=27.92$, $p=0.02$), but not in duration ($t=1.09$ $df=54$, $p=0.28$). When we examine each call type separately, we found six times smaller frequency differences within pairs than within the population in the PC1 of the 1SV (1SV$\chi2=6.62$, $df=21.67$, $p<0.01$; Figure 4.2A; Table 4.2). We also found three and a half times smaller differences in duration within pairs than within the population of the first call in the 2SV (2SV-1$\chi2=6.36$, $df=23.91$, $p<0.01$; Figure 4.2B

Table 4.2). Lastly, we found a trend for a smaller difference in the bandwidth within pair than within the population for the third call of the 3SV- (3SV-$3\chi2$=2.36, df=12.88, p=0.03). We found no difference between within pairs and within the population for duration in the 1SV, 2SV-call 2, and all calls in the 3SV, bandwidth in the 1SV, 2SV call 1 and 2, and 3SV call 1 and 2, and PC1 in all calls for 2- and 3SVs (p>0.05).

Similarities Among Pairs Based on Distance

Across all pairs, there was a trend for differences in frequency to be negatively related to the distance among pairs ($F_{1,13}$=4.47, p=0.05, R2 adj=0.20; Figure 4.3). We found no correlation between distance among pairs and duration ($F_{1,13}$=0.01, p=0.92, R2 adj=-0.08) or bandwidth ($F_{1,13}$=0.18, p=0.67, R2 adj=-0.06). When we examine each call type separately, we found no correlation between distance among pairs for any call characteristic of any call type (p>0.05).

Discussion

We found that California mouse SVs contain pair specific information. Calls produced by California mice are different among pairs, and the difference within a pair is smaller than the difference among pairs. Interestingly, we found a negative trend for frequency differences between pairs to correlate to the geographic distance between pairs. This trend suggests that neighboring pairs that are closer in the location are more different in call characteristics, whereas pairs that are further apart are more likely to have similar call characteristics. Our findings provide support for the vocal convergence hypothesis.

Differences in call characteristics can be reflected by habitat differences. Specifically, in dense habitats, individuals favor the production of lower frequency and less modulated calls because they are less susceptible to signal degradation (Nicholls and Goldizen 2006). Habitat structure unlikely contributed to the differences we observed among pairs. At our study site, all pairs occupied the same type of habitat which was primarily an oak woodland (*Quercus sp.*) with a thick understory of poison oak (*Toxicodendron sp.*).

We found a trend for greater differences in frequency between nearest neighbors, which is not what we expected. Furthermore, it appears that there is a threshold between 300 and 400m. This could potentially be attributed to the decreased likelihood of an interaction occurring between pairs that distance. The California mouse has female-biased dispersal while males remain in or near natal territories (Ribble 1992), therefore, males that are close in the environment could be related. We expected to observe greater similarities within pairs that are close together. However, vocal learning literature also suggests that within individuals and/or pairs, there can be a divergence between groups that are close together. Song sparrows (*Melospiza melodia*) are able to distinguish neighbors from strangers based on difference in song characteristics (Stoddard et al. 1991). In territorial song birds, individuals can respond to neighbors via song type matching or nonshared song type (Burt et al. 2001). In Diana monkeys (*Cercopithecus diana*) vocal divergence increases in the presence of neighbors (Candiotti et al. 2012). How do population dynamics and associated social interactions influence acoustic properties? Social interactions are important in vocal learning (Janki 1997). It would be

interesting to examine if pairs that share a territory boundary with multiple other pairs adjust vocal characteristics. Do individuals alter vocalizations based on arrivals of new neighbors or acquisition of a new mate? Is there vocal plasticity over the lifetime of an individual?

The difference in pairs varied among pairs, with some pairs showing more similar call characteristics than others. This difference between pairs could potentially be related to differences in duration of the pair bond. We do not understand how long it takes for call convergence within a pair to occur or when it starts. It would stand to reason that there is an adjustment period between members of a pair and production vocalizations with specific acoustic parameters requires time (Tyack 2020). In birds, vocal convergence within a pair takes a minimum of two weeks (Hile et al. 2000; Nowicki 1989). Alternatively, if all the pairs were bonded within the same time period, it is likely that the fine-tuning adjustment of vocal characteristics could be related to learning ability. Individuals can learn at different rates (Hile et al. 2000). It would be interesting to investigate call characteristics over the formation of a pair-bond.

We found that some calls were not different among pairs. The first call in the bout of the 2- and 3SV was not different among pairs but the other calls in the bout were. This difference among call types could potentially be explained by the functional significance of each call. Although we do not fully understand the function of each SV call in the California mouse, we do know that the SVs are often produced when a member of a pair is greater than 1m away and in this context to most likely serve as contact vocalizations (Briggs and Kalcounis-Rueppell 2011; Kalcounis-Rueppell et al. 2018). Alternatively,

our sample size potentially did not capture the full difference of each call. Lastly, the first call in the series may be a general attention-getting call while the other two call types contain the pair specific information. Zebra finches (*Taeniopygia guttata*) produce calls that contain individual signatures but they also produce calls that are less individualized (Elie and Theunissen 2018).

We did not find a sex difference in acoustic parameters for any call types in the California mouse. In several birds species, both the male or the female can adjusts calls to imitate the partner or a group (Mundinger 1979; Hile et al. 2000). It would be interesting to investigate which individual is altering the spectral characteristics, is it the male, the female or both? Do females have the same brain organization, physiology and neuromuscular control as the males to make the adjustment to call characteristics?

Overall, we found that in the California mouse there are greater similarities within a pair than among pairs for duration and frequency variables. Our results are consistent with other studies which have demonstrated that vocal production learning occurs in individuals that form life-long pair or social bonds to show similarities in acoustic characteristics (Hile and Striedter 2000; Candiotti et al. 2012; Crockford et al. 2004). Our results suggest that there is vocal plasticity in the monogamous California mouse. In a monogamous species, the shared acoustic properties most likely have adaptive significance to strengthen social bonds between members of a pair and to serve as pair distinctive identifiers. Rodent calls are used as behavioral responses for assessing neurobehavioral development associated with specific language-related diseases and

disorders (Fischer and Hammerschmidt 2011). The California mouse could be a good model system for studying vocal learning and the evolution of language.

Ethical Statement

All animal care and use guidelines were followed and research protocols for this study were approved by the University of North Carolina at Greensboro and University of Wisconsin-Madison Institutional Animal Care and Use Committees (IACUC; UNCG 12-004 and UWM L005047-A01) and by California Department of Fish and Wildlife under Scientific Collection Permits (SC-9663 and SC-13190).

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Figure 4.1. Among Pair Mean (± SE) A) Duration, B) Frequency at Maximum Amplitude, and B) Bandwidth from All Call Types Together. Pairs are arranged based on shortest geographic location between nest sites.

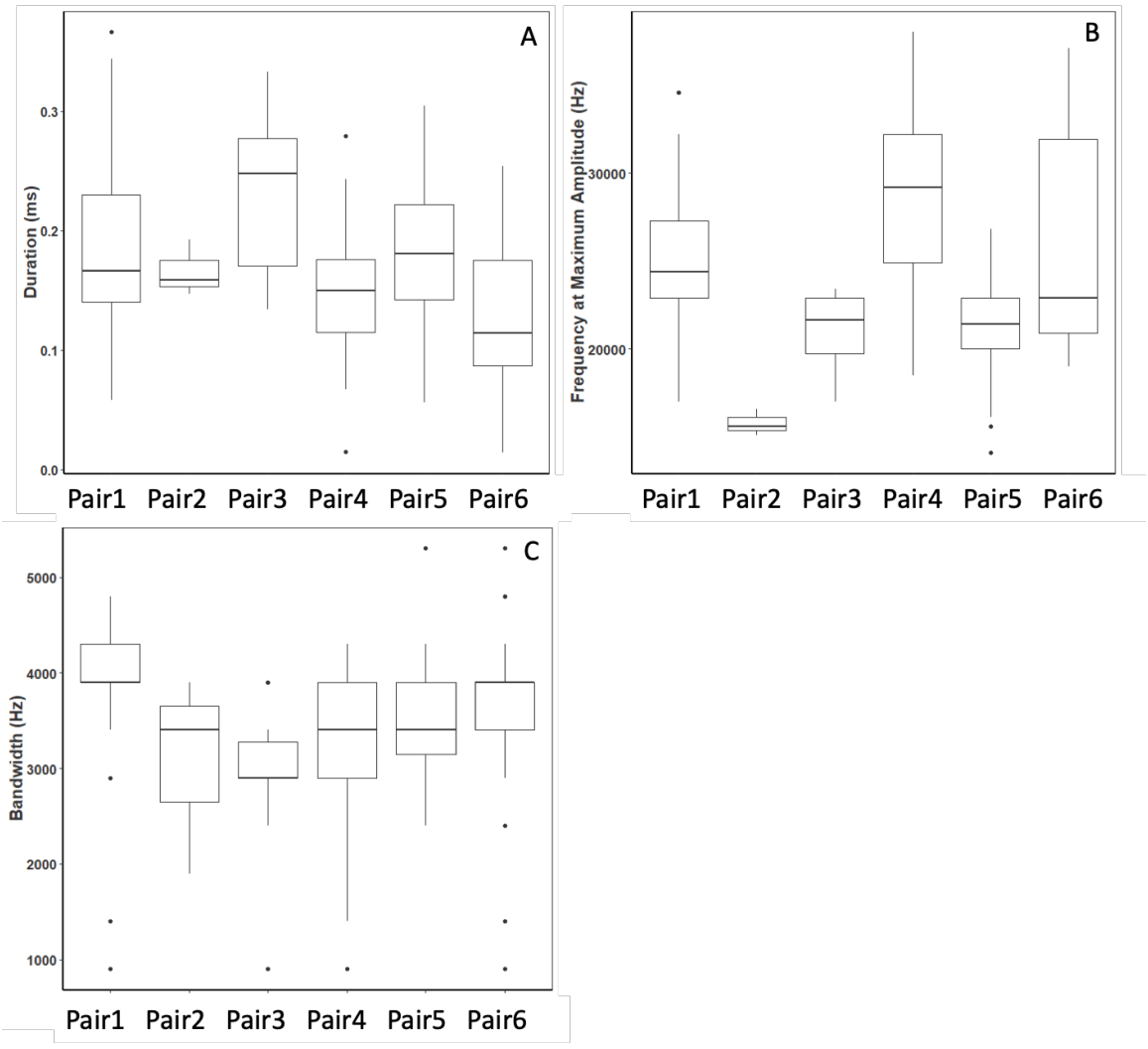

Figure 4.2. Mean (± SE) of Within Pair and Within Population Difference for 1SV Frequency at Maximum Amplitude and 2SV-Call 2 Duration Produced by Pair-bonded California Mice (*Peromyscus californicus*).

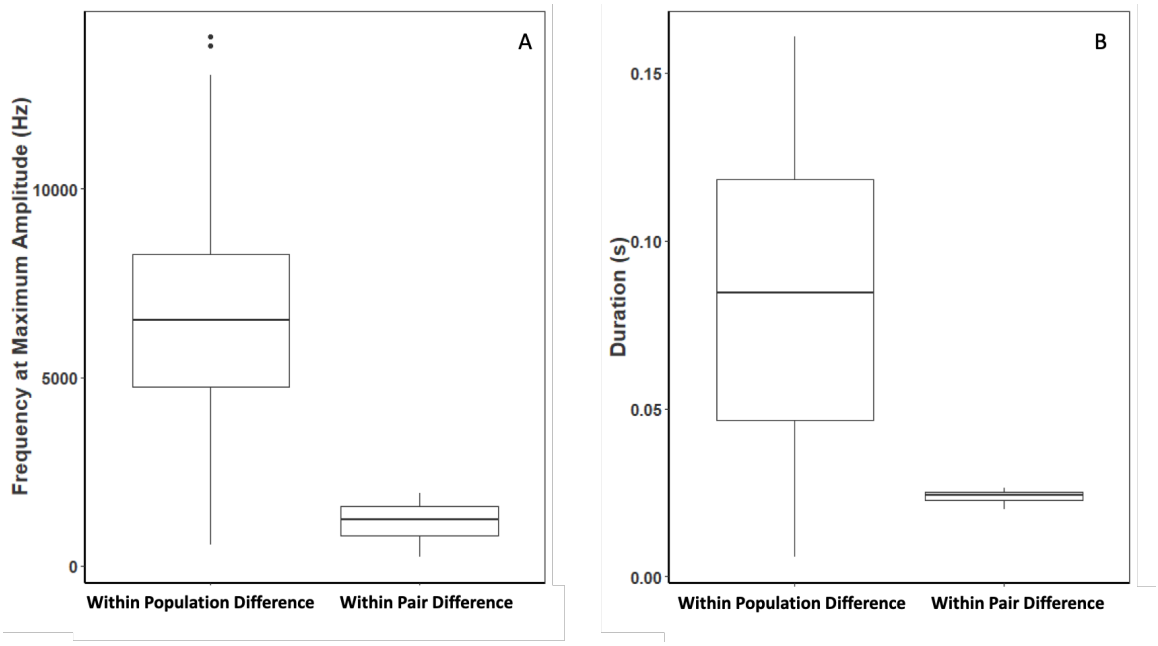

Figure 4.3. Scatterplot of the Difference in Frequency at Maximum Amplitude Between Pairs and Geographic Distance Between Pairs. Scatterplot is from all call types together produced by pair California Mice (*Peromyscus californicus*). Pairs are arranged based on shortest geographic distance between nest sites.

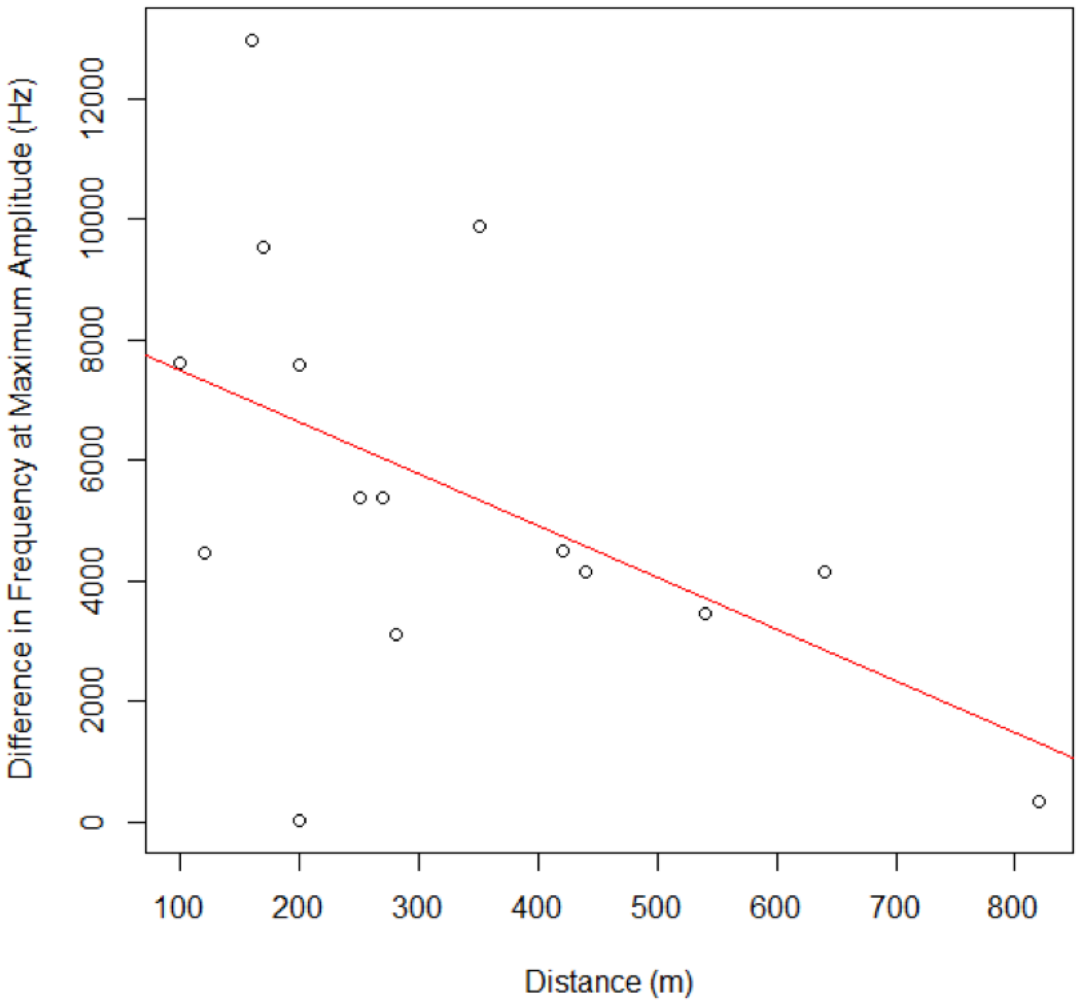

Table 4.1. Descriptive Statistics on All Call Types Combined from Paired California Mice (*Peromyscus californicus*). Pairs are arranged based on shortest geographic distance between nest sites.

All SV Call Types Combined (1-, 2-, and 3SV)							
Pair ID	**Pair**	**Minimum**	**Q25**	**Median**	**Mean**	**Q75**	**Maximum**
Duration (χ2=33.07, df=5, p<0.01)							
ML496	1	0.058	0.140	0.166	0.185	0.230	0.366
ML653	2	0.147	0.153	0.159	0.166	0.176	0.192
ML413	3	0.134	0.171	0.248	0.230	0.277	0.333
ML444	4	0.015	0.115	0.150	0.146	0.176	0.279
ML378	5	0.056	0.143	0.181	0.181	0.222	0.305
M273	6	0.014	0.087	0.114	0.127	0.175	0.254
Bandwidth ($1SV\chi2=33.13, df=5, p<0.01$)							
ML496	1	900	3900	3900	3786.21	4300	4800
ML653	2	1900	2650	3400	3066.67	3650	3900
ML413	3	900	2900	2900	2850	3275	3900
ML444	4	900	2900	3400	3261.54	3900	4300
ML378	5	2400	3150	3400	3576.92	3900	5300
M273	6	900	3400	3900	3728.57	3900	5300
Frequency at Maximum Amplitude							
ML496	1	17000	22900	24400	25300	27300	34600
ML653	2	15100	15350	15600	15766.7	16100	16600
ML413	3	17000	19750	21650	21140	22900	23400
ML444	4	18500	24900	29200	28753.9	32200	38000
ML378	5	14100	20000	21400	21161.5	22900	26800
M273	6	19000	20900	22900	25644.4	31950	37100
PC1 ($1SV\chi2=44.03, df=5, p<0.01$)							
ML496	1	-4.129	-1.448	-0.213	-0.123	1.201	5.786
ML653	2	1.696	1.987	2.278	2.134	2.353	2.428
ML413	3	-2.759	-2.143	-1.474	-1.484	-0.922	-0.172
ML444	4	-5.366	-0.599	0.961	0.423	1.932	3.375
ML378	5	-4.166	-2.602	-2.127	-1.890	-1.213	1.338
M273	6	-2.837	-1.664	-0.666	0.250	2.183	5.508

Table 4.2. Descriptive Statistics on Within Pairs and Within Population Differences in California Mouse (*Peromyscus californicus*) Call Characteristics by Each Call Type. Significant differences are in **bold**.

	Minimum	Q25	Median	Mean	Q75	Maximum	p-value
1SV							
Duration							
Within Population	0.004	0.023	0.040	0.040	0.056	0.085	0.25
Within Pair	0.005	0.009	0.016	0.023	0.030	0.057	
Bandwidth							
Within Population	25.000	343.750	650.000	650.000	881.250	1350.000	0.1
Within Pair	50.000	387.500	500.000	387.500	500.000	500.000	
Frequency at Maximum Amplitude							
Within Population	566.670	4754.167	6512.500	7083.333	8270.833	14025.000	**<0.01***
Within Pair	225.000	806.250	1237.500	1154.165	1585.415	1916.660	
PC1							
Within Population	0.740	2.483	2.907	3.031	3.331	5.815	**<0.01***
Within Pair	0.216	0.407	0.524	0.604	0.721	1.151	
2SV-Call 1							
Duration							
Within Population	0.006	0.047	0.085	0.082	0.118	0.161	**<0.01***
Within Pair	0.020	0.023	0.024	0.024	0.025	0.026	
Bandwidth							
Within Population	0.000	268.750	500.000	493.750	713.195	1175.000	0.55
Within Pair	122.222	180.556	250.000	374.306	443.750	875.000	
Frequency at Maximum Amplitude							
Within Population	233.330	2050.003	3666.665	4222.222	6108.335	10300.000	0.78
Within Pair	700.000	3700.000	5350.000	4683.333	6333.333	7333.330	
PC1							
Within Population	0.105	0.899	1.487	1.706	2.428	3.962	0.71
Within Pair	0.464	1.632	2.151	1.935	2.454	2.974	
2SV-Call 2							
Duration							

Within Population	0.002	0.036	0.088	0.086	0.145	0.181	0.51
Within Pair	0.001	0.030	0.053	0.063	0.086	0.144	
Bandwidth							
Within Population	19.047	419.048	650.001	815.079	1271.429	2066.667	0.61
Within Pair	333.334	458.334	576.191	688.096	805.953	1266.667	
Frequency at Maximum Amplitude							
Within Population	33.330	2112.500	4650.000	5244.642	8230.950	11300.000	0.42
Within Pair	1414.290	1591.072	3191.670	3824.408	5425.005	7500.000	
PC1							
Within Population	0.254	1.040	2.022	2.205	3.310	4.693	0.15
Within Pair	0.504	0.636	1.188	1.327	1.879	2.427	
3SV-Call 1							
Duration							
Within Population	0.001	0.030	0.048	0.054	0.071	0.124	0.95
Within Pair	0.004	0.022	0.028	0.052	0.059	0.150	
Bandwidth							
Within Population	20.000	57.143	240.000	238.393	357.143	607.143	0.67
Within Pair	0.000	230.357	319.643	292.322	381.607	530.000	
Frequency at Maximum Amplitude							
Within Population	246.670	3418.750	4458.930	5077.620	7695.000	12450.000	0.5
Within Pair	107.140	251.785	2908.930	3522.918	6180.062	8166.670	
PC1							
Within Population	0.267	1.146	1.855	2.658	3.640	7.264	0.63
Within Pair	0.085	0.090	1.316	1.979	3.206	5.200	
3SV-Call 2							
Duration							
Within Population	0.002	0.021	0.029	0.239	0.062	3.709	0.55
Within Pair	0.006	0.022	0.053	0.663	0.694	2.541	
Bandwidth							
Within Population	0.000	100.000	443.334	501.800	620.000	1645.000	0.06
Within Pair	2.541	25.635	71.667	78.135	124.167	166.667	
Frequency at Maximum Amplitude							

Within Population	1.169	2110.240	5227.625	5496.514	8382.855	13014.290	0.43
Within Pair	2.541	973.850	1708.810	3371.708	4106.668	10066.670	
PC1							
Within Population	0.026	1.439	2.250	2.446	3.486	5.699	0.62
Within Pair	0.460	0.535	1.550	1.935	2.951	4.180	
3SV-Call 3							
Duration							
Within Population	0.001	0.017	0.025	0.071	0.031	1.169	0.43
Within Pair	0.002	0.017	0.023	0.647	0.653	2.541	
Bandwidth							
Within Population	0.000	140.476	342.857	355.088	542.857	842.857	0.1
Within Pair	2.541	107.778	201.429	184.683	278.333	333.333	
Frequency at Maximum Amplitude							
Within Population	1.169	3008.750	5950.355	6024.343	9319.820	12933.330	0.24
Within Pair	2.541	454.205	1352.380	2951.825	3850.000	9100.000	
PC1							
Within Population	0.302	0.994	1.947	2.108	3.275	4.393	0.51
Within Pair	0.153	0.564	1.621	1.580	2.637	2.926	

Supplemental Table S4.1. Principal Component (PC) Analysis to Yield a Single PC Score to Represent Our Five Frequency Variables (Start, End, Min, Max, and Frequency at Maximum Amplitude).

All Calls Combined	
Acoustic Variable	**PC1 92.20%**
Start Freq (kHz)	0.82
End Freq (kHz)	0.83
Max Freq (kHz)	0.86
Min Freq (kHz)	0.85
Freq Max Amp (kHz)	0.86
1SV	
Acoustic Variable	**PC1 89.70%**
Start Freq (kHz)	0.84
End Freq (kHz)	0.79
Max Freq (kHz)	0.87
Min Freq (kHz)	0.87
Freq Max Amp (kHz)	0.87
2SV- Call 1	
Acoustic Variable	**PC1 95.30%**
Start Freq (kHz)	0.84
End Freq (kHz)	0.83
Max Freq (kHz)	0.86
Min Freq (kHz)	0.86
Freq Max Amp (kHz)	0.86
2SV-Call 2	
Acoustic Variable	**PC1 92.20%**
Start Freq (kHz)	0.83
End Freq (kHz)	0.83
Max Freq (kHz)	0.86
Min Freq (kHz)	0.85
Freq Max Amp (kHz)	0.86
3SV-Call 1	
Acoustic Variable	**PC1 89.70%**

Start Freq (kHz)	0.85
End Freq (kHz)	0.85
Max Freq (kHz)	0.84
Min Freq (kHz)	0.83
Freq Max Amp (kHz)	0.86
3SV-Call 2	
Acoustic Variable	**PC1 89.90%**
Start Freq (kHz)	0.82
End Freq (kHz)	0.84
Max Freq (kHz)	0.86
Min Freq (kHz)	0.86
Freq Max Amp (kHz)	0.86
3SV-Call 3	
Acoustic Variable	**PC1 93.10%**
Start Freq (kHz)	0.82
End Freq (kHz)	0.84
Max Freq (kHz)	0.85
Min Freq (kHz)	0.85
Freq Max Amp (kHz)	0.86

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CHAPTER V

ANTHROPOGENIC NOISE DECREASES ACTIVITY, FEEDING AND VOCALIZATION BEHAVIORS OF WILD MICE

This chapter was coauthored by Radmila Petric and Matina Kalcounis-Rueppell

Abstract

Anthropogenic noise is a pollutant that alters the natural soundscape. Animals rely on sound to mediate a myriad of daily activities. As human infrastructure expands, noise increases, and it is important to understand the effects of broadband (audible and ultrasonic) anthropogenic noise on the behavior of free-living animals. We broadcasted anthropogenic noise or familiar noise to wild deer mice (*Peromyscus maniculatus)* and woodland jumping mice (*Napaeozapus insignis*) to assess the effects of anthropogenic noise. We measured individual mouse activity, foraging behavior, and vocalizations at 25 sites in the Southern Appalachian Mountain Range of North Carolina, USA. Deer mice spent less time at sites with anthropogenic noise. Deer mice were less likely to approach food than woodland jumping mice in the presence of anthropogenic noise, and both species spent less time foraging and vocalizing in the presence of noise. Our results suggest anthropogenic noise affects activity, foraging and vocalization behaviors in nocturnal animals. Furthermore, we demonstrate species-specific responses to the same noise stimulus, and this is important to consider when implementing conservation efforts to mitigate the negative effects of noise across species.

Introduction

Anthropogenic noise is a global pollutant that alters the natural soundscape which animals rely on for foraging, navigation, exploration, predator avoidance and communication (Barber et al. 2010). To meet the high transportation demands of the expanding human population, the development of urban and agricultural areas with concomitant transportation networks are rapidly increasing (Dulac 2013). Road traffic and construction noises are recognized as the most prominent sources of noise pollution that affect a variety of animal species (Jamrah et al. 2006; McGregor et al. 2013). As human infrastructure expands, noise also increases, and it is important to understand the effects of broadband (audible and ultrasonic) anthropogenic noise on the behavior of free-living animals.

Animals rely on the soundscape to mediate a myriad of daily activities (Panyutina et al. 2016). Anthropogenic noise can have a detrimental effect on communication by directly and indirectly influencing the production and propagation of signals, which alter both senders and receivers' behavior, physiology, and survival (Babisch 2003; Jarup et al. 2008; Radford et al. 2014; Slabbekoorn and Ripmeester 2008). Signal impairment in any context could lead to decreased individual fitness which may lead to altered species composition (McLaughlin and Kunc 2013). In general, noise negatively affects communication in two ways: 1) by eliciting energetically costly anti-predator behaviors which shift energy away from exploring, foraging and vocal communication, and 2) by masking vocal signals which prevent successful propagation, detection, and interpretation of acoustic signals (Frid and Dill 2002).

Masking occurs when anthropogenic noise overlaps the spectral and temporal characteristics of animal signals (Slabbekoorn and Ripmeester 2008). This induces a sensory overload in the receiver by making decoding and differentiation between relevant and irrelevant signals difficult (Slabbekoorn and Ripmeester 2008). Noise interferes with the signal transmission which alters the message, perception of sound, and the response of the receiver (Bee and Swanson 2007). Animals have two choices for dealing with the masking effects of noise, either leave the area or adjust their behavior (McLaughlin and Kunc 2013).

In some species, the senders can cope with the masking effects of noise by adjusting spectral and/or temporal characteristics of vocalizations (Nelson et al. 2017). In the presence of anthropogenic noise, some animals have the vocal plasticity to shift duration, amplitude, frequency, and/or timing of the vocalizations (Nelson et al. 2017). For example, common marmosets (*Callithrix jacchus*) make each call last longer by adjusting the duration of vocalizations (Brumm et al. 2004). As noise pollution increases, other species sing louder by increasing signal amplitude (Brumm and Todt 2002; Lowry et al. 2012). To avoid signal masking and deterioration, birds may also vocalize with a higher minimum frequency producing calls above the frequency of the overlapping anthropogenic noise (McLaughlin and Kunc 2013). Lastly, if there are temporal shifts in anthropogenic background noise, then animals may utilize the "quiet" times to resume calling (Fuller et al. 2007). However, if background noise levels are high and changing the frequency, amplitude, or duration does not have an effect on successful signal transmission, vocal signaling may cease (Warren et al. 2006; Ophir et al. 2010). Other

species lack vocal plasticity and cannot mitigate the negative effects of noise by changing spectral and temporal characteristics of vocalizations. Therefore in the presence of noise, they stop vocalizing (Nelson et al. 2017). While senders can adjust some vocalizations aspects to avoid masking, receivers are not able to employ the same strategy (McLaughlin and Kunc 2013).

Some sounds are a byproduct of routine life-sustaining activities produced while breathing, walking, and eating. Animals use sounds as cues to listen for approaching predators or to hunt their prey (Knudsen and Konishi 1979). Anthropogenic noise can have a strong masking effect on sound cues which can negatively impact both prey and predators and this is important to considers in foraging ecology (Muhly et al. 2011). If the cue is masked by noise, it could lead to a missed feeding opportunity for the predator or a loss in predator detection by the prey, which leads to increased predation risk and decreased fitness (Schaub et al. 2008; Muhly et al. 2011; Leighton et al. 2010). For example, greater mouse-eared bats (*Myotis myotis*) rely on cues to passively locate invertebrates. In the presence of traffic noise, the overall search duration increased and hunting success decreased (Siemers and Schaub 2010). A study on fringe-lipped bats found that when audible traffic noise is present, bats shift from passively listening for their prey (gleaning) to producing prey detecting ultrasonic vocalizations (USVs) thus changing their sensory system (Gomes et al. 2016).

Noise disturbance has been identified as a form of predator risk stimulus. The behavioral responses exhibited by an animal in noisy habitats are similar to the behaviors observed in the presence of a predator (Grafe et al. 2002). Apprehension is defined as

increased attention toward predator detection (Kotler et al. 2004). The duration and location of an individual's activity is dependent on the perceived risk of predation (Kotler et al. 2004). In prey species, there is a tradeoff between food acquisition and predator avoidance (Bleicher et al. 2019). Thus, anthropogenic noise increases apprehension which in turn decreased activity and foraging effort (Waynert et al. 1999). These effects can be mild, short-term, and reversible or they can be permanent and irreversible (Torre and Snowdon 2002). The shift in the allocation of time and energy toward listening and being alert could lead to a significant increase in time spent hiding and decrease in foraging, mating, and vocal behaviors (Habib et al. 2007; Brown et al. 2012; Barber et al. 2010) which decrease the reproductive success of individuals. Anthropogenic noise can indirectly affect communication by altering spatial behavior, which results in altered individual, social, and reproductive behaviors (Habib et al 2007; Brown et al. 2012; Barber et al. 2010). Furthermore, anthropogenic noise can cause individuals to abandon breeding grounds which can then alter species abundance (Bunkley et al. 2017) and distribution (Habib et al. 2007; Brown et al. 2012; Barber et al. 2010).

Anthropogenic noise is pervasive in the audible range during the day and most of what we know about the effects of noise on animals comes from studies during the day. However, noise extends into the ultrasonic range and we were interested in studying free-living nocturnal animals that have not been examined for responses to broadband anthropogenic noise. The objective of this study was to understand how broadband anthropogenic noise influences behavioral responses in the free-living nocturnal deer mouse (*Peromyscus maniculatus*) and woodland jumping mouse (*Napaeozapus insignis*).

Deer mice are the most abundant and widely distributed native rodent species in North America, making anthropogenic noise results relevant to a wide geographic range. Furthermore, vocalizations are a component of their behavioral repertoire which serves to mediate social interactions and facilitate mating (Pomerantz and Clemens 1981; Kalcounis-Rueppell et al. 2018). We hypothesized that broadband anthropogenic noise would alter the allocation of time and energy spent toward exploring, foraging and vocalizing. We predicted that if there is an effect of anthropogenic noise then, in the presence of noise, mice would: 1) spend less in the study site, 2) forage less, and 3) produce fewer vocalizations or alter their vocalizations.

Methods

Study Paradigm

Our field work was conducted at the Highlands Land Trust Property (Brushy Face) (35°054'N, 83°188'W), Turtle Pond which is part of Nantahala National Forest (35°234'N, 83°559'W), and Highlands Biological Station (35°054'N, 83°188'W), North Carolina, USA. All sites were in Macon County, at least 2000 feet above sea level in the Southern Appalachian Mountains. At each study site, after determining the resident mice and their main areas of activity (focal area), we deployed radio telemetry, a foraging tray, full-spectrum audio recording, and a thermal imaging camera to measure behaviors. Specifically, we used radio telemetry to collect individual activity data from deer mice. We used foraging trays to collect foraging data from deer mice and jumping mice. We used audio recording to collect vocalization data from deer mice and jumping mice. We used the thermal imaging camera to collect activity and foraging activity because the

video allowed us to determine which species first entered the focal area and the foraging tray and how long each stayed in the focal area and the foraging.

We used a "before-during" experimental design. For the "before" behaviors, we eavesdropped on the resident mice for three consecutive nights (nights one to three) without noise manipulations. We then introduced a noise manipulation of either anthropogenic noise or familiar noise, at the same site by broadcasting noise for the next three nights (nights four to six). At any given site we only broadcast anthropogenic or a familial noise. Our broadcast manipulation was of road generator noise (ranging from 1-42kHz; anthropogenic noise) or sounds from a creek (ranging from 1-12kHz; familiar noise) recorded from the Brushy Face study site. At each focal area, noise type (anthropogenic or familiar) was randomly assigned by a third party. This study initially focused on deer mice but at our sites woodland jumping mice were common and we were able to measure and report some of the behavioral responses for both species.

To broadcast set up an AT-100 ultrasound speaker (Binary Vocal Technology LLC. Tucson, Arizona, USA) to broadcast sounds to the focal area. The speaker was set 2-meters from the edge of the focal area, and 40-cm off the ground. The speaker was connected to a small laptop (DELL Latitude E6230) and operated using G'Tools version 1.6 PLAY'R ultrasonic generation software (Binary Vocal Technology LLC). Using PLAY'R ultrasonic generation software, an hour-long recording of anthropogenic noise or familiar noise was broadcasted on a continuous loop for eight hours per night at a volume of approximately 90dB re 1 μPa. The broadcasting specifications (duration and sound level) were selected based on a laboratory rat study (Oliveira et al. 2009).

We measured seven response variables to our experiment as follows and for each variable, we calculated the differences between "during" broadcasting nights (nights four to six) and "before" broadcastings nights (nights one to three). The difference was calculated within each focal area in the following way: night four - night one; night five - night two; night six - night three. The difference was a measure of the effect of noise allowing for an effect of night, with a zero-difference indicating no effect of noise. We measured the following variables:

1) *Difference in total time spent in the focal area* – number of minutes a deer mouse spent in the focal area from sunset to sunrise was measured for deer mice through radio telemetry. We first determined resident deer mice by trapping at our study sites to identify individuals in the area. Trap stations across all study sites were set with a 10-meter spacing between stations. At Brushy Face, trap stations were set approximately in a 5x20 grid configuration, at Turtle Pond trap stations were set approximately in a 1 x 65 transect, and at Highlands Biological Station trap station were set in approximately in a 1x100 transect. At each trap station, we had two Sherman traps (7.6x8.9x22.9-cm) baited with a rolled oat and sunflower seed mixture and on cold nights we added a small piece of bedding. Traps were set an hour before sunset and checked at midnight and then again two hours before sunrise. Upon capture of a mouse, we recorded age, sex, reproductive condition and mass (for details see Petric and Kalcounis-Rueppell 2013). All newly captured mice were marked with a unique numbered ear tag and released at the site of capture after handling procedures. We identified resident mice as adult individuals captured two or more times in the same station or the surround five stations in any

direction over five trapping nights. We then selected a resident mouse and outfitted the individual with a 0.55g M1450 mouse style transmitter (Advanced Telemetry System [ATS]). We only outfitted and followed one individual at a time. The transmitter was tailored to fit the individual in a necklace style (as described in Petric and Kalcounis-Rueppell 2013). Using handheld radio telemetry, we determined the primary area of activity for the individual. Based on the primary area of activity of the mouse, we established a focal area for our experiment (approximate 3x5.5-meter area). To measure the total time a deer mouse spent in the focal area, we deployed the automated radio telemetry equipment R4500S DCC receiver/datalogger (ATS, Isanti, MN, USA) to record the number of minutes the deer mouse was present in the focal area. The datalogger was connected to an antenna in the center of the focal area and was programmed to continuously detect the transmitter frequency of the collared mouse. All detected signals were stored in the datalogger. The morning following data collection, the datalogger was removed from the field and brought to the lab to download the data and charge the unit. This allowed us to understand how individuals respond to noise.

2) *Difference in total food consumed* – we measured the number of seeds consumed in the foraging tray and the number of husks leftover at the foraging tray. We set out a single tray in the center of the focal area. Each tray had 20 whole sunflower seeds mixed into five cups of sand in a plastic plant saucer (30.48-cm diameter, 5.08-cm deep). The seed/sand mixture was protected from the rain with a clear, 40.64-cm diameter plate that was supported by four stakes and suspended 8 cm above the mixture. The following morning, we used a mesh strainer to separate the sand from the leftover

seeds/husks. The intact seeds were collected and counted. We also assessed and classified leftover husks into 3 categories 1) 0 husk pieces, 2) 1-5 husk pieces and 3) > 10 husk pieces remaining after each night. A zero in leftover husks indicated that the mice collected the seeds but did not take the time to consume the seeds in the foraging tray, whereas, >10 leftover husks indicated that mice took the time to consume the seeds in the foraging tray. This allowed us to understand how at each focal area noise affects overall foraging activity.

3) *Difference in latency to enter the focal area* – we measured, on our video recordings, the number of minutes post-sunset until the first mouse (deer mouse or a woodland jumping mouse) to appeared in the focal area.

4) *Difference in time spent in the focal area on the first visit* – we measured, on our video recordings, number of seconds the first mouse (deer mouse or a woodland jumping mouse) spent in the focal area after first entering the focal area.

5) *Difference in latency to start foraging* – we measured the number of minutes post-sunset until the first mouse (deer mouse or a woodland jumping mouse) entered a foraging tray.

6) *Difference in time spent foraging* – we measured, on our video recordings, the number of seconds the first mouse (deer mouse or a woodland jumping mouse) spent in the foraging tray. As highlighted by other anthropogenic noise studies, latency to - and time spent- being active and forage(ing) are important measures of the initial behavioral response which could have direct consequences for individual reproductive success and survival (Chan et al. 2010; Miller and Degn 1981; Barber et al. 2010; Schaub et al. 2008).

For video recording we used a thermal imaging lens (Photon 320 14.25 mm; Flir/Core By Indigo) set to record through the night in the focal area. The lens was mounted on a 2-meter tripod to capture the entire focal area in the field of view. The lens was connected to a JVC Everio HDD camcorder and powered by an external dry cell car battery connected to an inverter. The thermal imaging set-up was set to record continuously throughout the night. The camcorder was removed from the field the following morning.

To analyze video, we randomly selected a subset of 60 nights from our total data set to a target of 40% of nights. We watched the video footage in real-time to measure the time for each of the four described variables (variables 3-6). We were able to identify the species of the mouse, a deer mouse or a woodland jumping mouse, based on their movement. Deer mice move across the forest floor with little to no vertical movements whereas, woodland jumping mice propel their bodies at least 10-cm vertically as they move.

7) *Production* of vocalizations– we measured the number and types of calls produced by mice through audio recordings. To measure call production, we used 12 ultrasonic microphones (Emkay FG Series from Avisoft Bioacoustics, Berlin, Germany) connected to an UltraSoundGate system 1216H (Avisoft Biovocals, Berlin, Germany), which was connected to a small laptop (DELL Latitude E6230). The microphones were arranged in a 3x4-meter grid configuration approximately 1-2-m apart. Using Avisoft RECORDER Software, the system was set to record when sonic and ultrasonic sounds were detected by the microphone (s). Microphones were triggered and a .wav file was

recorded when sounds were detected. Each morning, files were downloaded. All files recorded were examined using Avisoft SAS Lab Pro (Avisoft Bioacoustics). All recorded mouse calls were identified and counted in Avisoft SAS Lab Pro. Using the time stamp on the recording we matched the vocalization with the time of the activity on the video footage (Briggs and Kalcounis-Rueppell 2011; Petric and Kalcounis-Rueppell 2013), allowing us to assign the vocalization to species (deer mouse or woodland jumping mouse) based on movement. This allowed us to understand how at each focal area noise affects the overall mouse calling behavior.

This work was approved through the following permissions: UNCG IACUC 16-002, NC Wildlife Resource commission 17-ES00336 and 17-SC00162, North Carolina Park Services 2720, Highlands Biological Station IACUC 16-08, and Highlands-Cashers Land Trust.

Statistical Analyses

All data were checked for normality and equality of variances. We used the package lem4 (Bates et al. 2015) to fit Generalized Linear Mixed Models (GLMM) with the site as the random term, noise type as the fixed term, and night as a covariate to the following variables: difference in total time spent in the focal area, difference in total food consumed, difference in number of husks leftover, difference in total USVs produced, difference in latency to enter the focal area, difference in time spent in the focal area on the first visit, latency to start foraging, and difference in time spent foraging.

We were also interested in species-specific responses to noise type. We used the Chi-squared test of Independence to examine the relationship between first species to appear (deer mouse or woodland jumping mouse) and the presence of noise ("before" or "during"). For additional species-specific analyses, we could not calculate the differences between "during" broadcasting nights (nights four to six) and "before" broadcastings nights (nights one to three) within a species because the effect of noise on deer mice reduced their activity during noise (see below). Thus, we used Mann-Whitney U tests for video derived variables. All data are represented using box plots to show skewness and outliers in the data. We used an alpha level of $p<0.05$. All data were analyzed using R software version 3.2.2 (R Core Team 2018).

Results

Sample Size

We ear tagged 107 deer mice and 48 woodland jumping mice which were recaptured 449 times from June to August 2016, 2017, and 2018. We radio-collared and broadcasted sounds to the 21 sites with collared deer mice (anthropogenic noise=13, familiar noise=8) for a total of 126 days. We broadcasted noise to additional 4 sites that did not have a radio-collared mouse (anthropogenic noise=1, familiar noise=3) for a total of 25 focal areas with broadcasting noise (anthropogenic noise=14, familiar noise=11). The thermal imaging equipment was set-up in 25 focal areas (anthropogenic noise =15, familiar noise =10) and we analyzed a subset of 60 recording nights from 10 focal areas (anthropogenic noise =5, familiar noise =5). The foraging tray was set-up at 25 focal areas (anthropogenic noise=15, familiar noise=10). Due to raccoon disturbance, we did

not collect data from one night at two different anthropogenic broadcasting sites. Our final foraging dataset consisted of 148 recording nights from 25 focal areas (anthropogenic noise=15, familiar noise=10).

1) Difference in Total Time Spent at the Focal Area

Difference in time spent in the focal area was affected by noise type and deer mice spent roughly 90% less time during the broadcasting of anthropogenic noise compared to familiar noise (GLMM Estimate 71.43±28.13, p=0.02; Figure 5.1). Difference in time spent in the focal area was not influenced by night (night five GLMM Estimate 11.95±27.96, p=0.67; night six GLMM Estimate 29.29±27.96, p=0.30; nights five and six are compared to night four for this type of analysis). Total time deer mice spent in the focal area on each night at each site is shown in table 5.1.

2) Difference in Total Food Consumed

The number of seeds consumed did not differ between familiar and anthropogenic noise broadcasts (GLMM Estimate 7.42±4.77, p=0.13) and there was no night effect (night five GLMM Estimate -0.86±5.80, p=0.88; night six GLMM Estimate 8.20±5.67, p=0.16). However, 97% fewer husks were leftover during broadcasts of anthropogenic noise when compared to familiar noise (GLMM Estimate 1.69±0.13, p<0.01; Figure 5.4). There was no night effect for the number of husks leftover (night five GLMM Estimate -0.14±0.13, p=0.39; night six GLMM Estimate 0.20±0.13, p=0.13).

3 and 4) Difference in Latency to Enter the Focal Area and Time Spent in the Focal Area on the First Visit

Latency for any mouse to enter the focal area was similar during the broadcasting of familiar and anthropogenic noise (GLMM Estimate -5.73±22.40, p=0.80). There was no night effect on latency for a mouse to enter the focal area (night five GLMM Estimate 6.10±27.44, p=0.83; night six GLMM Estimate -18.6±27.44, p=0.50). However, at anthropogenic noise sites, the first mouse to appear on the before nights was more likely to be a deer mouse, whereas, during the broadcasting nights the first mouse to appear was more likely to be a woodland jumping mouse ($\chi 2$=9.5, df=1, p< 0.02).

The length of the first bout in the focal area did not differ between familiar and anthropogenic noise (GLMM Estimate 66.00±161.58, p=0.69) and there was no night effect (night five GLMM Estimate 175.00±176.64, p=0.34; night six GLMM Estimate 125.30±176.64, p=0.49).

5 and 6) Difference in Latency to Forage

Latency to forage was roughly 60% longer during anthropogenic noise compared to familiar noise (GLMM Estimate -79.93±31.70, p<0.04; Figure 5.2). There was no night effect in latency to forage (night five GLMM Estimate 15.00±33.63, p=0.66; night six GLMM Estimate 1.90±33.63, p=0.96). Furthermore, within anthropogenic noise, the first mouse to start foraging was more likely to be a deer mouse, whereas, during broadcasting nights the first mouse to start foraging was more likely to be a woodland jumping mouse ($\chi 2$=9.5, df=1, p<0.02).

Time spent foraging was 89% shorter during anthropogenic noise (GLMM Estimate 222.73±97.29, p=0.05; Figure 5.3) compared to familiar noise. There was no night effect for time spent foraging (night five GLMM Estimate -110.30±121.72, p=0.37; night six GLMM Estimate -43.50±121.72, p=0.72).

7) Vocalization Production

We recorded 353 mouse calls and assigned 204 calls to deer mice and 149 calls to woodland jumping mice. We only recorded 6 vocalizations during the broadcasting nights (anthropogenic noise=2, familiar noise=4) and the remaining 347 were recorded during the three nights before broadcasting. There was no significant difference in the number of vocalizations produced between anthropogenic and familiar noise sites (GLMM Estimate 7.71±4.96, p=0.13) and there was no night effect (night five GLMM Estimate -6.16±5.97, p=0.31; night six GLMM Estimate 0.44±5.97, p=0.94).

Discussion

Our study is the first to demonstrate a direct impact of broadband anthropogenic noise on individual responses of nocturnal rodents. In the field, using playbacks of anthropogenic noise and familiar noise, we provide evidence that 1) deer mice individuals spend less total time in the focal area in the presence of anthropogenic noise but not familiar noise; 2) woodland jumping mice are more likely to enter the focal area during broadcasting of anthropogenic noise than deer mice 3) during broadcasting of anthropogenic noise, mouse latency to enter the focal area increases and mice spend less time foraging; 4) woodland jumping mice and deer mice decrease vocal communication during broadcasting nights at both anthropogenic noise and familiar noise sites. The

presence of broadband anthropogenic noise introduced to a natural soundscape alters the behaviors expressed by native species, which could have long-term implications for the fitness of individuals.

In the presence of anthropogenic noise, individuals decreased time spent in the focal area. Our results support the hypothesis that anthropogenic noise reduces animal abundance (Reijnen et al. 1995). Individuals may shift spatial preferences and allocate less time in areas with elevated anthropogenic noise because these sites may hinder the ability for an individual to detect predators, communicate, defend a territory, attain a mate, and reproduce (Barber et al. 2010). Animals mitigate the negative effects of noise by altering space use or by dealing with the noise or they risk becoming locally extinct (Slabbekoorn and Ripmeester 2008). Noisy environments can reduce individual awareness of predators as observed in Ambon damselfish (*Pomacentrus amboinensis*) where the fish were twice as likely to preyed upon in the presence of noise (Simpson et al. 2016). Due to the effects of anthropogenic noise on animals, it is expected that individuals may experience disruption and avoid environments with high noise levels. We found that deer mice exhibit spatial avoidance by spending less time in the physical environment with elevated sound level. It would be interesting to investigate where the mice are going and what they are doing. Are they going back to their nest or are they allocating more time to different areas of their territory? It would also be interesting to investigate predation risk in the presence of noise.

In the presence of anthropogenic noise, mice alter their foraging activity. The time it takes a deer mouse to initially approach the foraging tray increases with the onset of

broadcasting. However, we did not find a difference in the total number of seeds consumed over the night and this lack of difference could potentially be attributed to species. The woodland jumping mouse appears to be more tolerant of noise. During the broadcasting nights, the woodland jumping mouse was more likely to approach the foraging tray than the deer mouse. However, both species respond to the broadcasting of anthropogenic noise. Although the number of seeds consumed did not differ between the sound type or broadcasting nights, there was a difference in the amount of time a mouse spent in the foraging tray independent of species. In the presence of anthropogenic noise but not familiar noise, the number of leftover shells decreases, which also corresponds to our thermal imaging footage which shows mice spend less time in the foraging tray during anthropogenic noise broadcasting nights. Together, our results suggest that anthropogenic noise alters the perception of risk and fear, and mice become more vigilant. Our foraging and video results are consistent with the risk disturbance hypothesis in which animals exposed to noise spend less time foraging and more time being vigilant (Evans et al. 2019; Morris-Drake et al. 2017; Brown et al. 2012; Shannon et al. 2014). Furthermore, if individuals are spending more time being vigilant, then they are spending more time listening than vocalizing. Interestingly, any change in the soundscape alters vocal production in both species of mice. In our study, familiar noise did not affect spatial preference or foraging effort, but it did alter vocalization production. This suggests that mouse vocalizations are potentially sensitive to signal degradation and individuals may recognize the additional sounds in the soundscape as unsuitable conditions for successful propagation of acoustic signals.

Why there is a species difference in response between woodland jumping mice and deer mice? One proposed explanation is the ear morphology differences between the two species (Preble 1956; Blair 1950) and therefore, there is potentially a sensitivity difference to the perception of external noise. There should be a cost for individuals that engage in vigilance because they are more likely to mount a stress response and coupled with decreased foraging, the overall energetic intake and nutrition would be altered. For example, in tree swallows (*Tachycineta bicolor*), traffic noise-induced greater oxidative stress, lower body weight and delayed fledging (Injaian et al. 2018). Whereas, in other species like the great tits (*Parus major*), noise decreases the clutch size and the number of chicks that fledge (Halfwerk et al. 2011), showing a direct fitness cost. It would be interesting to understand the physiological response of wild mice to noise and if there are any long-term effects on survival and reproduction.

Another potential explanation for differences between woodland jumping mice and deer mice is boldness. Activity during the presence of anthropogenic noise is risky behavior (Chan et al. 2010), and boldness is the inclination of an individual to engage in risky behavior. We collected anecdotal data from trap releases during our last field season by examining latency to exit a trap in the presence of a potential threat. These results suggest that there are personality differences between species, however, this would require further investigation. By examining species personalities in response to environmental challenges like anthropogenic noise, we can make predictions about changes in species composition and the ecological and evolutionary consequences of those changes. Furthermore, if individual presence decreases in an environment in

response to anthropogenic noise, the species may disappear which can alter the overall landscape and opens the area for potentially invasive species that are much better adapted to anthropogenic influences. Understanding how rapid changes in the soundscape leads to altered time allocation, foraging and vigilance are important when developing conservation strategies.

Overall, broadband anthropogenic noise reduces activity, foraging and vocalization production in deer mice and woodland jumping mice, a group of animals that were not previously studied. Our results are consistent with previous research from other taxonomic groups, which demonstrate that anthropogenic noise negatively impacts multiple aspects of animal behaviors (Barber et al. 2010). In nature, rodents provide important ecosystem services by directly and indirectly influencing the abundance and distribution of other species, from plants to carnivores (Fischer et al. 2018; Tschumi et al. 2018; Davidson et al. 2008; Zhang et al. 2003). Rodents are important components of ecosystems and our research suggests that anthropogenic noise negatively impacts this group of animals. Furthermore, the species examined in this research respond differently to the same stimulus and the species-specific differences are important for consideration when implementing conservation efforts to mitigate the negative effects of noise. Overall, our results highlight the importance of assessing the effects of noise across the frequency spectrum on multiple species within a community.

Figure 5.1. Difference in Total Time the Deer Mouse (*Peromyscus maniculatus*) Spent in the Focal Area by Broadcasting. Deer mice spent the same amount of time in the focal area during familiar noise but roughly 90% less time during anthropogenic noise (anthropogenic noise=13, familiar noise =8; GLMM Estimate 71.43±28.13, p=0.02).

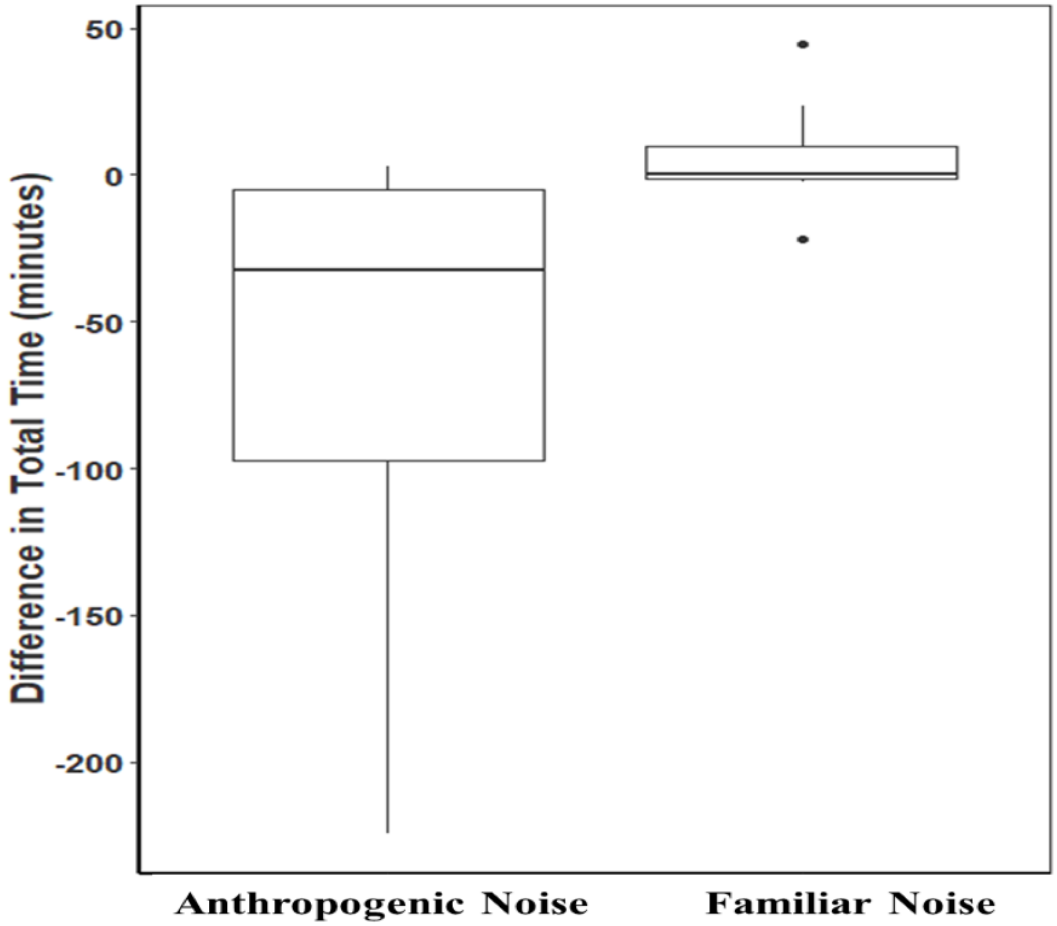

Figure 5.2. Difference in Latency a Deer Mouse (*Peromyscus maniculatus*) and a Woodland Jumping Mouse (*Napaeozapus insignis*) to Start Foraging by Broadcasting Type. Latency to forage was similar during familiar noise but roughly 60 % longer during anthropogenic noise (anthropogenic noise=5, familiar noise=5; GLMM Estimate -79.93±31.70, $p<0.04$).

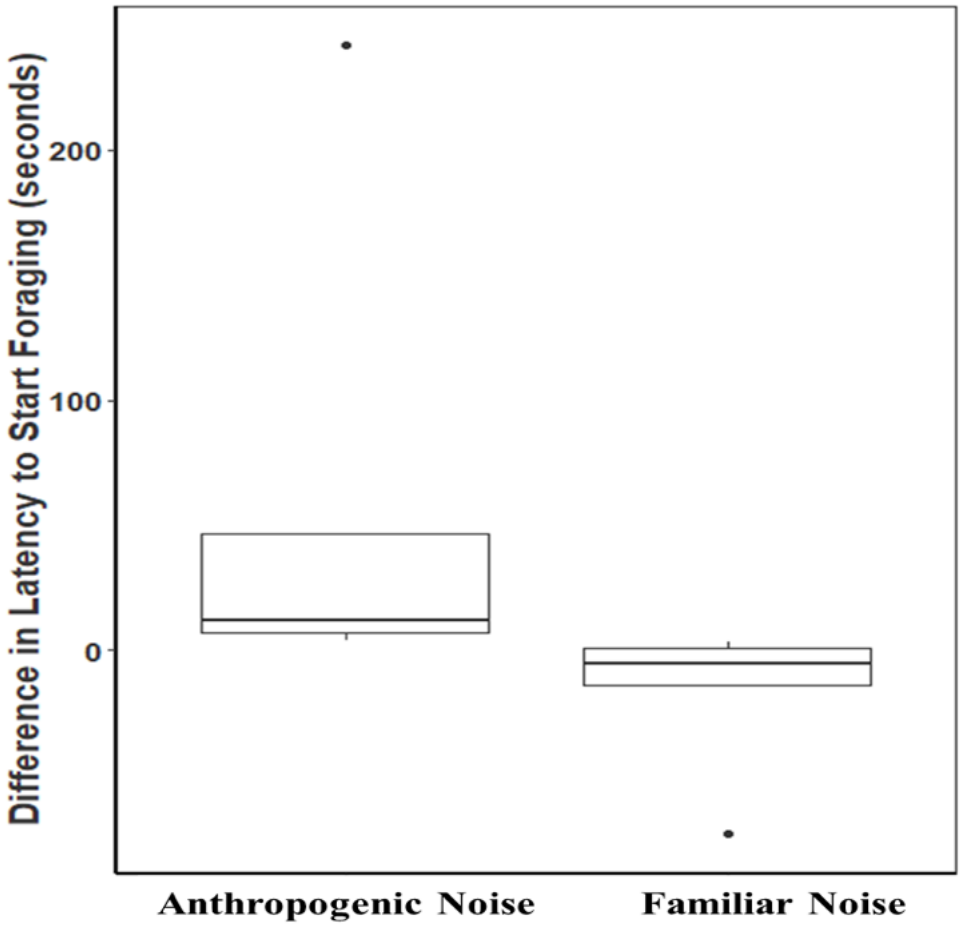

Figure 5.3. Difference in Time Spend Foraging by a Deer Mouse (*Peromyscus maniculatus*) and a Woodland Jumping Mouse (*Napaeozapus insignis*) by Broadcasting Type. Time spent foraging was similar during familiar noise but roughly 89 % shorter during anthropogenic noise (anthropogenic noise=5, familiar noise=5; GLMM Estimate 222.73±97.29, p=0.05).

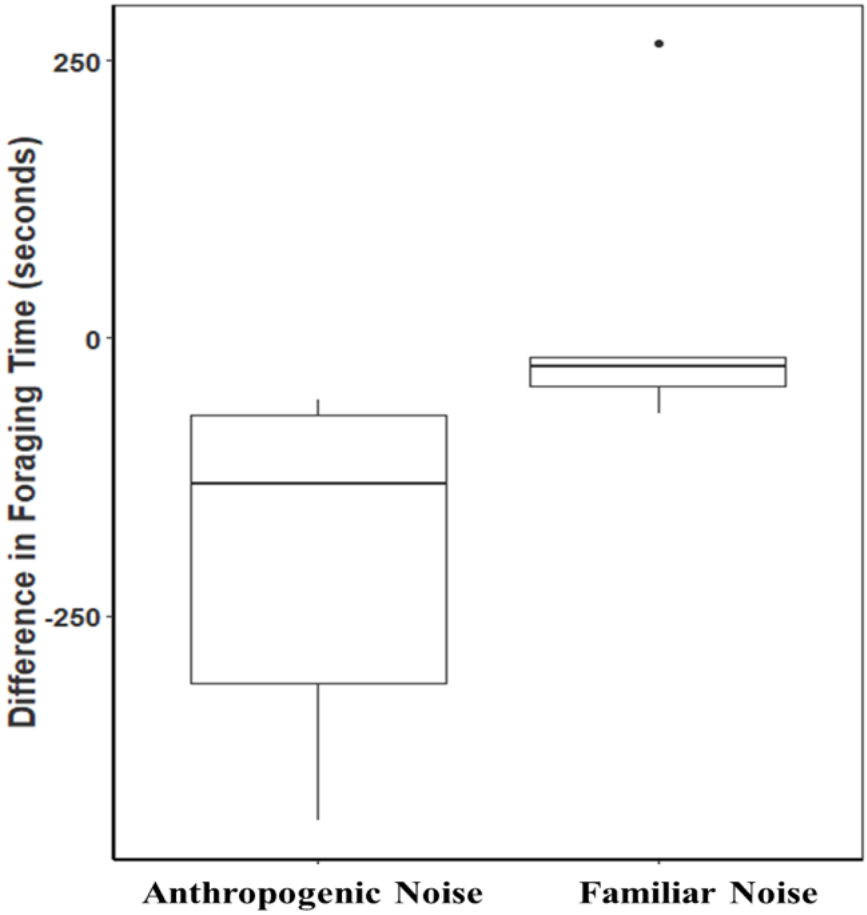

Figure 5.4. The Number of Husks Left Over in the Foraging Tray by Night and Treatment Type. Leftover husks were similar during familiar noise but 97% fewer husks were leftover during anthropogenic noise (anthropogenic noise=15, familiar noise=10; GLMM Estimate 1.69±0.13, $p<0.01$).

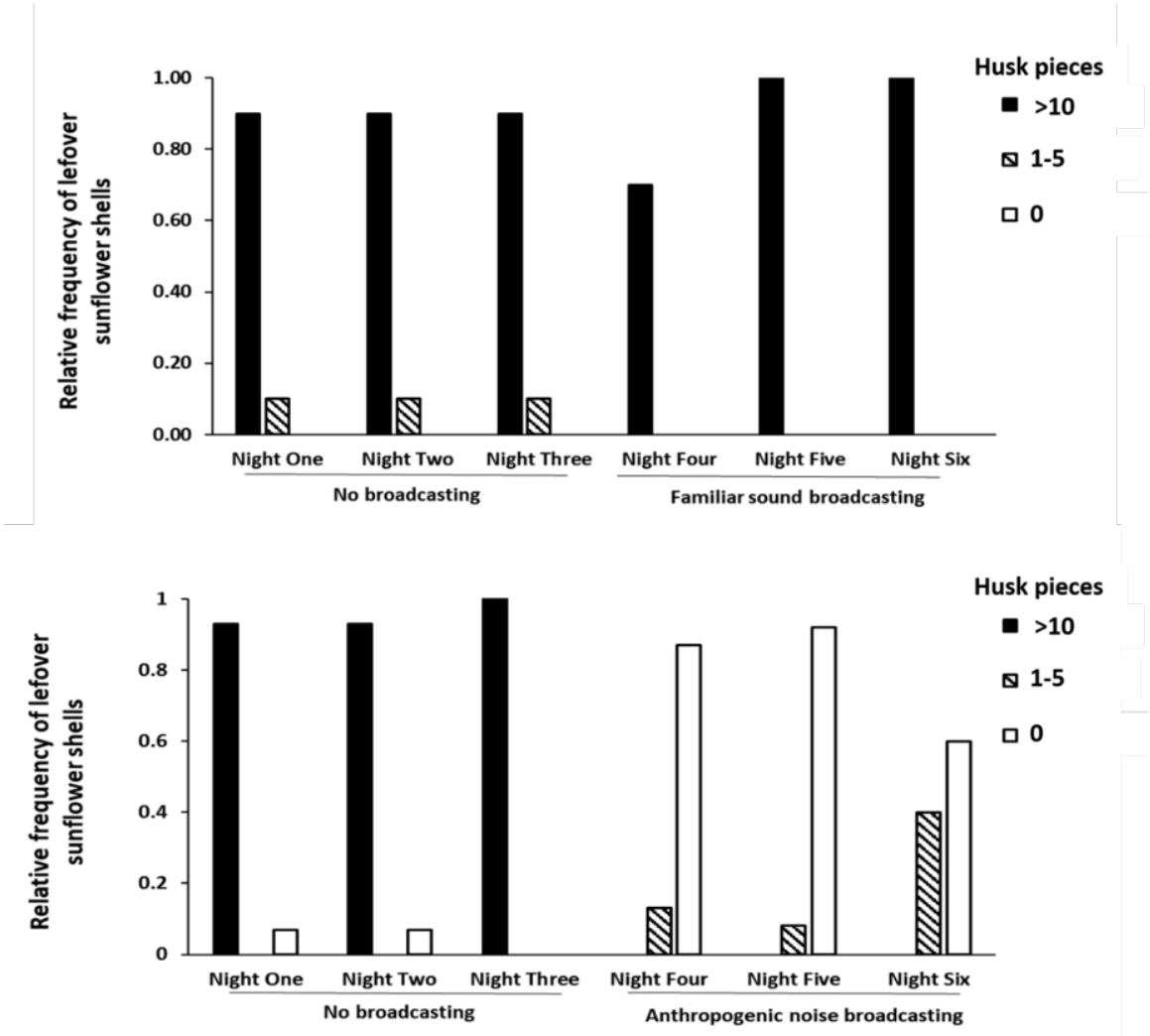

Table 5.1. Descriptive Statistics on the Amount of Time the Deer Mouse (*Peromyscus maniculatus*) Individuals Spent in the Focal Area by Treatment Type and Recording Night. At each site, spatial data was recorded for three consecutive nights without introducing additional noise followed by three additional nights at the same site while broadcasting either anthropogenic noise or familiar noise.

		Broadcasting	n	Mean	SE	Median	Min	Max
Time at the Focal Area	Anthropogenic Noise – Before	No	39	80.5	19.1	34	0	641
	Anthropogenic Noise – During	Yes	39	15.3	4.3	7	0	125
	Familiar Noise – Before	No	24	22.8	8.3	6	0	153
	Familiar Noise – During	Yes	24	28.7	11.1	2	0	205
Time at the Focal Area and Recording Night	Anthropogenic Noise- Night One	No	13	101.5	48.3	34	0	641
	Anthropogenic Noise- Night Two	No	13	72.8	21.8	43	0	244
	Anthropogenic Noise- Night Three	No	13	67.3	24.5	24	0	294
	Anthropogenic Noise- Night Four	Yes	13	12.1	4.9	9	0	67
	Anthropogenic Noise- Night Five	Yes	13	11.2	4	5	0	53
	Anthropogenic Noise- Night Six	Yes	13	22.8	11.3	5	0	125
	Familiar Noise - Night One	No	8	27.1	14.7	6	0	118
	Familiar Noise - Night Two	No	8	17.5	10.1	5	0	78
	Familiar Noise - Night Three	No	8	23.6	18.6	5	0	153
	Familiar Noise - Night Four	Yes	8	36.3	21.1	2	0	152
	Familiar Noise - Night Five	Yes	8	13	7.7	4	0	63
	Familiar Noise- Night Six	Yes	8	36.8	25.6	2	0	205

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CHAPTER VI

CONCLUSION

Overall, my results suggest that in complex field settings where multiple competing stimuli occur simultaneously both the physiological (T-pulses) and environmental (anthropogenic noise) factors influence behavior. The goal of my research was to understand how both factors alter the allocation of time and energy to influence vocal output, signal structure, spatial preference, and other reproductively related behaviors in free-living animals. I found that T-pulses were a positive stimulus that increased vocal communication and space allocation, whereas, the anthropogenic noise was a negative stimulus that decreased vocal communication and space allocation.

Testosterone, the Physiological Factor

I found that in paired-bonded, wild, California mouse males three exogenously administered testosterone pulses at the nest and territory over a five-day period can have long-term effects on the behavioral response of individual males and his non-injected mate. In the male, T-pulses induced preference for the nest location through conditioned place preference (CPP). Males that received testosterone spend more time at the nest and males with pups that received testosterone spent the most time at the nest. Given these results, in the California mouse, testosterone pulses at the nest may be the mechanism for inducing and maintaining paternal care. In response to altered time allocation at the nest by the male, the female in the pair spent less time at the nest. Furthermore, more calls

were produced at the testosterone nests and these calls had a smaller bandwidth, most likely to increase the propagation of the call and attract the mate back to the nest. Overall, in the complex field setting, transient T-pulses can alter the allocation of time to a specific location within a territory (the nest) and not toward behaviors such as aggression, pair bonding, or mate guarding.

Contrary to our T-pulse at the nest results at which T-males spent more time at the nest via CPP, at the territory boundary the males did not allocate more time to a specific location of focused activity at the territory boundary and do not support the CPP hypothesis. T-males and their mates allocated less time at the territory boundary and the males traveled further outside the original boundary suggest that T-pulses at the territory boundary likely promote territory expansion. T-males produce shorter duration calls which are likely associated with aggression. Results from the male and females show that the effects of T-pulses on placed preferences are context-dependent.

I found that members of the same pair show vocal similarities in frequency and duration and these differences were independent of T-pulses. Pairs that are closer to one another are more different in call characteristics that pairs that are further apart. Suggesting that California mouse individuals have control of the vocal organ and the neural circuitry to coordinate the fine-tuning of the vocalizations. Thus, the modification of spectral and temporal characteristics between members of a pair to show similarities in call structure suggests there is vocal convergence in a rodent species that forms a lifelong pair-bond. California mice form lifelong pair-bonds, are social, and have plasticity associated with their vocal behaviors making them a potential model system for

understanding the development and evolution of vocal communication, especially in research associated with speech disorders.

In rodents, behavioral endocrinology studies have demonstrated that exogenous administration of long-lasting T implant restores vocalization production and here I found that T-pulse administration is correlated with changes in call production, spectral characteristics of vocalizations, and interpretation and behavioral response by the receiver. Overall, in the complex field setting transient T-pulses alter both location preference and social behavior in the form of ultrasonic vocalizations in the field. The variation of T in inducting CPPs at the nest but not at the territory boundary suggests there is plasticity in the sensitivity to T based on the physical location. Environmental location dictates the effects of T-injections suggesting the effects of T-pulses are highly context dependent. In both males and females, vocalizations convey information about sender identity. Vocal recognition is documented based on spectral and temporal characteristics, including frequency, duration, and bandwidth.

Anthropogenic Noise, the Environmental Factor

Broadcasted broadband anthropogenic noise, but not familiar noise decreases activity and foraging behavior of wild deer mice (*Peromyscus maniculatus)* and woodland jumping mice (*Napaeozapus insignis*). Furthermore, anthropogenic and familiar noise negatively affect call production in both species. Interestingly, I found species-specific responses to the same type of noise, with the deer mice being more sensitive to anthropogenic changes in the soundscape that the woodland jumping mouse. My study is the first to demonstrate a direct impact of broadband anthropogenic noise on

individual responses of nocturnal rodents. Rodents are an essential component of a functioning ecosystem and anthropogenic changes in the soundscape negatively affect a myriad of rodent behaviors necessary for survival and reproduction. Overall, changes in the natural soundscape due to anthropogenic noise negatively affects vocal communication, activity, and foraging.

Vocal communication is a crucial component of animal behavior that contains information and elicit predictable behavioral responses by the receivers. My research shows that animals rely on vocal signals to mediate routine social behaviors. Through a different mechanism, both the environmental and physiological stimuli influenced space allocation and vocal communication. My research reinforces the idea that an animals behavior is three dimensional and that context is important. Communication and location preferences are influenced by where an individual is in the environment, who they are, and the social interactions occurring at that time. The complex field environment is important for testing ideas to understand the evolution of vocal communication and associated behaviors because in a natural setting where these behaviors are most important.