PETRIC, RADMILA, M.S. Individual Context of Ultrasonic Vocalizations Produced by Free-living Brush Mice (*Peromyscus boylii*) with an Emphasis on Differences Between Males and Females. (2010)
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Muroid rodents regularly use ultrasonic vocalizations (USVs). The majority of research work on USV communication in rodents comes from laboratory strains of rats (*Rattus norvegicus*) and mice (*Mus musculus*). The objective of my project was to examine the individual context of USVs produced by wild *P. boylii* with a specific focus of examining differences between males and females. USVs were recorded during the breeding season; however there was no correlation between the number of USVs produce and the proportion of reproductive adult in the population (Pearson's Correlation=0.582, 0.470). There were individual differences between males based on duration and frequency of USVs. Adult *P. boylii* males with scrotal testis produced USVs when alone and when in the presence of an estrous female. Adult, *P. boylii* females residents produced USVs in the presence of another female and when pups are emerging from the nest. There were individual differences between females based on frequency and bandwidth of USVs. Females produce more 3 syllable vocalizations than males and the mean overall modulation and bandwidth were lower in males than females. My results suggest that vocalizations produced by males may serve to attract females and facilitate copulation. Vocalizations produced by females may serve to mediate social interactions with other females and as warning signals for newly weaned pups. Furthermore, sex is communicated through motif type and spectral characteristics of USV.

INDIVIDUAL CONTEXT OF ULTRASONIC VOCALIZATIONS PRODUCED BY FREE-LIVING BRUSH MICE (*PEROMYSCUS BOYLII*) WITH AN EMPHASIS ON DIFFERENCES BETWEEN MALES AND FEMALES

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CHAPTER I

INTRODUCTION

Rodents regularly use acoustic communication as part of their behavioral repertoire (Costantini and D'Amato 2006). In the superfamily Muroidea (mice, rats, voles, hamsters, etc), all lineages investigated regularly produce ultrasonic vocalizations (USVs) (Geyer and Barfield 1979; Sales 1999). In the laboratory, rodent USVs have a communicative function (Sales and Pye 1974; Sales 1999). Ultrasonic vocalizations are emitted mainly during social situations, including: courtship, mating, aggression, and territoriality (Sales and Pye 1974; Arch and Narins 2009). The majority of research work on USV communication in rodents comes from extensive laboratory research on laboratory strains of rats (*Rattus norvegicus*) and mice (*Mus musculus*).

***Rattus norvegicus* USVs**

Adult *R. norvegicus* produces two main types of ultrasonic vocalizations: the 22 kHz and the 50 kHz vocalizations. The 22 kHz vocalization communicates negative affect that results from painful stimuli, fear, and defeat. The 22 kHz vocalizations are also produced by the male during and after ejaculation (Brudzynski et al. 1993; Knutson et al. 2002). The 50 kHz vocalization seems to communicate positive affect that results from sexual behavior, play fighting, tickling, and victory (Amsel et al. 1977; Brudzynski et al. 1993; Knutson et al. 2002).

In *R. norvegicus*, ultrasonic vocalizations are important in coordinating male–female reproductive behavior and both sexes produce USVs (Geyer and Barfield 1978; White and Barfield 1990; Costantini and D'Amato 2006). Before ejaculation, males vocalize at frequencies between 35 and 50 kHz with each vocalization lasting approximately 100 ms (White and Barfield 1990). Shortly after ejaculation, males produce longer vocalizations (500 ms – 3000 ms) at lower frequency (22 kHz) (White and Barfield 1990). During male-female interactions, females also emit 50 kHz vocalizations during early sexual encounters and copulation (Barfield and Geyer 1975). During sexual interactions, the 50 kHz vocalizations produced by females may advertise receptivity (Thomas and Barfield 1985; White and Barfield 1987). Female 50 kHz USVs are similar in spectral characteristics to those of males (Thomas and Barfield 1985).

***Mus musculus* USVs**

Adult *M. musculus* emit USVs which may serve both to facilitate and inhibit social interactions (Portfors 2007). In *M. musculus*, USVs range between 30 and 110 kHz and last approximately 300 ms (Sales and Pye 1974; Holy and Guo 2005; Portfors 2007). Vocalizations range from simple, single harmonic syllables to more complex frequency modulated syllable series (Holy and Guo 2005; Portfors 2007).

Both male and female *M. musculus* produce USVs, although, the function and number of vocalizations produced differ between the sexes. During male-female interactions, it is primarily the males that vocalize (Whitney et al. 1973; White et al. 1998). Vocalizations are emitted both before copulation and after ejaculation, and it has

been suggested that these vocalizations reduce female aggression (White et al. 1998; Costantini and D'Amato 2006). Males may also vocalize to attract females (Hammerschmidt et al. 2009; Musolf et al. 2010), retain females in close proximity (Pomerantz et al. 1983; Hammerschmidt et al. 2009) and to convey social status (Nyby et al. 1976). Vocalizations emitted during heterosexual interactions may also be used as an index of social recognition (Musolf et al. 2010).

Males emit different types of vocalizations and produce more complex syllables than females (Sales 1972; Whitney et al. 1973; Gourbal et al. 2004; Holy and Guo 2005). Complex USVs produced by males have characteristics of songs (Holy and Guo 2005). Males produce more USVs when presented with novel females than when presented with familiar females (Musolf et al. 2010). The number of calls produced by a male also correlates with his reproductive status and the reproductive status of his mate (Nunez et al. 1978). Males with scrotal testes produce more vocalizations than castrated males (Rose and Drickamer 1975; Nunez et al. 1978). The presence of female chemical cues alone is enough to elicit vocalizations from males (Hayashi and Kimura 1974; Rose and Drickamer 1975; Nyby et al. 1977; Musolf et al. 2010).

During female-female interactions, territory-holding females produce a large number of vocalizations in the presence of intruders (Maggio and Whitney 1985). These vocalizations may serve an affiliative function and for establishment of the social dominance hierarchy (Maggio and Whitney 1985; Moles and D'Amato 2000). Females also vocalize when pups are removed from the nest (Ehret 2005).

Individual male mice sing recognizably different songs (Holy and Guo 2005) and females may distinguish familiar from unfamiliar males based on their USVs (Musolf et al. 2010). This suggests that there should be individual characteristics in USVs that differ among individual males. However, individual variation based on spectral characteristics is not well documented in rodents. In songbirds and frogs, on the other hand, individual variation based on spectral characteristics is well documented and females appear to use the spectral characteristics of male vocalizations to assess genetic and physical quality (Duffy and Ball 2002). For example, in frogs, where large males are more viable, females rely on frequency, call duration and call complexity to assess male size (Akre and Ryan 2010; Baugh and Ryan 2009; Giacoma et al. 1997). Similarly in birds, call duration is correlated with the strength of the male's immune system (Duffy and Ball 2002). Therefore, female mice appear to use call duration, frequency and call complexity as good indicators of male genetic and physical quality. Furthermore, mice, like songbirds and frogs, are territorial, and unique vocalizations of individual mice may facilitate establishment and maintenance of territories (Nelson and Poesel 2009).

Peromyscus

North American deer mice in the genus *Peromyscus* are among the most abundant groups of mammals, and are distributed over most terrestrial habitats in North America (Findley 1987). The genus represents more than fifty species and can be found in a wide variety of ecosystems in North America (Kirkland and Layne 1989). *Peromyscus* make an excellent study animal because they are easily captured, marked, and recaptured

(Kirkland and Layne 1989). In the wild there is extensive variation in the behavior and ecology of *Peromyscus*. For example, mating behavior within *Peromyscus* is highly variable, where some species are known to be monogamous (*P. californicus* and, *P. polionotus*) while others are promiscuous or polygynous (*P. maniculatus, P. leucopus*, and *P. boylii*) (Millar 1989). The biological and geographic variation within the genus *Peromyscus* is ideal for studies of behavior.

An underappreciated component of *Peromyscus* behavior is the use of USVs. In the laboratory, all *Peromyscus* species studied regularly produce USVs (Sales 1999; Pomerantz and Clemens 1981; Wright and Brown 2004, Kalcounis-Rueppell et al. 2010). For example, *Peromyscus maniculatus* produce ultrasound during courtship and copulation and these vocalizations are an important component of male sexual behavior (Pomerantz and Clemens 1981). In the wild, *Peromyscus* frequently produce USVs (Kalcounis-Rueppell et al. 2006; Briggs 2009; Carney 2009; Kalcounis-Rueppell et al. 2010). Wild *Peromyscus* produce single and multi-syllabic vocalizations, with the various types referred to as motifs (Kalcounis-Rueppell et al. 2006; Briggs 2009; Carney 2009, Kalcounis-Rueppell et al. 2010). Motifs are distinguished according to the number of syllables in each vocalization, with syllables separated by a short interval of silence (intersyllabic interval) (Figure 1) (Briggs 2009; Carney 2009, Kalcounis-Rueppell et al. 2010). In *Peromyscus,* the most common motifs have 1-4 syllables (Kalcounis-Rueppell et al. 2006; Briggs 2009; Carney 2009, Kalcounis-Rueppell et al. 2010). Each syllable ranges in duration from 80 to 200 ms with an intersyllabic interval between syllables of approximately 200 ms (Kalcounis-Rueppell et al. 2006; Briggs 2009; Carney 2009).

Peromyscus boylii

Peromyscus boylii (the brush mouse) has one of the largest distributions in the genus *Peromyscus*, extending from southwestern Montana to southern Mexico and from California to Kansas (Kalcounis-Rueppell and Spoon 2009).Within its range, *P. boylii* inhabits almost all of the terrestrial regions above 1,500 meters elevation (Kalcounis-Rueppell and Spoon 2009). The species is well studied and there are extensive data on its behavior and ecology (Kalcounis-Rueppell and Spoon 2009). *Peromyscus boylii* is nocturnal and active year-round, with a breeding season that varies within populations and across its range (Kalcounis-Rueppell and Spoon 2009). Herein, I define the breeding season as those months in the year where adults are in reproductive condition (scrotal testis for males and females that have a perforate vagina, are pregnant, lactating or both pregnant and lactating).

The mating system of *P. boylii* is similar to that of other muroid rodents (Kalcounis-Rueppell and Ribble 2007; Kalcounis-Rueppell and Spoon 2009) and ranges from polygynous to promiscuous depending on population density (Hoffmeister 1986; Ribble and Stanley 1998; Kalcounis-Rueppell and Spoon 2009). As with other promiscuous mammals, *P. boylii* typically has male-biased dispersal and female natal philopatry (Dobson 1982). Dispersing males seem to prefer territories which are similar in habitat to their natal homes (Mabry and Stamps 2008a; Mabry and Stamps 2008b). Males have large home ranges (~0.49 ha) that overlap with other males and several

females. Females have smaller home ranges than males (~0.29 ha) that do not overlap with those of other females (Ribble and Stanley 1998; Kalcounis-Rueppell and Ribble 2007; Kalcounis-Rueppell and Spoon 2009).

Peromyscus boylii is sexually monomorphic, ranging in size from 22 to 36 g with a 1:1 sex ratio and a life span of 1-2 years (Schmidly et al. 1988; Kalcounis-Rueppell and Spoon 2009). Males and females do not form pair bonds or share nests and, litters of a given female can be sired by different males (Kalcounis-Rueppell 2000; Kalcounis-Rueppell and Spoon 2009). Females reach sexual maturity at approximately 4 months of age and produce up to 4 litters per year with an average of 3 pups per litter (Clark 1938; Zeveloff 1988). After parturition, females have a postpartum estrus and mate again. Thus, females can be both pregnant and lactating at the same time. The gestation period lasts an average of 29 days with inter-birth intervals of 25 to 31 days (Storer et al. 1944; Terman 1968; Kalcounis-Rueppell and Spoon 2009). Growth and development is rapid and pups are weaned and leave the nest in 21-27 days (Bradley and Schmidly 1999; Kalcounis-Rueppell 2000).

The behavioral ecology of *P. boylii* has been studied at the Hastings Natural History Reservation in California (Kalcounis-Rüppell and Miller 2002; Kalcounis-Rüppell et al. 2006). At this site, the breeding season of *P. boylii* is from December to May, which coincides with the rainy season of coastal California (Kalcounis-Rueppell and Spoon 2009). An earlier study demonstrated that the population of *P. boylii* at Hastings Natural History Reservation produces USVs in the wild (Kalcounis-Rueppell et

al. 2006). However, Kalcounis-Rüppell and colleagues (2006) passively recorded USVs and were not able to determine the context in which USVs were produced by *P. boylii* or which mouse produced USVs.

Peromyscus boylii is an excellent wild mouse model for studying ultrasound because its life history and behaviors are similar to the laboratory model *M. musculus*. *Peromyscus boylii,* like *M. musculus,* breeds in polygynous to promiscuous arrangaments, depending on population density and males have home ranges that overlap there of other males and females. We investigated the individual context of vocalizations in the wild. The objective of my project was to examine the individual context of USVs produced by wild *P. boylii* with a specific focus on examining differences between males and females. For this descriptive study, I formulated several non-mutually exclusive hypotheses to explain USV context, these hypotheses were based on previous work describing contexts of USVs production by adult *Mus musculus* in the laboratory.

My first hypothesis (**H_1**) is that males vocalize to attract mates. I can make several predictions (referred to here and throughout as **P_n**) that will occur if this hypothesis is true: If USVs produced by males serve to attract mates, then (**P_1**) males will produce USVs during the breeding season; (**P_2**) males will produce USVs when they are in breeding condition (i.e. have scrotal testis); (**P_3**) males will produce USVs when alone; (**P_4**) USV motifs and spectral characteristics will differ between males and females; and (**P_5**) USV spectral characteristics will differ among individual males.

My second hypothesis (**H_2**) is that males vocalize to facilitate copulation. If USVs produced by males facilitate copulation, then I predict that (**P_6**) males will produce USVs in the presence of estrous females.

My third hypothesis (**H_3**) is that resident females vocalize to mediate social interactions with other females. If USVs produced by females serve to mediate social interactions with other females, then I predict that as with **P_4** for **H_1**, USV motifs and spectral characteristics will differ between males and females; (**P_7**) females will produce USVs in the presence of another female; (**P_8**) USV spectral characteristics will differ among individual females; and (**P_9**) females producing USVs will be resident individuals.

My fourth hypothesis (**H_4**) is that female vocalizations serve as warning signals for pups that have recently emerged from the nest. If USVs produced by females serve as warning signals for pups, then, (**P_{10}**) when females are producing USVs they will have pups that were recently weaned.

CHAPTER II

METHODS

General Methods

Vocalizations were recorded from and assigned to wild *P. boylii* individuals using an integration of three remote sensing systems (microphone array, radio telemetry, and thermal imagery, described below). An array of 12 microphones recorded the USVs and, based on time delay of sounds arriving at the microphones, I was able to determine the location of the mouse that produced the USV. Resident mice and their neighbors in the focal study areas were trapped and collared with radio transmitters set at unique frequencies for each mouse. The radio telemetry system recorded signal strengths of the frequencies from radio-collared residents and their neighbors inside the microphone array. Using the signal strength from the radio telemetry data, I was able to identify the individual vocalizing at the location where the USV was recorded. The thermal imaging system provided video images from which I was able to determine the presence of a mouse in the study area at the time of the USV recordings and to see if there were any other mice near the position of the vocalizing mouse. Thus, my remote sensing system allowed me to assign USVs produced in the wild to individual mice. Each USV was then analyzed for spectral and temporal characteristics.

Study Area

Field work was conducted during the rainy season at the Hastings Natural History Reservation, Monterey County, in coastal California, USA (36º12'30"N, 121º33'30"W), from December 2007 to June 2008 and in January 2009. Trapping was conducted from December 2007 to June 2008 and in January 2009. Recording of USVs was conducted from February to June 2008 and in January 2009. The site is 500 m above sea level in the foothills of Santa Lucia Mountains. Canyon bottoms are dominated by live oak (*Quercus agrifolia*) and mixed deciduous trees with dense underbrush, which is the preferred habitat of *P. boylii* (see details in Kalcounis-Rüppell and Millar 2002).

Live Trapping and Focal Areas

Live trapping was conducted on two previously established trapping grids: Lower Robinson Creek and Upper Robinson Creek (Kalcounis-Rüppell and Millar 2002). Lower Robertson Creek consists of a 4 by 34 configuration of trap stations and Upper Robinson Creek consists of a 6 by 13 configuration of trap stations. Trap stations were approximately 10 m apart and at each trap station there were three live traps – one Longworth (Rogers Manufacturing Co, Peachland BC; box 14 × 16.5 cm; tunnel 4.5 × 4.5 cm) and two Sherman (AB Sherman Traps, Tallahassee FL; 7.6 × 8.9 × 23.3 cm) traps. Traps were baited with rolled oats and sunflower seed mixture and provided with a small piece of cotton.

At the start of the study, live trapping was conducted to determine the residency of individual mice and population density of the mice. Trapping sessions involved

trapping for three consecutive nights in one of the four sections of the Robinson Creek grids (one section in upper Robinson and three sections of Lower Robinson). Trapping sessions were conducted continually throughout the field season. Traps were opened one and a half hours before sunset. Traps were checked and deactivated three hours before sunrise. Traps remained on the grid throughout the field season, allowing the mice to acclimate to the presence of the traps and go freely in and out of the locked-open Longworth traps and explore closed Sherman traps when trapping was not in session for that section of the grid.

Upon capture, species, sex, age and reproductive condition (scrotal or abdominal testis for males; pregnant, lactating or perforate vagina for females) of each mouse were assessed. Males were determined to be reproductive (scrotal) if testis were descended and non-reproductive (abdominal) if testis were not descended. Pregnancy was determined by palpation. Lactation for captured females was determined by the observation of bare and enlarged nipples. Newly captured mice were tagged with uniquely numbered metal ear tags (Monel 1005 numeric, Nahad Band and Tag Co).

After each trapping session, captures were recorded in a relational database (Microsoft Access). From geographic coordinates of trap stations recorded in the database, I was able to determine home ranges of resident mice using Animal Movement software in ESRI ArcView GIS (version 3.2). I generated 50% and 95% contours of the fixed-kernel home range estimator with a smoothing factor of 5. I defined residents of the grid as individuals captured within a 30 m buffer of one trap station of the grid more than 3 times over the trapping season.

Using kernel home range data, I further defined residents of the area under observations (the focal area, described below) to be individuals whose 50% core home ranges were encompassed by the focal area. All other resident mice whose core home ranges were not encompassed by the focal area, but were captured around the focal areas, were considered neighbors of residents.

Observations were focused on a small (approximately 8 × 6 m) area of the grid at a time, the focal area, on which the remote sensing equipment was deployed. Observations were made at each focal area for about 14 days, and then the focal area was moved to a new location. The focal area was selected based on the number of resident (5 or 6 resident individuals) *P. boylii* in that area and the feasibility (considering canopy vegetation and understory) of assembling the remote sensing equipment. At each focal area, three remote-sensing pieces of equipment (a microphone array, a radio telemetry system, and a thermal imaging camera), were used to record and assign ultrasonic vocalizations to individual mice.

Prior to setting-up the remote sensing equipment, live trapping of the focal area (with 15 extra traps) and a buffer of three trap stations in every directions around the focal area was conducted for three consecutive nights to ensure all resident mice were captured. Upon capture, focal area residents and their neighbors were each outfitted with a unique frequency 0.55 g M1450 mouse-style transmitter (Advanced Telemetry System, ATS, Isanti, Minnesota). After focal area mice were outfitted with transmitters, the remote sensing equipment began recording. While remote sensing equipment was

recording, there was no trapping in the focal area or in a 30 m buffer around it. Remote sensing equipment was set up in the focal area as described below.

Remote Sensing in the Focal Area to Record USVs from Individuals

Microphone array was used to record and localize USVs. A 12 Emkay FG Series microphone array (Avisoft Bioacoustics, Berlin, Germany) was placed in a 3 by 4 grid configuration with approximately 1.5 m spacing. The 12 microphones were connected to an UltraSoundGate system 1216H (Avisoft Bioacoustics, Berlin, Germany) connected via USB 2.0 interface to a small laptop (Dell Latitude D410) running Recorder software (Avisoft Bioacoustics, Berlin, Germany). The microphone array was triggered when sound was detected at any of the 12 microphones and saved to .wav file. When triggered, all 12 microphones recorded sound. Based on the time delay of arrival of sound from each of the 12 microphones, I determined the location from which the sound was produced.

Radio telemetry was used to identify individual mice that produced the USVs recorded in the focal area. The unique frequencies of transmitters were coded into a data logger (DCC, DSU D50410; ATS) connected to a central receiver (4mHz R4000, ATS). The central receiver was connected to an antenna switch box and four small antennae (Sigflex 15 cm omni-directional, ATS). The antennae were placed on each of the 4 corners of the focal area. The receiver was programmed to search continuously for all transmitter frequencies in the focal area. As a collared mouse came near or through the focal area, the unique frequency was detected by one of the antennae. Once a signal was

detected by one antenna, all 4 antennae recorded the signal strength at the receiver. After the signal strength was recorded the receiver continued to search for the next frequency.

To determine the location of the mouse based on transmitter signal strength, I made a reference grid within each focal area to compare the signal strength data from radio-collared mice. Each transmitter was tested on the focal area prior to being secured to a mouse. The transmitter was tested for three minutes on the ground at the location of each of the 12 microphones from the Avisoft sound recording array. The transmitter signal strength from a collared mouse was manually compared to the reference database to assign the position of a mouse within the microphone array. If two mice were on the focal area at the same time, I identified the vocalizing mouse by overlaying the microphone position at which the vocalization was received in relation to the positions of the two mice, as determined by the video image, and their transmitter signal strengths. At the same time I was also able to determine the identity of the non-vocalizing mouse.

I used a thermal imaging camera to produce video images of the focal area and to determine when possible 1) the presence of a mouse at the time the USV was produced, 2) to determine if there were any other mice in the focal area at the time the USVs were produced, and 3) to determine the amount of time mice spend in the focal area when alone and in the presence of another mouse. The thermal imaging lens (Photon 320 with 14.25 mm lens; Flir/Core by Indigo) was suspended at least 9 m above the forest floor using a rope and pulley system between two trees to video record the entire focal area. The lens was connected to a ground-based JVC Everio hard disk drive camcorder using standard video cables.

The microphone array, telemetry system, and thermal imaging were all synchronized for time and turned on at dusk for night recording. All equipment ran through the night continuously and recorded in real time. In the morning, the laptop computer, DCC data logger and the camcorder with recordings were taken back to the laboratory where the recordings from that night were examined. All data were uploaded and backed-up on hard-drives (Data Robotics). Each piece of the remote sensing equipment in the focal area was powered by a single 12V, 33amp/hour dry cell battery attached to 150 W inverters. The focal area was set up for recording each night for at least 14 continuous days. At the end of the 14 day period, a 3 day live trapping session was conducted to capture the mice on the area and remove transmitters. During the time transmitters were being removed from a given focal area, a new focal area on the grid was being established, using methods described above, at different location, and the process was repeated.

Analysis of the Remote Sensing Data from Focal Area to Assign USVs to Individuals

Spectrographs of all sound (.wav) files from the microphone array were visually examined using Avisoft SASLab Pro (Avisoft Bioacoustics, Berlin, Germany). Spectrographs were generated using an fast fourier transform (FFT) length of 512, and 100% Frame size with Hamming window. Window overlap was 50%. Frequency range was 5 -125 kHz with a frequency resolution of 488 Hz and a temporal resolution of 1.024 ms. All spectrographs that appeared to be mouse USVs were further examined by playback at 4.4% of recording speed to render the sound audible. Sound files with mouse

USVs were examined at 12 channels (from the 12 microphones in the array) to determine the order at which the sound arrived at each of the microphones. Using a map of the focal area in which the USV was recorded and the time delay of arrival, I determined the approximate location (i.e., within a quadrant of microphones) at which the USV was emitted.

To assign a USV to a mouse, I examined telemetry data for five minutes before and five minutes after the USV was produced. I determined if any of the mouse locations detected by transmitters matched USV locations detected by the microphone array. If a transmitter location matched the location of the USV, the USV was assigned to the mouse carrying the transmitter. If the mouse produced a vocalization in the presence of another mouse I used the telemetry data to determine the identity of the second mouse. For this method of assigning USVs to mice the transmitter on the mouse had to be working. If the battery on the transmitter failed, we were not able to assign the USV to a mouse and this is why we could not assign all recorded USVs to individual mice.

I examined the video recorded by the thermal imaging camera in two ways. First, I examined the video during the minute that included the USV to determine if there was a mouse at the location where the USV was recorded. Since the start time of each video file was shown only to the minute, video and sound (.wav) files could not be synchronized to the second when a USV was recorded. Therefore, a full one minute clip of the video was made, encompassing the entire minute during which the USV was recorded. I will refer to this method throughout as the one-minute video analysis. Analyzing one minute of video

in this manner was not possible for every USV assigned to a mouse because not all assigned USVs occurred on camera (e.g., mouse was acoustically and telemetrically localized at the edge of the array off camera). Analysis of the one-minute video allowed me to determine if there was a second mouse on the focal area at the time the vocalization was recorded.

Second, I examined a one hour segment of video to quantify the proportion of time that individuals spent vocalizing both when alone and in the presence of other mice, I will refer to this method throughout as the one-hour video analysis. For this analysis I sampled one hour of video from each of two days of each focal area that had more than 1 USV recorded and assigned to individuals. This analysis was done on 9 focal areas and amounted to a total of 18 hours of video examined. I selected the days for which I was sure all the resident and neighboring mice had working transmitters, so that, I could identify all the mice on the grid. All one hour segments were examined beginning one hour after sunset (e.g., if sunset was at 1800 hrs. video for 1900 – 2000 hrs was examined). Each time a mouse or mice first entered and then exited the thermal imaging view I recorded the time. I refer to this period when there was one or more mouse on the focal area as an on-camera interval. I recorded start and end time (to calculate total time), rounded to the nearest minute of on-camera intervals. I also counted the number on-camera intervals for each hour. I also overlaid the telemetry data for each on-camera interval to determine the identity of individual mice and the acoustic data to determine if any USVs were produced during that on-camera interval.

Determining identity of individual mice for each USV was performed by three independent researchers. After all vocalizations were assigned, the results were compared between the three researchers. If there was a disagreement in the assignment, the vocalization assignment was checked once more and either a consensus of agreement was reached or the USV was not assigned to a mouse and not included in the analysis (see results for rate of agreement).

For each USV that could be assigned to an individual, I determined the sex and reproductive condition of the mouse and analyzed spectral and temporal characteristics of the USV using Avisoft SASLab Pro. For the spectral and temporal analysis of USVs, I selected from the 12 channels the recording with the clearest waveform and of highest amplitude. Using the automated detection feature I measured the following parameters for each vocalization: start frequency (kHz), end frequency (kHz), maximum frequency (kHz), minimum frequency (kHz), frequency at maximum amplitude (kHz), duration of each syllable (ms), bandwidth (number of frequencies the vocalizations passes through) (kHz), time from start to maximum frequency (ms), intersyllabic interval (silent period between syllables; ms), internal modulation (change from the point of maximum frequency to the point of minimum frequency divided by duration; kHz/ms) , and overall modulation (change from start frequency to end frequency divided by duration; kHz/ms) (Figure 1**)**.

Statistical Analysis

For the analysis of spectral and temporal characteristics, I examined the four common motifs 1 syllable vocalizations [1SV], 2 syllable vocalizations [2SV], 3 syllable

vocalizations [3SV], and 4 syllable vocalizations [4SV] separately because they are unique motifs (Kalcounis-Rueppell et al. 2006). The five frequency variables (start frequency, end frequency, maximum frequency, minimum frequency, frequency at maximum amplitude) were highly correlated with one another and therefore I reduced them to a single variable (PC1) using principle components (PC) analysis (**Table 1**). The other five variables (duration, bandwidth, internal modulation, overall modulation and start to maximum frequency) were not subjected to PC analysis. Thus a total of 6 variables were used in my analysis (duration, bandwidth, internal modulation, overall modulation, start to maximum frequency and PC1 score from the frequency variables).

To determine whether, as I had predicted, USVs were produced during the breeding season (**P_3**), I correlated the total number of USVs recorded with proportion of the population that was in reproductive condition each month in 2008 for which I had recordings (February to June). To determine whether mice vocalized alone or in the presence of another mouse (**P_2, P_6, P_7**), I used the one-minute video analysis. To determine whether individuals produced USVs more frequently when another mouse was present on the grid (**P_2, P_6, P_7**) comparing to when the mouse was alone, I used a chi-square test of independence.

To determine the proportion of time mice spent in the focal area either alone or in the presence of another mouse and to determine the proportion of time spent vocalizing in different contexts (male alone, female alone, male with male, male with female, female with male) (**P_6, P_7**), I used the one-hour video analysis I examined the number of USVs

produced in each context during on-camera intervals in relation to total on-camera interval time and number of on-camera intervals using a chi-squared test with post-hoc analyses.

To determine to determine whether of not, males vocalize in the presence of estrous females (**P_6**) or females vocalize when they have weaned pups (**P_{10}**), I determined from trapping records when females gave birth, based on a decrease in their body mass. Based on the literature (Kalcounis-Rueppell et al. 2009), I estimated females to be in postpartum estrus one to two days postpartum, and weaning date to be 21 days postpartum. Dates when males were scrotal were also determined from trapping records.

To determine if there were sex-specific signatures in vocalizations (**P_4**), I compared motif type and spectral characteristics of USVs between males and females. To determine if USVs of males and females differed in spectral and temporal characters (**P_4**), I tested spectral characters for homogeneity of variances using a Levene's test of homogeneity of variance and compared spectral characters between sexes using a Mann-Whitney U test. To determine if motif type was independent of sex (**P_4**), I used a chi square test of independence. To determine if there was individual variation in USVs for males (**P_5**) and for females (**P_8**), I tested spectral characters of vocalizations within each sex for homogeneity of variance using a Levene's test of homogeneity of variance and compared spectral characters among individuals using a Kruskal-Wallis test.

Because I was using multiple Mann-Whitney U and Kruskal-Wallis tests I used a Bonferroni corrected rejection criterion of $p<0.008$. Chi square post hoc tests were

performed using the crosstabs analysis feature in SPSS, whereby residual (the difference between the actual frequency and the expected frequency) and standardized residuals (z-score) were selected from crosstabs. Significance for the post hoc test was determined by observing the variable with the largest residual and by determining if the standardized residual was smaller than the critical value (-1.96). All analyses except for chi square tests of independence were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL). Chi square tests were calculated using standard formulae (Ramsey and Schafer, 1997) in Excel 2007.

CHAPTER III

RESULTS

Data were collected on 131 nights at 11 focal areas for an average of 13.6±4.8 nights at each focal area. In addition, I collected data during the day for 7 days at 3 different focal areas. I attached 36 transmitters to 33 *P. boylii* individuals (21 adult females and 12 adult males). Three mice received transmitters in more than one focal area. On average 3.3±1.9 *P. boylii* individuals were outfitted with a transmitter at each focal area.

I recorded 1,417 hours of thermal imaging videos, 1,748 hours of remote radio telemetry data, and 127,629 acoustic files (.wav files). Over the study period 198 *P. boylii* vocalizations were recorded of which 170 vocalizations (86%) were assigned to individual mice in unanimity by all three researchers.

The 170 assigned vocalizations belonged to 25 adult *P. boylii* individuals (16 female and 9 male). Of the 170 assigned vocalizations, 76 were produced by females with an average ± SD of 4.8±4.0 vocalizations per female and 86 produced by males with an average ± SD of 9.6±10.6 vocalizations per male. Of 170 USVs, 162 were long modulated multisyllabic vocalizations and 8 were modulated barks (**Figure 2a-f**). The 162 long modulated multisyllabic vocalizations were categorized by number of syllables: one-syllable vocalizations (1SV, n=40; **Figure 2b**), two-syllable vocalizations (2SV, n=71; **Figure 2c**), three-syllable vocalizations (3SV, n=36; **Figure 2d**), four-syllable

vocalizations (4SV, n=13; **Figure 2e**), and five-syllable vocalizations (5SV, n=2; **Figure 2f**).

Although the equipment was set up and running during some of the day (n=7), no vocalizations were recorded during the day on the focal area (i.e., at the time mice were in their nests). Vocalizations were recorded only between 1800 hours and 0500 hours.

Most mice were in reproductive condition in February (92%), March (80%), and April (92%), with the percentage declining in the following months in May (43%) and Jun (10%) (**Figure 3**). The majority of USVs were produced during the months when over 40% of individuals were reproductive. The peak number of USVs for both males and females were produced in April, and the number of vocalizations declined as percentage of reproductive mice declined. However, there was not a significant relationship for the period of February to June between the number of USVs produced per month and the proportion of individuals in reproductive condition during the month (R=0.58, df=3, p=<0.30).

***Peromyscus boylii* males**

Males produced vocalizations in the breeding season, as would be expected ($\mathbf{P_1}$) if males vocalize to attract mates. All *P. boylii* males that produced USVs were adults with scrotal testes at the time the USVs were recorded, as I had predicted ($\mathbf{P_2}$). Of the 86 assigned vocalizations produced by males, 16 (31%, 16 out of 52 vocalizations) were produced when alone, as predicted ($\mathbf{P_3}$), and 36 (69%, 31 out of 52 vocalizations) were produced when in the presence of another mouse, but the difference was not significant (χ^2 =0.028, p=0.867; Chi-square by sex and context). The remaining 34 assigned

vocalizations were assigned based only on telemetry and there was no mouse visible (mouse was at the edge of the grid) and thus it could not be determined if it was alone or with another mouse. Thus, as predicted (**P_1, P_2, P_3**) during the breeding season, males in reproductive condition vocalize when they are alone, supporting the hypothesis (**H_1**) that males vocalize to attract mates.

Of all the assigned USVs for males that occurred in the presence of a second mouse (n=36), the identity of the second mouse could be determined for 18 vocalizations (based on telemetry and the one minute video analysis). Males vocalized in the presence of another male 22% of the time (4 out of 18 vocalizations) and in all cases all the males had scrotal testes. Males also vocalized in the presence of a female 78% of the time (14 out of 18 vocalizations). Of the 14 male vocalizations made in the presence of a female, 11 were produced in the presence of 4 different females that had a new litter of pups and were in postpartum estrus at the time vocalizations were recorded. Thus, as predicted (**P_6**) males vocalize in the presence of estrous females, supporting the hypothesis (**H_2**) that males vocalize to facilitate mate attraction.

The one-hour video analysis showed that number of on-camera intervals with USVs was dependant on context (χ^2 =14.65, p=0.001; **Figure 4; Table 2**) with time males spent with another mouse yielding proportionally more vocalizations (67% of intervals had vocalizations; 2 out of 3) than when males were alone (27% of intervals had vocalizations; 4 out of 15 intervals). In addition, I found from the one-hour video analysis that males did not vocalize in the presence of other males, and when males and females

were together only the males vocalized (67% of intervals had vocalizations; 2 out of 3 intervals; **Figure 4; Table 2**). These results also support my prediction (**P_6**) that males vocalize in the presence of estrous females supporting the hypothesis (**H_2**) that males vocalize to facilitate mate attraction.

There were individual differences in vocalizations of males based on frequency (PC1) and duration, as expected (**P_5**) if males have individual signatures in their vocalizations. There was a significant difference in duration of 1SVs among individual males (U=14.04, p=0.003) (**Table S1**). There were no significant differences among individual males for syllable 1 of 2SVs (**Table S2**). There were significant differences in duration (U=16.17, p=0.003) and PC1 (U=16.23, p=0.003) among individual males for syllable 2 of 2SVs (Table S3). Due to small sample size, differences between male individuals were not compared for 3SVs and 4SVs. These results support my prediction (**P_5**) that there are differences among individual males in spectral (frequency) and temporal (duration) characters of USVs, supporting my hypothesis (**H_1**) that USVs facilitate mate attraction.

***Peromyscus boylii* females**

Fourteen of 16 *Peromyscus boylii* females from whom I recorded vocalizations were residents, as predicted (P_9). *Peromyscus boylii* females produced 76 vocalizations that were recorded. Of these 76 vocalizations, 17 were produced when alone and 41 when in the presence of another mouse, but the difference was not significant (χ^2 =0.028, p=0.867; Chi-square by sex and context). The remaining 18 assigned vocalizations were

assigned only based on telemetry and there was no mouse visible (mouse was at the edge of the grid) and thus it could not be determined if they were alone or with another mouse. Thus, the majority of females producing USVs were resident, supporting my hypothesis (H_3) that females vocalize to mediate social interactions.

Of all the assigned USVs for females where there was a second mouse present (n=41), the identity of the second mouse was determined for 11 vocalizations (based on telemetry and the one-minute video analysis). Females vocalized in the presence of a male 73% of the time (8 out of 11 vocalizations). The 8 female vocalizations made in the presence of a male were made by 3 different females. Of these 3 females, 2 had 21 day old pups that were newly weaned and were emerging from the nest, a context I had expected (**P_{10}**). The age of the pups for the third female could not be determined. Thus, 2 of 3 females vocalized when they had emerging pups, supporting my hypothesis (**H_4**) that female vocalizations serve as warning signals for pups. Females also vocalized in the presence of a female, as expected (**P_7**) if females vocalize to mediate social interactions with other females. Females vocalized in the presence of another female 27% of the time (3 out of 11 vocalizations). Three different females produced these 3 vocalizations. Additionally, the second female was, in all cases, a neighbor to the vocalizing resident female. That females vocalized in the presence of other females, who were their resident neighbors, supports my hypothesis **(H_3)** that females vocalize to mediate social interactions with other females.

The one-hour video analysis showed that number of intervals with USVs was dependant on context (χ^2 =14.65, p=0.001; **Figure 4**; **Table 2**) with time females spent

with another mouse (always a female, never a male) yielding proportionally more vocalizations (36% of intervals had vocalizations; 4 out of 11 intervals) than when females were alone (8% of intervals had vocalizations;; 11 out of 62 intervals). The significant difference of number of intervals with USVs on context (χ^2 =14.65, p=0.001; **Figure 4**; **Table 2**) is driven by the pattern of lone females having fewer intervals with USVs than expected (standard residual = -2.0). In addition, I found from the one-hour video analysis that when females and males were together, females never vocalized in the presence of a male (**Figure 4**, **Table 2**). That females rarely vocalize on their own, never vocalize with males, and vocalize with other females (**P_7**), further supports my hypothesis (**H_3**) that females vocalize to mediate social interactions with other females.

There were individual differences in vocalizations of females based on frequency (PC1) and bandwidth of motifs as expected (**P_8**) if females have individual signatures in their vocalizations. There was a significant difference in PC1 of 1SVs among individual females (U=9.85, p=0.007; **Table S4**).There were significant differences in bandwidth among individual females for syllable 1 of 2SVs (U=16.96, p=0.005; **Table S5**). There were no significant differences among individuals for any spectral characters for syllable 2 of 2SVs (**Table S6**) or for any syllables of 3SVs (**Tables S7 – S9**). Due to small sample size, differences among females were not compared for 4SVs. These results support my prediction (**P_8**) that there are differences among individual females in spectral (frequency and bandwidth) characters of USVs supporting my hypothesis (**H_3**) that USVs produced by females mediate social interactions.

Motif and spectral differences between males and females

There were sex differences between males and females based on motif type, and modulation and bandwidth of the USVs, as predicted (**P_4**). Motif type was dependent on sex (χ^2=11.61, p=0.009; **Figure 5**). Females produced more 3SVs than expected by chance (standard residual = -3.7). There were no significant differences in spectral characters between sexes for 1SVs (**Table 3**). Syllables 1 and 2 of 2SVs produced by males had less overall modulation than females (**Tables 4 and 5)**. There were no significant differences between males and females in spectral characters for syllables 1 and 2 of 3SVs (**Tables 6 and 7**), however, in syllable 3, male bandwidth was lower than female bandwidth (**Table 8)**. There were no significant differences in spectral characters between males and females for 4SVs (**Tables 9-12**). Thus, as predicted (**P_4**), there are motif types (i.e., 3SVs produced by females) and spectral characters (i.e., lower overall modulation in 2SVs and lower bandwidth in the 3rd syllable of 3SVs produced by males compared with females) that demonstrate that sex can be conveyed through USVs, supporting my hypothesis (**H_1**) that males vocalize to attract mates.

CHAPTER IV

DISCUSSION

Overall my method of assigning ultrasonic vocalizations to *P. boylii* individuals was effective and I had a high agreement rate (86%) between the three independent researchers. However, because of transmitter failure I was not able to assign all USVs to individual mice. Nonetheless, my methods were adequate to record USVs from free-living, adult, breeding, male and female *P. boylii*. I found that adult males and females regularly produce ultrasonic vocalizations. Males on average produce more vocalizations than females. The most common motifs produced were 1SVs, 2SVs and 3SVs. My results on the types of motifs produced by *P. boylii* are consistent with those previously recorded for wild and laboratory *Peromyscus* (Kalcounis-Rueppell et al. 2006; Briggs 2009; Carney 2009, Kalcounis-Rueppell et al. 2010) and suggest that these are common motifs produced by *Peromyscus* mice. Furthermore, all vocalizations were recorded during the night when mice were active and never during the day when mice were in their nest. Thus, I suggest that USVs are an important component of communication in *P. boylii* individuals when active.

Vocalizations were recorded from February to June when I had equipment set up in focal areas. However, the majority of USVs were during months when over >40% of residents in the population were reproductive. These months correspond with the latter part of the breeding season for *P. boylii*. Although the majority of USVs were recorded

during months that correspond with the breeding season, I found no correlation between the number of USVs produced in a month and the proportion of reproductive adults during that month. However, because the number of vocalizations recorded were greatest during (March and April) months that correspond with the breeding season, and declined abruptly as the breeding season ended (June; number of USVs=3), I conclude that vocalizations are associated with breeding behaviors.

***Peromyscus boylii* males**

My results on USVs produced by *P. boylii* males are consistent with those of *M. musculus* males. As I predicted, I found that males vocalize when alone (prediction, $\mathbf{P_3}$) and in the presence of an estrous female ($\mathbf{P_6}$). These results are consistent with my hypotheses that male USVs facilitate mate attraction and copulation. Furthermore, I found individual differences in male USVs ($\mathbf{P_5}$), suggesting that spectral and temporal characteristics of USVs may contain information about individuals that facilitates mate attraction.

From the one-minute video analysis, I found that context during which USVs were produced (alone or not alone) was independent of sex. Therefore, lone males vocalize as often as lone females (31% and 29%), and males vocalize just as often in the presence of another mouse as do females (69% and 71%). However, when we compare the two contexts for only males, males seem to vocalize more often when in the presence of another mouse (69%) than when alone (31%). Furthermore, from the one-hour video analysis, I found that intervals with USVs were dependent on contexts (male alone, male

with male, male with female, female with female, and female alone). Taken together, my results suggest that males vocalize in all contexts, but more frequently when another mouse is present. Thus, male vocalizations may serve an important communications function between the vocalizing male and the second mouse.

Peromysucs boylii males with scrotal testes produce vocalizations during the breeding season, as predicted (**P_1**, **P_2**). Furthermore, based on two different analyses (one minute and one hour video analysis) I found that males vocalize when alone, as predicted (**P_3**). From the one-minute video analysis, 31% of the time males vocalized when alone. Furthermore, from the one-hour video analysis, males vocalized 27% of the time when alone. In *M. musculus,* female chemical cues alone are enough to elicit vocalizations from a solitary male (Musolf et al. 2010). Vocalizations by lone males may serve to inform the resident female of his presence and of his sexual arousal (Hammerschmidt et al. 2009; Musolf et al. 2010). It has been suggested that vocalizations emitted by *M. musculus* males when alone are part of their courtship behavior and are emitted to attract females (Musolf et al. 2010). Thus, I suggest that vocalizations from lone *P. boylii* males serve to attract mates. This hypothesis could be tested using playbacks of male calls.

Contrary to what I expected, I found evidence that males vocalize in the presence of another scrotal male. From the one-minute video analysis, 22% of the USV produced by males were produced in the presence of another male. However, in the one-hour video analysis, males were not observed to interact or vocalize with other males. Although these two results appear contradictory, the discrepancy may be an artifact of the two

approaches. Analysis of the one-minute videos, by definition, includes analysis of all USVs produced, whereas the analysis of the one-hour video only captures a small portion of video data (18 out of 1417 recorded hours of video). Therefore, capturing interactions between males during the 18 hours of video analysis is less likely, especially if the behavior is uncommon. Based on my results, I conclude that interactions between two males are not very common. In *M. musculus*, males do vocalize in the presence of another male, and these vocalizations may indicate the male's presence and convey social status to the second male (Nyby et al. 1976). In the wild, interactions between males should be rare because each is a territory holder; however, on the rare occasion when *P. boylii* males vocalize in the presence of another male it may also be to indicate presence and covey social status of the second (non-territory holding) male.

Males vocalized in the presence of an estrous female, as predicted ($\mathbf{P_6}$), as I observed in both video analyses (one-minute and one-hour video analysis). From the one minute video analysis, males vocalized 78% of the time. From the one-hour video analysis, when males vocalized in the presence of a second mouse it was always in the presence of a female. In *M. musculus,* vocalizations produced by a male in the presence of a female may serve to coordinate reproductive behavior (Costantini and D'Amato 2006), reduce female aggression (White et al. 1998; Costantini and D'Amato 2006), and retain the female in close proximity (Hammerschmidt et al. 2009). These are all situations which would increase the chance of successful mating (Pomerantz et al. 1983; Hammerschmidt et al. 2009). Similarly, in golden hamsters, *Mesocricetus auratus*, males

produce more vocalizations when presented with an estrous as opposed to a non-estrous female (Floody and Bauer 1987). Thus, vocalization produced by *P. boylii* males in the presence of an estrous female may serve to facilitate copulation.

I found individual differences among males in spectral and temporal characters of USVs, as predicted (**P_5**). Specifically, differences occurred in syllable duration and sound frequency. The individual differences I found suggest that individual recognition and individual quality may be communicated through duration and sound frequency of USVs. In *M. musculus,* females showed more self-grooming to indicate arousal when presented with USV recordings from non-sibling males than those from sibling males, suggesting individual and kin differences in USVs (Musolf et al. 2010).

Individual differences in USVs of *P. boylii* males may also be used for assessing the quality of potential mates by females. The rates of male *M. musculus* USV production and variability among individuals appear to be influenced by genetic variability, and genetic variability influences individual quality (Holy and Guo 2005; Musolf et al. 2010). In satin bowerbirds (*Ptilonorhynchus violaceus*), females seem to prefer older males that sing long and high-quality bouts (Loffredo and Borgia 1986). In male starlings (*Sturnus vulgaris*), the number of vocalizations produced in an hour is directly correlated with the strength of the male's immune system (Duffy and Ball 2002). In several anuran species, male size and male quality may be assessed by females through frequency, call duration and call complexity of male vocalizations (Akre and Ryan 2010; Baugh and Ryan 2009). In tungara frogs (*Engystomops pustulosus*), females gather information about male

quality through complexity of male vocalizations (Akre and Ryan 2010; Baugh and Ryan 2009). Male gray tree frogs (*Hyla versicolor*) that produce long duration calls are fast growing, with shorter larval period and high survival rate compared to males with short calls (Welch et al. 1998). In grey tree frogs (*H. versicolor*), males with long calls have a higher phenotypic quality (Welch et al. 1998). Thus, consistent with all of these other vertebrate species, *P. boylii* females could rely on call duration and frequency as indicators of male genetic and physical qualities and thus, individual differences in male vocalizations may serve to facilitate mate choice and copulation.

***Peromyscus boylii* females**

My results on USVs produced by *P. boylii* females are consistent with observations on *M. musculus* females. As I predicted, I found that resident *P. boylii* females vocalize (**P_9**), that these vocalizations are produced in the presence of another female (**P_7**), and when pups are emerging from the nest (**P_{10}**). These results support my hypotheses that USVs produced by females mediate social interactions between females and that vocalizations serve as warning signals for pups. Furthermore, I found individual differences in females USVs (**P_8**), which further support my hypothesis that female USVs mediate social interactions.

From the one-minute video analysis I found that context during which USVs were produced (alone or not alone) was independent of sex. That is, lone females vocalize just as often as lone males (29% and 31%), and females vocalize just as often in the presence of another mouse as do males (71% and 69%). However, when comparing the two

contexts for females only, females vocalized more often when in the presence of another mouse (71%) than when alone (29%). Furthermore, from the one-hour video analysis, I found that on-camera intervals with USVs were dependent on context (male alone, male with male, male with female, female with female, and female alone). Based on the one-hour analysis, lone females produced fewer vocalizations, compared to mice in other situational contexts. Both results suggest that they vocalize in all different contexts, however, females vocalize more when in the presence of another mouse and females rarely vocalize when alone. Thus, female vocalizations appears to be important for communication between mice.

Peromyscus boylii females vocalize in the presence of a male. From the one-minute video analysis, 73% of female USVs were produced in presence of a male. Furthermore, females that vocalized in the presence of a male had newly weaned pups at the time the USVs were produced. However, from the one-hour analysis, during male - female interactions, females never vocalized. These contradicting results could be attributed to the time during which the interactions occurred and data were analyzed. During the one-minute video, vocalizations produced by females were recorded when the females had emerging pups. Whereas, when the one-hour video analysis was examined, the pups may not have been weaned. Thus, I suggest that when females with newly weaned pups vocalized in presence of a male, these vocalizations may serve as warning signals for pups emerged from the nest.

Females vocalized in the presence of another female, as predicted (**P_7**). Furthermore, *P. boylii* females that vocalized were residents, also as predicted (**P_9**).

Fourteen of the 16 resident females (87%) in the study were recorded vocalizing in the presence of another mouse. From the one-minute video analysis, 27% of the USVs produced by females were produced in the presence of another female. From the one-hour video analysis, females vocalized 36% of the time. In *M. musculus*, females produce vocalizations during female-female encounters (Maggio and Whitney 1985; Moles and D'Amato 2000). *Mus musculus* vocalizations produced by a resident female may serve to establish social dominance hierarchy as well as having an affiliative function (Maggio and Whitney 1985; Moles and D'Amato 2000). Likewise, I suggest that vocalizations produced by resident *P. boylii* females may serve to mediate social interactions with other females.

Peromyscus boylii females occasionally produced vocalizations when they are alone. From the one-minute video analysis, 31% of the vocalizations were produced by lone females. From the one-hour video analysis, lone females vocalized 8%, of the time on the on-camera interval. *Mus musculus* females vocalize when they have pups emerging from the nest regardless of presence or absence of a second mouse (Ehret 2005). These vocalizations may serve as warning signals for the pups to indicate presence of danger (Sales and Pye 1974). *Mus musculus* females are territorial, especially when they have pups in the nest and therefore act aggressively, which may reduce infanticide (White et al. 1998; Costantini and D'Amato 2006). Although I could not see the nests of resident females, it is possible that *P. boylii* females vocalized when pups were just emerging from the nest. Based on my trapping data, the time during which the resident

females vocalized corresponded to the time the pups were being weaned. Thus, I suggest that vocalizations produced by lone *P. boylii* females may serve as warning signals for pups as pups emerge from the nest.

I found individual differences among females in spectral characters USVs, as predicted (**P_8**). Specifically, differences occurred in sound frequency and bandwidth. The individual differences I found in *P. boylii* female USVs may provide evidence of individual identification and serve in recognition of neighbors. Individual recognition may be important because females are territorial (Kalcounis-Rueppell and Spoon 2009). In addition, neighbor recognition could be important because neighbors are less of a threat than non-neighbors, as they already hold a territory and are therefore are less likely to try and seize the territory of their neighbor (Brunton et al. 2008). Therefore, I suggest that differences in spectral characters among individual *P. boylii* females may facilitate individual and neighbor recognition which would be important for mediating social interactions among females.

Motif and spectral differences between males and females

I found two lines of evidence to suggest that identification of sex is communicated through USVs, as predicted (**P_4**). First males produce different motifs from females. Females produce more 3SVs than expected. *Peromyscus boylii* and *M. musculus* share a similar breeding system. Both species are promiscuous or polygamous, depending on population density (Kalcounis-Rueppell and Spoon 2009; Sales and Pye 1974). Male *M. musculus* emit vocalizations as part of their courtship behavior which

may attract females (Sales 1972; Whitney and Nyby 1979; Musolf et al. 2010). Therefore, it may be beneficial to advertise sexual identity through motif type when searching for a new mate. Second, sexes differed based on modulation and bandwidth of vocalizations. The mean overall modulation of 2SVs was lower in males than females, and mean bandwidth of 3SVs as lower in males than females. Male *M. musculus* vocalize when approaching females (White et al. 1998; Costantini and D'Amato 2006; Hammerschmidt et al. 2009), which may reduce female aggression and keep females in close proximity. Vocalizations may indicate to the female that the male is not aggressively motivated but instead sexually motivated (Sales and Pye 1974). Advertising sex through acoustic characteristics is therefore beneficial for sexually mature males in reduceing female aggression. I suggest that *P. boylii* may advertise identity of sex through motif type, bandwidth and modulation of USVs. Thus, demonstrating sex and potential mate quality through USVs may serve to attract mates and facilitate copulation.

Overall, my data are compatible with my hypotheses that the vocalizations produced by *P. boylii* are important for communication in reproductively active mice and may serve as sexual, territorial and warning signals. However, further experiments in this field are necessary to distinguish among these hypotheses. If USVs serve as sexual signals, then males who vocalize in the in the presence of an estrous female will have a higher reproductive success than males who do not. If USVs serve a territorial function, then resident females will more often produce vocalizations in the presence of a strange female as opposed to the presence of a female neighbor. If USVs serve as warning signals

for the pups, than females who vocalize during the time that their pups are emerging from the nest will have higher reproductive success than females who do not. Future studies could examine whether there is a functional significance of the different motifs.

Although vocalizations from *P. boylii* have been previously recorded in the wild (Kalcounis-Rueppell et al. 2006), this is the first and only study conducted on USV production by known *P. boylii* individuals. I found that *P. boylii* males and females regularly produce ultrasonic vocalizations in the wild and they differ with respect to motif type produced and acoustic characteristics. Moreover, there is individual variation in the characteristics of USVs, suggesting that characteristics of individuals might be communicated through USVs. My results suggest that vocalizations produced by males may serve to attract females and facilitate copulation. Moreover, vocalizations produced by females may serve to mediate social interactions with other females and as warning signals for newly weaned pups.

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APPENDIX A: TABLES

Table 1. Number of intervals, minutes during each interval and number of USVs during each interval for the five different contexts during the 18 hours of video.

Table shows number of intervals (each time a mouse or mice entered and exited the camera screen), total time for intervals (total number of minutes the mouse or mice were on the screen), number of intervals with USVs, total time for intervals with USVs, and total number of USVs produced during the interval. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February -June 2008 and January 2009.

	Number of Intervals	Total Time for Intervals (minutes)	Number of Intervals with USVs	Total Time for Intervals with USVs (minutes)	Total Number of USVs Produced
Male Alone	15	32	4	7	4
Female Alone	62	179	5	11	11
Male with Male	0	0	0	0	0
Female with Female	11	30	4	15	10
Female with Male	3	32	2	31	2

Table 2. Principle Component Score (PC) for the first syllables of the 1, 2, 3, and 4 syllable vocalizations.

Table shows the first principle component score (PC1) of the first syllable for the five original frequency variables. The five frequency variables (start frequency, end frequency, maximum frequency, minimum frequency, frequency at maximum amplitude) were highly correlated with one another and therefore were reduced to a single variable using a principle components (PC) analysis. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February -June 2008 and January 2009. All frequency variables are in kilohertz (kHz).

Motif Type	Sample Size n=	Start Freq (kHz)	End Freq (kHz)	Max Freq (kHz)	Min Freq (kHz)	Freq at Max Amp (kHz)	PC1
1-SV	40	0.94	0.97	0.98	0.97	0.98	-93.87%
2-SV	71	0.92	0.93	0.96	0.95	0.97	-88.64%
3-SV	36	0.94	0.95	0.97	0.96	0.97	-92.18%
4-SV	13	0.98	0.98	0.98	0.99	0.99	-96.72%

Table 3. Comparison of 1SVs from male and female *Peromyscus boylii* using Mann-Whitney U Statistic

Comparison of 1 syllable vocalizations produced by female and male *Peromyscus boylii*. Descriptive statistics are used on the original acoustic variables and PC1 score. The PC1 score incorporates the 5 original frequency variables. The Mann-Whitney U test is significant (*) at $p<0.008$. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, February -June 2008 and January 2009. Duration is in milliseconds (ms) and all frequency and bandwidth variables are in kiloHertz (kHz).

	Female (n=18)	Male (n=22)	Mann-Whitney U	
Acoustic Variable	**Mean±SD**	**Mean±SD**	**U**	**P**
Duration (ms)	180±63	160±61	166.0	0.396
Start Freq (KHz)	26.78±6.65	29.45±4.84		
End Freq (KHz)	25.24±6.14	27.79±4.24		
Max Freq (KHz)	29.48±6.32	31.06±4.85		
Min Freq (KHz)	23.97±6.34	26.78±4.17		
Freq Max Amp (KHz)	28.39±6.63	30.33±4.80		
Start to Max Freq (ms)	50±36	30±20	132.0	0.075
Bandwidth (KHz)	5.52±2.07	4.28±2.19	131.5	0.070
Internal Modulation	95500.82±95471.85	98532.37±157468.99	178.0	0.600
Overall Modulation	20309.45±15034.48	17854±11430.96	188.5	0.798
PC1	-0.23±1.17	0.19±0.81	157.0	0.274

Table 4. Comparison of 2SVs from male and female *Peromyscus boylii* using Mann-Whitney U Statistic

Comparison of 2 syllable vocalizations produced by female and male *Peromyscus boylii*. Descriptive statistics are used on the original acoustic variables and PC1 score. The PC1 score incorporates the 5 original frequency variables. The Mann-Whitney U test is significant (*) at p<0.008. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, February -June 2008 and January 2009. Duration is in milliseconds (ms) and all frequency and bandwidth variables are in kiloHertz (kHz).

	Female (n=29)	Male (n=7)	Mann-Whitney U	
Acoustic Variable	**Mean±SD**	**Mean±SD**	**U**	**P**
Syllable 1				
Duration (ms)	110.93±47.98	134.57±72.62	342.5	0.003
Start Freq (KHz)	26.58±5.78	23.47±5.25		
End Freq (KHz)	24.51±5.40	20.60±6.20		
Max Freq (KHz)	28.84±5.58	24.66±6.43		
Min Freq (KHz)	23.53±5.43	20.171±5.78		
Freq Max Amp (KHz)	27.95±5.52	24.10±6.84		
Start to Max Freq (ms)	43.53±38.14	50.14±56.85	433.0	0.056
Bandwidth (KHz)	5.32±2.82	4.86±3.20	456.0	0.101
Internal Modulation	166057.89±225472.81	75687.38±85125.05	468.5	0.137
Overall Modulation	30655.84±20407.20	13917.19±12578.70	274.7	0.000*
PC1	0.13±0.96	0.11±0.75	553.0	0.627
Syllable 2				
Duration (ms)	145.47±50.26	173.24±67.26	416.0	0.035
Start Freq (KHz)	27.59±4.91	26.60±5.13		
End Freq (KHz)	29.30±4.12	26.46±4.56		
Max Freq (KHz)	33.17±3.58	30.51±4.82		
Min Freq (KHz)	26.87±4.96	24.99±4.85		
Freq Max Amp (KHz)	32.26±3.65	29.69±4.58		
Start to Max Freq (ms)	52.39±25.94	59.99±25.75	519.5	0.377
Bandwidth (KHz)	6.31±2.86	5.53±1.93	417.5	0.036
Internal Modulation	487863.41±1606302.55	101730.11±93177.02	584.0	0.906
Overall Modulation	18961.01±18486.13	11181.11±67471.51	371.0	0.008*
PC1	0.11±0.96	-0.44±1.13	558.0	0.670

Table 5. Comparison of 3SVs from male and female *Peromyscus boylii* using Mann-Whitney U Statistic

Comparison of 3 syllable vocalizations produced by female and male *Peromyscus boylii*. Descriptive statistics are used on the original acoustic variables and PC1 score. The PC1 score incorporates the 5 original frequency variables. The Mann-Whitney U test is significant (*) at $p<0.008$. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, February -June 2008 and January 2009. Duration is in milliseconds (ms) and all frequency and bandwidth variables are in kiloHertz (kHz).

	Female (n=29)	Male (n=7)	Mann-Whitney U	
Acoustic Variable	**Mean±SD**	**Mean±SD**	**U**	**P**
Syllable 1				
Duration (ms)	110.93±47.98	134.57±72.62	79.0	0.387
Start Freq (KHz)	26.58±5.78	23.47±5.25		
End Freq (KHz)	24.51±5.40	20.60±6.20		
Max Freq (KHz)	28.84±5.58	24.66±6.43		
Min Freq (KHz)	23.53±5.43	20.171±5.78		
Freq Max Amp (KHz)	27.95±5.52	24.10±6.84		
Start to Max Freq (ms)	43.53±38.14	50.14±56.85	100.0	0.969
Bandwidth (KHz)	5.32±2.82	4.86±3.20	79.5	0.387
Internal Modulation	166057.89±225472.81	75687.38±85125.05	64.0	0.142
Overall Modulation	30655.84±20407.20	13917.19±12578.70	86.0	0.557
PC1	0.13±0.96	0.11±0.75	64.0	0.142
Syllable 2				
Duration (ms)	145.47±50.26	173.24±67.26	83.0	0.480
Start Freq (KHz)	27.59±4.91	26.60±5.13		
End Freq (KHz)	29.30±4.12	26.46±4.56		
Max Freq (KHz)	33.17±3.58	30.51±4.82		
Min Freq (KHz)	26.87±4.96	24.99±4.85		
Freq Max Amp (KHz)	32.26±3.65	29.69±4.58		
Start to Max Freq (ms)	52.39±25.94	59.99±25.75	88.0	0.611
Bandwidth (KHz)	6.31±2.86	5.53±1.93	94.0	0.784
Internal Modulation	487863.41±1606302.55	101730.11±93177.02	63.0	0.131
Overall Modulation	18961.01±18486.13	11181.11±67471.51	78.0	0.366
PC1	0.11±0.96	-0.44±1.13	77.0	0.345
Syllable 3				
Duration (ms)	115.97±37.05	107.57±65.61	85.0	0.531
Start Freq (KHz)	27.93±4.78	27.99±73.07		
End Freq (KHz)	30.04±4.02	27.21±5.21		
Max Freq (KHz)	33.09±3.51	30.03±5.05		
Min Freq (KHz)	27.48±4.99	26.94±5.03		
Freq Max Amp (KHz)	32.51±3.53	29.39±5.09		
Start to Max Freq (ms)	36.78±18.57	22.04±15.34	51.0	0.044
Bandwidth (KHz)	5.60±3.57	3.09±0.91	29.5	0.002*
Internal Modulation	205471.22±138994.71	78089.83±54368.79	40.0	0.012
Overall Modulation	26256.48±28746.68	16131.54±11935.67	89.0	0.639
PC1	0.10±0.92	0.79±0.82	81.0	0.433

Table 6. Comparison of 4SVs from male and female *Peromyscus boylii* using Mann-Whitney U Statistic

Comparison of 4 syllable vocalizations produced by female and male *Peromyscus boylii*. Descriptive statistics are used on the original acoustic variables and PC1 score. The PC1 score incorporates the 5 original frequency variables. The Mann-Whitney U test is significant (*) at p<0.008. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, February -June 2008 and January 2009. Duration is in milliseconds (ms) and all frequency and bandwidth variables are in kiloHertz (kHz).

	Female (n=10)	Male (n=3)	Mann-Whitney U	
Acoustic Variable	**Mean±SD**	**Mean±SD**	**U**	**P**
Syllable 1				
Duration (ms)	106.20±29.96	144.67±35.64	7.0	0.217
Start Freq (KHz)	26.66±6.49	28.77±3.38		
End Freq (KHz)	23.29±5.29	24.87±2.24		
Max Freq (KHz)	27.62±5.84	30.23±1.27		
Min Freq (KHz)	22.51±5.80	24.03±2.51		
Freq Max Amp (KHz)	26.94±5.84	28.43±2.03		
Start to Max Freq (ms)	30.80±20.15	44.67±7.1	6.0	0.161
Bandwidth (KHz)	5.11±1.81	6.20±2.04	9.0	0.371
Internal Modulation	62169.60±37620.18	51486.39±25138.05	12.0	0.692
Overall Modulation	33641.70±2329.23	30328.84±26510.02	15.0	1
PC1	-0.08±1.12	0.28±0.40	9.0	0.371
Syllable 2				
Duration (ms)	136.40±21.54	123.33±30.07	12.0	0.692
Start Freq (KHz)	28.27±2.92	28.47±3.91		
End Freq (KHz)	29.39±3.72	26.00±1.78		
Max Freq (KHz)	33.24±2.86	32.00±2.04		
Min Freq (KHz)	27.31±3.62	25.33±1.95		
Freq Max Amp (KHz)	31.73±3.87	30.70±2.55		
Start to Max Freq (ms)	51.80±32.36	35.67±18.82	9.0	0.371
Bandwidth (KHz)	5.93±1.74	6.67±0.64	7.0	0.271
Internal Modulation	164846.60±95763.230	70499.62±25917.31	6.0	0.161
Overall Modulation	18282.20±11094.44	24529.02±37095.41	10.0	0.469
PC1	0.12±1.07	-0.39±0.72	11.0	0.573
Syllable 3				
Duration (ms)	128.10±12.98	125.33±47.43	11.0	0.573
Start Freq (KHz)	29.00±3.07	28.10±4.61		
End Freq (KHz)	30.28±3.36	26.60±2.25		
Max Freq (KHz)	33.84±2.58	31.67±1.95		
Min Freq (KHz)	28.27±3.06	25.67±2.97		
Freq Max Amp (KHz)	32.37±3.59	31.33±2.20		
Start to Max Freq (ms)	58.00±37.09	26.00±18.00	6.0	0.161
Bandwidth (KHz)	5.57±1.52	6.00±1.01	10.5	0.469
Internal Modulation	152821.87±111914.208	163569.44±187414.56	14.0	0.937
Overall Modulation	19316.24±10336.56	32839.27±37166.33	14.0	0.937
PC1	0.17±1.02	-0.56±0.86	8.0	0.287
Syllable 4				
Duration (ms)	99.90±25.62	89.33±10.50	11.5	0.573
Start Freq (KHz)	29.93±3.02	29.27±2.24		
End Freq (KHz)	30.72±3.16	25.17±0.23		
Max Freq (KHz)	33.65±2.58	31.23±2.24		
Min Freq (KHz)	28.81±3.80	25.03±0.23		
Freq Max Amp (KHz)	32.67±2.36	30.07±2.97		
Start to Max Freq (ms)	34.70±26.31	35.33±24.83	14.0	0.937
Bandwidth (KHz)	4.84±2.62	6.20±2.46	8.0	0.287
Internal Modulation	227417.81±258015.21	81613.15±24894.51	8.0	0.287
Overall Modulation	25595.54±22156.31	47147.04±29718.16	7.0	0.217
PC1	0.26±0.97	-0.87±0.52	5.0	0.112

APPENDIX B: FIGURES

Figure 1. Example of a spectrograph of a 3 Syllable Vocalization from a free-living *Peromyscus boylii* Hastings Natural History Reserve, Carmel Valley, CA.

An example of a 3-syllable vocalization showing parameters measured **a**) start frequency (kHz), **b**) maximum frequency (kHz), **c**) frequency at maximum amplitude, **d**) end frequency (kHz), **e**) minimum frequency (kHz). Calculations from these annotations as follows: syllable duration= ׀time of **a** – time of **e**׀; bandwidth= frequency of **b** – frequency of **e**; internal modulation= frequency of **b** – frequcncy of **e**/ ׀time between **b** and **e**׀; overall modulation= ׀frequency of **a**-frequency of **d**׀/duration. Parameters of the spectrograph include FFT length of 512, and 100% frame size with Hamming window. Window overlap was 50%. Frequency is measured in kHz on the y-axis and time is measures in seconds on the x-axis.

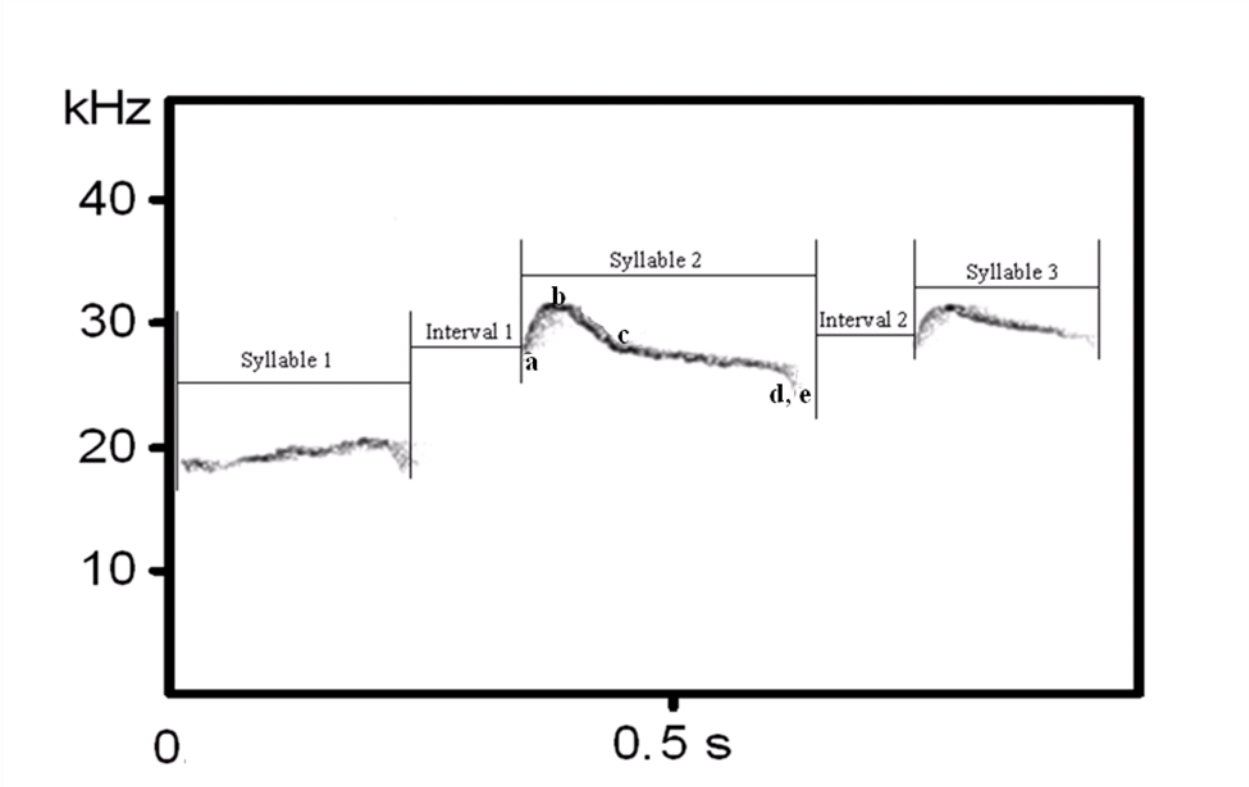


Figure 2. Spectrograph of the 6 motifs produced by *Peromyscus boylii.*

Vocalizations were recorded from wild populations of *Peromyscus boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February – June 2008 and January 2009. Frequency is measured in kHz on the y-axis and time is measures in seconds on the x-axis. a) frequency modulated bark, b) 1SV, c) 2SV, d) 3SV, e) 4SV, f) 5SV. Parameters of the spectrograph include: FFT length of 512, and 100% Frame size with Hamming window. Window overlap was 50%.

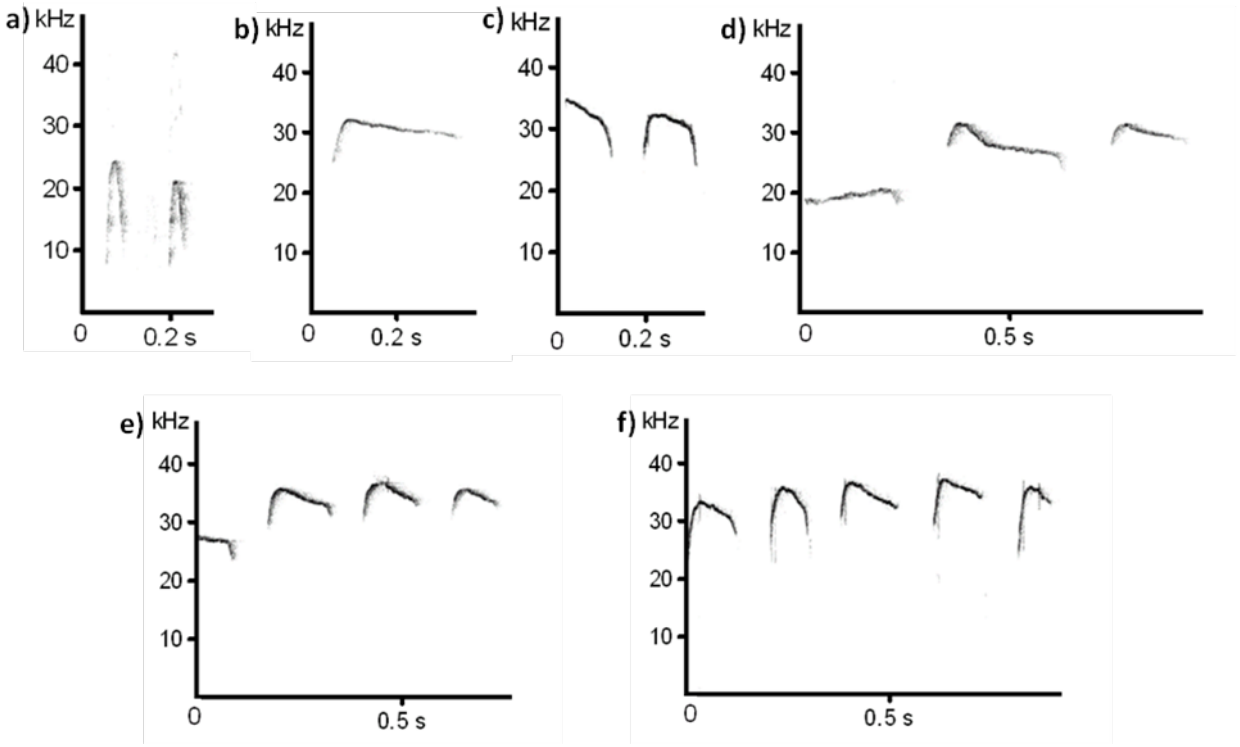


Figure 3. Histogram of the number of vocalizations produced by each sex during the month.

The number of vocalizations (n=170) produced each month, throughout the field season. Numbers of vocalizations produced were counted within the month. Vocalizations were recorded from a wild population of *P. boylii* at HastingsNatural History Reserve, Carmel Valley, CA, February-June 2008.

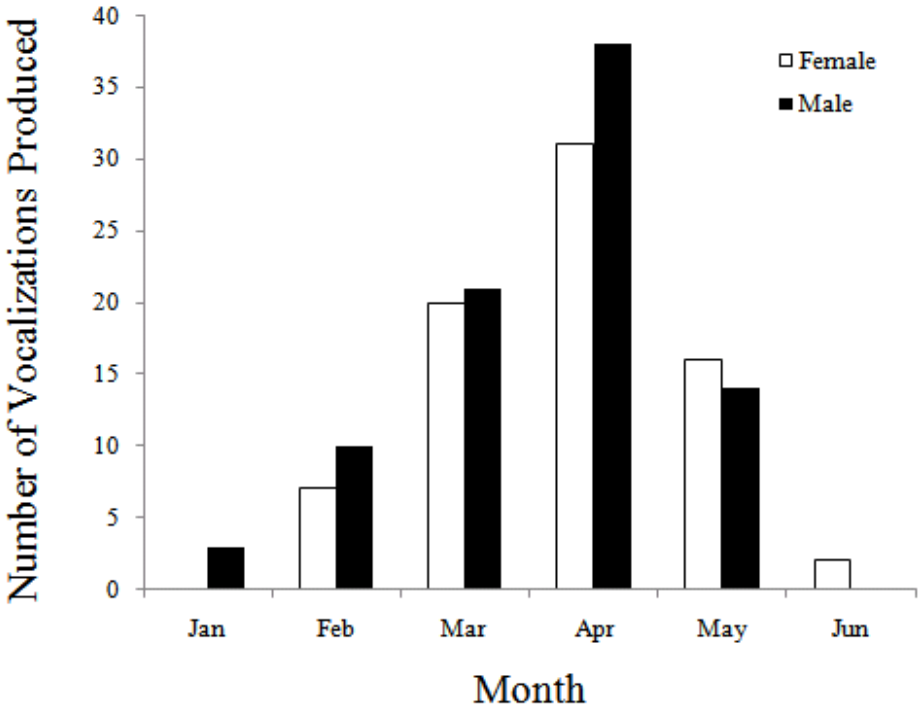


Figure 4. Histogram of the number of intervals and vocalizations produced for the five different contexts

The number of intervals (n=91) and vocalizations (n=25) produced during the 18 hours of video data. Vocalizations were recorded from a wild population of *P. boylii* at HastingsNatural History Reserve, Carmel Valley, California.

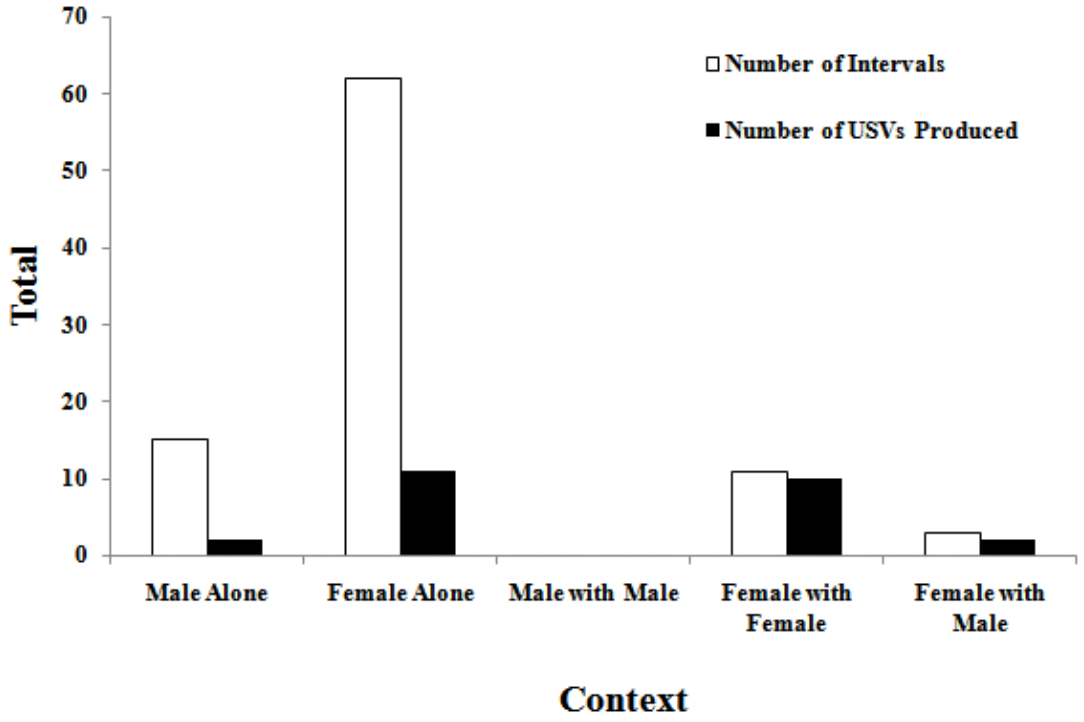
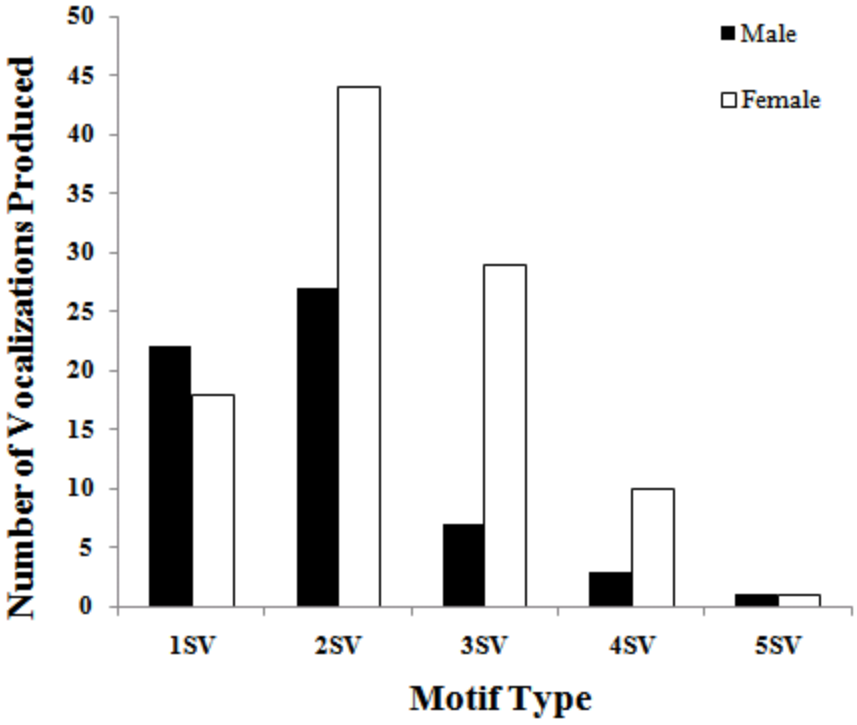


Figure 5. Motif distribution by sex of *Peromyscus boylii*.

Total number of vocalizations produced for the five motifs by each sex of *Peromyscus boylii*. Vocalizations were recorded from a wild population of *Peromyscus boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February – June 2008 and January 2009.



APPENDIX C: SUPPLEMENTAL INFORMATION

Table S1. Comparison of 1SV Among Individual Males Using Mann-Whitney U Statistics

The means, plus and minus standard deviations for the 1 syllable vocalizations produced by 4 different. *P. boylii* males. Descriptive statistics are used on the original acoustic variables and PC1 score (First principle component of Frequency Variables). The Kruskal-Wallis test is significant (*) at p<0.008. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February -June 2008 and January 2009. Duration is in milliseconds and all frequency and bandwidth variables are in kilohertz.

Tag Number	**1045 (n=3)**	**1210 (n=4)**	**1253 (n=9)**	**1264 (n=3)**	Kruskal-Wallis **U**	**P**
Acoustic Variable						
Duration (ms)	99.93±13.02	120.525±37.62	207.567±48.71	186.5±16.55	14	0.003*
Start Freq (KHz)	24.23±6.47	30.1±4.03	30.26667±3.61	31.7±2.55		
End Freq (KHz)	23.23±4.39	30.175±4.85	28.3±3.25	27.63333±1.60		
Max Freq (KHz)	25.17±5.68	32.4±4.77	32.23333±3.15	32.8±2.25		
Min Freq (KHz)	22.77±4.37	28.375±4.72	27.06667±3.56	27.3±1.50		
Freq Max Amp (KHz)	25.00±5.39	31.925±5.02	31.3±3.30	31.53333±1.53		
Start to Max Freq (ms)	40.03±25.06	39.575±18.03	31.1±19.41	46.9±11.43	1.9	0.589
Bandwidth (KHz)	2.40±1.32	4.025±0.85	5.16667±2.01	5.5±3.63	4.9	0.183
Internal Modulation	54482.71±9810.48	140901.59±122617.59	52350.58±51820.36	63519.6±39987.81	5.8	0.124
Overall Modulation	13155.52± 19009.72	19304.40±7080.58	17321.79±9446.48	20731.18±18393.58	0.9	0.822
PC1	-0.6548639±1.00	0.6023333±0.89	0.451886±.58	0.515941±0.14	5.2	0.158

Table S2. Comparison of 2SV Syllable 1 Among Individual Males Using Mann-Whitney U Statistics

The means, plus and minus standard deviations for the 2SVs syllable 1 vocalizations produced by 5 different *P. boylii* males. Descriptive statistics are used on the original acoustic variables and PC1 score (First principle component of Frequency Variables). The Kruskal-Wallis test is significant (*) at $p<0.008$. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February -June 2008 and January 2009. Duration is in milliseconds and all frequency and bandwidth variables are in kilohertz.

Tag Number	**1026 (n=8)**	**1045 (n=4)**	**1210 (n=9)**	
Acoustic Variable				
Syllable 1				
Duration (ms)	219.00±75.83	243.75±22.91	135.57±22.89	
Start Freq (KHz)	30.35±2.48	30.48±1.45	29.02±4.17	
End Freq (KHz)	29.61±2.11	27.65±1.77	28.9±3.82	
Max Freq (KHz)	33.58±2.43	33.28±1.87	31.24±3.10	
Min Freq (KHz)	29.01±2.15	27.05±1.76	27.34±3.24	
Freq Max Amp (KHz)	32.61±2.91	32.30±2.10	32.88±2.61	
Start to Max Freq (ms)	65.25±35.45	49.50±28.48	21.34±1.87	
Bandwidth (KHz)	4.56±1.13	6.23±0.22	5.53±1.94	
Internal Modulation	41921.36±45646.89	28473.89±2478.18	639793.67±1598842.30	
Overall Modulation	5635.22±1846.57	11797.24±9146.02	19896.89±14561.90	
PC1	0.62±0.53	0.42±0.36	0.52±0.47	
Tag Number	**1253 (n=5)**	**1264 (n=6)**	**Kruskal-Wallis U**	**P**
Duration (ms)	148.06±80.35	206.98±58.76	12.9	0.012
Start Freq (KHz)	26.78±4.26	26.83.348±		
End Freq (KHz)	26.24±4.30	25.25±3.16		
Max Freq (KHz)	29.14±3.69	29.00±2.86		
Min Freq (KHz)	24.76±4.42	24.43±3.21		
Freq Max Amp (KHz)	27.60±4.59	28.03±2.91		
Start to Max Freq (ms)	28.52±19.29	57.62±45.55	9.1	0.058
Bandwidth (KHz)	4.38±1.80	4.57±0.88	7.8	0.099
Internal Modulation	88909.09±47754.54	91233.30±104745.22	10.6	0.032
Overall Modulation	20393.55±8859.49	13071.85±8525.13	10.7	0.031
PC1	-0.33±0.91	-0.37±0.67	8.27	0.082

Table S3. Comparison of 2SV Syllable 2 Among Individual Males Using Mann-Whitney U Statistics

The means, plus and minus standard deviations for the 2SVs syllable 2 vocalizations produced by 5 different *P. boylii* males. Descriptive statistics are used on the original acoustic variables and PC1 score (First principle component of Frequency Variables). The Kruskal-Wallis test is significant (*) at $p<0.008$. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February -June 2008 and January 2009. Duration is in milliseconds and all frequency and bandwidth variables are in kilohertz.

Tag Number	**1026 (n=8)**	**1045 (n=4)**	**1210 (n=9)**	
Acoustic Variable				
Syllable 2				
Duration (ms)	156.88±26.31	186.25±13.43	84.79±27.98	
Start Freq (KHz)	31.51±2.25	28.25±2.32	30.60±2.48	
End Freq (KHz)	31.70±0.85	29.38±1.26	32.23±2.84	
Max Freq (KHz)	34.74±0.81	33.53±1.65	34.02±1.89	
Min Freq (KHz)	30.40±1.75	27.88±2.12	30.48±2.48	
Freq Max Amp (KHz)	34.44±0.84	32.80±1.07	33.41±1.76	
Start to Max Freq (ms)	36.00±28.82	49.50±24.28	21.34±1.87	
Bandwidth (KHz)	4.34±1.35	5.65±0.87	3.53±1.50	
Internal Modulation	131112.85±118682.58	209856.77±142216.14	157820.02±53207.65	
Overall Modulation	11480.75±9561.81	7349.34±4708.96	27343.19±31423.79	
PC1	0.83±0.40	-0.01±0.66	0.49±0.93	
Tag Number	**1253 (n=5)**	**1264 (n=6)**	**Kruskal-Wallis U**	**P**
Duration (ms)	169.12±63.38	155.98±51.59	16.2	0.003*
Start Freq (KHz)	29.54±1.47	26.12±2.90		
End Freq (KHz)	29.06±2.58	28.27±2.19		
Max Freq (KHz)	32.56±1.09	31.13±2.20		
Min Freq (KHz)	28.10±2.10	26.12±2.90		
Freq Max Amp (KHz)	31.70±1.58	29.82±1.64		
Start to Max Freq (ms)	55.08±40.71	60.90±38.01	5.3	0.058
Bandwidth (KHz)	4.46±1.47	5.02±1.92	6.3	0.260
Internal Modulation	66136.00±88142.63	206616.40±138179.76	7.0	0.177
Overall Modulation	10875.05±8699.19	15025.98±8616.97	2.7	0.611
PC1	-0.17±0.66	-0.94±0.89	16.2	0.003*

Table S4. Comparison of 1SV Among Individual Females Using Mann-Whitney U Statistics

The means, plus and minus standard deviations for the 1 syllable vocalizations produced by 3 different. *P. boylii* females. Descriptive statistics are used on the original acoustic variables and PC1 score (First principle component of Frequency Variables). The Kruskal-Wallis test is significant (*) at $p<0.008$. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February -June 2008 and January 2009. Duration is in milliseconds and all frequency and bandwidth variables are in kilohertz.

Tag Number	**1118 (n=4)**	**1163 (n=4)**	**1247 (n=4)**	Kruskal-Wallis **U**	**P**
Acoustic Variable					
Duration (ms)	194.925±71.8	141.45±76.07	197.75±45.64	2.3	0.309
Start Freq (KHz)	19.625±0.63	33.4±3.03	21.925±3.81		
End Freq (KHz)	16.925±0.43	30±4.07	23.65±0.87		
Max Freq (KHz)	21.525±0.25	33.9±3.29	26.55±0.87		
Min Freq (KHz)	16.225±0.48	29.375±4.67	21.2±3.01		
Freq Max Amp (KHz)	20.2±2.4	32.775±3.54	25.475±0.90		
Start to Max Freq (ms)	54.65±51.69	45.625±21.23	63.25±26.17	1.4	0.491
Bandwidth (KHz)	5.3±0.41	4.525±1.44	5.35±3.43	0.1	0.944
Internal Modulation	43549.87±8970.38	44994.41±16233.35	144459.15±133959.72	0.1	0.943
Overall Modulation	16377.36±10201.66	30226.04±24394.33	13575.88±7236.07	1.7	0.437
PC1	-1.65205±1.00	0.8417135±1.00	-0.704548±0.25	9.8	0.007*

Table S5. Comparison of 2SV Syllable 1 Among Individual Females Using Mann-Whitney U Statistics

The means, plus and minus standard deviations for the 2SVs syllable 1 vocalizations produced by 6 different *P. boylii* females. Descriptive statistics are used on the original acoustic variables and PC1 score (First principle component of Frequency Variables). The Kruskal-Wallis test is significant (*) at $p<0.008$. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February -June 2008 and January 2009. Duration is in milliseconds and all frequency and bandwidth variables are in kilohertz.

Tag Number	1027 (n=11)	1032 (n=7)	1033 (n=3)	1063 (n=3)
Acoustic Variable		**Mean±SD**		
Syllable 1				
Duration (ms)	147.73±33.44	144.39±46.28	100.00±52.05	59.20±40.65
Start Freq (KHz)	29.02±3.33	33.64±2.033	23.87±5.91	29.90±4.98
End Freq (KHz)	26.72±5.45	30.21±3.25	21.47±2.54	26.13±3.23
Max Freq (KHz)	32.31±2.86	34.70±1.52	25.50±4.08	30.07±5.19
Min Freq (KHz)	25.21±4.83	29.54±3.11	19.97±3.44	25.97±3.44
Freq Max Amp (KHz)	29.77±4.62	33.56±2.10	23.07±2.65	28.43±3.50
Start to Max Freq (ms)	34.39±48.64	31.66±18.48	66.33±59.48	32.10±44.5
Bandwidth (KHz)	7.10±3.10	5.16±1.93	5.53±0.71	4.10±4.50
Internal Modulation	96218.64±60218.35	42623.56±16545.04	86666.67±15275.25	71262.94±15214.90
Overall Modulation	29263.72±17832.11	25711.98±29434.89	42606.06±29434.89	50566.10±28058
PC1	0.07±0.86	0.92±0.82	-1.27±0.819	-0.05±0.84
Tag Number	**1190 (n=7)**	**1247 (n=7)**	**Kruskal-Wallis U**	**P**
Duration (ms)	184.95±41.27	180.00±90.27	10.6	0.060
Start Freq (KHz)	31.03±2.35	24.64±7.40		
End Freq (KHz)	24.10±2.78	22.84±7.40		
Max Freq (KHz)	32.58±2.47	26.31±6.59		
Min Freq (KHz)	23.72±2.64	22.41±7.57		
Freq Max Amp (KHz)	31.77±2.36	25.19±7.14		
Start to Max Freq (ms)	25.12±6.95	71.00±48.95	6.1	0.293
Bandwidth (KHz)	8.87±1.97	3.90±1.83	17.0	0.005*
Internal Modulation	56124.51±12687.20	48595.07±36082.69	10.9	0.054
Overall Modulation	38607.80±7723.30	12321.32±12037.37	13.3	0.031
PC1	0.24±0.44	-0.93±1.63	13.6	0.018

Table S6. Comparison of 2SV Syllable 2 Among Individual Females Using Mann-Whitney U Statistics

The means, plus and minus standard deviations for the 2SVs syllable 2 vocalizations produced by 6 different *P. boylii* females. Descriptive statistics are used on the original acoustic variables and PC1 score (First principle component of Frequency Variables). The Kruskal-Wallis test is significant (*) at $p<0.008$. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February -June 2008 and January 2009. Duration is in milliseconds and all frequency and bandwidth variables are in kilohertz.

Tag Number	**1027 (n=11)**	**1032 (n=7)**	**1033 (n=3)**	**1063 (n=3)**
Acoustic Variable		**Mean±SD**		
Syllable 1				
Duration (ms)	147.73±33.44	144.39±46.28	100.00±52.05	59.20±40.65
Start Freq (KHz)	29.02±3.33	33.64±2.033	23.87±5.91	29.90±4.98
End Freq (KHz)	26.72±5.45	30.21±3.25	21.47±2.54	26.13±3.23
Max Freq (KHz)	32.31±2.86	34.70±1.52	25.50±4.08	30.07±5.19
Min Freq (KHz)	25.21±4.83	29.54±3.11	19.97±3.44	25.97±3.44
Freq Max Amp (KHz)	29.77±4.62	33.56±2.10	23.07±2.65	28.43±3.50
Start to Max Freq (ms)	34.39±48.64	31.66±18.48	66.33±59.48	32.10±44.5
Bandwidth (KHz)	7.10±3.10	5.16±1.93	5.53±0.71	4.10±4.50
Internal Modulation	96218.64±60218.35	42623.56±16545.04	86666.67±15275.25	71262.94±15214.90
Overall Modulation	29263.72±17832.11	25711.98±29434.89	42606.06±29434.89	50566.10±28058
PC1	0.07±0.86	0.92±0.82	-1.27±0.819	-0.05±0.84
Tag Number	**1190 (n=7)**	**1247 (n=7)**	**Kruskal-Wallis U**	**P**
Duration (ms)	184.95±41.27	180.00±90.27	5.8	0.319
Start Freq (KHz)	31.03±2.35	24.64±7.40		
End Freq (KHz)	24.10±2.78	22.84±7.40		
Max Freq (KHz)	32.58±2.47	26.31±6.59		
Min Freq (KHz)	23.72±2.64	22.41±7.57		
Freq Max Amp (KHz)	31.77±2.36	25.19±7.14		
Start to Max Freq (ms)	26.12±6.95	71.00±48.95	10.9	0.054
Bandwidth (KHz)	8.87±1.97	3.90±1.83	11.1	0.049
Internal Modulation	56124.51±12687.2	48595.07±36082.69	12.4	0.030
Overall Modulation	38607.80±7723.30	12321.32±12037.37	5.1	0.399
PC1	0.08±0.52	-0.93±1.63	9.0	0.110

Table S7. Comparison of 3SV Syllable 1 Among Individual Females Using Mann-Whitney U Statistics

The means, plus and minus standard deviations for the 3SVs syllable 1 vocalizations produced by 3 different *P. boylii* females. Descriptive statistics are used on the original acoustic variables and PC1 score (First principle component of Frequency Variables). The Kruskal-Wallis test is significant (*) at $p<0.008$. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February -June 2008 and January 2009. Duration is in milliseconds and all frequency and bandwidth variables are in kilohertz.

Tag Number	**1107 (n=3)**	**1118 (n=3)**	**1247 (n=4)**	**Kruskal-Wallis U**	**P**
Acoustic Variable					
Syllable 1					
Duration (ms)	119.33±50.93	75.10±15.85	101.00±69.06	0.7	0.705
Start Freq (KHz)	33.50±4.14	32.67±0.95	26.05±8.68		
End Freq (KHz)	29.57±1.96	30.03±0.29	24.50±9.49		
Max Freq (KHz)	36.10±1.32	32.67±0.95	29.15±9.59		
Min Freq (KHz)	29.23±2.54	29.53±0.58	23.25±8.52		
Freq Max Amp (KHz)	34.30±1.21	32.03±1.04	28.40±10.03		
Start to Max Freq (ms)	50.00±36.29	17.37±11.28	63.00±67.28	2.2	0.328
Bandwidth (KHz)	6.87±1.27	3.13±0.55	5.90±4.84	2.2	0.333
Internal Modulation	70846.37±23061.56	51078.87±13934.72	275193.12±284743.22	1.1	0.585
Overall Modulation	45526.18±45011.53	35871.31±15408.57	72212.56±121316.27	0.7	0.689
PC1	0.91±0.39	0.69±0.13	-0.30±1.73	1.8	0.408

Table S8. Comparison of 3SV Syllable 2 Among Individual Females Using Mann-Whitney U Statistics

The means, plus and minus standard deviations for the 3SVs syllable 2 vocalizations produced by 3 different *P. boylii* females. Descriptive statistics are used on the original acoustic variables and PC1 score (First principle component of Frequency Variables). The Kruskal-Wallis test is significant (*) at $p<0.008$. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February -June 2008 and January 2009. Duration is in milliseconds and all frequency and bandwidth variables are in kilohertz.

Tag Number	**1107 (n=3)**	**1118 (n=3)**	**1247 (n=4)**	**Kruskal-Wallis U**	**P**
Acoustic Variable					
Syllable 2					
Duration (ms)	155.00±24.02	112.37±6.03	150.75±103.78	3.3	0.196
Start Freq (KHz)	32.00±2.46	30.20±1.73	24.48±7.72		
End Freq (KHz)	32.83±1.55	31.53±0.58	29.50±5.09		
Max Freq (KHz)	36.73±1.48	33.80±0.72	33.18±3.63		
Min Freq (KHz)	30.87±1.53	29.87±1.15	24.48±7.72		
Freq Max Amp (KHz)	35.10±1.50	32.83±1.00	32.30±3.93		
Start to Max Freq (ms)	69.00±37.00	67.37±22.86	26.25±20.61	4.6	0.102
Bandwidth (KHz)	5.87±2.59	3.93±0.50	8.70±4.37	4.8	0.089
Internal Modulation	2957261.90±5059962.03	126224.10±76101.71	380318.74±74260.54	3.1	0.215
Overall Modulation	14835.03±13656.46	11504.21±9971.74	39779.87±25222.68	5.7	0.058
PC1	0.77±0.22	0.09±0.28	-0.66±1.71	4.7	0.095

Table S9. Comparison of 3SV Syllable 3 Among Individual Females Using Mann-Whitney U Statistics

The means, plus and minus standard deviations for the 3SVs syllable 3 vocalizations produced by 3 different *P. boylii* females. Descriptive statistics are used on the original acoustic variables and PC1 score (First principle component of Frequency Variables). The Kruskal-Wallis test is significant (*) at $p<0.008$. Vocalizations were recorded from a wild population of *P. boylii* at Hastings Natural History Reserve, Carmel Valley, CA, from February -June 2008 and January 2009. Duration is in milliseconds and all frequency and bandwidth variables are in kilohertz.

Tag Number	**1107 (n=3)**	**1118 (n=3)**	**1247 (n=4)**	**Kruskal-Wallis U**	**P**
Acoustic Variable					
Syllable 3					
Duration (ms)	134.33±45.62	91.20±9.11	120.25±43.80	3.1	0.215
Start Freq (KHz)	31.53±1.44	29.57±1.98	22.18±7.89		
End Freq (KHz)	32.53±0.76	30.70±1.32	30.10±2.24		
Max Freq (KHz)	35.60±1.50	32.83±0.71	33.38±1.84		
Min Freq (KHz)	31.37±1.15	29.57±1.98	22.18±7.89		
Freq Max Amp (KHz)	35.30±1.97	32.03±1.04	33.03±1.66		
Start to Max Freq (ms)	41.00±22.61	57.17±11.03	40.50±29.65	.6	0.727
Bandwidth (KHz)	4.23±1.89	3.27±1.52	11.20±7.27	4.0	0.137
Internal Modulation	152119.88±105303.94	143053.42±33254.75	395188.49±96595.05	6.7	0.035
Overall Modulation	9039.36±4346.02	11736.42±10340.15	56826.88±42646.77	5.6	0.062
PC1	0.72±0.52	-0.36±0.49	-0.85±1.13	4.0	0.132