

Quality and physiological state encoded in the
territorial vocalization of an asocial mammal

Undergraduate Senior Thesis

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Matt Sehrsweeney

Abstract:

Acoustic signalling in many species is critical for the communication of information. A call may encode multiple layers of information, informing the receiver on a range of stable and labile traits in the signaller. This paper explores stable and labile information communicated in the rattle, the territorial vocalization of the North American red squirrel (*Tamiasciurus hudsonicus*), specifically exploring their capacity to communicate quality and physiological state, in terms of acute stress, energetic state, chronic stress, and age. These acoustic effects are tested for in several experiments and one post-hoc test. Our results provide evidence that all four traits alter rattle acoustic parameters in a manner that presents a notable acoustic signature; the most robust signature among these traits is that of acute stress. We conclude that rattles have the capacity to communicate information on quality and acute physiological state, and suggestions are made for examining the ability of conspecifics to discern this information.

Introduction:

Vocalizations, across animal taxa, communicate a significant amount of information about the signaller. Calls can be individually distinct (Beer 1970, Beecher 1989, Blumstein and Munos 2004), and often carry stable information about body weight and size (Fitch 1997, Bee et al. 1999, Reby and McComb 2001, Blumstein and Munos 2004, Koren and Geffen 2008), sex (Ey et al. 2007, Blumstein and Munos 2004), and social rank (Koren and Geffen 2008, Briefer et al. 2010, Terleph 2016, Muller et al. 2003). This information communicated in a signal--in this case, a vocalization--is important for receivers to decipher, informing them of the signaler's quality and viability as a potential mate or competitor (Zahavi 1975, Grafen 1990, Bradbury 1998). Clutton Brock and Albon (1979) demonstrated this principle in a now-classic case study on red deer, *Cervus elaphus*, finding that certain characteristics of the male "roar" correlate tightly with fighting ability, making them honest indicators of quality. The capacity to discern information encoded in calls, and thus information on signaller quality, then, is selectively advantageous (Seyfarth and Cheney 2003). In asocial animals, such as North American red squirrels, *Tamiasciurus hudsonicus*, acoustic signaling is critical for the transfer of information.

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

Red squirrels are solitary, diurnal rodents, and defend individual territories throughout the year, interacting physically only during mating, and for females, pup rearing (Smith 1968). They advertise their defense of these territories through vocalizations called rattles (Smith 1978). Rattles are known to be individually distinct (Digweed et al. 2012), and though some research indicates that individuals can discriminate between kin and non-kin and neighbors and non-neighbors, this discrimination ability may be context-dependent (Wilson et al. 2015, Shonfield et al. 2016).

Research in recent decades has illuminated the physiological mechanisms that produce vocalizations. In mammals, implicated in production are the lungs, which generate airflow, the larynx, which converts the airflow into acoustic energy, and the various morphological features that make up the rest of the vocal tract, which serve to enhance certain frequencies and cause others to attenuate, altering the structure of the sound produced (Fitch and Hauser 2001). This general paradigm for vocal production was first articulated by Fant (1960), which later came to be known as source-filter theory. These morphological features impose constraints upon the production of vocalizations; this is the physiological basis for understanding calls as honest indicators of quality. For example, larger animals, which are expected to be stronger competitors and better mates, have larger lungs and longer vocal tracts, and tend to produce louder vocalizations with decreased fundamental frequencies or formant dispersion (meaning spacing between successive resonances) (Hinds and Calder 1971, Martin 1972, Fitch 1997, Reby and McComb 2003).

Along with the stable traits discussed, current physiological state can also be reflected in animal vocalizations. Thus, calls can encode information about both an animal's long term and labile traits. Among these labile states reflected is stress, defined most cohesively by Romero

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

(2009) as the combination of disruptions to predictive and reactive homeostasis, termed reactive scope. This definition encompasses physiological reactions to acute and chronic stressors in the environment. When encountering a stressor, the hypothalamic-pituitary-adrenal axis activates and releases a steroid hormone (cortisol or corticosterone) to evoke a metabolic response allowing the animal to react to the stressor. The HPA axis then acts to restore homeostasis, allowing stress hormones to decline back to baseline levels once the acute stressor is no longer present. Chronic stress, caused by repeated exposure to stressors, and thus high levels of GCs, can cause long term damage to the HPA axis, eventually diminishing its functionality. This can have significant deleterious effects on long term body condition (Boonstra 1998, Romero 2004).

Acute stress is known to present an acoustic signature in birds (Perez et al. 2012) and mammals (Esch et al. 2016), both when stress is induced by environmental stimuli, such as isolation, and when it is induced by exogenous stress hormones. Perez et al. (2012) present a study that examines the effects of both isolation and GCs: zebra finches *Taeniopygia guttata*, were fed exogenous stress hormones, and their calls were significantly altered in both patterns of the vocalizations and the fine scale structure of the vocalizations themselves. The same stress induced variations in call traits also arose as a result of isolation.

The relationship between vocal characteristics and affect, or acute emotional arousal, which encompasses stress along with all other heightened emotional states, has been more thoroughly studied, across mammalian taxa (Briefer et al. 2015, Lemasson et al. 2012, Bastian and Schmidt 2008, Rendall 2003). Bastian and Schmidt (2008) found that in bats, arousal produced numerous changes in call patterns, and in fine scale acoustic structure. The literature concerning affect related changes in vocalizations in primates is robust. The changes tend to manifest in source related features, such as fundamental and peak frequencies, generally

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

increasing with affect (Rendall 2003). On the subject of the influence of chronic stress on vocalization structure, the literature is almost entirely silent; the results on chronic stress presented here are thus unique. Overall, the role of stress in influencing the acoustic structure of vocalizations, and thus, the capacity for vocalizations to communicate stress, is understudied, as is the potential perception and response to that information.

Though also understudied (in species besides humans), literature suggests that vocalizations are also significantly impacted by age across animal taxa (Kipper 2010, Verburgt 2011, Terleph et al. 2016). Repertoire size and complexity tends to stay constant, and in many bird species, increases throughout ontogeny (Kilper and Kiefer 2010). Senescence instead generally manifests in changes in temporal characteristics, most often a decline in time, and thus, energy spent calling (Verburgt 2010, Fitzsimmons and Bertram 2011), and changes in motor performance traits, such as altered dominant frequencies--in some taxa a positive correlation is seen, in some taxa negative (Terleph et al. 2016, Verburgt and Ferguson 2011) --and decreased call rates (Cooper et al. 2012). These age-related changes in vocalization structure are interpreted as a result of degradation in the anatomy and physiology of vocalization apparatus, and thus a decline in body condition (Terleph et al. 2016, Torre and Barlow 2009). Descamps et al. (2008) found evidence for senescence in red squirrels, manifesting as age related declines in fitness.

Lastly, energetic state can also be reflected robustly in calls- energy in the form of stored food can have a significant impact on the capacity for production of vocalizations. When given supplementary food, the silvereye, *Zosterops lateralis*, a songbird, performed dawn choruses the following morning of extended length and increased complexity (Barnett et al. 2007). This is but one among many studies that found increased song output in fed birds (Cuthill and McDonald 1990, Berg et al. 2005). Other studies, also in songbirds, show that low overnight temperatures,

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

which eat up fat stores, can temper song output the next morning (Gottlander 1987, Reid 1987).

Significantly less research has been conducted on the relationship between energetic state and vocalization structure in mammals.

To assess whether red squirrel vocalizations communicate more than just individual identity and relatedness, we explored the relationships between various stable and labile traits and acoustic structure of the rattle, analyzing multiple acoustic parameters for signatures of: acute stress, chronic stress, age, and energetic state. We hypothesized that if rattles have the capacity to communicate information on physiological state, then acute trap-induced stress and experimental elevations in circulating glucocorticoids would be reflected in a rattle's acoustic parameters. If rattles also communicate body condition, age, and chronic, density induced stress will be reflected in rattle characteristics as well. Thematically, this research aims to provide evidence that red squirrel rattles have the capacity to communicate information on physiological state and body condition.

Methods:

Study Site and Subjects

We conducted research on a population of red squirrels in the Kluane region of the Yukon (61° N, 138° W) that has been studied continuously since 1989, a region of northern boreal forest that is dominated by white spruce trees. All study squirrels are tagged at birth with ear tags with distinct letter-number combinations unique to the individual. We also attached short segments of pipe cleaner or wire to the ear tags in unique combinations to make individual identification possible from afar. We live trap the squirrels periodically throughout their lives to track female reproductive state and territorial ownership, using tomahawk traps baited with peanut butter (McAdam et al. 2007). Both male and female squirrels own territories individually;

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

female squirrels only share their territories with her offspring while they remain in the natal nest, and with males on the one day of the year that she is receptive to matings (Smith 1968). These territories serve as caches for spruce cones, which comprise 50-80% of a red squirrel's annual caloric intake (Donald and Boutin 2011). The spruce trees that dominate the forest follow a masting life history pattern, producing cones en masse only once every few years synchronously across a region (Lamontagne and Boutin 2007). Each squirrel's territory centers on a midden, an underground food storage stache that can contain thousands of cones, accumulated over the course of years, and overwinter survivorship without ownership of a midden is near zero (Larsen and Boutin 1994).

Experimental Design

Rattles were collected in five separate years between 2005 and 2016, all between April and August, across four study grids (Table 1). Though I drew rattles from a database compiling rattles from every year collected, I contributed to this database as well, collecting 49 rattles in the summer of 2016 as a field tech for the Kluane Red Squirrel Project. To examine the effect of trap induced stress on rattle structure, rattles collected opportunistically were compared to rattles collected immediately after the squirrel had exited a trap, henceforth "trap rattles." Opportunistic rattles were collected by standing on a squirrel's midden at a distance of no greater than 5 meters from the squirrel until it produced a rattle. Rattles were recorded with a Marantz digital recorder (model PMD 660; 44.1 kHz sampling rate; 16 bit accuracy; WAVE format) and a shotgun microphone (Senheiser, model ME66 with K6 power supply; 40-20,000 Hz frequency response (± 2.5 dB); super-cardioid polar pattern).

To validate this method of inducing acute stress, we trapped a subset of squirrels and took blood cortisol measurements, which showed increased cortisol levels after 3 minutes (the

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

amount of time it takes cortisol to enter the bloodstream) when compared to a baseline sample (van Kesteren et al., unpublished data). These rattles were also compared to rattles induced by playback. Playback induced rattles were elicited by playing the rattle of another squirrel to a focal squirrel using a stereo (GPX, model BCDW9815CNP) approximately 10 meters away from the center of the midden. Response rattles from the focal squirrel were then recorded (Wilson et al. 2015).

The effect of chronic stress was tested in two ways: in the first, chronic stress was induced by exogenous glucocorticoids (GCs). Dantzer et al (2012) found that squirrels living in higher densities have high fecal cortisol metabolites (FCMs), so this experiment was intended to simulate density induced stress. The effects of GCs were measured by comparing rattles of squirrels split into two treatment groups: in the experimental group, squirrels were fed 8 grams of peanut butter mixed with 2 grams of wheat germ and 12 mg GCs per day for ten days, and in the control group, squirrels were fed control peanut butter with no GCs. To treat the squirrels, peanut butter doses were placed in buckets hanging from a central tree on their midden. Each day of treatment, buckets were checked to verify consumption of the previous dose and individuals that were found to not be consuming peanut butter were dropped from the experiment. In both treatment groups, rattles were collected opportunistically between 1 and 15 days prior to the initiation of treatment, during the course of treatment (between days 1 and 25), and between 9 and 35 days after cessation of treatment.

For the second test for the effects of chronic stress, we directly tested the effects of density on rattle structure by comparing rattles collected from squirrels living in varying densities. Squirrel density varies for a number of reasons, mostly due to food availability, and fluctuates between years (Dantzer et al. 2013). Because squirrels are so territorial, higher density

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

confers higher stress levels. This density-stress relationship has been validated with FCMs: squirrels on the high density grids present as appreciably higher levels than those on the lower density grids (Dantzer et al. 2013). To analyze the effects of density on rattle acoustic structure, we examined the effect of local squirrel density, defined as the number of occupied middens within one hundred and thirty meters of the focal squirrel (Dantzer et al. 2012), on the acoustic parameters measured in the other experiments. Twice a year, a midden census is conducted, in which the ownership and exact location of each midden on every grid is determined and recorded; from these records we were able to extract the immediate density around each midden from which a rattle was collected.

Longitudinal data on rattles were not available for this experiment because in very few squirrels did we have rattles from more than one year, so in order to assess the effects of senescence on vocalization characteristics, a cross sectional analysis was conducted. 423 rattles from 280 unique individuals were collected and analyzed from squirrels ranging in age from several months and eight years old. All squirrels below one year old appear as “0” years old on graphs.

The influence of energetic state on rattle structure was examined in a post-hoc analysis conducted with squirrels that had been part of the GC experiment that were fed 8 grams of control peanut butter with 2 grams of wheat germ (with no GCs) per day for 25 days. Rattles of these squirrels were compared to opportunistically collected rattles of squirrels outside of this experiment that had not been given supplementary food. Thus, the control squirrels in the GC experiment became the experimental squirrels in this post hoc analysis-- the experimental variable being supplementary food provided to one group, theoretically providing them with a boost in energy.

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

Table 1: Number of rattles collected by year, grid, date range, and collector. In parentheses, rattles are split up by sex: (male, female). Rattles from 2015 were fed exogenous GCs and were thus excluded from analysis of long term data.

Year	Grid: AG	Grid: KL	Grid: SU	Grid: JO	Collection Method	Date Range
2005	0	10 (7,3)	15 (7,8)	0	Opportunistic: 1 Trap: 3 Playback: 21	6/7/05-7/31/05
2006	0	126 (71,55)	100 (55,45)	0	Opportunistic: 205 Trap: 2 Playback: 19	6/13/06-8/14/06
2009	43 (24,19)	66 (22,34)	13 (9,4)	0	Opportunistic: 54 Trap: 37 Playback: 31	3/26/09-7/26/09
2015	0	0	0	91 (37,48)	All opportunistic Treatment: Control: Pre- 12 Pre- 20 Treat- 13 Treat- 19 Post- 14 Post- 12	5/10/15-7/28/15
2016	24 (12,12)	25 (14,11)	0	0	Opportunistic: 49 Trap: 0 Playback: 0	6/16/16-8/2/16

Table 2: Total number of rattles collected by age.

Age (yr)	<1	1	2	3	4	5	6	7	8
Number of Rattles	25	155	125	38	45	15	11	5	2

Rattle acoustic parameters, listed and briefly described in Table 3, were then extracted using AviSoft-SASLab Pro, using the extraction protocol employed in Wilson et al. 2015. Because of the broadband and atonal (noisy) nature of rattles, the less acute characteristics of the frequency spectrum, such as 1st, 2nd, and 3rd quartile frequencies proved most effective for analysis, as opposed to more subtle characteristics such as formants. Statistical analysis was

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

conducted using R package lme4 (Bates et al. 2015) to generate linear mixed effects models, and graphed using ggplot2 (Wickham 2009) We combined all analyses into one model, which analyzed local density, collection method (trap, playback and opportunistic), sex, age, and Julian date with left eartag number and year included as random variables.

Figure 1: Spectrogram of a red squirrel rattle

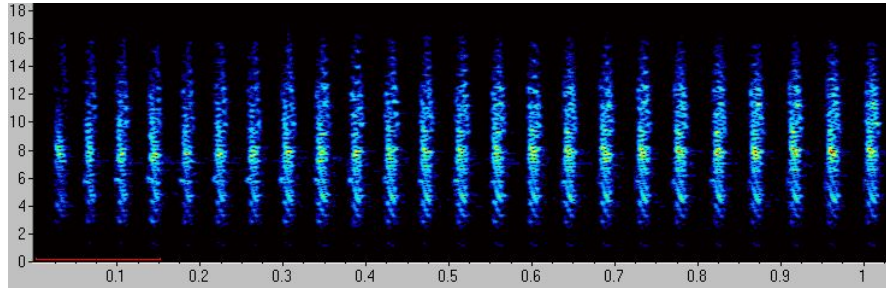


Table 3: Rattle acoustic parameters analyzed.

Acoustic Parameter	Description	Acoustic Parameter	Description
Duration	Length (s)	1st Quartile Frequency	Frequency below which lies 25% of the energy of the call (Hz)
Call Rate	Pulses per second	2nd Quartile Frequency	Frequency the divides the call into intervals each containing 50% of the energy of the call (mean frequency) (Hz)
Min Frequency	Lowest frequency band present in call (Hz)	3rd Quartile Frequency	Frequency below which lies 75% of the energy of the call (Hz)
Max Frequency	Highest frequency band present in call (Hz)	First Frequency Peak	Lowest dominant frequency band (fundamental frequency) (Hz)
Bandwidth	Frequency range (min to max) (Hz)	Entropy	Noisiness of call

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

Results:

Effects of trap-induced acute stress

A general trend emerges when examining the differences between trap and opportunistic rattles: under trap-induced stress, rattles broaden in frequency bandwidth, expanding upward toward higher frequencies in the spectrum. Rattles also speed up and lengthen, increasing in not just the overall number of pulses, but also the number of pulses per second. Lastly, calls increase in entropy, meaning they are noisier, or less tonal. All results are presented in figure 2.

The call rate was significantly higher in trap rattles than those collected opportunistically ($p = 0.0056$, $t = 2.8$, $df = 169.9$). Trap rattles had significantly higher maximum frequency ($p = 0.0182$, $t = 2.68$, $df = 225.4$), though they did not differ in minimum frequency or first frequency peak. The average duration of trap rattles was found to be longer than those collected opportunistically ($p=0.0182$, $t = 5.7$, $df = 166.38$). Trap rattles also had significantly broader bandwidths ($p = 0.0176$, $t = 2.964$, $df = 27.78$), meaning they contained a broader range of frequencies.

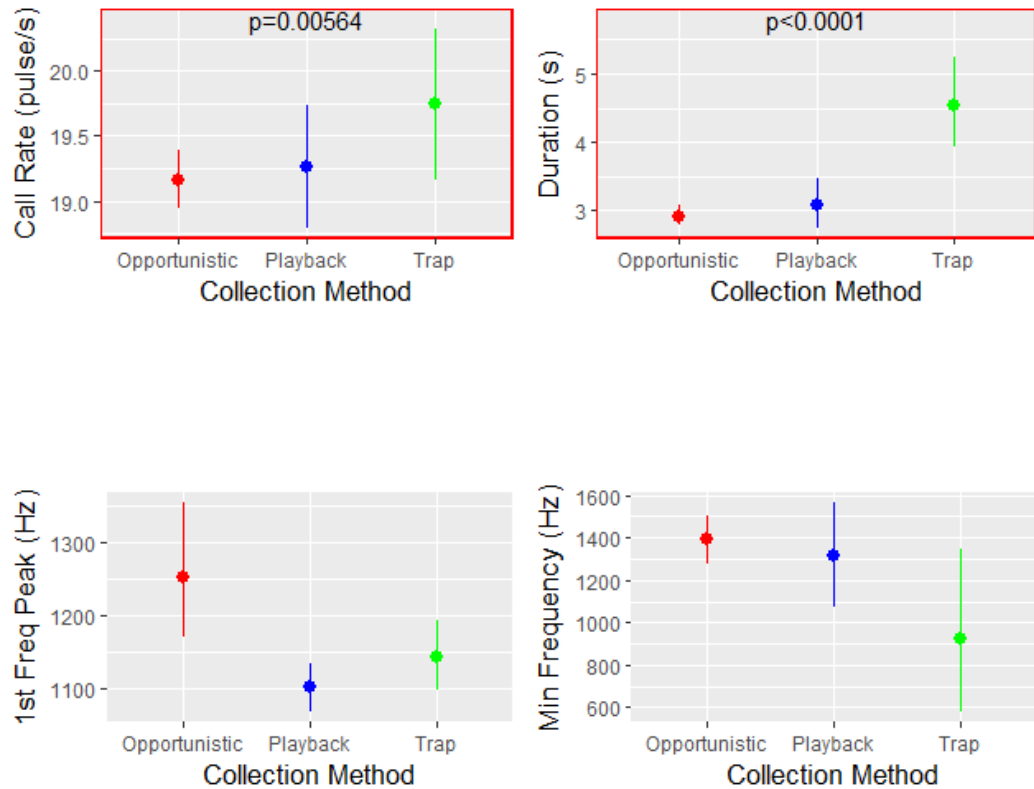
Trap rattles had higher 2nd quartile frequencies, meaning the frequency that divided the call into frequency intervals each containing 50% of the energy was higher on average ($p = 0.0082$, $t = 2.86$, $df = 271.1$) and the same was found with 3rd quartile frequencies ($p = 0.0031$, $t = 3.23$, $df = 29.5$), which divide the call into intervals containing 75% and 25% of the energy of the call. Entropy, in this context best understood as a measure of a call's "noisiness", differed as well between trap rattles and opportunistic rattles, with higher entropy found in trap rattles ($p<0.0001$, $t = 4.63$, $df = 123$).

Though no such significant differences were found between rattles collected opportunistically and those collected after a playback, a trend of playback-induced rattles falling in between opportunistic and trap induced rattles emerged in several acoustic parameters.

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

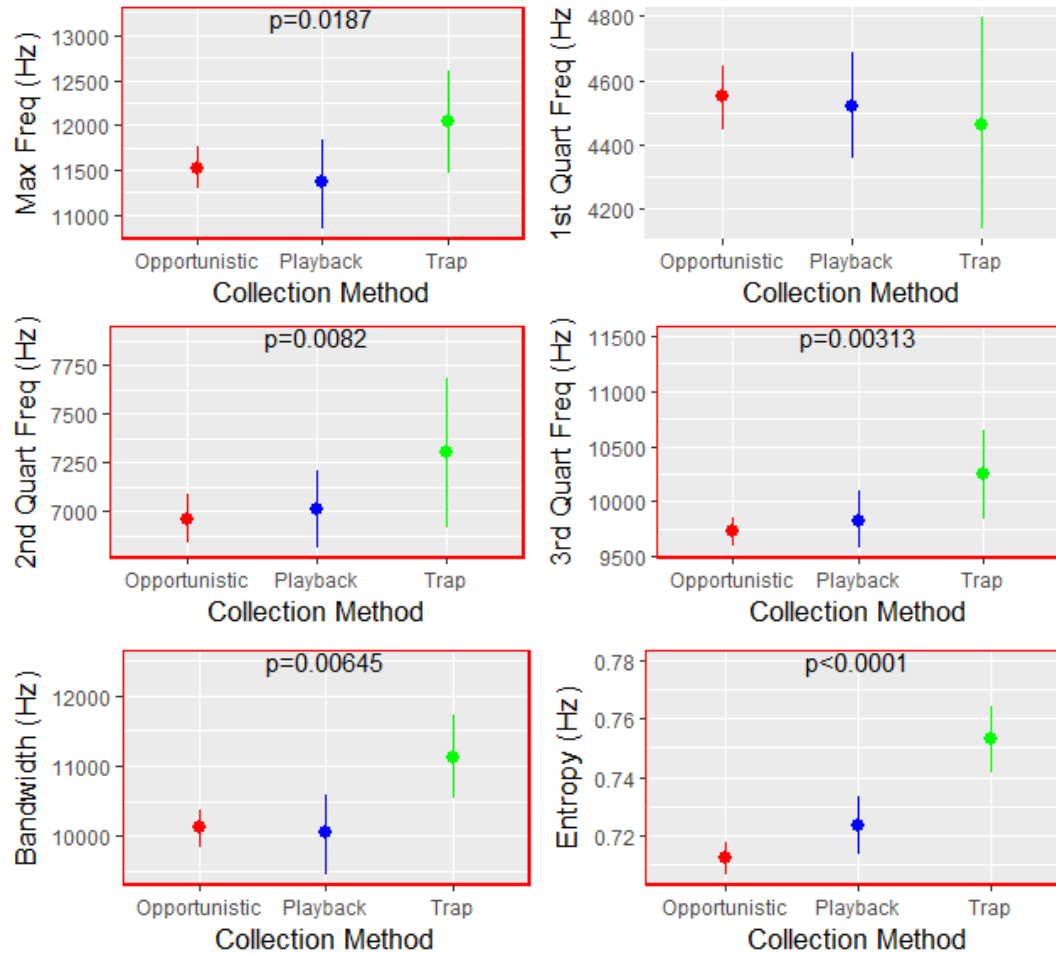
Matt Sehrsweeney

Figure 2: Comparison of difference in acoustic parameters between of rattles collected opportunistically, collected upon release from a trap, and induced by playback. Most of the acoustic parameters measured different by collection method: call rate, duration, maximum frequency, 2nd quartile frequency, 3rd quartile frequency, bandwidth and entropy were all significantly higher in trap rattles than in opportunistic rattles. Parameters of rattles induced by playback often fell between trap and opportunistic, though no differences were significant. P values on graphs indicate difference between trap and opportunistic rattles. A red frame indicates significant results.



Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrensweeny



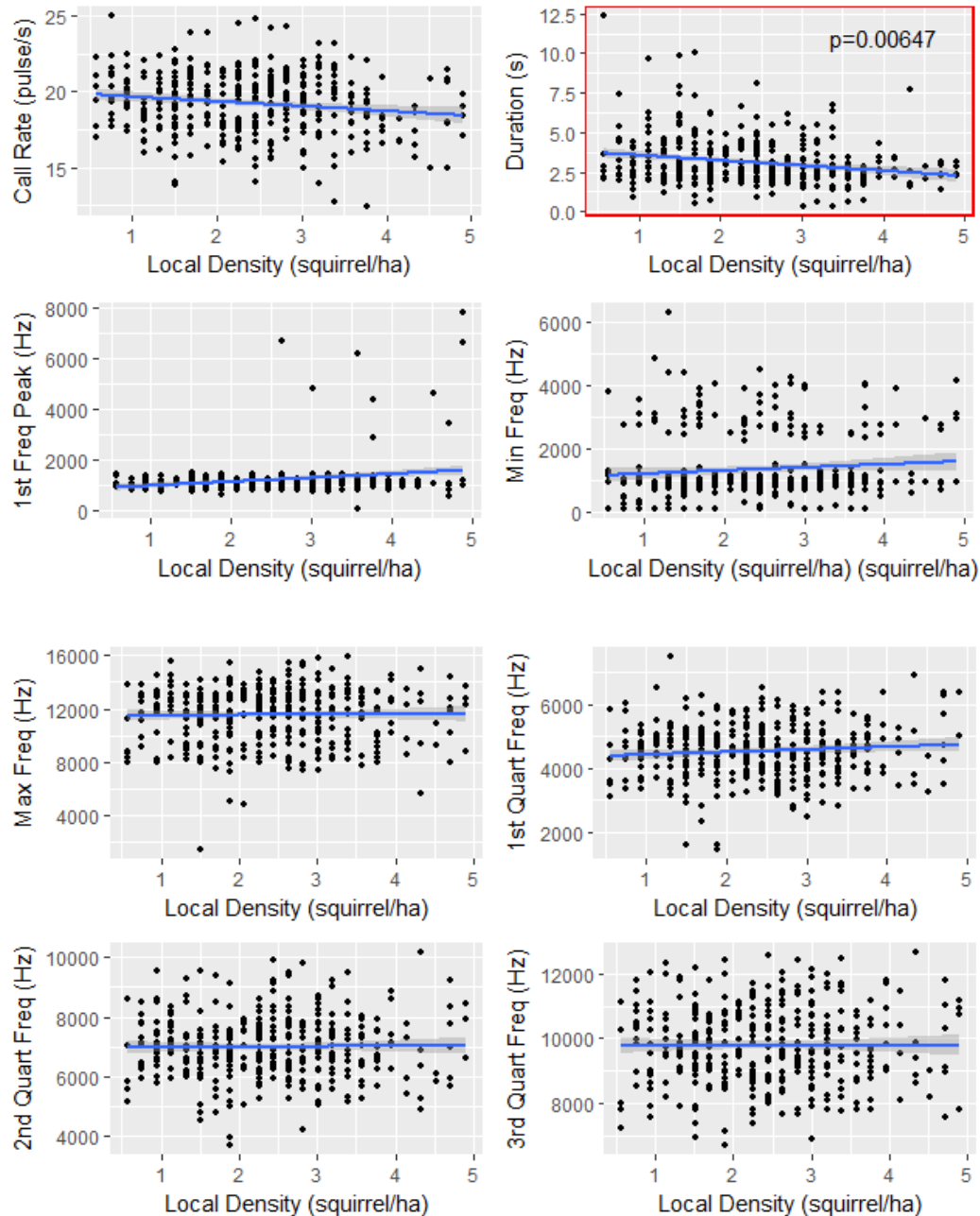
Effects of density-induced chronic stress

Our findings also support the hypothesis that increases in glucocorticoids induced by experimentally increasing population densities are to some degree reflected in rattle acoustic characteristics. The only parameter impacted by chronic stress was duration, which decreased with density ($p=0.0067$, $t = 5.0$, $df = 553.6$). No other parameters exhibited significant relationships with local density. Results are presented in figure 3.

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

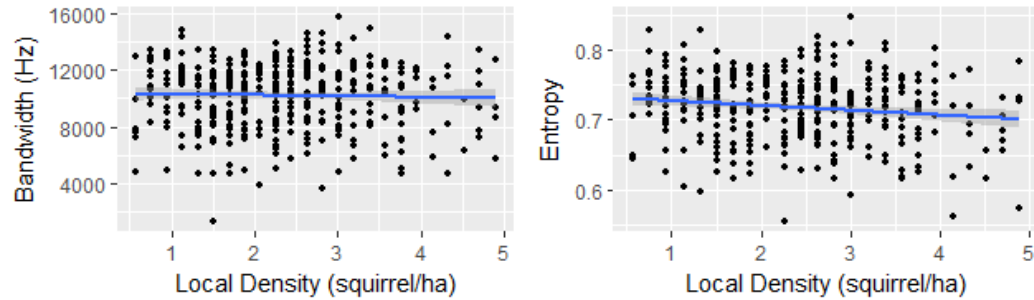
Matt Sehrsweeney

Figure 3: Effects of chronic, density induced stress on all rattle parameters. The only significant effects were a negative correlation with between local density and call duration ($p=0.003$, $t = 6.3$, $df = 553.6$). A red frame indicates significant results. The gap between data points seen in the minimum frequency graph is a results of an inconsistency in acoustic parameter extraction by a previous researcher- some of the data was sent through a “high pass filter,” filtering out the lowest frequencies, thus raising minimum frequency, and some was not. This filtering, however, has no notable effect on other acoustic parameters.



Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrensweeney



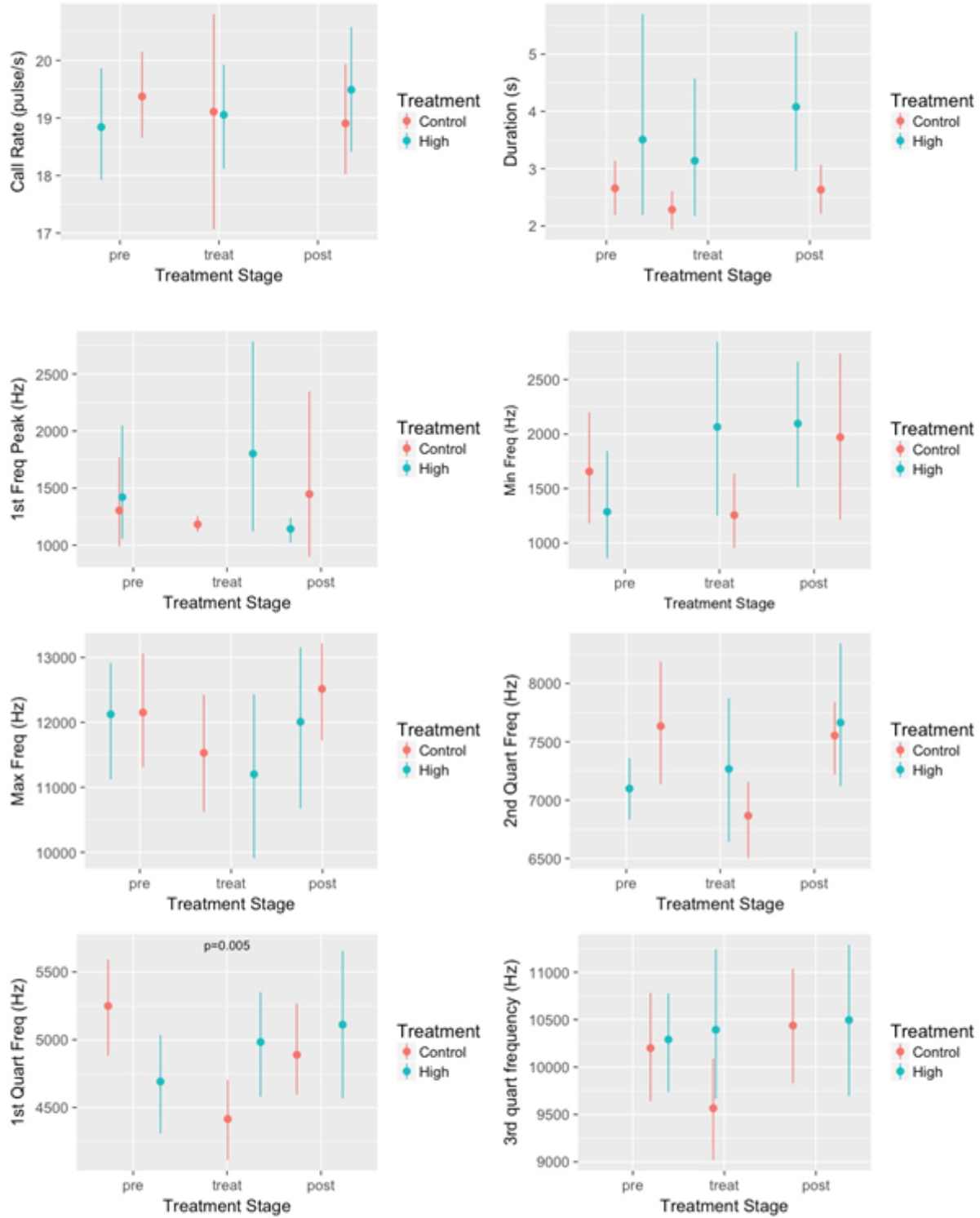
Effect of stress induced by exogenous glucocorticoids

The impacts of exogenous glucocorticoids on acoustic parameters were not clear cut. We expected the rattles of squirrels fed GCs collected during and after treatment to resemble rattles of chronic, density induced stressed squirrels; however this was largely not the case- none of the parameters that we expected to change between treatment stages changed. The only parameter that did present a significant difference was the first quartile frequency. In squirrels treated with GCs, the mean first quartile frequency in rattles collected after treatment was significantly higher than those taken before treatment ($p = 0.005$, $t = 6.7$, $df = 3$), with the frequency during treatment falling between them. Though not significant, similar trend emerges in the graphs of second and third quartile frequencies- in treated squirrels, these frequencies step up during treatment and step up again after treatment. This trend is also seen in call rate. Results are presented in figure 4.

Figure 4: Rattle acoustic parameter measures before, during and after GC treatment. Treatment squirrels were feed PB with exogenous glucocorticoids once a day for twenty five days, and control squirrels received normal peanut butter once a day for twenty five days. The only significant difference is found in the 1st quartile frequency in treatment squirrels, between rattles collected before and during GC treatment ($p = 0.005$, $t = 6.7$, $df = 3$).

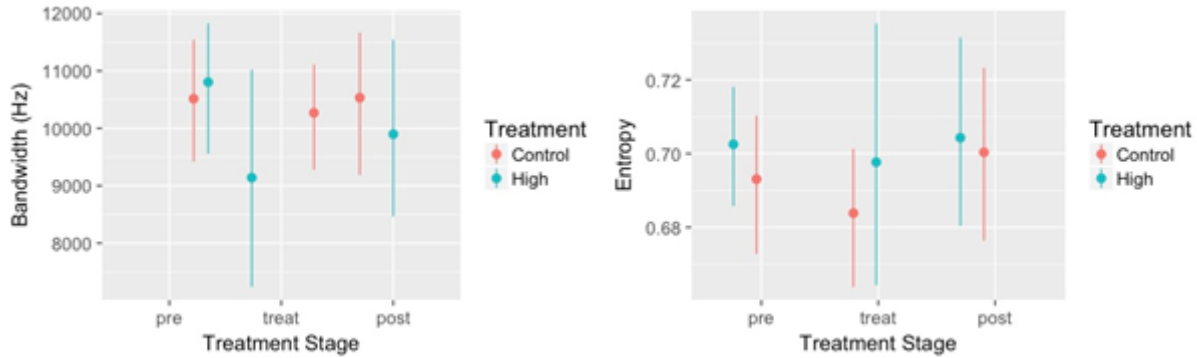
Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney



Quality and physiological state encoded in the territorial vocalization of an asocial mammal

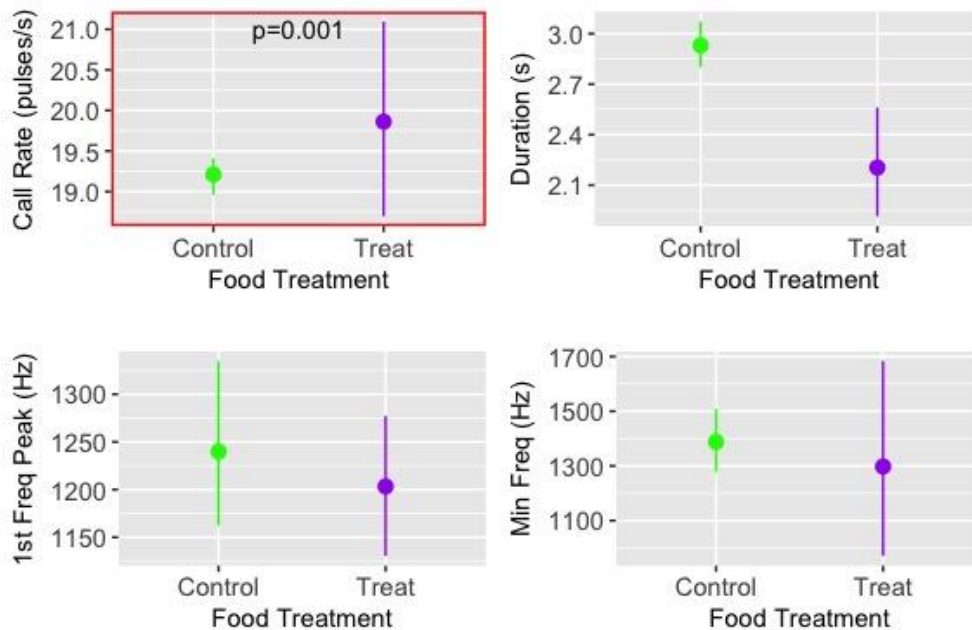
Matt Sehrsweeney



Effect of energetic state

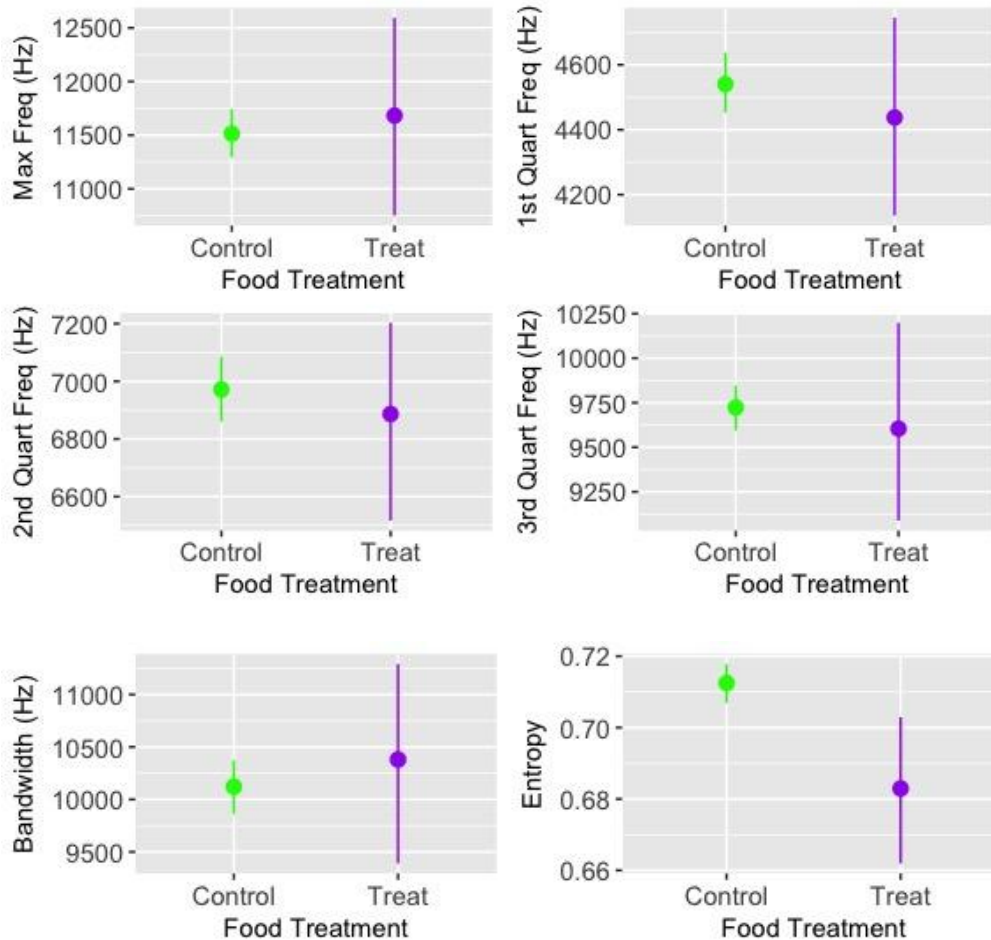
Energetic state was found to have an influence on the rattle structure as well, though minor. Squirrels that were fed 10 grams of supplemental food every day for 25 days had, on average, higher call rates than control squirrels ($p=0.001$, $t = 3.5$, $df = 44.35$). No other significant changes in acoustic structure were found. Results are presented in figure 5.

Figure 5: Effects of energetic state on acoustic parameters. Squirrels that were fed 10 grams of peanut butter on the day a rattle was collected had significantly higher call rates ($p = 0.001$, $t = -3.577$, $df = 44.35$). No other rattle parameters were significantly affected by energetic state. A red frame indicates significant results.



Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney



Effect of age

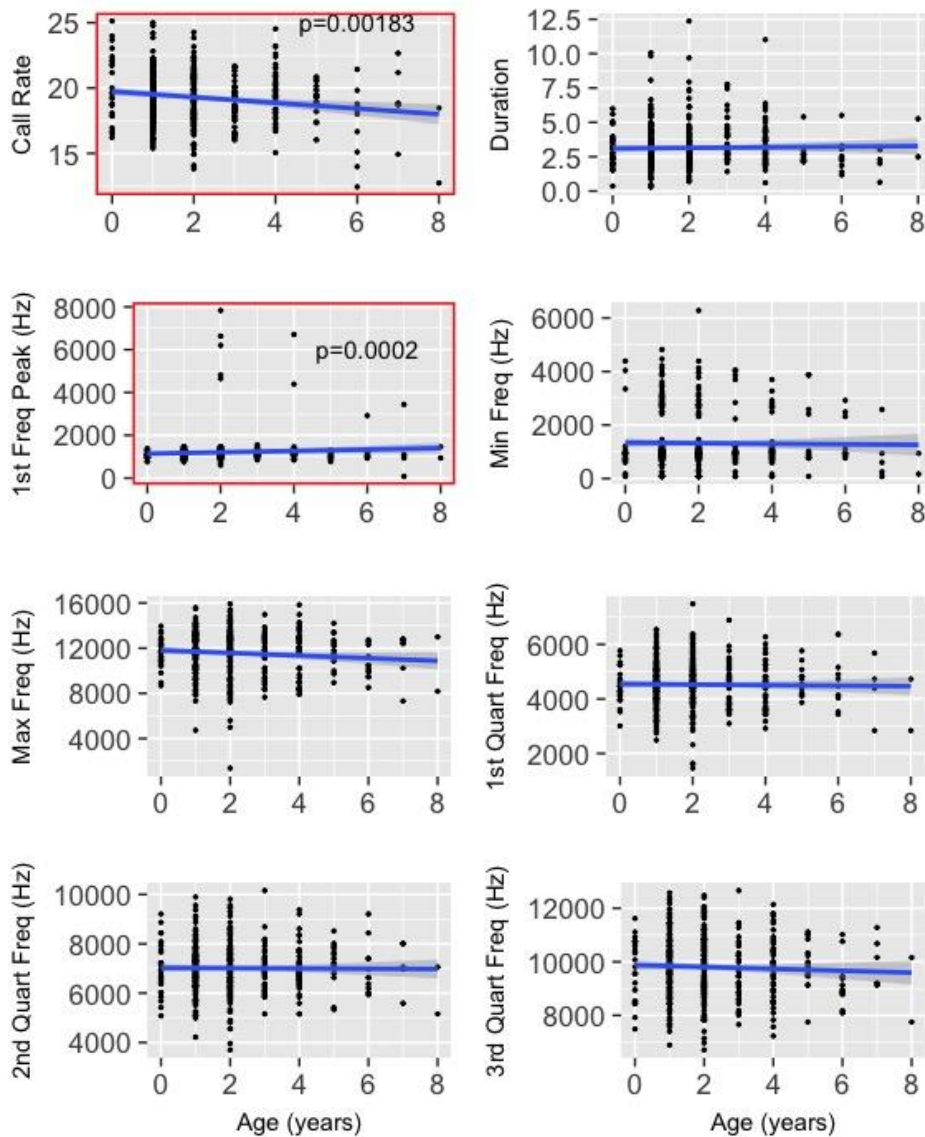
A squirrel's aging process also seems to have an impact on the acoustic structure of a rattle, though not in exactly the manner predicted. The effect of aging on the acoustic structure does not fit the model typically predicted by senescence, in which a given trait improves as an organism grows, optimizes at the age at which the organism is maximally fit, and then degrades as the organism ages, creating a trend best represented by a quadratic model. However, the quadratic model did not fit the data for any of the acoustic parameters. However, a simple linear

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrensweeny

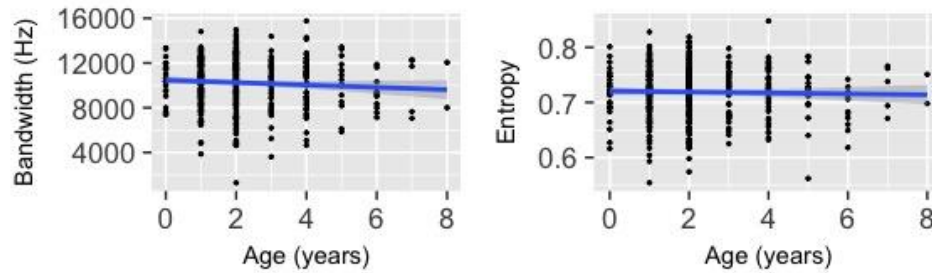
regression model assessing the relationship between call rate and age did fit the data, finding call rate to be negatively correlated with age ($p < 0.0001$). Thus, call rate decreases over the course of a squirrel's life, suggesting a steady degradation of the trait. Frequency of the first peak also decreases as a function of age, though neither minimum nor maximum frequency either increases or decreases.

Figure 6: Effect of age on all acoustic parameters. The only significant effects were a negative correlation between age and call rate, and a positive correlation between age and 1st frequency peak. The same issue with minimum frequency presents here as well.



Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrensweeny



Discussion

Our results discern deeper complexity to the information encoded in the rattle, the territorial vocalization of the red squirrel, than previously understood. They indicate that the rattle has the capacity to communicate physiological state, in terms of acute stress and energetic state, along with physiological quality, as determined by chronic stress and age. Thus, a rattle contains not only stable information about the caller's individual identity and potential kin relationship to nearby squirrels, but also information about physiological quality, and labile information about physiological state. This layering of stable and labile encoded information is not uncommon; major strides in bioacoustic research in the past several decades have uncovered this capacity across animal taxa (Cheney and Seyfarth 2003, Rendall 2003, Blumstein and Munos 2005, Soltis 2005, 2011, Koren and Geffen 2009, Terleph et al. 2016).

Several bioacoustic theories serve as useful interpretive tools for analyzing the influences of various phenomena on acoustic structure presented here. Source-filter theory predicts that affective and physiological state alter vocal characteristics in a predictable manner across taxa; these traits tend to exert a more significant influence on source related characteristics, such as mean frequency and entropy, and indicators of individual identity and semantic information tend to arise from alterations to filter related characteristics, such as formant dispersion (Fant 1960, Taylor et al. 2010). Motivational theory, which describes a set of rules about how vocalizations should change in situations of emotional arousal, also applies: it predicts that in the victim of an

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

antagonistic encounter, the animal experiencing significant distress, call frequency will increase, and that overall call entropy, a measure of a call's noisiness, will increase, are broadly applicable to acute stress inducing situations (Morton 1977, Ordonez-Gomez et al. 2014). Our findings fit predictive models set forth by both of these theories. The call features altered by physiological state and quality are the source-related characteristics predicted to be influenced in these contexts, and features that motivational theory predicts will change in such context are altered as well. Taken together, these support the assertion that mammalian vocalizations vary with some consistency across taxa when influenced by certain internal or external factors (Taylor and Reby 2009).

The modifications in rattle characteristic due to acute stress found here, specifically, increased frequency related characteristics, call rate, bandwidth, and duration, align with expectations drawn from similar stress related alterations in other species, and fit the paradigm dictating that affect tends to influence source-related features of calls, described by source-filter theory and motivational theory. In the only other study to examine the impact of exogenous glucocorticoids on vocalizations, zebra finches that were fed one dose of GCs (thus subjected to acute stress) produced vocalizations of higher frequency and longer duration, and both characteristics increased in finches subjected to isolation-induced stress as well (Perez et al. 2012). In stressful contexts, the whistles of bottlenose dolphins increase in both rate and frequency (Esch 2009). Low ranking female elephants, when interacting with dominant individuals (a state of emotional arousal), generate rumbles with increased fundamental frequency and duration (Soltis et al. 2009). In non-human primates, a significant amount of research examining affect and vocalizations focuses on infant calls. In both infant rhesus macaques and squirrel monkeys in isolation, call duration and pitch increased, and the same

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

acoustic features vary in hunger driven food calls (Masataka et al. 1986, Bayart 1990, Hauser and Marler 1993). These trends are present in vocalizations of adult primate as well. For example, in baboons, high arousal situations involved in group movement and infant interaction produced changes in the same set of source-related call characteristics (Rendall 2003). The trend of elevation in dominant frequencies, or pitch, under stress, is conserved in humans (Fairbanks and Pronovost 1939, Murray and Arnott 1993, Sondhi et al. 2015).

Also notable is the fact that entropy, predicted by motivational theory to increase in stressful situations, increases in squirrels experiencing acute stress. An analogous trend is found in many non-human primate species: the production of screams, very high entropy vocalizations, in high-arousal contexts, is well documented across primate species (Slocombe and Zuberbuhler 2009, Rendall et al. 2009, Alfaro 2008). Research on screams in rhesus macaques has revealed more subtle relationships between entropy and arousal, finding that entropy increases proportionately with affect level of the situational context in agonistic encounters (Gouzoules et al. 1984, 1998).

In this context, the significance of communicating stress in this way lies not in the intention of the sender but the discernment of the receiver. Our findings are especially interesting in when placed within the context of recent research on kin discrimination in rattles conducted by Shonfield (2016). Her work indicates that receivers can indeed discriminate between stressed and non-stressed calls: in her playback study, squirrels only discriminated between rattles of kin and nonkin (rattling more readily in response to a nonkin rattle) when the playbacks were trap rattles (Shonfield 2016); as we have shown, rattles that present a stress signature. Thus, our findings corroborate her hypothesis on the presence of a “stress signature” in a rattle; however significance of context-dependent kin discrimination requires further research. Because

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

Shonfield's (2016) findings emerged from a post-hoc analysis, a study designed explicitly to test the relationship between stress and kin discrimination, in which focal squirrels are played trap and opportunistic rattles of both kin and non-kin, in order. A similarly useful experiment would do this same with neighbor and non-neighbor rattles. These experiments could more acutely reveal the interactions between the layers of information in rattles simultaneously discerned by squirrels.

This study found that chronic, density induced stress confers unique alterations in rattle acoustic parameters when compared to acute stress. This signature however is much subtler, manifesting primarily as a decrease in call duration. Forthcoming research from David Wilson shows evidence that squirrels face an energetic trade-off between rattle duration and pulse rate--also understood as a performance constraint (Wilson, unpublished data). Performance constraints are known to define the limits of vocalizations of other small mammals, and in singing mice, are to some degree influenced by hormones (Pasch et al. 2012). A possible interpretation of this decline in call duration due to density induced stress is that chronic stress induces physiological effects that degrade the musculature implicated in vocal production, pushing the performance constraint lower, making it more difficult for squirrels to produce long rattles with high call rates. Another possible explanation is ecological, and unrelated to stress or decline in body condition--a higher density neighborhood means more squirrels rattling, and thus more information to discern. Any time spent rattling is time lost in collecting acoustic information on neighboring squirrels; thus rattle duration may plastically decrease in high density regions.

Sustained stress can also have a deleterious impact on the hypothalamic-pituitary-adrenal axis, weakening adrenal function and thus diminishing acute stress responses (Romero 2009), potentially including stress related alterations in rattle characteristics. A future study could assess

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

this acute plasticity of rattles by examining opportunistic rattles and trap rattles collected from the same squirrel, comparing the magnitude of the changes in rattle characteristics between squirrels in high and low density regions.

The experiment aimed at inducing chronic stress through exogenous GCs produced ambiguous results, with almost no significant differences in rattle structure between treated and untreated squirrels. This ambiguity is likely attributable to a number of methodological inconsistencies. Unpublished data by Freya Van Kesteren shows that the effect of the GC treatment on circulating cortisol is not as straightforward as anticipated: consumption of the GC laced peanut butter causes a spike in circulating cortisol, and then subsequent steady decline over the course of 24 hours. In Van Kesteren's experiment, the buckets containing the treatment peanut butter were checked every two hours to determine a window in which the peanut butter was consumed in order to determine time between consumption and taking the blood sample. However, in the experiment described in this paper, buckets were checked only once a day, leaving a 24-hour window in which the peanut butter could have been consumed. Furthermore, recorded rattles were not timestamped, making it very difficult to judge the stress state of the individual at the time of rattle collection. Van Kesteren's findings are unpublished, and should be regarded with trepidation, but still may shed light on the inconclusive results here. However, because of the significant potential of this experiment (experimentally altering rattle acoustic structure by inducing stress through exogenous GCs), an updated study has been designed and will be implemented this April by technicians working for the KRSP.

The GC experiment, though not fine-tuned enough to provide adequate insight on the role of exogenous GCs on rattle acoustic structure, did shed light on the influence of another physiological state on acoustic structure: energetic state. Rattles collected from squirrels that

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

were fed control peanut butter (the experimental group in the context of this experiment) had significantly higher call rates when compared with those not provided supplementary food. This finding dovetails with literature in other species on the role of energetic state on vocalizations (Gottlander 1987, Reid 1987, Cuthill and McDonald 1990, Berg et al. 2005, Barnett et al. 2007). Gillooly and Ophir (2010) present another theory useful for contextualizing these results, which attributes much of the heterogeneity in animal vocal signals to the energetic constraints of sound production, predicting that for all species, acoustic communication is primarily controlled by individual metabolism. Our results reflect that energetic state does have an influence on vocalization structure, one of the only studies to find such a relationship in mammals. Others that have been conducted focus primarily on fatigue (Pitcher et al. 2014). However, my analysis is limited by the nature of it being performed post hoc. A study directed at examining the role of energy in rattle production more explicitly could provide a more thorough picture of the influence of energetics on rattle structure.

Our results also found a significant relationship between senescence and changes in various acoustic parameters. As squirrels age, the call rate decreases, as does the frequency of the first peak. These trends do not cleanly match up with existing literature on the effects of senescence on acoustic call rate and peak frequency (comparable to frequency of the first peak in terms of tracking such trends); however, the literature is sparse and contradictory. In red deer, dominant frequencies increase as males age, but in Norway rats, peak frequency decreases (Briefer et al. 2010, Basken et al. 2012). In humans, age related changes in vocal pitch differ between males and females. Males vocal pitch tends to rise after middle age under the end of life; in females, vocal pitch tends to lower slightly after menopause (Linville 1996).

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

Decrease in call rate seen here matches with an analogous, though insignificant, trend of decreased call rate chronically stressed squirrels ($p=0.37$, $t=-0.9$, $df=292.4$), suggesting a similar mechanism of change in acoustic structure relating to decline in body condition. Research indicates that these age-related declines in vocalization quality result from deterioration of the microstructure of the larynx (Peterson et al. 2012). Oxidative damage likely contributes significantly to this decline. Velando et al. (2008) propose that this plays a prominent role in the evolution of mate signalling; if secondary sexual characters, such as vocalizations, are honest indicators of male quality, then age-induced oxidative damage will cause these traits to deteriorate, and the calls of older males with significant oxidative stress damage will be selected against by females. This hypothesis was supported by Hoikkala et al. (2008), who found that the songs of *Drosophila montana* males deteriorate with age, as does reproductive success. Casagrande et al. (2016) reviewed the literature on the influence of oxidative stress on bird song, finding significant evidence that it does mediate song characteristics in many species, and recommending more pointed research on the impact of oxidative stress specifically caused by senescence. This oxidative stress paradigm could have significant implications in understanding rattles as honest indicators of quality. If a future study on oxidative stress in red squirrels found that oxidative damage due to senescence does significantly impacts the physiological mechanisms implicated in call production at similar rates to the impact seen in other aspects of the squirrel's physiology, this hypothesis would be supported.

The ability for other squirrels to discern age related differences in rattles structure could have significant ramifications in this system. Ownership of a midden, as mentioned earlier, is virtually essential for overwinter survival; competition for these middens is thus extremely strong, especially in the late summer and fall, as juveniles recruit into the population and attempt

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

to supplant established squirrels from their middens (Boutin 1993). Were squirrels to have the capacity to discern the effects of senescence in a rattle, a juvenile could focus its energy on taking over the midden of an older squirrel less fit to defend its territory, instead of attempting to unseat a younger, fitter squirrel, thus minimizing energy expenditure.

In order to cushion their own food stores, squirrels are known to pilfer spruce cones from neighboring middens. In more extreme cases, individuals will take control of a neighboring midden, securing a significantly larger available store of food; this is referred to as a secondary midden. Females with offspring still on their natal midden are the most likely to do this successfully; in one study in which vacancies were created experimentally by removing squirrels from their middens, females with offspring still on their midden took over twice as many vacant middens as juveniles. This allowed their offspring to remain on their natal territory, increasing offspring survivorship (Boutin 1993). In the case of both pilfering and acquiring secondary middens, squirrels might target squirrels that they know to be older and less fit, using a deteriorating rattle as an indicator.

One weakness to the senescence-related component of this study emerges from the fact that it was a cross sectional, rather than a longitudinal study. This could theoretically explain why the traits that were altered by senescence did not follow the typical senescence predictive quadratic, model, but instead declined linearly with age. Because call rate decreases across age, and is elevated in stressed squirrels, it is theoretically possible these stressed squirrels are dying younger, and thus the six, seven, and eight-year-old cohorts are more dominated by low-stress squirrels who exhibit rattle characteristics typical of individuals not experiencing stress. This could be tested by examining the relationship between lifetime FCMs and mortality.

Conclusion

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrsweeney

The research presented here contributes to the accumulating body of bioacoustic research uncovering an increasingly complex understanding of the multiple layers of information communicated in animal vocalizations. The findings that physiological state, in terms of acute stress and energetic state, and body condition, in terms of chronic stress and senescence, present acoustic signatures in red squirrel territorial vocalizations suggests that these calls encode much more information than previously understood. These findings, paired with the knowledge that rattles are individually distinct (Digweed et al. 2012) and are used for context based kin discrimination (Shonfield et al. 2016), present a thoroughly nuanced picture of the complexity of a call initially assumed to serve exclusively for territorial defense. This analysis is a case study, illuminating the fact that animal vocalizations, even in an asocial species, are complex communicative channels, in which multiple levels of information can be simultaneously encoded. Bioacoustics researchers should take this cue to initiate further investigation on two fronts: first, the capacity for vocalizations, territorial and otherwise, in other asocial species to carry multiple layer of information, and second, the ability for conspecifics to discern these complex acoustic cues making up their local acoustic landscape. Following these two exciting channels of research should produce a much more thorough understanding of the subtleties of animal acoustic communication systems.

Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrensweeney

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Matt Sehrensweeney

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Matt Sehrsweeney

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Quality and physiological state encoded in the territorial vocalization of an asocial mammal

Matt Sehrensweeney

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Matt Sehrsweeney

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