INDIVIDUAL VARIATION IN THE CALL

CHARACTERISTICS OF AMERICAN TOADS

(*ANAXYRUS AMERICANUS*)

By

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Abstract:

Recent studies in animal behavior have demonstrated that behaviors are often repeatable. Variation in behaviors arise at many levels, including the level of a single individual, and between individuals in a population. Characterizing these differences in behavior among and within individuals can provide information about the repeatability of the behavior; how consistent the behavior is over multiple measurements of the same individuals. In this study, I examined how call characteristics of the American toad (*Anaxyrus americanus*) vary both within an individual and among individuals in a population. More specifically, I measured five call characteristics (dominant frequency, call duration, pulse rate, pulse number, and call rise time) and examined how they varied across various environmental and social factors. I then estimated repeatability for these call characteristics, through = repeated sampling periods of the same individuals. Overall, call characteristics were affected by multiple environmental variables. In addition, the number of males present in a breeding chorus affected the variation in calls: with more males present in a chorus, larger males had lower dominant frequencies. Both dominant frequency and call duration were significantly repeatable, indicating that individuals are consistent in their display of these two call characteristics. My results demonstrate that individuals differ in their display of reproductive behaviors over multiple environmental factors. Competition among males might be occurring via overlap of calls Repeatable call traits (dominant frequency and call duration) are assumed to be characteristics determined by individual factors such as size, age, metabolism, parasite load, etc. Furthermore, these consistent individual differences shown through high repeatability may indicate the potential for these mating behaviors to respond to selection.

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CHAPTER I

INTRODUCTION

A key component in the mechanism of natural selection is variation. Genotypic variation within a
population leads to phenotypic variation, and when these phenotypic differences affect survival or
reproduction, then natural selection can act on these fitness differences. Factors such as
environmental fluctuations, migration, and mutation work to maintain genetic variation in a
population. In the case of ever-changing environmental conditions, populations with higher
genetic variation are more likely to persist (Hedrick 1986; Hedrick et al. 1976; Hedrick 2006).
This is simply because variation provides the population with "options" in the face of
environmental fluctuations. Traits that are closely associated with fitness are hypothesized to
have lower genetic variance (Mousseau and Roff 1987). This is because alleles associated with
promoting fitness will become fixed by selection. However, fluctuating environmental conditions
may increase genetic variance in traits associated with behavior, physiology, or even fitness
(Stirling et al. 2002, Ewing 1979). When environmental shifts do occur, traits that increase fitness
in these new conditions are likely to be selected for (Allentoft and O'Brian 2009; Wei et al.
2020). Similarly, populations that display higher phenotypic plasticity in their response to
environmental fluctuations are more likely to persist (Hutchings et al. 2007; Charmantier et al.
2008). By observing these responses to environmental variation, we can understand the functions,
adaptations, and heritability of traits.

Studying genotypic and phenotypic variation within a population is therefore necessary to understanding the ability of populations to respond to unstable or diverse environments. Ultimately, our goal is to determine the genetic and environmental effects of trait variation. However, without performing genetic analyses and long-term multi-generational experiments, this is difficult to do directly. Measuring the variation of traits within and between individuals, and the consistency of that variation over time, can serve as a more accessible proxy to genetic studies.

Studies in animal behavior have demonstrated that behavioral traits are often repeatable, and there is growing interest in understanding the causes and consequences of this consistent individual behavioral variation (Bee 2004, Bee et al. 2001, Sullivan and Wagner 1988, Howard and Young 1998, Bell et al. 2009, Laskowski et al. 2022, Dochtermann et al. 2015). Individual behavioral variation can be partitioned into within-individual and among-individual sources. Within-individual variance is the variation of behaviors within an individual and more specifically, how inconsistent individuals are in their display of repeated behaviors. Among-individual variance refers to consistent differences among individuals in behavior (Laskowski et al. 2022). For example, if two male anurans are repeatedly displaying behaviors that are different from one another, within-individual variation refers to the variation of behaviors within one of those individuals. Among-individual variation is therefore the variation between the two males in average behavior (Figure 1). A combination of stable individual characteristics and plasticity due to varying environmental conditions contribute to behavioral variation. Individual state variables such as body condition or energetic rates (metabolism) could cause individuals to behave consistently even in varying environmental conditions, leading to among-individual behavioral variation (Holtman et al. 2017, Niemel ä & Dingemanse 2018, Mathot et al. 2019). Measuring the consistency of behaviors over fluctuating conditions can potentially indicate the genetic component of variation (Dingemanse & Wright 2020, Niemelä & Dingemanse 2017. Studying the

variation within the individual and accounting for environmental factors (temperature, humidity, breeding habitat, size of the breeding chorus, etc) can quantify how much these behaviors are affected by environmental conditions, which could lead to temporary differences in behavior depending on the conditions an individual is in. Selection acts on genetic traits that are consistently repeated within an individual and consistently different among individuals (Boake 1989). Measuring repeatability of behaviors can therefore potentially indicate traits that are likely to respond to selection, if there is indeed a genetic basis to this repeatability.

Within a population, heritability can be estimated as the proportion of total phenotypic variation that is due to the additive genetic variance. Estimates of heritability generally cannot be determined solely from field observations without accounting for genetic and environmental contributions or performing observational studies over multiple generations. However, estimates of trait repeatability can be acquired in the field from repeated measures of individuals. Repeatability is the proportion of behavioral variation that is due to differences among individuals and can be calculated as the ratio of among-individual variance to total phenotypic variance (Boake 1989; Bell et al. 2009). Estimates of repeatability are calculated by measuring the extent to which behaviors are consistently repeated within an individual and different among individuals. By accounting for these variations across environmental differences, some estimates of the genetic contribution to variability can be assessed (Dochtermann et al. 2015) Understanding the repeatability of certain traits can give some indication of heritability and show which behaviors are consistent over fluctuating environmental conditions.

Both the maintenance and the effects of variation are driven by selection pressures. Maintenance of behavioral variation in a population may be the result of multiple behavioral strategies achieving similar fitness, or environmental variation favoring different strategies under different conditions (Laskowski et al. 2022). Consistent individual behavioral variation can affect fitness fitness, if traits that are consistently expressed within an individual lead to variation in survival or

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reproductive success. Population-level consequences of behavioral variation may have ecological impacts on species or community dynamics; consistent behavioral variation among individuals can affect responses to environmental shifts, dispersal, anti-predator responses, mating displays, and feeding (Okuyama 2008; Bernal et al. 2005; Bee 2001). Understanding repeatable behaviors in the same individuals and the consistent variation among individuals may give insight as to which behaviors determine fitness (and how fitness influences behaviors). (Laskowski et al. 2022; Dochtermann et al. 2015).

Acoustic signaling plays an important role in social communication; signals can be used to locate potential mates, identify conspecifics, and communicate territory ownership (Littlejohn 1977; Gerhardt and Huber 2003). Because animal signals vary at multiple levels (within individuals, between individuals, between populations, etc), characterizing the differences at these various levels is an important step in understanding the function, perception, patterns of signal selection, and heritability of signals (Bee et al. 2010). Characterizing sources and patterns of individual variation in anuran acoustic signals can help derive some information about the consequences of mate choice and sexual selection.

American toads (*Anaxyrus americanus*) are an explosive breeder with their peak breeding season occurring during late March to May. Mate choice in American toads is determined predominantly by females choosing males based off their acoustic signals. Acoustic signals are used by females to locate males and select them as mates by initiating amplexus (Howard 1998; Sullivan and Hinshaw 1992). While some males call from a stationary position to attract mates, others have been documented approaching females and grasping indiscriminately to initiate amplexus (Waldman 2001).

The advertisement call of *A. americanus* consists of a long train of pulses with a narrow frequency bandwidth. American toads typically produce long calls with low call rates, with 2-3

calls being produced per minute (Cocroft and Ryan 1995). In some *Bufonid* species, the advertisement call of one male has been shown to stimulate the call of nearby males (Wells 1977). When female *Anaxyrus americanus* are choosing a mate, they may evaluate male body size through certain call parameters (Howard and Young 1998, Sullivan et al. 1992). However, results from previous studies assessing female *A. americnaus* preferences for certain call parameters have been variable. Sullivan (1992) found that females prefer increased calling efforts shown in longer call durations and higher call rates and that dominant frequency (a call characteristic strongly associated with snout-vent length (SVL)) was not as important. However, Howard and Young (1998) discovered that females were repeatable in their preference for lower dominant frequency calls when the lower frequency call preceded a higher frequency call. Similarly, female mate preferences can be influenced by the number of males present at a breeding site; when more males were detected at a site, females preferred larger males (Howard and Young 1998).

Call characteristics generally are influenced by environmental factors such as temperature, as well as phenotypic characteristics like body size variation. While temperature has been shown to affect whether or not some anuran species call on given nights, *Anaxyrus americanus* will call over a broad range of temperatures. However, temperature still plays a role in reproductive behavior of *A americanus* as it can affect calling characteristics. For example, call duration has been shown to decrease in increasing temperatures, probably because pulse rate has a strong positive correlation with temperature (Sullivan 1992; Howard and Young 1998). Dominant frequency, a call property that is often negatively correlated with SVL, has not been shown to be influenced by temperature (Sullivan 1992, Howard and Young 1998, Cocroft and Ryan 1995). Additionally, longer call durations might be associated with increased body size in some toad species (Sullivan 1992; Zweifel 1968, Sullivan and Wagner 1988), Therefore, it is important to

account for temperature and size when making comparisons of call characteristics of different individuals.

Social aspects of breeding choruses can affect mating calls of males; for instance, overlap between multiple calls may cause males to reduce the dominant frequency of their calls (Howard and Young 1998). Some evidence suggests that larger males may be able to overcome these vocal interactions by displaying decreased dominant call frequency in the face of increased male competition (Howard and Young 1998). This is likely because longer vocal tracts associated with larger body sizes are optimal for producing lower frequencies (Gingras et al. 2013). Studying the variation in *A. americanus* acoustic signals provides a good study system for quantifying levels of variation in phenotypic traits. By understanding some of the sources of this variation, we can derive some implications about the genetic and environmental components of these behaviors and make predictions about how these traits might be subject to selection pressures.

Hypotheses and Predictions

In this study I quantified the effects of environmental and social factors on the call properties of *Anaxyrus americanus* males. Because a subset of males were re-sampled on subsequent nights, I also estimated repeatability of these call characteristics. The main questions I addressed with this study were:

1. How do call parameters change in varying environmental and social conditions?

2. How do these call properties vary within and among individuals? How repeatable are the call properties?

I expected that call characteristics would be highly dependent on the environmental conditions during measurement. Based on previous studies with *A. americanus* advertisement calls, I

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predicted that temperature and humidity would influence the call properties that are the most energetically costly or most under the control of metabolic processes (pulse rate, call duration, and rise time). Dominant frequency has been shown to be influenced most by body size and I expected to find similar results in this study. Interactions between males in a chorus might impact call characteristics through intra-specific competition, and therefore I predicted that increases in the number of males in a chorus would cause males to invest more into calling leading to lower dominant frequency and longer call durations. Because pulse rate has been shown to be crucial for species recognition, I predicted that the variation among individuals would be lower for pulse rate than for other call characteristics. Characteristics that have been previously classified as static call properties (dominant frequency and pulse rate) will likely display lower levels of variation within an individual (Gerhardt 1991). In addition, call characteristics that are highly tied to morphology are expected to be highly repeatable.

CHAPTER II

METHODOLOGY

Capture and Recording Methods:

Data collection and experimentation occurred during Spring 2021 and 2022 (March 23, 2021 –

April 25, 2021 and March 28, 2022 – May 5, 2022). Opportunistic road cruising was used to

identify potential breeding locations of toads. During these road cruises, we used auditory surveys

to determine if toads were present at these locations (Table 1). Once toads were heard in an area,

visual surveys were used to confirm the presence of toads. Because of the explosive breeding

pattern of this species, surveys occurred on the majority of nights during the breeding period, but

especially after heavy rainfall and warm temperatures. On nights in which *A. americanus* were

calling, we recorded frogs using a directional microphone positioned no more than 2 meters away

from calling males. Calls were recorded using a Sennheiser ME-67 microphone (K6-U power

module) with a Marantz PMD661 recorder at a sampling rate of 48 kHz. A wind-screen was used

to prevent wind noise from interfering with recordings. Calls were recorded until each male had

given five consecutive calls. The temperature of each recorded American toad was taken in the

water before they were handled in order to reduce any stress or handling-related temperature

fluctuations.

A non-contact thermal thermometer was pointed at the dorsum of the male to record their body temperature, avoiding pointing the thermometer into the pond water surrounding the toad. After that, individuals were captured, measured (snout-vent length) and toe-clipped for individual identification, after which they were released. At locations where *A. americanus* were calling, distance to the closest road from the middle of the breeding pond was recorded using Google Maps. The average environmental humidity for each day of sampling was collected using MesoNet (Daily Data Retrieval, McPherson et al. 2007; Brock et al. 2007). The number of calling males in each sampled chorus population was collected to estimate the effects of social interactions on individual call structures. This was determined by sampling every calling male in the chorus until all calling males were recorded. Small population sizes for this study ensured confidence in estimating the number of calling males in a chorus. On subsequent nights, individuals were re-recorded to determine whether individual differences in call parameters persisted over time, and the extent to which they varied based on environmental conditions.

Toe-clipping:

Toe clips were obtained from all captured animals using sterile surgical scissors. The skin and toes of the incision site were rinsed thoroughly with clean water before toe-clipping. Three toes were clipped at the second phalange for each animal and the location of the toes needed to be clipped for each animal was pre-determined before field work began (Brannelly et al. 2014). Each individual was given a unique toeclip for identification. All animals were handled under state wildlife permits and with IACUC approval (IACUC-21-14).

Audio Analysis using Raven Pro Software:

The software I used for the sound analysis is Raven Pro 1.6 (Laboratory of Ornithology, Cornell University). I measured 5 call properties (Figure 2):

1. Call Duration – The time from the onset to the offset of each individual call.

2. Pulse Number – The number of pulses per call.

3. Pulse Rate – The repetition rate of pulses within a call which is Pulse Number/Call Duration.

4. Dominant Frequency – The frequency (Hz) with the highest amplitude in the call.

5. Call Rise Time – The amount of time to reach a plateaued amplitude of the call.

Statistical Analyses:

All statistical analyses were performed using R Version 2022.07.2. Environmental effects on call characteristics were analyzed using generalized linear mixed models (GLMM) (package lmerTest) with individual ID number nested within and site as random effects (Kuznetsova et al. 2017). Each call characteristic was tested in a separate model, which included all fixed effects (temperature, SVL, number of males, distance to road, humidity, and day in the season). For dominant frequency, we additionally fitted interactions between SVL and temperature, and SVL and number of males, based on previous studies showing interactions between these variables for dominant frequency (Howard and Young 1998). Model sets were formed using the MuMIn package and "dredge" function to evaluate all possible subsets of the full model (containing all fixed effects) and choose the top models (Barton 2014). Models were ranked by the amount of support they received from the Akaike Information Criterion (AIC). Models within seven AIC values of the top model were retained for model averaging. When two or more models were retained, a model averaging function was used to average the parameter estimates across multiple models; we used the full coefficients from model averaging (i.e. terms not included in the model were set to zero).

Repeatabilities of call characteristics were analyzed using the top model from the AIC tables for each call characteristic (package rptR) (Stoffel et al. 2017). Repeatability is calculated as the

variance among group means over the total variance (group variance and residual variance).

Short-term repeatability was analyzed by assigning individuals a variable called 'series',

corresponding to the combination of their ID number and the night they were recorded. Series

was used as an additional random effect in the model. For short-term repeatability, the among-

individual variance and the series, $(V_{ID} + V_{Series}) / V_{Total}$, was used to estimate the repeatability of

call characteristics within a single night of calling (Araya-Ajoy 2015). I calculated long-term

repeatability across multiple nights of calling as the ratio of among-individual variance over the

total variance: V_{ID} / V_{Total} .

CHAPTER III

RESULTS

Environmental Effects

Over the two field seasons, I recorded and analyzed calls from 118 *A. americanus* males. Of those

118 males, I took repeated measures of 34 of them on subsequent nights (and one male on a third

night).

For dominant frequency, there were three models within 7 dAICc units (Table 2A). These models

included distance to road, humidity, number of males, day in the season, SVL, and temperature as

contributing factors affecting dominant frequency. Interactions between SVL and temperature,

and SVL and number of males, also contributed to variation in dominant frequency. After model

averaging, I found significant positive effects of humidity, number of males, and day in the

season on dominant frequency (Figure 3). There was also a significant negative effect of the

interaction between SVL and number of males on the dominant frequency (Figure 4), where

larger males displayed lower dominant frequencies and this trend was intensified by an increased

number of males at the pond (Table 3).

For pulse rate, there were three models within 7 dAICc units (Table 2B). These models included humidity, SVL, temperature, and day in the season. After model averaging, I found significant positive effects of day in the season and temperature on the pulse rate (Figure 5); with increased temperatures and later in the season, pulse rates were higher (Table 3).

For call duration, there were two models within 7 dAIC units and they included SVL and temperature (Table 2C). After model averaging, I found significant negative effects of temperature on the call duration (Figure 6), where call duration decreased with body temperature (Table 3).

For rise time, there were eight models within 7 dAICc units that included humidity, number of males, day in the season, SVL, and temperature (Table 2D). After model averaging, I found a significant positive effect of humidity on the rise time (Figure 7), where rise times were longer on more humid nights (Table 3).

For pulse number, there were 24 models within 7 dAICc units. These models included distance to road, humidity, number of males, day in the season, SVL, and temperature. After model averaging, I found no significant effects of any of these variables on the pulse number (Table 3).

Repeatability

Repeatability for each call characteristic was separated into short-term repeatability, the repeatability of calling within the same night, and long-term repeatability, the repeatability across multiple nights of measurements (Table 4). There was significant long-term repeatability for dominant frequency ($R = 0.5$, SE = 0.121, P = 0.0011) (Figure 8) and call duration ($R = 0.291$, *P* = 0.000562, SE = 0.078) (Figure 9), but not for pulse number ($R = 0.17$, SE = 0.09, P = 0.0753) (Figure 10), pulse rate ($R = 0.07$, SE = 0.128, P = 0.355) (Figure 11), or rise time ($R = 0.07$, P = 0.42) (Figure 12). Short-term repeatability for call characteristics was always higher than long-term repeatability. Short-term repeatability was judged to be significant if the 95% confidence

interval did not cross zero. There was significant short-term repeatability for all call characteristics: dominant frequency (R = 0.93, 95% CI = (0.903 - 0.942), SE = 0.129), call duration (R = 0.36, 95% CI = (0.271 – 0.453, SE = 0.946), pulse rate (R = 0.82, 95% CI = (0.772 - 0.86), SE = 0.128), pulse number (R = 0.32, 95% CI = (0.227 - 0.411), SE = 0.087), and rise time (R = 0.66, 95% CI = (0.583 - 0.727), SE = 0.122).

CHAPTER IV

DISCUSSION

Environmental Effects

These results demonstrate that environmental and social factors significantly influence call

characteristics in *A. americanus*. Dominant frequency, a call trait that is closely associated with

size in many anuran species, was influenced by SVL. However, there was a significant interaction

between SVL and number of males; as more males were present in a chorus, larger males had

lower dominant frequencies than smaller males. Therefore, with an increase in number of calling

males, the effect SVL on dominant frequency increased. One possible explanation for the

interaction between size and number of males on dominant frequency could be because larger

males may have the energy to divert to increased calling efforts by participating in more breeding

events (more nights calling) than smaller males. Therefore, on nights with few males, only large

males may have been present, whereas on nights with many males, more small males may have

been present. Many studies have found dominant frequency to correlate with body size in *A.*

americanus, but the strength of the correlation may rely on social interactions with other males if

males adjust the frequency of their calls in response to competition (Howard and Young 1998).

This study found similar results: the number of males in a chorus had a significant effect on the

relationship between SVL and dominant frequency. Some studies have found no correlation

between SVL alone and dominant frequency, but this may be because they did not account for the

number of competitors (Sullivan 1992; Bee et al. 2000, Zweifel 1968). Intraspecific competition

may be occurring between males through calling. Males may be displaying plasticity in their calling frequency depending on the level of social competition. Previous studies on intra-specific competition in *A. americanus* have shown that overlapping acoustic signals can cause males to lower their dominant frequency within a single sampling period (Howard and Young 1998). My observations from this field study indicated that the call of one male stimulates the call of other males, aligning with findings in previous studies (Wells 1977). As females have been shown to prefer males with lower dominant frequencies, males with less attractive call frequencies may be intentionally overlapping calls in a chorus in order to interfere with the calls of conspecifics and disrupt this trend. Larger males may be able to overcome this acoustic overlap by lowering their dominant frequency. Alterations in dominant frequency during vocal interactions have been reported in other anuran species: *Leptodactylus albilabris* (Lopez et al. 1988), *Acris crepitans blanchardi* (Wagner 1989b), *Hyla versicolor* (Reichert and Gerhardt 2013), and *Rana clamitans* (Perrill and Bee 1996).

Previous studies have found that females are able to discriminate among individuals using male advertisement calls and choose high-quality mates (Licht 1976). This may be the case in some anuran species such as *A. americanus* and *Hyla chrysoscelis* where studies have found that larger males were more successful in mating (Gatz 1981; Morris and Yoon 1989). However, another study found no significant difference between the SVL of mated and unmated males in *A. americanus* (Kruse 1981). Females may prefer larger males for their initial mate, but then may subsequently mate with males of various sizes (Howard and Young 1998). However, larger males do not necessarily produce better offspring (larger at metamorphosis, earlier metamorphosis, greater survival to metamorphosis) (Howard et al. 1994). Females typically prefer lower frequency calls, but this preference may be disrupted when calls alternate or temporally overlap (Howard and Palmer 1995). Although overlapping calls might interrupt female choice for lower frequencies, when more males were present, females preferred larger males (Howard and Young

1998). The interactive effects of SVL and increased competition on dominant frequency might reveal how larger males are able to overcome overlapping calls in a chorus to attract mates.

Number of males, humidity, and day in the season had positive relationships with dominant frequency. When breeding opportunities are optimal (such as later in the season or with increased humidity), these trends in dominant frequency might be due to the sheer presence of smaller males only on these optimal nights, leading to biased sampling of smaller males and therefore higher dominant frequencies on those nights with the most males present.

Pulse rate was positively correlated with body temperature and days since the start of the breeding season. As ectotherms, increased temperatures would allow for an increase in metabolic rate of the muscles used to produce pulses (Gayou 1984, Gerhardt 1988). Variation in pulse rate is largely determined by the rate of muscular contractions in the trunk muscles. For this reason, pulse rate is often used to recognize conspecifics (Cocroft and Ryan 1995; Martin 1972), but is usually not a great indicator of body size or individual fitness (Sullivan 1982, Zimmerman 1983). The relationship between body temperature and pulse rate might be explained by this energetic mechanism that causes males to produce more pulses per second in higher temperatures.

Pulse rates were found to increase as days in the season increased. Field observations from this study found that near the end of the breeding season, other anuran species started entering the same breeding habitats as *A. americanus* such as *Hyla chrysoscelis, Hyla versicolor,* and *Anaxyrus woodhousii*. Studies of female preferences in *Hylid* species and *Bufotes viridis* for pulse rates found that preferences are stabilizing and typically fall within a narrow range of pulse rates that represents the average pulse rate for a population (Gerhardt and Doherty 1988; Gerhardt 1991; Castellano and Giacoma 1998, respectively). However, these studies were with other anuran species, and no studies have quantified female preference for pulse rates *in A. americanus*.

The only factor significantly affecting call duration was body temperature; calls were shorter in warmer temperatures. This relationship is in the opposite direction to the trend seen between pulse rate and temperature. As temperatures increase, males increase the rate of muscle contractions responsible for creating pulses and, as a byproduct, lengths of calls are shorter. Previous studies have found similar negative effects of increasing body temperature on call duration (Sullivan 1992, Gayou 1984). Many female anuran species, including *A. americanus*, have been shown to prefer high call efforts either through high call rates or longer call durations (Sullivan 1992, Ryan and Keddy-Hector 1992, Gerhardt and Huber 2002).While this study found no significant effects of temperature on pulse number, males that can overcome the energetic costs of producing longer calls by adding pulses in increased temperatures may be at a selective advantage. However with this data showing low repeatability for pulse number, future studies are needed to investigate individuals differences and the relationship between temperature, pulse rate, and pulse number.

Repeatability

Dominant frequency and call duration were the only call characteristics that were significantly repeatable over the long term, between nights of recording. Dominant frequency, a call characteristic that was greatly influenced by size and the number of conspecific males in the chorus, was consistently expressed, despite the high variation in environmental and social conditions. The analyses accounted for environmental and social factors that influence dominant frequency (i.e., these were adjusted repeatability estimates), therefore high repeatability indicates that there are potentially intrinsic individual-level differences or other unmeasured permanent environmental effects contributing to variation in dominant frequency. The interaction between chorus size and SVL revealed how larger males displayed lower dominant frequencies with increased intraspecific competition. Although the dominant frequency was repeatable, there may be some variation within individuals due to plasticity, but average frequencies may be limited by

the size of the individual. As females have been shown to prefer lower dominant frequencies in advertisement calls, this would put larger males at a selective advantage.

Gerhardt (1991) categorized acoustic properties based on within-individual variation as static, dynamic, or intermediate, where static properties had a coefficient of variation of less than 5% within a single bout of calling. Dominant frequency was determined to be a static property in many anuran species (Gerhardt 1991). Previous studies analyzing individual variation in other anuran call properties found that dominant frequency exhibited very high repeatability and low within-individual variation (Bee et al. 2010; Bee et al. 2000, Howard and Young 1998). With significant short-term and long-term repeatability for dominant frequency, *A. americanus* males may be able to adjust dominant frequency based on social interaction with other males, but their range of frequencies may be size-dependent or determined by the female-preferred average dominant frequency of a population (Gerhardt 1991; Bee et al. 2010). Bee (2004) found that dominant frequency was among the call characteristics that contributed most to statistically discriminating among individuals, further supporting dominant frequency as a highly repeatable trait. Previous studies showing a female preference for lower dominant frequencies indicate that this call characteristic is subject to selection, and the high repeatability of dominant frequency means that there could potentially be a response to selection, if there is indeed a heritable genetic basis to this repeatability (Howard and Palmer 1995; Castellano and Giacoma 1998).

Pulse rate, another static property, was not significantly repeatable between nights of recording. The relationship between temperature and pulse rate is presumably driven by the muscle contractions responsible for producing pulses; with increased temperatures, males produce faster pulse rates. Low repeatability indicates that pulse rate is not highly variable between individuals and that, although pulse rate is variable within an individual across varying temperatures, males in a population are similarly affected by temperature, and therefore their pulse rates are affected similarly to each other. My repeatability analyses accounted for temperature variation by

including body temperature as a fixed effect. Previous studies on pulse rate have found similar results and determined that this call characteristic is important for species recognition (Gerhardt 1991, Gayou 1984, Sullivan 1992, Cocroft and Ryan 1995, Bee 2004). However, these results contradict those of Gerhardt (1991) who found pulse rate to be highly repeatable in *Hyla versicolor* and Howard and Young (1998) who found high repeatability for *A. americanus*. Low repeatability for this call characteristic in *A. americanus* indicates that there are not consistent differences in male displays of this behavior across different temperatures.

Call duration, a characteristic shown to be negatively affected by temperature, displayed significant repeatability between nights of recording. Long-term repeatability for this call characteristic (and not pulse rate) demonstrates that individuals may be responding to this trade-off in different ways; some individuals are displaying longer calls than others over varying temperatures. This indicates some individual-level contribution to the variability in this call characteristic. However, since SVL did not influence call duration, body size is likely not the reason for the variability between males. Gerhardt (1992) classified call duration as a dynamic property with high repeatability and indicated how this call characteristic can be used to distinguish between males. High variability and high repeatability suggests that there is a potential for a response to selection due to female choice because the repeatability may indicate some heritable genetic variation.

My results demonstrate that individuals differ in their display of reproductive behaviors over multiple environmental factors. Competition among males might be occurring via overlap of calls and warmer temperatures might reveal individual differences among their abilities to produce longer calls. Measures of repeatability correspond with previous studies except for pulse rate. Repeatable call traits (dominant frequency and call duration) are assumed to be characteristics determined by individual factors. These consistent individual differences shown through high repeatability may indicate the potential for these mating behaviors to respond to selection. Since

call characteristics depend on environment and individual characteristics, selection pressures are subject to change over fluctuating environments. Future studies may explore whether genetics or stable individual factors contribute to repeatable variation in call characteristics (metabolism, age, diet, reproductive history, etc) and if female preference repeatability aligns with the call characteristics we found to be repeatable.

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Table 1. Dates and locations of sampling locations including the number of males in each breeding chorus for each night and the distance of the breeding location to the road.

Date(s)	Number of Males in Chorus	Location	Distance to Road (m)	GPS coordinates
3/23/21 3/25/21	2 5	Babcock Park	853.35	(36.0129792, -97.0910737)
3/24/21 3/26/21 3/27/21 3/28/21 3/29/21 4/19/21 4/24/21	3 6 7 2 1 11 11	May Show Cattle	457.52	(36.1347827, -97.1251065)
4/6/21 4/8/21	10 1	Oklahoma State Dairy Center	1404.53	(36.1339037, -97.0998136)
4/25/21	9	Turning Point Therapeutic Horsemanship	218.61	(36.1191456, -97.1224709)
3/28/2022 3/29/2022	9 10	Western/McElroy Rd	159	(36.1317586, -97.0866410)
4/5/2022 4/24/2022 4/28/2022	12 5 27	Byard Farm – Small Pond	891.49	(36.1328148, -97.1181466)

4/29/2022 5/5/2022	4 8			
4/29/2022	8	Byard Farm – Large Pond	1056.39	(36.1337935, -97.1180204)
4/21/2022	14	The Links Apartments	927	(36.1713928, -97.0639864)

Table 2. The results of AICc analysis for each call characteristic. Only models within 7 delta AICc ('delta') units were included. a). dominant frequency, b) pulse rate, c) call duration, d) rise time, e) pulse number.

a)

Intercept	Dist. Road	Humidity	No. Males	Season Day	SVL	Temp	Male:SVL	SVL:Temp	df	logLik	AICc	delta	weight
1523	-	2.128	34.89	1.247	-0.9806	18	-5.097	-1.567	11	-2959.17	5940.8	0	0.773
1655	-	2.152	37.65	1.24	-22.22	8.138	-5.535	-	10	-2961.754	5943.9	3.08	0.165
1515	0.002613	2.155	35	1.259	-0.1465	17.98	-5.11	-1.577	12	-2961.466	5947.5	6.68	0.027

b)

Intercept	Dist. Road	Humidity	No. Males	Season Day	SVL	Temp	Males:SVL	SVL:Temp	df	logLik	AICc	delta	weight
-3.904	-	-	-	0.07434	-	1.745	-	-	6	-1019.947	2052	0	0.659
-5.313	-	-	-	0.07447	0.2232	1.746	-	-	7	-1019.864	2053.9	1.88	0.257
-2.114	-	-0.02407	-	0.07698	-	1.733	-	-	7	-1021.592	2057.4	5.34	0.046

c)

Intercept	Dist. Road	Humidity	No. Males	Season Day	SVL	Temp	Males:SVL	SVL:Temp	df	logLik	AICc	delta	weight
20.31	-	-	-	-	-	-0.563	-	-	5	-1517.096	3044.3	0	0.622
18.55	-	-	-	-	0.2858	-0.564	-	-	6	-1516.734	3045.6	1.32	0.322

d)

Intercept	Dist. Road	Humidty	No. Males	Season Day	SVL	Temp	Males:SVL	SVL:Temp	df	logLik	AICc	delta	weight
0.8547	-	0.0133	-0.01255	-	-	-0.038	-	-	7	-179.3	372.8	0	0.368
1.133	-	0.01253	-	-	-	-0.058	-	-	6	-180.588	373.3	0.52	0.284
0.1154	-	0.01543	-0.01867	-	-	-	-	-	6	-181.21	374.6	1.76	0.152
2.124	-	-	-	-	-	-0.066	-	-	5	-183.268	376.7	3.83	0.054
0.1628	-	0.01685	-0.01563	-0.00779	-	-	-	-	7	-181.445	377.1	4.29	0.043
1.922	-	0.01337	-0.01146	-	-	-0.048	-	-	6	-183.068	378.3	5.48	0.024
0.9672	-	0.01261	-0.01261	-	-0.0189	-0.037	-	-	8	-181.074	378.4	5.61	0.022
1.199	-	0.01422	-	-	-0.0114	-0.058	-	-	7	-182.402	379	6.2	0.017

e)

Intercept	Dist. Road	Humidity	No. Males	Season Day	SVL	Temp	Males:SVL	SVL:Temp	df	logLik	AICc	delta	weight
182.3	-	-	-	-	8.306	2.827	-	-	6	-3392.08	6796.3		0.136
187.4	-	-	0.2202	-	8.343	2.384	-	-	7	-3391.259	6796.7	0.4	0.111
199.2	-	-0.168		-	8.285	2.507	-	-	7	-3391.492	6797.2	0.9	0.088
179.6	-	-		0.3621	8.705	2.377	-	-	7	-3391.538	6797.3	1	0.084
207	-	-0.1997	0.2651		8.406	1.978	-	-	8	-3390.651	6797.6	1.3	0.073
184	-	-	0.1318	0.3415	8.637	2.095	-	-	8	-3390.727	6797.7	1.4	0.068
209.3	-	-0.3422		0.4359	8.811	1.86	-	-	8	-3390.818	6797.9	1.6	0.062
214.9	-	-0.3561	0.176	0.4143	8.819	1.507	-	-	9	-3389.993	6798.3	2	0.05
231.8	-	-		-	8.22	-	-	-	5	-3394.297	6798.7	2.4	0.041
224.6	-	-	0.5568	-	8.471	-	-	-	6	-3393.369	6798.9	2.6	0.038
252.7	-	-0.3322		-	8.444	-	-	-	6	-3393.601	6799.4	3.1	0.03
220.2	-	-		0.4711	8.457	-	-	-	6	-3393.66	6799.5	3.2	0.028
244.6	-	-0.3147	0.542	-	8.671	-	-	-	7	-3392.686	6799.6	3.3	0.027
216.5	-	-	0.4174	0.392	8.63	-	-	-	7	-3392.805	6799.8	3.5	0.024
248.9	-	-0.4931		0.5591	8.856	-	-	-	7	-3392.823	6799.8	3.5	0.023
243.8	-	-0.4599	0.367	0.4828	8.979	-	-	-	8	-3391.994	6800.2	3.9	0.019
235.6	-	-		-		2.757	-	-	5	-3395.755	6801.6	5.3	0.01
240.4	-	-	0.1985	-		2.366	-	-	6	-3394.94	6802	5.7	0.008
250.8	-	-0.1548		-		2.484	-	-	6	-3395.17	6802.5	6.2	0.006
236.3	-	-		0.3384		2.295	-	-	6	-3395.234	6802.6	6.3	0.006
258.3	-	-0.1825	0.2388	-		2.014	-	-	7	-3394.337	6802.9	6.6	0.005
239.6	-	-	0.1193	0.3214		2.051	-	-	7	-3394.424	6803	6.7	0.005
185.5	0.01949	-	-	-	7.665	2.048	-	-	7	-3394.465	6803.1	6.8	0.005
264.4	-	-0.3217	-	0.41		1.829	-	-	7	-3394.523	6803.2	6.9	0.004

Table 3. Environmental factors affecting variability in male advertisement calls. Models within 7 dAICc units were averaged using a model averaging function to obtain a single value ('full' estimate).

Call Characteristics	Predictor Variable	Estimate	P-Value
Dominant Frequency (full average)	SVL	-0.9806	0.95
	Humidity	2.13	<.001
	Number of Males	35.4	<.001
	Season Day	1.247	0.002
	Temperature	16.31	0.58
	Males and SVL	-5.172	0.001
	SVL and Temperature	-1.299	0.78
	Distance to Road	0.00007399	0.99
Pulse Rate (full average)	Season Day	0.074	<.001
	Temperature	1.74	<.001
	SVL	0.06	0.78
	Humidity	-0.001	0.84
Pulse Number (full average)	SVL	8.108	0.52
	Temperature	1.745	0.44
	Number of Males	0.123	0.84
	Humidity	-0.116	0.81
	Season Day	0.15068	0.7
	Distance to Road	0.00009249	0.97
Call Duration (full average)	Temperature	-0.56	<.001
	SVL	0.097	0.755
Rise Time (full average)	Humidity	0.012	0.01
	Number of Males	-0.009	0.24
	Temperature	-0.038	0.1
	Season Day	-0.0003	0.84
	SVL	-0.0006	0.96

Table 4. Repeatability estimates for each call characteristic. Only the top model from the AICc testing was used for repeatability estimation.

Call Characteristic	Short term repeatability (CI 95%)	Short-Term Repeatability CI (95%)	Long term repeatability	P-Value (long term)
Dominant Frequency	0.93 (0.903 - 0.942)	(0.9027905 - 0.9423209)	0.5498865	0.00143
Pulse Rate	0.82 (0.772 - 0.86)	(0.7720523 - 0.8595248)	0.07016322	0.355
Pulse Number	0.32 (0.227 - 0.411)	(0.2271118 - 0.4111728)	0.1704435	0.0753
Call Duration	0.36 (0.271 - 0.453)	(0.2714144 - 0.4534796)	0.291329	0.000562
Rise Time	0.66 (0.583 - 0.727)	(0.5828174 - 0.7269011)	0.0665326	0.42
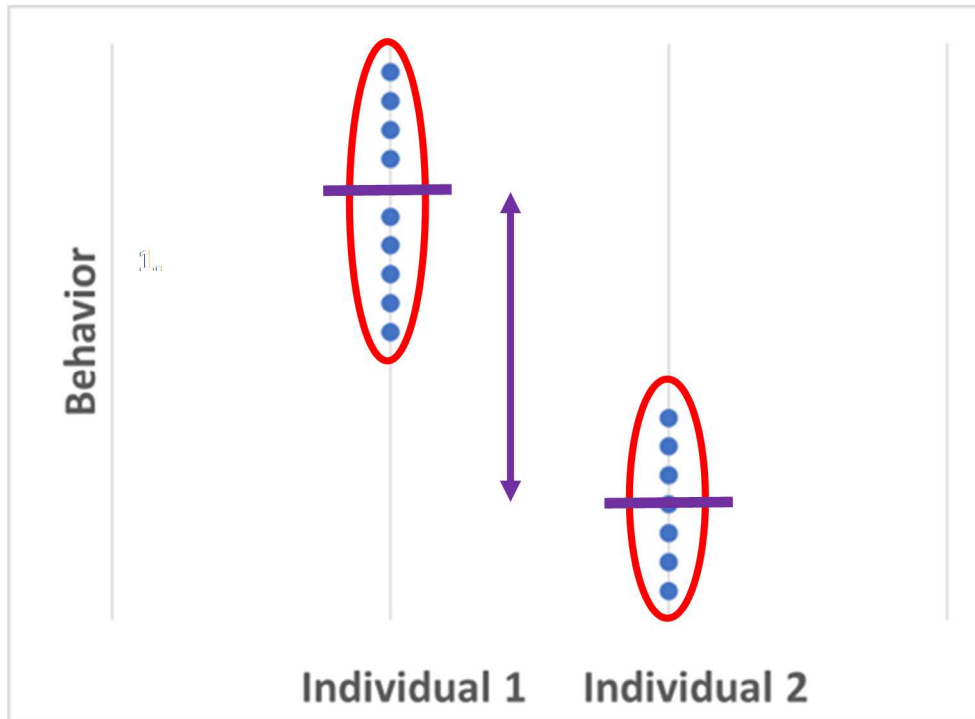
Figure 1. Conceptual illustration of a) within-individual variation characterized by the variation in repeated measurements of behaviors within a single individual (red ovals) and b) among-individual variation (arrow) characterized by the variation among the average behaviors (horizontal lines) for two (or more) individuals.
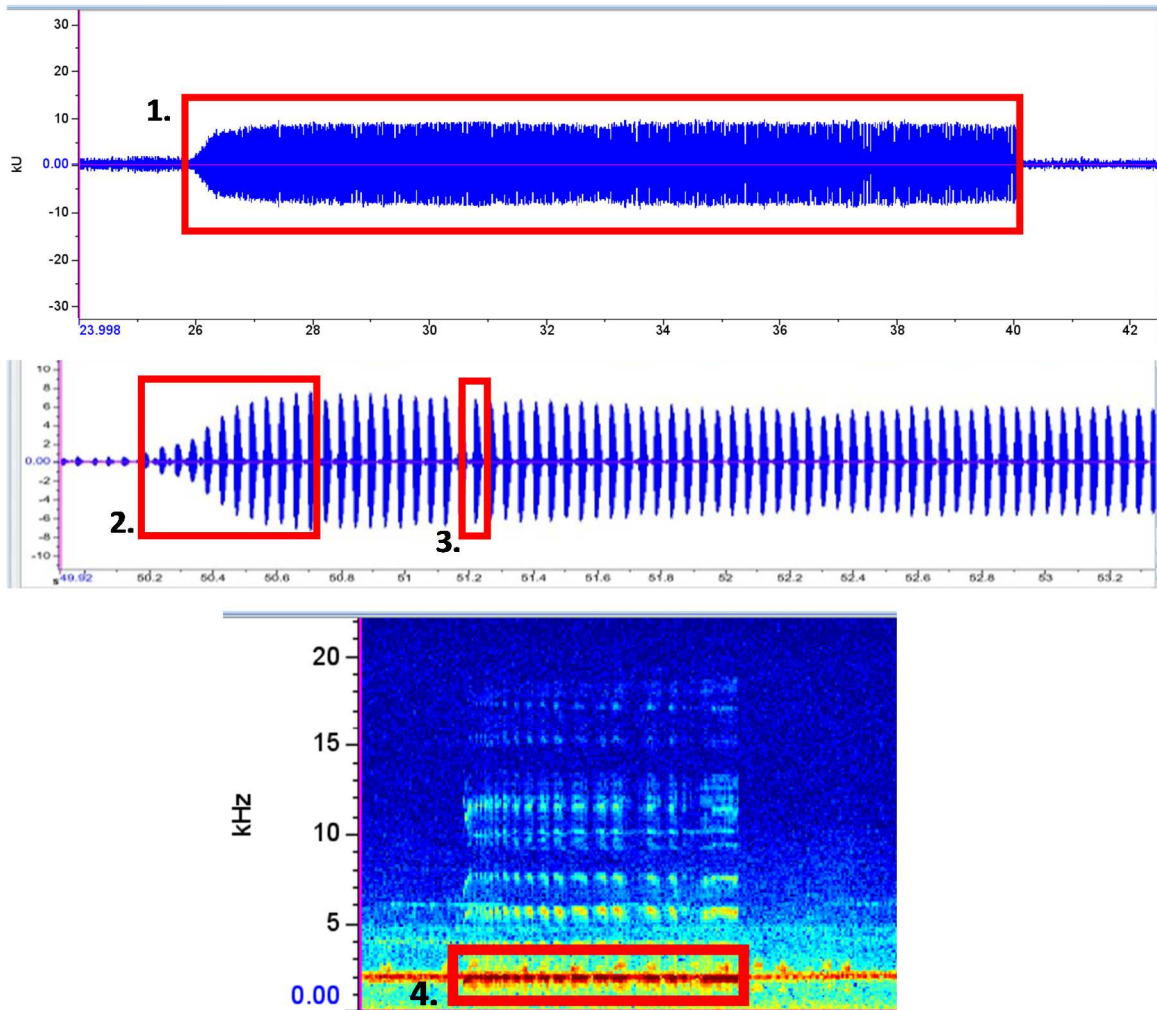
Figure 2. Waveform and spectrogram displays (x-axis shows time in s) of the call characteristics measured in American toads. 1. Call duration, 2. Rise time, 3. An individual pulse, 4. The dominant frequency
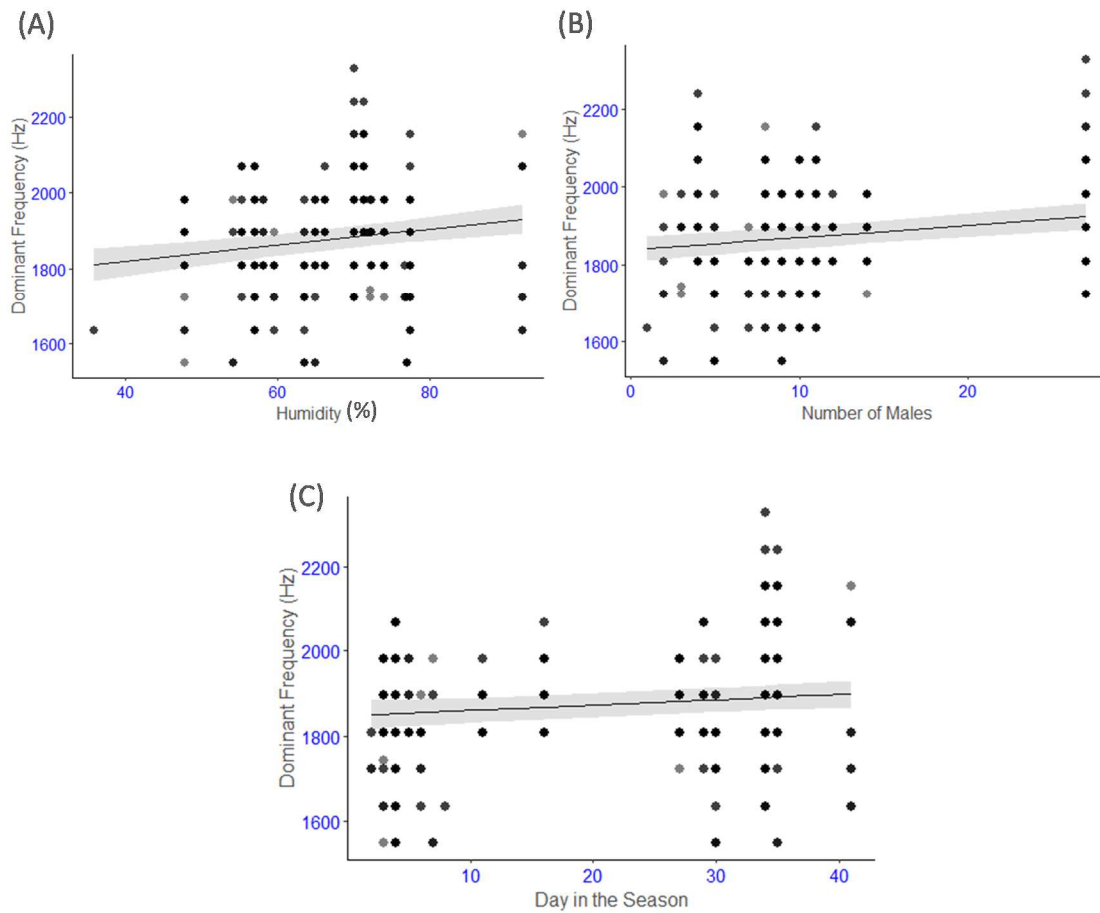
Figure 3. The effects of a)humidity, b) number of males in a breeding chorus, and c) day in the season on dominant frequency. Individual data points are represented by black points – darker points display overlap. Lines represent predicted effects (shading-95% confidence intervals). Data points are predicted values from the top model.
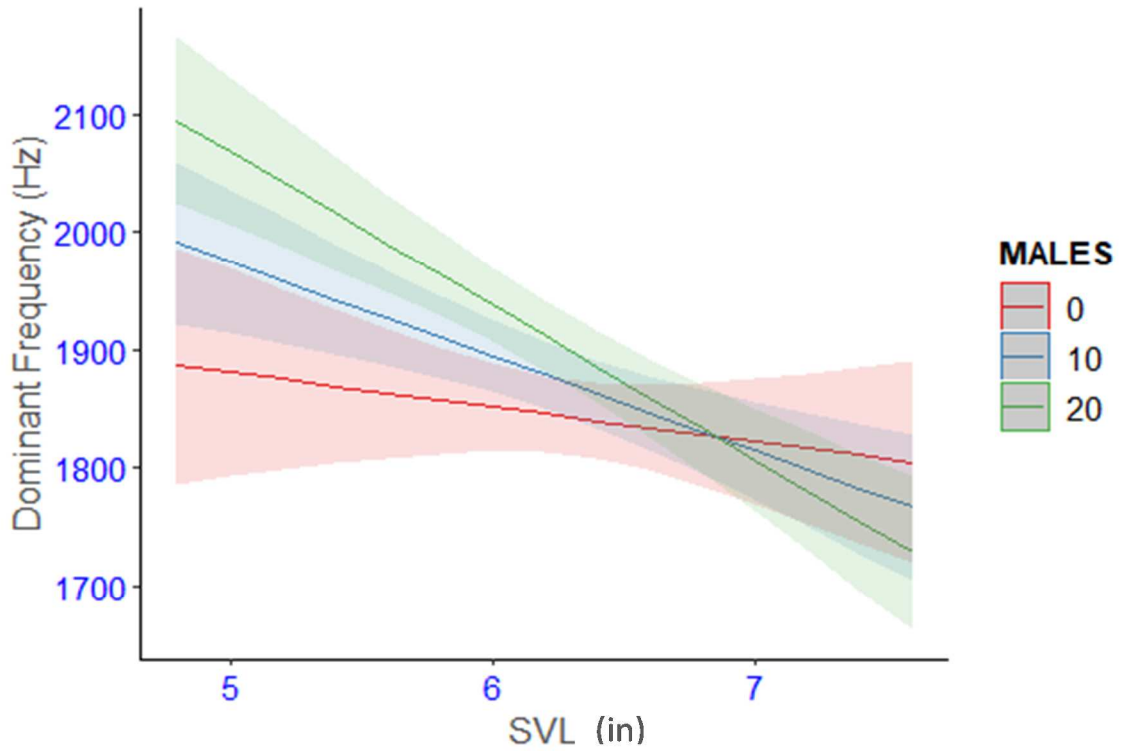
Figure 4. The interaction effect of SVL and chorus density on dominant frequency. Lines represent predicted effects (shading-95% confidence intervals) for three different chorus densities: (red) zero males, (blue) 10 males, and (green) 20 males.
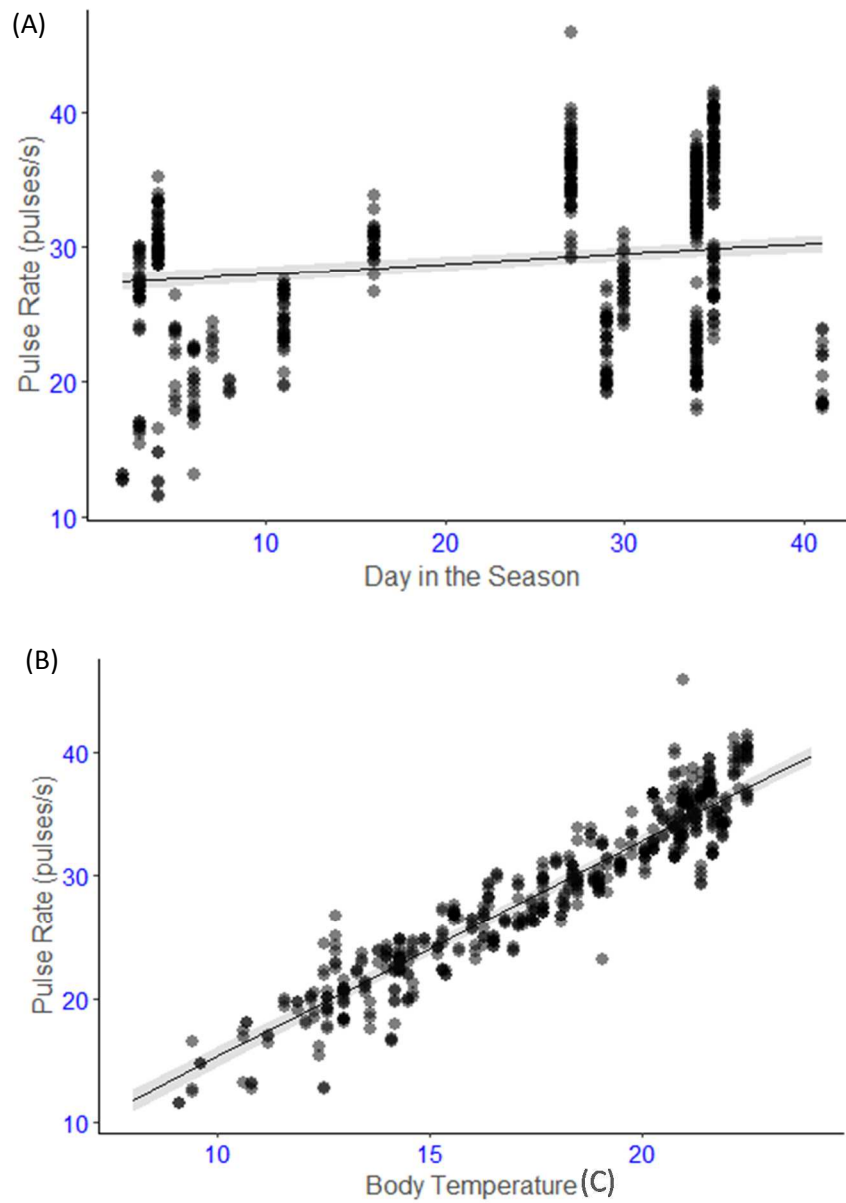
Figure 5. The effects of the a) day in the season and b) body temperature on pulse rate. Individual data points are represented by black points – darker points display overlap. Lines represent predicted effects (shading-95% confidence intervals). Data points are predicted values from the top model.
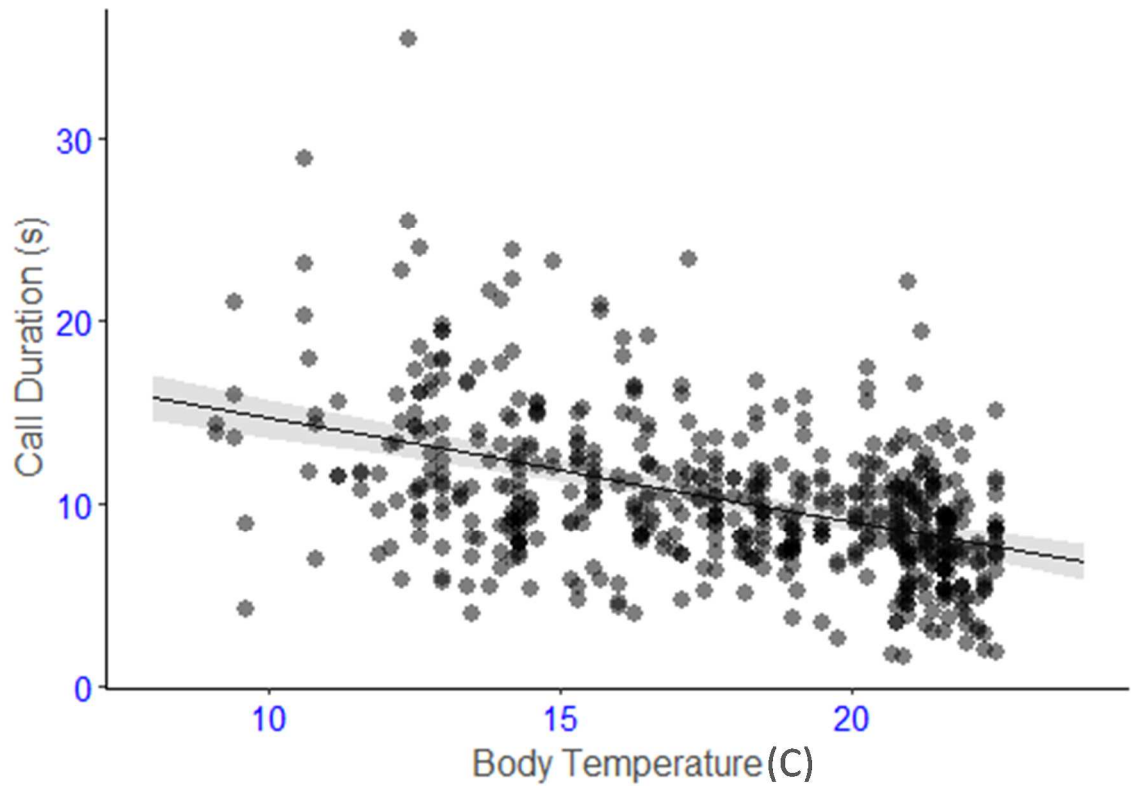
Figure 6. The effects of body temperature on call duration. Individual data points are represented by black points – darker points display overlap. Lines represent predicted effects (shading-95% confidence intervals). Data points are predicted values from the top model.
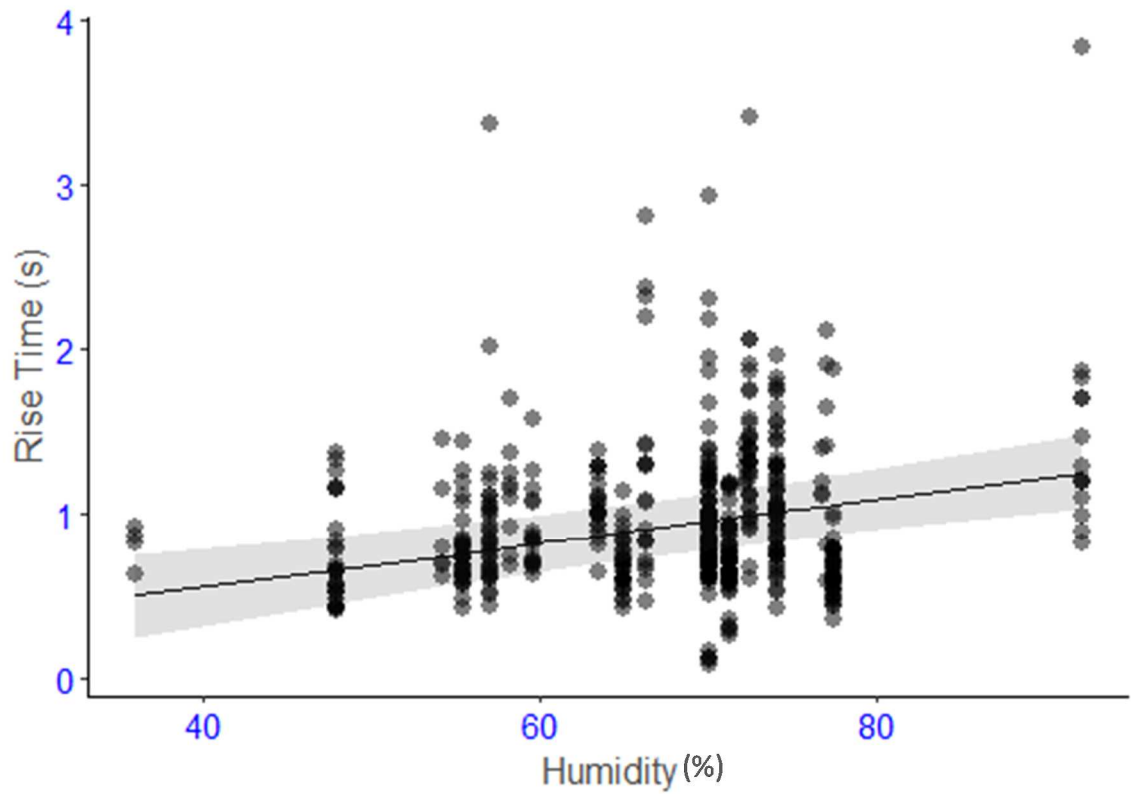
Figure 7. The effects of humidity on rise time. Individual data points are represented by black points – darker points display overlap. Lines represent predicted effects (shading-95% confidence intervals). Data points are predicted values from the top model.
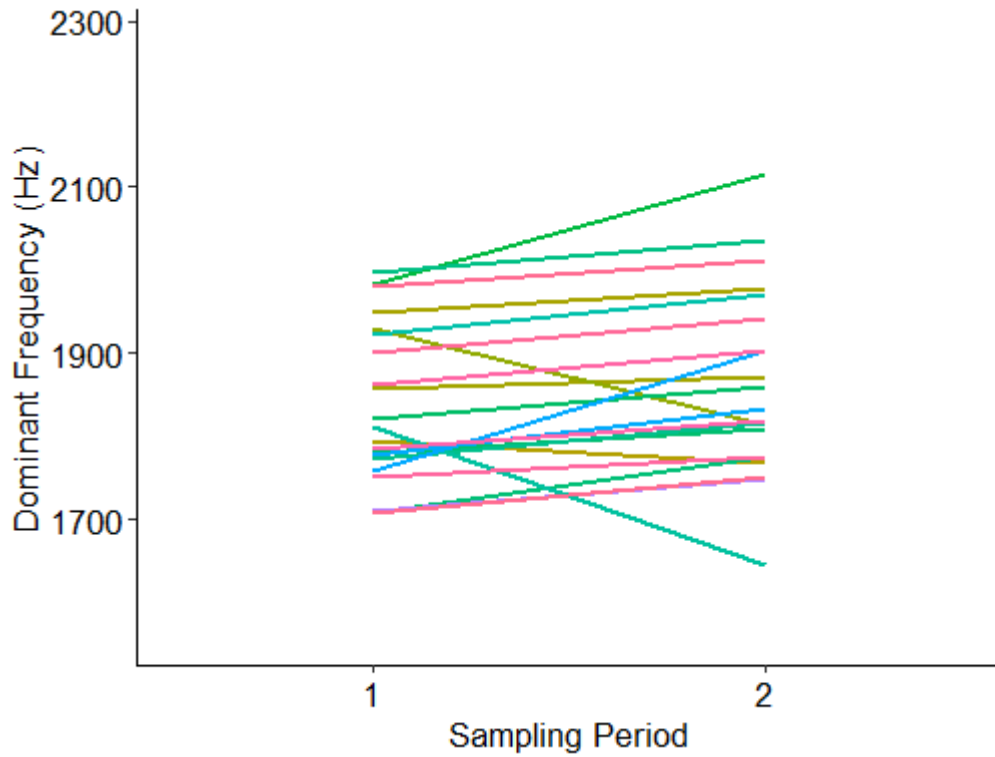
Figure 8. Repeatability of dominant frequency over two sampling periods. Each line represents an individual's average dominant frequency from the first sampling night and the average dominant frequency of the second sampling period. Individuals are represented by different color lines. Data points are predicted values from the top model.
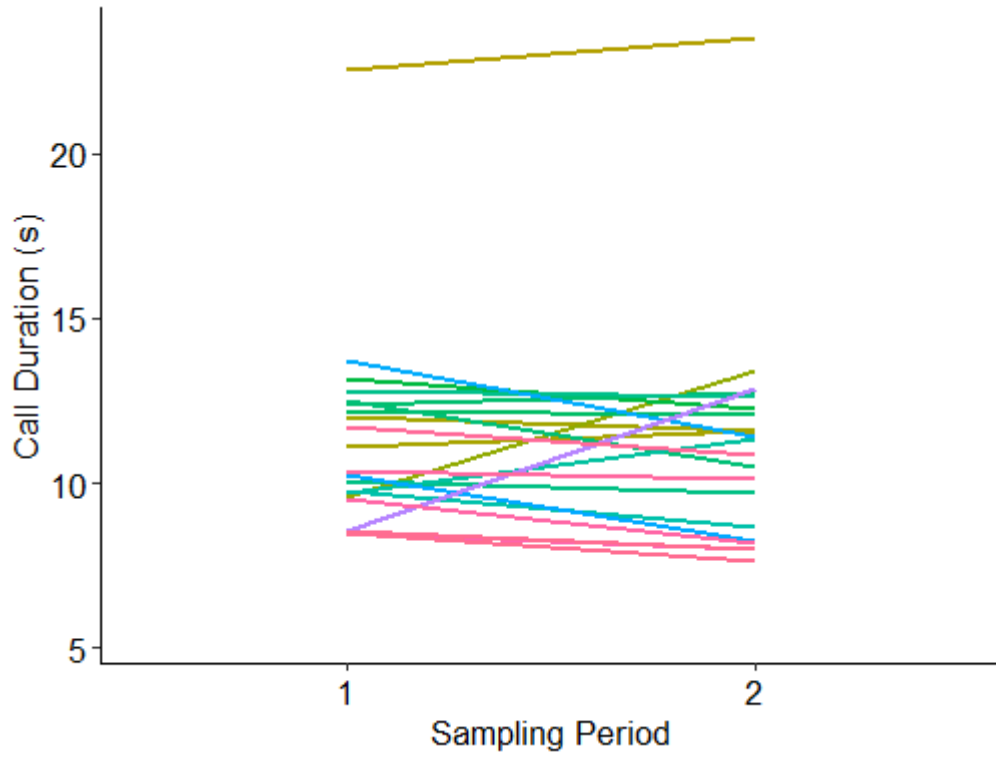
Figure 9. The repeatability of call duration over two sampling periods. Each line represents the average call duration from the first sampling night to the average call duration of the second sampling period. Individuals are represented by different color lines. Data points are predicted values from the top model.
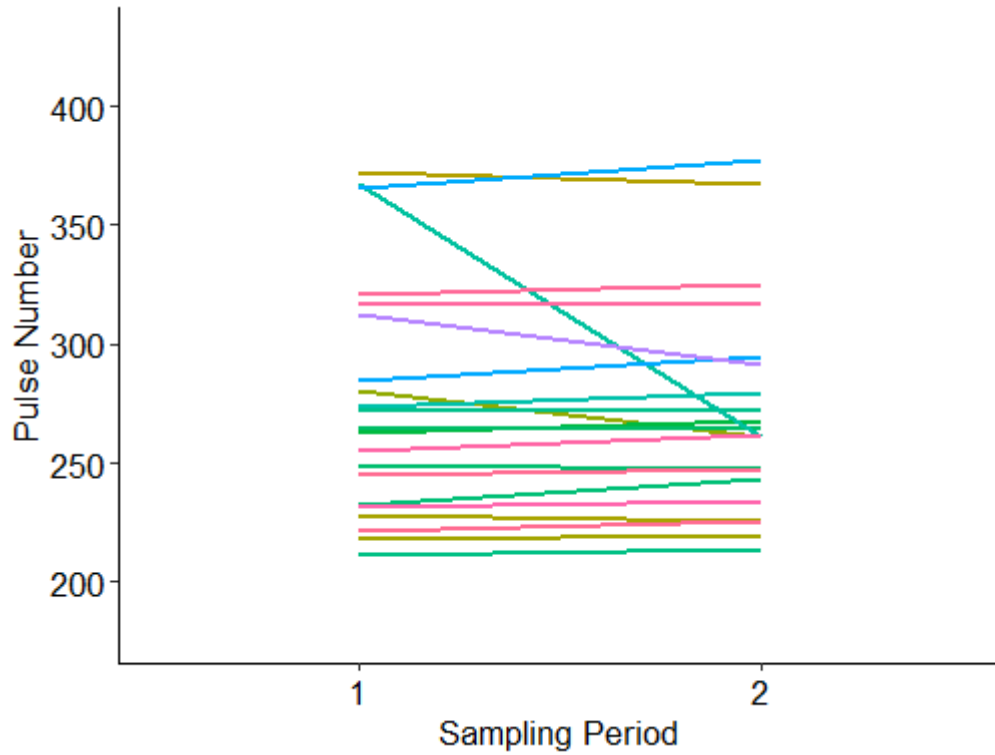
Figure 10. The repeatability of pulse number over two sampling periods. Each line represents the average pulse number from the first sampling night and average pulse number of the second sampling period. Individuals are represented by different color lines. Data points are predicted values from the top model.
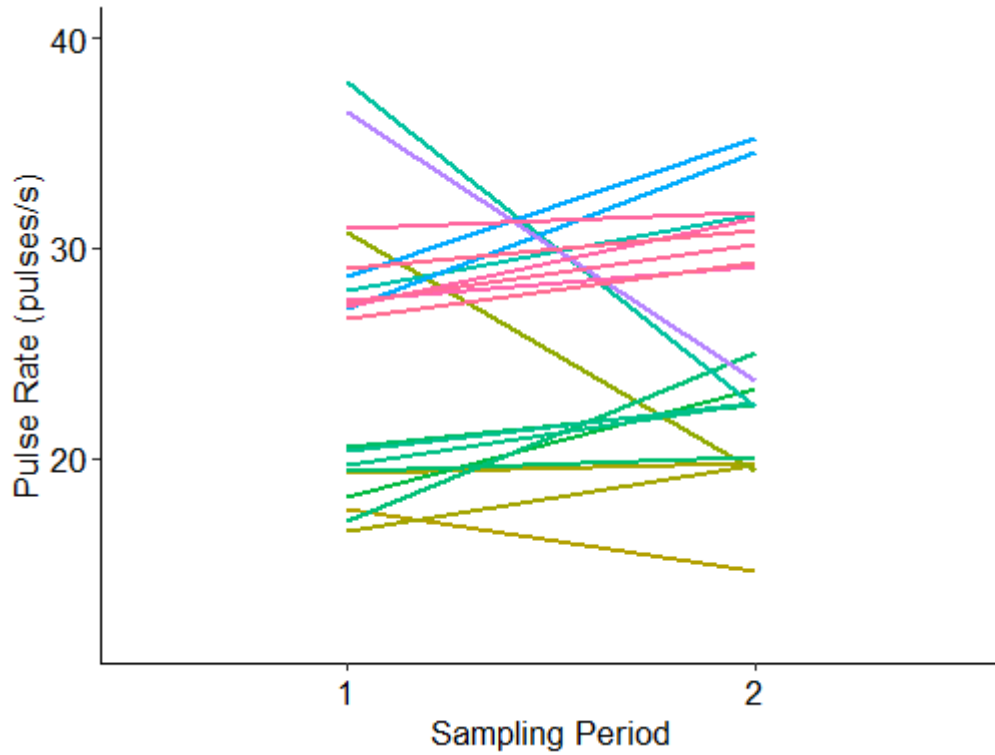
Figure 11. The repeatability of Pulse Rate over two sampling periods. Each line represents the average pulse rate from the first sampling night to the average pulse rate of the second sampling period. Individuals are represented by different color lines. Data points are predicted values from the top model.
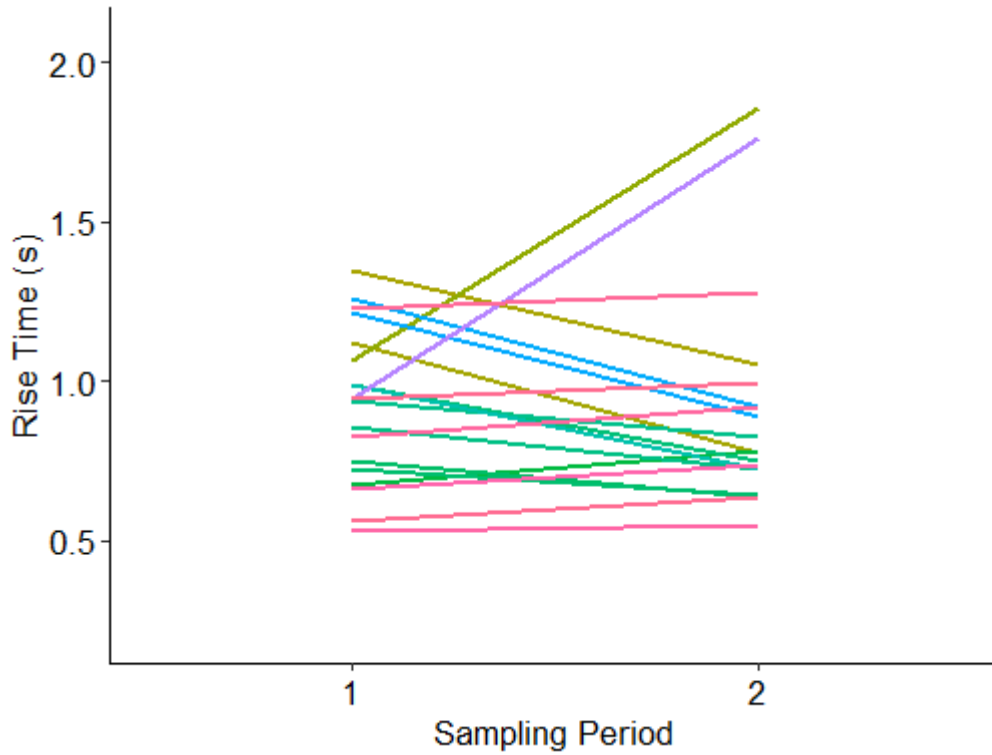
Figure 12. The repeatability of rise time over two sampling periods. Each line represents the average rise time from the first sampling night to the average rise time of the second sampling period. Individuals are represented by different color lines. Data points are predicted values from the top model.

VITA

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