Title

A Study on the Direct and Indirect Relationship between Calling Song and Morphology in the sand cricket, Gryllus firmus

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A STUDY ON THE DIRECT AND INDIRECT RELATIONSHIP BETWEEN CALLING SONG AND MORPHOLOGY IN THE SAND CRICKET, *GRYLLUS FIRMUS*

By

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Abstract

The sand cricket, *Gryllus firmus*, is dimorphic with respect to flight capability. Some individuals possess a fully functional flight apparatus (hereafter, LW), whereas others have reduced wings, lack flight muscles and are incapable of flight (SW). The LW morph can colonize new habitats but the energy diverted into flight capability (flight muscles, flight fuel) reduces the amount of time it can call, thereby reducing its success at attracting a female relative to the SW. Previous research has shown that female preference results in sexual selection for the call song of the male, SW males are preferred both because they call more and because of some song component(s) that differ from the LW males. This experiment tested the relationship between the calling song, morphology, and wing morph to understand whether the female preference of this species' calling song has direct or indirect effects on the morphological structures that generate this call. Statistical analyses suggested 25 possible relationships between a morphological trait or wing morph and a song component. Of these significant relationships, one in particular, resonator area and mean volume, is thought to be a causal relationship. The remaining relationships could be causations, correlations, or randomly associated errors. Even in the absence of direct, causal relationships between morphology and calling song, correlational selection exists in this species, and at the very least, the known preference on song indirectly affects the size and relative output of sound-producing morphological traits.

Acknowledgments

I would like to thank Dr. Roff for not only allowing me to conduct research in his lab but also for his constant guidance, support, and patience. It has truly been a pleasure to be able to be a part of his research for these last two years and I have learned so much about both intraspecies evolution and the research environment as a whole. I came into his lab with few expectations and little applicable knowledge and am leaving with so much more, and for that I am very grateful.

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Introduction

A general principle in the field of evolution is the understanding that an individual's fitness, the competency in survival and reproduction, is directly dependent on the visual, behavioral, and auditory cues that facilitate the attraction between the sexes. When sexual selection exists in a species, the use of and proficiency at courtship displays determines an individual's reproductive success. Among the varying forms of courtship, the use of mating songs in auditory-signaling species is an especially complex behavior because it involves the interplay of two systems, the morphological traits that generate the call and the call itself. In this research, the anatomical structures involved in the formation of calling songs were studied within the sand cricket, *Gryllus firmus*, to understand the relationship between these two systems, and ultimately to determine if the sexual preference that acts on a male's mating call also has an effect on that individual's morphology.

In *Gryllus firmus*, males adopt three different mating calls, each in their own situation. An agonistic call exists in this species to vocalize aggression between males. *Gryllus firmus* males also have regular, repetitive calling songs that through duration, amplitude, and frequency, attract neighboring females within earshot. And lastly, highly variable courtship songs are used when females and the possibility of upcoming copulation are near (Webb & Roff, 1992). This study focused on this second, calling song because of its repetitive, quantitative nature and its implicit relationship with preference. The duration and amplitude of these calls are both understood to be positively correlated with female preference, and even though both factors are crucial to the fitness of a calling song, the repetitive components within the song are additionally important in this form of preference (Crnokrak & Roff, 1995). Each calling song can be broken down into components consisting of individual pulses that compromise individual chirps. This study measured the characteristic song components of chirp length, chirp repetition rate, average pulses per chirp, pulse width (also called pulse length), pulse period, and pulse repetition rate as well as several other distinguishing factors to understand the evolutionary mechanisms behind preference encoded in these basic song values (refer to Figure I).

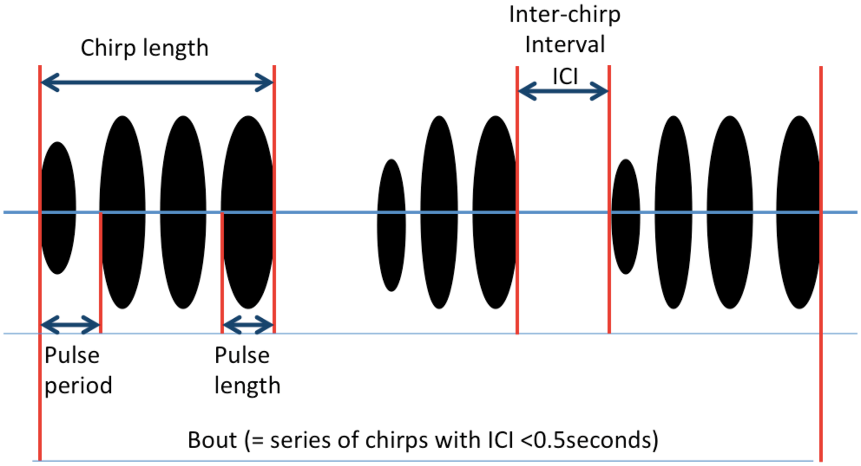


Figure I: Song component parameters (Roff, 2016)

The differences in song and morphological components in *Gryllus firmus*, is due both to the genetic variation among individuals and to a dimorphism within each sex. Two wing morphs exist in this cricket species, each with their own evolutionary advantage. Macropterous, “long-winged” (LW) individuals have comparatively larger wing areas, which serve to benefit this morph in flight capability (Roff, 1984). In laboratory conditions, females show significant preference towards micropterous, “short-winged” (SW) males, largely due to their relative advantage in call duration, but also preference in song components, independent of wing morph, has been experimentally demonstrated (Roff, 2016). It is assumed that preference for one or more of the call components is inherited, but presently this cannot be confirmed because of insufficient knowledge of the preference and heritability of the sound-producing structures in *Gryllus firmus*.

The mating calls indicative of species within the genus *Gryllus* are produced when the inferior plectrum scrapes against the overarching, superior file. This process occurs when the two tegmina relax, closing their previously elevated forewing position (Bennet-Clark & Bailey, 2002). The file and plectrum are not the only sound-producing structures; the harp and resonator also contribute to the sound frequency and amplitude of the calling song, respectively (Bennet-Clark & Bailey, 2002; Webb & Roff, 1992). In the present experiment, I measured the area of both the harp and resonator as well as the length of the file to compare the morphological variation in these wing regions to the variation in the calling songs. In doing so, this study tests for the existence of a preferred morphology that is a consequence of the preference for both wing morph and the components of the call song. Presumably, there should be a quantitative relationship between call, morphology, and wing morph due to the interplay between each and all of the three systems. The interaction between the resonator area and volume of the calling song is a key predictor to whether direct relationships exist because of the known role of the resonator in amplifying the loudness of the call.

Methods

The *Gryllus firmus* lineage used in this experiment was initially collected from one area in Northern Florida in 2013. After the approximately 100-individual parental generation, the proceeding generations were bred in cages under a consistent 15:9 hr. photoperiod and a constant temperature of 28 °C. Crickets were housed with sufficient Purina rabbit chow and a water reservoir made accessible through a soaked cheese cloth. Oviposition was ensured using earth dishes, and after eclosion, nymphs were stored in a large stock cage under the same temperature and photoperiod. After metamorphosis from nymph to adult, both males and females were randomly selected and placed into the apparatus shown below to record and measure the preference of calling songs.

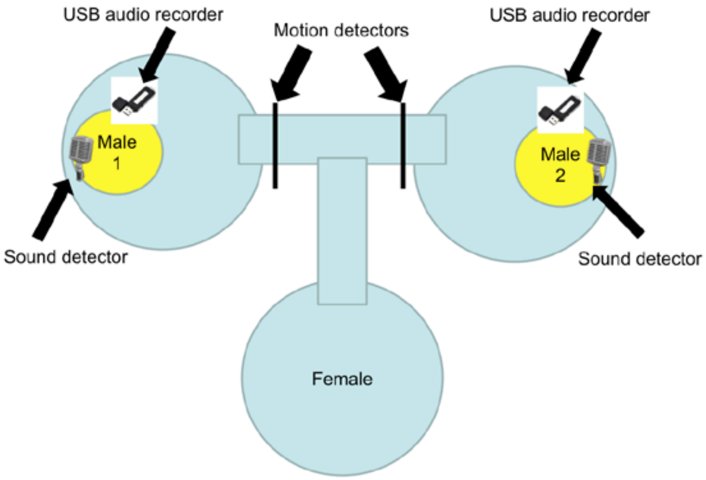


Figure II: Apparatus used to measure preference and record calling song (Roff, 2016)

Three cages, two containing one male each and the other with a female, were connected using PVC pipe (as seen in Figure II). The female was able to move throughout the tubes and motion sensors measured the frequency of visitation at either end, closer to one of the male cage than the other. The female was unable to directly interact with the male as he was in a closed container that permitted the female to hear the call but not see or touch the male. Light was turned on at midnight, simulating dawn and initiating mating calls, and shut off at 3 pm. A USB audio recorder was placed in each male cage to obtain an audio recording of the particular male. Calling songs were quantified using RAVEN interactive sound analysis software to acquire peak frequency, center frequency, pulses per chirp, the proportion of triple pulses, pulse width, pulse period, pulse rate, chirp length, chirp rate, and call duration. While the recorders used were not designed to measure the loudness of the call, tangible values for average RMS amplitude, mean volume, and maximum volume were also collected.

The superior tegmen and right femur (left if the right was missing) of audio-recorded males were dissected and placed on slides for morphological analysis. Digital images were made through a LEICA Wild M3C light microscope and ToupView computer software, and these images were then measured using SigmaScan Pro 5 software. Femur length was measured from coxal notch to femur-tibial node. In apportioning the song-producing structures in the tegmen, the resonator area was well defined, the harp area was designated up to the 5th cross vein, and the file was measured from the 5th cross vein to the plectrum. An illustration of both leg and wing measurements is shown in Figure III. A total of 120 males were dissected, 51 SWs and 69 LWs. All standard errors and statistical analyses were performed through SYSTAT 10.

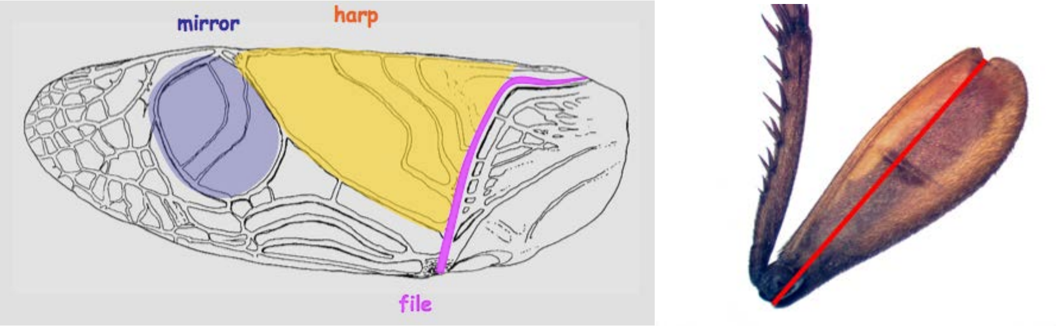


Figure III: Wing and femur structures with measured regions labeled

Results

To test for a relationship between morphology, song components, and wing morph, I used the general linear model, a least squares regression approach. All individual song components were compared to the three different morphological predictors (resonator area, harp area, and file length) and the wing morph predictor to statistically test for a linear relationship between each variable. The general linear model was used in a hierarchical fashion to determine if an additive or saturated model (which involves an interaction term) was most satisfactory. In the additive model, the dependent variable, the song component (y), was compared to both independent variables, morphology (x_1) and wing morph (x_2) using the relationship $y=a+bx_1+bx_2$. Similarly in the saturated model, the variables were related using $y=a+bx_1+cx_2+dx_1x_2$. If an interaction term existed then there was an explicit, significant relationship between the individual song component and morphology as well as that song component and the wing morph. If the interaction term was not significant, the model was reduced to the additive model, which assumes a parallel relationship of variables (see above equation).

Through the use of the general linear model, significant relationships between song components, morphologies, and wing morph were found. Of all song components studied, only the average number of pulses per chirp, average chirp length, and average chirp rate showed no significant association with any morphology or wing morph (Table I). The analysis between average pulse period and the resonator size showed a highly significant relationship between wing morph and average pulse period but no relationship between the resonator area and average pulse period, suggesting that the wing morphs differ in the pulse period in their call but this difference is not due to their respective resonator areas. Similarly, the average RMS amplitude showed a highly significant relationship to the harp area and no relationship with wing morph, signifying the covariance of the RMS amplitude with respect to the size of the harp but no distinguishable differences between the wing morphs. In the case of average pulse period and harp area, the presence of a significant interaction term indicated the relationship between the harp and pulse period as well as different relationships in pulse period in the two wing morphs, even though the individual comparisons themselves showed no such relational significance.

The numerous associations derived from this statistical approach indicate that a relationship does exist between this species' call, wing anatomy and morph, but further analyses of the nature of this relationship needed to be later explored. A Bonferroni correction was not used in determining statistical significance, and were this method used to limit the probability of a type I error, it would have predicted a lower significance level for all interactions studied (i.e. significance level of 0.05/n, where n is the number of tests). Whenever multiple comparisons are performed using one test, there is an increased likelihood that significant relationships result from chance alone (Mittelhammer, Judge, & Miller, 2000). Even though 39 different comparisons were prepared, the sheer number of weighted relationships (15 significantly related to wing morph, 10 to morphology) was far more than the 2 significant relationships expected by chance, upholding the overall consensus that call, morphology, and wing morph are systemically related. In addition to the total number of relationships, the interactions between the systems that tested significant also aligned with the functional role of the different components. The resonator area and mean volume were highly related using this model, which is consistent with the known role of the resonator in controlling the loudness of the call (Michelsen & Noche, 1974). This likely implies a direct relationship between the resonator and mean volume, in which preference of song volume directly influences resonator size. The distribution of resonator area, mean volume, and wing morph showing this relationship is seen in Figure IV. Clearly, mean volume tends to increase with resonator size, and a distinct difference between the morphs exists.

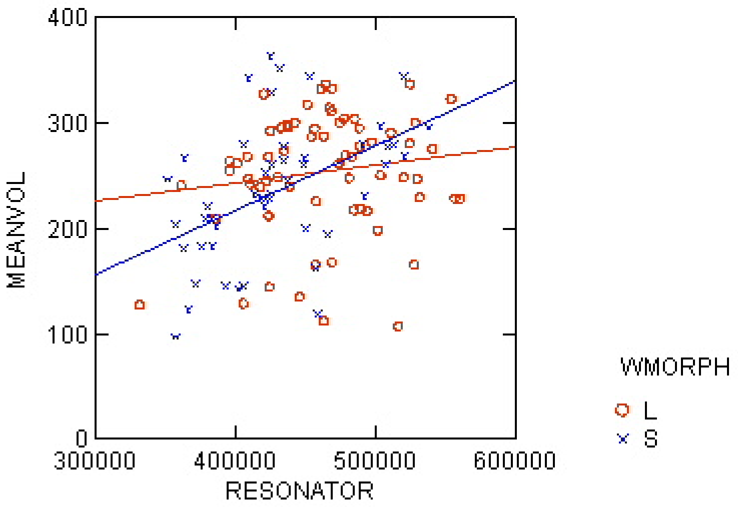


Figure IV: Scatterplot of the resonator and mean volume illustrating their relationship between the morphs

To understand the nature of the individual relationships between song components, morphologies, and wing morph, correlational matrices were performed to identify and separate possible causal and indirect associations. Through the use of a simple correlation, the interactions within and among all tested morphological structures and call components were analyzed giving the likelihood that these components co-vary. The high correlation coefficients observed between all morphological wing structures indicated that these regions were highly related, and therefore, direct preference of one structure could lead to correlated selection of the others (Table IIa). This strong correlation also suggests that a causal relationship between one morphology and a call component would result in the indirect relationship between another, correlated morphology and that same song variable. It is speculated that because a known causation exists between the resonator and mean volume, the relationship between the harp or file length and mean volume is strictly correlational due to their high correlation coefficients (0.711 and 0.550 respectively). The song components also were found to have some key correlations, revealing why some seemingly unconnected variables showed significant relations (Table IIb). As expected, average peak frequency and average center frequency were highly correlated, as both are measurements of song pitch. Average pulse width, which showed relationships to all morphological structures, also significantly correlated with a number of other components including average pulses, proportion of triple pulses, average pulse period, average chirp length, average RMS amplitude, mean volume, and maximum volume. Whether or not average pulse width is itself causally related to any morphological trait is unknown, but these relationships are at the very least correlational.

To interpret how this network of relationships factors into the preference of an individual, the averages of all components studied were sorted by wing morph (Table III). Through these simple statistics, a known preference for specific call components can be constructively associated to the probability of selecting one wing morph over the other. The known preference for call loudness favors macropterous males because LWs not only have higher mean volumes than SWs, they also have significantly larger resonators. Through these basic statistics, the relationships between song components and morphologies can be identified on the distinct wing morphs.

List of Tables

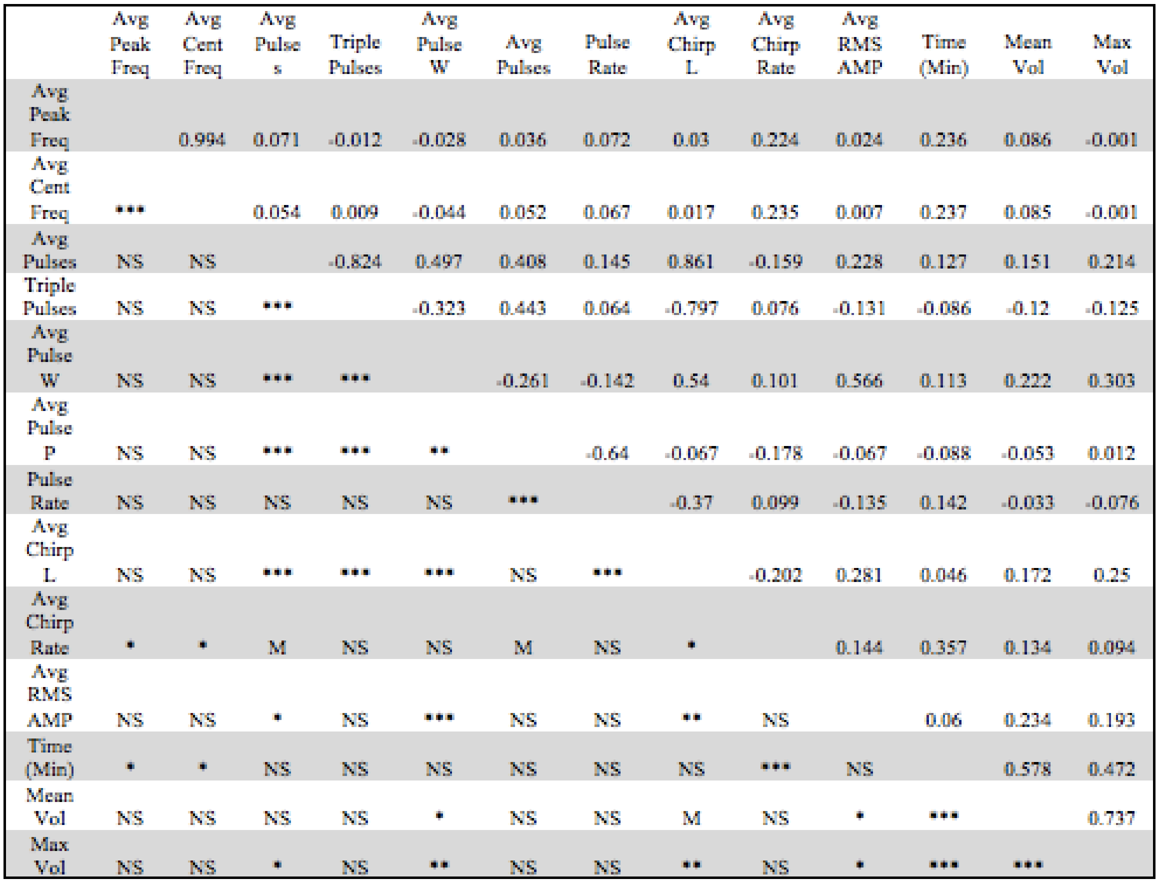
Table I: Relationships between song components, traits, and wing morph using the general linear model (Measure of significance: ***- p-value <0.001, **- <0.01, *- <0.05, M- <0.1, NS- not significant)

Song Component	Trait	Wing Morph	P-value for Trait	Interaction Term
Avg Pulse Period	Resonator	***	NS	M
Avg Center Freq	Resonator	**	M	NS
Avg Peak Freq	Resonator	*	NS	NS
Avg Peak Freq	Harp	*	NS	NS
Avg Peak Freq	File	*	NS	M
Avg Center Freq	Harp	*	NS	M
Avg Center Freq	File	*	NS	M
Triple Pulses	Resonator	*	NS	NS
Triple Pulses	File	*	NS	NS
Avg Pulse Width	Resonator	*	*	NS
Avg Pulse Width	Harp	*	*	NS
Avg Pulse Width	File	*	M	NS
Avg Pulse Period	File	*	NS	**
Time (min)	Resonator	*	M	*
Mean Vol	Resonator	*	***	M
Avg RMS AMP	Harp	NS	***	M
Avg RMS AMP	Resonator	NS	*	NS
Time (min)	File	NS	*	NS
Mean Vol	Harp	NS	*	NS
Mean Vol	File	NS	*	NS
Max Vol	Resonator	NS	*	NS
Max Vol	File	NS	*	NS
Avg Pulse Period	Harp	M	NS	*
Avg Pulses	Resonator	NS	NS	NS
Avg Pulses	Harp	NS	NS	NS
Avg Pulses	File	NS	NS	NS
Triple Pulses	Harp	M	NS	NS
Pulse Rate	Resonator	M	NS	M
Pulse Rate	Harp	NS	NS	M
Pulse Rate	File	NS	NS	NS
Avg Chirp Length	Resonator	NS	NS	NS
Avg Chirp Length	Harp	NS	NS	NS
Avg Chirp Length	File	NS	NS	NS
Avg Chirp Rate	Resonator	NS	NS	NS
Avg Chirp Rate	Harp	NS	M	NS
Avg Chirp Rate	File	NS	NS	NS
Avg RMS AMP	File	NS	M	NS
Time (min)	Harp	NS	NS	NS
Max Vol	Harp	NS	M	NS

Table IIa: Correlations between morphologies (correlation coefficients on the right, measure of significance on the left)

	Resonator	Harp	File Length
Resonator		0.711	0.55
Harp	***		0.671
File Length	***	***	

Table IIb: Correlations between song components



	Avg Peak Freq	Avg Cent Freq	Avg Pulses	Triple Pulses	Avg Pulse W	Avg Pulses	Pulse Rate	Avg Chirp L	Avg Chirp Rate	Avg RMS AMP	Time (Min)	Mean Vol	Max Vol
Avg Peak Freq		0.994	0.071	-0.012	-0.028	0.036	0.072	0.03	0.224	0.024	0.236	0.086	-0.001
Avg Cent Freq	***		0.054	0.009	-0.044	0.052	0.067	0.017	0.235	0.007	0.237	0.085	-0.001
Avg Pulses	NS	NS		-0.824	0.497	0.408	0.145	0.861	-0.159	0.228	0.127	0.151	0.214
Triple Pulses	NS	NS	***		-0.323	0.443	0.064	-0.797	0.076	-0.131	-0.086	-0.12	-0.125
Avg Pulse W	NS	NS	***	***		-0.261	-0.142	0.54	0.101	0.566	0.113	0.222	0.303
Avg Pulse P	NS	NS	***	***	**		-0.64	-0.067	-0.178	-0.067	-0.088	-0.053	0.012
Pulse Rate	NS	NS	NS	NS	NS	***		-0.37	0.099	-0.135	0.142	-0.033	-0.076
Avg Chirp L	NS	NS	***	***	***	NS	***		-0.202	0.281	0.046	0.172	0.25
Avg Chirp Rate	*	*	M	NS	NS	M	NS	*		0.144	0.357	0.134	0.094
Avg RMS AMP	NS	NS	*	NS	***	NS	NS	**	NS		0.06	0.234	0.193
Time (Min)	*	*	NS	NS	NS	NS	NS	NS	***	NS		0.578	0.472
Mean Vol	NS	NS	NS	NS	*	NS	NS	M	NS	*	***		0.737
Max Vol	NS	NS	*	NS	**	NS	NS	**	NS	*	***	***	

Table III: Component means and standard errors

	SW	**LW**
Resonator	0.06 [0.001]	0.07 [0.001]
Harp	0.14 [0.002]	0.15 [0.002]
Total	0.48 [0.004]	0.50 [0.004]
Leg	1.26 [0.008]	1.26 [0.007]
Avg Peak Freq	4.2 [0.02]	4.2 [0.02]
Avg Cent Freq	4.2 [0.02]	4.2 [0.02]
Avg Pulses	3.7 [0.08]	3.5 [0.06]
Triple Pulses	0.3 [0.05]	0.4 [0.04]
Avg Pulse W	29.8 [1.00]	28.0 [0.78]
Avg Pulse P	49.0 [0.51]	52.2 [0.55]
Pulse Rate	22.0 [0.20]	21.4 [0.23]
Avg Chirp L	166.6 [3.66]	166.2 [3.25]
Avg Chrip Rate	1.9 [0.07]	1.8 [0.06]
Avg RMS AMP	11526.1 [920.17]	11612.5 [867.25]
Time (min)	173.1 [19.0]	167.1 [12.49]
Mean Vol	235.3 [9.32]	253.1 [7.06]
Max Vol	458.9 [13.7]	471.6 [11.05]

Conclusions

In *Gryllus firmus*, there is a significant relationship between song components and morphology, but the specific causal interactions used in these two systems for sound production are still somewhat ambiguous due to random associations, indirect effects and correlations. In past experiments, these specific song components have been found to interact with different forms of preference. Females from previous generations of this same lineage have been shown to prefer lower pulse periods and chirp lengths (negative preference), higher pulse rates, call durations, and mean volumes (positive preference), and intermediate chirp rates (stabilizing preference) (Roff, 2016). This suggests that preference significantly affects the size of the sound-producing morphology, through both causal and correlational selection, and that this complex network of direct and indirect relationships is highly controlled. Because the wing dimorphism in this species also determines the intensity and proportion of the morphology, the indirect preference of wing morph also factors into selection.

Further research into the functions of the independent sound-producing morphological structures is required to determine the explicit causal preference of wing morphology. While this experiment, provided foundational evidence of relationships between mating call, morphology, and wing morph, the specific associations between independent characteristics is far from clear. The comprehensive system of sound-production needs to be better understood to consider the heritability of advantageous morphological structures, and maintenance of optimal sizes in the gene pool.

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