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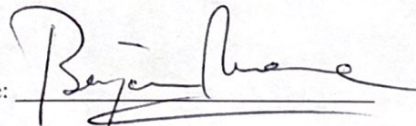
Evaluation of Phonotactic Behavior in Male-exposed Female Cricket *Acheta domesticus*

Janelle An

04/5/2021

Advisor: Dr. Benjamin Navia

Primary Advisor Signature:

A handwritten signature in black ink, appearing to read "Benjamin Navia", written over a horizontal line.

Andrews University Department of Biology

Abstract

Phonotaxis is defined as the movement of organisms in response to an auditory stimulus. It plays an important role in helping animals find a potential mate, and thus it is worthwhile to understand what factors may influence phonotaxis. Phonotactic behavior in virgin female cricket *Acheta domesticus* has been the focus of multiple studies, which have reported a degree of variability among individuals. Several factors that have been reported to influence such variability include age, environmental temperature, and the presence of certain neuromodulators. This study investigated possible changes in the behavioral responses of young females when raised with males but not allowed to mate. The results of this study showed a significant difference in the behavioral response of young male-exposed females compared to young virgin females. These results suggest that factors other than mating may alter selectivity in the females' phonotactic response and its underlying neural elements controlling behavior.

Introduction

The ability to produce sound as well as process sounds emitted from other individuals and their surroundings plays a vital role in the survival and reproduction of living organisms. For instance, bats use echolocation to locate food and avoid obstacles, dolphins generate whistles and clicks to communicate with each other, and birds produce songs to defend their territory or attract a mate. Crickets serve as a model organism in the study of acoustic communication and auditory processing, and the female's capacity to discriminate and move towards the call of conspecific males has been the focus of intense studies at both the behavioral and neuronal level (Atkins *et al.*, 2008; Stout *et al.*, 2010; Choi *et al.*, 2012; Samuel *et al.*, 2013; Atkins *et al.*, 2020). Females exhibit phonotaxis (movement in response to an auditory stimulus) to calling songs emitted by males.

Males produce sound by rubbing file-like serrations on their wings together, emitting a series of sound pulses that comprise a single chirp. The length of time between the onset of two consecutive sound pulses is known as the syllable period, and it is has been shown to be the most important temporal parameter of the chirp to which females respond phonotactically to (Stout & McGhee, 1988).

In 2010, Stout *et al.* surveyed hundreds of crickets from 4 different species – including *Acheta domesticus*, *Gryllus bimaculatus* DeGeer, *Gryllus pennsylvanicus* Burmeister and *Gryllus velveticus* Alexander – for the phonotactic behavior of females in response to model calling songs. They reported a considerably greater degree of variability in the number and range of syllable periods that females responded towards. Early studies (Thorson *et al.*, 1982) reported cricket phonotactic behavior as fixed, and Stout *et al.* (2010) attributed this understanding to be partly due to relatively small sample sizes and pre-selective screening that was conducted in such studies.

In addition, Stout *et al.* (2010) found that female phonotaxis varied based on the age of the cricket. In *A. domesticus*, Stout *et al.* (2010) classified 90% of young (5-7 day old) virgin females as

selective, defined as responding to 5 or less of the 7 model calls presented (see materials and methods). The remaining 10% of young female crickets were unselective, responding to 6 or all syllable periods presented. In contrast, old (21-31 day old) virgin female crickets were significantly less selective (53%).

Age is not the only factor that has been reported to influence syllable period-selective phonotaxis. In 2015, Navia *et al.* reported the effects of temperature on song production and its relation to song processing and selective phonotaxis. As temperature increased from 17°C to 33°C, the syllable period of the male's calling song decreased in length by 2.5 ms/°C. Females also displayed significantly greater levels of phonotaxis towards syllable periods that were most likely to be produced by males at similar temperatures. Similarly, processing in the L3 auditory neuron showed peak levels of decrement correlated with calls that females found most attractive and were shown to vary across a range of temperatures (22-33°C). The plasticity of decrement levels demonstrated that female phonotactic preferences were influenced by temperature.

Several studies have reported the influence of nanoinjection of neurochemicals into the prothoracic ganglion (PTG) and its effects on selective phonotaxis and neuronal activity. In Atkins *et al.* (2008), females (5-30 days old) injected with juvenile hormone III (JHIII) were attracted to significantly fewer syllable periods compared to their response when tested before injection. Based on its short latency of action, exerting its effects as quickly as 15 minutes after injection, JHIII was proposed to act as a direct modulator on mechanisms regulating phonotaxis (Atkins *et al.*, 2008). In both species *A. domesticus* and *G. bimaculatus*, JHIII injections 'tuned' females' phonotactic responses to center in on syllable periods found within the range of the call of the conspecific male (35-50 ms in *G. bimaculatus*, 50-70 ms in *A. domesticus*), similar to those found in young virgin females (Atkins *et al.*, 2008; Choi *et al.*, 2012). Recently, Atkins *et al.* (2020) studied the effects of five different neurotransmitters injected into the PTG of *A. domesticus* to better understand what neurotransmitters

may be involved in the signaling pathways and inhibition mechanisms regulating female phonotaxis. As a result, histamine was found to significantly narrow female phonotactic choice to the syllable periods of the conspecific male (Atkins *et al.*, 2020).

Only recently have we begun to explore how exposure to males might affect female behavior. In the past, studies involving female crickets were conducted using virgin females that had not come into contact or mated with males (Stout & McGhee, 1988; Atkins *et al.*, 2008; Stout *et al.*, 2010; Navia *et al.*, 2015; Atkins *et al.*, 2020). Because most females are likely to come in contact with males at one point during their lives, it is beneficial to study whether mating may alter the female's phonotactic behavior.

In an unpublished, preliminary study, Kent *et al.* (2018) reported that in comparison to young virgin females, young females placed in an environment where they could mate were less able to discriminate between attractive and unattractive calls. Although it appears that exposure to males modified female phonotaxis much like temperature and JHIII, due to the nature of their experimental conditions, it is not yet clear whether this effect was caused by direct mating or whether some other signal, such as a pheromones, may have been responsible for the change in behavior (Atkins *et al.*, 2008; Navia *et al.*, 2015). The methodology of Kent *et al.* (2018) allowed males and females to mate. However, because they could not verify whether the tested animal had mated, they were unable to rule out what factors affected phonotaxis.

The current study discriminates between direct (mating) and indirect male-exposure and seeks to clarify Kent's research (2018) by documenting whether mating is necessary to alter selective phonotaxis.

Materials and Methodology

Establishing non-direct exposure to males

A. domesticus nymphs were obtained from Fluker Farms, Inc. (Port Allen, LA) and kept in 100-mL containers stocked with cricket food, water and egg cartons for shelter. Crickets were kept in a growth chamber at 22-24°C in a 12:12 hour light/dark cycle (on at 06:00 hr) and adults were sorted from nymphs on a daily basis. Within 1-2 days following the final molt, adult males and females were placed in a plastic container and segregated by gender using a perforated divider. This divider prevented the crickets from mating but allowed indirect forms of interaction such as acoustic communication and pheromone signaling. After a minimum of three days of exposure, young females (4-10 days old) were tested for phonotactic behavior.

Testing of phonotaxis

Phonotaxis was tested using the procedure described in Samuel *et al.* (2013). Female crickets were fixed onto a non-compensating spherical treadmill that gave them free mobility to move and turn in all directions on the rotating ball. This was accomplished by attaching a metal pin unto the cricket's back with wax and then mounting the pin onto a metal rod, which positioned the cricket on the north pole of a styrofoam ball. The treadmill was kept in a dark, soundproof chamber at a temperature of 20-22°C. After crickets were given 5 minutes to adjust to the new environment, their movements in response to computer-generated model calls (three syllable chirps at 5kHz, 85 dB) were tracked and quantified using the computer software Optical Kugel. Seven different syllable periods ranging from 30-90 milliseconds were played in a nonsequential order (50, 90, 70, 30, 60, 80, 40). For each syllable period, phonotactic behavior was recorded for 5 minutes, with a 3-minute break in between recording sessions. Crickets were said to exhibit phonotaxis when they displayed both an average angular orientation between -60 and +60 degrees and movement towards the speaker that was twice the distance of that away from the speaker. These criteria indicated that the

cricket was moving intentionally towards the speaker and did not arrive closer to the source of sound through random motion.

Classification of behavior

After cricket phonotactic behavior was recorded in response to all 7 syllable periods, crickets that responded to 5 or less of the calls presented were classified as selective, while crickets that responded to more than 5 were classified as unselective according to Stout *et al.* (2010).

Statistical analysis

The ratio between selective and unselective females was determined for male-exposed females. This ratio was then compared to data provided by Stout *et al.* (2010), which characterized the ratio between selective and unselective non-exposed (virgin) females. A one-sample Chi-squared test was used to establish significant difference between male-exposed and non-exposed females.

Results

A total of 41 young male-exposed female crickets (4-10 days old) were tested for phonotactic behavior in this study. Of this total, 5 crickets did not respond phonotactically to any syllable periods presented. Therefore, they were not included in the results of this study. A total of 36 crickets were phonotactically active and responded to at least one syllable period presented (Fig. 1). Twenty-four crickets responded to 5 or less of the syllable periods presented and were classified as selective, a term specified by Stout *et al.* (2010). The remaining 12 crickets responded to 6 or all syllable periods presented and therefore were classified as unselective.

Considerable variation was seen in the responses of individual crickets, with some responding to a small and continuous range of syllable periods while others responded to a wide range of syllable periods in a non-continuous fashion (Fig. 1). Responses per syllable period are shown in figure 2, where a similar pattern of responses was observed in both selective and unselective crickets. Model calls with syllable periods of 50 ms and 60 ms elicited the highest

number of responses. For syllable periods outside the typical range of attractive calls (50-70 ms), females preferred longer syllable periods (80-90 ms) over shorter syllable periods (30-40 ms).

Of the 36 male-exposed females that were tested, 24 females or 66.7% were selective, while 33.3% were unselective. Data from Stout *et al.* (2010) found 90% of young (5-7 days old) virgin females to be selective, while only 10% were unselective (n=190). Figure 3 shows the frequencies of selective versus unselective females in both Stout *et al.* (2010) and this study. A one-sample Chi-square test revealed a significant difference in the proportions of selective versus unselective females between virgin and male-exposed groups ($X^2_{(1)} = 21.78, p = 3.06 \times 10^{-6}$).

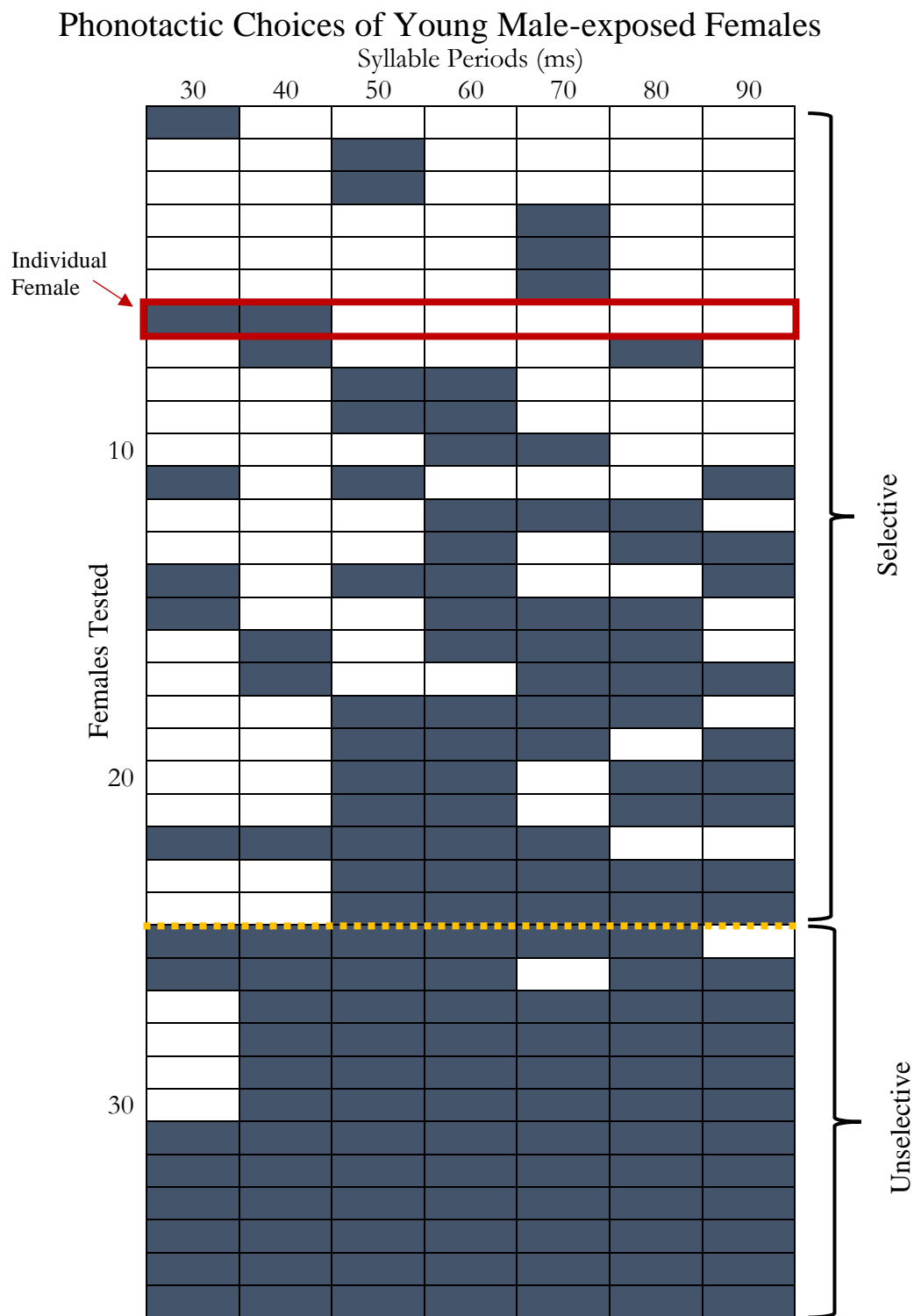


Figure 1. Phonotactic Choices of Young Male-exposed Females. Table summarizes phonotaxis for 36 females. Columns indicate a specific syllable period used. Rows represent phonotaxis of an individual cricket. Females are organized by the number of syllable periods they responded to. Colored cells indicate phonotaxis towards the model call. Rows found above the yellow dotted line indicate selective crickets, while rows below indicate unselective crickets.

Syllable Period Preference of Male-exposed Females

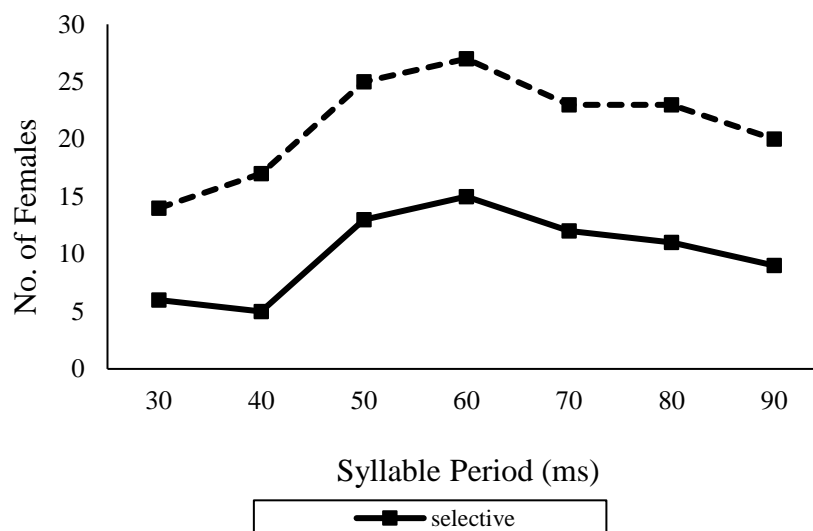


Figure 2. Syllable Period Preference of Male-exposed Females. A similar pattern of SP preference is observed when comparing selective to unselective females. Crickets responded most to model calls with 50 ms or 60 ms syllable periods, and in general preferred longer syllable periods (80-90 ms) over shorter syllable periods (30-40 ms).

Selectivity of Virgin vs. Male-exposed Females

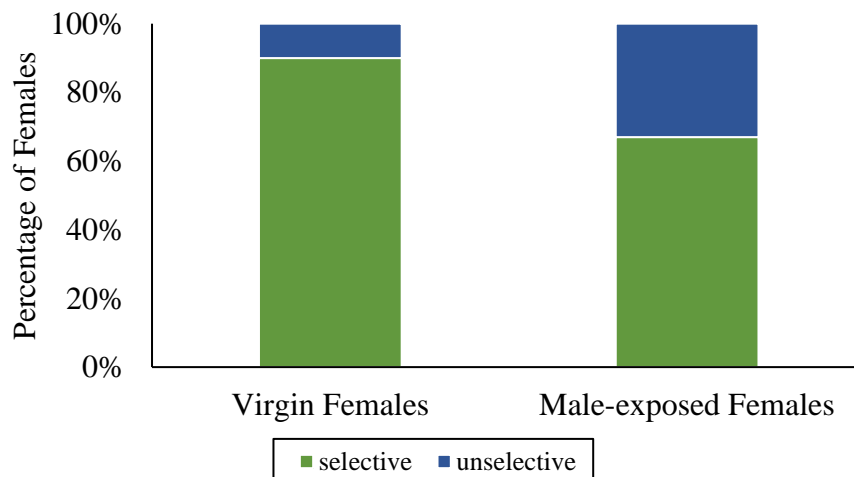


Figure 3. Selectivity of Young Male-exposed Females. Depicted in the bar on the left, data from Stout *et al.* (2010) found 90% of young virgin females are selective while only 10% are unselective ($n=190$). Depicted in the bar on the right, this study found 67% of young male-exposed females are selective while 33% are unselective ($n=36$). A one-sample Chi-square test revealed a significant difference in the proportions of selective versus unselective females between virgin and male-exposed groups ($X^2_{(1)} = 21.78$, $p = 3.06 \times 10^{-6}$).

Discussion

These results clearly indicate that even in the absence of mating, young females that are exposed to males exhibit a significantly different level of selectivity compared to that of females that have been raised in isolation to males (virgin). The current results support the notion that mating is not required for males to exert an influence on female phonotactic behavior. Similar to previous studies, male-exposure is reported to decrease selectivity in females, making them more likely to respond to a greater number of syllable periods presented (Kent *et al.*, 2018). Several potential factors related to male-exposure that are hypothesized to have elicited this change in female selectivity include chemical communication such as pheromone signaling or acoustic communication such as exposure to mating calls. Further studies are required to determine which specific factor relating to male-exposure can influence female phonotaxis.

It is not yet clear why male-exposure elicits a decrease in female selectivity. One possibility could be that interaction with males might alter neural synapses in a manner that modifies the pathways involved in auditory processing and subsequent call song recognition. The mechanism by which this would happen remains to be evaluated (Atkins *et al.*, 2008; Samuel *et al.*, 2013). One proposed hypothesis is that male-exposure prompts a decrease in the production of JHIII in young females. This would alter their behavior to become like that of older females who have a reduced production of JHIII (Atkins *et al.*, 2008). This would agree with what was reported in Choi *et al.* (2012) in *Gryllus bimaculatus*, in which old females were induced to become more phonotactically selective (behaving similarly to young females) after being injected with JHIII.

In an effort to provide greater accuracy in assessing whether virgin and male-exposed females differ significantly in their phonotactic behavior, it would be beneficial to establish a comparison of central tendencies rather than classifying crickets in a binary system as selective or unselective. This new test would compare the average number of syllable periods an individual

cricket responded to within virgin and male-exposed groups. Instead of using the Chi-squared test, which measures frequencies such as percent selectivity, a Mann-Whitney U test would be invoked to compare central tendencies such as the average number of responses per individual. To use the Mann-Whitney U test for this study, access to all responses for individual virgin females tested in Stout *et al.* (2010) would be required.

This study provides further clarity to the findings of Kent *et al.* (2018) through implying that mating is not necessary to significantly influence the selectivity of young male-exposed females. It also broadens the scope of studies on cricket auditory processing by moving beyond the well-defined behavior of virgin females into investigating the behavior of females raised in the presence of males. While this study explored the behavioral component of auditory processing and phonotaxis, it opens the door to evaluate whether such changes in behavior are also expressed at the neuronal level. Future studies could evaluate the physiological response of auditory neurons such as L3 in females that have been raised in similar conditions as those described in this study.

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