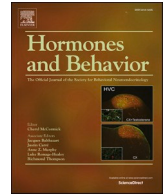




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Octopamine affects courtship call structure in male *Acheta domesticus* crickets

Fadeke Adeola^{a,*}, James Keen^b, Simon Lailvaux^a

^a Department of Biological Sciences, University of New Orleans, 2000 Lakeshore Drive, New Orleans, LA 70148, USA

^b Department of Physics, University of New Orleans, 2000 Lakeshore Drive, New Orleans, LA 70148, USA

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ABSTRACT

Secondary sexual displays vary considerably in both type and structure both within and across animal species. Although such variation is of keen interest to evolutionary biologists, the functional factors driving variation in male displays are poorly understood. In crickets, acoustic calls are produced by muscular contractions via stridulation of file and scraper wing components. We tested the effect of varying octopamine, an important biogenic amine neurohormone in invertebrates, on call production in male *Acheta domesticus* house crickets by blocking the octopamine receptors that influence skeletal muscle function with epinastine, a synthetic octopamine antagonist. We then measured male courtship calls and analyzed the call structure to quantify the differences in call structure based on the changes in carrier frequency, and whether chirps or ticks are a more prevalently expressed frequency in treated vs untreated males. Males treated with epinastine exhibited clear differences in call structure compared to untreated controls, such that epinastine-treated males were more likely to produce simpler calls and to exhibit their carrier frequencies as ticks rather than chirps. Thus, we were able to directly modify male courtship calling performance during mating interactions by altering the neuropharmacological milieu, demonstrating the potential role of biogenic amines in contributing to the diversity of call types in nature.

1. Introduction

Secondary sexual signals and displays are common throughout the animal kingdom and are key determinants of reproductive success (Andersson, 1994). For this reason, a large literature exists documenting the consequences of such displays in ecological contexts ranging from predator-prey interactions (e.g. Leal, 1999) to male combat (e.g. Brandt, 2003; Jenssen et al., 2005) and female choice (Brooks and Endler, 2001; Candolin, 2003; Hunt et al., 2004). Although researchers have long sought ultimate explanations for the evolution of male displays, the proximate mechanisms affecting the structure, variation, and degree of stereotypy exhibited by such displays, as well as the intrinsic factors influencing display production, remain poorly understood.

A theme common to most animal displays is conspicuousness, and examples exist of species enhancing the conspicuousness of their displays via each of the main sensory modalities (Searcy and Nowicki, 2005). Visual and auditory displays specifically are frequently characterized by dynamic, often spectacular movements that either constitute the display itself in the former case, or serve to produce sounds of a

particular frequency and temporal structure in the latter (Johnstone, 1998). Because such dynamic displays are often mediated by locomotor activities, potentially allowing signal receivers to assess aspects of the display for errors and irregularities in motor skill (Johnstone, 1998), recent research on the mechanical and physiological basis of signalling has focused on aspects of the musculoskeletal system that bolster signal production within vertebrates in particular. For example, Fuxjager (2013) showed that androgen receptors and their activation play an important role in sustaining abilities necessary for performing elaborate high-speed displays in the golden-collared manakin, *Manacus vitellinus*. However, despite recent interest in the mechanisms underlying motor skill competency, these studies remain largely limited to vertebrate species.

Invertebrates exhibit a variety of visual and auditory signals and displays that function within the contexts of female choice or male combat. Similar to vertebrates, these displays are often linked to dynamic movements involving rapid muscular contraction and are thus subject to similar mechanical constraints. In invertebrates specifically, there is evidence that muscle function can be regulated by various

* Corresponding author.

E-mail address: fiadeola@my.uno.edu (F. Adeola).

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intrinsic pharmacological agents (Evans and Maqueira, 2005). Octopamine (OA), a biogenic amine, functions as a neurotransmitter, neurohormone, and neuromodulator and along with tyramine, an OA precursor, regulates physiological and behavioral processes such as courtship, locomotion, cognition, and reproduction in invertebrates ranging from crustaceans to arthropods (Hana and Lange, 2017). Octopamine also has regulatory effects on energy metabolism and homeostasis (Fields and Woodring, 1991; Roeder, 2020) and aggression (Bubak et al., 2014; Hoyer et al., 2008). Consequently, behaviors altered by manipulation of OA include the modulation and initiation of locomotor patterns, and the ability of individuals to engage in complex social interactions (Rillich and Stevenson, 2015). In addition to affecting function within specific ecological and behavioral contexts, OA also affects muscle function directly; in the desert locust, *Schistocerca americana gregaria*, for example, twitch and tetanic tensions in flight muscles of adult males treated with OA yielded an increase in mechanical power output, a result which was reversible with the application of an OA antagonist (Malamud et al., 1988). Given this array of effects, OA is a likely candidate agent for exerting regulatory control over the emergent properties of muscle function, such as signal production, as well.

Crickets are a useful model system for understanding both the evolutionary and functional ecology of calling. Males of most species call in both male-male and male-female interactions, and the structure of cricket calls is subject to both sexual and natural selection (Gray and Cade, 1999a, 1999b). Male *Teleogryllus commodus* crickets, for example, experience multivariate stabilizing selection on the properties of the advertisement call, used to attract females over long distances, that is driven by female preferences for specific call components (Bentsen et al., 2006; Brooks et al., 2005). By contrast, advertisement calls in the congener *Teleogryllus oceanicus* on the island of Kauai have been strongly selected against by an acoustically orienting parasitoid fly to the point that they have been lost entirely (Zuk et al., 2006). In between these two extremes are a range of diverse call types and structures, yet the neuromuscular and neuropharmacological factors underlying variation in cricket call structure are seldom considered. Consequently, despite the attention paid to the fitness effects of cricket calls, we lack an understanding of the functional mechanisms affecting cricket call structure.

We used a synthetic OA receptor antagonist, epinastine, to dampen muscular activity in male *Acheta domesticus* house crickets. Because the stridulatory apparatus enabling sound production is driven by muscular contractions, variation in the neuromuscular activity is likely to drive variation in call structure both among and within call types. Altering muscular function should change overall calling frequency because of the effect muscular contractions have on the file and scraper mechanism involved with male sound production. House crickets exhibit three distinct calls (advertisement calls, courtship calls, and aggressive calls) which vary in frequency structure (Stevenson et al., 2005). The courtship song specifically comprises three types of sound pulses: two alternating low-frequency, low-intensity pulses of ~5 kHz (i.e. “chirps”), and occasional higher-frequency (15 to 20 kHz) “ticks”, which replace the low-frequency pulses (Nelson and Nolen, 1997). We tested the hypothesis that blocking OA receptors affects call structure by recording the calls of courting males supplemented with epinastine and comparing the resulting call structures to those of courting control males. Specifically, we predict that males treated with epinastine will exhibit simpler calls that are easier to produce with a compromised signalling apparatus. Furthermore, we also predict that the calls of epinastine-treated crickets will comprise more ticks than chirps because ticks, although higher in frequency than chirps, are produced at lower amplitudes than the more complex chirps, and consequently are likely to be energetically cheaper and thus easier to produce.

2. Methods and materials

The *A. domesticus* crickets we used for this experiment were bred in

our lab at the University of New Orleans from 1000 count cricket stocks originating from Fluker's Cricket Farm, Baton Rouge, Louisiana. We maintained our cricket supply in separate containers of similar densities of crickets with mesh nets in the lid for ventilation, similar to previous studies (e.g. Condon and Lailvaux, 2016). We provided Purina dry cat feed for food, and water via tube every three days. We isolated males at the 7th instar to ensure virginity and increase the motivation of males to court the paired female. We measured the width at the base of the pronotum under a Leica dissection scope as a proxy for body size to test whether size was a factor in call production.

2.1. Behavioral trials

We induced courtship behavior and calls in males by pairing them with females in experimental trials. Before pairing with females, males were placed for 2 h inside a plastic container with either plain pureed sweetcorn for control crickets, or sweetcorn supplemented with epinastine at a concentration of 15 mg/ml for the epinastine treated crickets (as in Bubak et al., 2013). At the end of those 2 h, we recorded male courtship calls during pairings with females to determine differences in call structure of carrier frequencies of males that were supplemented with epinastine. To allow cricket pairs to engage in mating encounters, we placed pairs comprising one male and one female in 17 × 11.5 × 6 cm containers with clear lids. We placed a damp paper towel covering the base in each container to provide favorable mating conditions (as in Hall et al., 2013). Males and females were only permitted to engage in mating attempts with their assigned partner once the trial began. Pairs were given an average of approximately 2.5 h to initiate mating.

2.2. Courtship call recording and analysis

We recorded male courtship calls during the trials with a linear microphone (TASCAM DR-40 Linear PCM Recorder) as uncompressed audio files. Our sample size was 92 male crickets. We observed 47 males in the control group and 45 males in the epinastine treatment group. Overall, although we obtained 47 recorded calls from individual crickets in the control group, we found that supplementation with epinastine tended to eliminate calling entirely in many of the treatment crickets. However, we recorded calls from 15 individual crickets in the epinastine-treated cricket group. We clipped each of our audio samples into the clearest 8-second interval and visually expressed our audio data as waveforms and power spectral density charts which we used to identify the maximum frequency density within the sound (Figs. 1, 2). Using Audacity 2.3.3 (audacityteam.org) and Raven Lite 2.0.1 (Cornell Lab of Ornithology) software we analyzed the call structures of individual male crickets in each experimental group by applying a high-pass bandpass filter of 3200 Hz to remove lower frequency instrumental noise within the laboratory setting. Frequencies higher than the 3200 Hz cut-off were processed post-filtering. Once we filtered the files, we used the seewave package in R (Sueur et al., 2018) to create a Power Spectral Density plot from which we determined the frequencies of the signals present throughout the recorded call and, ultimately, the peak frequency of dominant signals throughout each call (Fig. 1). We determined call structure by analyzing ranges of carrier frequencies crickets produced in the high range consisting of ticks (10 kHz+) and low range consisting of chirps (4 kHz–5.5 kHz) for each of the treatment types (control or epinastine). We were thus able to determine if the stridulatory courtship calls produced by males are within the detection range of the female auditory neurons after manipulating male locomotor function. To test quantitatively whether epinastine might dampen propensity to call at all, we also documented whether each of the tested males produced calls.

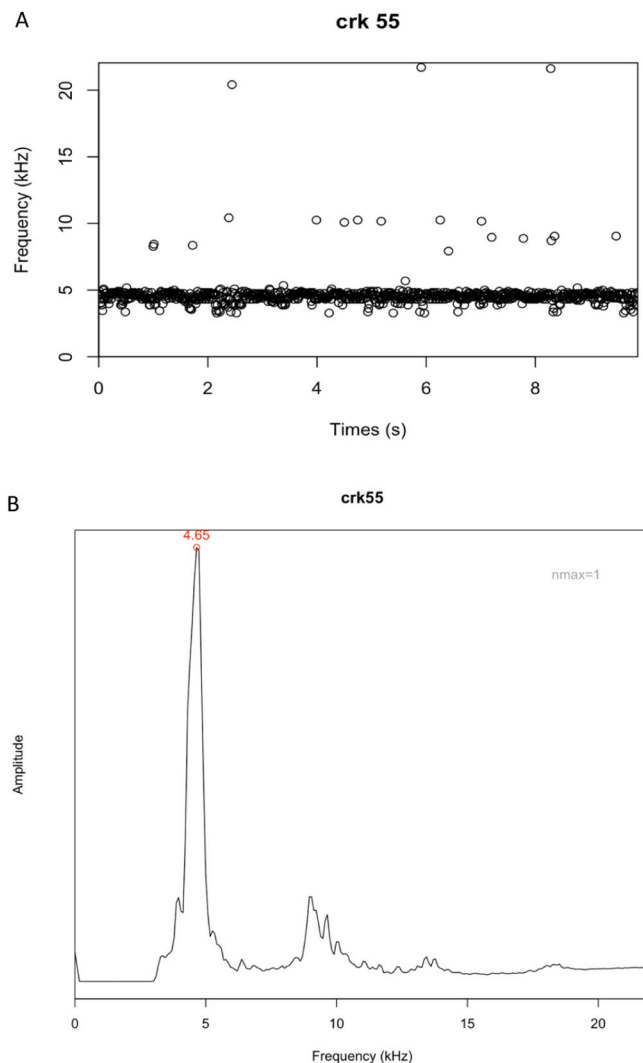


Fig. 1. Individual frequencies shown in a cricket (crk55) recorded throughout the call duration. On the left (A) each of the frequencies from the cricket's produced call are plotted on by frequency over time. On the right (B) the labelled peak is the frequency that is dominantly expressed throughout the call duration of the power spectral density chart. We use the dominant frequency for range classification where amplitude is the distance of a wave moved from an equilibrium position.

2.3. Statistical analysis

We conducted all analyses using R version 4.0.4 (R Development Core Team, <http://www.R-project.org>). To test whether epinastine treatment affected the propensity to produce a courtship call, we used a logistic regression model (i.e. a generalized linear model with binomial errors) with calling or not calling as a binary dependent variable and treatment; pronotum width; and the interaction between treatment and pronotum width as factors. Of the crickets that produced calls, we performed an ANCOVA to test whether supplementing the crickets with epinastine affected the average number of syllables the crickets produced during courtship while controlling for size by including pronotum width as a covariate. To characterize how the differences between treatment types affected the dominant carrier signal within the subset of crickets that produced courtship calls, we again fit a logistic model to the data, with carrier frequency coded as a binomial variable (i.e. dominantly in the ticks (10 kHz+) or chirps (4 kHz–5.5 kHz) range) and treatment; pronotum width; and the interaction between treatment and pronotum width as factors.

For all GLMs we used log-likelihood ratio reduction tests to determine the significance of individual terms in the model and arrive at the minimum adequate model [that is, the simplest model that explains the most amount of variation; (Crawley, 2012; Quinn and Keough, 2002)] in each case. We used the ggplot2 package (Gómez-Rubio, 2017) in R (R Core Team 2021, version 4.0.4) for data visualizations.

3. Results

We observed 47 males in the control group and 45 males in the epinastine treatment group, of which we recorded courtship calls from all 47 control crickets, but only 15 males treated with epinastine. Our minimum adequate model for propensity to call retained an effect of treatment, such that males supplemented with epinastine were significantly less likely than control males to call at all (Table 1). Of the filtered dominant carrier frequencies we measured, the minimum carrier frequency of the control group was 3.467 kHz and the maximum was 11.822 kHz, where 2 of the 47 control crickets sampled expressed their carrier frequency in the higher range. Within the epinastine treated group, the minimum carrier frequency recorded was 3.790 kHz and the maximum was 12.489 kHz, where 8 of the 15 males expressed their carrier frequencies in ranges consistent with tick production rather than chirp production. The frequency ranges of the chirps we recorded here fell within the range of previously observed chirp frequencies (Nelson and Nolen, 1997; Stout et al., 1988).

Our logistic regression results show that while size (as measured by pronotum width) is not a significant factor in changes of carrier frequency ranges of calls, treatment type does affect dominant carrier frequency expressed by males (Table 2). Specifically, crickets treated with epinastine were more likely to dominantly produce ticks at higher frequencies as carrier signals as opposed to producing chirps during their courtship song. Crickets treated with epinastine were also less likely to produce calls at all during trials (Fig. 3). Control males were more likely to produce chirps in the 4–5 kHz range frequency as opposed to producing ticks in the 10 kHz+ frequency range as their carrier signal (Fig. 4). Finally, the calls of epinastine-treated and control crickets differed significantly in complexity, with those of treatment males comprising fewer syllables on average than those of control males (Figs. 2, 5). Despite this difference in complexity, overall calling patterns did not differ, as the calls were expressed with repeated down sweep patterns, where the frequency decreases towards the end of every syllable produced, regardless of treatment, chirp period, or syllable period repetition (Fig. 6).

4. Discussion

Sexual displays may vary in conspicuousness and in component structure, yet despite considerable inter- and intraspecific variation in dynamic signalling, the proximate mechanisms contributing to that variation are poorly understood. We tested the hypothesis that the biogenic amine octopamine (OA), which acts as a neurotransmitter, neurohormone, and neuromodulator of muscular action, affects the expression of courtship calls in the house cricket *A. domesticus* crickets by altering muscle function. Specifically, we tested the predictions that blocking OA receptors via epinastine supplementation would cause male crickets to produce less complex courtship calls, and that those calls would also be higher in frequency, comprising predominantly “ticks”, compared to untreated controls.

Acheta domesticus courtship calls exhibit a general down sweep pattern (Fig. 6A) consisting of a chirp period comprising two to three syllables per period (Henley et al., 1992; Stout et al., 1988) and a tick that is produced at a variable rate throughout the duration of the courtship song (Nelson and Nolen, 1997). Our first prediction was supported, as we found that the courtship calls produced by males treated with epinastine were simpler than those of control males, independent of size effects, comprising overall fewer syllables on average

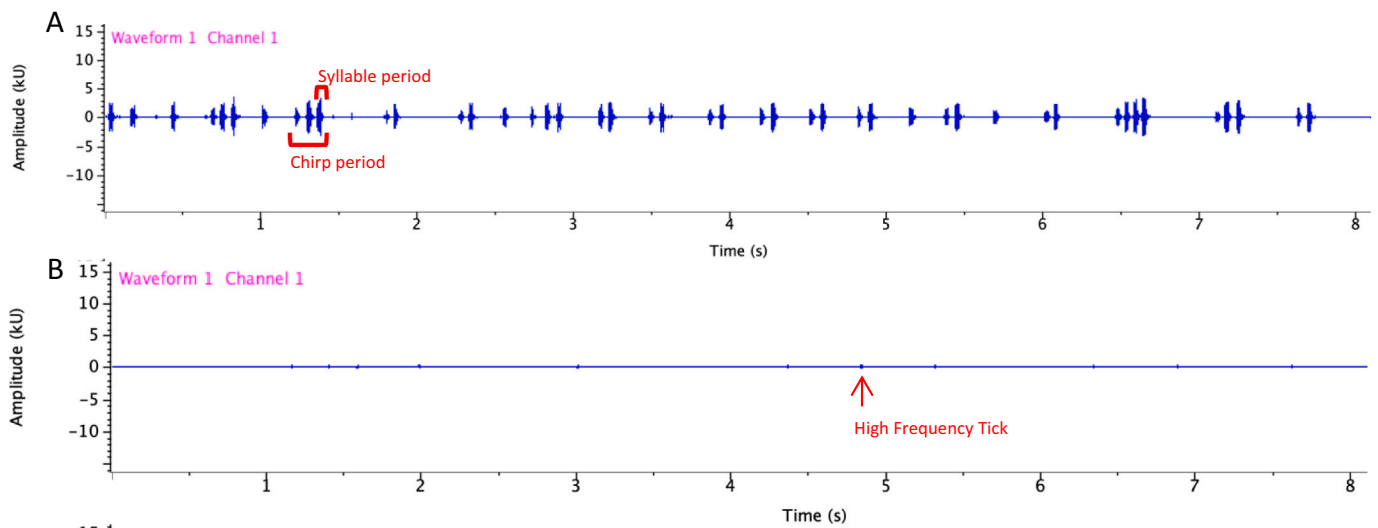


Fig. 2. Chirp period and syllable period of a cricket (crk87) within the control group (A). For each cricket we obtained the count data for number of syllables in a chirp period. Chirp period and syllable period of a cricket (crk135) within the epinastine treatment group (B).

Table 1

Term	Estimate	Std. error	Statistic	Conf. low	Conf. high
1 (Intercept)	6.83	0.437	4.40	3.13	17.9
2 Type epinastine	0.0434	0.558	-5.47	0.00146	0.133

Results of logistic regression analysis testing the propensity of epinastine-treated males to produce courtship calls compared with untreated controls. The above model is the minimum adequate model which retained only an effect of treatment, and did not retain either an effect of cricket size or an interaction between size and treatment. The baseline level for the treatment factor is “Control”.

Table 2

Effect on sound production.

Term	Estimate	Std. error	Statistic	Conf. low	Conf. high
1 (Intercept)	0.0769	0.599	-4.28	0.0186	0.212
2 Type epinastine	15.6	0.852	3.23	3.16	96.1

Results of logistic regression analysis examining the effect of treatment type on male-produced frequencies. This table represents the minimum adequate model which retained only an effect of treatment and did not retain either an effect of cricket size or an interaction between size and treatment. The baseline level for the treatment factor is “Control”.

per call period in epinastine-treated crickets compared to untreated controls (Table 3; Fig. 5). Our second prediction regarding the overall call frequency was also supported, with the calls of epinastine-treated males exhibiting more high-frequency chirps as opposed to the low-frequency ticks which dominate the calls of control males, again independent of the size of the calling cricket (Table 2). Thus, the calls of control and treated males differ markedly in key components of call structure, even while other aspects of the courtship call are clearly observable in both experimental groups.

This difference in call complexity could be attributed to at least two non-mutually exclusive effects of our pharmacological intervention that our current data do not allow us to distinguish between. In the cricket *Teleogryllus oceanicus*, increasing octopamine levels increased twitch amplitude contraction rate and relaxation rate of metathoracic longitudinal muscles (O’Gara and Drewes, 1990) which are associated with rapid wing movements during both stridulation and flight. While we did not directly measure these muscular contractions, the clear differences in average syllable number and frequency observed between treatment crickets and untreated controls indicate the strong possibility that it is

these same muscles powering stridulation that are targeted by the OA antagonist in *A. domesticus*, and that are responsible for the altered call structure. However, these likely effects of an OA antagonist on muscle function do not preclude a simultaneous effect of call motivation on call structure and expression. Specifically, although our experimental results suggest that epinastine administration altered the action of these stridulatory muscles as opposed to eliminating it entirely, there were nonetheless instances in the experiment where epinastine treated males did not produce any calls; did not move; or both remained stationary and did not call. Indeed, males treated with epinastine in our study were significantly less likely than control males to produce a courtship call at all under the same experimental conditions (Table 1; Fig. 3). Males treated with epinastine in our study also did not successfully mate with females.

Previous studies using vertebrates have implicated brain function in the propensity to express locomotor behavior (Baldo et al., 2003; Cardinal et al., 2002; Rhodes et al., 2003), and experimental studies using mice have shown that selection for voluntary wheel running results in mice who are incentivized, and thus more motivated, to exhibit wheel-running via the dopamine based reward system (Rhodes et al., 2005). The observed reluctance of epinastine treated crickets in our dataset to engage in courtship mirrors the reduced likelihood of engaging in aggression exhibited by individuals treated with OA antagonists in other insect species (Hoyer et al., 2008; Stevenson and Rillich, 2012). Furthermore, *A. domesticus* males are subject to the “loser effect” (Hack, 1997), an OA-mediated phenomenon in insects whereby males who have recently lost a fight experience a decrease in circulating OA and are more likely to lose subsequent fights compared to males who have not recently lost (Stevenson and Rillich, 2012). Condon and Lailvaux (2016) found that such “loser” *A. domesticus* males also are also less likely to exert their maximum bite forces when measured, and a recent study by Bubak et al. (2022) found that this “motivation” to bite is in fact eliminated in *A. domesticus* by dietary supplementation with epinastine, and restored by supplementation with OA. Collectively these findings both implicate OA in mediating courtship song structure and production, and suggest an important role for OA in affecting not just the absolute ability to perform integrated neuromuscular tasks, such as biting, calling, and courting, but their motivational capacity to perform such tasks to their maximum functional capacity. Although our data here do not speak to “motivation” in the incentivized reward sense in which the term is used in vertebrate species, the notion that OA is involved in such a motivation system in crickets, perhaps in conjunction with dopamine (Bromberg-Martin et al., 2010) and/or serotonin (Bubak et al., 2016), is a testable

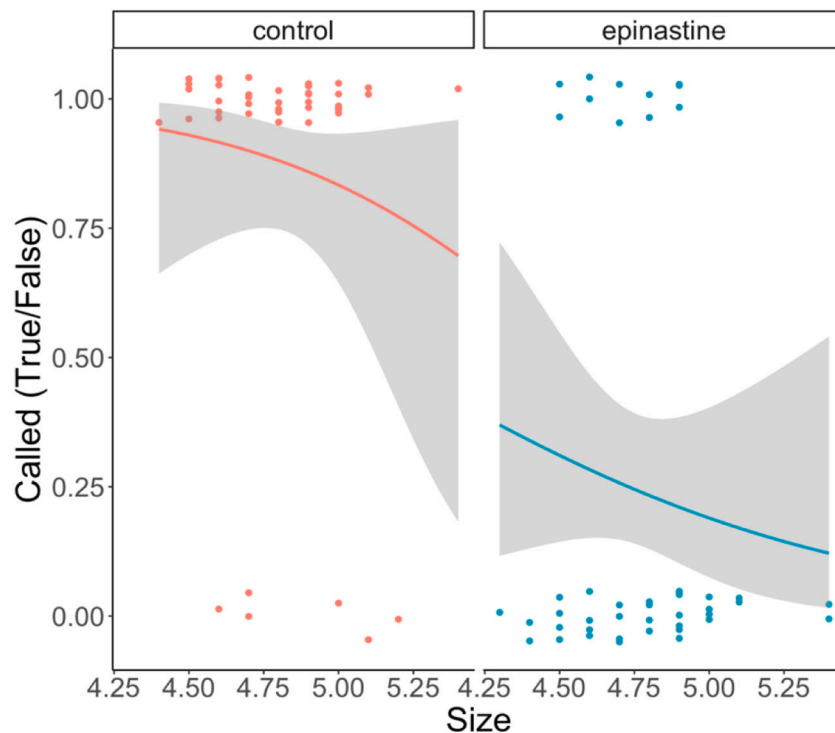


Fig. 3. Treatment type affects whether crickets of each group were able to produce any call during courtship call trials. Males treated with epinastine were less likely to produce calls at all. Size is not a significant factor in the calling model, but is shown here as the x-axis for clarity.

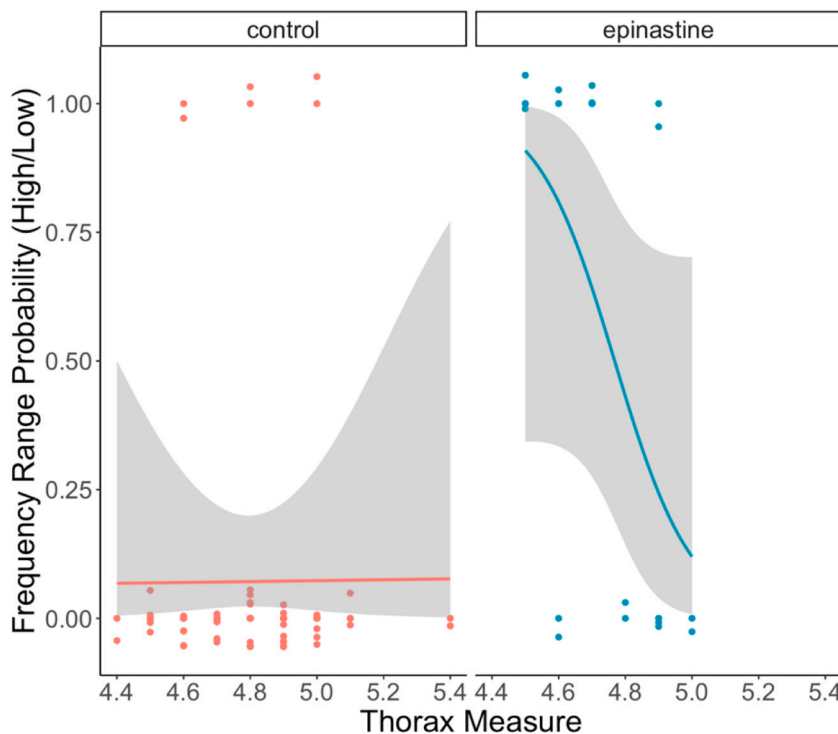


Fig. 4. Treatment type alters dominant carrier frequencies produced by male cricket. The relationship between control and epinastine treatments and the probability of dominant carrier frequencies being expressed as chirps (~4–5 kHz) at lower frequencies or ticks (10 kHz) at higher frequencies during courtship interactions. Circular symbols depict sounds in ranges expressed by individual males. Size is not a significant factor in the frequency model, but is shown here as the x-axis for clarity.

one.

Our findings here regarding the impact of epinastine treatment on the production and complexity contradict those of several previous studies. For example, Zhou et al. (2008) found that mutant fruit flies lacking OA nonetheless express normal courtship, and blocking OA receptors with epinastine did not affect courtship call production in *Gryllus*

bimaculatus crickets (Rillich et al., 2019). The reasons for these contrasting results here are unclear; it could be that our “bulk” method of epinastine supplementation, although not novel and commonly used in other invertebrates (Bubak et al., 2013) resulted in a greater OA receptor blocking efficacy than the injection approach of Rillich et al. (2019). However, Solari et al. (2018) also found that OA application modulates

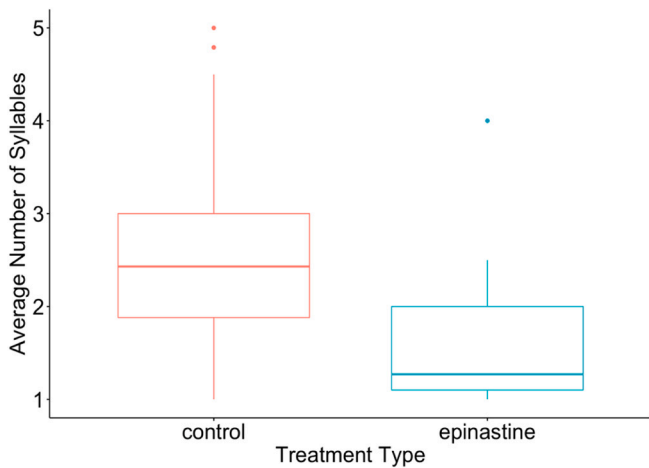


Fig. 5. Average number of syllables within each chirp period of the courtship call duration produced by crickets of each group.

the activity of motoneurons that affect calling behavior in *Lymantria dispar* gypsy moths, particularly enhancing the activity of the terminal abdominal ganglia that control calling behavior, which suggests that blocking OA activity might affect aspects of calls in this species as well. Given the complexity of courtship interactions, as well as the known stage-specific action of OA during different phases of aggressive escalation (Brown et al., 2007) it might be that certain components of courtship are affected to a greater extent than others, perhaps in a taxon-specific fashion, although our current data offer no insight into this possibility. From our results, males produced calls within female courtship call hearing sensitivity ranges though there were differences between experimental groups in their call expression at those frequency

Table 3

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Type epinastine	1	7.50	7.505	7.584	0.008 **
Size	1	2.66	2.664	2.692	0.107
Residuals	50	49.48	0.990		

Signif. codes: 0 '****' 0.001 '***' 0.01 '**' 0.05 '.' 0.1 '1'.

ANCOVA of the average number of syllables produced by each cricket throughout their produced number of calls. Size is not a significant factor.

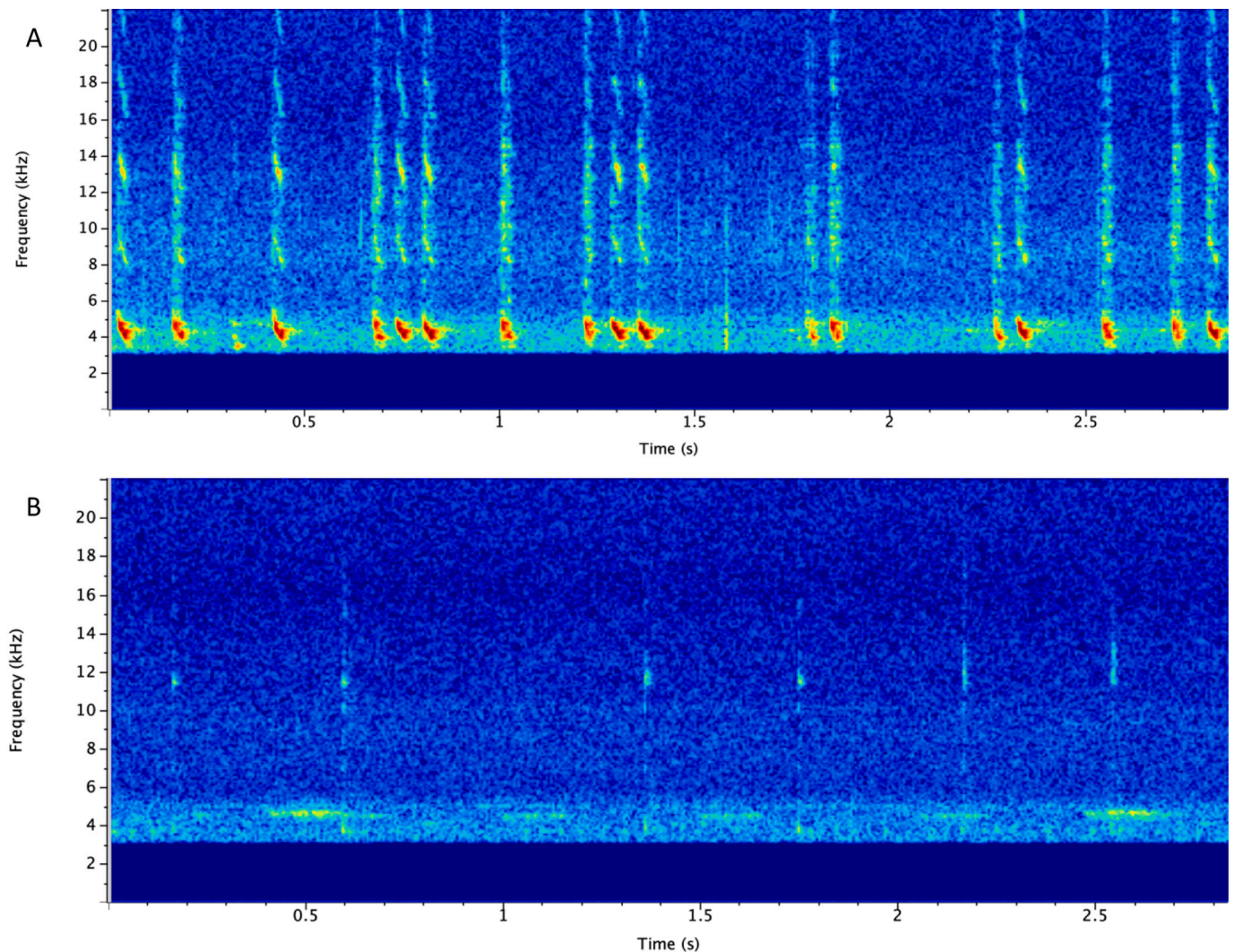


Fig. 6. Spectrogram a cricket (crk87) within the control group (A). In the first 3 s of the calling song there are repeated down sweep patterns (~4–5 kHz) at lower frequencies. (B) The below spectrogram (crk119) shows frequencies produced by crickets treated with epinastine. We use the same time interval and magnification in both images. u Frequencies below 3.2 kHz were filtered out for environmental laboratory noise.

ranges.

Octopamine is present in invertebrates in high concentrations and has significant functionality in the peripheral nervous system and central nervous system along with other tissues (Farooqui, 2007). Consequently, changes in circulating OA could, and likely do, affect multiple systems, phenotypes, and, potentially, behaviors simultaneously, yet may do so in different ways or to different extents (Husak and Lailvaux, 2022). For example, a study conducted on octopaminergic neuromodulation within *Drosophila* resulted in differential effects to both song and flight such that increased OA levels stabilized flight rather than song motor patterns, even though *Drosophila* flies use their wings both for flight and for courtship (O'Sullivan et al., 2018). Our results here constitute further evidence for the conserved nature of OA function in insects, but also point towards the complexity of that function, implicating it as a proximate mechanism affecting call production and structure in addition to the known effects on aggression and locomotion. The multivariate functional nature of OA, as well as the multiple ecological and social effectors of OA dynamics, could provide insight into the context-specific nature of courtship activity in these insects, and ultimately into the various factors affecting call expression. Indeed, given our finding that fundamental aspects of courtship calls are altered by the neuropharmacological milieu in these animals, similar experiments considering OA dynamics might provide further insight into the neuromuscular control of both other song types within *A. domesticus*, and the same song types in other cricket species.

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