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Selective Processing by the L3 Auditory Interneuron in Acheta Domesticus : Juvenile Hormone III and Protein Kinase C

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ABSTRACT

SELECTIVE PROCESSING BY THE L3 AUDITORY INTERNEURON IN *ACHETA DOMESTICUS*: JUVENILE HORMONE III AND PROTEIN KINASE C

by

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ABSTRACT OF GRADUATE STUDENT RESEARCH

Thesis

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College of Arts and Sciences
(Department of Biology)

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Juvenile Hormone III (JHIII) is a prolific and essential hormone in insects, controlling many aspects of insect physiology such as egg development, nymphal and larval maturation, diapause and metamorphosis. In female crickets, *Acheta domesticus,* JHIII increases selectivity in phonotactic behavior, narrowing phonotactic choices for Syllable Periods (SPs) of the Calling Songs (CSs) that most closely resemble the natural call of the male. JHIII has been suggested to work through a pathway that activates the protein kinase C (PKC) molecule. Chelerythrine Chloride (CC) is a potent inhibitor of PKC action. This study analyzes the responses of the L3 auditory interneuron, which has been suggested as an important neuron in the prothoracic ganglion for filtering CSs and inducing phonotaxis, in response to JHIII and CC. We recorded neuronal responses extracellularly before and after

nanoinjection with JHIII and CC in order to analyze the molecular effects of the two substances on the selective processing of the L3 neuron. JHIII increases SP-selective decrement in a subgroup of our crickets by significantly reducing decrement at the shortest SPs (30 and 40 ms) and centering the response around the SPs most similar to the natural call. It has no effect on a smaller, subgroup of crickets. CC decreases the selectiveness of decrement in L3. It is suggested that both the mechanism for sharpening the females' phonotactic behavior is expressed through molecular pathways in the L3 neuron, and that JHIII carries out at least part of its effects on the L3 neurons through a PKC mediated pathway, the effects of which can be reversed or blocked following treatment with CC.

Keywords: JHIII, Protein Kinase C, Chelerythrine Chloride, L3 Auditory Interneuron, Selective Processing

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A Thesis

Presented in Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

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LIST OF ABBREVIATIONS

CS	Calling Song
SP	Syllable Period
PTG	Prothoracic Ganglion
JHIII	Juvenile Hormone III
CC	Chelerythrine Chloride
PKC	Protein Kinase C
SD	Syllable Duration
PLC	Phospholipase C

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CHAPTER 1

INTRODUCTION

Many species of insects are capable of stridulation, a sound produced by rubbing parts of their bodies together, and several studies have shown that this ability is commonly used by males to attract females (*e.g.* Regen, 1913 - Orthoptera; Haskell, 1953 - Orthoptera; Walker, 1957 – Orthoptera; Tychsen, 1978 - Diptera; Surlykke & Gogala, 1986 - Lepidoptera). Females hear and move toward the sounds produced by conspecific males, a behavior known as phonotaxis. This process has been observed and well documented in various species of the order Orthoptera such as *Gryllus kerkennesis*, *G. campestris, G. bimaculatus, Melanogryllrus desertus, Teleogryllus oceanicus,* and *Acheta domesticus* (Popov & Shulavov 1977; Pollack & Hoy, 1979; Thorson, 1982; Stout *et al.*, 1983; Pollack, 1986; Ritchie, 1991; Hennig & Weber, 1997; Hennig, 2009;) whose calling songs (CSs) are easily recorded and modeled. Since phonotaxis was first described, several labs have used this behavioral system in an attempt to understand the neuronal networks that underlie and regulate the females' choices in response to model calls.

Initial studies were conducted on various species of crickets (Family Gryllidae) to determine which temporal and spectral parameters of the males' CS were the most important for eliciting phonotaxis (Walker, 1957; Pollack & Hoy,

1979; Stout *et al.,* 1983; Pollack, 1986). In *A. domesticus,* the most important parameter was determined to be the syllable period (SP), the time between the onset of successive syllables in the chirp (Stout & McGhee, 1983). The lengths of model SPs were altered, to present a range from 30 to 90 ms in experimental studies. Those SPs that most closely matched the SPs of the natural male's call most often elicited positive phonotaxis (50 – 70 ms for *A. domesticus,* Stout *et al.,* 1983), and were defined as the most attractive SP range.

Two categories of behavior were recognized for female *A. domesticus*. Selective females were defined as those that responded to 5 or fewer SPs of the 7 presented, and unselective females responded to 6 or more (Walikonis *et al.,* 1991; Stout *et al.,* 1992; Stout *et al.,* 2010). Further studies showed considerable plasticity within these categories, which was not initially expected. A study of 4 species from two genera of field crickets (Family Gryllidae) indicated that selective females can respond to SPs that are not commonly found in the natural conspecific male's calling song (Stout *et al.*, 2010). Additionally males show considerable plasticity in their calling rate dependent upon certain environmental factors (i.e. temperature) of their microenvironment. This plasticity is paralleled by changes in female preference for SPs (Navia *et al.*, 2015).

Interest in the neuronal basis for this behavior and its plasticity has been centered on networks of auditory neurons in the prothoracic ganglion (PTG), where auditory afferents terminate and connect with first order auditory interneurons (Esch *et al.*, 1980; Wohlers & Huber 1985) and on the brain where several of these first order auditory neurons terminate and synapse with higher order auditory

neurons (Schildberger, 1984; Kostarakos & Hedwig, 2012; Schoneich *et al.,* 2015). Various interneurons were identified and described such as the AN1 and AN2 interneurons of *G. bimaculatus* (Wohlers & Huber, 1985)**,** the omega neurons (ON1 and ON2) of *G. bimaculatus* and *A. domesticus* (Atkins *et al*., 1984; Schildberger & Horner, 1988) and the "L-shaped neurons" L1 and L3 of *A. domesticus* (Stout *et al*., 1985; Atkins, *et al*, 1992).

Atkins *et al.* (1984, 1992) found that unilaterally killing one of the pair of L-shaped (L1 and L3) auditory interneurons in the PTG of *A. domesticus* resulted in significant errors in phonotaxis at all SPs. Further work centered on the L3 interneuron which unlike L1 responds in a selective manner to SPs that are most attractive phonotactically with a reduction in the number of action potentials to the 2nd and 3rd syllables, termed "decrement" (Atkins *et al.,* 1989). Furthermore, L3 has exhibited significant plasticity in its SP-selective responses that closely matches the plasticity described phonotactically (Navia, 2005; Stout *et al.,* 2010). Each of these studies supported the proposal that L3 played a significant role in phonotactic choices made by females.

Additionally, the AN2 auditory interneuron (homolog of L3) in female *G. bimaculatus* also responds selectively to the SPs of models of the males' CS that match those found in the natural male's call (Stout *et al*., 2011) in ways that are similar to the L3 of female *A. domesticus* described by Navia (2005). When measurement of phonotaxis by individual female *G. bimaculatus* was immediately followed by recording the SP-selective responses of the AN2 neurons, there was a greater than 90% concordance between SPs that resulted in high AN2 decrement

and the SPs chosen for positive phonotactic response (Samuel *et al.,* 2013). That study linked for the first time, selective processing of the AN2 (L3) neuron and phonotactic behavior in the same animal.

Juvenile Hormone plays an essential role in regulating many aspects of insect physiology such as egg development, nymphal and larval maturation, diapause and metamorphosis (Wigglesworth, 1963; Koch & Hoffmann, 1985; Riddiford, 1994; Wyatt & Davey, 1996; Riddiford, 2007). Work done in crickets showed that Juvenile Hormone III (JHIII) peaks in females on the fourth day following the imaginal molt (Renucci & Strambi, 1983; Koch & Hoffman, 1985; Walikonis *et al.,* 1991) which coincides with the day on which phonotaxis has been reported to start (Stout *et al.,* 1992). JHIII can induce phonotaxis in female *A. domesticus* when topically applied as young as 1 day old (Stout *et al.* 1992). Topical application (Henley *et al.*, 1992) as well as nanoinjection into the PTG (Atkins *et al.,* 2008) initiate or restore behavioral selectivity in crickets that were previously unselective phonotactically. It has been proposed that JHIII may have some of its effects through a membrane bound receptor and PKC mediated pathway (Wheeler & Nihjout, 2003; Liu *et al.,* 2015).

In a preliminary study Chelerythrine Chloride (CC), a potent protein kinase C (PKC) blocker (Chao *et al.,* 1998) has been suggested to block the effects of JHIII behaviorally (Byssainthe, 2008). No work has been done yet in *A. domesticus* on the modulatory effects of JHIII or CC in the neuronal networks of the PTG.

In order to evaluate whether JHIII influences selective processing by the L3 neuron in a manner that is similar to its effects on SP-selective phonotaxis by female *A. domesticus*: 1.) JHIII is nanoinjected into the PTG and its influences on selective

processing by the L3 neuron are evaluated. 2.) JHIII injection will be followed by injection of the PKC blocker CC, and subsequent changes in L3's selective processing will be evaluated in some females, as a check on the possible involvement of PKC in mediating JHIII's effects.

CHAPTER 2

METHODS AND MATERIALS

Animal Care

Four-week-old *A. domesticus* nymphs were obtained from Fluker Farms, Inc. New colonies were housed in 100-L plastic containers and given water and Cricket Chow (Fluker's Cricket Farm) *ad libitum*. Egg cartons were provided for shelter. Colonies were maintained in climate-controlled incubators at 22 - 24° C, with a 12 hour light dark cycle (on at 06:00). Females completing their imaginal molt were removed, isolated, and kept under identical conditions in 16-L containers. Adult males were also removed and discarded daily.

Specimen Preparation

Females between 5 and 28 days old were selected from the colonies for use. The tegmina and inner wings as well as the mesothoracic and metathoracic legs were removed. Females were secured to a wax block using a 3:1 beeswax to resin mixture warmed by a Digital Wax Carving Pencil (Whip Mix). They were secured ventral side up with wax dots to the pronotum, dorsal thorax, and abdomen. Each cricket's head was also secured to the wax block to prevent movement. The prothoracic legs were positioned so that the posterior tibial tympani were facing laterally toward each speaker and secured with pieces of molding clay. Care was

taken to not cover the thoracic spiracles or prothoracic leg joints. Small microscissor incisions were made through the exoskeleton on the prothoracic area just ventral to the prothoracic ganglion and neck connectives. The exoskeleton and the ventral trachea were removed to expose the PTG. Auditory trachea (important for sound localization, Schmidt & Romer, 2013) on either side of the PTG were left intact. Fielden (1960) saline was immediately placed over the exposed tissue to keep it moist. Temperatures during each experiment ranged between 22 - 24° C

Sound Stimulation

Computer generated model calling songs were played using Sound Studio (version 3.5.7) through laterally located speakers, (82 cm apart, Optimus 2x6" Horn Tweeter) attached to the outside of the Faraday cage (0.6 x 0.7 x 0.8 m) with sound insulated walls. The sound emitting from each speaker was calibrated at a location midway between the cricket's ears (at the middle of the cage, approximately 41 cm from either speaker) to 85 +/- 2 DB using a Radio Shack ® Sound Level Meter (model no 33-256).

Calling songs were produced with three syllables (20 ms syllable duration (SD), 6 ms rise and fall times) and a chirp period of 666 ms at 5 KHz (within the range of typical carrier frequencies for *A. domesticus,* (Stout *et al.* 1983). Pre- and postinjection calling song SPs were presented in one-minute durations in a standard non-sequential order (50ms, 90ms, 70ms, 30ms, 60ms, 80ms, and 40ms). One minute of rest was given between each CS. Sound was directed through either the left or right speaker. The last 30 chirps of each recording were used for analytic purposes.

Extracellular Recordings

Prepared specimens were placed at the center of a Faraday cage. Suction electrodes were custom made from plastic polyvinyl chloride tubing, heated with a mounted Weller Soldering Iron, and pulled to an approximate inner and outer diameter of 0.40 and 1.30 mm respectively. The suction electrodes were filled with Fielden (1960) saline and used to obtain electrophysiological recordings from inside the split neck connectives (split using the broken tip of a glass microelectrode; Jeffery, 2003). A silver wire was inserted into the saline as a ground electrode.

Criteria previously established were used to locate and isolate the L3 neurons (Navia, 2005). L3s were identified using their unique sensitivity to 5 KHz (65 – 75 db) and 16 KHz (55 – 65 db, Stout *et al.*, 1985) as well as by the existence of a reduction in spiking (decrement) and the presence of a prolonged response (Navia, 2003).

The response of the L3 neurons to model CSs were recorded and digitized using a P55 A.C. Pre-Amplifier(Grass Products, Warwick RI), ADInstruments Powerlab 2/20, and Chart 5 V.5.54 software.

Following the pretests, various solutions (50 pg of JHIII in 9.2 nL of acetone, 9.2 nL of acetone as a control, 9.2 nL of 10^{-5} M CC (Sigma) in saline, and 9.2 nL of saline as a control) were injected into the PTG using a nanoinjector (Drummond Nanoject II; Drummond Scientific Co, Broomal, PA). Post-test experiments were conducted following injections of JHIII and again following injection of CC as well as following injections of CC only. Model calling songs were presented after each

injection in the manner described and used for the pretests, and responses of the L3 were digitized using identical methods. To control for repeated exposure to auditory stimulus, CSs were also played through twice without injecting the animal.

Analysis And Decrement Determination

PostStimulus Time Histograms (PSTs) with 1 ms bins were generated from the last 30 chirps of each digitized response of recordings that were clear and distinct enough to analyze. Recordings and parts of recordings that were unable to be resolved were discarded or excluded. Spike numbers in response to each syllable were determined by counting the number of spikes in a number of bins of the PST histograms that matched the time frame of the SP being studied. The count began with the distinct onset of spikes usually after a latency of 10 – 20 ms (Fig. 1). Procedures used match those described by Samuel *et al.* (2013). Percent decrement for each SP was calculated using the equation (1-(#of spikes to syllable 3/# of spikes to syllable 1))*100. Decrements were calculated and analyzed for each SP in each pretest, test or control test. Additionally, the total number of spikes from the 30 chirps of each SP was counted and the average found per 30 chirps.

Statistical Analysis

Error calculation and averages were done in Excel 2011 for Mac version 14.0.0. Analysis of variance – repeated measures was computed on decrement values within the pretests and treatment groups as well as between groups using SPSS version 12. Significant differences between means are all based on p values

equal to or less than 0.05. Independent t-tests were computed on the average of spike numbers for each SP between treatment groups and controls using Excel.

CHAPTER 3

RESULTS

Order Of Experiment And Effect On L3 Response

Given that the experimental design used in this study involved a pretest followed by nanoinjection of a neurohormone or neurochemical (JHIII, acetone, CC, or saline), a control for the sequence of experiments was performed in which model calling songs (CSs) were played through twice with no injection into the prothoracic ganglion (PTG). The second presentation of the CSs yielded similar results (no significant differences), indicating that repeated exposure of virginal females to auditory stimulus did not independently affect the outcome (n=10, Fig. 2).

JHIII Effect On L3's Decrementing Response To Model CSs

L3 interneuron's response to the SPs of model CSs was evaluated in 16 females. Two types of responses from the L3 interneuron were described based on their characteristics. In one group of crickets, the L3s responded with significantly higher decrements to 30 ms than to longer SPs and displayed low spiking rates (hereafter referred to as Group 1, n = 5 Fig. 3A, B). The second group of crickets (hereafter referred to as Group 2, n=11, Fig. 3A, B) contained L3s whose decrements to 30 or 40 ms were the same as or lower than to subsequent SPs (see

also Fig. 5D) and had significantly higher spiking rates than Group 1(t test, $p<0.0005$).

The Group 1 neurons displayed SP-selective decrement that was not noticeably influenced by JHIII (Fig. 4). This is illustrated by recordings from an individual L3 in a Group 1 female in which JHIII had no effect on the decrementing response to SPs of 40 (Fig. 4A) or over all to the 7 SPs presented (Fig. 4B). There was no significant difference following JHIII injection in the averaged response (ANOVA, $p>0.05$ Fig. 4C)

L3s in Group 2 responded with higher spiking rates (see Fig. 3B) and decrements that were responsive to JHIII (Fig. 5A-E). The decrement pattern from an individual in Group 2 was sharpened (Fig. 5C) by JHIII, with the largest decrements following injection given in response to SPs of 50 to 70 ms. Before JHIII injection, decrements in response to SPs of 80 and 90 ms were significantly lower than to 70 ms (ANOVA $p<0.05$), but the response to shorter SPs were not significantly different (ANOVA, $p>0.05$, Fig. 5D). On average, JHIII reduced the decrements to SPs of 30 and 40 ms resulting in significantly smaller decrements at these SPs (t test, 30 ms $p=.076$ and 40 ms $p=.052$) than the decrement to 70 ms SPs (Fig. 5D, E). The overall effect of JHIII was that decrements of Group 2 L3s in 11 females were significantly tuned to the SPs contained in the males' CSs (Navia *et al.* 2015, Fig. 5E). Results following injection of acetone (the solvent and control for JHIII) showed no significant change in L3's decrement response before and after treatment (ANOVA, $p>0.05$, $n=11$, Fig. 5F).

CC's Effect On L3's Decrementing Response To Model CSs

For a female in Group 2, treatment with CC after treatment with JHIII resulted in a clear increase of decrement (Fig. 6A, B, 40 ms shown) at all SPs presented. L3s on average significantly increased their decrement (19% vs 28%, t test, $p<0.004$, Fig. 6C) and tended to decrement more uniformly following treatment with CC (the greatest difference in decrements before CC treatment was 17%, following CC treatment the greatest difference was less than 10%).

Seven additional crickets were chosen and the SP- selectiveness of their L3 interneurons was analyzed before and after CC nanoinjection. For an individual in this group, the L3's decrementing response following treatment became more uniform and consistent to all SPs (Fig. 7A, B). The high selective decrement for 70 ms (45%) was significantly decreased (to 30%, ANOVA, $p<0.05$, Fig. 7A, B). The average decrements of this group (Fig. 7C) show a similar pattern. After treatment with CC the decrementing response is adjusted higher at some SPs, lower at others (significantly higher at 40 ms and significantly lower at 70 ms, ANOVA, $p<0.05$) such that the L3 does not decrement particularly highly to any one SP or SP range, but on average decrement reduces for SPs longer than 50 ms. Though the averaged decrements were not significantly different following CC injection (t test, $p = 0.627$ Fig. 7C), the CC response is more uniform. The decrementing response following CC injection at most differed by less than 14%, while before injection the greatest difference in decrement was greater than 20%. Nanoinjection of Fielden Saline (1960), the control for CC, did not significantly change the decrements at any SP for the six individuals tested (ANOVA, $p>0.05$, Fig. 7D).

There are very notable differences between the average L3 decrement following CC injection that was preceded by JHIII injection (32%) and that following only CC injection (22%) (Fig. 6C and 7C). The average difference in decrement between CC only injection and JHIII - CC injection is significant (t test, $p < 0.004$).

Effects Of JHIII And CC On L3's Excitation

For each group analyzed (Group 1, Group2, and CC only), evaluation of the average number of spikes per 30 repetitions of the model CS for each SP demonstrated clear trends during the pretest as well as following treatment with JHIII and CC. First, during the pretest the average number of spikes at each SP are significantly lower in Group 1 than in Group 2 (t-test, $p<0.0005$, Fig. 3B). Second, treatment with JHIII does little to change the average number of spikes/30 chirps at each SP in either Group 1 or Group 2 (t-test, $p>0.05$, Fig 8A, B,). Finally, treatment with CC significantly increases the total number of spikes for both groups treated with CC (Fig. 8B, C, Group 2 $p< 0.05$; Just CC $p<0.005$). Variability (as measured by the standard error of the means) is minimal in all groups. The location (timing) of the CC effect was evaluated by determining the amount of increase at each syllable. CC caused the greatest increase in spike numbers to the first syllable of each chirp. The increase is less to the second syllable, and for most SPs, least to the third syllable (Fig. 8D). No significant change was seen in the average number of spikes when CSs were played through twice, or with injection of either control substance (t test, $p>0.05$, Fig. 8E).

Figure 1 Sample PST histogram demonstrating the method used to count and allocate spikes in response to different SPs of the model CS. The example shown is for 30 chirps of L3's response to a 50 ms SP CS. Spikes are counted with the first distinct onset of spiking, usually after some latency, and the number of spikes from 50, 1 ms bins are assigned as the response to each syllable of the chirp. The timing for the onset of the response to the third syllable is calculated as the average latency of the response to the first syllable plus two times the SP (in this case 50 ms = 114 ms).

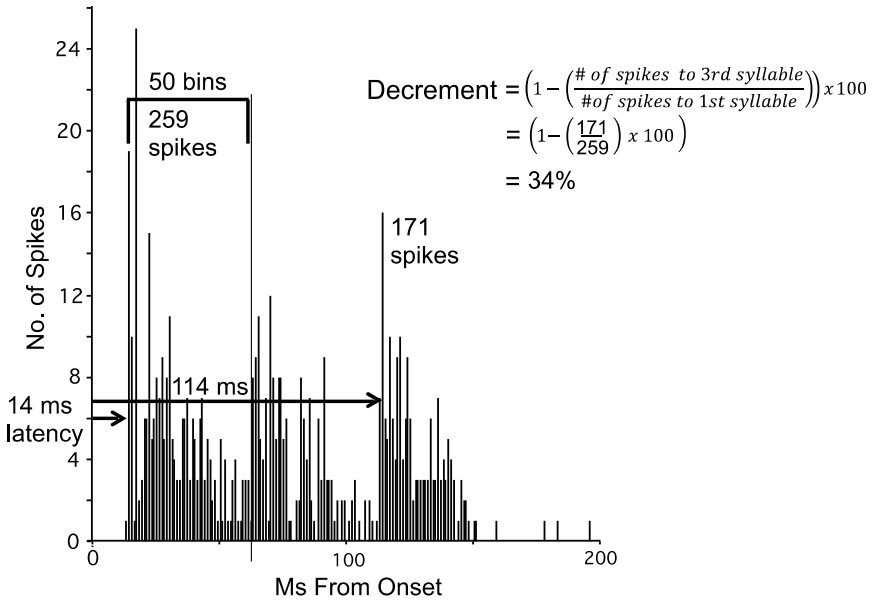

50 bins
259
spikes
171
spikes
114 ms
14 ms
latency
No. of Spikes
Ms From Onset
0
4
8
12
16
20
24
0
100
200
Decrement = $\left(1-\left(\frac{\#\ of\ spikes\ \ to\ 3rd\ syllable}{\#of\ spikes\ to\ 1st\ syllable}\right)\right)x100$
= $\left(1-\left(\frac{171}{259}\right)x\ 100\right)$
= 34%

Figure 2 SP-selective decrement showing response of L3s (n=10) in female *A. domesticus* after model CSs were played through twice. The similar responses show that the order of experiments and repeated exposure to auditory stimulus has no effect on the L3's decrementing response.

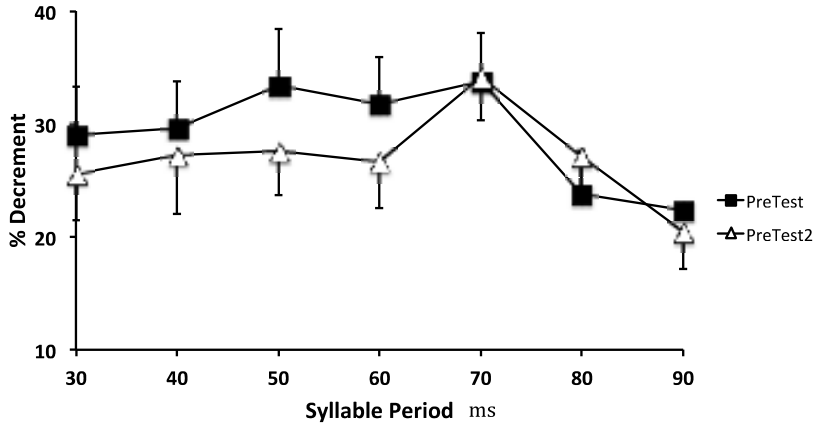
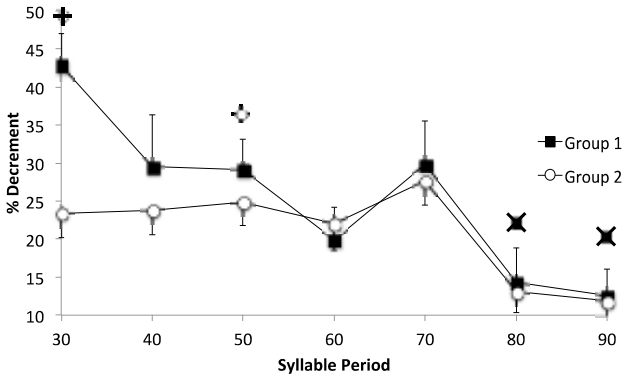

40
30
20
10
% Decrement
30
40
50
60
70
80
90
Syllable Period ms
PreTest
PreTest2

Figure 3 Two types of L3 responses based on percent decrement values and average spike numbers per SP. **A** Group 1 (n =5) percent decrement values were higher to 30 or 40 ms than to subsequent syllable periods while Group 2 (n = 11) had decrement values that were equal to or lower than to subsequent SPs. Unique symbols(ie ◇ and ✚) indicate significant differences between SP means in Group 1. **B** Average spike numbers per SP were lower for Group 1 neurons when compared to Group 2 neurons.

A


50
45
40
35
30
25
20
15
10
% Decrement
30
40
50
60
70
80
90
Syllable Period
Group 1
Group 2

B

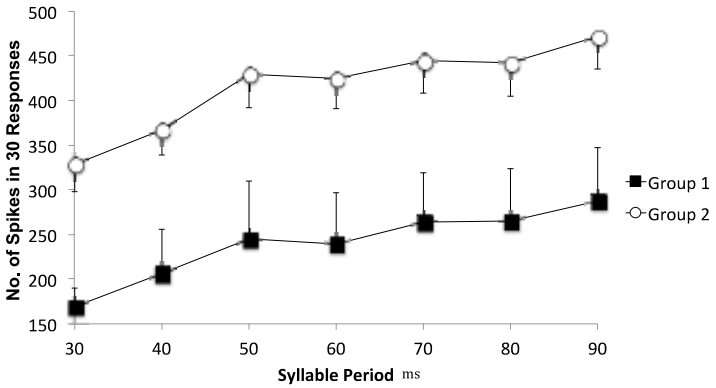
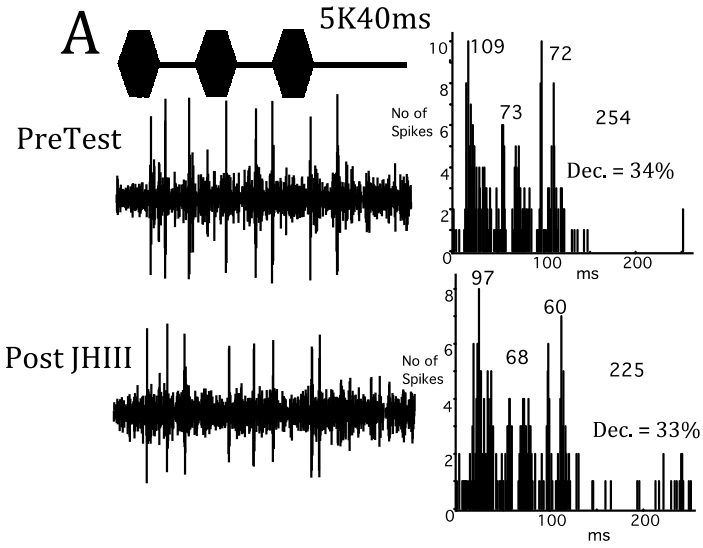
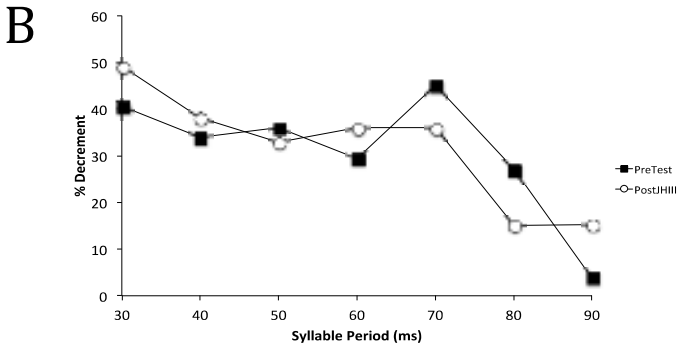

500
450
400
350
300
250
200
150
No. of Spikes in 30 Responses
30
40
50
60
70
80
90
Syllable Period ms
Group 1
Group 2

Figure 4 Decrement graphs, traces, and PST histograms showing SP - selective decrement of L3s before and after treatment with JHIII on Group 1 neurons. **A** Recordings and PST histograms from an individual in Group 1 show no difference following treatment with JHIII at 40 or **B** to any of the 7 SPs presented. **C** No effect was observed following JHIII injection in the group of L3s with initially higher decrements to the shortest SPs (Group 1, n=5).


A
5K40ms
PreTest
No of Spikes
109
72
73
254
Dec. = 34%
ms
Post JHIII
97
60
68
225
Dec. = 33%
ms


B
% Decrement
Syllable Period (ms)
PreTest
PostJHIII

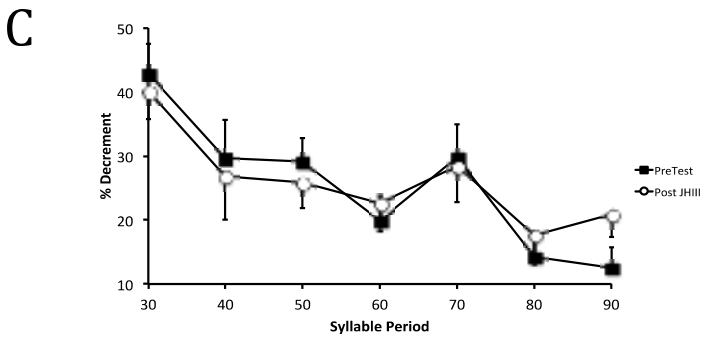

C
% Decrement
Syllable Period
PreTest
Post JHIII

Figure 5 Decrement graphs, traces, and PST histograms showing SP - selective decrement of Group 2 L3s before and after treatment with JHIII. **A** Recordings and PST histograms from an individual in this group show the decreased decrement at 40 ms, and **B** the unchanged decrement at 50 ms. **C** The decrement graph of all SPs presented to the same individual shows the sharpening effect of JHIII with largest decrements for 50 - 70 ms following injection. **D,E** The SP - selective response of all Group 2 L3s (n=11) was sharpened following treatment with JHIII, with smaller decrements for 30 and 40 ms after injection. Unique symbols (ie and) indicate significant difference between Group 2 SP means. **F** No change was seen following acetone injection into the PTG.

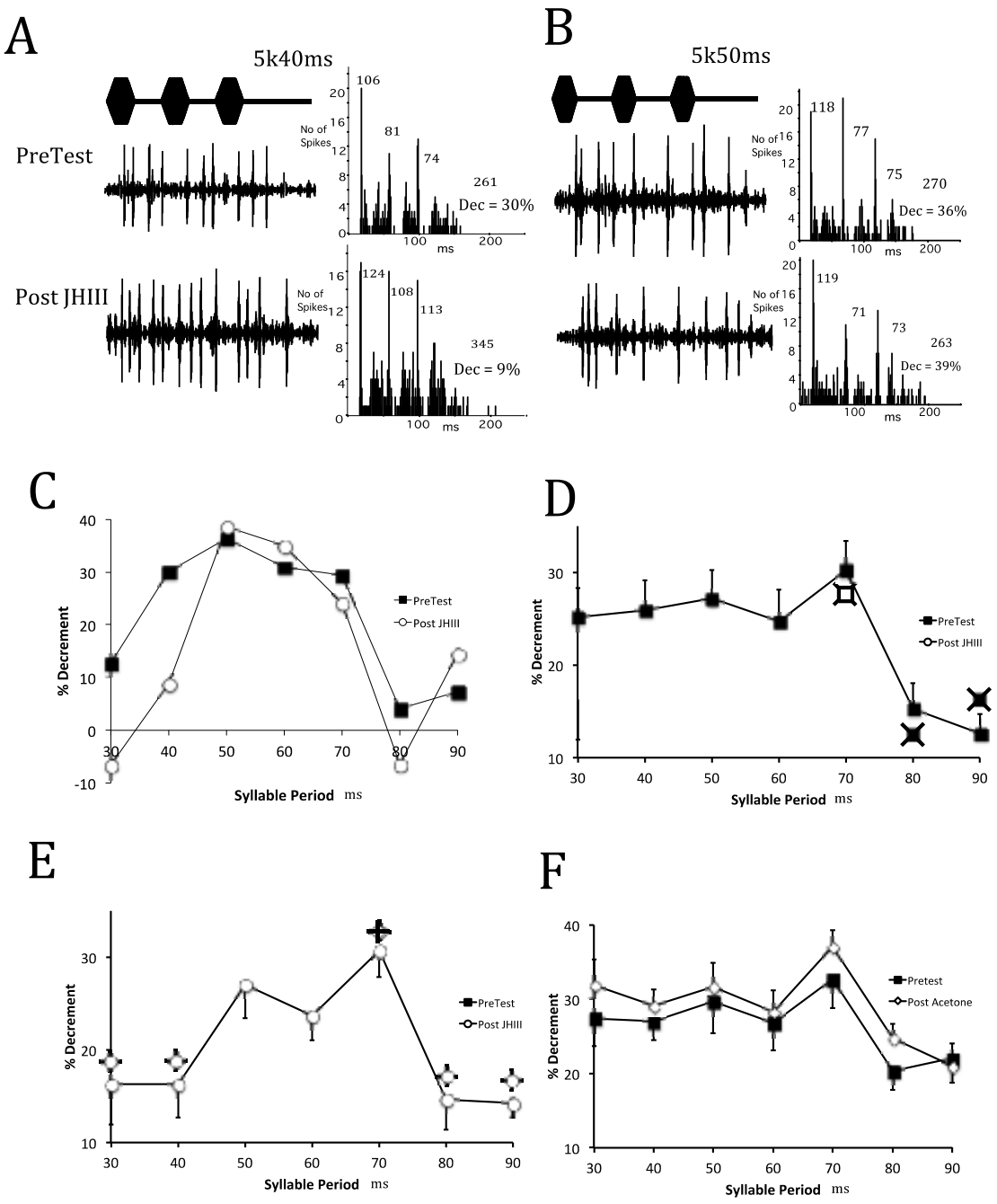
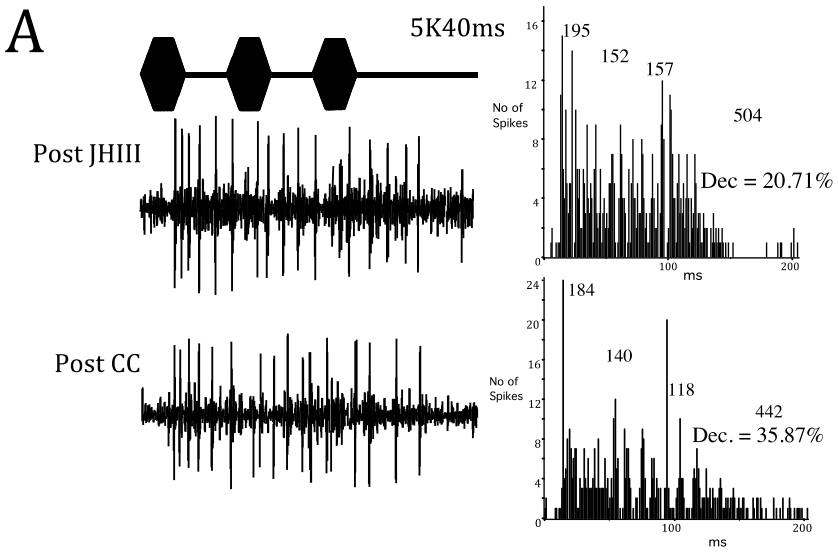
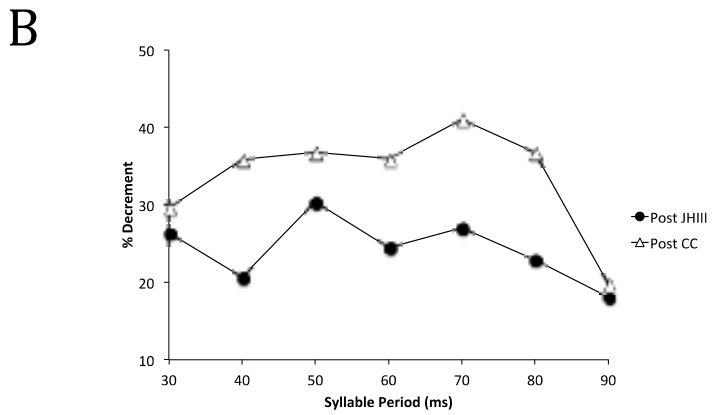

A
5k40ms
PreTest
Post JHIII
No of Spikes
106
81
74
261
Dec = 30%
124
108
113
345
Dec = 9%
ms
B
5k50ms
118
77
75
270
Dec = 36%
119
71
73
263
Dec = 39%
C
% Decrement
Syllable Period ms
PreTest
Post JHIII
D
% Decrement
Syllable Period ms
PreTest
Post JHIII
E
% Decrement
Syllable Period ms
PreTest
Post JHIII
F
% Decrement
Syllable Period ms
Pretest
Post Acetone

Figure 6 SP - selective decrement graphs illustrating the change in decrement following JHIII and CC injection into the PTG for Group 2 females (n = 8). **A** A sample recording from an individual in Group 2 shows an increase in decrement at 40 ms following CC injection and **B** across all SPs presented, with a clear loss of SP-selective decrements in the unit. **C** On average, CC removes the SP-selective decrement induced by JHIII.


A
5K40ms
Post JHIII
Post CC
No of Spikes
195
152
157
504
Dec = 20.71%
ms
184
140
118
442
Dec. = 35.87%
ms


B
% Decrement
Syllable Period (ms)
Post JHIII
Post CC

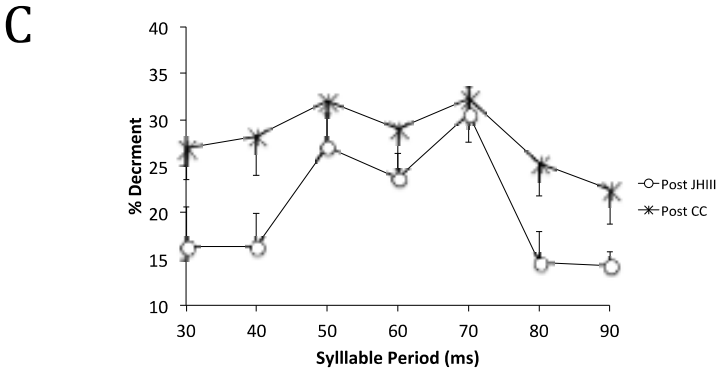
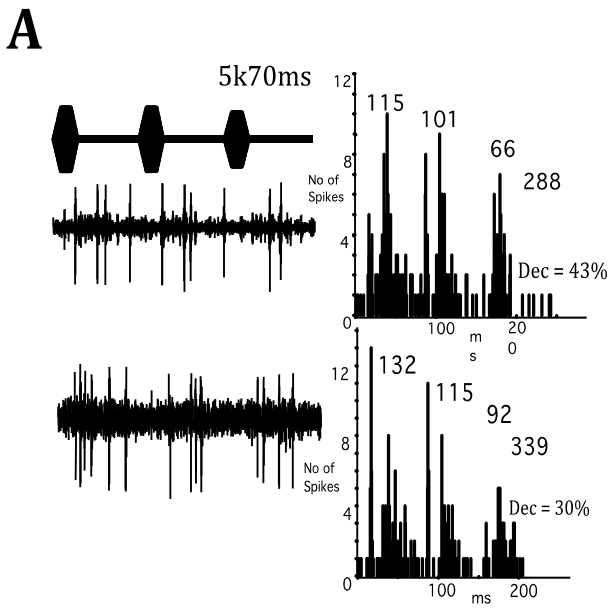
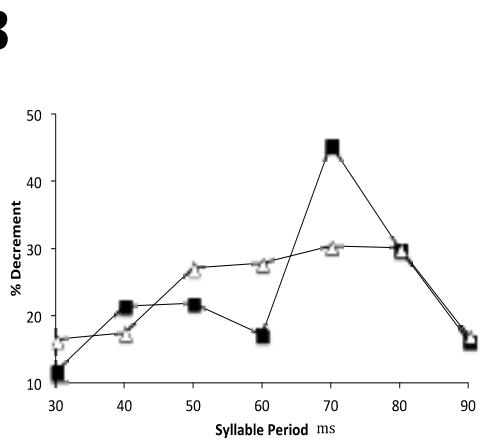
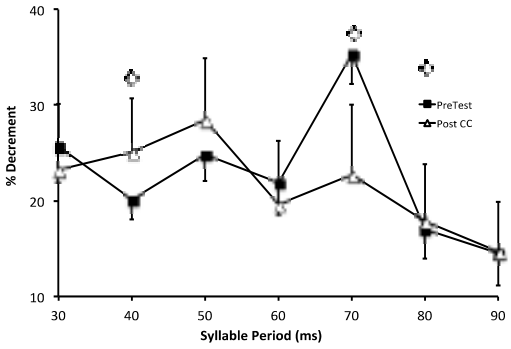

C
% Decrment
Syllable Period (ms)
Post JHIII
Post CC

Figure 7 Decrement graphs, traces and PST histograms showing SP-selective decrement response before and after only CC injection. **A** An individual in this group shows a decrease in decrement at 70ms, and **B** a more uniform response across all SPs. **C** The average of this group (n=7) shows a change in decrement (significant at 40 and 70 ms) and a more uniform, consistent response following CC treatment. **D** Injection of saline did not alter the decrementing response of L3s (n=6).


A
5k70ms
No of Spikes
115
101
66
288
Dec = 43%
100 ms 200
132
115
92
339
Dec = 30%
100 ms 200


B
% Decrement
Syllable Period ms


C
% Decrement
PreTest
Post CC
Syllable Period (ms)

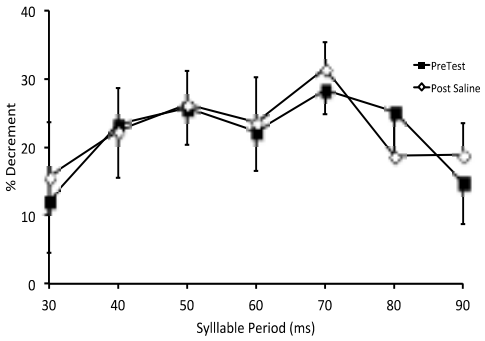
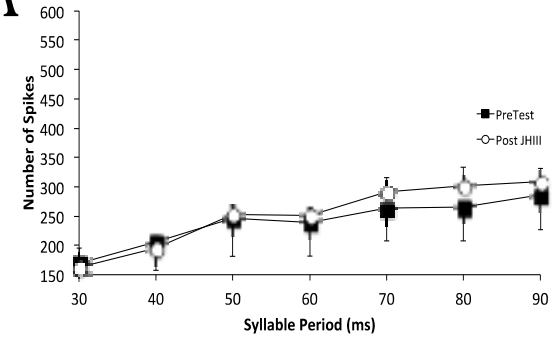
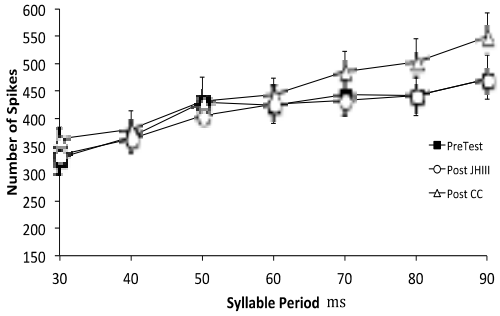

D
% Decrement
PreTest
Post Saline
Sylllable Period (ms)

Figure 8 Average total number of spikes for 30 chirps at each SP for all treatment and control groups. JHIII has no effect on the number of spikes for **A** Group 1 and **B** Group 2. While CC increases the average number of spikes in Group 2 as well as **C** treatment with CC only. **D** The difference in the number of spikes following CC in Group 2 is greatest at the first syllable and less for the second and third. **E** No difference was found in the number of spikes in any control group.

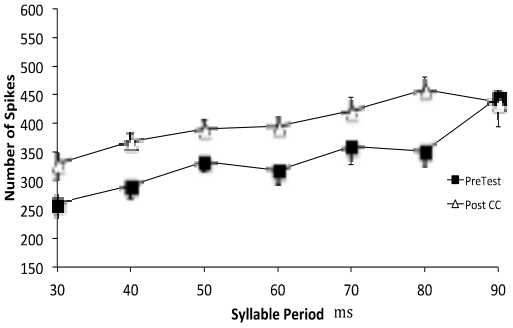
A


Number of Spikes
600
550
500
450
400
350
300
250
200
150
30
40
50
60
70
80
90
Syllable Period (ms)
PreTest
Post JHIII

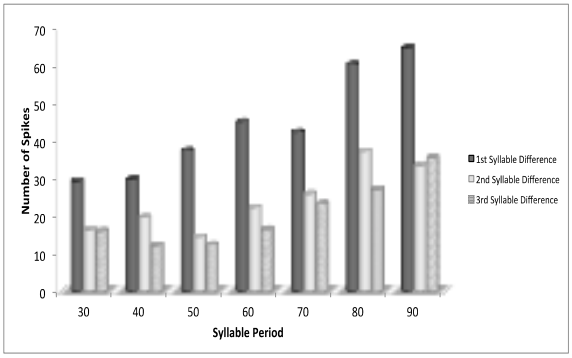
B


Number of Spikes
600
550
500
450
400
350
300
250
200
150
30
40
50
60
70
80
90
Syllable Period ms
PreTest
Post JHIII
Post CC

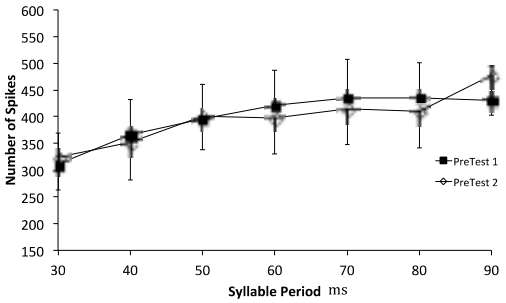
C

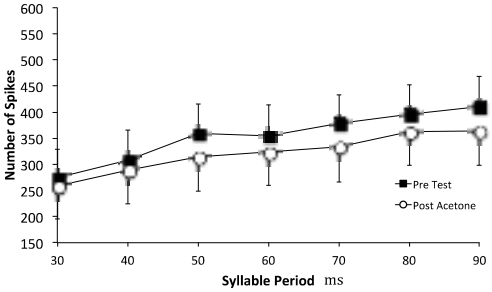

Number of Spikes
600
550
500
450
400
350
300
250
200
150
30
40
50
60
70
80
90
Syllable Period ms
PreTest
Post CC

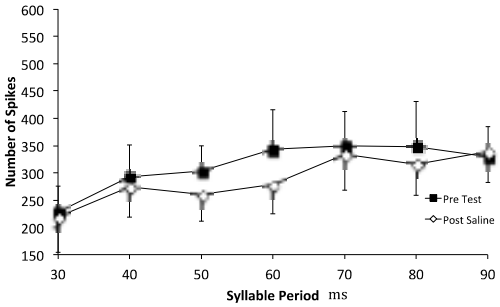
D


Number of Spikes
70
60
50
40
30
20
10
0
30
40
50
60
70
80
90
Syllable Period
1st Syllable Difference
2nd Syllable Difference
3rd Syllable Difference

E


Number of Spikes
600
550
500
450
400
350
300
250
200
150
30
40
50
60
70
80
90
Syllable Period ms
PreTest 1
PreTest 2


Number of Spikes
600
550
500
450
400
350
300
250
200
150
30
40
50
60
70
80
90
Syllable Period ms
Pre Test
Post Acetone


Number of Spikes
600
550
500
450
400
350
300
250
200
150
30
40
50
60
70
80
90
Syllable Period ms
Pre Test
Post Saline

CHAPTER 4

DISCUSSION

Atkins *et al.* (1989) first showed that the L3, a prothoracic, first order, auditory interneuron, responded selectively to the SPs of model CSs. This SP-selective response was characterized by a reduced excitation to the second and third syllables of each chirp in the CS and was termed "decrement". Decrement in spiking (measured as percent decrease) was greatest in response to computer generated CSs with SPs most similar to the male's natural call (50 to 70 ms at the temperatures (20° - 22° C) used during the current study (Navia *et al.*, 2015). Since Stout & McGhee (1983) had already shown that 50 to 70 ms was the most phonotactically attractive SP range for female *A. domesticus,* interest was focused on the L3 and its possible involvement in the behavioral recognition of attractive CS SPs.

A series of subsequent studies reinforced the possibility that L3 played a pivotal role in the phonotactic choices made by female *A. domesticus* by demonstrating that the largest decrements of the L3 neurons matched behaviorally attractive SPs in females (Henley *et al.,* 1992; Atkins *et al.,* 1992; Stout *et al.*, 1997; Stout *et al.,* 2002; Navia, 2005). In recent work, the SP-selective decrementing responses of the L3 homolog, AN2, were demonstrated to be strongly correlated with the phonotactic choices made by female *G. bimaculatus* (Stout *et al.,* 2011).

Furthermore, Samuel *et al.* (2013), studied phonotactic choices and decrement of the AN2 neuron sequentially in the same female, and reported that female *G. bimaculatus* were most likely to choose SPs for phonotaxis to which their AN2s responded with the highest decrements. These results together strengthen the possibility that L3/AN2 plays a central role in the regulation of phonotactic choices made by both female *A. domesticus* and *G. bimaculatus*.

Higher rates of JHIII biosynthesis in female *A. domesticus* are strongly correlated with an increase in SP-selectiveness during phonotaxis (Walikonis *et al.,* 1991). Topical application of JHIII to crickets that were not phonotactically selective caused a significant increase in responses that were selective (Henley *et al.*, 1992). For both female *A. domesticus* (Atkins *et al.,* 2008) and *G. bimaculatus* (Choi *et al.*, 2012) nanoinjection of JHIII into the PTG, significantly increased the phonotactic selectiveness within 10 minutes by reducing the number of SPs responded to phonotactically, and centering this behavior on the SPs most commonly produced by their conspecific males.

The strong correlation between L3's SP-selective response and behavior by *A. domesticus* females as well as JHIII's role in behavior and phonotactic selectivity let to the study of JHII's role in the SP-selective processing of the interneuron. The proposed mechanism of JHIII's effects through a protein kinase C (PKC) mediated pathway (Wyatt & Davy, 1996; Wheeler & Nijout, 2003; Liu, *et al.,* 2015), led to the evaluation of the effects of a PKC blocker CC (Chao *et al.,* 1998) and its role in the selective processing by L3.

JHIII's Role In Selective Processing Of The L3 Interneuron

Analysis of the L3s' responses to the model SPs led to the discrimination between crickets with Group 1 and Group 2 neurons. It was clear that the L3s were producing two different responses; one that decremented more to the shortest SPs (30 and 40 ms, Group 1 neurons, n = 5) than to the subsequent SPs, and one that decremented less or equal to the shortest SPs than longer SPs (Group 2 neurons n=11, Fig. 3A). It was discovered that the Group 1 neurons also displayed significantly lower spiking rates across all SPs than Group 2 neurons (Fig. 3B).

This finding is paralleled by a recent study on *G. bimaculatus* females. Samuel *et al.* (2013) found that AN2 in *G. bimaculatus* also produced two types of responses: a. one group that respond with higher decrement to the shortest SPs (25 and 35ms) b. another group that respond with lower or equal decrement to the shortest SPs. Samuel *et al.* (2013) additionally reported that the selection for longer SPs was identical between the two groups, an observation that is also noted in this study, as Group 1 and 2 decrement percentages and overall trends are very similar from 50 ms to 90 ms (Fig. 3A.) and both experience a drop in decrement values past 70 ms. The parallels between the two studies (current study and Samuel *et al.,* 2013) are not complete though, as Samuel *et al.* (2013*)* shows a distinct behavioral correlate to the "Group 1" neurons, a subgroup that is also phonotactically selective for the shortest SPs only. To date, there are no well-defined behavioral subgroups in *A. domesticus* that match with the Group 1 neurons from this study.

In the present study, JHIII treatment had very different effects on the two groups of neuronal responses. JHIII left Group 1 neurons relatively unchanged (Fig.

4) but significantly narrowed selectivity in Group 2 neurons (Fig. 5D, E) to more closely match the SPs of the males natural CS (Navia *et al.,* 2015). This largely results from reduced L3 decrement in response to SPs of 30 and 40 ms (Fig. 5D, E). While the p values for 30 and 40 ms are significant at the p=0.08 and p=0.06 level respectively, they stand in sharp contrast to comparison of the L3 responses to all of the other SPs. JHIII also has a narrowing effect on the SP-selective responses of AN2 neurons in *G. bimaculatus* females (Stout *et al*., 2011b), that is similar to the effects of JHIII on the phonotactic selectiveness of this species (Choi *et al.* 2012). Thus, JHIII effects on behavior match closely the effect we see in the Group 2 neurons of *A. domesticus* females (Fig. 5D, E; Atkins *et al*., 2008) and *G. bimaculatus* females (Stout, 2011; Choi *et al*., 2012).

Atkins et al. (2008) and Choi *et al.* (2012) both showed that injection of JHIII into the metathoracic ganglion had no effect on phonotactic selectivity, and control data from the current work show that injection of acetone into the PTG (Fig. 5E) has no effect on the decrementing response of L3s.

A possible explanation for the Group 1 findings is given by the fact that L3 as well as AN2 in *G. bimaculatus,* consistently responds vigorously to the first chirp and rapidly habituates, so that the response to the 10th or later chirp is much lower (Stout *et al.,* 2011). Additionally, the neurons in Group 1 of the current study were shown to be experiencing lower levels of excitation than the Group 2 neurons, as exhibited by the significantly lower spiking rate of Group 1 neurons in response to PreTest SPs (Fig 3B). It is possible that inhibitory input, coupled with the normal habituation experienced by these neurons results in lower number of spikes to the

3rd syllable, and causes substantially higher levels of decrement in response to the shortest SPS. JHIII's method of action, and its ability to narrow the selective decrement at these SPs might be impeded somewhat by lower levels of excitation.

In summary of the current findings for Group 2 neurons, the parallel studies on female *A. domesticus* (Atkins *et al.,* 1989; Henley *et al.,* 1991; Atkins *et al.,* 2008; Stout *et al.,* 2010) and on female *G. bimaculatus* (Stout *et al.,* 2010; Stout *et al.,* 2011a; Stout *et al.,* 2011b; Choi *et al.,* 2012; Samuel *et al.,* 2013), demonstrate the following for both species: a. Females respond with SP-selective phonotaxis to models of the male's natural CSs that are tuned to the SPs most common in the conspecific's call, b: L3/AN2s respond to model CSs with SP-selective responses that closely parallel the female's SP-selective phonotactic behavior; c. Nanoinjection of JHIII into the PTG sharpens SP-selective phonotaxis, tuning it more closely to the SPs of the conspecific males' CSs; d. Nanoinjection of JHIII into the PTG also tunes the SP-selective response of the L3/AN2 neurons more closely to the SPs found in the conspecific males' CSs.

The parallel roles that JHIII plays in both species' behavior and neuronal processing suggest that the mechanism for sharpening the females' phonotactic behavior is expressed through molecular pathways in the L3 neuron.

CC's Role In Selective Processing Of The L3 Interneuron

In insect orders, the search for mechanisms of JH's diverse actions have led to the study of membrane, cytosolic, and nuclear receptors, since JH is quite nonpolar, lipid soluble, and can easily pass through cell membranes (Li *et al.* 2007). A model for JH action through a PKC pathway for insects was suggested by Wheeler &

Nijhout (2003) who cited numerous examples of JHIII action by this pathway in other insect species. This pathway for JH action was recently confirmed by Liu *et al.* (2015) with the demonstration that in mosquitos, JH activated the phospholipase C (PLC) pathway and upregulated the molecular components that leads to activation of PKC molecules. A method to test JHIII's use of the PKC mediated pathway in the L3 neuron was given by CC, a potent inhibitor of PKC (Chao *et al.*, 1998).

Chao *et al.* (1998) demonstrated that CC works by inhibiting the translocation of PKC from the cytosol to the plasma membrane, which would impede the activation of PKC as a review paper on the regulation of PKC (Newton, 1995) stated that the PKC molecule must be present at the cell membrane to be activated. Historically, CC has been used in many different studies to modulate PKCs effects in different molecular pathways including evaluation as an anti tumor agent (Chmura et al., 2000; Yang *et al.*, 2008).

Recently CC was used in a study of phonotaxis and SP-selectivity (Byssainthe, 2008), which showed that CC could block the selective effects of JHIII injection in *A. domesticus,* when injected sequentially. A second study by Creighton (2012) suggested that crickets that were selective during the pretest exhibited less SP-selective phonotaxis following injection with CC.

Results from the present study indicate that the L3 decrementing response follows a similar pattern. Data show a clear loss of decrements that are selective for individual SPs following nanoinjection of CC into the PTG (Fig. 6, and 7A-C), and no change when injecting the control, saline (Fig. 7 D). Decrements by L3s treated with both JHIII and CC in Group 2 crickets were unselectively and significantly increased

(Fig. 6C) and the responses became much more uniform by decrementing more similarly to all SPs (Fig. 6), which demonstrates a reversal of the selective decrement induced by treatment with JHIII. Additionally, SP-selective decrement is decreased (most noticeably at 70 ms) following CC only injections into the PTG (Fig. 7A-C).

It has been shown in this study, that JHIII, possibly through a PKC mediated pathway, increases the selectivity of L3, by tuning decrements to be highest for the SPs most similar to the conspecific males call (Fig. 5 A-E). CC, a known PKC inhibitor reduces SP-selective decrement following JHIII injection (Fig. 6) or when injected singularly (Fig. 7A - C). This suggests an integral role for PKC in inducing selectivity, and supports the possibility that JHIII works through a PKC mediated pathway.

JHIII in *A. domesticus* (current study) and in *G. bimaculatus* (Stout *et al.,* 2011b) sharpens the decrementing response of L3/AN2 to SPs by reducing decrement to the range of SPs that are outside the range of SPs in the conspecific males' CSs. CC alone, in this study flattens SP-selective decrement by L3 without increasing the overall decrement (Fig. 7). These results support the importance of PKC mediated pathways within the network of neurons in the prothoracic ganglion that influence SP-selective decrement by L3. However, the significant increase in decrement by JHIII-CC treated L3s in response to all SPs might seem anomalous, or could be interpreted to suggest that JHIII might also operate on this network through other pathways not dependent on the uninhibited activity of PKC. A reduction in SP-selective decrement by L3 as a result of the blocking PKC's activity with CC is consistent with preliminary results in which JHIII injection into the PTG,

followed by CC injection did not sharpen the phonotactic selectiveness of female *A. domesticus* (Byssainthe, 2008), while injection of only JHIII did sharpen the females' phonotactic responses.

JHIII And CC And The Excitation Of L3

L3's SP-selective decrement has been shown to result from both excitatory modulation of its response to the first syllable as well as a delayed inhibition that cuts down its response to subsequent syllables. The delayed inhibition is timed such that the decrement in *A. domesticus* is maximal in response to 50 – 70 ms (Henley *et al.,* 1991, Navia, 2005).

In this study, the number of spikes produced in response to each SP following JHIII was no different, on average, from pretest values in both Group1 and Group 2 crickets (Fig. 8A, B). This suggests that JHIII does not primarily alter SP-selective decrement in L3 by changing its overall excitation. JHIII then may be more subtly altering the selective excitation and delayed inhibition documented by Henley *et al.* (1991) and Navia (2005).

The average number of spikes following CC treatment is increased significantly in Group 2 and CC Only Group (Fig. 8B, C) though not as dramatically in Group 2, whose average number of spikes during the pretest were noticeably higher than in Group 1. As there are no other relevant data yet collected on CC's effect on the spiking rate of L3s, it is suggested only that CC increases the excitation of these neurons unselectively. The increase in decrement, for Group 2, as discussed earlier (Fig. 5), is possibly due to a larger increase in the number of spikes in response to

the first syllable, and a lesser increase in response to the third (Fig. 8 D). No change is seen in the excitation of L3s in any control group (Fig. 8E).

L3 As The First Filter In A Pathway

Schieldberger (1984) described neurons in the brain of *G. bimaculatus* that responded with tonic output to SPs that represented the natural call, and proposed that they were the filter neurons responsible for the females' selective phonotactic behavior. L3 is one of the auditory interneurons that send an axon to the brain and synapse with higher order auditory neurons (Kostarakos & Hedwig, 2012; Schoneich *et al.* 2015). The results of the current study support the suggestion that the L3's decrementing response may be the first step in filtering and selective processing, which is further carried out by the brain neurons that are also responsive to SPs of the model CSs.

Further Studies

In an effort to further elucidate the roles of CC and JHIII in SP-selective behavior and decrement the following studies are suggested: First, an increase in the sample size for phonotactic behavior following CC only injections. Second, CC injections before and after JHIII injections – followed by behavior studies and neuronal recordings. This may shed light on how CC influences phonotactic behavior and if JHIII's effect requires that PKC be present at the membrane. Thirdly, it is suggested that Ca^{++} channel blockers be used in both types of studies, as Ca^{++} is necessary for PKC's activation. Finally, intracellular recordings of L3 under the

influence of both JHIII and CC should be conducted to better understand the effect of these compounds on the PSPs influencing L3.

Reference List

Atkins, G., Henley, J., Handysides, R. & Stout, J. (1992). Evaluation of the behavioral roles of ascending auditory interneurons in calling song phonotaxis by the female cricket (*Acheta domesticus*). *Journal of Comparative Physiology A,* **170**, 363-372.

Atkins, G., Kilmer, J., Scalfani, M., Navia, B. & Stout, J. (2008). Modulation of syllable period-selective phonotaxis by prothoracic neurones in crickets (*Acheta domesticus*): juvenile hormone, picrotoxin and photoinactivation of the ON1 neurones. *Physiological Entomology,* **33**, 322-333.

Atkins, G., Ligman, S., Burghardt, F. & Stout, J. (1984). Changes in phonotaxis by the female cricket *Acheta domesticus* L. after killing identified acoustic interneurons. *Journal of Comparative Physiology A,* **154**, 795-804.

Atkins, S., Atkins, G., Rhodes, M. & Stout, J. (1989). Influence of syllable period on song encoding properties of an ascending auditory interneuron in the cricket *Acheta domesticus. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology,* **165**, 827 - 836.

Byssainthe, P. (2008). Hormonal Control of Female Cricket Phonotaxis. *Honors Thesis, Andrews University*.

Chao, M., Chen, I. & Cheng, J. (1998). Inhibition of protein kinase C translocation from cytosol to membrane by chelerythrine. *Planta medica,* **64**, 662-663.

Chmura, S., Dolan, E., Cha, A., Mauceri, H., Donald W. & Weichselbaum, R. (2000). In vitro and in vivo activity of protein kinase C inhibitor chelerythrine chloride induces tumor cell toxicity and growth delay in vivo. *Clinical Cancer Research,* **6**, 737-742.

Choi, R., Atkins, G. & Stout, J. (2012). The effects of injecting Juvenile Hormone III into the prothoracic ganglion on phonotaxis by female crickets *Gryllus bimaculatus*. *Physiological Entomology,* **37**, 201-205.

Creighton, C. (2012). Determination of the Biosynthetic Pathway Used by Juvenile Hormone III in the Phonotactic Response of the Female Cricket *Acheta domesticus*. *Honors Thesis, Andrews University*.

Esch, H., Huber, F. & Wohlers, D. (1980). Primary auditory neurons in crickets: physiology and central projections. *Journal of Comparative Physiology,* **137**, 27-38.

Fielden, A. (1960). Transmission through the last abdominal ganglion of the dragonfly nymph, *Anax imperator*. *Journal of Experimental Biology,* **37**, 832-844.

Haskell, P. (1953). The stridulation behavior of the domestic cricket. *The British Journal of Animal Behaviour,* **1**, 120-121.

Henley, J., Greenwood, J., Stout, J. & Atkins, G. (1992). Age-correlated changes and juvenile hormone III regulation of the syllable period specific responses of the L3 auditory interneurons in the cricket, *Acheta domesticus*. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology,* **170**, 373-378.

Hennig, R. & Weber, T. (1997). Filtering of temporal parameters of the calling song by cricket females of two closely related species: a behavioral analysis. *Journal of Comparative Physiology A,* **180**, 621-630.

Hennig, R. (2009). Walking in Fourier's space: algorithms for the computation of periodicities in song patterns by the cricket *Gryllus bimaculatus*. *Journal of Comparative Physiology A,* **195**, 971-987.

Jeffery, J. (2003). Selective processing of calling songs by auditory interneurons in two species of female crickets: possible roles in behavior. *Honors Thesis, Andrews University.*

Kostarakos, K. & Hedwig, B. (2012). Calling song recognition in female crickets: temporal tuning of identified brain neurons matches behavior. *The Journal of Neuroscience,* **32**, 9601-9612.

Koch, P. & Hoffmann, K. (1985). Juvenile hormone and reproduction in crickets, *Gryllus bimaculatus* De Geer: corpus allatum activity (in vitro) in females during adult life cycle. *Physiological Entomology,* **10**, 173-182.

Li, Y., Zhang, Z., Robinson, G. & Palli, S. (2007). Identification and characterization of a juvenile hormone response element and its binding proteins. *Journal of Biological Chemistry,* **282**, 37605-37617.

Liu, P., Peng, H-J. & Zhu, J. (2015) Juvenile hormone-activated phospholipase C pathway enhances transcriptional activation by the methoprene-tolerant protein. *Proceedings of the National Academy of Sciences*. **112**, E1871 – E1879.

Navia, B. (2005). Plasticity in the selectivity of phonotaxis and neuronal responses in crickets. *PhD Thesis, Loma Linda University*.

Navia, B., Stout, J. & Atkins, G. (2003). Prolonged response to calling songs by the L3 auditory interneuron in female crickets (*Acheta domesticus*): Intracellular evaluation. *Journal of Experimental Zoology Part A: Comparative Experimental Biology,* **296**, 63-71.

Navia, B., Burden, C., Steely, T., Hasegawa, H., Cha, E., Henson, S. M., Stout, J. & Atkins, G. (2015). Temperature and its effect on the call and response of the female cricket. *Physiological Entomology* **40**, 113 - 122.

Newton, A. (1995). Protein kinase C: structure, function, and regulation. *Journal of Biological Chemistry,* **270**, 28495-28498.

Pollack, G. (1986). Discrimination of calling song models by the cricket, *Teleogryllus oceanicus*: the influence of sound direction on neural encoding of the stimulus temporal pattern and on phonotactic behavior. *Journal of Comparative Physiology A,* **158**, 549-561.

Pollack, G., & Hoy, R. (1979). Temporal pattern as a cue for species-specific calling song recognition in crickets. *Science,* **204**, 429-432.

Popov, A., & Shuvalov, V. (1977). Phonotactic behavior of crickets. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology,* **119**, 111-126.

Regen, J. (1913). Ueber die Anlockung des Weibchens von *Gryllus campestris* L. durch telephonisch √ºbertragene Stridulationslaute des M√§nnchens. *Pfl√ºgers Archiv European Journal of Physiology,* **155**, 193-200.

Renucci, M. & Strambi, C. (1983). Juvenile hormone levels, vitellogenin and ovarian development in *Acheta domesticus*. *Experientia,* **39**, 618-620.

Riddiford, L. (1994). Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. *Advances in Insect Physiology,* **24,** 213-274.

Riddiford, L. (2007). Juvenile hormone action: a 2007 perspective. *Journal of Insect Physiology,* **54**, 895-901.

Ritchie, M. (1991). Female preference for 'song races' of *Ephippiger ephippiger* (Orthoptera: Tettigoniidae). *Animal Behaviour,* **42**, 518-520.

Samuel, L., Stumpner, A., Atkins, G. & Stout, J. (2013). Processing of model calling songs by the prothoracic AN2 neurone and phonotaxis are significantly correlated in individual female *Gryllus bimaculatus*. *Physiological Entomology,* **38**, 344-354.

Schmidt, A. & Romer, H. (2013). Diversity of acoustic tracheal system and its role for directional hearing in crickets. *Frontiers in Zoology*. **10**, 61-70.

Schildberger, K. (1984). Temporal selectivity of identified auditory neurons in the cricket brain. *Journal of Comparative Physiology.* **155**, 171 – 185.

Schildberger, K. & Horner, M. (1988). The functioning of auditory neurons in cricket phonotaxis. *Journal of Comparative Physiology*. **163**, 621 – 631.

Schoneich, S., Kostakaros, K. & Hedwig, B. (2015). An auditory feature detection circuit for sound pattern recognition. *Science Advances.* **1,** 1-7

Stout, J., Atkins, G., Bronsert, M., Hao, J. & Walikonis, R. (2002). Influence of juvenile hormone III on the development and plasticity of the responsiveness of female crickets to calling males through control of the response properties of identified auditory neurons. *Hormones, Brain and Behavior,* **3**, 167-193.

Stout, J., Atkins, G., & Burghardt, F. (1985). The characterization and possible important for phonotaxis of "L" shaped ascending acoustic interneurons in the cricket (*Acheta domesticus*). In Kalmring, K. & Elsner, E. (Eds.) *Acoustic and Vibrational Communication in Insects* (89 – 99). Paul Verlag: Berlin & Hamburg.

Stout, J., Carlson, N., Bingol, H., Ramseier, J., Bronsert, M. & Atkins, G. (1997). The L3 neuron and an associated prothoracic network are involved in calling song recognition by female crickets. *Invertebrate Neuroscience,* **3**, 145-153.

Stout, J., Hayes, V., Zacharias, D., Henley, J., Stumpner, A., Hao, J. & Atkins, G. (1992). Juvenile hormone controls phonotactic responsiveness of female crickets by genetic regulation of the response properties of identified auditory interneurons. In Mauchamp, B., Coulluad, F., & Baehr, J.C. (Eds.) *Insect Juvenile Hormone Resaerch: Fundamental and Applied Approaches.* Institut National Recherche Agronomique: Paris. (265 – 283)

Stout, J. & Huber, F. (1981). Responses to features of the calling song by ascending auditory interneurones in the cricket *Gryllus campestris*. *Physiological Entomology,* **6**, 199-212.

Stout, J. & McGhee, R. (1983). Attractiveness of the male *Acheta domesticus* calling song to females. II: The relative importance of syllable period, intensity, and chirp rate. *Journal of comparative physiology. A, Sensory, Neural, and Behavioral Physiology,* **164**, 277-287.

Stout, J., Navia, B., Jeffery, J., Samuel, L., Hartwig, L., Butlin, A. & Atkins, G. (2010). Plasticity of the phonotactic selectiveness of four species of chirping crickets (Gryllidae): Implications for call recognition. *Physiological Entomology,* **35**, 99-116.

Stout, J., Samuel, L. & Atkins, G. (2011b). Selective processing of calling songs by AN2 interneurons of female *Gryllus bimaculatus:* roles in a temporally selective neuronal network. *Proceeding of the Eleventh International Invertebrate Sounds and Vibration Conferences.* Abstract 85.

Stout, J., Stumpner, A., Jeffery, J., Samuel, L. & Atkins, G. (2011a). Response properties of the prothoracic AN2 auditory interneurone to model calling songs in the cricket *Gryllus bimaculatus*. *Physiological Entomology,* **36**, 343-359.

Stout, J., DeHaan, C. & McGhee, R. (1983). Attractiveness of the male *Acheta domesticus* calling song to females. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology,* **153**, 509-521.

Surlykke, A. & Gogala, M. (1986). Stridulation and hearing in the noctuid moth *Thecophora fovea* (Tr.). *Journal of Comparative Physiology A,* **159**, 267-273.

Thorson, J., Weber, T. & Huber, F. (1982). Auditory behavior of the cricket. *Journal of comparative physiology,* **146**, 361-378.

Tychsen, P. (1978). Mating behaviour of the Queensland fruit fly, *Dacustryoni* (Diptera: Tephritidae), in field cages. *Australian Journal of Entomology,* **16**, 459-465.

Walikonis, R., Schoun, D., Zacharias, D., Henley, J., Coburn, P. & Stout, J. (1991). Attractiveness of the male *Acheta domesticus* calling song to females. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology,* **169**, 751-764.

Walker, T. (1957). Specificity in the response of female tree crickets (Orthoptera, Gryllidae, Oecanthinae) to calling songs of the males. *Annals of the Entomological Society of America,* **50**, 626-636.

Wheeler, D. & Nijhout, H. (2003). A perspective for understanding the modes of juvenile hormone action as a lipid signaling system. *Bioessays,* **25**, 994-1001.

Wigglesworth, V. (1963). The action of moulting hormone and juvenile hormone at the cellular level in *Rhodnius prolixus*. *Journal of Experimental Biology,* **40**, 231-245.

Wohlers, D. & Huber, F. (1985). Topographical organization of the auditory pathway within the prothoracic ganglion of the cricket *Gryllus campestris* L. *Cell and Tissue Research,* **239**, 555-565.

Wyatt, G. & Davey, K. (1996). Cellular and Molecular Actions of Juvenile Hormone. ll. Roles of Juvenile Hormone in Adult Insects. *Advances in Insect Physiology,* **26,** 1.

Yang, R., Piperdi, S. & Gorlick, R. (2008). Activation of the RAF/mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase pathway mediates apoptosis induced by chelerythrine in osteosarcoma. *Clinical Cancer Research,* **14**, 6396-6404.