5-1-1998

Regulation of the Phonotactic Threshold of the Female Cricket, Acheta domesticus: Juvenile Hormone III, Allatectomy, L1 Auditory Neuron Thresholds and Environmental Factors

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J Comp Physiol A (1998) 182: 635–645

ORIGINAL PAPER

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Regulation of the phonotactic threshold of the female cricket, *Acheta domesticus*: juvenile hormone III, allatectomy, L1 auditory neuron thresholds and environmental factors

Accepted: 29 September 1997

Abstract Juvenile hormone III (JHIII), when applied to the abdomen of 1-day-old female *Acheta domesticus* (in quantities that would create JHIII titers in the hemolymph that were within the range measured in females of this species) caused a significant decrease in phonotactic thresholds (Fig. 1). Removal of the corpora allata from 5-day-old females with low phonotactic thresholds caused significantly increased phonotactic thresholds 2–5 days later. After a temporary increase (24 h) of, on average, about 25 dB, the phonotactic thresholds drop to about 10 dB above preallatectomy levels (Fig. 2), but remain significantly higher than controls. Application of JHIII to allatectomized females, with a mean increase in thresholds of 20 dB, results in significantly decreased thresholds (mean of about 20 dB) over the next 6 h (Fig. 3). Exposure to males 1 week before the imaginal molt causes the phonotactic thresholds of postimaginal females to drop 1–2 days significantly earlier than controls (Fig. 4). One- and 3-day-old females, phonotactically tested only once, exhibit lower thresholds in the early morning than they do in the late afternoon (Fig. 5). Five-day-old females do not exhibit such a diurnal rhythm. Phonotactically testing females more than once a day significantly influences their phonotactic thresholds (Figs. 6, 7). In 1-day-old females, with high (above 70 dB) phonotactic thresholds, the threshold of their L1 auditory interneurons can be 30 dB or more below their phonotactic threshold (Fig. 8). In females with phonotactic thresholds of 70 dB or lower, the L1 threshold is within 10 dB of their phonotactic threshold. Both JHIII and allatectomy influence phonotactic and L1 thresholds in a similar manner.

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Key words Phonotactic threshold · Juvenile hormone III · Allatectomy · Environmental influences · Auditory interneuron

Abbreviations *CS* calling song · *JHIII* juvenile hormone III

Introduction

Female crickets recognize and respond phonotactically to the calling song of a conspecific male. In *Acheta domesticus*, the sensitivity to and selectiveness for the conspecific male's call are age-related (Walikonis et al. 1991; Stout et al. 1991; Atkins and Stout 1994) and influenced by juvenile hormone III (JHIII; Koudele et al. 1987; Stout et al. 1991). Phonotaxis begins 2–4 days following the imaginal molt as a result of declining behavioral thresholds in response to the male's call. During this time, the rate of JHIII synthesis increases. Since topical application of JHIII on the day of or the day following the imaginal molt, when female phonotactic thresholds are high, usually lowers the threshold for positive phonotaxis within the next 12 h (2–4 days earlier than thresholds drop naturally, Koudele et al. 1987; Stout et al. 1991), JHIII influences the onset of the female's phonotactic response to the male's calling song (Koudele et al. 1987; Stout et al. 1991; Walikonis et al. 1991). In adult female *A. domesticus* (Walikonis et al. 1991) and in females of several other gryllid species (Loher et al. 1992), phonotactic thresholds remained at their lowest values (generally 45–60 dB) for several weeks following the onset of this most sensitive phonotaxis. Removal of the corpora allata, and thus the source of JHIII, from female *A. domesticus* that are already phonotactically responsive to the male's call does not eliminate phonotaxis (Loher et al. 1992), but decreases phonotactic sensitivity within 2–3 days (Atkins and Stout 1994). Topical application of JHIII to these allatectomized females restores phonotactic sensitivity within 6 h of application (Koudele et al. 1987).

Atkins et al. (1992) demonstrated that in female *A. domesticus* the L1 auditory interneuron's response is necessary for phonotaxis in response to calling songs produced at low intensities (60 dB). This interneuron is located in the prothoracic ganglion, receives excitatory input from the ears, projects to the brain, encodes the temporal structure of the male's calling song is tuned to calling song carrier frequencies (4–6 kHz) and is the functional analog of the AN1 neuron of other cricket species (Stout et al. 1985; Stumpner et al. 1995). JHIII influences the threshold of the L1 auditory interneuron's response to model calling songs (Stout et al. 1991) by increasing its excitatory responses to afferent input from the ears. This hormone's influence is mediated through processes regulated, at least in part, within L1's soma (Stout et al. 1991; Atkins and Stout 1994). Since L1's response is necessary for phonotaxis by the female (Atkins et al. 1992) at low calling song intensities, Stout et al. (1991) proposed that regulation of the female's phonotactic threshold was hormonally mediated and achieved through control of L1's sensitivity to calling songs.

It is the purpose of this study to more carefully evaluate the regulation of the female cricket's phonotactic threshold. The studies cited above were carried out with females that were kept under rather constant environmental conditions and were physically and acoustically separated from males. To illustrate possible environmental modulation of phonotactic threshold, data relating to diurnal changes in threshold, male exposure and previous phonotactic testing are presented. To more fully investigate the role of JHIII in threshold regulation, the effects of both hormone addition and allatectomy are presented more extensively and the results are compared with our previous studies (Koudele et al. 1987; Stout et al. 1991) and with the more recent study by Loher et al. (1992). Finally, the roles of the L1 auditory neuron in the phonotactic sensitivity of 1-day-old and older adult females are reconsidered in light of new data on: (1) the correlation between L1 and phonotactic thresholds, (2) hormonal influences on L1 and phonotactic thresholds, and (3) environmental influences on phonotactic thresholds. The hypothesis that the female's phonotactic thresholds are largely controlled through regulation, by JHIII, of the L1 auditory interneuron's threshold (Stout et al. 1991) is reconsidered.

Since the female cricket's phonotactic response to the male has been developed by many laboratories as a productive model system for understanding the control of behavior, the factors that control the sensitivity of that behavioral response contribute importantly to understanding the biology and the neurobiology of this model system.

Materials and methods

Animals and care

Female *A. domesticus*, purchased as 3- to 4-week-old nymphs from Fluker's Cricket Farm, were raised to adults at 22 °C with a 12:12 LD light cycle (on at 0600 hours). Cricket chow and water or potatoes were supplied ad libitum. Every day, females that had molted to adults within the previous 24 h were isolated and maintained under the above conditions but separate from the nymphs. Newly molted adult males were removed each day.

Behavior experiments

Phonotaxis of female crickets was tested in a circular arena (Walikonis et al. 1991) with a centrally located, omnidirectional loudspeaker. Phonotactic threshold (in response to a model calling song (CS) with carrier frequency = 5 kHz, syllable period = 50 ms, syllable duration = 22 ms, 3 syllables/chirp, chirp rate = 1.5 chirps/s) was measured at intensities ranging between 40 dB and 90 dB, in 10-dB increments. Threshold was determined as the minimum CS intensity (measured at the edge of the arena) which caused the female to leave the edge and walk directly toward (without turning away with an angle greater than 90°) and approach (within one body length) the centrally located sound source within 5 min of sound onset. This method is presented in detail by Stout et al. (1991) and validated by comparison with the responses of females on a treadmill, in a constant sound field, to the same stimuli.

Juvenile hormone application

The phonotactic behavior of 1-day-old females with 80 dB or higher phonotactic thresholds was tested after applying 1 ng–10 μg of JHIII in 2 μl of acetone topically to the abdomen following an initial test of phonotaxis. The behavioral thresholds of these females were measured 4–12 h later. A control group was treated with only 2 μl of acetone.

Allatectomies

Females were pretested for phonotactic threshold, cold anesthetized, the head was bent forward and the paired corpora allata were removed through a small slit in the soft neck tissue at the posterior dorsal margin of the head. One hour following allatectomy, the phonotactic threshold was measured; thresholds were also tested over the next 7 days. For some allatectomized females, JHIII was applied after 3 days and changes in their phonotactic thresholds were followed over the next 24 h. Sham-operated females were used as controls in all of these tests.

Effects of males

Some nymphs were raised in 1.6-l containers divided in half with a screen. Five males (either muted by removing their wings, or normal males) were placed in the lower half (with food and shelter as described above). Ten females were placed in the upper portion of the container (with food and shelter) and raised to adults (at least 7 days) and maintained this way until phonotaxis testing.

Diurnal changes in threshold

Crickets of known ages were tested once at either 0600, 1200, 1800 or 2400 hours. For these tests, crickets were sorted from the nymphal colony every 6 h at the times above, so that 1-day-olds are really 1–1.25 days old when tested.

Effects of repeated testing

Crickets 1–1.25 days old or 5 days old were tested every 6, 12 or 24 h for 4–5 days.

Statistical evaluation

Experimental results were first evaluated with analysis of variance (ANOVA). If the resulting F ratio resulted in $P < 0.05$, individual

means were compared with Fisher's protected least significant difference test. In several cases the variability of the test results were compared with an F calculated from the sample variances being compared. In all tests, results were considered to be statistically significant only if the test yielded $P < 0.05$.

Electrophysiology

Following determination of a female's phonotactic threshold (described above) the threshold (defined as production of at least one spike in response to each syllable of the CS) of the L1 auditory interneuron was determined in the same individual, using either intracellular or extracellular recording techniques.

Intracellular recordings

Recordings, lucifer-yellow staining, and morphological identification of the auditory interneurons were performed as described by Atkins et al. (1989).

Extracellular recordings

A small incision was made on the ventral surface of the neck to expose the neck connectives after the cricket was immobilized, ventral side up, on a wax block in a manner similar to that described for intracellular recordings (Atkins et al. 1989). The tip of a suction electrode was placed along side of a desheathed connective and positioned to record from the most sensitive 5-kHz-tuned unit. Of the three known prothoracic, ascending units in the neck connective, only L1 is most sensitive to 5 kHz – thus L2 and L3 (Stout et al. 1985) could be easily found and avoided. For those L1s recorded extracellularly with thresholds of 70 dB or higher (Fig. 8), its response dynamics clearly separated it from the L3 neuron. L3 neurons were also recorded from the same female in a majority of these cases, confirming the accuracy of discriminating between the L1 and L3 neurons based on response dynamics.

Results

Effect of the amount of JHIII applied on phonotactic thresholds of 1-day-old females

Topical application of a series of JHIII concentrations to different 1-day-old females, starting with a 10-μg dose, and reducing the amount applied by factors of 10, significantly increased the drop in phonotactic thresholds ($P < 0.0001$, $F = 9.92$) as compared to the controls treated with only acetone for all except the two smallest (0.01 and 0.1 ng) dosages. The 100-ng (1/100 of 10 μg) and 1000-ng doses were just as effective as (were not significantly different from) the original 10-μg dose (used by Stout et al. 1991; Fig. 1) in reducing the threshold for phonotaxis. The significant reduction in the phonotactic thresholds of these 1-day-old females that resulted from the application of a 1-ng dose (1/10000 of the typical 10-μg dose used routinely in our laboratory) demonstrates that the effect of JHIII on phonotactic thresholds happens at physiological levels of this hormone (see Discussion). In general, significant differences ($P < 0.05$) between the changes in phonotactic thresholds resulted from doses that differed by 100 times or more (with the exception of the three largest dosages). Overall, there

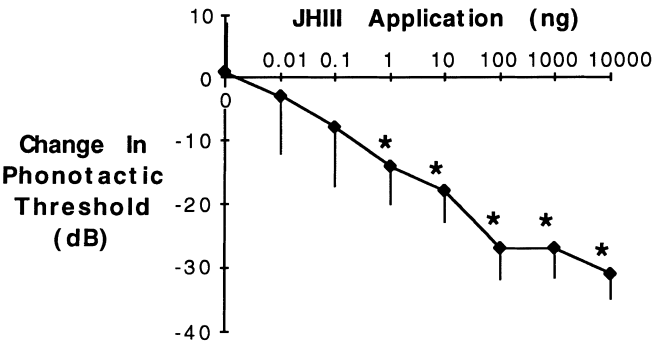

Fig. 1 The effect of topically applying 0.01–10000 ng of juvenile hormone III (JHIII) (in 2 μl of acetone) to the abdomens of ten (in each group) 1-day-old females (with initial thresholds of 80 dB or higher) on the change in mean phonotactic thresholds 12 h later. Fisher's protected least significant difference test was applied to the data at the 0.05 significance level after it was determined, using an ANOVA test (see text) that significant differences existed between the groups of females. An *asterisk* indicates significant difference between that group and the group receiving only acetone (0 ng JHIII). Error bars indicate the standard error of each mean

was a significant correlation (0.626, $P < 0.0001$) between the mean changes in phonotactic thresholds of 1-day-old females and the log of JHIII dosage, demonstrating a quantitative, physiological influence of the amount of JHIII applied on phonotactic thresholds of 1-day-old females.

Effect of allatectomy and JHIII replacement on the thresholds of 5-day-old females

When compared to sham-operated controls, removal of the corpora allata from female crickets (with 60 dB or lower phonotactic thresholds during pretests) resulted in a progressive increase in mean phonotactic threshold (to an average maximum increase of 25–30 dB) between 3 days and 5 days following allatectomy (Fig. 2A). Since the maximum threshold following allatectomy occurred on different days, the 1st day of the maximum threshold for each female was synchronized. Changes in threshold before and after are shown with respect to the day of maximum threshold (day 0, Fig. 2B) and the values between −96 h and +96 h showed significant changes in threshold ($P < 0.0001$, $F = 6.77$). Data presented this way demonstrate that mean thresholds increased over 2 days by about 30 dB to a maximum of approximately 85 dB that was significantly higher ($P < 0.0001$) than any other mean threshold in the histogram. Thresholds then immediately dropped to a level that was approximately 10 dB above and significantly different from ($P < 0.002$ to $P < 0.02$) both the mean thresholds preceding the allatectomy induced increase in threshold and the thresholds of sham controls (Fig. 2). Synchronizing the phonotactic thresholds of the sham controls in a similar way resulted in an increase that was significantly higher ($P < 0.02$ to $P = 0.05$) by 7 or 8 dB than the preceding mean thresholds for sham females at −24, −48 and

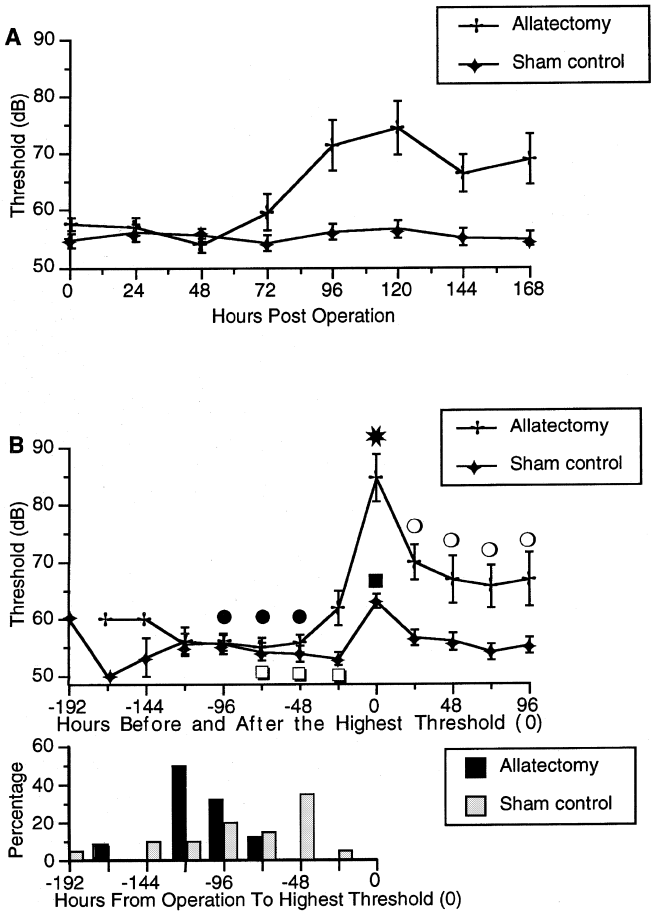

Fig. 2 **A** The effect of removal of the corpora allata on phonotactic thresholds of 5-day-old females which had low (50–60 dB) behavioral thresholds before allatectomy. **A** Mean phonotactic thresholds of 5-day-old females ($n = 16$) following allatectomy and sham controls ($n = 16$) that were tested every 24 h. **B** The first day the female reached the highest threshold following allatectomy was synchronized at time 0 and the thresholds were averaged and shown relative to this highest threshold before and after time 0. The lower graph indicates the number of hours that elapsed between allatectomy and time 0 for the indicated percentage of females. Error bars represent the standard errors of the means. The *asterisk* (★) indicates that the indicated mean at time 0 was significantly different from all other values in the histogram. The pair of symbols (●,○) indicate two groups of means that are significantly different from each other. The pair of symbols (■,□) indicate another two groups of means that are significantly different from each other

−72 h (Fig. 2). Thus about 7 or 8 dB of the increased thresholds shown by allatectomized females could be accounted for by the synchronization of highest thresholds without regard to allatectomy. However, it should be emphasized that the highest mean threshold for the allatectomized females was significantly higher ($P < 0.0001$) than the highest mean threshold for the sham controls (Fig. 2B). The bar graph associated with Fig. 2B indicates that most allatectomized females experienced their highest thresholds 3–5 days following allatectomy, while sham-operated controls varied more widely with the greatest percentage having their highest threshold 2 days following surgery.

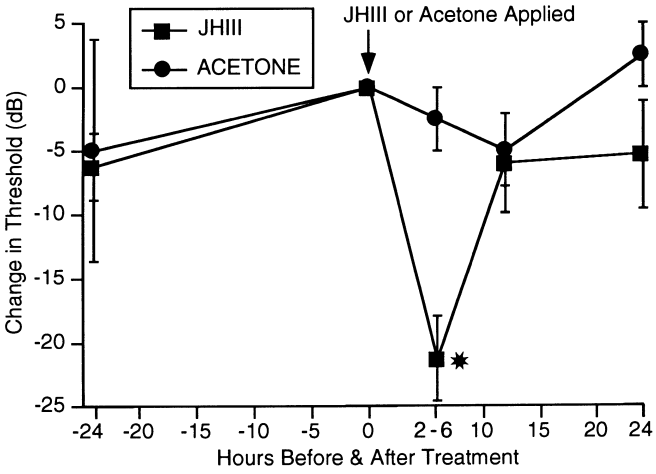

Fig. 3 Mean changes in phonotactic thresholds of 15 females (whose thresholds had increased over 3 days following allatectomy) as the result of topical application of JHIII (10 μg in 2 μl of acetone). The lowest threshold for each female in the 6 h (measured at 2, 4 and 6 h) following the application of JHIII was used to calculate the mean threshold for the time period indicated as 2–6. The symbol (★) indicates a mean that is significantly different from all other means in the graph

Topical application of JHIII to allatectomized females whose thresholds had risen at least 20 dB, resulted in a significant decrease ($P = 0.0002$, $F = 6.42$) of about 20 dB in threshold (that occurred between 2 h and 6 h following JHIII application, Fig. 3) in response to calling songs, while acetone-treated, allatectomized females maintained their higher thresholds (Fig. 3). Thresholds returned to approximately their presurgery levels within 12 h of JHIII application.

Effect of exposure to males on threshold over 5 days following the imaginal molt

Nymphal females, either exposed to males for 7–10 days before the imaginal molt or unexposed control females when isolated from males on the day of the imaginal molt all experienced significant decreases in phonotactic thresholds ($P < 0.0001$, $F = 4.53$) 1–3 days following isolation. Females with olfactory exposure to muted males responded phonotactically to model calls with thresholds that were significantly lower (about 25 dB, $P = 0.001$) on the 2nd day following the imaginal molt than the thresholds of unexposed control females (Fig. 4). Females tested under the same conditions, but exposed to unmuted (normal) males, experienced a significantly lower mean threshold (about 20 dB, $P < 0.02$) than the control females on the 1st day following the imaginal molt, 1 day sooner than the females exposed to muted males and 2 days sooner than the largest decline in the mean threshold of unexposed control females.

Effect of time of day on the thresholds of 1-, 3- and 5-day-old females tested once

Females, 24–30 h old (at the time of testing), tested once at 0600, 1200, 1800 or 2400 hours, exhibited thresholds that were influenced by the time of day the testing took place (Fig. 5). Thresholds were lowest at 0600 and highest at 2400 hours. Although the differences between thresholds were not significant, the trend toward lowest thresholds at 0600 hours will be seen in other testing situations (see below).

Three-day-old females had thresholds that were significantly different ($P < 0.03$, $F = 3.38$) The lowest mean threshold (approximately 57 dB) occurred at 0600 hours and was significantly different ($P = 0.004$) from the highest mean thresholds (approximately 73 dB) at 1800 hours. Mean thresholds at 2400 hours were intermediate and were not significantly different from the other values (Fig. 5). Overall the thresholds of 3-day-old females were lower than those for 1-day-olds and higher that the thresholds for 5-day-old females.

Five-day old females had the lowest and least variable thresholds, in that means varied at different times of day around 55 dB and were not significantly different. They showed no evidence for a diurnal cycle of thresholds that was apparent for 3-day-old females (Fig. 5).

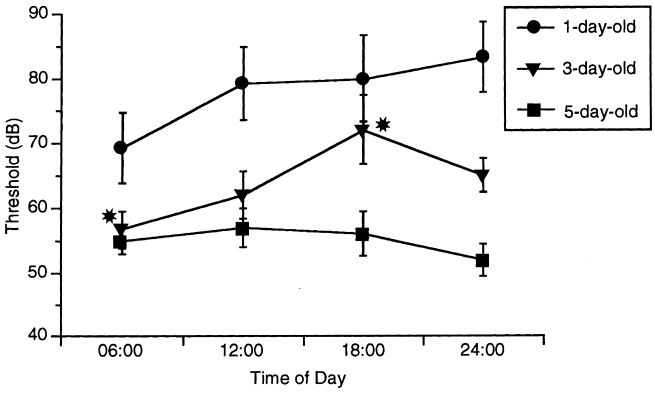

Fig. 5 One- ($n = 12$)-, 3- ($n = 10$)- and 5-day-old ($n = 10$) females were tested once for phonotactic thresholds at the indicated time of day (+ or −1 h). The two means indicated with *asterisks* (★) are significantly different from each other. Error bars represent the standard errors of the means

Effect of repeated phonotactic testing on threshold

Females that were initially tested at 1800 hours on the day following their imaginal molt, and then tested every 12 h over the next 5 days (Fig. 6) showed very different changes in phonotactic threshold than did females in this same age range that were only tested once (Fig. 4). Their mean thresholds did not show a clear progression from relatively high to relatively low over the first 5 days, but remained relatively high and quite variable for the first 2 1/2 days, dropped significantly ($P = 0.01$, $F = 2.50$) on average over 20 dB by the 0600 test on the 3rd day (all other means except for the 0600 tests on the next 2 days were significantly different with significance levels ranging between $P = 0.001$ and $P = 0.008$), returned to the higher levels over the next three tests and dropped again about 15 dB by 0600 hours on the 5th day (which was significantly different from four other means with significance levels ranging between $P = 0.02$ and $P = 0.04$, see Fig. 6). Very similar results were shown by females initially tested at 0600 hours on the day following the imaginal molt and then every 12 h over a similar time period with a clear drop to the lowest threshold at 0600 hours on the 3rd day (data not shown). One-day-

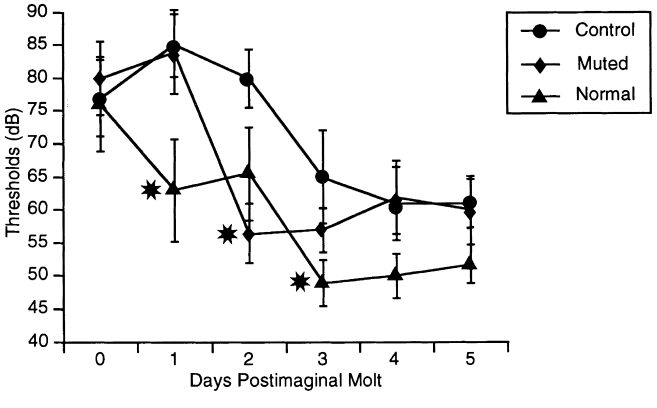

Fig. 4 Females were exposed to normal males, muted males or no males (control) for at least 7 days prior to the imaginal molt, and continuously following the molt up until the single test of phonotactic threshold on each female ($n = 10$ for each point). The *asterisks* (★) indicate means that are significantly different from the mean of the controls at the same time period. Error bars represent the standard errors of the means

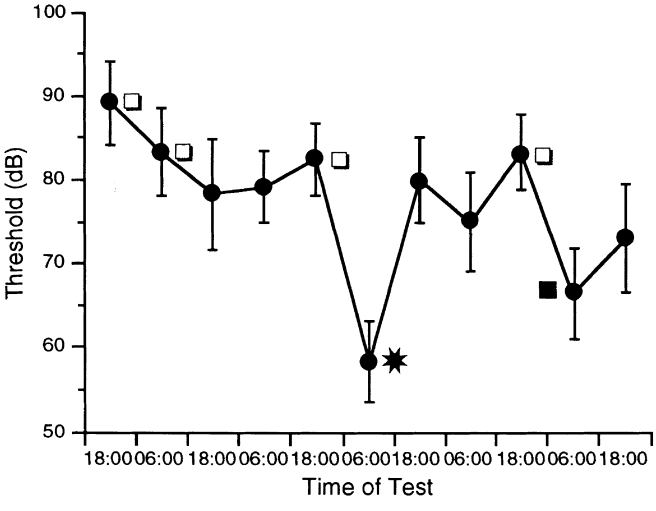

Fig. 6 One-day-old females ($n = 11$) were repeatedly tested for phonotactic threshold every 12 h starting at 1800 hours. The mean indicated with an *asterisk* (★) is significantly different from all other means in the histogram except the one indicated by a (■). The mean indicated with the *symbol* (■) is significantly different from the means indicated with the *symbol* (□). Error bars represent the standard errors of the means

old females that were initially tested at 1200 hours on the day following their imaginal molt, and then tested every 12 h over the next 5 days did not show significant differences between their thresholds at any time (data not shown).

Testing 5-day-old females at 0600 hours and then every 12 h over the next 4 days did not result in significant changes in the mean thresholds of their phonotactic responses. The most apparent effect of repeated testing at these intervals was in the increased variability of the thresholds of individual females (Fig. 7A). The large asterisks shown just above the *x*-axis of Fig. 7A indicate significant increases in the variability of the female's thresholds (variance ratios for the asterisked time periods, calculated by comparing the variance of the females tested at subsequent time periods with the variance of the first test, yielded F values ranging between 5.1 and 13.1, and P values < 0.05). Five-day-old females, that were initially tested at 1200 hours and then every 12 h for 4 days yielded similar results (data not shown).

Repeating the tests on 5-day-old females at 12-h intervals (starting at 1400 hours) also yielded significant increases in the variability of the female's thresholds at 12, 60 and 72 h following the initial test (Fig. 7B). Testing other groups of females at either 6-h or 24-h intervals did not significantly effect the variability of the female's phonotactic thresholds (Fig. 7B). Testing 5-day-old females every 6 h resulted in significant changes in mean phonotactic threshold ($P = 0.006$, $F = 2.28$). Mean thresholds that were significantly higher than all other thresholds tested occurred at 0800 hours on the first 2 days of testing, with lowest thresholds taking place at 2000 hours (Fig. 7B). Females tested every 24 h when they were 5 days old had phonotactic thresholds that were significantly higher ($P = 0.002$ to $P = 0.03$) at the initial test than for all of the subsequent tests for this group. However, inspection of the data demonstrated that the higher mean threshold for the group was largely the result of 80- to 90-dB thresholds for three of the ten females, whose thresholds dropped and remained low for all of the subsequent tests. A larger group of 28-day-old females, tested every 24 h, had mean phonotactic thresholds between 55 dB and 60 dB (data not shown) at all testing periods.

Comparing the thresholds of the three groups of females (Fig. 7B) tested every 6, 12 or 24 h at 24-h intervals demonstrated significant differences ($P < 0.02$, $F = 2.1$) between the thresholds of the females in these groups. Significantly higher thresholds for the group tested every 12 h in comparison with the group tested every 24 h occurred at 24 ($P = 0.04$) and 48 ($P = 0.003$) hrs following the first test for these two groups.

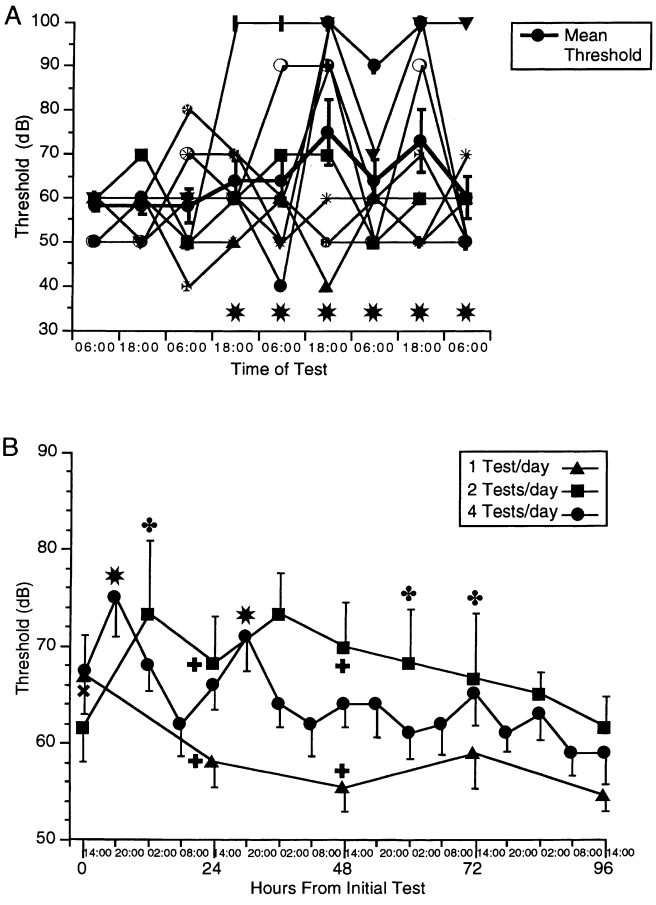

Fig. 7 **A** Five-day-old females ($n = 10$) were repeatedly tested for phonotactic threshold every 12 h starting at 0600 hours. The *individual, thinner lines* represent the behavior of individual females, while the *heavy black line* with error bars represents the mean threshold of all females. The *asterisks* (★) indicate significantly greater variability at the indicated period as compared to the first test. Error bars represent the standard errors of the means. **B** Five-day-old females were repeatedly tested for phonotactic thresholds every 6, 12 or 24 h as indicated. For the females tested every 6 h ($n = 9$) thresholds were significantly higher at 6 h and 30 h (★) than at all other time periods (except for 12 h and 30 h). For the females ($n = 10$) tested every 12 h, three time periods (+) had significantly larger variability than the variance during the first test. For females tested every 24 h ($n = 11$), the mean threshold (×) at the first test was significantly higher than at the other time periods. At 24 h and 48 (+) h, the mean thresholds of the females tested every 12 h were significantly higher than for females tested every 24 h. The mean thresholds for each group are shown with error bars (standard errors)

L1 auditory interneuron thresholds – correlations with behavioral thresholds

In 1-day-old females Stout et al. (1991) found that L1 neuron thresholds were frequently high (70+ dB) and correlated well with behavioral thresholds measured in other females of the same age. As part of this investigation, L1 thresholds in 1-day-old females, whose behavioral thresholds were known, were measured in five groups of females under different conditions; three involved different approaches to recording the L1 threshold, and two involved manipulating the female's JHIII level. Initially (group 1, Fig. 8), L1 thresholds in 1-day-old females with behavioral thresholds ranging from 40 dB to 90 dB were recorded intracellularly and it was found that L1 thresholds corresponded very well

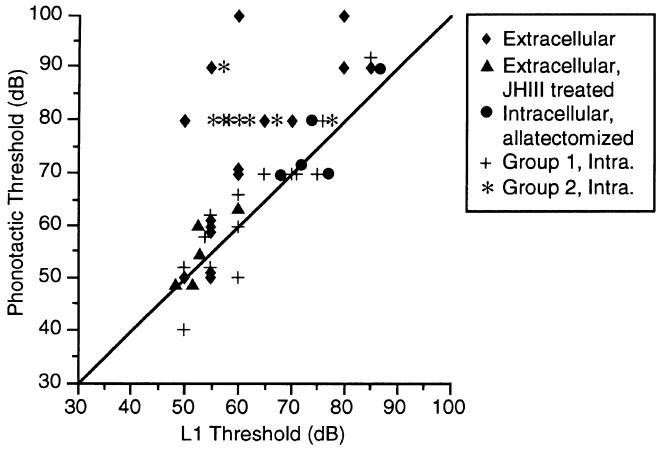

Fig. 8 The phonotactic and L1 thresholds of individual 1-day-old (43) and 8-day-old (5) females are plotted against each other for the indicated groups of females. A point falling to the left of the diagonal line represents an L1 threshold that is lower than the phonotactic threshold of the same female

with behavioral thresholds. However in this group, only two females had behavioral thresholds that were higher than 70 dB. Group 2, using only females whose behavioral thresholds were 80 dB or 90 dB, demonstrated that L1 thresholds could range from just below behavioral threshold to as much as 35 dB lower than behavioral threshold. Intracellular recordings used for the first two groups resulted from penetrating the L1 neuron in, or immediately adjacent to its integrative areas within the prothoracic ganglion. In order to evaluate whether the differences between these two sets of data resulted from different techniques by two different investigators, another group (extracellular) of 1-day-old females was tested. Extracellular recordings of the axons of putative L1 neurons within the cervical connectives were used which left the structure of the L1 neuron in its integrative areas untouched. Results from this group, which included females whose behavioral thresholds ranged from 50 dB to 100 dB, demonstrated that for females with behavioral thresholds of 70 dB or lower, the threshold of these putative L1 neurons was no more than 10 dB lower than the same female's behavioral threshold. In females whose behavioral thresholds were 80 dB or higher, the threshold of putative L1 neurons ranged from just below the behavioral threshold to as much as 40 dB lower than behavioral threshold (Fig. 8). Manipulating 1-day-old females' JHIII levels by applying 10 μg of JHIII resulted in L1 (recorded extracellularly) and behavioral thresholds, measured 12 h later, that were all between 50 dB and 60 dB (Fig. 8). Allatectomizing 5-day-old females that had phonotactic thresholds of 60 dB or lower, waiting 3 days for the threshold to increase at least 20 dB, resulted in L1 thresholds (recorded intracellularly) that were between 70 dB and 90 dB, and were within 5 dB of the female's phonotactic threshold (Fig. 8).

Discussion

From the results presented above, it is clear that a number of external and internal factors influence the phonotactic thresholds of female *A. domesticus*. While the determination of external and internal changes that cause phonotactic thresholds to rise or fall is important, for this study the theme that integrates these rather diverse results is to evaluate the proposal by Stout et al. (1991) – see also Koudele et al. (1987) and Walikonis et al. (1991) – that phonotactic thresholds are regulated by changes in JHIII levels expressed through the influence of JHIII on the thresholds of the L1 auditory interneuron. Consideration below of the individual results leads to a reconsideration of the role of JHIII in regulating phonotactic thresholds of female *A. domesticus*.

Effect of applied JHIII on the development of phonotaxis by 1-day-old females

JH titers in hemolymph, in addition to the synthesis of JH by the corpora allata, is influenced by changes in the levels of juvenile hormone esterase (Ichinose et al. 1992) resulting in different half-lives for JH in vivo for insects of the same species under different circumstances. Possible sequestering of a JH pool outside the hemolymph (Tobe et al. 1985) could also make JH available to the insect tissues in spite of low hemolymph titers. Thus, in this study, these conditions could cause differences in the resulting JHIII titers in the *A. domesticus* females receiving the same dose of applied JHIII even if the JHIII were to enter the hemolymph at approximately the same rates. However, in spite of these intervening variables, application of JHIII to 1-day-old female *A. domesticus* with high phonotactic thresholds had, as discussed below, rather consistent and quantitative effects on increasing their responsiveness to models of the male's calling song.

One-hundred ng of JHIII applied in acetone to the integument of 1-day-old female *A. domesticus* (1/100 of the 10-μg dose used by Stout et al. 1991 to demonstrate the influence of JHIII on the phonotactic thresholds of 1-day-old females, and in more than 100 experiments done since then) caused the same degree of reduction in phonotactic thresholds as the 10-μg dose (Fig. 1). A reduction to 1/10000 (1 ng) of the original 10-μg dose still caused a significant average reduction in the phonotactic thresholds of 1-day-old females when compared to the acetone-treated controls.

One ng of JHIII dispersed in 50 μl of hemolymph (the amount of hemolymph that could frequently be extracted from an adult female *A. domesticus* – unpublished results) would give a concentration of 2 ng or 7.5 pmols of JHIII/100 μl of hemolymph (and a 100-ng dose would give JHIII concentrations of 750 pmol/100 μl) if all of the JHIII applied to the surface moved across the integument and into the hemolymph simultaneously. Renucci and Strambi (1983) found that JHIII

titers in the hemolymph of 1- to 5-day-old female *A. domesticus* varied between a minimum of about 6 pmol/100 µl and a maximum of approximately 30 pmol/100 µl with concentrations in most measurements at or above 10 pmol/100 µl. Measurements of titers in females in this age range from our colony (A. and C. Strambi, unpublished data) also fell within this range, but measurements of titers in three female *A. domesticus* that were 17 or 18 days old were lower (Loher et al. 1992). While we do not know the rate of entry of JHIII into the hemolymph of these females, it will not all enter at once, and some of the entering JHIII will be inactivated by JH esterases (Woodring and Sparks 1987). Therefore, based on available information, the 1-ng dose applied to the integument would probably produce lower than normal JHIII titers, while the 100-ng dose, if approximately 1/20 of the applied JHIII were in the hemolymph at any one time, would produce titers at the high end of the range measured by Renucci and Strambi (1983).

Since these experiments (Fig. 1) involved 80 1-day-old females, they extend the initial results reported by Stout et al. (1991) and clearly demonstrate that the level of JHIII available in the body of a 1-day-old female is an important factor determining the female's phonotactic threshold. The significant correlation between the mean phonotactic thresholds of 1-day-old females and the log of JHIII dosage (Fig. 1) demonstrates a quantitative influence of the amount of JHIII applied on phonotactic threshold. The effectiveness of low, physiological doses suggests that this applied JHIII influences the phonotactic thresholds of 1-day-old females in ways that represent a normal developmental effect of JHIII on the onset of phonotaxis in young females of this species. The increasing levels of JHIII titers (Renucci and Strambi 1983) and in JHIII production by the corpora allata of 1- to 4-day old females of this species (Walikonis et al. 1991) during a time when the phonotactic thresholds of these same females were decreasing further supports a developmental role of JHIII for the onset of phonotaxis in post-imaginal females. However, females, allatectomized as nymphs, still become phonotactically responsive as adults (Loher et al. 1992), suggesting that although the presence of JHIII in females during the 2–3 days following the imaginal molt influences the time of development of phonotaxis, its absence does not prevent the female from becoming phonotactically responsive. Unfortunately, Loher et al. (1992) did not compare the time and dynamics of the onset of phonotaxis in females allatectomized as nymphs with normal females following the imaginal molt. Thus their data only indicate that other unidentified factors can cause the development of phonotaxis in the absence of JHIII.

Effects of allatectomy on phonotactic responsiveness by females

The phonotactic thresholds of 5-day-old female *A. domesticus* significantly increased by 20–30 dB, 3–5 days following allatectomy and then dropped within 24 h to a level about 10 dB higher than before allatectomy (Fig. 2B). Sham-operated controls did not exhibit such changes in behavioral threshold. Topical application of JHIII to the allatectomized females significantly reduced their phonotactic thresholds by an average of about 20 dB within 6 h (Fig. 3). These results clearly demonstrate the importance of JHIII for the maximum sensitivity of phonotactic responsiveness by mature, sexually responsive females. Thus they substantiate a role for JHIII in regulating phonotactic responsiveness of female *A. domesticus*, as suggested by Koudele et al. (1987).

However, the animals deprived of their corpora allata continued to exhibit phonotaxis at higher thresholds (Fig. 2). Loher et al. (1992) also reported that allatectomized female *A. domesticus* continued to respond phonotactically, in spite of the absence of JHIII in the hemolymph (Loher et al. 1992). In comparing these results with ours, it is not clear that their procedures would have "caught" the short-term (24–48 h) relatively large increases (20–30 dB) in thresholds, 3–5 days following allatectomy. They describe the behavior of one female in detail whose behavioral thresholds were followed for several days. This female showed increases (apparently 10–20 dB) in threshold several days following allatectomy. These results are consistent with the significant, longer-term increases in threshold of, on average, 10 dB that are demonstrated for 16 female *A. domesticus* in the present study (Fig. 2B). Loher et al. (1992) do not indicate quantitative measures of the degree of change in threshold following allatectomy of a number of females, or variability of that change, and do not indicate the daily testing paradigms for more than one female. They report only the number of tests (a maximum of seven spread over 5–12 days for females allatectomized as adults and a maximum of five spread over 10–21 days for females allatectomized as nymphs) with or without positive phonotaxis for all but one female. Since each test typically used stimuli that ranged from lower to higher intensities, their results could reflect phonotaxis only in response to the most intense stimuli and it would only be apparent in the data of the one female reported in detail (where it was seen – see above). Thus, their data cannot be compared in detail with the results this study describes from testing 16 females daily following allatectomy.

However, Loher et al. (1992) correctly point out that:
1. Koudele et al. (1987) over-interpreted the reduced responsiveness demonstrated for allatectomized female *A. domesticus* with the suggestion that allatectomy eliminated phonotaxis.
2. Phonotaxis continues in the absence of JHIII (at higher thresholds in our study and apparently in theirs). The present data demonstrate a short-term, large increase in threshold of phonotaxis, and a longer term, smaller increase following allatectomy. However, both the present data and those described by Loher et al. (1992) demonstrate that factors other than the avail-

ability of JHIII also control the development and regulation of the phonotactic response of female *A. domesticus*. Unfortunately, neither their study nor ours sheds light on what these other factors might be.

Since this study has been devoted exclusively to female *A. domesticus*, no comparisons with the reported failure of JHIII to influence phonotactic thresholds of other cricket species (Loher et al. 1992) are possible. However, it should be noted that the influence of JH on adult insect reproductive behavior and physiology varies widely, even in closely related species (Raabe 1989).

Effect of exposure to males on the development of phonotaxis

Exposing females to muted males, starting 7–10 days preceding the imaginal molt, strongly suggests that olfactory contact (the females were unable to touch the muted males) with males significantly accelerates (by 1 day) the normal developmental decrease in phonotactic thresholds experienced by unexposed (control) females (Fig. 4). Exposing females in the same way to males that could produce the calling song (normal males) resulted in significant decreases in phonotactic thresholds 2 days sooner than the control females, thus suggesting an additive effect of both olfactory and auditory exposure on the females developmental onset of phonotaxis (Fig. 4). Exposing the females only after their imaginal molt to males had little effect on the developmental onset of phonotaxis (data not shown). Since the ear of the female is only fully formed and on the surface of the prothoracic legs of adults and the thresholds of auditory interneurons that receive sensory input from the ears are much higher (>90 dB, Atkins and Pollack 1986) in last-stage instar crickets than adults, our results suggest that olfactory exposure to the males during larval development is effective in accelerating the development of phonotactic behavior and probably sets the stage for the effects of hearing the calling song during the 1–2 days of adult life that preceded the onset of low threshold phonotaxis. Koudele et al. (1987) demonstrated an influence of exposure of adult virgin females to males (under conditions that also prevented visual or tactile contact) on phonotaxis that resulted in orientation that was not as direct to the source of model calling songs. This effect was also greatest when females were exposed to unmuted (normal) males. However, this exposure to males did not influence the female's readiness to mate.

Diurnal changes in phonotactic thresholds

Measuring the phonotactic thresholds of 1-, 3-, and 5-day-old females (only once for each female) at 0600, 1200, 1800 and 2400 hours indicated a daily change in phonotactic thresholds for 3-day-olds with lowest thresholds in the early morning and significantly highest thresholds in the late afternoon (Fig. 5). Although mean phonotactic thresholds for 1-day-old females were also lowest in the early morning, the diurnal changes in threshold were quite variable and thus not significantly different. Five-day-old females did not exhibit a diurnal rhythm in phonotactic thresholds. Overall thresholds were highest in 1-day-olds, intermediate in 3-day-olds and lowest in 5-day-females as would be expected from the results reported by Stout et al. (1991) and from the data provided from control experiments shown in Fig. 4. The diurnal rhythm in thresholds observed in 3-day-old females might suggest an underlying rhythm in JHIII titers and/or L1 auditory neuron thresholds as a basis for the observed daily changes in threshold; however, at present there is no information to evaluate those possibilities. Since phonotaxis develops (Loher et al. 1992) and persists (Fig. 2) in the absence of the corpora allata, other, as yet unidentified, regulatory mechanisms may underlie these diurnal changes in phonotactic threshold.

Effect of repeated testing on phonotactic thresholds

Repeated testing of the phonotactic thresholds of females every 12 h, starting when the females were 1 day old, resulted in a very different progression in the development of low thresholds over the first 5 days of adult life (Fig. 6A) than were observed in females of the same ages tested only once (Figs. 4, 5; Stout et al. 1991). The well-synchronized, significant decrease in thresholds at 0600 hours on the 3rd day of testing, followed by a return to higher thresholds seen in females repeatedly tested at 1800 and 0600 hours (and those tested initially at 0600 hours followed by 1800 hours) were not seen in 3- and 5-day-old females tested once (Fig. 5). At the end of the 5-day testing period, the average thresholds were 10–20 dB higher than the thresholds of females of the same age that had only been tested once. Interestingly, the low threshold observed at 0600 hours on the 3rd day of testing, was at the same time of day that 1 day- and 3-day-old females, tested only once (Fig. 5) exhibited their lowest thresholds. Females repeatedly tested at 1200 and 2400 hours (or 2400 and 1200 hours) did not show a well-synchronized, single, clear decrease in thresholds on any of the 5 days of testing (data not shown).

It is apparent from these data that repeated testing of females every 12 h, beginning on day 1 (Fig. 6), changes the developmental onset of phonotactic sensitivity, as compared to females of the same ages tested only once (Fig. 5). The repeatedly tested females showed significantly more interfemale variability in phonotactic thresholds than did females tested only once on that same day. Repeatedly tested females demonstrated thresholds of 60 dB or lower from 1 to several days earlier than females that were tested only once but returned to higher thresholds following this low-threshold response (data not shown).

Five-day-old females with initially low phonotactic thresholds also became quite variable in phonotactic thresholds as the result of phonotactic testing every 12 h (Fig. 7A). Testing 5-day-old females every 24 h, every 12 h or every 6 h, demonstrated that the effect of repeated testing was dependent on the testing period (Fig. 7B). Females tested every 12 h experienced significantly greater variability in thresholds while those tested every 6 or 24 h showed little change in variability. Females tested every 6 h experienced diurnal changes in threshold during the first 2 days, with thresholds significantly highest in the evening (2000 hours) and lowest in the morning (0800 hours). The significantly higher thresholds during the first test for females tested every 24 h were largely the result of several 5-day-old females with high thresholds during the initial test which dropped to values equivalent to the other females in subsequent tests. Since this initial high threshold has not been observed in older females tested every 24 h, it is most likely that this result was based on 5-day-old females whose thresholds had not yet reached their lowest values.

In summary, the effects of repeated testing on the phonotactic thresholds of both 1- and 5-day-old females are complex with increased variability in thresholds as the most consistent effect of multiple tests repeated at 12-h intervals. Since females tested every 24 h did not become more variable in thresholds, the effect of phonotactically testing a female apparently diminishes somewhere within the next 12–24 h in 5-day-old females. Testing 5-day-old females every 6 h results in a diurnal rhythm with lowest thresholds in the morning and highest thresholds in the evening. While it is tempting to suggest that the effects of multiple testing on phonotactic thresholds are the result of auditory exposure and/or a phonotactic response by the female, the data at this point do not allow us to distinguish between the effects of handling the female crickets before and after testing and the auditory response produced by the females. It seems unlikely that the effects of multiple testing of female crickets are expressed solely, or primarily through underlying changes in the availability of JHIII. This is especially so for increasing thresholds as the result of multiple testing since depriving females of the source for JHIII by allatectomy (Fig. 2) requires 3 or more days for the effects to become apparent as increasing phonotactic thresholds.

Correlation between phonotactic and L1 neuron thresholds in the same females

Atkins et al. (1992) demonstrated that the response of the L1 neuron to model calls produced at intensities below 70 dB was necessary for phonotaxis to occur. In more than 400 intracellular recordings and stainings of ascending auditory interneurons, the L1 neuron (100+ recordings; Stout et al. 1989; Atkins et al. 1992; Stumpner et al. 1995 and numerous unpublished recordings) and L3 neuron (300+ intracellular recordings and stainings; Atkins et al. 1988; Atkins et al. 1989; Henley et al. 1992 and numerous unpublished recordings), were the only ascending auditory interneurons encountered whose responses to 5-kHz calling songs suggested that they were important elements in the neuronal network that underlies calling song phonotaxis in female *A. domesticus*. The failure to encounter other types of ascending neurons (during this large number of recordings and stainings), with these response properties, but different morphology, implies that L1 was the only ascending pair of neurons that encoded the structure of the male's call at intensities below 70 dB at 5 kHz. For L1 recordings with thresholds of 70 dB or higher, the response dynamics were consistently similar to the responses of units with lower thresholds (Stout et al. 1988; Stumpner et al. 1995) and readily distinguishable from the L3 neuron (Atkins et al. 1988; Henley et al. 1992). Identification of the L1 was confirmed with the cell's morphology. In this study extracellular recordings of neurons with thresholds lower than 70 dB (Fig. 8) could only have been from the L1 neuron. For those recordings with thresholds of 70 dB or higher, response properties of the units included in Fig. 8 were clearly typical of L1 and readily distinguishable from the L3 neuron which was frequently recorded from the same animal.

Stout et al. (1991) proposed that control of phonotactic thresholds was largely the result of hormonal regulation (JHIII) of the threshold of the L1 auditory interneuron. This hypothesis was reasonable in light of Atkins' et al. (1992) demonstration that the response of the L1 neuron to a calling song was necessary for a female to respond to calling songs produced below 70 dB and the demonstration that JHIII could induce parallel decreases in phonotactic and L1 thresholds in the same 1-day-old females (Stout et al. 1991). However, in this study intracellular and extracellular recordings demonstrated that L1 auditory interneurons can have thresholds that are 20–30 of dB lower than the phonotactic thresholds of the same females (with behavioral thresholds that are 80 dB or higher, Fig. 8). Thus, a factor(s) in addition to L1's threshold is also involved in controlling phonotactic thresholds.

Both L1 threshold (Stout et al. 1991; Fig. 8) and the unidentified factor(s) (Figs. 1, 8) are responsive to JHIII. Unfortunately there is, at present, no evidence suggesting what the mechanisms in addition to L1 threshold might be. However, topically applying JHIII on the prothoracic ganglion, or microinjecting it into the prothoracic ganglion induced a 20-to 30-dB decrease in phonotactic thresholds within 2 h in 1-day-old females with high behavioral thresholds (control applications to other ganglia had no effect, Stout et al. 1991). Thus, input into mechanisms which can translate increased JHIII availability into lower phonotactic and L1 thresholds must exist within the prothoracic ganglion.

Since L1s whose thresholds are more than 10 dB below the female's behavioral threshold are only found in 1- to 2-day-old females, before the decline in the females' phonotactic thresholds that usually occurs between 2 and 5 days of age (Fig. 4), JHIII treated 1-day-old females have both phonotactic and L1 thresholds that are between 50 dB and 60 dB (Fig. 8, Stout et al. 1991), and allatectomized 5-day-old females have L1 and phonotactic thresholds between 70 dB and 90 dB, it is possible that although JHIII influences both L1 and phonotactic thresholds, L1 threshold is more responsive to JHIII levels than is the, as yet unidentified, mechanism(s) that can keep the 1-day-old female's phonotactic threshold above the L1 threshold.

Regulation of phonotactic thresholds in female *A. domesticus*

In newly molted adults, increased levels of JHIII accelerate the onset of phonotactic responsiveness in a dose-dependent manner (Fig. 1). After phonotactic thresholds have reached their minimum values and the females are most sensitive to the male's calls, maintenance of maximal sensitivity is dependent on the presence of JHIII (Figs. 2, 3). The reduction of phonotactic thresholds that followed within 12 h of applying JHIII to the integument of 1-day-old females (Fig. 1) suggests that increased JHIII production could have been responsible for the accelerated reduction in phonotactic thresholds which resulted following exposure of nymphal females to males (Fig. 4). However, the 4–5 days that it took for the phonotactic thresholds of most allatectomized females to maximally increase (Fig. 2B) indicates that the 6–12 h over which phonotactic thresholds increased diurnally (Figs. 5, 7B) or as the result of repeated testing every 12 h (Fig. 7A) are too fast to be explained solely by an externally induced reduction in JHIII titers.

Although L1 response is necessary for phonotaxis (in response to low intensity CSs, Atkins et al. 1992) it is clear that other factors in young females can keep phonotactic thresholds above the L1 threshold. Thus we propose that phonotactic thresholds in female *A. domesticus* are regulated by JHIII and other influences on the both the threshold of the L1 auditory interneuron and on other, as yet unidentified, neurons.

Acknowledgements This research was supported by National Science Foundation grant IBN 92–22129, and by Andrews University faculty research grants to J.S. and G.A. Several undergraduate coauthors were supported by N.S.F. Undergraduate Fellowships. Reviews of the manuscript by Prof. Dr. Franz Huber, Dr. Andreas Stumpner and an anonymous reviewer are gratefully acknowledged.

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