EXPOSURE TO MALE PHEROMONES ENHANCES *ANASTREPHA SUSPENSA* (DIPTERA: TEPHRITIDAE) FEMALE RESPONSE TO MALE CALLING SONG

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ABSTRACT

Anastrepha suspensa (Loew) females are attracted to traps baited with male pheromone and/or broadcast calling song, but a high variability in female responsiveness has hindered attempts to use such attractants in practical trapping systems. Prior experience is one factor that may contribute to variability in female responses. To investigate this possibility, female responses to male calling song were compared after 38-40-h prior exposure to different combinations of live males, live females, synthetic pheromone components, and broadcast song. The broadcast song, obtained from a sexually successful male, contained a series of wing-fanning pulse trains averaging 0.31-s in duration, separated by 0.36-s quiet intervals. Within the pulse trains, the frequency rose quickly from ~125 to 148 Hz and then slowly declined to ~120 Hz. The proportions of females responding to the broadcast calling song were greatest when females were first exposed to live males or pheromone components. These proportions significantly exceeded 0% difference between the proportions under silent and broadcasting speakers. The proportion of females that responded after prior exposure to broadcast song alone was significantly higher than the proportion that responded after no prior exposure to sexual stimuli, but it did not significantly exceed 0%.

Key Words: calling song, pheromone, *Anastrepha suspensa*, attraction

RESUMEN

Hembras de *Anastrepha suspensa* Loew son atraídas a trampas con señuelo de feromonas masculinas y/o transmisión de canción de llamado, pero una alta variabilidad de reacción en la hembra ha impedido intentos para desarrollar sistemas prácticos de trampas. Un factor que puede contribuir a la variabilidad de reacción es experiencia previa a señales sexuales. Para investigar este efecto, respuestas de hembras a canción de llamado fueron comparadas después de 38 a 40-h de pre-exposición a diferentes combinaciones de machos, hembras, feromona masculina, y transmisión de canción. El estimulo de canción fue estandarizado por copias concatenadas de una grabación de 7.2-s de un macho sexualmente exitoso, y contenía trenes de pulsos de abanicos de ala promediando 0.31-s de duración, separados por intervalos de silencio de 0.36-s. Dentro de los trenes de pulso, la frecuencia subió rápidamente de ~125 a 148 Hz y después declinaron lentamente a ~120 Hz. Las proporciones de hembras que respondieron a las transmisiones de canción de llamada fueron las mayores cuando las hembras fueron pre-expuestas a machos o feromona masculina. Estas proporciones significativamente excedieron niveles nulos. Las proporciones de hembras que respondieron después de pre-exposición solo a transmisión de canción fueron significativamente mayores que las proporciones de hembras respondientes que no habían tenido pre-exposición al estimulo sexual, pero en ningún grupo las proporciones excedieron niveles nulos.

Sexual courtship in the Tephritid pest, *Anastrepha suspensa* (Loew) ('caribfly'), involves male-produced pheromonal (Nation 1972), acoustic (Webb et al. 1976), and visual signals (Burk 1981) that are attractive to females and consequently have potential uses in trapping and monitoring programs. These signals are usually presented to females by males that aggregate in leks on larval host plants in late afternoon (Burk 1983, Norrbom & Kim 1988). Males compete for single-leaf territories on which they emit pheromone, produce a calling song generated by repeated bursts (trains) of wing-fanning pulses, and semaphore with patterned wings (e.g., Sivinski & Burk 1989, Aluja et al. 2000). When a female lands on his leaf, he approaches and mounts her if she permits, producing an intense precopulatory song while attempting to engage her genitalia (Sivinski et al. 1984). The precopulatory song is continuous rather than pulsed, and is more intense and higher in frequency than the calling song (Webb et al. 1984). As in most systems where courtship occurs in aggregations (Alexander et al. 1997), males vary considerably in their mating success.

Bioassays designed to identify the signal characteristics that distinguish sexually successful males from nonmaters have yielded ambiguous results. For example, initial laboratory studies comparing female responses to different combinations of pheromone and calling song suggested that calling song combined with pheromone was more attractive than pheromone alone (Webb 1973, Chambers 1975). In field-cage studies, however, statistically significant numbers of females were captured in traps baited with live males, pheromone alone, and broadcast calling song alone, but not in traps with a combination of broadcast song and pheromone (Webb et al. 1983).

Additional ambiguities have appeared in studies comparing responses to songs that contained systematic differences in acoustic parameters. Sexually successful caribfly males produce songs generated from precisely featured bursts of wing fanning pulses (example in Fig. 1). The bursts have a mean pulse-train duration (PTD) of ~0.32 s and are separated by ~0.34-s pulse train intervals (PTI) (Sivinski & Webb 1986). The mean inter-pulse interval (IPI) is ~7.14 ms, corresponding to a frequency of ~140 Hz (Sivinski & Webb 1986). Changes in the magnitudes of one or more of these parameters often result in reduced responsiveness of females. For example, Sivinski et al. (1984) found that female caribflies failed to respond to songs recorded from conspecific males with pulse trains of typical frequency and duration (~140-Hz pulses, 0.273-s PTD) but atypically long PTI (1.115s). Female caribflies were also unresponsive to male precopulatory song and to song produced by a male Queensland fruit fly, *Bactrocera neohumeralis* Hardy. The Queensland fruit fly produces song with the correct PTI but a higher frequency (~379 Hz) and shorter PTD (~0.12 s) (Sivinski et al. 1984). Such results suggest that the songs most attractive to female caribflies are those with low frequencies, long PTDs, and short PTIs. Indeed, Burk & Webb (1983) had reported that females mate preferentially with larger males, and that larger males produced lower frequency songs with shorter PTIs than smaller males. Other studies, however, did not always find that larger males produce song with shorter PTIs (Webb et al. 1984).

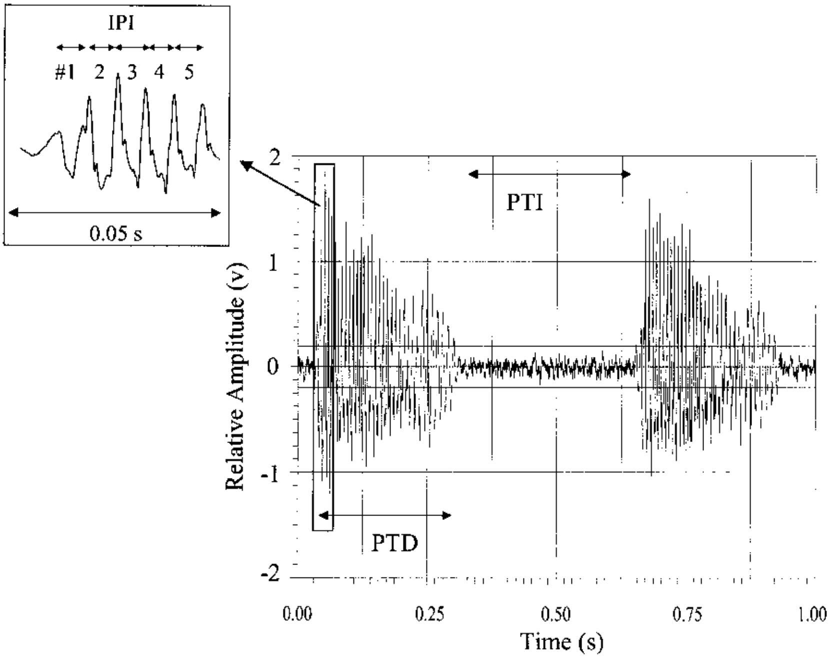

Fig. 1. Example of 2 pulse trains in a recording of male caribfly calling song, with inset showing 6 individual pulses at the start of 1st train. The horizontal axis shows time in seconds. The vertical axis shows the microphone signal on a relative scale (see text for amplification details): PTD, pulse train duration; PTI, pulse train interval; IPI, interpulse interval; horizontal axis shows time (s); vertical axis shows microphone signal in relative scale.

Some of this ambiguity may have resulted from a lack of understanding of potentially important features of caribfly pulse trains. Historically, calling songs were described simply by their mean frequencies (e.g., Webb et al. 1983, Sivinski & Webb 1986). However, Webb et al. (1987) and others observed later that the frequency is not constant within pulse trains, but decreases toward the end of each train. The magnitudes of these frequency changes have not been characterized and their effects on female response are unknown.

Additional ambiguity may be explained by variability in the levels of female responsiveness (*cf.* Searcy & Andersson, 1986). Prior experience with sexual signals is one potential contributor to this variability. Caribfly males are known to change their calling patterns in the presence of females and other males (Sivinski & Webb 1986). However, the effect of prior experience on female caribfly behavior has not been investigated.

The objective of this study was to investigate the effect of prior exposure to sexual stimuli on female caribfly responsiveness to broadcast song. We conducted a series of bioassays during which we exposed virgin females to different combinations of pheromone and/or male calling song prior to experiments, and then assessed their responses to play-back of precisely characterized song recorded from a sexually successful male.

MATERIALS AND METHODS

Insects

Caribflies used in this study were obtained as pupae from the Florida Department of Agriculture, Division of Plant Industry in Gainesville, Florida. After eclosion, adult flies were given water and a 3:1 mixture of refined cane sugar and hydrolyzed brewer's yeast. They were maintained in a laboratory with a photoperiod of 12:12 h (L:D) at room temperature and ambient humidity. Adult flies were sorted by sex 3-4 days after eclosion, placed in cubic screen cages (30-cm per side), and the females thereafter were kept in a separate "female room". Because most males do not signal sexually until after 5-7 d (Sivinski 1994), the sorted females were unlikely to have been exposed to significant amounts of male pheromones.

Sexually mature females (10-18 d old) were used for all experiments. They had no exposure to adult live males from time of sorting until use in an experiment unless otherwise stated.

Male Calling Song

The acoustic signals were generated by concatenating multiple copies of a 7.2-s segment of song from a sexually successful male (Fig. 2) onto an endless loop tape. Webb et al. (1983) used this same song segment to produce a continuous-loop recording that successfully captured female caribflies when it was broadcast from a trap in a field cage. The first two pulse trains of the signal are shown on smaller time scales in Figure 1.

Webb et al. (1976, 1983) measured temporal patterns and the mean frequencies and Sound Pressure Levels (SPLs) of *A. suspensa* calling song bursts, but frequency patterns and SPLs within bursts could not be measured using the technology then available. For this report, we analyzed the dynamic features of the song bursts using a Bruël and Kjaer (B & K) model 4145 microphone, a model 2639 preamplifier, and a model 2610 measuring amplifier (Mankin 1994). Signals were amplified 20-40 dB (where dB = 20 $\text{Log}_{10}(V_{out}/V_{inp})$, and V_{out}, V_{inp} are the amplifier output and input voltage levels, respectively) and digitized at 25 kHz using a 12-bit MetraByte (Keithley/MetraByte Inc., Taunton, MA) DAS-16G A/D converter installed in a Pentium 350-mHz microcomputer. The digitized signals were analyzed with custom-written software (Mankin 1994, Mankin et al. 1996a). The customized software located the peak of each wingbeat pulse, marked its time within the recording, and measured the inter-pulse interval (IPI, Fig. 1). A pulse train was identified as a series of unbroken pulses separated by an IPI of no more than 20 ms (approximately 3 typical pulses). A custom-written subroutine noted the order of each pulse within the train and calculated the instantaneous frequency (1/IPI). Another subroutine noted the beginning and end of each pulse train for calculations of pulse train duration (PTD) and interval (PTI).

The mean frequency within the pulse train was calculated as an average for each IPI measurement in the train, based on its order number from the beginning of the train (e.g. IPI #'s 1-5 in Fig. 1). The mean frequency at the 5th IPI for example, was the average of all values of 1/IPI between the 5th and 6th pulse.

Sound Pressure Levels were calibrated as in Mankin et al. (1996b), and the speaker output was adjusted to produce 55 dB SPL (relative to 20 (Pa) mean signal level at a distance of 12 mm. This is the level used in previously successful trapping

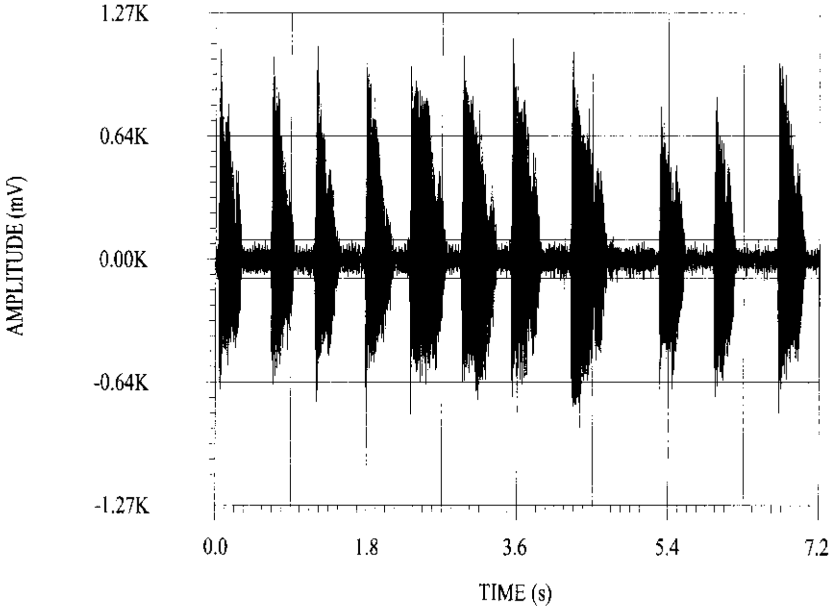

Fig. 2. Oscillograph of 7.2-s repeated section of male caribfly calling song used in female attraction bioassay. Horizontal and vertical axes, same as in Figure 1.

studies (Webb et al. 1983). Such sounds are audible to humans over distances of 2-4 m in the laboratory, but the range of detectability by female caribflies has not been measured.

Bioassay Arenas and Response Measurements

Separate exposure treatments were conducted in a laboratory and a wind tunnel. The effect of prior exposure to live males was tested by moving some of the caged virgin females into a 1.8 × 1.8 × 2.5 m "male room" containing several hundred caged males of all ages. These females were adjacent to, but physically isolated from males, and were exposed to sight, sound, and pheromone from males. After 38-40 h exposure in the "male room", 20 females were moved to a (20 × 20 × 20 cm) screen cage in a separate room for the acoustic bioassay.

In the acoustic bioassays, 2 pairs of monaural headphones (Realistic or Archer, both including the foam pads) were placed on top of the cage, facing down. One pair was silent and the other was connected to a recorder (Realistic CTR-62 or CTR-66) playing the endless-loop tape. The signals were broadcast during the peak of the daily courtship-signaling period, 8.5 h after the start of the 12-h photophase. The females standing directly under each speaker were counted every five minutes, a total of 13 times for each 1-h replicate. The locations of silent and broadcasting speakers were alternated at least once during each trial.

Attraction to male song was measured as the mean of the 13 measurements of the difference between the proportions of females under the broadcasting and the silent speakers. The responses of females exposed in the "male room" prior to assays were compared with the responses of females kept continuously in the “female room”. Thirteen replications were done in the “female room” tests and seven replications in the “male room” tests.

In the second experiment, females were exposed in a wind tunnel (Heath et al. 1993) to different combinations of male courtship signals for 38-40 h before the acoustic bioassay. The combinations were filtered air in a “clean tunnel”, air from the “male room”, ”live males” (10 males in a cage, 1.1 m upwind), “pheromone” (exposure to putative synthetic pheromone), or “sound only” (calling song broadcast continuously from speakers on top of the cage). The putative synthetic pheromone was composed of ~5% ocimene; ~2% nonenols ((*Z*)-3-nonen-1-ol and (*Z*,*Z*)-3,6-nonadien-1-ol);~10% suspensolide ((*E*,*E*)-4,8-dimethyl-3,8-decadien-10-olide); ~5% *E*,*E*-α-farnesene; ~35% ß-bisabolene; ~10% anastrephin (*trans*-hexahydro-*trans*-4,7α-dimethyl-4-vinyl-2-(3H)-benzofuranone); and ~33% epi-anastrephin (*trans*-hexahydro-*cis*-4,7α-dimethyl-4-vinyl-2-(3H)-benzofuranone), which approximated the ratio of components that are released under natural light condition in late afternoon (Heath et al. 1993). Synthetic components were formulated in glass capillaries (ocimene and nonenols) and on rubber septa (remaining components) using protocols reported previously (Weatherston et al. 1985a, 1985b, and Heath et al. 1986, respectively). The putative synthetic pheromone was formulated to release ~900 ng per h or the equivalent of release from 10 male caribflies in late afternoon (Heath et al. 1993).

The cages used for the wind tunnel treatments had solid sides (14.2 × 30 cm) and bottoms (13.3 × 30 cm) with single-screen covered circular openings (9 cm diameter) cut into the front and back pieces (13.3 × 14.2 cm). Two screen-covered circular openings were cut into the top for placement of the speakers. The foam pads were removed to prevent oviposition on the speakers in the “sound only” experiment. Attractiveness of broadcast songs after the wind tunnel treatment was measured as in the previous experiment. This experiment had 10 replications.

Behavioral Response Analysis

Nonparametric statistical analyses were used due to nonnormal frequency distributions of the raw data. Dunn’s multiple comparison (based on Kruskal-Wallis rank sums) (Hollander & Wolfe, 1973) was used to compare the treatments in the second experiment.

RESULTS

Female Acoustic Attraction Response

Male caribfly calling song elicited a detectable attraction response when females were in the presence of synthetic pheromone or had been exposed to males. In the first experiment, females that had been kept in the male-room responded at significant levels to calling song, but the responses of females that had been kept in the female room were not significantly different from zero (Fig. 3). The two treatments differed in the proportion of females under broadcasting speakers ($z = 2.774$, $p < 0.005$, Mann-Whitney *U*-test). In the second experiment, females that had been kept in the male room, exposed to live males, or exposed to synthetic pheromone in the wind tunnel were attracted to male calling song (Fig. 4). Females that had been kept in the clean tunnel or exposed only to male calling song showed no attraction toward broadcast

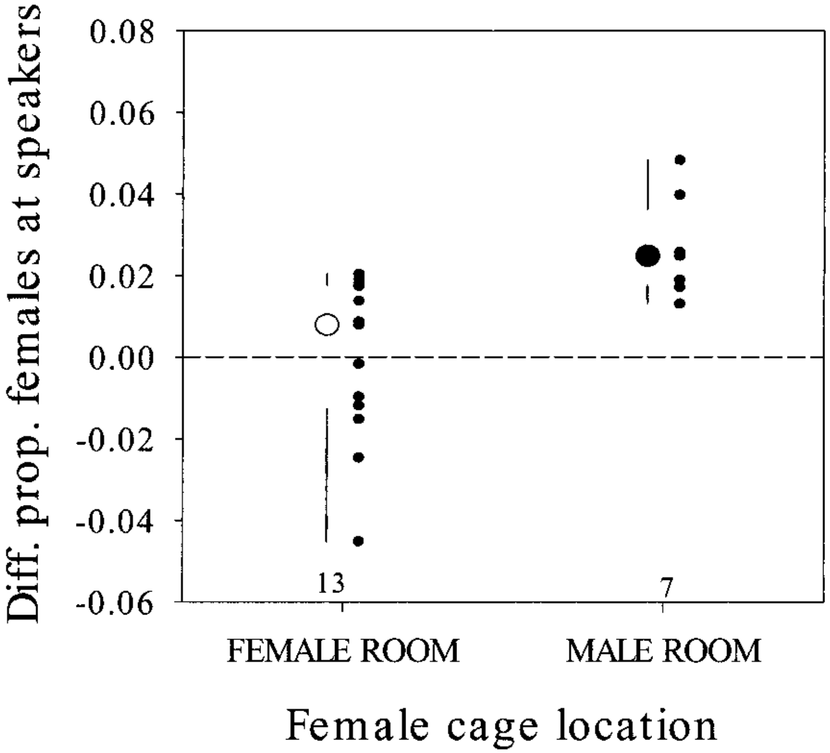

Fig. 3. Comparison of responses to calling song by females kept only with other females or kept in a room with caged males. The vertical axis shows the difference in the proportion of females at the broadcasting and the silent speakers, with positive values indicating a greater proportion of females observed under the broadcasting speakers. Medians are indicated by the larger circles and vertical lines indicate the 0-25th and 75-100th percentiles. Smaller dots (right) show the measured proportion differences. Filled median circles indicate that the median differs significantly from zero according to the sign test ($p < 0.016$). The numbers underneath list the number of cages (replicates) in each treatment.

songs. The “clean tunnel” and “male room” tests in the second experiment were essentially equivalent to the “female room” and the “male room” tests in the first experiment. The results of the two experiments were comparable.

Dynamic Features of Male Calling Song

The 7.2-s segment of male calling song broadcast in these recordings (Figs. 1 and 2) consisted of 11 pulse trains with a mean frequency ± Standard Error (SE) of 140.38 ± 3.86 Hz. The pulse trains had a mean duration (PTD) of 0.31 ± 0.02 s, separated by silent intervals (PTI) of 0.36 ± 0.05 s. The frequency varied within the pulse train. On average, the train began with pulses of ~125 Hz (Fig. 5). The frequency increased rapidly to a maximum of ~148 Hz by the 5th IPI (~36 ms into the train), and then gradually declined to below 120 Hz by the 30th IPI (~225 ms into the train).

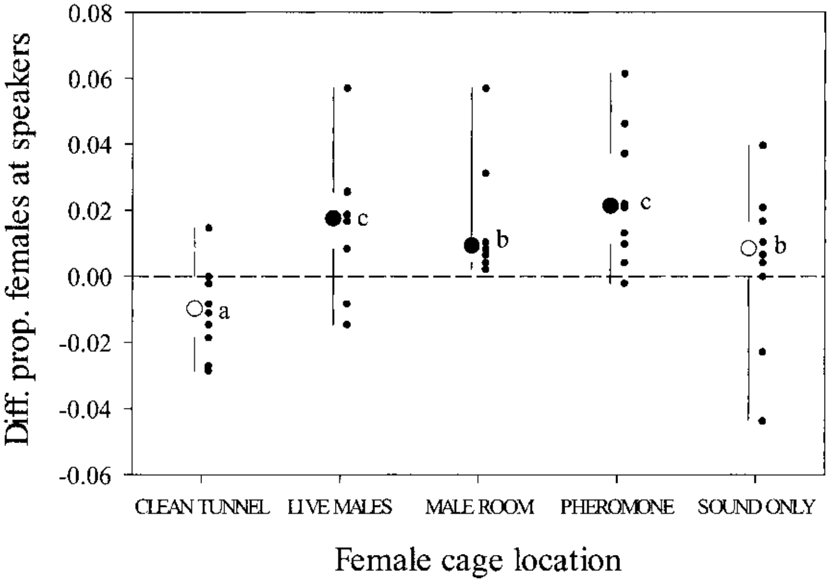

Fig. 4. Comparison of female responses to male calling song after previous exposure in a wind tunnel to clean air, caged males, putative synthetic male pheromone, or calling song. Positive values indicate that proportionally more females were observed under the broadcasting speakers. Medians are indicated by the larger circles and vertical lines indicate the 0-25th and 75-100th percentiles. Smaller dots (right) show the measured proportion differences. Filled median circles indicate that the median differed significantly from zero according to the sign test. Treatments with the same letter had medians that were not significantly different according to Dunn's multiple comparison (experimentwise error rate = 0.09).

DISCUSSION

The result that females exposed to male pheromone before a bioassay were more responsive to calling song than unexposed females is consistent with at least two alternative hypotheses. One is that exposure to pheromone is necessary to trigger a response to calling song. A second is that experience with the male courtship repertoire may increase the responsiveness to subsequent courtship signals. The first hypothesis is plausible because, in nature, (*cf.* Sivinski & Burk, 1989) females usually smell the males before they hear or see them. However, Sivinski et al. (1984) found that virgin females with no experience of male pheromone increased their levels of activity when they heard broadcast male calling song. In this study, the responses of females that had been exposed only to broadcast song were significantly greater than the responses of females that had never been exposed to any sexual stimuli. Such results suggest that acoustic attraction can occur without exposure to pheromone, but the attraction is weaker than that to pheromone.

The results here and those of Sivinski et al. (1984) are most consistent with the second hypothesis, that prior experience of the female with the male courtship repertoire increases female responses to newly encountered courtship stimuli. This effect

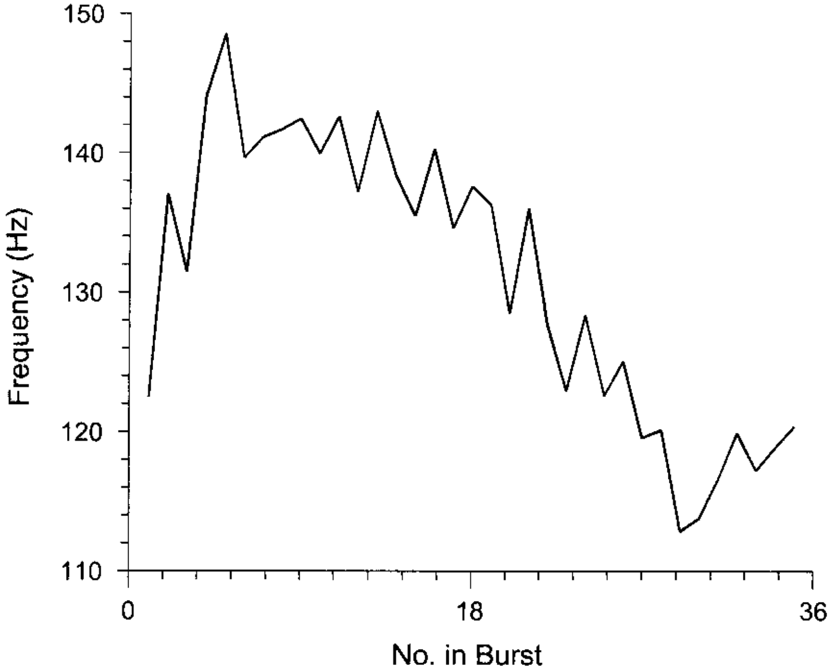

Fig. 5. Temporal pattern of wing-beat frequency within calling song pulse trains of a sexually successful caribfly male. The vertical axis indicates the wing-beat frequency (1/IPI) in s^{-1}. The horizontal axis indicates the number of pulses from the beginning of the pulse train, counted as in the inset in Figure 1.

might be similar to one observed in wind tunnel experiments with female parasitoid attraction to host larvae (e.g. Drost et al. 1986; Eller et al. 1988; Turlings et al. 1989). If the second hypothesis is correct, however, the nonsignificant result for females that had been exposed to calling song but not pheromone suggests that exposure to pheromone has a greater effect on subsequent responsiveness.

Because the female responses to the broadcast calling song in these bioassays were too low for practical applications, the main benefit of this study is some insight for improvements in the design of fruit fly acoustic bioassays. First, female responsiveness to acoustic signals can be increased by exposure to pheromone, either before or during the acoustic testing. Second, the broadcasting of acoustic stimuli by speakers may present an inadequate stimulus to female fruit flies. The adequacy of acoustic stimuli for attraction has been a problem in many other insect bioassays as well (Searcy & Andersson 1986).

The response in this bioassay was low despite the use of song generated by a sexually successful male. The mean frequency is lower than the 149-Hz average of sexually successful males in Webb et al (1984), but a lower frequency may correlate with larger size and improved mating propensity (Burk & Webb 1983). The PTI and PTD are comparable to measurements from other sexually successful males (Webb et al. 1984). Consequently, it is not likely that the signal pattern itself was deficient but some other stimulus feature. One potential contributor to the low female responsiveness was that the sound was produced by a speaker rather than a vibrating wing. The speaker generates a signal of larger spatial extent and lower air velocity than the vibrating wing. In addition, the vibrating wing has visual components that have not yet been demonstrated as affecting caribfly female mate choice (Aluja et al. 2000) but have been shown to affect mate choice in other Diptera (e.g., Lunau 1992). The continued development of new acoustic signal analysis and signal presentation capabilities may improve our future ability to elicit an attraction response of female fruit flies to synthetic courtship stimuli.

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