

Evaluation of amplitude in male song: female waxmoths respond to fortissimo notes

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SUMMARY

Female evaluation of male signals in the context of sexual selection is often made on the basis of signal energy. Particularly in acoustic species, females may prefer male song that is broadcast at greater amplitude or power. However, song amplitude may be represented by various parameters, and the specific one(s) that are evaluated are not clear. We addressed this problem in an acoustic moth, *Achroia grisella* (Lepidoptera: Pyralidae), where males attract females with trains of paired ultrasonic pulses. Previous studies showed that females prefer songs that include pulse pairs that have greater mean peak amplitude and that are delivered with greater power ($\text{mean peak amplitude} \times \text{pulse-pair rate}$). Here, we report that given male songs of equal acoustic power, females prefer songs in which some pulses attain peak amplitudes that exceed the mean value and that this preference depends largely on the magnitude of amplitude fluctuation. We measured significant variation among males in their degree of amplitude fluctuation, and we note that males that broadcast with lower acoustic power typically show greater relative fluctuations and attain relatively higher amplitude maxima. We discuss the potential role of multiple integration time constants in female evaluation of mean song amplitude and amplitude maxima. We then consider the possibility that the variation observed in the male population is a response to female choice, but we also indicate that mechanical factors constraining song production may be responsible for such variation.

Key words: acoustic power, fluctuating signal, integration time constant, signal evolution, song amplitude.

INTRODUCTION

The general female preference for and orientation toward male advertisements broadcast at high signal intensity is well known in the contexts of neuroethology and sexual selection (Andersson, 1994; Bradbury and Vehrencamp, 1998). Minimum thresholds of signal intensity that are necessary for evoking a neural or behavioral response in females normally exist. Moreover, females often exhibit higher levels of response as signal intensity increases, and they may display a distinct behavioral preference for the more intense of several signals (Kirkpatrick and Ryan, 1991; Ryan and Keddy-Hector, 1992). These generalizations are derived from laboratory and field studies of signaling in acoustic, vibrational and visual modalities, and they may represent a universal phenomenon (Greenfield, 2002). At a neuroethological level, increased responses to more intense signals may simply reflect that higher stimulus energy evokes an increased likelihood of messages from sensory neurons and a greater rate of action potentials in those messages (Ewert, 1980). From the perspective of sexual selection, females exhibiting such graded responses may obtain genetic or material benefits by virtue of mating with a male that can or does expend greater energy on his advertisements (Lande, 1981; Pomiankowski, 1988; Kokko et al., 2002). This fundamental response to signal intensity, however, can mask some critical details in the processing that females use to evaluate signals and how such evaluations might have shaped the evolution of male signaling efforts. Specifically, signal intensity normally fluctuates over different temporal scales, and it is not clear how females process and evaluate intensity given such fluctuations.

Neurophysiologists have addressed one aspect of this problem through investigations of 'integration time constants' in perception. For example, acoustic species, particularly among insects and

anurans, may integrate the sound energy that has arrived over a brief time interval (Tougaard, 1996; Tougaard, 1999; Ronacher et al., 2000; Gerhardt and Huber, 2002; Wyttenbach and Farris, 2004). This interval extends backward from the present time to a given moment in the past, and it is thus continually updated. The animal then processes a running integral of acoustic energy (sound amplitude summated over time) and can therefore detect and evaluate short pulses of sound and brief silent gaps, as well as other features, in longer broadcasts of song (e.g. Schiolten et al., 1981; Hennig et al., 2004). But depending on the parameters of the integration time constant, the animal may also fail to detect shorter pulses and briefer gaps.

Despite the above studies on neuroethological function, relatively few behavioral studies have probed the manner in which time constants might influence the various processes of sexual selection. That is, we have very little information on the specific way in which females respond to the amplitude of male song. Do females simply assess overall mean amplitude levels and orient toward songs with the highest mean values, or are females unduly influenced by occasional maximum values that greatly exceed the mean? And, do the amplitude properties of male song show evidence of selection pressure imposed by the specific protocol of female assessment?

We addressed the question of female evaluation of amplitude in male song in an acoustic moth, *Achroia grisella* Fabricius (lesser waxmoth; Lepidoptera: Pyralidae: Galleriinae). Male *A. grisella* attract females with a calling song that consists of a continuous train of ultrasonic pulses (Spangler et al., 1984). The males produce their song for 6–10 h each night until death while remaining stationary on the substrate and fanning their wings at 35–50 cycles per second (measured at 25°C). This activity causes a pair of small tymbal structures situated at the base of each forewing to resonate, once

on the upstroke of the wings and once on the downstroke. Each resonance yields an 80–130 μ s pulse of sound consisting of frequencies from 70–130 kHz. The pulses are relatively intense [peak amplitude=90–95 dB SPL (sound pressure level) at 1 cm; 0 dB SPL=20 μ Pa], and their envelope shows a characteristic sudden rise and exponential decay. Because the left and right tymbals do not resonate in perfect synchrony, two pulses, separated by a brief ‘asynchrony interval’, are normally produced during both the upstrokes and downstrokes of the wings. Thus, a wing-fanning male generates pulse pairs and does so at 70–100 pulse pairs s^{-1} , twice the rate of cycles of wing movement. Peak amplitude of the sound pulses typically fluctuates by several decibels between pulse pairs and between the two pulses within a pair.

Female *A. grisella* normally run, rather than fly, toward singing males, and they may exhibit such phonotaxis over a distance up to 1 m (Greenfield and Coffelt, 1983). Playback experiments in which females were attracted to loudspeaker broadcasts of digitized song recordings indicate that phonotaxis thresholds may be as low as 50–60 dB SPL [peak amplitude (Brandt et al., 2005) and unpublished data]. Studies of several *A. grisella* populations showed that females prefer male songs with pulses of greater peak amplitude that are delivered at a faster rhythm, and that include longer asynchrony intervals within the pulse pairs (Jang and Greenfield, 1996; Jang and Greenfield, 1998). A major portion of female evaluation of male song may be based on acoustic power, defined here as the product of mean peak amplitude and pulse pair rate: playback experiments using synthetic signals suggested that females were equally attracted to songs with low peak amplitude pulses delivered at a rapid pulse pair rate and to songs with high peak amplitude pulses delivered at a slow pulse pair rate, with acoustic power held constant in both songs (Greig and Greenfield, 2004).

The above experiments, however, were conducted in the absence of the fluctuations in peak amplitude that occur naturally in *A. grisella* male song. Given these natural fluctuations and our incomplete understanding of amplitude evaluation in this species, we asked whether females show a strict adherence to preference for song based on acoustic power or whether they prefer songs that fluctuate and include some pulses that attain maximum amplitudes considerably higher than the mean. We report that females prefer the latter and that this preference is influenced by an integration time constant that may last for approximately 10 ms, one pulse-pair period. In turn, we note that some males do exhibit marked fluctuations in their amplitudes, and it remains possible that this feature represents a response to selection pressure imposed by female choice.

MATERIALS AND METHODS

Population studied and measurement of acoustic parameters

We studied *A. grisella* from a laboratory population derived from several hundred individuals collected in Indre et Loire, France in October 2007. The moths were reared on a standard diet containing wheat, corn and rye flours, water, glycerol, nutritional yeast, honey and beeswax [modified from Dutky et al. (Dutky et al., 1962)]. Both larvae and adults were kept in an environmental chamber at 26 \pm 1 $^{\circ}$ C and a 12:12 L:D photoperiod. Generation duration, measured from oviposition to oviposition, lasted 40–45 days under these conditions.

Our test insects were sampled from the population during the pupal stage and kept individually in 30-ml plastic cups to ensure that the eclosing adults experienced a standard social environment. This measure was particularly essential for the females, who usually mate but once and become sexually unreceptive thereafter. We conducted all tests of female responses and recordings of male song

during the initial 6 h of the photoperiodic night, the diel interval during which mating activities in *A. grisella* are maximum. Because *A. grisella* adults neither feed nor drink and do not survive long (5–7 days for females and 7–14 days for males), we only tested adults within 30 h of their eclosion to avoid measuring senescing individuals.

We sampled the mating songs of 25 males to obtain a standard song representative of the population. Males were kept in small screen cages (1.5 cm diameter, 2.0 cm height) placed in an acoustically insulated chamber that was maintained under environmental conditions identical to those during rearing except that diffuse red light (25 W, incandescent) provided illumination. Earlier recordings confirmed that males sing normally in these cages and that the screen does not modify the acoustic parameters of the song (Jang et al., 1997). We placed a barrier of acoustic insulation foam between neighboring males and separated them by 30 cm to ensure that a male’s song was not influenced by acoustic interactions with neighbors (see Jia et al., 2001). This measure also permitted clear recordings of a focal male without neighbors’ songs in the background. We allowed the males a minimum of 15 min acclimatization in the chamber prior to recording. We used a condenser ultrasound microphone (model CM16/CPMA; Avisoft Bioacoustics; Berlin, Germany; frequency response: \pm 3 dB, 20–150 kHz), positioned 20 cm from the male and oriented toward him to record his song. The microphone output was digitized with an analogue/digital converter (model UltraSoundGate 416–200; Avisoft Bioacoustics) at 16 bits and 500,000 samples s^{-1} , and we saved a 30-s sample of this digitized song to a file on a personal computer using signal processing software (BatSound Pro 4.0; Petterson Elektronik AB; Uppsala, Sweden).

From the file saved from each of the 25 sampled males we randomly selected a 1-s segment in the middle of the recording for analysis of acoustic parameters. Our only criterion was that the 1-s segment did not include brief silent gaps that reflected missing pulse pairs in an otherwise continuous train. We determined the repetition rate of pulse pairs (PR) for the entire segment and then randomly selected 20 consecutive pulse-pair periods for computing the asynchrony intervals (AI), measured from the onset of the first pulse to the onset of the second pulse of a pair. Because the repetition of pulse pairs includes an alternating succession of longer and shorter periods as well as an alternating succession of longer and shorter AIs, we measured the average ratio of the longer to shorter periods. We also measured the average of the longer and shorter AIs. Finally, we note that peak amplitude (PA) often fluctuates regularly between the two pulses of a pulse pair and also between successive pulse pairs. Thus, we measured PA, in arbitrary linear units, of each pulse in the 20 consecutive periods and thereby determined mean PA values for each of four consecutive pulses (two consecutive pulse pairs; see Fig. 1). We then used these several measures, calculated with BatSound Pro 4.0, to create a standard signal the temporal and energy features of which represented the average values for male song in the population. This standard signal had a PR=80 pulse pairs s^{-1} , a period ratio=1.405 (longer: shorter period), longer and shorter AIs=554 and 530 μ s, respectively, and relative PAs that fluctuated successively from 1.00 to 1.23, 1.17 and 1.09 among the four pulses that constituted two consecutive pulse pairs (Fig. 1).

Playback experiments

General procedure

All of our playback experiments used a basic choice protocol in which we released a female in the center of an 80-cm diameter screen

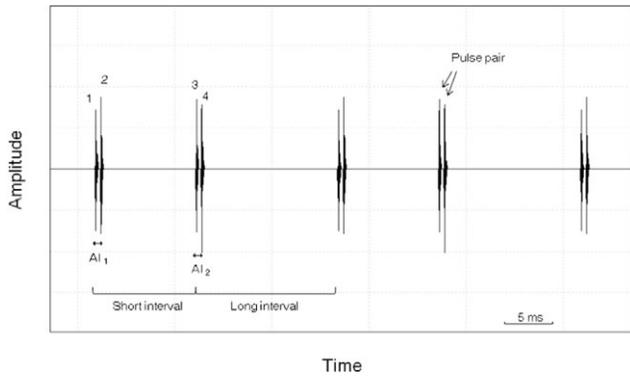


Fig. 1. Oscillogram of four consecutive pulse-pair periods of the standard male signal used for testing female preference. The standard signal represents average temporal and energy values measured in the population; these values include: short pulse-pair period=10.4 ms; long pulse-pair period=14.6 ms; long asynchrony interval (AI_1 =554 μ s); short asynchrony interval (AI_2 =530 μ s); peak amplitude of pulse 1=1.00 arbitrary linear units; peak amplitude of pulse 2=1.23; peak amplitude of pulse 3=1.17; peak amplitude of pulse 4=1.09. The four numbered pulses comprise two consecutive pulse pairs or two consecutive pulse-pair periods, which are repeated for the duration of a playback trial. All call models are generated from a single pulse; vertical displacements from the baseline on the oscillogram accurately reflect maximum and minimum amplitude values of a pulse and are not subject to aliasing or pixelization, which could bias the oscillogram toward depicting lower values.

arena and presented simultaneous broadcasts of synthetic male song stimuli from two loudspeakers situated just outside the arena and separated by an azimuth of 120°. The central axes of the loudspeakers were level with the female in the center of the arena, and each loudspeaker was oriented directly toward her. The female was given 120 s in which to orient and arrive within a 10-cm radius of a loudspeaker and remain in that sector for a minimum 10 s. For release, the female was placed within a small, covered cell depressed 1 cm below the surface of the arena center. The cover was then removed, the cell was raised such that its floor was level with the arena surface, and the playback stimuli were broadcast. The cell did not have sides, which could have hindered the female's movement or her perception of song stimuli. All playback tests were conducted in a second acoustically insulated chamber maintained under conditions similar to the chamber used for song recording. Females were brought to the chamber at least 30 min prior to testing, and they were held in an acoustically insulated box, isolated from males and synthetic male song, at all times except during its playback tests.

We created our playback stimuli from a single male pulse, recorded at 16 bits and 500,000 samples s^{-1} as described above, that had an envelope and spectral properties average for the population. Using BatSound 4.0, we digitally repeated this pulse at precise intervals and with precise adjustments in relative amplitude in order to fashion the standard signal on one channel of a 30-s stereo sound file. The standard signal was then copied to the other channel and its relative peak amplitudes were modified appropriately. During a playback test we continuously looped the stereo file on a personal computer, converted the digital signal to analog with an input/output card (DAQcard 6062E; National Instruments; Austin, Texas, USA), and sent the analog signals at 214,285 samples s^{-1} to the two loudspeakers (model ScanSpeak; Avisoft Bioacoustics; frequency response: ± 2 dB, 60–120 kHz), each amplified by a multi-channel power amplifier (Avisoft Bioacoustics).

In all experiments we adjusted the peak amplitude, as presented at the location of the female in the arena center, of the loudest pulse of the standard signal to 75 dB peSPL (peak equivalent sound pressure level; 0 dB=20 μ Pa). Thus, the other pulses were 73.2 (pulse 1), 74.6 (pulse 3), and 74.0 dB peSPL (pulse 4; see Fig. 1). Adjustment was made with the aid of a sound pressure level meter (model CEL-430/2; Casella, Kempston, UK; flat frequency response from 30–20,000 Hz), confirmed with a calibrator (model CEL-110/2; Casella). We implemented the method of peak equivalents by relating the millivolt output of a continuous 20 kHz broadcast, as measured by the condenser ultrasound microphone, to the SPL of this broadcast, as registered by the SPL meter. We then noted the millivolt output of the synthetic song stimulus broadcast as measured by the microphone, and we adjusted the gain on the loudspeaker amplifier until this millivolt output was equivalent to 75 dB peSPL (see Jang and Greenfield, 1996). This peSPL value was roughly equivalent to the song of a male *A. grisella* 10 cm distant, and it was 6–10 dB higher than average thresholds observed for female orientation toward male song.

Amplitude discrimination

Our first experiment served to evaluate the basic degree at which females in the population discriminated amplitude levels of male song. We judged that this information was necessary before proceeding to the tests of fine-tuned assessment of amplitude fluctuation. We conducted four different tests in which females were presented with playback of the standard signal *versus* a modified signal in which the peak amplitude of each pulse was either decremented or incremented by a given percentage: (1) decrement by 50% (−6 dB); (2) decrement by 75% (−12 dB); (3) increment by 50% (+3.5 dB); (4) increment by 100% (+6 dB) (see Fig. 2A for depiction of the first test). The positions of the loudspeakers broadcasting the standard and modified stimuli were switched on successive tests to preclude a side bias from confounding our results. Individual females were tested once with each of the four tests, which were presented in random sequence with at least 30 min between successive tests to avoid habituation. Only data from females that responded to a song stimulus in each of the four tests were retained for analysis.

Discrimination of constant *versus* fluctuating song

We began our study of discrimination of amplitude fluctuation by conducting an experiment that included six different tests in which we presented females with a choice of the standard signal *versus* a modified one in which peak amplitudes fluctuated over a greater range. The modification was effected over a cycle of either 16 pulse-pair periods (two tests), 8 pulse-pair periods (two tests), 4 pulse-pair periods (one test), or 2 pulse-pair periods (one test). In each case the pulses were decremented by a given amplitude, either 3 dB (two tests; modification cycles of 16 and 8 pulse-pair periods) or 6 dB (four tests; modification cycles of 16, 8, 4 and 2 pulse-pair periods), for half of the modification cycle and incremented by the same amount for the other half of the cycle (see Fig. 2B for depiction of test presenting ± 6 dB with a modification cycle of 8 pulse-pair periods). These modification cycles were then repeated for the duration of the playback test. Thus, peak amplitudes of the loudest pulses of the standard signal were raised to either 78 or 81 dB peSPL in the incremented half of the modification cycle, and they were lowered to either 72 or 69 dB peSPL in the decremented half. The modified peSPLs of the three other pulses of the standard signal were adjusted accordingly, to levels slightly lower than the values noted above (cf. Fig. 1).

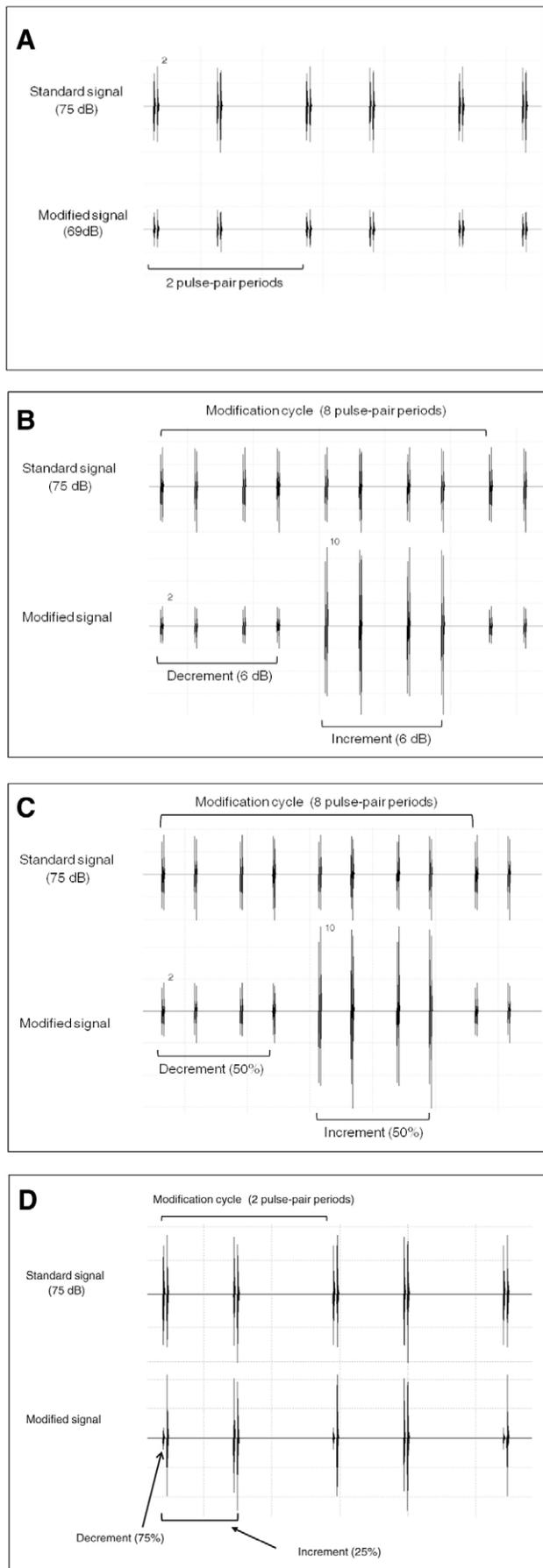


Fig. 2. Oscillograms showing standard and modified signals presented in the four playback experiments. In each experiment the amplitude of the second, most intense pulse (2) of the standard signal (see Fig. 1) is adjusted to 75 dB peSPL at the position of the tested female; the modified signal is created by adjusting the standard signal by specific decrements and increments in amplitude. (A) Experiment testing amplitude discrimination: modified signal in this example is adjusted such that the peak amplitudes of each pulse are decremented by 6 dB, i.e. the second, most intense pulse is broadcast at 69 dB peSPL. (B) Experiment testing discrimination between fluctuating and standard song: the modified signal in this example is adjusted such that the peak amplitudes of the pulses in the first half of the 8-period modification cycle are each decremented by 6 dB (50%) whereas the pulses in the second half of the modification cycle are each incremented by 6 dB (100%); i.e. the second (2) and tenth (10) pulses of the modified signal are broadcast at 69 and 81 dB peSPL, respectively. (C) Experiment testing discrimination between fluctuating and standard song: the modified signal in this example is adjusted such that the peak amplitudes of the pulses in the first half of the 8-period modification cycle are each decremented by 50% (6 dB) whereas the pulses in the second half of the modification cycle are each incremented by 50% (3.5 dB), thereby preserving the same power as broadcast in the standard signal; i.e. the second (2) and tenth (10) pulses of the modified signal are broadcast at 69 and 78.5 dB peSPL, respectively. (D) Experiment testing discrimination between fluctuating and standard song at shorter time scales: modified signal in this example is adjusted such that the peak amplitude of the first of four pulses is decremented by 75% and the following three are incremented by 25%, thereby preserving the same power as broadcast in the standard signal. Vertical displacements from the baseline accurately depict maximum and minimum amplitude values of a pulse; see Fig. 1.

Acoustic perception may normally scale logarithmically (see Wytenbach and Farris, 2004), and we made the above modifications to reflect the expectation that the difference between 75 dB peSPL and $75+x$ dB peSPL may be perceived as equivalent to the difference between 75 dB peSPL and $75-x$ dB peSPL. However, we note that when this procedure is used the acoustic power of the modified signal slightly exceeds the power of the standard signal. For example, when $x=6$ dB, the mean peak amplitude of the modified signal = 0.1403 Pa, whereas the mean peak amplitude of the standard signal = 0.1125 Pa. Thus, in a second experiment we repeated the above tests while broadcasting equivalent acoustic power in both standard and modified signals. As above, we conducted six different tests in which the modification was effected over a cycle of either 16 pulse-pair periods (two tests), 8 pulse-pair periods (two tests), 4 pulse-pair periods (one test), or 2 pulse-pair periods (one test). But here, the pulses were modified by a given amplitude differential measured along a linear scale, a 50% lowering (two tests; modification cycles of 16 and 8 pulse-pair periods) or a 75% lowering (four tests; modification cycles of 16, 8, 4 and 2 pulse-pair periods) in the decremented half of the modification cycle and a 50% or 75% raise in the incremented half (see Fig. 2C for depiction of test presenting $\pm 50\%$ with a modification cycle of 8 pulse-pair periods). Expressed in terms of dB peSPL, signals with $\pm 50\%$ fluctuations were broadcast at 69 and 78.5 dB peSPL, whereas those with $\pm 75\%$ fluctuations were broadcast at 63 and 79.9 dB peSPL.

In a third experiment we examined whether females might discriminate among different degrees of amplitude fluctuation that occur at a finer temporal scale. We repeated the protocol of the above tests in which acoustic power was held constant in both signals except that here we incremented and decremented peak amplitudes of the modified signal within 2 pulse-pair periods (two consecutive pulse pairs). Four different tests were conducted in which we: (1)

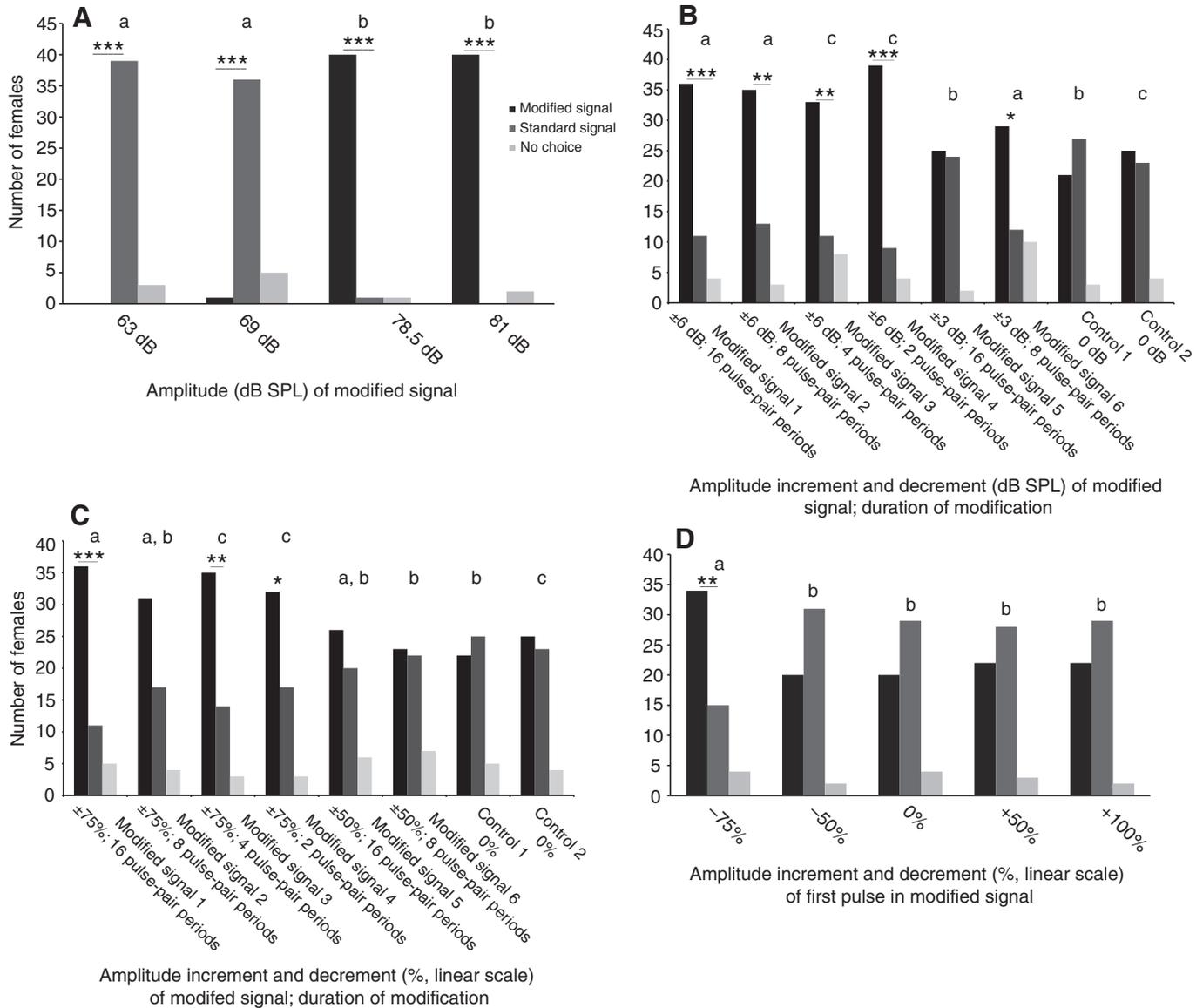


Fig. 3. Female discrimination between standard and modified signals presented in the four playback experiments. In each experiment the dark, medium, and light vertical bars represent the numbers of females orienting toward the loudspeaker broadcasting the modified signal, the standard signal, or failing to orient to either loudspeaker, respectively. (A) Experiment testing amplitude discrimination between the standard signal (75 dB peSPL) and the modified signal (peak amplitude of second, most intense pulse is indicated along x-axis). (B) Experiment testing discrimination between standard and fluctuating song (magnitude and modification cycle of fluctuation indicated along x-axis). (C) Experiment testing discrimination between standard and fluctuating song where power is conserved in both songs (magnitude and modification cycle of fluctuation indicated along x-axis). (D) Experiment testing discrimination between standard and fluctuating song at shorter time scales (amplitude decrement or increment of first pulse in fluctuating song is indicated along x-axis; the following three pulses were incremented or decremented accordingly to conserve the power broadcast in the standard song; see text). * $P < 0.05$, two-tailed binomial test; ** $P < 0.01$; *** $P < 0.001$; underlined asterisks indicate $P < 0.05$ following Holm correction for multiple tests (Holm, 1979). Within each of the four experiments, modified signals with the same letters above the vertical bars do not have significantly different levels of discrimination; $P > 0.05$, McNemar's test for comparison of dependent proportions (Zar, 1999). In A and D, an individual female was tested with all four modified signals and the control. In B and C, an individual female was tested with either modified signals 1, 2, 5 and 6 and control 1 (five tests) or with modified signals 3 and 4 and control 2 (three tests).

incremented the first of four pulses by 100% and decremented the following three pulses by 33%; (2) incremented the first of four pulses by 50% and decremented the following three pulses by 17%; (3) decremented the first of four pulses by 50% and incremented the following three pulses by 17%; (4) decremented the first of four pulses by 75% and incremented the following three pulses by 25% (Fig. 2D). In each test the four-pulse modification cycle was repeated for the duration of the playback trial.

All three experiments evaluating female discrimination of constant *versus* fluctuating song included an additional, control test in which the two loudspeakers broadcast identical, standard signals. As in the experiment on basic amplitude discrimination, individual females were tested once with each of several different modified signals plus the control (see Fig. 3 for specific design). The several tests were presented to a female in random sequence with at least 30 min between successive tests. Once a female failed to move from

the release point during a test in the sequence her data was eliminated from the analysis.

Measurement of natural fluctuation in peak amplitude of male song

We systematically analyzed male song from our population to evaluate whether maximum amplitude features showed any evidence of selection pressure imposed by specific female preferences. We sampled 30-s song recordings from 82 males using the procedure described above for determining the standard playback signal. First, we selected a 1-s segment from the recording of each individual and determined the repetition rate of pulse pairs (parameter 1). Then, from each 30-s recording we selected three segments, 16 pulse pairs in length and uninterrupted by silent gaps, from the beginning, middle, and final 10 s, for study using the ‘pulse train analysis’ and ‘root mean square’ (r.m.s.) functions in Avisoft SASlab Pro. In addition to the song recordings, we weighed each male to the nearest 0.01 mg on the day of adult eclosion.

From each 16-pulse-pair segment we determined the following energy parameters: (2) mean peak amplitude, measured in Pa, of pulse pairs; i.e. mean peak amplitude of the louder pulse of each pulse pair; (3) mean r.m.s. amplitude, measured in Pa, of pulse pairs; i.e. mean r.m.s. amplitude of the louder pulse of each pulse pair; (4) acoustic power, as measured by the repetition rate of pulse pairs multiplied by the mean peak amplitude of pulse pairs; i.e. parameter 1 \times parameter 2; (5) acoustic power, as measured by the repetition rate of pulse pairs multiplied by the mean r.m.s. amplitude of pulse pairs; i.e. parameter 1 \times parameter 3. We also determined the following parameters of amplitude fluctuation: (6) coefficient of variation (=standard deviation/mean) of peak amplitudes of pulse pairs; (7) coefficient of variation of r.m.s. amplitudes of pulse pairs; (8) standardized difference between maximum and minimum peak amplitude of the pulse pairs [= (peak amplitude of the pulse pair having the maximum value – peak amplitude of the pulse pair having the minimum value)/mean peak amplitude of pulse pairs]; (9) standardized difference between maximum and minimum r.m.s. amplitudes of the pulse pairs [= (r.m.s. amplitude of the pulse pair having the maximum value – r.m.s. amplitude of the pulse pair having the minimum value)/mean r.m.s. amplitude of pulse pairs]. For each of these eight parameters, we averaged the three values determined from an individual to estimate his mean level.

To evaluate whether any correlations existed between temporal or energy parameters (1–5) and parameters measuring levels of amplitude fluctuation (6–9), we determined the Spearman rank correlation coefficient between individual mean levels of each parameter in the first set *versus* individual mean levels of each parameter in the second set. This non-parametric statistic was measured because most of the parameters were not normally distributed. Thus, we considered the hypotheses (1) that males who sustained a relatively high pulse-pair rate, acoustic power, or mean peak or r.m.s. amplitude level in their song also produced peak or r.m.s. amplitude maxima, and an alternative (2) that males with relatively low pulse-pair rate, peak or r.m.s. amplitude, or acoustic power compensated for their inferior song by producing peak or r.m.s. amplitude maxima.

RESULTS

The experiment on amplitude discrimination showed that females in our population clearly preferred higher SPL levels of male song and that this preference remained very strong for a 3.5 dB difference (75 vs 78.5 dB peSPL; Fig. 3A; two-tailed binomial test, $P < 0.001$).

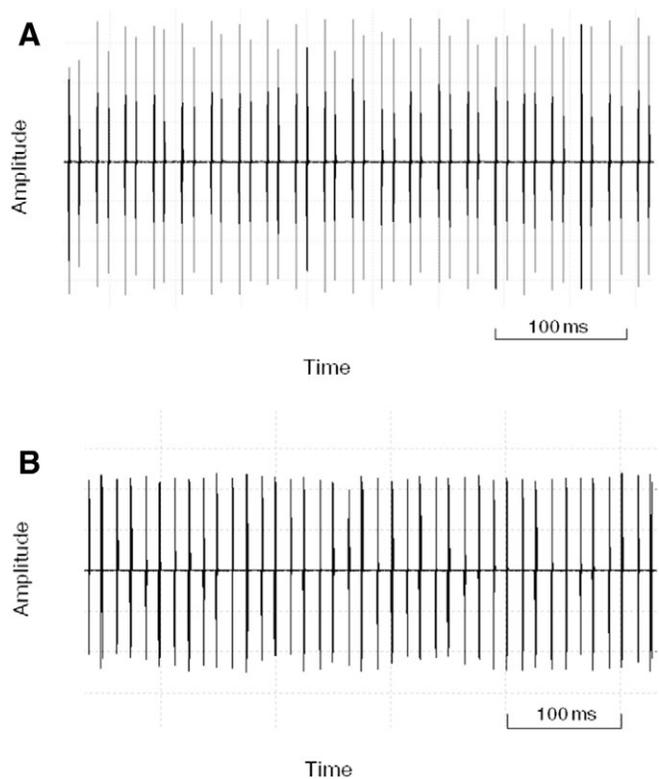


Fig. 4. Oscillograms showing two representative males singing (A) with marked amplitude fluctuations between successive pulse pairs and (B) at relatively constant amplitude. In both A and B, each apparent pulse actually represents a pulse pair (see Fig. 1). Vertical displacements from the baseline accurately depict maximum and minimum amplitude values of a pulse pair; see Fig. 1.

Based on these findings, we proceeded to the next experiments in which we tested female discrimination of song with constant vs fluctuating SPL.

Overall, our study of discrimination of constant (standard signal) vs fluctuating SPL (modified signal) showed that females preferred fluctuating male songs, wherein some pulses attain greater peak amplitude. The first of the three experiments in this study demonstrated that females clearly preferred song with 6-dB amplitude fluctuations (69 and 81 dB peSPL) over songs of constant amplitude (75 dB peSPL; Fig. 3B; two-tailed binomial test, $P < 0.01$), but that the preference was much weaker when 3-dB amplitude fluctuations were tested. In the latter case, the preference for fluctuating song was seen only when an 8-period modification cycle was used ($P < 0.05$). On the whole, the magnitude of the fluctuations influences female preference more strongly than the length of the modification cycle does: highly significant preferences were observed in four of four tests with 6-dB fluctuations, whereas only weak significance was observed in one of two tests with 3 dB fluctuations. However, highly significant preference was observed in one of two tests with 16-period modification cycles, in one of two tests with 8-period cycles, and in the tests with 4-period and 2-period cycles; we observed a weak preference in the second test with 8-period cycles.

In the second experiment, where acoustic power was held constant, we found some female preference for fluctuating vs constant song but at a reduced level in comparison with the

experiment above. Females showed a significant preference for the fluctuating song only in the tests where the modified signal included fluctuations of $\pm 75\%$ over a 16-period, 4-period, or 2-period modification cycle (Fig. 3C; two-tailed binomial test, $P < 0.05$). To facilitate comparison with the experiment above, we note that this modified signal eliciting preference included pulses with peak amplitudes of 63 and 79.9 dB peSPL. Again, we note that fluctuation magnitude influences female preference more strongly than the length of the modification cycle does: we observed a highly significant preference in two of four tests with 75% fluctuations and a weak preference in a third test, whereas we observed no preferences in tests with 50% fluctuations. However, we observed a highly significant preference in one of two tests with 16-period modification cycles, a statistically similar preference (McNemar's test for comparison of dependent proportions, $P > 0.05$) in one of two tests with 8-period cycles, and significant preferences in the tests with 4-period and 2-period cycles.

The third experiment, in which we tested discrimination over a shorter time interval, showed that females preferred the fluctuating song only when peak amplitude of the first of four pulses was decremented 75%, with the following three incremented 25% (Fig. 3D; two-tailed binomial test, $P < 0.01$). Otherwise, significant preferences for constant or fluctuating song were not observed,

although we note that here a greater number of females oriented toward the constant song.

Our analyses of male song in the sampled population show that certain individuals produce fluctuations in amplitude that greatly exceed the relatively modest fluctuations included in the standard signal (Fig. 4). We observed that approximately 25% of the sampled population had a coefficient of variation for peak amplitude of pulse pairs (parameter 6) greater than 0.13: four times the coefficient of variation measured in the standard signal (Fig. 5A), and 70% of the sampled population had a standardized difference between maximum and minimum peak amplitude of pulse pairs (parameter 8) greater than 0.19, four times the index measured in the standard signal (Fig. 5C). Moreover, when r.m.s. amplitudes were measured, we found that 56% of the sampled population had a coefficient of variation (parameter 7) greater than 0.40, a level equivalent to that in the ± 3 dB modified signal (Fig. 5B), and 65% of the sampled population had a standardized difference between maximum and minimum amplitude (parameter 9) greater than 1.1, a level equivalent to that in the ± 6 dB modified signal (Fig. 5D). Overall, peak and r.m.s. amplitudes of pulse pairs were significantly correlated ($r = 0.88$, Spearman rank correlation, $P < 0.01$).

We found significant negative correlations between measures of signal energy (parameters 2–5) and the coefficient of variation of

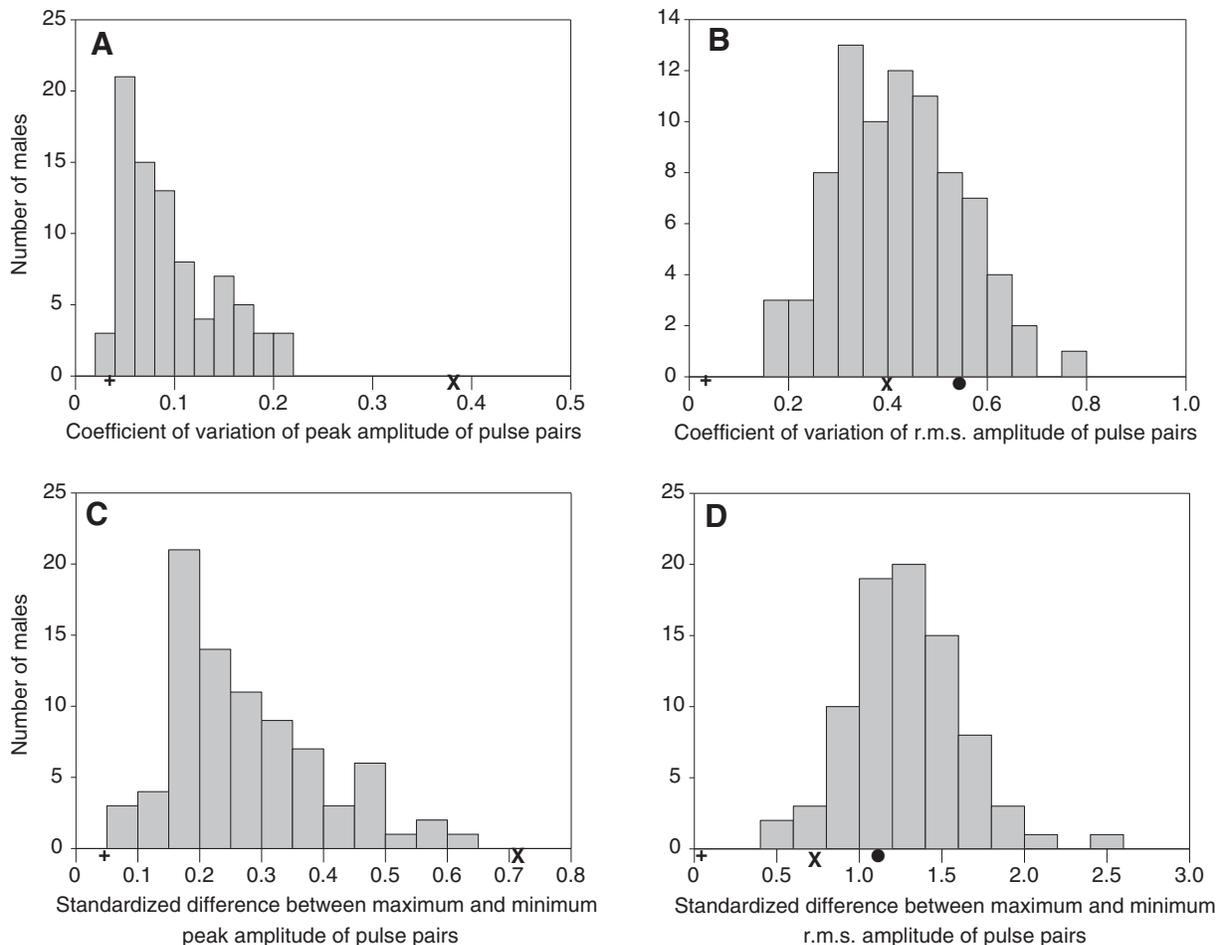


Fig. 5. Indices of amplitude fluctuation observed among 82 males sampled from the laboratory population. (A) Coefficient of variation of peak amplitude of pulse pairs (parameter 6). (B) Coefficient of variation of root mean squares (r.m.s.) amplitude of pulse pairs (parameter 7). (C) Standardized difference between maximum and minimum peak amplitude of pulse pairs (parameter 8). (D) Standardized difference between maximum and minimum r.m.s. amplitude of pulse pairs (parameter 9). See text for definitions of parameters. +, value of index in standard signal; X, value of index in ± 3 dB modified signal; ●, value of index in ± 6 dB modified signal.

Table 1. Spearman rank correlations between parameters of amplitude fluctuation (rows 1–4) and parameters of temporal and song energy (columns 1–5) as determined from measurements of 82 males.

	Mean amplitude of pulse pairs			Acoustic power		Mass
	Pulse pair rate (parameter 1)	Peak value (parameter 2)	r.m.s. value (parameter 3)	(parameters 1×2 =parameter 4)	(parameters 1×3 =parameter 5)	
Coefficient of variation of peak amplitude of pulse pairs (parameter 6)	0.0807 (0.470)	−0.351 (0.00129)*	−0.359 (0.00)*	−0.299 (0.00643)*	−0.312 (0.00443)*	−0.318 (0.00372)*
Coefficient of variation of r.m.s. amplitude of pulse pairs (parameter 7)	0.0118 (0.916)	0.164 (0.140)	−0.166 (0.136)	0.171 (0.124)	−0.130 (0.244)	−0.0461 (0.680)
Standardized difference between maximum and minimum peak amplitude of pulse pairs (parameter 8)	0.121 (0.278)	−0.359 (0.00)*	−0.324 (0.00)*	−0.296 (0.00711)*	−0.268 (0.0149)*	−0.211 (0.0568)
Standardized difference between maximum and minimum r.m.s. amplitude of pulse pairs (parameter 9)	−0.0193 (0.863)	0.197 (0.0764)	−0.135 (0.226)	0.180 (0.105)	−0.118 (0.291)	−0.0539 (0.630)
Mass	0.0435 (0.697)	−0.00702 (0.950)	0.117 (0.296)	−0.00692 (0.951)	−0.0886 (0.428)	

Correlations between male body mass, measured on the day of adult eclosion, and parameters of song energy and parameters of amplitude fluctuation are also shown (row 5 and column 6, respectively).

r.m.s., root mean squares.

P-values of correlation coefficients are shown in parentheses; *P-value <0.05 following Holm adjustment for multiple tests.

amplitude of pulse pairs (peak value; parameter 6) and the standardized index of maximum–minimum difference (peak value; parameter 8; Table 1, Fig. 6). In general, body mass was not correlated with the parameters of song energy or of fluctuation in song energy. We observed a single (negative) correlation between mass and the coefficient of variation of maximum amplitude of pulse pairs (peak value; parameter 7; Table 1).

DISCUSSION

Findings from our playback experiments show that female *A. grisella* discriminate finely between male signals that differ in mean peak amplitude, and that they also discriminate between male signals broadcast at the same mean peak amplitude but that differ in the extent to which peak amplitude varies over the course of a song. Females clearly prefer the higher of two signals differing by as little as 3.5 dB in peSPL, which is consistent with previous findings – obtained from a different *A. grisella* population – that some discrimination even occurs for amplitude differentials of 2 dB peSPL (Jang and Greenfield, 1996). When given male signals in which mean peak amplitude is held constant, females also prefer those signals that exhibit marked fluctuations in peak amplitude over those that remain relatively constant (Fig. 3). This preference is more pronounced where the standard signal (75 dB peSPL; 0.1125 Pa) is incremented and decremented by x dB than where the increments and decrements are represented by $x\%$, measured on a linear scale; i.e. 0.1125 Pa $\pm x\%$. Nonetheless, the preference for fluctuating signals does remain in the latter case, wherein a mean peak amplitude of 0.1125 Pa was preserved in both standard and fluctuating signals. In both cases we note that the magnitude of the fluctuations exerts a stronger influence on female preference than the length of the modification cycle does.

We observed that a substantial proportion of the sampled male population exhibits a much higher level of variation in amplitude of pulse pairs than that represented by the standard signal. This variation is more striking for measures of variation based on r.m.s.

values (parameters 7 and 9) than on peak values (parameters 6 and 8; Fig. 5). Consistent with one initial expectation, we found that the males exhibiting greater fluctuations in peak amplitude of pulse pairs were generally those individuals broadcasting at lower mean peak amplitudes of pulse pairs (Fig. 6).

Behavioral responses to fluctuating *versus* standard signals may be influenced by sensitivity thresholds, and we analyze the preferences for fluctuating signals noted above to discern whether they might represent artifacts of such thresholds. That is, average sensitivity thresholds in the population for female orientation toward male song range from 65–70 dB peSPL (unpublished data), which implies that some tested females had higher threshold values. Presented with a fluctuating song, e.g. 75±6 dB peSPL or 0.1125 Pa ±75%, these latter females would have perceived a slower pulse-pair rate, as some pulse pairs would have fallen below their threshold. But female *A. grisella* are expected to reduce their response as pulse-pair rate slows (Jang and Greenfield, 1996; Greig and Greenfield, 2004), indicating that this potential artifact did not confound the result that fluctuating signals are more attractive than the standard. Similarly, we consider the possibility that sensory adaptation to maximum peak amplitudes might have interfered with our tests of female discrimination between standard and fluctuating song. Here, females might adapt to the peak amplitude level of incremented pulse pairs in fluctuating song (PA_{inc} dB peSPL) and thereby ignore the decremented ones because these weaker pulse pairs fail to attain the insect's sliding threshold (amplitude within y dB of the maximum stimulus level) for response, i.e. decremented pulse pairs do not exceed (PA_{inc-y}) dB peSPL. But again, females subject to adaptation would be expected to perceive a slower pulse-pair rate and reduce, rather than increase, their response to fluctuating song.

Previous tests (Greig and Greenfield, 2004) have shown that *A. grisella* females evaluate acoustic power (pulse-pair rate multiplied by mean peak amplitude) of male song. Our present findings do not contradict this but rather indicate that additional song features are

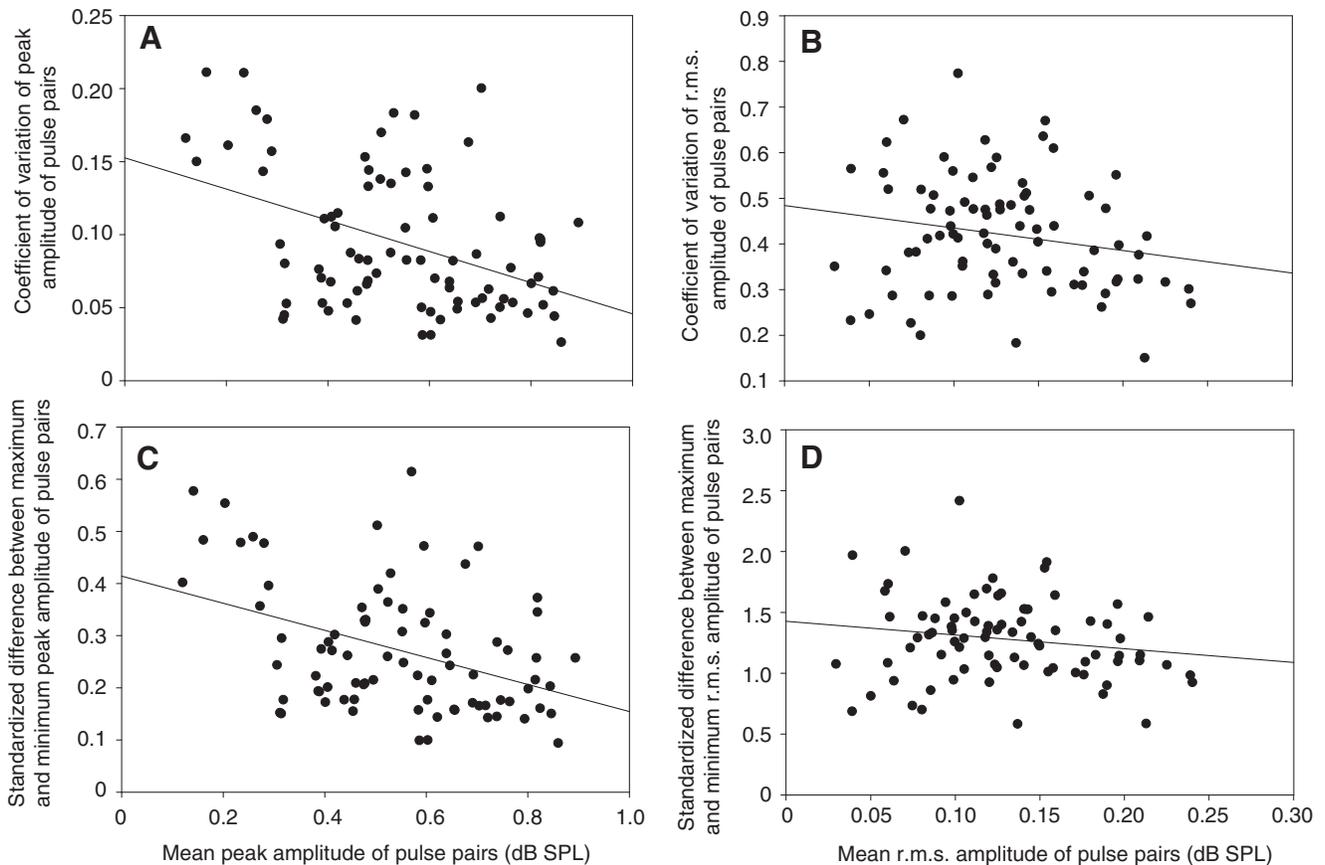


Fig. 6. (A) Coefficient of variation of peak amplitude of pulse pairs *versus* mean peak amplitude of pulse pairs. (B) Coefficient of variation of root mean squares (r.m.s.) amplitude of pulse pairs *versus* mean r.m.s. amplitude of pulse pairs. (C) Standardized difference between maximum and minimum peak amplitude of pulse pairs (see text) *versus* mean peak amplitude of pulse pairs. (D) Standardized difference between maximum and minimum r.m.s. amplitude of pulse pairs *versus* mean r.m.s. amplitude of pulse pairs. Data points represent 82 males sampled from the laboratory population. See Table 1 for correlation statistics.

evaluated. Given songs of equivalent acoustic power, female *A. grisella* appear to evaluate the maximum amplitudes that are attained during the course of a song and then orient preferentially toward the song with higher maxima; at present, we cannot distinguish whether this evaluation is based on peak or r.m.s. values of the maxima. One interpretation of this refinement is that song is evaluated by several processing mechanisms that operate simultaneously, each triggered by a different threshold amplitude level or summated over a different time constant (see Tougaard, 1998). Possibly, the trigger level of one processor is set rather high, such that it is only released by pulse pairs with peak or r.m.s. amplitudes that greatly exceeds the mean. We note that *A. grisella* have only four receptor neurons in each tympanum (Knopek and Hintze-Podufal, 1986), but we also note that each of these neurons could have different sensitivities (cf. Roeder, 1967). Thus, the hypothetical processors that are released by different trigger levels might be represented by the several receptor neurons. Subsequent to this peripheral neural processing, overall song evaluation would then occur via central processing of these several sensory inputs.

The various results of our three experiments testing female discrimination of standard *versus* fluctuating songs suggest that the time constant for evaluating amplitude maxima is approximately 10ms, one pulse-pair period in length. We observed that modification cycles as short as of 2 pulse-pair periods in length elicited preferences for fluctuating song (Fig. 3B,C), whereas

modifications made within a single pulse-pair generally did not (Fig. 3D). That is, females appeared to respond to maxima that lasted for 1 to 8 pulse pairs but not to shorter maxima. Females did not respond preferentially to fluctuating songs in which a single pulse was incremented followed by three slightly decremented pulses, and they showed only a weak preference for fluctuating songs in which a single pulse was decremented followed by three slightly incremented pulses. This latter finding suggests that amplitude maxima may be evaluated primarily at the level of the pulse pair rather than the individual pulse: in this test (Fig. 3D), females may have perceived two consecutive pulse pairs the peak amplitudes of which were each incremented by 25% (2dB) because they were relatively uninfluenced by the weaker pulse of a pulse pair.

Does female preference for fluctuating song merely reflect the outcome of physiological mechanisms of signal processing, or do females also profit from orienting toward these songs and from mating with males who produce them? Our data do not directly address this question, and we can only speculate on potential indirect (genetic) benefits obtained via phonotaxis and mate choice (Andersson and Simmons, 2006; Kotiaho and Puurtinen, 2007). Concerning female preference for male song broadcast at higher peak amplitudes, aside from the superior neural stimulation evoked by louder song and the more direct orientation that may result, one could readily propose that peak amplitude is a reliable indication of a male's 'condition'. This condition may represent his capacities to acquire and assimilate

food resources during larval development in a competitive social environment. Or, the condition may simply represent a heritable capacity to produce an attractive song, which assures a female that she will likely produce attractive male offspring. These two possibilities represent the 'good genes' (indicator) and Fisherian (arbitrary) mechanisms of sexual selection, respectively (see Kokko et al., 2002). We now ask whether one can propose analogously that a male's demonstrated ability to attain amplitude maxima – either peak or r.m.s. measures – that greatly exceed mean values offers similar assurance to a discriminating female? Perhaps these maxima cannot be produced in the absence of superior condition and the requisite genotype? Here, we suggest that the negative correlations observed between a male's mean peak amplitude and his coefficient of variation of peak amplitude pose a problem for the hypothesis that amplitude maxima serve as reliable indicators of male quality (see Searcy and Nowicki, 2005). On the one hand, we could expect that males who are deficient in some way and relegated to singing at low amplitudes might compensate for their inferior broadcasts by producing brief amplitude maxima. However, assuming that mean song amplitude is a reliable indication of male quality, females will receive conflicting information on such quality if they also evaluate amplitude maxima and orient toward males who produce them. But our experiments have not examined the relative weighting of mean amplitude and amplitude maxima in female evaluation of male song, and it is possible that evaluation of amplitude maxima merely refines the overall assessment of song. Thus, the several hypothetical processing mechanisms of song amplitude may provide hierarchical information on male competitive capacity and quality. Although we observed no significantly positive correlations between male mass and either amplitude maxima or the coefficient of variation of amplitude maxima, we suggest that mass does not necessarily represent an index of competitive capacity and quality (see Kasumovic and Andrade, 2009; Lehtonen and Lindstrom, 2009).

Our experimental findings and analyses motivate us to ask whether the markedly fluctuating amplitude levels observed in the songs of some male *A. grisella* reflect selection imposed by female choice? Such sexual selection would be consistent with the female preference observed for fluctuating as opposed to standard signals, but it is at odds with the absence of positive correlations between mean amplitude and the indices of amplitude fluctuation, and the expectations of signal reliability. Moreover, although the range of fluctuations observed for the r.m.s. amplitude of pulse pairs does surpass the magnitude of fluctuations in modified signals that elicited female preference in choice tests (Fig. 5B,D), the range of fluctuations observed for the peak amplitude of pulse pairs does not (Fig. 5A,C). This latter discrepancy could reflect mechanical constraints on the production of amplitude maxima. Thus, until we learn more about female evaluation of amplitude maxima, we cannot reject an alternative possibility that fluctuating amplitude levels simply represent a byproduct of the kinesiology of wing beating and the mechanics of song production.

Failure to resolve the various evolutionary questions notwithstanding, our findings reveal the complexity with which signal amplitude may be evaluated in animal communication. Several mechanisms of evaluation may occur simultaneously, and they may have selectively favored certain processes by which signals are generated. Evaluation of signal amplitude by the receiver, as well as its control by the signaler, clearly deserve further attention at both neuroethological and behavioral levels.

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