Double meaning of courtship song in a moth

Ryo Nakano¹, Fumio Ihara¹, Koji Mishiro¹, Masatoshi Toyama² and Satoshi Toda¹

¹Breeding and Pest Management Division, Institute of Fruit Tree Science, National Agriculture and Food Research Organization, 2-1 Fujimoto, Tsukuba, Ibaraki 305-8605, Japan
²Grape and Persimmon Research Division, Institute of Fruit Tree Science, National Agriculture and Food Research Organization, 301-2 Mitsu, Akitsu, Higashi-hiroshima, Hiroshima 739-2494, Japan

Males use courtship signals to inform a conspecific female of their presence and/or quality, or, alternatively, to ‘cheat’ females by imitating the cues of a prey or predator. These signals have the single function of advertising for mating. Here, we show the dual functions of the courtship song in the yellow peach moth, Conogethes punctiferalis, whose males generate a series of short pulses and a subsequent long pulse in a song bout. Repulsive short pulses mimic the echolocation calls of sympatric horseshoe bats and disrupt the approach of male rivals to a female. The attractive long pulse does not mimic bat calls and specifically induces mate acceptance in the female, who raises her wings to facilitate copulation. These results demonstrate that moths can evolve both attractive acoustic signals and repulsive ones from cues that were originally used to identify predators and non-predators, because the bat-like sounds disrupt rivals, and also support a hypothesis of signal evolution via receiver bias in moth acoustic communication that was driven by the initial evolution of hearing to perceive echolocating bat predators.

1. Introduction

Courtship displays, including courtship songs, sexual ornaments, pheromones, vibrations, tactile stimuli and nuptial gifts, have evolved in association with mate recognition and mate preference, and serve only as attractive signals in most animals [1–3]. Therefore, revealing the evolutionary origins of courtship signals is fundamental to understanding the evolution of sexual communication, sexual selection, speciation and biodiversity [4,5]. With respect to the origin of sexual communication, studying the ultrasound-based mating strategies in acoustic moths offers opportunities to better understand the evolution of courtship signals [1,6–10]. Moths have tympanal hearing organs that are sensitive to ultrasound and used primarily to detect bat predators emitting echolocation calls. These organs were not used originally to perceive mating partners producing calling/courtship songs, as has been confirmed by (i) palaeontological studies of moth fossils suggesting that tympanal organs evolved after the emergence of bats, (ii) structural homogeneity of the tympanal organs within the same superfamilies and heterogeneity of sound-producing organs within the same families, and (iii) auditory degeneration in moths in bat-free habitats or seasons [11–13].

After hearing organs evolved, some moth species developed acoustic sexual communication to enhance mating success via male calling or courtship songs [1,7,8,12–23]. Singing male moths are thought to use ultrasonic signals to attract females; however, how the females have developed a mating preference for the male song from the ancestral evasive response against echolocating bats remains unclear. Thus, understanding the moth mating system involving ultrasonic songs could lead to a better comprehension of the coevolution of male signals and female preferences, in addition to the prey–predator (moth–bat) interaction and its arms race [1,6,7,10–17,24–26].
Although increasing numbers of moths in which the males generate ultrasonic courtship songs have been reported, the detailed functions and evolutionary processes of these songs have not been studied in most of these species [1,6–10,23]. On the basis of receiver’s response, the functions of moth courtship songs can be divided into two types: ‘deceptive’ courtship song or ‘true’ courtship song. In some moths with the deceptive song, a male emits ultrasonic signals that provoke a female’s freeze response, which is also seen as a defence against attacking bats. For example, to increase their opportunities for copulation, singing males of the Asian corn borer, Ostrinia furnacalis (Pyraloidea, Cramibidae), exploit the female’s freeze response to ultrasound [7,18,19,21]. This mechanism is probably also present in the common cutworm, Spodoptera litura (Noctuoidea, Noctuidae), because mating is achieved even when bat-like sounds are broadcast instead of male songs [20]. By contrast, in other species with the true courtship song, such as the tiger and lichen moths (Noctuoidea, Arctiidae), females assume a mate-acceptance posture as a positive response to a male producing courtship song [7,10,14–17,27]. Unlike the corn borer, females of, for example, the Japanese lichen moth, Eilema japonica, do not show bat-avoidance responses to male courtship songs but do show evasive behaviour against bat echolocation calls; females develop mate preferences for male songs [7]. Male courtship songs in these species work inversely to one another—a negative or positive function. However, a courtship song with both functions has never been discovered.

In this report, we demonstrate a novel double-functioning courtship song in the yellow peach moth, Conogethes punctiferalis (Pyraloidea, Cramibidae). This species of nocturnal moth is a trivoltine agricultural pest in Asia; the larvae infest peach fruits in late spring–summer and chestnut fruits in summer–autumn, suggesting adult dispersion, and hence exposure to predatory bats. During courtship, after approaching a stationary female releasing sex pheromones during late scotophase, males hovering around the female produce a series of brief pulses and then one long pulse (figure 1a) [8,9]. In response, females assume a mate-acceptance posture, with raised wings (figure 1b), which is essential for mating to progress via the male landing and attempting to copulate. This female response is also evoked by a playback of the full male song, which is composed of short pulses and a long pulse (figure 1a); however, which elements of the song promote female acceptance are unknown [8,9]. Here, we hypothesized that female behavioural responses to the short and long pulses of the male courtship song are different. We predicted that the short-pulse components of the song do not serve as a mating signal for female acceptance because acoustic characteristics of the male short pulse are similar to attacking calls of insectivorous horseshoe bats [8]. In other words, we expected the male long pulse to induce female mate acceptance by her assuming a position with raised wings. To prove that the wing-raising is a mate-acceptance response, we then tested whether the wing-raising behaviour was limited to receptive females (i.e. sex-pheromone-releasing females). In addition, to demonstrate that the male long pulse is a positive mating signal, we checked the duration of pulses that induced receptive females’ wing-raising. To confirm a function of the male short pulse, we further compared the effects of male flight disruption by male song components and by bat echolocation cries.

2. Material and methods

(a) Animals

We collected larvae of the yellow peach moth, C. punctiferalis (Gueneé) (Pyraloidea, Cramibidae, Pyraustinae), from young peaches (Prunus persica (L.) Stokes) in orchards of the NARO Institute of Fruit Tree Science (36.05°N, 140.10°E; Tsukuba, Japan) in June 2011 and 2012. The larvae and their offspring were reared on an artificial diet of Silk-mate 2 M (Nosan Corp., Yokohama, Japan), oak sawdust and water at 24°C under conditions of a 16 L : 8 D photocycle and 50% relative humidity. We designated newly emerged adult moths as 0 days old, sexed them on the basis of genital morphology, and housed males and females individually in nylon mesh cages (30 × 30 × 30 cm) with a supply of 10% glucose solution until used in the following experiments.

(b) Activity of releasing sex pheromone in female

We introduced virgin female adults (1–3 days old) into the mesh cage and examined when the pheromone gland at the abdominal tip was exposed to release a sex pheromone. We directly observed females’ abdominal tips every 1 h in the late scotophase, 5–8 h into an 8 h scotophase (D5–D8), under a dim red light (0.6 lux). For 3-day-old females, which most often copulate with males [8], we checked the pheromone gland exposure every 1 h from L16 (1 h before the onset of scotophase) to L2 (2 h after the end of scotophase). Because the same females were used in each experiment, we statistically analysed these data by performing a likelihood ratio (LR) test in analysis of deviance in a generalized linear mixed model (GLMM; we used binomial error distribution with logit link function in all linear models) with a random effect of individual identity in R v. 2.14.0 [28].

(c) Activity of raising wings in female

We examined the frequency of female wing-raising (figure 1b) in response to male song playbacks (see below; figure 2a) in 1–3-day-old virgin females in late scotophase (D5–D8). In preliminary observations, some females who were not exposing a pheromone gland raised their wings to singing males and copulated with them. We therefore did not directly test whether females exposing the gland showed the wing-raising response. For 3-day-old females, we observed wing-raising behaviour every 1 h from L16 to L2. These females were individually
We edited male songs using the software DASYLAB BASIC looped for 2 s with realistic inter-pulse intervals (IPIs) of 25 ms. (figure 2).

**Figure 2.** Sound stimuli used in behavioural experiments. Oscillograms of 1 s are shown. (a) Representative short pulse (28.3 ms PD with 25 ms IPI) and long pulse (304.1 ms PD with 25 ms IPI) of male courtship song. These two stimuli were separately played back for 2 s to individual females to examine the correlation between pheromone-gland-exposing and wing-raising behaviours. See also §§5c and 3a, and figure 3b,c,d. (b) Eleven typical playbacks of male courtship song components. These stimuli were separately broadcast to individual females for 2 s. See also §§2d and 3b, and figure 3f. In experiments for male flight disruption, these stimuli were separately broadcast to individual flying males for 2 s in the cage and for 30 min in the flight tunnel. See §§2e and 3c, and figure 4a.c. (c) Fifty kilohertz simulations of male short pulse (28 ms PD with 26 ms IPI, 51.85% duty cycle (DC, proportion of PD per summation of PD and IPI), 18.52 pulse s⁻¹), male long pulse (339 ms PD with 26 ms IPI, 92.88% DC, 2.74 pulse s⁻¹), approach phase of CF pulse emitted from the greater horseshoe bat, R. ferrumequinum (30 ms PD with 30 ms IPI, 50.00% DC, 16.67 pulse s⁻¹) and approach phase of FM pulse emitted from the big-footed myotis bat, M. macrochrysus (5 ms PD with 11 ms IPI, 31.25% DC, 62.50 pulse s⁻¹). These stimuli and simulation of 50 kHz background noise were presented for 15 min in the flight tunnel. See §§2f and 3d, and figure 5a.

**Figure 2.** Sound stimuli used in behavioural experiments. Oscillograms of 1 s are shown. (a) Representative short pulse (28.3 ms PD with 25 ms IPI) and long pulse (304.1 ms PD with 25 ms IPI) of male courtship song. These two stimuli were separately played back for 2 s to individual females to examine the correlation between pheromone-gland-exposing and wing-raising behaviours. See also §§5c and 3a, and figure 3b,c,d. (b) Eleven typical playbacks of male courtship song components. These stimuli were separately broadcast to individual females for 2 s. See also §§2d and 3b, and figure 3f. In experiments for male flight disruption, these stimuli were separately broadcast to individual flying males for 2 s in the cage and for 30 min in the flight tunnel. See §§2e and 3c, and figure 4a.c. (c) Fifty kilohertz simulations of male short pulse (28 ms PD with 26 ms IPI, 51.85% duty cycle (DC, proportion of PD per summation of PD and IPI), 18.52 pulse s⁻¹), male long pulse (339 ms PD with 26 ms IPI, 92.88% DC, 2.74 pulse s⁻¹), approach phase of CF pulse emitted from the greater horseshoe bat, R. ferrumequinum (30 ms PD with 30 ms IPI, 50.00% DC, 16.67 pulse s⁻¹) and approach phase of FM pulse emitted from the big-footed myotis bat, M. macrochrysus (5 ms PD with 11 ms IPI, 31.25% DC, 62.50 pulse s⁻¹). These stimuli and simulation of 50 kHz background noise were presented for 15 min in the flight tunnel. See §§2f and 3d, and figure 5a.

confined in polypropylene cylinders (diameter 3 cm, length 5.5 cm) with nylon mesh screens 1 h before the experiments. Effect of age on wing-raising and its time course were analysed by a generalized linear model (GLM) and GLMM, respectively.

In addition to normal virgin females, we checked pheromone-gland-exposing behaviour and wing-raising behaviour in 3-day-old females that were copulating with males, that had mated on the previous day and that were virgin but had been decapitated on the previous day. Mating was conducted in the nylon mesh cage in D5–D8; immediately after pair formation, we confined a copulating couple in a polypropylene cylinder and examined the female’s wing-raising response to male song playbacks. For post-copulation females, we observed pheromone gland exposure and wing-raising behaviour in D7 after confinement in the cylinder in D6.

To test wing-raising behaviour in females, we broadcast representative short pulses (28.3 ms pulse duration, hereafter PD) and long pulses (304.1 ms PD) of male courtship songs (figure 2a) that we had recorded previously [8]. Each pulse was looped for 2 s with realistic inter-pulse intervals (IPIs) of 25 ms. We edited male songs using the software DASYLAB Basic v. 12.0 (Measurement Computing, Norton, MA) and played them with DASYLAB, a D/A converter (USB-1604HS, 16-bit, 192 kHz sampling; Measurement Computing) and an electrostatic speaker (ES1 speaker, ± 11 dB from 4 to 110 kHz, with ED1 speaker driver; Tucker-Davis Technologies, Alachua, FL). Because high-frequency (more than 90 kHz) sounds could not be reproduced with the broadcasting devices used here, we down-sampled .wav files from the original 300 to 192 kHz with R v. 2.14.0. The .wav files were converted into .asc files to play them. We broadcast the ultrasonic pulses of male courtship song at 100 dB peSPL (peak equivalent sound pressure level in decibel, 0 dB = 20 μPa) at 10 cm from the speaker membrane [29] and maintained a 10 cm distance between the speaker and a subject moth by moving the speaker manually. This sound level (100 dB peSPL), which we confirmed with an ultrasound recording system (type 4939 1/4-inch microphone, type 2670 preamplifier, type 2690 Nexus conditioning amplifier with a 0.02–100 kHz band-pass filter, type 4231 sound calibrator; Bruel & Kjær, Naerum, Denmark; USB-1604HS A/D converter with 16-bit, 320 kHz sampling), was consistent with the natural male song (103 dB peSPL at 1 cm) that a female receives in actual courtship [8].

**Figure 2.** Sound stimuli used in behavioural experiments. Oscillograms of 1 s are shown. (a) Representative short pulse (28.3 ms PD with 25 ms IPI) and long pulse (304.1 ms PD with 25 ms IPI) of male courtship song. These two stimuli were separately played back for 2 s to individual females to examine the correlation between pheromone-gland-exposing and wing-raising behaviours. See also §§5c and 3a, and figure 3b,c,d. (b) Eleven typical playbacks of male courtship song components. These stimuli were separately broadcast to individual females for 2 s. See also §§2d and 3b, and figure 3f. In experiments for male flight disruption, these stimuli were separately broadcast to individual flying males for 2 s in the cage and for 30 min in the flight tunnel. See §§2e and 3c, and figure 4a.c. (c) Fifty kilohertz simulations of male short pulse (28 ms PD with 26 ms IPI, 51.85% duty cycle (DC, proportion of PD per summation of PD and IPI), 18.52 pulse s⁻¹), male long pulse (339 ms PD with 26 ms IPI, 92.88% DC, 2.74 pulse s⁻¹), approach phase of CF pulse emitted from the greater horseshoe bat, R. ferrumequinum (30 ms PD with 30 ms IPI, 50.00% DC, 16.67 pulse s⁻¹) and approach phase of FM pulse emitted from the big-footed myotis bat, M. macrochrysus (5 ms PD with 11 ms IPI, 31.25% DC, 62.50 pulse s⁻¹). These stimuli and simulation of 50 kHz background noise were presented for 15 min in the flight tunnel. See §§2f and 3d, and figure 5a.

(d) Wing-raising behaviour of females to male ultrasonic pulses

After confirmation of exposure of their pheromone glands in D5 of scotophase, 3-day-old virgin females were individually confined in a polypropylene cylinder until the playback experiments were conducted in late (D7) scotophase. We selected 11 pulses (11.5, 16.2, 28.3, 40.6, 62.9, 83.7, 136.5, 213.1, 304.1, 396.1 and 521.0 ms PDs, including representatives of short and long pulses) from male courtship songs and played one type of pulse for 2 s with an IPI of 16–38 ms (figure 2b). We checked the presence of the female wing-raising response for the duration of the playback. The ES1 speaker was positioned 10 cm from a subject female to broadcast the male ultrasonic pulses of 100 dB peSPL (192 kHz sampling with the playback devices). Intervals between playbacks to the same individual were longer than 1 min. We observed whether the female raised her wings in response to the ultrasonic pulses. Because multiple stimuli were presented to each female in random order, data were analysed by a generalized additive mixed model (GAMM).

(e) Flight-stopping behaviour of males in response to male ultrasonic pulses

In D5–D8, we introduced a single virgin male (3–4 days old) into the mesh cage that already contained five virgin females. When the male started orientation flight towards a female, we presented one type of male courtship pulses (figure 2b) and background noise of 20–100 kHz in the experimental room. The ES1 speaker was held manually in the cage to maintain a distance of 10 cm between the speaker membrane and the moving male. Playback procedures were as described above (100 dB peSPL for male pulse and 24 dB peSPL for noise at 10 cm, 192 kHz sampling). We observed the male’s behavioural response (i.e. continued flight or stopping) within 2 s of the presentation of the pulses. Because different males were used each time, we used a generalized additive model (GAM) to analyse data with a consecutive independent variable.

Additionally, we confirmed the negative effect of male courtship pulses on rival male flight. To reproduce the male orientation flight for mating, we used a flight tunnel (0.25 m s⁻¹ wind speed; diameter 11.5 cm, length 66 cm) and a rubber septum impregnated with a synthetic sex pheromone (Sankei Chemical, Kagoshima, Japan). The lure was placed at the centre of a sticky square board (10 × 10 cm; Sumitomo Chemical, Tokyo, Japan) in the tunnel with screen steel meshes fitted at each end to restrict moth movement. We set the pheromone trap at the most upwind part of the tunnel and introduced five virgin males (3–4 days old) into the downwind end. The number of males...
Pulses, respectively (see also figure 3). Vertical bar: 95% CI of the mean rate (circle) to background noise playback. (PD (‘short pulse’ versus ‘long pulse’).

Male flight cessation in response to ultrasonic pulses of long duration (LR test with GAM, \( \chi^2 = 31.38, p < 0.0001 \)). Error bar indicates standard error of the mean. (f) Females (3 days old) raised their wings in response to playbacks of a series of male long pulses of 304.1 ms duration during late scotophase (LR test with GLMM for ‘short pulse’ versus ‘long pulse’, \( \chi^2 = 32.74, p < 0.0001 \)). Females did not raise their wings at all when exposed to 28.3 ms short pulses (data not shown; LR test with GLMM for ‘short pulse’ versus ‘long pulse’).

Figure 3. Correlation between pheromone-releasing and wing-raising in female. (a) Probability of exposing the sex-pheromone gland (y-axis in a,b) increases in frequency with age (x-axis in a,b) in females during late scotophase (7 h into 8 h scotophase, D7) (LR test in analysis of deviance with GLMM for age, \( \chi^2 = 8.25, p = 0.0041 \)). Females did not display wing-raising behaviour at all to series of male short pulses with 28.3 ms duration (data not shown; LR test with GLMM for ‘short pulse’ versus ‘long pulse’, \( \chi^2 = 8.25, p = 0.0041 \)). Females did not raise their wings at all when exposed to 28.3 ms short pulses (data not shown; LR test with GLMM for ‘short pulse’ versus ‘long pulse’).

Figure 4. Male flight cessation in response to ultrasonic ‘short pulses’ of the male courtship song. (a) Males showing orientation flight towards a female stopped flying when they heard male ultrasonic pulses of short duration (LR test with GAM for ‘duration’, \( \chi^2 = 49.92, p < 0.0001 \)), y-axis: probability of male flight cessation given a PD (x-axis). Each circle represents the mean rate. Broken lines indicate 95% CI of the mean. Left and right grey horizontal bars: ranges of 95% CI of short pulses and long pulses, respectively (see also figure 3). Vertical bar: 95% CI of the mean rate (circle) to background noise playback. (b) Males oriented towards a synthetic sex-pheromone lure on a sticky trap during late scotophase (7 h into 8 h scotophase, D7) (LR test with GLMM for ‘time of day’). Each bar represents mean (± s.e.) rate of males captured by the trap in L16—L2. Open and solid bars indicate pheromone and scotophase, respectively. (c) Fewer males were captured by the pheromone trap when male short pulses were played back for 30 min in D7 (LR test with GAM for ‘duration’, \( \chi^2 = 29.61, p < 0.0001 \)). Circles, broken lines, horizontal bars and vertical bar with circle are the same as those in (a). (Online version in colour.)
Figure 5. Disruption of male orientation flight by male courtship pulses and bat echolocation pulses. (a) Compared with short pulses of male song, more males were captured by the pheromone trap when male long pulses and background noise were presented in 15 min trials in the flight tunnel (LR test with GLM, male long pulse, \(\chi_1^2 = 7.00, \text{critical } ** p = 0.0085\); background noise, \(\chi_1^2 = 7.21, \text{critical } * p = 0.015\)). Approach phases of CF echolocation calls of \(R. \text{ferrumequinum}\) and FM echolocation calls of \(M. \text{macrodactylus}\) disrupted the flight of males and were equivalent to the male short pulse (CF call of \(R. \text{ferrumequinum}\), \(\chi_1^2 = 8.44, p = 0.0019\) (n.s.); FM call of \(M. \text{myotis}\) bat, \(\chi_1^2 = 2.36, p = 0.087\) (n.s.)). Each bar represents mean (+ s.e.) rate of males captured by the trap. (b) Distribution of duration of male short pulses and CF bat echolocation pulses (x-axis, PD; y-axis, number of pulse). The duration of short pulses emitted by male moths, \(C. \text{punctiferalis}\) (i), was within the range of that of the CF attacking pulses during the approach phase of \(R. \text{ferrumequinum}\) (ii). Laboratory data for the echolocation calls of \(R. \text{ferrumequinum}\) were derived from a previous paper by Hiryu and co-workers [32]. (Online version in colour.)

3. Results

(a) Correlation between releasing sex pheromones and raising wings in females

The frequencies of pheromone gland exposure and wing-raising (figure 1b) in response to male song playback (figure 2a) increased with age in 1–3-day-old virgin females (‘pheromone-gland exposure’, LR test in analysis of deviance with GLMM, \(\chi_1^2 = 31.38, p < 0.0001, n = 126\) from 42 females; figure 3c; ‘wing-raising’, LR test with GLMM, \(\chi_1^2 = 8.25, p = 0.0041, n = 190\) from 27–40 females; figure 3b). Females raised their wings when the long pulse (304.1 ms PD with 25.0 ms IPI) was played back but never in response to the short pulse (28.3 ms PD with 25.0 ms IPI; figure 2a).

Three-day-old females that were most responsive to male calls when they were 1–3 days old (figure 3c) vigorously exposed their pheromone glands 6–8 h into an 8 h scotophase (D6–D8; LR test with GLMM, \(\chi_1^2 = 110.35, p < 0.0001, n = 305\) from 100 females; figure 3c). Wing-raising responses to long pulses of the male song were also confirmed in the later scotophase but never in the photophase (LR test with GLMM, \(\chi_1^2 = 18.13, p = 0.0028, n = 228\) from 19 females; figure 3d). In addition, females that were engaged in copulation (\(n = 28\) females), that had mated the previous day (\(n = 19\)) or that had been decapitated (\(n = 21\)) never showed wing-raising in response to playback of either pulse. The female wing-raising response to male song was thus positively correlated to the behaviour of exposing the pheromone gland (Spearman’s rank correlation, \(\rho = 0.81, p = 0.0081, n = 9\); figure 3e).

(b) Female wing-raising response to long pulses of male courtship song

The long pulses that appeared in the late phase of the male courtship song (figure 1a) induced wing-raising in females captured on the sticky board was counted after 30 min. First, we checked male flight activity in one night (L16–L2). Second, in peak flight activity (D7; figure 4b), we examined the effects of playbacks of male song components (figure 2a) and background noise on male flight. The ESI1 speaker was placed outside the upwind end of the tunnel and directed downwind. Playback stimuli were the same as described above and looped for 30 min during the experiment. Data were analysed by GLM.

(f) Flight-stopping behaviour of males to simulations of bat echolocation calls

At the site where the moths were collected (Ibaraki, Japan), the typical moth-eating greater horseshoe bat, \(Rhinolophus ferrumequinum\) (Schreber), and the big-footed myotis, \(Myotis macrodactylus\) (Temminck), are reported [30,31]. For comparison with the disruptive effects of short pulses of male courtship song, we synthesized five stimuli (figure 2c): (i) a short pulse of male song (28 ms PD with 26 ms IPI), (ii) a long pulse of male song (339 ms PD with 26 ms IPI), (iii) an \(R. \text{ferrumequinum}\) approach-phase echolocation call (30 ms PD with 30 ms IPI) [32,33], (iv) an \(M. \text{macrodactylus}\) approach-phase echolocation call (5 ms PD with 11 ms IPI) [34], and (v) background room noise that had been band-pass filtered to 50 ± 1 kHz. These pulses (with the exception of the background noise) were formed with a rise/fall time of 1 ms. Because moths cannot discriminate sound frequencies [11–13], we used pure tones of 50 kHz to synthesize these stimuli. Each pulse was continuously broadcast at 100 dB peSPL (noise was broadcast at 22 dB peSPL) at a distance of 10 cm from the speaker.

Fifteen minutes after introducing four to five males into the downwind end of the tunnel, we checked the number of males captured by the pheromone trap at the upwind end. Data were analysed by GLM, and we calculated critical \(p\)-values by controlling the false discovery rate based on a two-stage linear step-up procedure to compare each effect of stimulus (ii)–(v) with that of stimulus (i) (male short pulse) in multiple testing [35].

To compare the duration of male short pulses and moth-attacking calls of laboratory \(R. \text{ferrumequinum}\), we generated a histogram (i.e. a distribution of PDs). PD data for \(Rhinolophus\) echolocation calls (taken from 16 moth-capturing flights of four bats in a flight room) were obtained from S. Hiryu (Doshisha University, Kyoto, nabe City, Japan). We used echolocation pulses of the approach phase emitted by an attacking bat after emission of the long duration pulse (more than 60 ms) [32]. The 95% intervals of duration of moth short pulses and bat echolocation calls were calculated.
exposing the pheromone gland (figures 1b and 3f). On the other hand, females did not raise their wings significantly frequently in response to the short pulses (LR test with GAM, $\chi^2 = 288.03, p < 0.0001, n = 428$ from 78 females; figure 3f). The representative short pulses (28.3 ms PD) and long pulses (304.1 ms PD) used here caused wing-raising behaviour in 2.5% and 77.5% of females, respectively. Pure tone (25 kHz) of roughly 500 ms PD produced by a commercial dog-whistle also elicited female wing-raising (figure 1b; electronic supplementary material, movie S1).

(c) Male flight disruption by short pulses of male courtship song

Short pulses of male song made males more frequently stop their orientation flight than did long pulses (LR test with GAM, $\chi^2 = 49.92, p < 0.0001, n = 391$ males; figure 4a). A large percentage (71.4%) of flying males ceased orientation flight in response to a representative short pulse (28.3 ms PD), whereas a moderate percentage (45.7%) of males did so when hearing a representative long pulse (304.1 ms PD). In response to noise playback, fewer (22.2%) males stopped their flight ($n = 18$ males; red bar with open circle in figure 4a).

In the flight tunnel with a synthetic sex pheromone, males showed the highest activity (64.0%) of flight at D7 of late scotophase (LR test with GLM, $\chi^2 = 26.80, p = 0.0015, n = 250$ males; figure 4b). Again, in the playback experiment with the flight tunnel at D7 only, fewer males were attracted to the pheromone lure when we broadcast short pulses (LR test with GAM, $\chi^2 = 29.61, p < 0.0001, n = 275$ males; figure 4c) as long ones. The trap captured 24.0% and 72.0% of the males that heard short and long pulses of male song, respectively. When background noise was played, 68.0% of males were captured ($n = 25$ males; red bar with open circle in figure 4c).

(d) Male flight disruption by bat echolocation call

Simulation of constant-frequency (CF) echolocation calls of the greater horseshoe bat, R. ferrumequinum, frequently disrupted male flight in the flight tunnel, as did short pulses of male song (LR test with GLM with false discovery rate adjusted by two-stage linear step-up procedure [35], $\chi^2 = 0.84$, critical $p = 0.19, n = 128$ males in total; figure 5a). Also, a relatively short pulse of frequency-modulation (FM) echolocation calls of the big-footed myotis bat, M. macrodactylus, another insectivorous bat that is sympatric to the moth, slightly inhibited the male orientation flight ($\chi^2 = 2.36, p = 0.087$). Simulated long pulses of male song and of background noise, however, did not affect male flight (male long pulse, $\chi^2 = 7.00, p = 0.0085$; noise, $\chi^2 = 7.21, p = 0.015$; figure 5a). Acoustic analyses revealed that the duration of the male short pulse of the yellow peach moth and that of approach-phase calls emitted by laboratory R. ferrumequinum overlapped (95% quantile, 22.27–34.64 ms for moth courtship short pulse, $n = 120$ pulses from 24 males; 17.31–47.34 ms for bat approach pulse, $n = 260$ pulses from four bats; figure 5b).

4. Discussion

In this study, we demonstrated that the male courtship song of the yellow peach moth has dual functions—short pulses emitted in the early phase are repulsive, whereas long pulses in the late phase of a single courtship song are attractive. Our results indicate that animals can have evolved manifold signals, including mutually opposite effects in a single courtship behaviour that has thus far been believed to be simply attractive to a partner. Additionally, recognizing the multiple functions of courtship signals provides a better understanding of the origins of sexual communication; prey–predator interactions have driven ultrasonic communication in moths.

A long pulse in male song is essential to induce the female to assume a mate-acceptance posture (i.e. wing-raising) that only occurs in virgin receptive females (figures 1b and 3a–c). We therefore concluded that the long pulse serves as a signal for mate recognition by females. Because a courting male can attempt to grasp a female’s genitalia shortly after landing beside her raised wings, female wing-raising is indispensable for successful copulation [8,9]. Response frequency of wing-raising peaked at a long PD of more than 200 ms (figure 3f). Nearly all males produce a long pulse of 297–381 ms duration (95% confidence interval (CI), $n = 66$). Therefore, females of this species, as well as those of the Asian corn borer, accept most of the normal singing males [7,18,19,21]. As shown in figure 1b and the electronic supplementary material, movie S1, which depict female mate acceptance to artificial long ultrasound, wing-raising seemed to be an inevitable response to a long pulse of more than 200 ms. We cannot confirm the mechanism of this response, but signal processing via the central nervous system must be involved because decapitated or mated females never raise their wings.

Male short pulses do not directly affect a female’s mate acceptance (figure 3), but are likely to contribute to successful mating by influencing the behaviour of rival males. In the European corn borer, Ostrinia nubilalis, which belongs to the same subfamily (Pyraustinae) as the Conogethes used in this study, a single sex-pheromone-releasing female might be courted simultaneously by multiple males [36]. If a similar situation occurs in our moth in the wild, the males could not successfully copulate, because they can land beside her and attempt to mate only when she is raising her wings in response to a male long pulse [8,9]. Thus, the presence of other courting males obstructs copulation by impeding timing/position of his landing. The first male that arrives near a female for mating, therefore, tries to deter rivals from approaching her by generating short pulses (28 ms PD with 26 ms IPI) like the attacking calls (30 ms PD with 30 ms IPI) of horseshoe bats (figures 4a,c and 5a). Male short pulses and horseshoe bat calls equivalently suppressed male orientation flight towards a sex pheromone (figure 5a). Singing males evidently exploit the bat-avoidance behaviour of their conspecifics to deter rival males and more successfully court the female. Actually, we have never seen multiple males of Conogethes simultaneously court the same female, although several males were flying at the same time in the laboratory cage. The effective range of the male courtship song is unknown, but it appears to be greater than 30 cm, because females and males in a cage showed wing-raising and flight-stopping, respectively, when a pioneer male sang in another cage located that distance away.

The dual function of the male courtship song in the yellow peach moth reflects its evolutionary origin. The evolution of moth acoustic communication is explained by receiver bias or sensory exploitation of ultrasound detection, which originally evolved to perceive bat predators, and enabled moths to evolve to use novel sound signals [1–3,37,38]. In the yellow peach moth, male short pulses (repulsive) exploit only the
original state of the receivers. In females, wing-raising possibly originated from a power dive to avoid bats. Flying moths may dive to the ground by raising their wings and keeping them motionless. In preliminary experiments, however, male long pulses evocative of female wing-raising did not substantially suppress female or male flight, unlike male short pulses (figures 4 and 5). In addition, wing-raising was never found in virgin females in photophase, mated females or males, and was not found even in pheromone-releasing females when any bat calls were presented. All these observations imply that, at present, female wing-raising is not a bat-avoidance response. We could not determine how females acquired the preference for the long pulse (more than 200 ms PD) in this study, but female acceptance (wing-raising) and the male attractive trait (long pulses) evolved together through sexual selection processes such as in the lesser wax moth [39–42]. Eared moths, which have already evolved evasive responses to bat echolocation calls, can independently evolve a sexual response to male song [25,26]. Therefore, male signals currently attractive to females might not have originally exerted negative effects on the ancestral species.

Acknowledgements. We thank S. Hiryu (Doshisha University) for providing the data of R. ferrumequinum echolocation calls and E. Morley (University of Toronto Scarborough) for helpful comments on this study.

Funding statement. This study was supported by a Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science (R.N., no. 23780053), the Oso Acoustic Research Fund (R.N.), a Kurata grant (R.N., no. 1172) and an internal research grant from the NARO Institute of Fruit Tree Science (R.N.).

References


