The odour makes the difference: male moths attracted by sex pheromones ignore the threat by predatory bats

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The odour makes the difference: male moths attracted by sex pheromones ignore the threat by predatory bats

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Many animals have to trade off predator avoidance and other important activities influencing fitness, such as foraging and mate finding (Dukas 2002). Whereas several studies have focused on how prey alter their foraging behaviour in relation to the risk of predation (Metcalfe et al. 1987, Hamilton and Heithaus 2001), analyses of behavioural trade-offs between reproduction activities and predator avoidance are rare (Jones et al. 2002). In addition, few studies have focused on predator-prey systems where the conflicting signals originate from different sensory modalities (Skals et al. 2003).

Ultrasonic hearing has evolved independently in several families within Lepidoptera as a response to the selection pressure imposed by echolocating, insectivorous bats (Roeder 1962, Scoble 1992, Miller and Surykke 2001). Ultrasound-sensitive ears in combination with an array of defensive manoeuvres enable moths to increase the probability of surviving attacks by bats. A lot of research has focused on the hearing ability and predator avoidance in moths (Fullard and Yack 1993, Miller and Surykke 2001). However, in the majority of cases, ultrasound-triggered evasive reactions have only been analysed using tethered moths or free flying moths presumably not engaged in other adaptive behaviours associated with fitness, e.g. foraging or mate searching, at the time of sound exposure (Roeder 1962, 1967, Rydell et al. 1997, Skals and Surykke 2000).
Male finding in moths is often guided by olfaction, generally by females releasing long-range sex pheromones to attract males (Cardé and Baker 1984). Thus, the search flight of males is associated with potentially increased vulnerability to predatory bats. Due to their short life span and constraints that limit opportunities for mating, males are faced with a trade-off between reproduction activities and predator avoidance. To date, however, few studies have analysed how increased predation risk by bats alters the sexual behaviour in moths (Baker and Cardé 1978, Acharya and McNeil 1998).

We studied how odour cues that signal presence of a mate influence the avoidance behaviour elicited by ultrasounds mimicking echolocating bats in two species of moths: *Agrotis segetum* Denis and Schiffermüller (Noctuidae) and *Plodia interpunctella* Hübner (Pyralidae). These species belong to families with independently evolved hearing systems. Whereas noctuid moths use a pair of tympanic organs (containing two sensory cells) located dorso-laterally on the metathorax for ultrasound detection (Roeder 1964, Fullard 1988), the hearing organs (including four sensory cells) of pyralid moths are located on the pleural-ventral side of the first abdominal segment (Mullen and Tsao 1971). Males were exposed to ultrasound when orienting in pheromone plumes in a flight tunnel.

We hypothesised that a male’s response to the increased predation risk would depend on the relative quality of the pheromone signal. This was investigated by measuring the proportion of moths locating the pheromone source after ultrasound exposure. We predicted (i) no difference in the proportions reaching the odour source between ultrasound-exposed and unexposed males when responding to high-quality odour cues, i.e. a female pheromone gland extract or the complete synthetic pheromone blend at high dose but that (ii) fewer ultrasound-exposed males would locate the pheromone source when flying in odour plumes of low quality, i.e. incomplete pheromone blends or the complete blend at a low dose because of “time-consuming” avoidance behaviours.

**Material and methods**

**Insects**

Male *A. segetum* were obtained from a laboratory culture maintained at the Dept of Ecology in Lund, Sweden. The culture was established 25 years ago from field-collected moths from southern Sweden and Denmark and genetically refreshed on a yearly basis by mating of ca 100 wild males and females from the culture with ca 100 field-collected moths. Larvae were reared on a bean diet described by Zhu et al. (1996) at 25°C and 60% RH.

Male *P. interpunctella* were obtained from a laboratory culture maintained at the Dept of Ecology at Lund University. The culture was established five years ago from a laboratory culture at the Danish Pest Infestation Laboratory at Lyngby (Denmark), and mixed with about 100 males and 100 females from the Central Science Laboratory, Slough (England) after two years. Larvae were reared on a diet described by Zhu et al. (1999) at 22°C and 60% RH. Pupae of both species were separated by sex and placed in a L17:D7 photoperiod at 22°C and 60% RH.

**Chemicals and dispensers**

Compounds were purchased from DLO Plant Research International PRI, Wageningen (the Netherlands), or from Bedoukian Research Inc, Danbury (USA). Pheromone stimuli used were categorised as “high-quality” or “low-quality” based on their attractivity on male moths in earlier flight tunnel studies. For *A. segetum*, two pheromone blends were used: (i) a high-quality four-component blend of (Z)-5-decenyl acetate (Z5-10:OAc), (Z)-7-dodecencyl acetate (Z7-12:OAc), (Z)-9-tetradecenyl acetate (Z9-14:OAc), and (Z)-5-dodecencyl acetate (Z5-12:OAc), in the proportions 1/5.2/5.0/25.0 (Löfstedt et al. 1982, Wu et al. 1995), and (ii) a low-quality two-component blend of Z5-10:OAc and Z9-14:OAc, in the same relative proportions as in the four-component blend, and shown to be less attractive to males compared to the four-component blend (Wu et al. 1995). Blends were prepared in distilled hexane and applied on rubber septa (red sleeve, 16 × 9 mm, catalogue no. 1780J07, Thomas Scientific, Swedesboro, USA) corresponding to 100 ng of Z5-10:OAc. A lower dose stimulus (10 ng of Z5-10:OAc) of the four-component blend was also used, which is less attractive to males compared to the 100 ng dose (Valeur et al. 2000). Septa were put in a fume hood for 3 h to allow the hexane to evaporate. Between experiments septa were stored at −18°C.

For *P. interpunctella*, three pheromone stimuli were used: (i) a high-quality four-component blend of (Z,E)-9,12-tetradecadienyl acetate (Z9,E12-14:OAc), (Z,E)-9,12-tetradecadienol (Z9,E12-14:OH), Z9-14:OAc and (Z,E)-9,12-tetradecadienal (Z9,E12-14:Ald) in the ratios 100/18/12/11 (Kuwahara et al. 1971, Zhu et al. 1999), (ii) a low-quality stimulus of only Z9,E12-14:OAc and (iii) a pheromone gland extract from 15 females (2–4 days old), dissected 1–3 h into the scotophase and extracted in distilled hexane. The aldehyde was synthesised from Z9,E12-14:OH at the Dept of Chemistry, Royal Institute of Technology, Stockholm, Sweden. For emission of pheromone stimuli, a modified version of the sprayer device (Skals et al. 2003) originally described by El-Sayed et al. (1999) was used. The piezo ceramic disc was driven by a Tektronix function gen-
Sound emission system

The acoustic stimuli were created by multiplication of signals from a pulse generator (Berkeley Nucleonics Corporation 555) and a sine wave generator (Agilent 33120A) in a custom built trapeze modulator. The signal was attenuated (Kay 865 step attenuator), amplified (Xelec power amplifier) and broadcast through a Technics leaf tweeter (EAS10TH400B). Different sound stimuli were used for the two moth species, as they differ in sensitivity to ultrasound. The sound regimes used were those eliciting strong and consistent evasive responses by the moths in preliminary experiments.

For A. segetum, the stimulus consisted of 1 ms long pulses with a repetition rate of 180 pulses/s and a total stimulus length of 1 s, at 20 kHz, corresponding to the best frequency of hearing in this species (Surlykke and Miller 1982, Waters and Jones 1996). The highest sound pressure level that could be produced by the loudspeaker at this frequency (102 dB SPL at 30 cm) was used, and this intensity matched those emitted by sympatric bat species (e.g. 103–113 dB SPL (RMS) Rydell et al. 1999). Hence, the intensity of our stimuli was 50 to 60 dB above the threshold for the most sensitive sensory cell (A1) and 30 to 40 dB above the A2 threshold of A. segetum (Surlykke and Miller 1982, Waters and Jones 1996).

For P. interpunctella, the sound stimulus consisted of 10 ms long pulses with a repetition rate of 30 pulses/s and a total stimulus length of 2.5 s. The carrier frequency of the pulses was 50 kHz, which corresponds to the frequency with lowest threshold for flight cessation of moths in tethered flight (Svensson et al., unpubl.). The highest sound pressure level emitted by the loudspeaker at this frequency (95 dB SPL at 30 cm) was used.

The loudspeaker was positioned 1 m downwind from the odour source at the same height (30 cm) as the pheromone plume. The opposite wall of the flight tunnel was covered with a 30 cm wide portion of sound absorbing material to minimise sound reflections. Calibration of the loudspeaker was done several times during the experimental period by a 0.25-inch G.R.A.S. (Vedbaek, Denmark) microphone (Type 40 BF) without grid, a G.R.A.S. preamplifier (Type 26AC), and a measuring amplifier (Type 12 AK). In order to calculate the intensity of the sounds, the microphone (with grid on) was calibrated against a G.R.A.S. sound calibrator (Type 42AB).

Flight tunnel experiments

The behavioural response of male moths to the conflicting olfactory and auditory stimuli were observed in a 0.9 × 0.9 × 3 m Plexiglas flight tunnel (Valeur and Löfstedt 1996). During flight experiments the conditions were: temperature 21–22°C, humidity 28–32%, wind speed 0.3 ms⁻¹ and light intensity 4 lux. Experiments were conducted one to four hours into the scotophase, using two to four day old males of A. segetum and one to six day old males of P. interpunctella. Each male was introduced in the downwind end of the flight tunnel in cylindrical screen cages (and thus exposed to the pheromone plume) for approximately five seconds, and then allowed to take off from the cylinder. Males not taking flight within three minutes were excluded from the protocol. When a moth was orienting in the plume at the position of the loudspeaker the acoustic stimulus was switched on manually, hence targeting the moth at its right side in relation to the flight direction. Moths were then observed for three minutes. The proportion of males reaching the pheromone source was scored as well as the source location time, i.e., the time spent to reach the odour source from the position of the loudspeaker. To calibrate for differences in attraction to the different odour stimuli, control males without sound stimulation were also tested. Each male was only used once. Differences in proportion reaching the odour source between ultrasound-exposed and unexposed moths were analysed using χ² statistics.

Results

General avoidance behaviour

Ultrasound exposure elicited different anti-predator responses in the two species. Male A. segetum showed a broad repertoire of defensive flight manoeuvres. The most common response involved looping (on about 50% of trials), but other behaviours, e.g. dives, backward flight and accelerated forward flight were also observed. In contrast, male P. interpunctella showed a more stereotypic ultrasound-triggered response pattern with cessation of flight and dropping to the ground in the majority of cases (≈ 70%). No detailed estimations
of response pattern differences between groups of males were performed due to the difficulties in categorising the evasive responses, which were often combinations of several behaviours, e.g. loops and forward flight.

**Effect of pheromone blend on male avoidance behaviour**

When males of *A. segetum* were flying towards the four-component blend, there was no difference between ultrasound-exposed and unexposed moths in the proportion locating the odour source (exposed: 18 out of 20, unexposed: 19 out of 20, $\chi^2 = 0.35$, d.f. = 1, $P > 0.05$, Fig. 1). The source location time was $54 \pm 14$ s for exposed and $25 \pm 8$ s for unexposed males, respectively. In contrast, when moths were flying towards the two-component blend significantly fewer males with ultrasound exposure (seven out of 16) located the pheromone source within the time limit compared to control moths (17 out of 19, $\chi^2 = 8.55$, d.f. = 1, $P < 0.01$). The source location time was $118 \pm 20$ s for exposed and $34 \pm 12$ s for unexposed moths, respectively.

All ultrasound-exposed ($n = 27$) and control ($n = 29$) males of *P. interpunctella* flying towards the synthetic four-component blend reached the pheromone source within the time limit with source location times of $12 \pm 1$ s and $7 \pm 1$ s, respectively. Similarly, no difference in source contact level was observed between ultrasound-exposed (24 out of 25) and unexposed (26 out of 26) moths ($\chi^2 = 1.03$, d.f. = 1, $P > 0.05$) when flying towards the female gland extract stimulus. The source location time for moths exposed to ultrasound was $26 \pm 7$ s and for control moths $12 \pm 2$ s. However, when males were flying towards Z9,E12:14:OAc alone, significantly fewer ultrasound-exposed moths (eight out of 28) reached the pheromone source compared to unexposed ones (18 out of 28, $\chi^2 = 7.18$, d.f. = 1, $P < 0.01$). Source location times were $142 \pm 13$ s and $72 \pm 16$ s for exposed moths for control moths, respectively.

**Effect of pheromone dose on male avoidance behaviour**

The dose of the high-quality pheromone blends also influenced the risk-taking behaviour of moths (Fig. 2). No difference was observed in source contact level between groups of male *A. segetum* when flying towards the four-component blend at the dose of 100 ng of Z5:10:OAc (exposed: 21 out of 24, unexposed: 23 out of 24, $\chi^2 = 1.09$, d.f. = 1, $P > 0.05$). Males exposed to ultrasound reached the odour source after $41 \pm 12$ s whereas unexposed moths did so after $25 \pm 9$ s. However, whereas all of the 26 unexposed males except one reached the odour source within 180 s when flying towards the dose containing 10 ng of Z5:10:OAc, a significantly lower proportion (18 out of 24) did so after ultrasound exposure ($\chi^2 = 4.48$, d.f. = 1, $P < 0.05$). The source location time was $66 \pm 15$ s and $17 \pm 7$ s for ultrasound-exposed and unexposed moths, respectively.

All ultrasound-exposed ($n = 24$) and unexposed ($n = 24$) males of *P. interpunctella* reached the pheromone source when the four-component blend was emitted at a rate of 50 pg/min of Z9,E12:14:OAc, with source location times of $13 \pm 1$ and $10 \pm 1$ s, respectively. In contrast, significantly fewer ultrasound-exposed males (two out of 24) compared to unexposed ones (13 out of 24) located the odour source when flying towards the pheromone source released at the rate of 50 fg/min of Z9,E12:14:OAc ($\chi^2 = 11.73$, d.f. = 1, $P < 0.001$). Ex-
Fig. 2. Percentage of ultrasound-exposed (grey bars) and unexposed (white bars) males of *Agrotis segetum* and *Plodia interpunctella*, reaching pheromone sources of the same composition but different quantities in a flight tunnel. The blend of *A. segetum* included Z5-10:OAc, Z7-12:OAc, Z9-14:OAc and Z5-12:OAc in the proportions 1/5/2.5/0.25. The blend of *P. interpunctella* included Z9,E12-14:OAc, Z9,E12-14:OH, Z9,E12-14:Ald and Z9-14:OAc in the proportions 100/18/11/12. Between 24 and 26 males were tested in each group. Differences between groups were compared using *t*-tests (significance levels: * P < 0.05, ** P < 0.01, *** P < 0.001).

Discussion

This study shows how the auditory-based predator avoidance behaviour of male moths is altered depending on the quality of the olfactory cue signalling a mate. The majority of males of both species responded to the acoustic cue by strong evasive reactions that took them out of the odour plume but large differences were observed in the subsequent ability to relocate the pheromone plume. Most males flying towards high-quality odour sources (pheromone gland extract or complete synthetic blend at high dose) quickly relocated the plume after ultrasound exposure and reached the odour source at a similar level as controls. However, those flying towards low-quality blends or doses showed stronger reactions to the sound, and hence fewer reached the pheromone source. Thus, a male moth flying towards an odour stimulus of high quality seems to take higher risks than those flying towards odour sources of low quality.

The risk-taking behaviours of the two moth species were similar although the ultrasonic hearing systems of Noctuidae and Pyralidae have most likely evolved independently. Interestingly, male *P. interpunctella* showed strong reactions to the ultrasound cues which suggests that this indoor pest has retained ultrasonic hearing and associated defence behaviours in spite of the lack of predation pressure from echolocating bats in indoor facilities. This is in contrast to other moths that have lost their hearing capacity after becoming isolated from bats temporally or geographically (Fullard et al. 1997).

In dose experiments, the difference in evasive reactions to the ultrasound was more pronounced for *P. interpunctella* than for *A. segetum*. This was probably due to a 1000-fold difference in dose between odour stimuli used for *P. interpunctella* compared with only a 10-fold difference between stimuli for *A. segetum*. In preliminary tests a 1 ng instead of a 10 ng stimulus was used to get a larger difference in dose between stimuli, but less than 5% of *A. segetum* males located the 1 ng lure.

Most studies on trade-offs between mating activities and predator avoidance have included cases where the conflicting stimuli are linked to the same sensory modality. Rohr and Madison (2001) observed a trade-off in behaviour of male salamanders when exposed to conflicting olfactory cues signalling reproduction and predation. Behavioural trade-offs between reproduction and predation risk based on visual cues have been observed in gobid fish (Forsgren 1992) as well as water striders (Sih et al. 1990). Analyses of systems where the conflicting cues represent different sensory modalities are rare. Acharya and McNeil (1998) observed an increase in aborted flights of male *Ostrinia nubilalis* Hübner (Pyralidae) and *Pseudaletia unipuncta* Haworth (Noctuidae) in a sex pheromone plume with increased predation risk level. However, even at the highest predation risk level, a significant proportion (> 30%) of exposed males for both species remained in the
pheromone plume, indicating that the evasive behaviour was modified by the chemical stimulus. The length of the sound stimulus used in that study was ten seconds, i.e. much longer than the stimulus length used in the present study, and much longer than moths would be exposed to the echolocation calls of a passing bat under natural conditions (Schnitzler and Kalko 2001). Thus, the relatively stronger reactions of moths to ultrasound observed in that study compared to our study may be explained by the use of a super-stimulus of sound. We intended to stimulate the moths when they were in the odour plume only and used a relatively short sound stimulus.

Baker and Cardé (1978) observed the evasive behaviour of male Lymantria dispar (L. (Lymantriidae) after exposure to high-frequency sound from jingling keys. Although the majority of males lost their orientation towards sex pheromone traps in the field after sound exposure, about 25% returned to the odour plume within seconds, again indicating high risk-taking of males flying towards high-quality odour cues.

We only studied the risk taking behaviour of male moths and it may be hard to extrapolate our data to female behaviour. According to sexual selection theory males should take higher risks than females due to the asymmetry in reproductive potential between the sexes (Magnhagen 1991). Radar tracking of male A. segetum in the field has shown that their flight routes can range over several kilometres during a single night (Svensson et al. 2001), indicating a potentially high risk of predation by foraging bats. However, female calling behaviour in moths is also affected by acoustic cues mimicking a bat predator (Acharya and McNeil 1998, Jones et al. 2002, Svensson et al. 2003). Furthermore, Skals et al. (2003) have recently shown that if male and female moths are in the same behavioural context when exposed to bat sounds, their risk-taking willingness is equal. Thus, the sex-biased predation rate in moths found by Acharya (1995) is probably caused by different flight activity rather than different risk-taking behaviour between the sexes.

Using flight tunnel experiments, the possibility to standardise the ultrasound exposure of moths is greatly enhanced compared to field conditions. However, due to the enclosed nature of the flight tunnel it may be difficult to assess the importance of exposed males’ manoeuvres occurring after the initial response to the acoustic predator cue. In the field, evasive flight behaviours may take a moth several meters away from the pheromone plume, which should decrease the possibility to relocate the odour plume compared to a flight tunnel situation where the distance between the moth and the plume is always less than a meter. Although there are limitations involved in using a flight tunnel for this kind of experiments, the behavioural differences observed in the present study were distinct and would probably be even more pronounced under natural conditions when relocation of odour plumes is further constrained due to turbulent wind, vegetation etc.

We have demonstrated how the predator avoidance behaviour of male moths is altered depending on the quality of the olfactory cue signalling a mate. Studies on the trade-off between predation risk and mate finding in other families of eared moths are needed, as well as analyses of evasive behaviour when both predation and mate cues are manipulated, to get a better picture of the dynamic integration of these conflicting sensory signals.

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