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SOUND PRODUCTION BY ABDOMINAL TYMBAL ORGANS IN TWO MOTH SPECIES: THE GREEN SILVER-LINE AND THE SCARCE SILVER-LINE (NOCTUOIDEA: NOLIDAE: CHLOEPHORINAE)

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Summary

Male moths of the chloephorine species *Pseudoips prasinana* and *Bena bicolorana* produce clicks (approximately 100 dB peSPL at 10 cm) using ventral tymbal organs located in a cleft in the second abdominal sternite. Large muscles insert on the dorsal part of the tymbal frame and rhythmically flex a thin sheet of cuticle. Normally, each sound-production cycle contains four clicks, the left and right tymbals producing clicks both on active buckling caused by muscle contraction and on the passive elastic return from buckling. Histochemical staining indicated the presence of elastic resilin-like proteins in the tymbals. Obvious differences between the click patterns of the two species reflect differences in their tymbal morphology. *P. prasinana* has smooth tymbals and produces a single click (300 μs, 40 kHz) for each tymbal buckling. In contrast, *B. bicolorana* has striae on the medial part of the tymbals. Accordingly, it produces many clicks per buckling. The click pattern is a heterogeneous mixture of large clicks at 52 kHz, resembling those of *P. prasinana*, interspersed with series of broad-band clicks (20–100 kHz) of lower intensity (15–20 dB). Thus, in chloephorine moths, there is a correlation between the structure and function of the smooth and striated tymbals that is strikingly similar to that in arctiid moths, although the two types of tymbals have evolved independently. The hearing of *P. prasinana* is tuned to its own sounds with lowest threshold (38 dB SPL) at 40–60 kHz. We suggest that sound production in male chloephorines plays a part in sexual acoustic communication.

Key words: moth, Chloephorinae, Noctuoidea, *Pseudoips prasinana*, *Bena bicolorana*, sound production, tymbal, hearing threshold, ultrasound, acoustic communication.

Introduction

Many moths, including most Noctuoidea, Geometroidea and Pyraloidea, have simple ears that are sensitive to ultrasound. Most moths are silent, and their acoustic behaviour is limited to different evasive manoeuvres elicited by bat sonar signals. Therefore, it is commonly assumed that the ears have evolved as a defence against echolocating bats (Fullard, 1988). However, a few moths employ sounds as part of their intraspecific communication (for a review, see Conner, 1999). In Pyralidae, there are several examples of ultrasonic signalling, probably involved in mate formation (Gwynne and Edwards, 1986; Spangler, 1988; Heller and Krahe, 1994; Trematerra and Pavan, 1995). Different sound-producing mechanisms have been described in Noctuoidea, including stridulation in *Thecophora fovea* (Surlykke and Gogala, 1986), percussive sound production using the forewings in the Australian whistling moth *Hecatesia* sp. (Bailey, 1978) and buckling of the tymbals either on the body (Lymantriidae; Dall'Asta, 1988) or on the wings (*Amyna natalis*; Heller and Achmann, 1993).

The most widespread sound-producing organs in moths are the tymbals of the arctiid moths, which are air-filled blisters of cuticle on the thoracic metepisterna surrounded by an almost oval ring of heavily sclerotized cuticle (Blest et al., 1963; Fullard, 1977). Contraction of the coxal branch of the basalar muscle buckles the tymbal (Dawson and Fullard, 1995). When the muscle relaxes, the elasticity of the tymbal reverses and the tymbal buckles back to the resting position, thus completing one whole modulation cycle. In most species, clicks are produced during both the active and passive phases, but not by the left and right tymbal simultaneously. Each inward and outward buckling movement produces either one or a series of clicks depending on whether the arctiid tymbal is smooth or striated, i.e. containing a number of so-called microtymbals. Arctiid moths produce clicks as part of their defence against bats (see, however, Sanderford et al., 1998).

In this paper, we describe yet another type of sound production in noctuoids, namely that of the males of the subfamily Chloephorinae. The Chloephorinae was previously

placed in the Noctuidae (Skou, 1991), but is currently considered to be a subfamily within the Nolidae (Kitching and Rawlins, 1999). Our interest was triggered by the short anecdotal descriptions given by Lorimer (1983) and Madsen (1987) of sound production by green silver-line moths *Pseudoips prasinana* (L.) (formerly *P. fagana*) while flying high among the tops of beech trees at dusk. While hunting for *P. prasinana*, we were lucky enough to catch a few specimens of another and less common chloephorine, the scarce silver-line *Bena bicolorana* (Fuessly) (formerly *B. prasinana*). *P. prasinana* occurs from Japan to Western Europe. *B. bicolorana* is distributed throughout most of Europe, west of the Ural mountains and south of southern Scandinavia (Skou, 1991).

Most chloephorine species occur in the Old World tropics, and sound production had been noted in some Asian chloephorine moths in the last century (Moore, 1867; White, 1872). A ventral organ, the 'basal pouches', presumed to produce sound, has been found in males of some, but not all, of the chloephorine species described (Kobes, 1988).

Materials and methods

Animals

We captured 23 male (wing span 35–38 mm) and seven female (wing span 38–43 mm) *Pseudoips prasinana* (L.) and three male and one female *Bena bicolorana* (Fuessly) (wing span 45–47 mm) in light traps in the vicinity of Odense University on the island of Fyn, Denmark, in July and August 1997. We also reared approximately 100 *P. prasinana* of both sexes from eggs and used them after storage as pupae at approximately 5 °C for 3–10 weeks.

Anatomical methods

The morphology of the tymbal organ was studied using both light microscopy and scanning electron microscopy. Both males and females were prepared for examination by cutting the tymbal organ caudally and/or frontally. Fluorescence microscopy was used to test for resilin-like proteins (Andersen and Weis-Fogh, 1964). We also employed various staining techniques. Bromophenol Blue (Menzies, 1961) was used to reveal muscle insertions. Heidenhain's Azan (Mallory, 1968) stains both connective and muscle tissue. Toluidine Blue and Light Green (Andersen and Weis-Fogh, 1964) were used as an indicator of the presence of resilin. Glutaraldehyde-fixed specimens were examined by scanning electron microscopy after drying and sputter-coating with platinum. Some dried museum specimens of *B. bicolorana* were macerated in KOH and stored in alcohol before drying and coating with platinum for scanning electron microscopy.

Sound stimuli

Constant-frequency sound pulses were generated using a Hewlett Packard function generator (3314A), a pulse generator (Hewlett Packard 8011A) and custom-built envelope shaper producing linear rise and fall times. The stimuli were amplified (Xelex) and broadcast either through a Technics leaf tweeter (EAS-10TH400B) or through a custom-built electrostatic loudspeaker (6 cm in diameter). Both loudspeakers were calibrated several times during the experimental period by means of a 1/4 inch Brüel & Kjær (B&K) microphone (type 4135), a preamplifier (type 2619), a measuring amplifier (type 2607) and a calibrator (type 4231). However, the sound intensity (94 dB SPL rms at 1 m at 40 kHz) from this equipment was often not sufficient to elicit clicks from the male moths. We therefore used an electronic dog whistle (Pet Trainer) that produced a sound intensity of 110 dB SPL rms at 1 m at 26 kHz when activated (for further details, see Rydell et al., 1997).

Sound levels are given as dB pe (peak equivalent) SPL (sound pressure level), i.e. re 20 μPa rms (root mean square) (Stapells et al., 1982) unless stated otherwise.

Sound recordings

The animals were lightly anaesthetised in CO_2, and a thin syringe needle was waxed (Senco Softceal Tackiwax) to the pro- and mesonotum. The moths were placed ventral side up to facilitate sound recordings from the ventrally located sound-producing organ. A gentle puff of air over their antennae initiated flight. The moths flew relatively steadily and for a long time, although up-side down.

The intense ultrasonic stimulus from the electronic dog whistle was used to elicit clicks in *P. prasinana*. In *B. bicolorana*, sound production was elicited by tactile stimulation of the abdomen. The clicks were recorded using a 1/4 inch B&K microphone (type 4135) without grid. The microphone was positioned 2, 5 or 8 cm above the sound-producing organ. Since the sound recordings were made close to the moth, we checked that we were not recording in the near field by measuring the intensity of the sounds at distances of 28, 16, 8, 4, 2 and 1 cm from the tymbal organ. The inverse square law was valid down to 1 cm, i.e. the sound pressure level decreased by 6 dB every time the distance doubled.

The signals from the microphone were recorded on a Racal Store 7D tape recorder running at 32 inches s^{-1}. Later, the sounds were replayed at 1/32 speed and sampled at 44.1 kHz clock rate (i.e. effectively digitising at 1.4 MHz). In some cases, the sounds were digitised online (Iotech Wavebook A/D converter) at 850 kHz (12 bits) and stored directly on an AST Pentium notebook. Because of the high effective digitising rate, the frequency response of the whole recording chain was mainly determined by the properties of the microphone and the tape recorder and, hence, was flat (±2 dB) up to approximately 120 kHz. The sounds were analysed using commercial software (BatSound, Pettersson Elektronik AB). The fast Fourier transformation (FFT) window (Hanning) used for the spectra was 1024 points, giving a frequency resolution of 1378 Hz at 1.4 MHz or 830 Hz at 850 kHz clock rate. The bandwidth (BW) of the signals was determined as the BW_{-10dB}, i.e. the bandwidth of the spectrum 10 dB below the peak.

To measure the directionality of sound radiation, *P. prasinana* males were mounted with the tymbal organ in the centre of a ring having a diameter of 72 cm. Sounds were

recorded by two B&K 1/4 inch microphones (type 4135) fastened to the ring such that the tip of the microphone was 8 cm from the tymbal organ. One microphone was stationary and served as the reference microphone. The other microphone was moved around the insect in steps of 30 °. The moth was placed in three different positions relative to the ring to measure the sound radiation pattern in all three dimensions, the horizontal and the two vertical (longitudinal and transverse) planes (see Fig. 7).

The microphone signals were digitised directly on two channels of the Iotech system described above at a sampling rate of 350 kHz for each channel. The average (linear) sound intensity of the first click in the three last sound-production cycles in a click train was calculated relative to the reference microphone.

Muscle activity

Muscle potentials were measured from both the right and left tymbal muscles in *P. prasinana*. Differential electrodes of diameter 25 µm made of Pt–Ir wires insulated except at the tip were inserted through the cuticle into the muscles. The electrodes were fastened using wax or tissue glue. The moth's sounds were recorded using a 1/4 inch B&K microphone (type 4135) placed in front of the moth, 4 cm from the tymbal organ. Simultaneously, wingbeat frequencies were measured using a gramophone pick-up attached to the pro- and mesonotum of the moth. All signals were recorded on separate channels on a Racal Store 7D instrumentation tape recorder. Flight was initiated by disrupting the moth's tarsal contact. The flying moth was stimulated with sound from the dog whistle.

The muscles were also stimulated directly through the electrodes. The moths used for measuring muscle potentials were left overnight with the electrodes in place and used on the following day in muscle stimulation experiments. The electrodes were connected to a DISA stimulator (type 14 E 11) *via* a current-to-voltage converter. The stimulus pulses were 40 µA direct current pulses of various durations and repetition frequencies. The stimulus intensity was adjusted so that a single stimulus would result in a click response. The moth sounds were recorded simultaneously with the stimulus signals. We stimulated muscles of both flying and sitting males. Generally, these preparations were viable and could still click after up to 4 days.

Tymbal vibrations

Tymbals from male and female *P. prasinana* were exposed by removing the abdomen caudal to the organ. The passive vibrations of the tymbal were measured using a DISA laser Doppler vibrometer (see Michelsen and Larsen, 1978) while driving the tymbal with short frequency-modulated sound pulses (90 dB SPL) sweeping from 1 to 100 kHz. The sounds were delivered from a Technichs leaf tweeter (4288) with a flat frequency response from 5 to 50 kHz and a shallow drop of 10 dB between 50 and 80 kHz. The tymbal was covered with 10 µm diameter silver-coated hollow glass spheres to obtain good reflections from the tymbal surface. Control experiments showed that the load of these particles was too small to affect the vibrations. The laser spot (approximately 50 µm in diameter) was focused at points over the whole tymbal to locate the area with maximal vibration. We tried to measure the vibrations of actively vibrating tymbals (by stimulating the muscles), but it proved impossible to keep the laser focused on the tymbal because of the large movements of the whole organ.

We also studied tymbal vibrations using light microscopy. We covered the tymbals of some males with *Pinus* pollen grains and recorded particle movements through a binocular microscope connected to a Panasonic CCD camera and a Panasonic video recorder (AG-6200). An electrostatic loudspeaker mounted 20 cm above the tymbal delivered pure tones driving the tymbal. As the sound frequency was changed towards the tymbal's resonant frequency, the particles began to move.

Hearing

The tympanic nerve in *P. prasinana* was exposed using a dorsal approach (Roeder, 1966) and hooked onto a tungsten electrode. The preparation was placed 30 cm from the loudspeaker with the ear facing the loudspeaker. Tympanic nerve activity was bandpass-filtered, amplified (using custom-built equipment) and displayed on an oscilloscope and through an audio monitor. The stimuli were pulses 10 ms long with a rise/fall time of 0.5 ms repeated at 1 Hz. Threshold was defined as the sound pressure level necessary to elicit 1–2 spikes in at least eight out of ten stimulations. Hearing sensitivity was tested in 5 kHz steps in the frequency range 5–150 kHz. The frequencies were tested in random order.

Results

Morphology

The sound-producing organ of green silver-lines and scarce silver-lines is found only in the males. It is located ventrally in a pair of pouches on the base of the abdomen (Fig. 1). When a moth is viewed from the ventral side, the tymbal organ is seen as a cleft at the level of the second abdominal sternite. The sternite is modified into a triangular cleft with strongly sclerotized walls (Fig. 1, scanning electron micrograph). The cleft is deep in males, but an indication of a similar structure is seen at the same position in females. The inner anterior surface of this cleft bears two white, stiff tymbal membranes. When the abdomen caudal to the organ is removed and the moth is viewed end-on, the very characteristic organ is easily visible in males (Fig. 2). Each tymbal is surrounded by a frame of rigid sclerotized cuticle and is backed by an air-filled cavity on the caudal side. Ventrally, the left and right tymbal frames are fused in the middle. The tymbals are placed transversely in the abdomen and tilt slightly forwards. The tymbal surface is not flat, but slightly undulating. Generally, the tymbal is rather stiff, except for a small oval region in the middle, which appears thinner, softer and more translucent than the rest of the membrane. The oval region was easy to move and perforate with a needle. This part of the tymbal stained brilliant blue with the Toluidine Blue/Light Green solution, indicating the

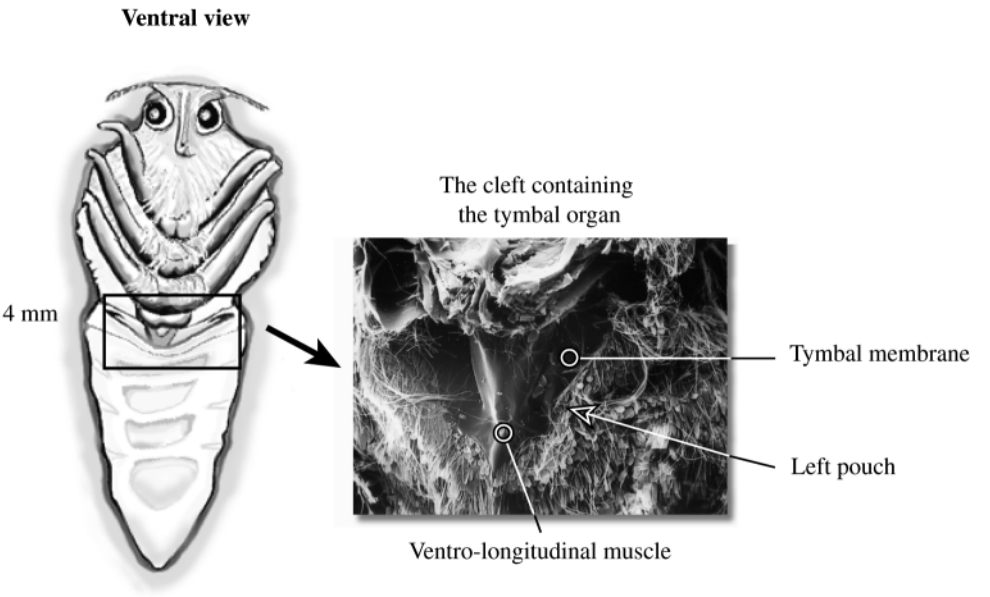

Fig. 1. Location of the sound-producing organ. Drawing of a *Pseudoips prasinana* male seen from the ventral side. The legs have been removed at the tibia–femur joint. A scanning electron micrograph (right) shows the V-shaped cleft that forms the external part of the tymbal organ. The tymbal membrane is seen deep in the pouches. The ventro-longitudinal muscle inserts at the position indicated and extends frontally beneath the cuticle that separates the two pouches.

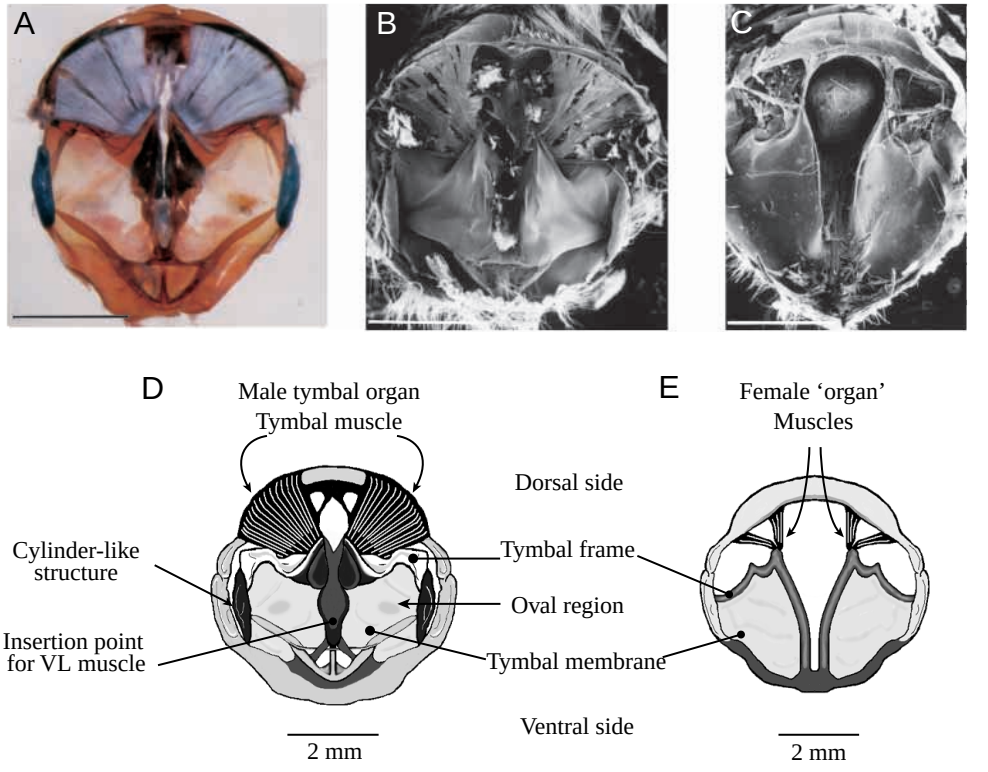

Fig. 2. The tymbal organ of *Pseudoips prasinana*. (A) Caudal view of a cross section of the male abdomen at the level of the tymbal organ (stained with Heidenhain’s Azan). Dorsally, the two fan-shaped tymbal muscles are inserted on the tymbal frame. The cylinder-shaped structures containing resilin (stained blue) are visible lateral to the tymbal membranes. The oval region is seen best on the left tymbal membrane and is stained weakly blue. Scale bar, 2 mm. (B) Scanning electron micrograph of a cross section as in A. The surface of the tymbal membrane is smooth, without striae. Scale bar, 2 mm. (C) Scanning electron micrograph of the corresponding cross section in the female. Two tiny muscles forming a V insert dorso-medially on each ‘tymbal’ frame (see also E). Scale bar, 2 mm. (D) Drawing of the male tymbal organ. The grey shading indicates the areas with a high resilin content: the darker the shading the greater the resilin content (except for the tymbal muscles). (E) Drawing of a cross section of the female second abdominal segment showing the structures corresponding to the tymbals in the male. VL, ventro-longitudinal.

presence of resilin-like proteins. The other parts of the tymbal were not coloured by any of the stains. The tymbal frame did not stain except for a cylinder-shaped structure on the lateral side, which stained intensely blue with Heidenhain’s Azan (Fig. 2A). Furthermore, it stained brilliant blue in the Toluidine Blue/Light Green solution and fluoresced strongly in ultraviolet light, thus strongly suggesting the presence of resilin.

Several muscle groups attach to the frame. Most conspicuous are large fan-shaped muscles inserted on the dorso-medial part of the frame and attaching at their other end to the second tergum (Fig. 2A,B,D). We termed these muscles the tymbal muscles because of their size, direct insertion and functional correlation to sound production (see below). Fluorescence microscopy and staining indicated that the tymbal organ contains resilin at the areas where these large muscles insert.

Another pair of prominent muscles are longitudinal muscles on the ventral side attaching the heavily sclerotized ventro-medial part of both tymbal frames. These muscles run anteriorly towards the thorax. Some smaller muscles also insert dorsally on the anterior part of the tymbal frame.

In females, we found a vestige of a corresponding organ (Fig. 2C,E) located in a shallower pouch. It is a tymbal-like structure, but the 'tymbal area' is smaller, much thicker, less transparent, almost flat and without the cylinder-like structure found lateral to the male tymbal. We did not measure the volume of the air cavity behind the tymbal organ, but it was obvious that it was smaller in females than in males. The muscle supply to the female 'tymbal' is very sparse, consisting only of a V-shaped pair of tiny muscles connected to the dorsal part and attached to the second tergum at their other end (Fig. 2E). Neither fluorescence tests nor histological stainings indicated high concentrations of resilin in the female organs.

We also studied the morphology of the tymbal organ in male *B. bicolorana*, both in the three specimens we caught and in some dried museum specimens from the Zoological Museum at the University of Copenhagen. The overall structure of the tymbal organ is the same as in male *P. prasinana*, but *B. bicolorana* has striae on the ventro-medial part of the tymbals, whereas the same area in *P. prasinana* is smooth (Fig. 3). Seven or eight striae were clearly visible in all the *B. bicolorana* males we examined (Fig. 3C).

Staining indicated high concentrations of resilin in a circular region in the middle of the tymbal, which corresponds to the oval region in the tymbals of *P. prasinana*. *B. bicolorana* also has a pronounced structure containing resilin lateral to the tymbal but, compared with *P. prasinana*, this structure is considerably smaller and placed more dorsally. The large dorsal tymbal muscles in *B. bicolorana* attach to a smaller, more lateral area of the tergum compared with those of *P. prasinana*, and they appear less fan-shaped (Fig. 3A). The ventral longitudinal muscles are very similar in both species. Furthermore, *B. bicolorana* has two minor muscles attached to the dorsal medial part of the tymbal frame extending caudad. Corresponding muscles are seen in *P. prasinana*.

Sound production in Pseudoips prasinana

At first it was very difficult to elicit sound production in *P. prasinana* using either acoustic or tactile stimuli. However, the intense sound level from an electronic dog whistle (Pet Trainer) proved to be effective, but only if the *P. prasinana*

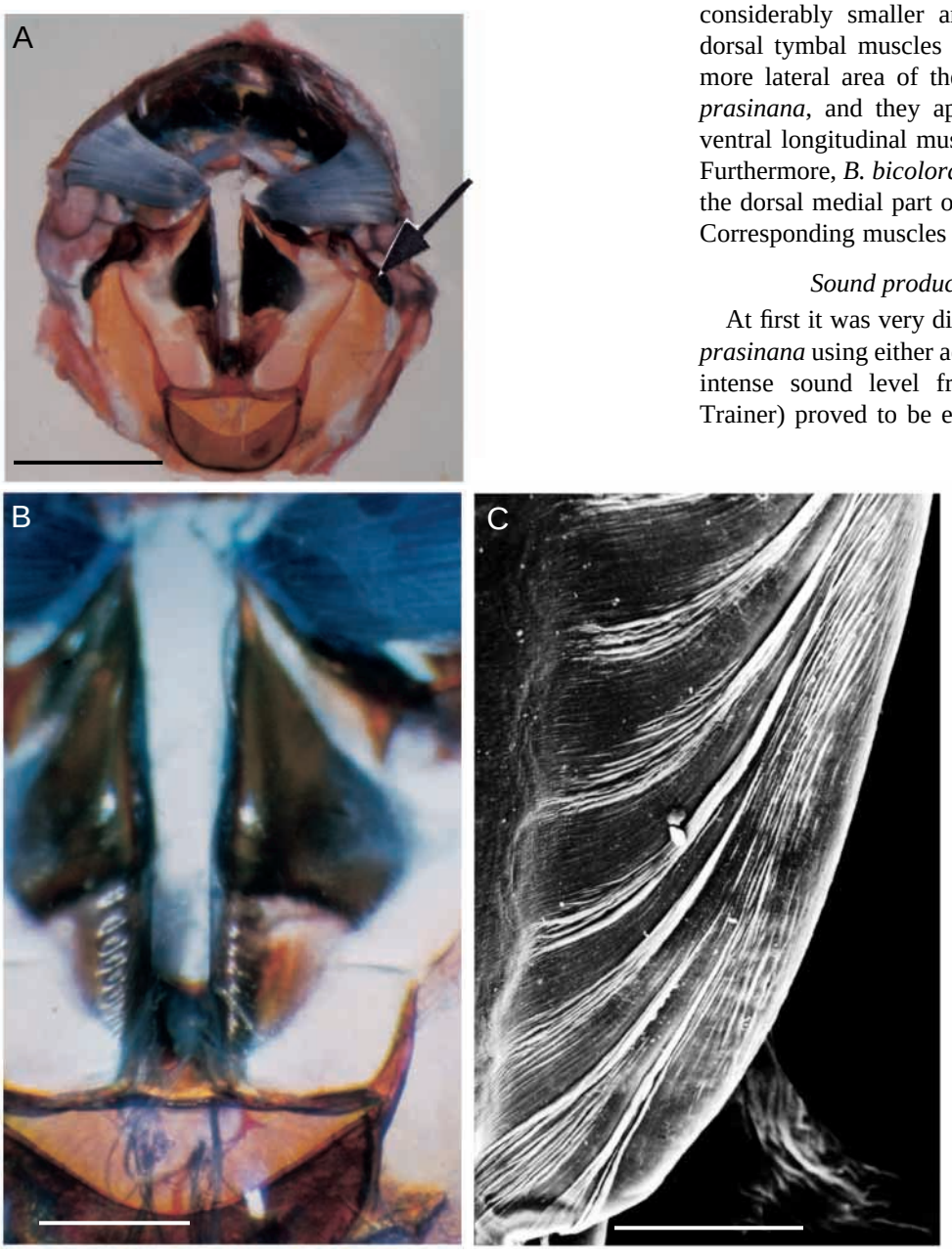

Fig. 3. The tymbal organ of *Bena bicolorana*. (A) Caudal view of a cross section of the male abdomen at the level of the tymbal organ (stained with Heidenhain's Azan). The tymbal muscles insert dorso-medially on the tymbal frame and extend dorso-laterally. A circular region in the middle of both membranes stains weakly blue. The dark (and weakly blue-stained) structure (arrow) on the dorso-lateral part of the tymbal frame corresponds to the resilin-containing cylinder-like structure in *Pseudoips prasinana*. Scale bar, 1.4 mm. (B) At greater magnification, the striae on the medial ventral part of the tymbals can be clearly seen. Scale bar, 0.4 mm. (C) Scanning electron micrograph of the area of the left tymbal membrane containing the striae. Seven striae are visible in this preparation. Scale bar, 0.1 mm.

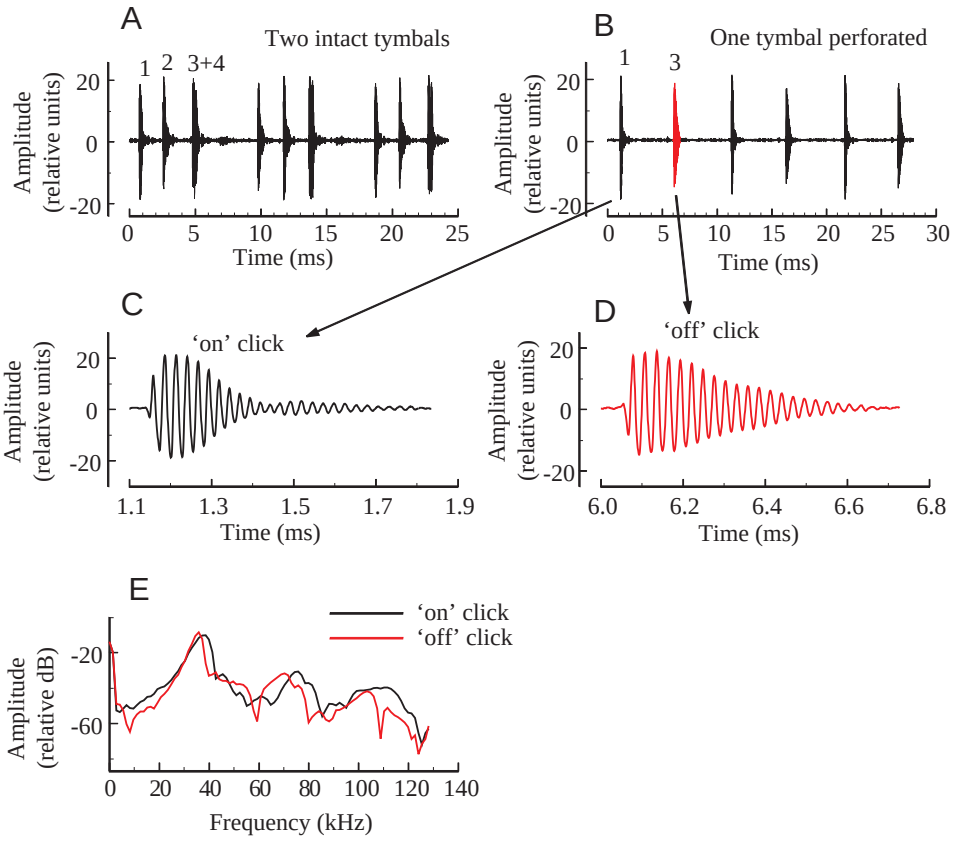

Fig. 4. Sound recordings from *Pseudoips prasinana*. (A) A sequence consisting of three sound-production cycles recorded from an intact moth clicking with both tymbals. The first two clicks (1, 2) in each cycle are caused by active buckling of the left and right tymbals ('on' clicks). The third click (3+4) is a double click caused by the almost simultaneous passive return buckling of the two tymbals ('off' clicks). (B) The unilateral sound-production cycle pattern from the same moth after one tymbal had been punctured. Click '1' is the 'on' click and click '3' is the 'off' click. (C,D) One 'on' click and one 'off' click on an expanded time scale. (E) The amplitude spectra of the on and off clicks.

males were flying (while tethered in the apparatus). However, sound production was not dependent on flight, since in two cases we recorded clicks from a sitting moth. Only very rarely did the animals click spontaneously. Some of the stimulated moths responded to the intense sound stimuli by flight cessation, sometimes preceded by a click train. However, flight-stop reactions were rare. The same applies to other evasive behaviours such as abdominal movements, changes in wingbeat, etc. We found no consistent reactions of these types. Females never produced sounds, although they were stimulated in the same manner as the males.

When stimulated with the dog whistle, *P. prasinana* males produced trains of intense short clicks caused by buckling of the paired tymbals. The latency from the start of the stimulus to the first click of the train was 50–600 ms. The click trains lasted 10–500 ms and normally consisted of repeated cycles (sound-production cycles) with four clicks (two pairs) in each cycle originating from the active and passive buckling of the left and right tymbal, respectively (Figs 4, 5).

The click duration was 300 µs on average (with values ranging from 100 to 700 µs among individuals) (Fig. 5) with a rapid onset and a slower decay (Fig. 4C,D). The intensity of the sound was up to 105 dB peSPL at 8 cm. The sound spectra were calculated from 11 individuals for three 'on' clicks and three 'off' clicks from each. The peak power frequency in the clicks (Fig. 4E) varied from 35.2 to 42.7 kHz among individuals with a mean of 39.2 kHz. The BW_{-10dB} around the peak frequency varied between 4.5 and 16.1 kHz, with a mean of 10.1 kHz.

Two different sound-production cycle patterns were seen (Fig. 5). The sound-production cycle that was observed in most cases (two out of three recordings) consisted of two separate 'on' clicks followed by two 'off' clicks coinciding more or less completely. We termed this pattern bilateral sound production. We interpret the click pair ('on' clicks) as the result of the active buckling of the left and right tymbals caused by muscular activity, and the two almost coincident clicks ('off' clicks) as the result of the almost simultaneous passive return buckling of both tymbals. The interval between the 'on' clicks from left and right tymbals within a cycle (t_{LR}; see Fig. 5) was 1.9±0.4 ms (mean ± S.D.; seven individuals, six click cycles). The interval between the 'on' and 'off' clicks for the tymbal buckling first (t_{AP}; Fig. 5) was 4.8±0.9 ms (mean ± S.D.; seven individuals, six click cycles) (see the Discussion for the interpretation of on and off clicks). The duration of the whole sound-production cycle (T) was 10.1±1.7 ms, so the mean repetition rate for the sound-production cycle was 99.0±14.4 Hz. Since each sound-production cycle contains three separate clicks (Fig. 4A), the overall click rate is approximately 300 Hz.

In some cases, we observed a different sound-production cycle pattern indicating that only one tymbal was active. We called this unilateral sound production (Fig. 5). The duration, T, of the sound-production cycle (11.0±1.5 ms, mean ± S.D.) and the interpulse interval, t_{AP} (3.9±0.7 ms, four individuals, six click cycles), were in the same range as for bilateral sound production. Thus, the pattern was repeated at 91.2±11.2 Hz,

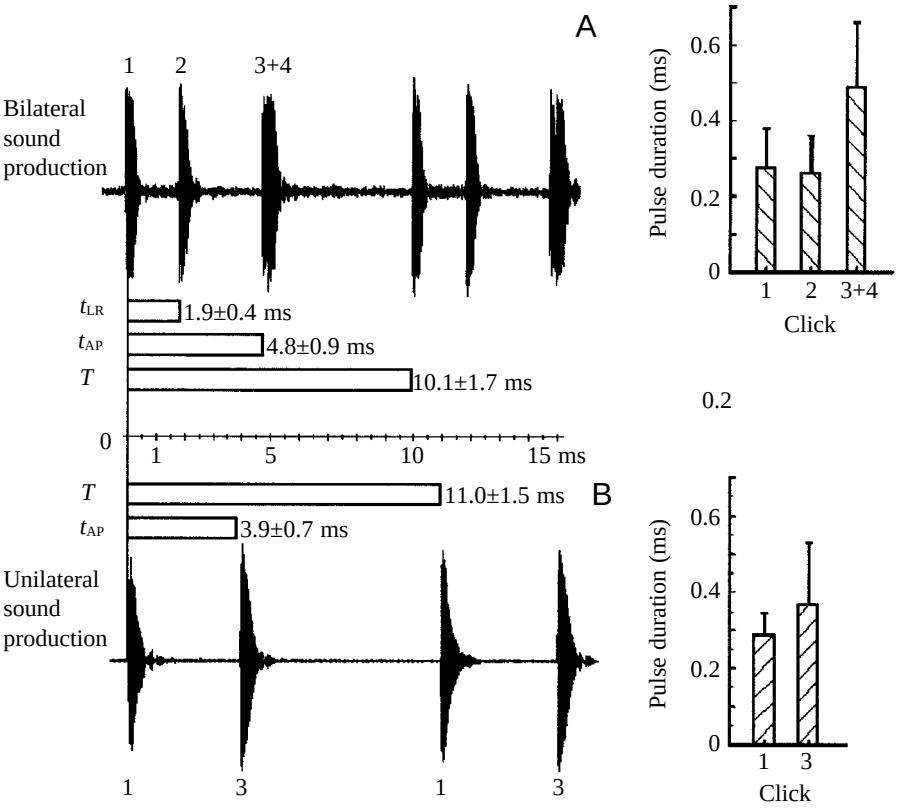

Fig. 5. Click patterns in *Pseudoips prasinana*. (A) Bilateral sound production. The sequence shows two cycles from a moth clicking with both tymbals. (B) Unilateral sound production. This naturally occurring click pattern is caused by the buckling of only one tymbal (see also text for explanation) and the time pattern is similar to the pattern seen when one tymbal is punctured (see Fig. 4B). The horizontal bars show the mean durations ± S.D. The vertical columns to the right show the mean click durations + S.D. Means for the bilateral pattern were calculated from six click cycles from seven individuals (N=42) and for the unilateral pattern from six cycles from four individuals (N=24).

giving an average click rate of twice this value, i.e. 182 Hz. We could convert the bilateral sound-production pattern to the unilateral pattern by perforating the tymbal on one side (Fig. 4B). This left two clicks per cycle, namely those of the remaining intact tymbal's active and passive buckling. Some individuals alternated between bilateral and unilateral click patterns. In these cases, the unilateral click often occurred at the beginning of the click train.

A few of the 20 specimens recorded produced other patterns, for example a single fused click first instead of the pair or, sometimes, *vice versa*, a pair of separate clicks instead of the two almost-fused clicks at the end of a sound-production cycle, indicating that the synchrony between the left and right sides was variable.

In a few preparations, we tried to manipulate the tymbal membrane mechanically by pulling the tymbal muscles with forceps. This caused the tymbal to buckle and to produce a short sound. When the muscles were released, the tymbal buckled back in place, often accompanied by a similar sound.

Sound production in Bena bicolorana

We succeeded in recording sounds from only one of the three male *B. bicolorana*. However, the recordings from that specimen revealed a very striking difference from those for *P. prasinana*, since the large clicks with characteristics resembling those of *P. prasinana* were interspersed by series of less-intense clicks. As shown in Fig. 6A, the sound-production cycles in a train could consist of large clicks alone (the last clicks), small clicks alone (the series of small clicks in the middle) or both large and small clicks simultaneously (the first two sound-production cycles in Fig. 6A). We never recorded a whole sequence consisting solely of either type of click. The typical pattern was a click train starting and ending with one or a few large clicks, and with several series consisting predominantly of small clicks in the middle. The duration of the large clicks was 200–400 µs (Fig. 6A, blue time trace), and the peak frequency was approximately 52 kHz with a BW −10 dB of approximately 10 kHz around the peak frequency (Fig. 6B). The amplitude of the large clicks was in the same range as that for *P. prasinana*, whereas the small clicks were approximately 15–20 dB less intense and had a shorter duration (approximately 100 µs) (Fig. 6A, red time trace) and a broader spectrum with the main energy content from 20 to 100 kHz (Fig. 6B). The number of small clicks produced by the active or passive phase of the sound-production cycles varied greatly. We counted 1–14 small clicks in one phase. *T*, the duration of the sound-production cycle, was approximately 17 ms in *B. bicolorana* thus longer than in *P. prasinana*, and hence the frequency of buckling the tymbal organs was somewhat lower, approximately 60 Hz. In *B. bicolorana*, as in *P. prasinana*, tymbal activation is not phase-locked to the wingbeat frequency.

Directionality of sound production

Fig. 7 illustrates the sound field around *P. prasinana* in stationary flight. The sound field was measured for three individuals. The two vertical radiation patterns showed cardioid characteristic (Fig. 7A,B), with sound pressure

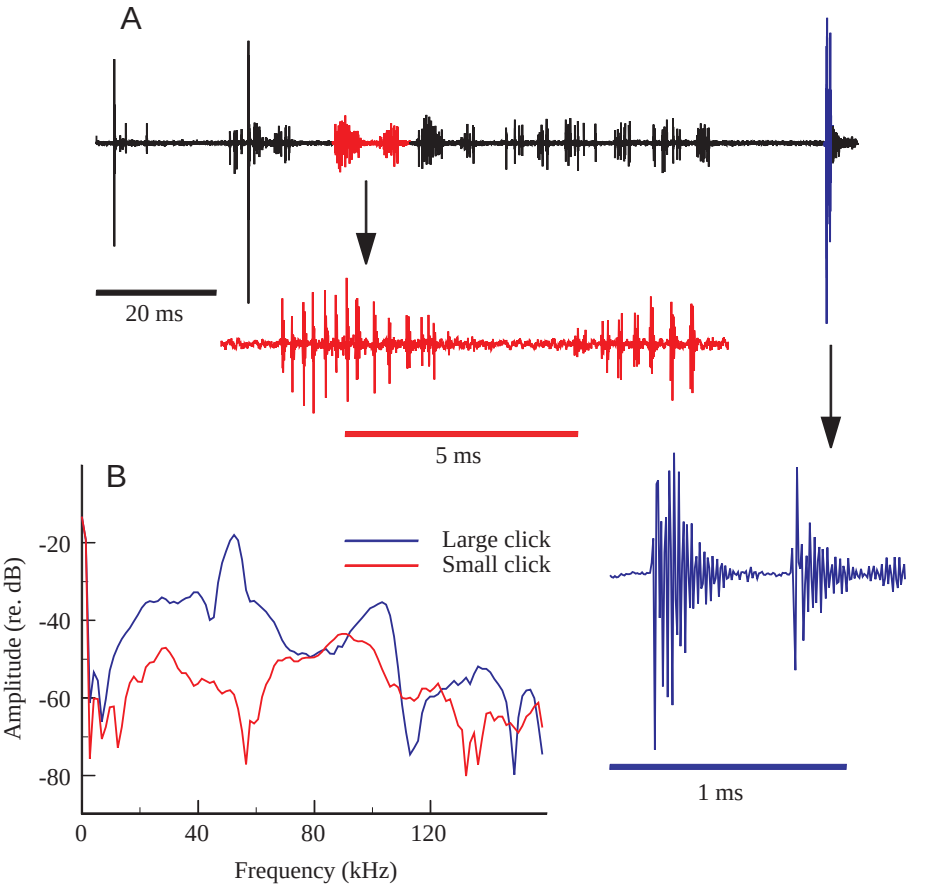

Fig. 6. Sound recordings from *Bena bicolorana*. (A) A typical click sequence including both large and small clicks. The red part of the time trace marks a whole sound-production cycle consisting only of small clicks. The blue part of the trace marks two large clicks. (B) The power spectrum of a large click (blue line) and a small click (red line) produced by active buckling.

15–20 dB higher below than above the moth. The horizontal sound radiation pattern was almost omnidirectional (Fig. 7C) with a sound pressure approximately 5 dB lower than the highest intensity measured below the organ (Fig. 7A,B).

Muscle activity in Pseudoips prasinana

The recordings of activity in the tymbal muscles showed muscle spikes with the same repetition rate (75–110 Hz) as that of the sound-production cycles in acoustic signals. We recorded these spikes in five animals every time we stimulated using the electronic dog whistle, but never spontaneously. Unfortunately, none of these animals with electrodes implanted in the tymbal muscles produced any acoustic output (clicks). Perhaps the insertion of the electrodes influenced the muscle efficiency. The electrodes also picked up the activity of the wing muscles, but these spikes were 15–20 dB smaller than the tymbal electromyograms (EMGs) and highly phase-locked to the wingbeat frequencies (approximately 50 Hz), measured using the pick-up, and therefore easily discriminated from the tymbal EMGs. The recordings showed no synchronisation between tymbal muscle activity and wing muscle activity.

Stimulation of the tymbal muscles through the implanted electrodes resulted in acoustic click production (Fig. 8). We could only elicit one-sided click patterns closely resembling the unilateral sound-production pattern produced spontaneously by some moths (see Fig. 5). The click rates in the elicited click trains followed the stimulation frequency. However, the efficiency in eliciting clicks decreased with decreasing stimulus frequency such that, close to the natural frequency (93 Hz), each stimulus pulse elicited clicks (Fig. 8A), at 52 Hz many clicks were skipped (Fig. 8B) and at 33 Hz we could elicit only single click pairs at unpredictable intervals, never trains of clicks. However, irrespective of stimulus frequency and stimulus duration, the clicks were always elicited in pairs with a constant interclick interval, t_{AP}, of approximately 5 ms. We interpret this pair as the ‘on’ click followed by the ‘off’ rebound click.

Tymbal vibrations

Laser vibrometry revealed large vibrations of the oval spot in the middle of the membrane (see Fig. 2), but not of other areas of the tymbal, when the organ was stimulated with short broad-band frequency-modulated sweeps. Peak frequencies (resonance frequency) ranged from 35 to 55 kHz in fresh preparations (Fig. 9) (three individuals). BW_{-10dB} ranged from 4 to 10 kHz. Approximately 1 h after dissection, the peak frequency of the vibration started to increase, probably because of drying of the tymbal membrane. The frequency increased by approximately 10 kHz after approximately 2 h and was then stable for many hours. When driving the tymbal covered with *Pinus* pollen with pure tones in the frequency range 35–55 kHz, only the grains around the oval area moved. In most experiments, the frequencies that elicited the largest movements were 10 kHz above the peak frequency of their

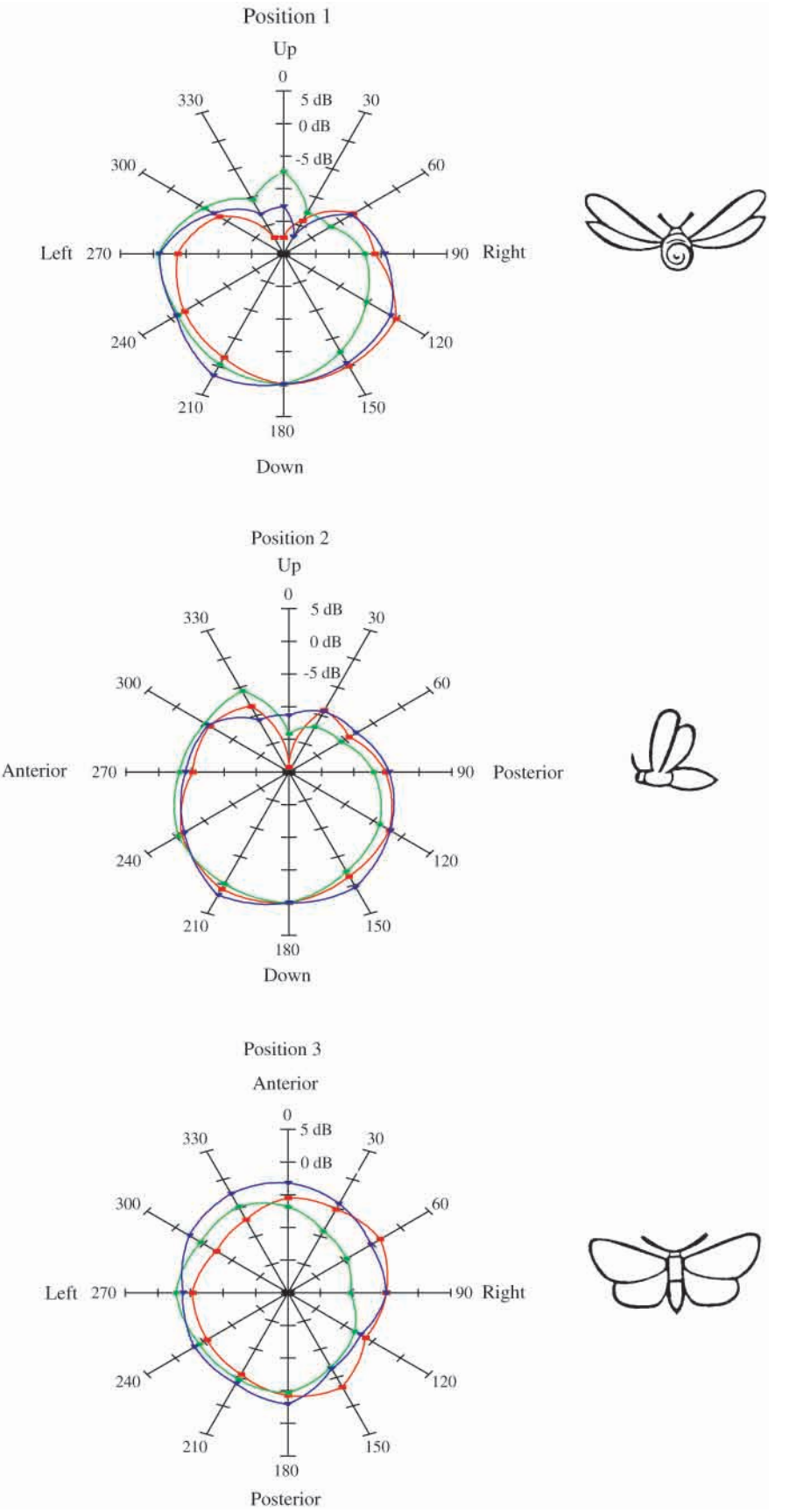

Fig. 7. Sound radiation. The polar plots show the sound radiation pattern for three animals (red, blue and green traces) measured in three different positions (A–C). The intensities on the *y*-axis are given in decibels relative to the sound pressure level measured by a stationary microphone placed 8 cm below the ventral pouches. Measurements were made in steps of 30°.

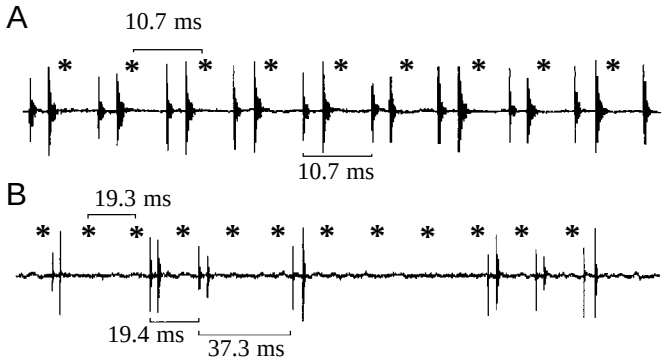

Fig. 8. Muscle stimulation. The muscles were stimulated electrically with 1 ms current pulses through wire electrodes implanted in the left and right tymbal muscles. The asterisks mark the stimuli, and the traces show the acoustic response. (A) Only one tymbal clicked in this case, and the click sound-production cycle is repeated at a rate of 93 Hz, corresponding to the stimulus frequency. The interval between 'on' and 'off' clicks varied between 2.8 and 3.3 ms. (B) Clicks from the same tymbal as in A stimulated at a frequency of 52 Hz. The interval between 'on' and 'off' clicks remained the same, but the intervals between sound-production cycles varied greatly.

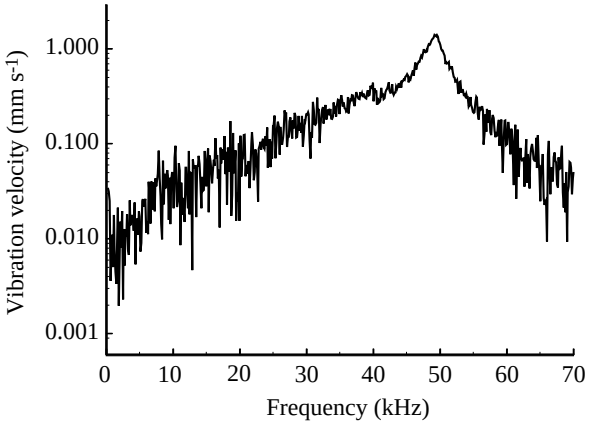

Fig. 9. Spectrum of vibration of the tymbal membrane measured using laser vibrometry. The curve shows the vibration in response to a laser beam focused on the oval region in the centre of the tymbal membrane, where maximum vibration velocity was measured. The peak frequency is 49 kHz. The tymbal was driven by broad-band clicks with a sound pressure level of approximately 90 dB SPL.

sounds (approximately 50 kHz), again probably because of drying of the membrane. Pollen grain movements were easily observed for more than 24 h.

Hearing

There was no difference in hearing thresholds between the two sexes of *P. prasinana* (two-sample *t*-test with unequal variances). The best frequency was between 40 and 60 kHz, with a mean threshold intensity of 38 dB SPL (Fig. 10). The roll-off towards lower frequencies was rather steep, such that the threshold between 20 and 30 kHz was 45–55 dB SPL. For comparison, we have plotted a typical audiogram for a

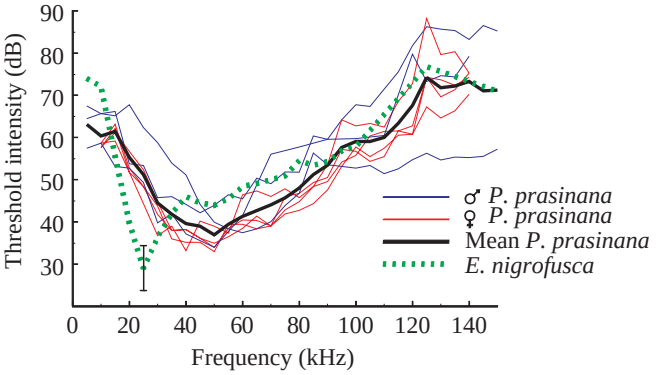

Fig. 10. Audiograms for *Pseudoips prasinana* for three males (blue lines) and four females (red lines). The mean audiogram is shown by the thick black line. A mean audiogram for a similarly sized sympatric moth (*Euxoa nigrofusca*, from Surlykke et al., 1999) with typical noctuid hearing is also plotted for comparison (dotted green line). The maximum S.D. for *E. nigrofusca* between 10 and 35 kHz is ±5.3 dB, as indicated by the black error bar.

similarly sized sympatric noctuid, *Euxoa nigrofusca* (the former *E. tritici*) (Surlykke et al., 1999).

Discussion

In this study, we describe a novel sound-producing mechanism in two moth species, male chloephorine moths of the species *Pseudoips prasinana* (green silver-line) and *Bena bicolorana* (scarce silver-lines), that use abdominal tymbal organs to produce trains of ultrasonic clicks.

Two striking anatomical features differentiate the two moth species we studied from other non-chloephorine noctuoid moths: (1) the paired abdominal pouches containing the tymbals on the ventral side of the intact male moth (Fig. 1), and (2) the large fan-shaped muscles attached to the dorsal part of the tymbals (Figs 2, 3). Such pouches have been described only in the subfamily Chloephorinae (Holloway, 1988; Kobes, 1988).

Various moths produce sound using tymbal organs (Conner, 1999). Not only the well-known tymbals of male and female arctiids but also, for example, the abdominal tymbal organs in males of the pyralid moth *Symmoracma minoralis* (Heller and Krahe, 1994) are used in sound production. However, interestingly, the insect tymbals that most resemble those of Chloephorinae are those of the very distantly related cicadas. Cicada tymbal organs are found only in males. They are located anteriorly on the abdomen and backed caudally by large air sacs (Pringle, 1954). Tymbal mechanics have been studied in detail in cicadas (e.g. Bennet-Clark, 1997; Young and Bennet-Clark, 1995). The sounds are produced first by inward buckling of the tymbal due to contraction of the tymbal muscle (Bennet-Clark, 1997). Elastic components in the tymbal, mainly resilin, are responsible for the passive return movement of the cicada tymbal, which also produces a click (Young and Bennett-Clark, 1995).

Although our data in this first study of chloephorine tymbals

are not sufficient to elucidate all the details of tymbal mechanics, our results strongly indicate that chloephorine tymbals also function by active buckling caused by muscle activity and passive return due to elastic components.

The experiments examining muscle activity were only preliminary. Since we did not succeed in eliciting acoustic responses from the moths using recording electrodes in the tymbal muscles, we can only draw a few conclusions from the results. It seems quite clear that the tymbal muscles play a crucial role in click production. Not only does the size of the muscles in males compared with females indicate this, but also the fact that stimulation with a dog whistle elicited muscle potentials with the same repetition rate as the clicks trains, and finally, that direct electrical stimulation of the muscles elicited clicks. The asymmetry between clicks from the left and right sides in natural bilateral sound patterns was not found in the EMG recordings, indicating that other muscles are involved in natural patterns. It may be that the tymbal muscles only deliver the main energy for sound production, while the fine control of the timing of the left and right tymbals depends on other less powerful muscles, e.g. the ventro-longitudinal muscles. In cicadas, it has been demonstrated that some muscles modulate the effect of others (e.g. Fonseca and Bennet-Clark, 1998). Many more experiments are needed to determine the role of all the muscles involved in chloephorine click production. However, the results on muscle activity do support is our interpretation of which clicks were on and off, since t_{AP} remained constant in all muscle stimulus experiments, irrespective of stimulus conditions, and was equal to the t_{AP} that we defined as the delay between active buckling and passive return-buckling in the natural sound recordings. The results of histochemical staining and fluorescence microscopy indicated high concentrations of resilin in the tymbal membrane and in parts of the frame, and we find it likely that the release of stored energy in these rubber-like proteins is responsible for the passive return of the tymbal. Resilin-like proteins are known to play a major role in insect cuticle, providing high elasticity in cuticle specialised for mechanical functions (Andersen and Weis-Fogh, 1964).

The oval region in the tymbal of *P. prasinana* may play an important role. Histochemical staining indicated larger amounts of resilin in this area than in the surrounding tymbal material, which means that the tymbal is highly flexible in this region. Since the oval region is very soft, flexible and located almost in the middle of the tymbal membrane, it could act as the point around which the tymbal is flexed when it buckles. Although the laser vibrometry studies showed that tymbal vibrations were maximal in the oval region (Fig. 9), this does not necessarily mean that it is the only sound-emitting structure. The clicks are rather intense, so it seems unlikely that sound is radiated only from this small oval region of the tymbals.

Our results show that, in chloephorine moths, both the left and right tymbals buckle in most of the sound-production cycles. In contrast, clicking arctiids moths all seem to buckle their left and right tymbals one at a time, either alternating between left and right or in less regular patterns, but seldom or never simultaneously (Fullard, 1992). However, chloephorine tymbals may also act independently. We interpret the ‘unilateral sound production’ (Fig. 5) as the output of only one of the tymbals for two reasons. First, puncturing one of the tymbals changed the bilateral pattern to the unilateral pattern without changing the overall duration of the whole sound-production cycle (*T*). Second, the click shapes and amplitudes (Fig. 4C,D) indicate that they are single clicks and not superimposed clicks from the left and right tymbals buckling in exact synchrony.

However, despite the differences between chloephorine and arctiid clicks, the similarities are the most striking features: short high-frequency clicks are produced with tymbals in both moth groups, and both groups also include species with either smooth or striated tymbals and show a correlation between tymbal morphology and sound structure. While being cautious because we only recorded sounds from a single *B. bicolorana*, we still think the results allow us to conclude that the characteristic differences between the tymbal morphology in *P. prasinana* and *B. bicolorana* explain the differences in the acoustic characteristics of their clicks. *P. prasinana* has a smooth tymbal surface and produces a single click for each inward or outward buckling phase, whereas *B. bicolorana* has striae on the medial part of the tymbal and produces series of clicks containing varying numbers of large and small clicks for each buckling phase. The sounds produced by *B. bicolorana* are very similar to the sounds of arctiids with microtymbals when examined by eye or by ear (e.g. http://www.ou.dk/Nat/biology/neuro/as-dk.html). Thus, chloephorine and arctiid tymbals, which have obviously evolved independently, form a remarkable example of convergent evolution.

Considering the similarity in spectral peaks, tuning and intensity, we believe that the mechanism of sound production described for *P. prasinana* is common to the large clicks of both species. The fact that *B. bicolorana* emits series of small clicks that may or may not coincide with the large clicks indicates that these are produced by an independent mechanism which may be controlled by another set of muscles, causing sequential buckling of the small striae on the medial part of the tymbals (Fig. 3B,C), which then directly or indirectly emit the small clicks. We recorded up to 14 small clicks in one buckling phase. Since there are only seven or eight striae on each side, this indicates that both the left and right tymbals are also active simultaneously in *B. bicolorana*. Tymbal organs with striae have been found in other chloephorines; e.g. the tymbal organ from *Parasinna diehli* depicted by Kobes (1988). The organ he described has microtymbals and, from our results, we would therefore expect *Parasinna diehli* to produce series of clicks. The number of microtymbals is smaller and they appear more pronounced than in *B. bicolorana*, perhaps causing fewer and relatively more intense ‘small’ clicks.

Although we have not observed either of the chloephorine species click and behave in the field, we suggest that the clicks function in a sexual context rather than as part of bat defence

as the clicks of most arctiid moths do. First, both males and females fly at night, since we caught them in the light traps, but only the males emit sounds, while click production in Arctiidae in general is common to both sexes. Second, bat-like sounds did not elicit clicks in either of the two species. We had to use either unphysiologically high stimulus intensities from the electronic dog whistle or tactile stimuli to elicit clicks. Third, the bandwidths of the sounds were rather narrow. Other noctuoid moths that communicate using sounds also produce narrow-band signals (Bailey, 1978; Surlykke and Gogala, 1986), whereas the clicks produced for bat defence by arctiid moths are of broader bandwidth (Surlykke and Miller, 1985; Bates and Fenton, 1988). It seems likely that the bandwidth of the sounds are correlated with their function. Using sounds for social communication allows for matched tuning of sender and receiver. A narrow bandwidth increases the communication distance and at the same time facilitates distinction between sympatric species. In contrast, a broader bandwidth is probably more advantageous, when sounds are produced for bat defence, since they are aimed at many different (bat) species. The hearing in both male and females matches the frequencies of the male sounds very well (Fig. 10). Compared with similarly sized sympatric noctuoid species with overlapping flight activity periods (Surlykke et al., 1999) (Fig. 10), the hearing is less sensitive at the best frequency and the best frequency is higher. However, the main frequencies of the sounds of both species are still within the frequency range emitted by insectivorous bats. Thus, we find it likely that the characteristics of the ear, which presumably originally functioned only as a bat detector in Chloephorinae, determined the frequency range of their communicative sounds.

Sound emission in *P. prasinana* is mainly directed downwards, and sound is therefore radiated more effectively while the moth is flying than while sitting. The maximal communication distance can be estimated to approximately 16 m on the basis of click intensity, hearing threshold, a spherical spreading loss and an atmospheric attenuation of $1\,dB\,m^{-1}$ (40 kHz).

How do these moths survive flying around at dusk emitting high-pitched sounds in the presence of insectivorous bats? Detailed field studies of the behaviour of sonorous chloephorine species are needed to solve this apparent enigma. Rydell (1998) suggested that the non-hearing ghost moths are protected from bats by flying close to the grass. Perhaps chloephorines are protected in a similar way by flying so close to the leaves of the trees in the clutter zone (Kalko and Schnitzler, 1993) that it makes it difficult for insectivorous bats to capture them.

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