J. exp. Biol. **118**, 185–208 (1985)
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A SYNCHRONOUS INSECT MUSCLE WITH AN OPERATING FREQUENCY GREATER THAN 500 Hz*

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Accepted 4 April 1985

SUMMARY

1. The male cicada, *Okanagana vanduzeei*, produces a calling song with a pulse repetition frequency of 550 Hz. This sound is produced by a pair of tymbals, each of which is buckled by a large tymbal muscle. Males sing in full sun and the operating temperature of the tymbal muscles is 40–45 °C.

2. Analysis of the songs of animals with the tymbal mechanism destroyed on one side, and of the sounds produced by directly manipulating a tymbal, indicates that the two tymbals normally buckle synchronously and that only one sound pulse is produced per tymbal muscle contraction. This implies that the contraction frequency of each tymbal muscle is 550 Hz.

3. Recordings of calling songs from animals with implanted electrodes show that there is usually synchrony between left and right tymbal muscle contractions and that each tymbal muscle can operate at a frequency of about 550 Hz. The recordings also show that there is a 1:1 correlation between muscle electrical and mechanical activity, i.e. these muscles are synchronous and not asynchronous muscles.

4. The ultrastructure of the tymbal muscle is clearly that of a very fast, synchronous muscle: the myofibrils are small, the sarcoplasmic reticulum is extraordinarily well developed, and the T-tubules lie at the 1/4 and 3/4 positions along the sarcomere.

5. When set up for isometric recording, with their nerve supply severed, the tymbal muscles often show spontaneous electrical and mechanical oscillations. The frequency of these oscillations is strongly temperature dependent, and at higher temperatures approaches the normal operating frequency of the muscle. The tendency to oscillate is so

*This paper is dedicated to the late Professor J. W. S. Pringle who was a pioneer in studies of cicada muscles and a major contributor to our understanding of insect muscle in general. We think he would have enjoyed the new perspective given by the tymbal muscles of *Okanagana vanduzeei*.

Key words: Insect muscle, tymbal muscle, cicada, sound production, contraction kinetics, muscle ultrastructure.

strong that single twitches could only very rarely be evoked by electrical stimulation. A twitch duration of under 6 ms was observed at 30 °C.

6. We conclude that the tymbal muscle of *O. vanduzeei* is indeed a synchronous muscle, albeit a highly modified one, capable of operating at 550 Hz. We suggest that the advantages of asynchronous muscles lie chiefly in their greater economy of structure and operation, rather than in an ability to operate at higher frequencies than synchronous muscles.

INTRODUCTION

Insect muscles are divisible into two classes on the basis of the neural control of contraction: synchronous muscles and asynchronous muscles. In insect synchronous muscle, as in vertebrate skeletal muscle, each contraction results from depolarization of the muscle fibres. The muscles are termed synchronous because of the one-to-one relationship between their electrical and contractile activity. In asynchronous muscle, by contrast, there need not be a one-to-one relationship between electrical and mechanical events. Muscle action potentials are required for contractile activity, but when activated by neural input an asynchronous muscle can contract in an oscillatory fashion if it is attached to a resonant load. The contraction frequency during oscillatory contraction is a function of the resonant frequency of the muscle and its load and is independent of the frequency of incoming motoneurone impulses (Machin & Pringle, 1959), hence the lack of congruence between electrical activity and contractions. Asynchronous muscles are the power-producing muscles of flight in some insects and the tymbal muscles of the sound-producing systems of certain cicadas (Pringle, 1981). From their taxonomic distribution, it appears that asynchronous muscles have evolved independently many times in insects (Cullen, 1974).

It is generally assumed that the asynchronous mode of muscle control has evolved as a mechanism allowing high contraction frequency (e.g. Cullen, 1974; Pringle, 1981). Most wing stroke frequencies exceeding 100 Hz in insects are achieved with asynchronous muscles. Asynchronous muscles may reach contraction frequencies of 1000 Hz or more (Sotavalta, 1953) while the maximum contraction frequencies hitherto reported for synchronous muscles are about 200 Hz in insects, fish and mammals (references in Josephson & Halverson, 1971; Young & Josephson, 1984). However 200 Hz, as it turns out, is nowhere near the maximum contraction frequency obtainable with synchronous muscle. The following describes a cicada tymbal muscle whose contraction frequency during sound production is about 550 Hz. From both electrophysiological recording during activity and ultrastructural analysis, the muscle is clearly a synchronous muscle, albeit a highly modified one. Very high contraction frequencies are thus available from synchronous muscles. The evolutionary advantages of asynchronous muscle, then, must be something more than simply an increase in the potential operating frequency.

MATERIALS AND METHODS

Specimens of the cicada, *Okanagana vanduzeei* Distant, were collected by net from chaparral slopes and open grasslands near the university campus at Irvine, California in July and August. All experiments were performed on the same day that the animals had been captured, usually within an hour or two of capture.

Sound recordings

Recordings were made of the calling songs of undisturbed animals in the field using a directional microphone (Sennheiser MKH 816) and a battery-powered tape recorder (Nagra IVS) at a tape speed of $19\,cm\,s^{-1}$. Similar recordings were made of the calling songs of animals in which one tymbal was ablated. These animals were captured while singing and one tymbal was immediately destroyed under a dissecting microscope in the field. To ensure that the destroyed tymbal could not participate in sound production, a circular cut was made around the outer rim of the tymbal, the apodeme attaching it to the tymbal muscle was severed, and the entire tymbal membrane was removed. The animal's wings were then clipped to prevent it flying off and the animal was placed on a short stick, about 50 cm high, driven into the ground. The stick was in full sunlight as are the usual locations at which these cicadas sing. Several such sticks were arranged in a semicircle so that several experimental animals could be monitored conveniently by the experimenter sitting in the centre of the semicircle with the tape recorder. This procedure was carried out in the same area of grassland in which the animals were captured.

Myogram and temperature recordings

Animals were captured as early as possible during the normal singing period and were taken to the laboratory. Each animal was restrained in a dissecting dish with pins or soft wax. Silver wire myogram electrodes, 50 μm in diameter and insulated to the tip, were inserted into one tymbal muscle through a small hole made in its ventral attachment, the chitinous V. The wires were secured with insect wax at the point of insertion, led around to the dorsal thorax, and there again secured with wax. An earth electrode of bare silver wire was inserted into the posterior abdomen and also secured with wax. The silver electrodes were soldered to fine leads of insulated copper wire, about 30–40 cm in length, each ending in a small connector.

The experimental animals, with wires attached, were returned to the field within an hour of capture and placed on the short sticks driven into the ground (described under sound recordings above). When one of the animals began to sing, a simple dynamic microphone was placed near it. Myograms and sound were fed through separate preamplifiers to a tape recorder (Nagra IVS).

The temperature of the tymbal muscle was measured in the field using implanted thermistor beads (0·18 mm diameter) attached to long, thin, insulated

wires. The procedure for mounting a thermistor bead in a tymbal muscle was exactly the same as that for inserting the myogram electrodes (described above). Recordings were made using a multichannel bridge circuit (Yellow Springs Telethermometer). Ambient temperatures were measured with a thermocouple transducer (Bailey BAT-9C).

Electron microscopy

Tymbal muscles were prepared for electron microscopy using the following solutions.

(1) Primary fixative. 30 ml 8 % glutaraldehyde, 6·0 ml 1 mol l^{-1} sodium cacodylate buffer, 60 ml locust saline (Usherwood, 1968), distilled water to 132 ml; pH adjusted to 6·8. Alternative batches of fixative were made up with sucrose added to give an overall concentration of either 0·2 or 0·4 mol l^{-1} sucrose.

(2) Buffer wash. 100 ml locust saline, 25 ml 1 mol l^{-1} sodium cacodylate buffer, distilled water to 220 ml; pH adjusted to 6·8. Alternative batches included 0·2 and 0·4 mol l^{-1} sucrose. Primary fixative and buffer wash solutions with added sucrose gave decidedly poorer fixation than solutions without sucrose and the sucrose-containing solutions were not used except in preliminary studies.

(3) Post-fixative. 8 ml 4 % osmium tetroxide, 3·2 ml 1 mol l^{-1} sodium cacodylate buffer, 10 ml locust saline, distilled water to 35 ml; pH adjusted to 6·8.

The part of the cicada containing the tymbal muscles was isolated quickly by cutting off the thorax in front and the remaining abdomen behind, leaving a ring of cuticle supporting the two tymbal muscles. This ring was plunged into the primary fixative and much of the tracheal air sac surrounding the muscles was picked away to facilitate penetration of the fixative. After about 15 min in fixative, the muscles were cut free of the cuticle and teased into thin bundles. To ensure optimum fixation, only bundles of superficial fibres were kept. These were transferred to fresh fixative and left for a total of 1 h in primary fixative. Then the fibre bundles were washed in two changes of buffer wash and stored in the buffer for a few days. They were then post-fixed in osmium tetroxide for 1 h, washed again with buffer, dehydrated in alcohol, and embedded in Epon. Throughout this procedure, care was taken to keep all the materials cold until fixation was complete. As soon as they were captured, the animals were placed in jars packed in ice and they remained there until dissection. The fixative and buffer solutions were kept on ice when in use and were stored in the refrigerator at other times.

Sarcomere length and Z-band width were measured from electron microscope prints of longitudinal sections at a total magnification of 6000–35 000. Five separate Z-bands or groups of serially-adjacent sarcomeres were measured for each muscle, each determination usually being from a different fibre. The individual values for Z-band width or sarcomere length were themselves averaged to obtain single values for each muscle. Measurements were obtained from individual muscles of five animals. Myofibril area, circumference and maximum diameter were measured with a digital planimeter (Zeiss MOP Digital Image Analyzer System) from transverse electron micrographs at a total magnification of

about 35 000. Five adjacent fibrils were measured from each of six micrographs for a given muscle. Only fibrils in which both thick and thin filaments were clearly visible were measured so as to exclude the distinctly narrower region of the fibril at the Z-line. The 30 sets of values obtained were averaged to give a single set of values for each muscle. Muscles from three different animals were so analysed. Stereological analysis was done from electron micrographs of muscle sections taken slightly oblique to the transverse fibre axis. The prints were at a total magnification of approximately 35 000. A transparent grid ruled at 1-cm intervals was laid over the prints and the number of grid intersections lying on selected fibre components was counted. The total number of grid intersections lying over a particular component, for example over mitochondria, divided by the total number of intersections in the whole field gives the fractional volume of the selected component within the field. Ten micrographs were analysed for each muscle. The fields represented by the micrographs were randomly selected during the original photography except that areas with nuclei and large intracellular tracheoles were avoided, so these components are under-represented in the analysis. With the grid used, approximately 450 intersections lay within the bounds of each micrograph, so the final volume density values for each muscle are based on approximately 4500 individual counts. Stereological analysis was done for muscles of three animals.

Mechanical recordings

The legs and wings were removed from the experimental animal and the tymbal muscle was denervated by cutting through the ventral tissue between the mesothoracic and metathoracic segments, a region through which the tymbal nerves course. The animal was fixed, dorsal surface uppermost, to a Lucite block with quick-setting epoxy resin. The left tymbal was cut in a circle centred on the insertion of the muscle apodeme and the cuticular elements around the dorsal portion of the left tymbal muscle were removed. This left the dorsal portion of the muscle isolated but still attached to its apodeme and a small piece of tymbal. Tension was recorded with a strain gauge made from a pair of semiconductor transducer elements ('Pixie' strain gauges, Endevco, San Juan Capistrano, California). A short segment of an insect pin was bent into a hook at one end and fixed to the strain gauge at the other end. The hook was slipped around the exposed apodeme to connect the transducer to the muscle. Mechanical responses were displayed on an oscilloscope screen and photographed for later analysis.

The muscle was stimulated through a pair of silver wires, 50 μm in diameter, inserted into the muscle near its ventral attachment. Similar wires connected to a differential, capacitor-coupled preamplifier were sometimes used to record spontaneous electrical activity from the muscle. Muscle temperature was monitored by a thermocouple probe (Bailey Instruments) inserted into the base of the contralateral, non-stimulated muscle. Muscle temperature was controlled by varying the intensity of a microscope lamp whose beam was directed equally at both tymbal muscles.

RESULTS

O. vanduzeei is a medium-sized cicada of typical appearance (Fig. 1A). The species is widely distributed in the western United State (Simons, 1954). In the males, sound is produced by a tymbal mechanism similar in morphology to that described for other species of cicada (e.g. Pringle, 1954*a*,*b*; Young, 1972). Each tymbal (Fig. 1B) consists of a cuticular membrane bearing alternating long and short ribs anteriorly and an irregularly-shaped tymbal plate posteriorly (terminology of Simmons & Young, 1978). The tymbal is buckled inwards by the large tymbal muscle, which inserts dorsally on the tymbal plate, and springs back to the resting position as the muscle relaxes.

The calling song

In the study area, males of *O. vanduzeei* produce their calling songs in chaparral and grassland habitats during the summer (July, August). Calling males were rarely found more than 1 m above the ground, and preferred calling sites appeared to be the stems of small, dead shrubs, upon which the cicadas are well camouflaged. Calling commenced each day as the sun became hot, usually about 09.00–10.00 h, and ceased after about 2–3 h. The ambient temperature during singing was generally 28–35 °C. Individual males would call for over an hour with only brief interruptions.

The calling song consisted of an uninterrupted train of sound pulses (Fig. 2) which were repeated at a constant frequency of about 550 Hz ($\bar{X}$ = 549·8 Hz; S.D. = 9·3; N = 10). These pulses were usually of a uniform amplitude (Fig. 2A),

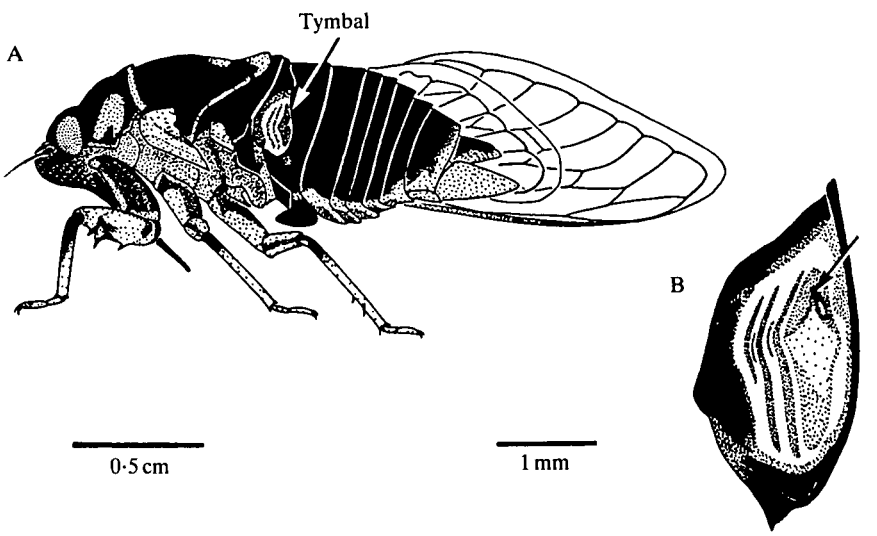


Fig. 1. (A) *Okanagana vanduzeei* male, lateral view, with left wings removed to show the location of the sound-producing tymbal. (B) The tymbal of *O. vanduzeei*; the arrow indicates the point of insertion of the tymbal muscle.

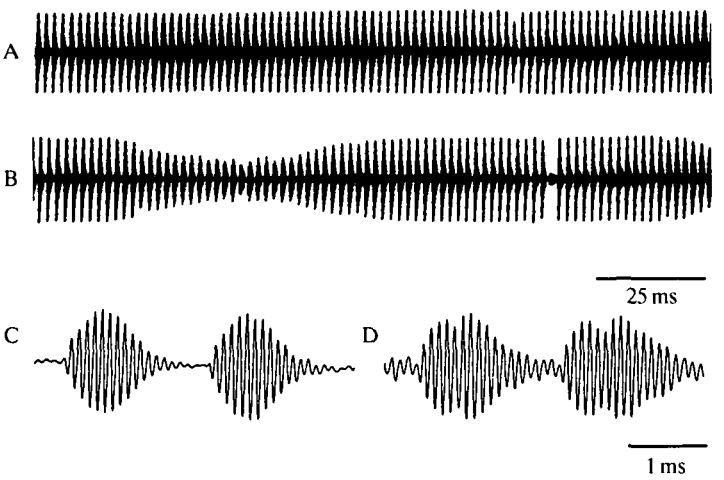


Fig. 2. Calling songs recorded from undisturbed males in the field. (A) Sample of song with pulses of uniform amplitude. (B) Sample in which song is amplitude-modulated. (C) High-resolution sample of pulses from uniform-amplitude song. (D) High-resolution sample from region of the amplitude-modulated song where the pulses are bimodal. In this figure, and in Figs 3, 5 and 6, low-resolution samples were obtained by filming the screen of a conventional oscilloscope, while high-resolution samples were obtained with an X/Y plotter from the output of a digital oscilloscope.

except for occasional smaller pulses that represented a momentary tymbal failure. At other times, the song was strongly amplitude-modulated (Fig. 2B). The occasional smaller pulses were more frequent in amplitude-modulated song. Individual males could change between uniform-amplitude and amplitude-modulated song more than once during the course of a morning.

Each sound pulse consisted of a train of sinusoidal sound waves (Fig. 2C). The fundamental frequency of this wave form was about 10·5 kHz ($\bar{X} = 10{\cdot}48$ kHz; S.D. $= 0{\cdot}34$, $N = 10$). Because of the high fundamental frequency, the song is heard by most human observers as a high-pitched whistle that is not very loud and is rather difficult to locate. Within each pulse, the amplitude of the sound showed a gradual increase to a peak, followed by an approximately exponential decay. When the song as a whole was of uniform amplitude, the individual pulses had a unimodal envelope (Fig. 2C), but in amplitude-modulated song the pulses from the low-amplitude region of the song often had a bimodal envelope (Fig. 2D).

Males of *O. vanduzeei* produce a courtship song when in the presence of females. The courtship song resembles the calling song but is more markedly amplitude modulated and is quieter, the sound pulses being noticeably damped. In most species of cicadas, calling songs cannot readily be obtained under laboratory conditions but protest songs can be elicited repeatedly and so can be used in experiments to estimate the contraction frequency of the tymbal muscle (e.g. Young & Josephson, 1983*a*). In *O. vanduzeei* the protest song is rare, but the calling song is produced under a variety of experimental conditions in the field and so it is possible to carry out the requisite experiments directly on the calling song.

Unilateral calling song

In the majority of cicada species studied so far, the frequency at which the tymbal muscle contracts is lower than the pulse repetition frequency of the song. This is because the left and right tymbal muscles contract alternately, or because each muscle contraction results in more than one pulse, or for both of these reasons (Hagiwara & Ogura, 1960; Aidley, 1969; Reid, 1971; Young & Josephson, 1983*a*,*b*). A simple test of the relative timing of contractions in the left and right tymbal muscles is to silence the tymbal on one side. If the two tymbal muscles normally contract alternately, the pulse repetition frequency of the song should be halved; if they normally contract synchronously, the pulse repetition frequency should remain unchanged. To be quite certain of silencing one side of a male *O. vanduzeei*, we removed the entire tymbal membrane from that side (see Methods). This rather drastic operation left a gaping hole in the side of the animal. Nevertheless most of the operated animals produced their calling song spontaneously within 15 min after the operation. The calling songs of the operated animals were essentially indistinguishable from those of normal animals (Fig. 3). The repetition frequency of the pulses (Fig. 3A) remained unchanged at about 550 Hz ($\bar{X}=541$ Hz; S.D. $=7$; $N=5$). The general form of the individual sound pulses and their fundamental frequency ($\bar{X}=10{\cdot}0$ kHz; S.D. $=0{\cdot}3$; $N=5$) also remained unchanged (Fig. 3C). These results imply that the right and left tymbal muscles contract synchronously and that the quality of the sound produced is not significantly affected by a large hole in the animal's side.

With the right and left tymbal muscles contracting synchronously, the contraction frequency of each muscle would equal the pulse repetition frequency if each muscle contraction were to produce one pulse. The contraction frequency would be less than the pulse repetition frequency if each muscle contraction were to produce more than one pulse. More than one pulse would be produced by each muscle contraction if more than one pulse were produced during the inward movement of the tymbal or if one pulse were generated during the inward

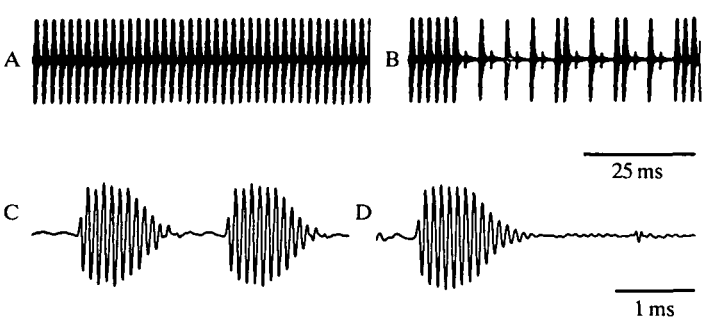


Fig. 3. Unilateral calling songs recorded from males after destruction of one tymbal. (A) Sample of uninterrupted calling song. (B) Sample in which the song falters. (C) High-resolution sample from uninterrupted, unilateral calling song. (D) A single pulse from a region where the unilateral song falters.

movement of the tymbal and another during the outward movement. If either of these possibilities were true for *O. vanduzeei*, one would expect to see indications of pulse grouping in the unilateral calling song. It is hardly possible that the intervals between successive pulses generated within one cycle of tymbal movement should always be the same as the interval between the last pulse of one tymbal cycle and the first pulse of the next tymbal cycle. Thus when a long succession of pulse intervals is measured in the animal's song, there should be a tendency for pulse intervals to be grouped according to the number of pulses generated by each cycle of tymbal movement. There is no sign of any such grouping when a succession of pulse intervals is measured in the unilateral calling song of *O. vanduzeei* (Fig. 4). On the contrary, the pulse intervals remain extraordinarily constant over long periods of time, with only a slight tendency to drift up or down. This regularity of pulse interval is evidence that each muscle contraction produces only a single sound pulse.

Further evidence that points to the same conclusion is obtained by examining regions where the unilateral song falters due to partial failure of the tymbal mechanism (Fig. 3B,D). In such a region, very small pulses are visible in the intervals where there are no large pulses. The very small pulses only occur singly, following one or more large pulses. The most plausible interpretation of these records is that each small pulse represents a quiet sound produced by the outward movement of the tymbal and each large pulse represents the loud sound produced by the inward movement of the tymbal. It is likely that the quiet outward sound has become visible as a separate entity through the tymbal sticking momentarily in the inward position and so delaying the outward movement. Normally the outward movement must occur after a much shorter latency and so the quiet

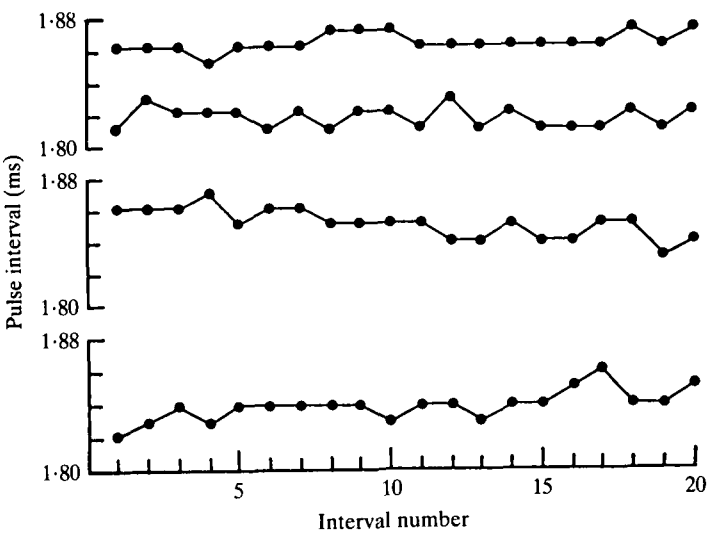


Fig. 4. Intervals between the onset of successive pulses in four samples of unilateral calling song. The intervals were measured with a resolution of 0·01 ms using a digital oscilloscope.

sound that it produces is largely lost in the powerful reverberations produced by the inward movement. If this is correct, one might expect to see indications of the outward sound during the decay of each large pulse, indications that would be absent where the outward pulse was delayed and therefore visible as a separate entity. Such observations were made in records of unilateral song on an expanded time scale (Fig. 3C,D). Normally, a disturbance of variable size was present in the exponential decay of each large pulse (Fig. 3C). Where the song faltered, the decay of the large pulse was perfectly smooth and a small pulse followed after some delay (Fig. 3D).

This interpretation was confirmed by directly manipulating the tymbal by hand in a dissected animal. The action of the tymbal muscle was mimicked by pushing on the point of muscle insertion with a fine probe at a slow speed at which the experimenter could be quite certain about the sounds associated with the inward and the outward movements of the tymbal. The sounds produced by hand-operated tymbal movements resembled those produced when unilateral song faltered (Fig. 5; cf. Fig. 3B,D). A succession of hand-operated movements produced a succession of alternating large and small pulses (Fig. 5A). The large pulses resulted from pushing the tymbal in. Though somewhat irregular and prolonged, each large pulse was essentially unimodal (Fig. 5B). The fundamental frequency of the sound was close to that of the natural song (about 10 kHz). The small pulses represented the barely audible click that the tymbal made as it returned to its resting position after being pushed in. Each small pulse consisted of a brief, highly damped, train of sound waves (Fig. 5C). Thus these experiments support the view that each pulse in the calling song results from a separate muscle contraction.

Muscle temperature during singing

A number of brief experiments were done to estimate the temperature at which the tymbal muscle operates in the field. On one day temperature recordings were

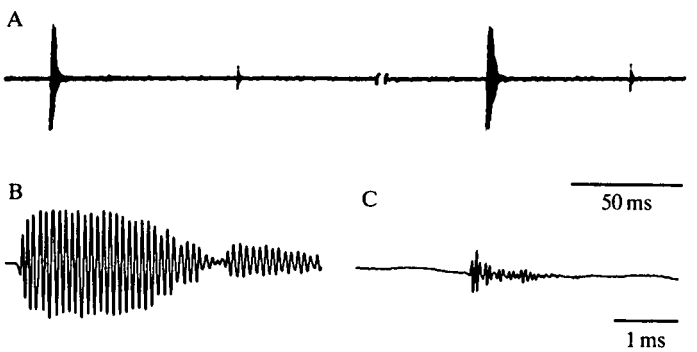


Fig. 5. Sounds produced by hand-operated movements of the tymbal. (A) Sounds produced by two successive in and out movements of one tymbal. (B) High-resolution record of the sound produced by the inward movement of the tymbal. (C) High-resolution record of the sound produced by the outward movement.

made in direct sunlight, to which a singing cicada is normally exposed, with a dead cicada impaled on the end of the temperature probe. On this occasion the ambient temperature fluctuated about 28 °C; in two separate trials with a dead cicada on the probe the temperature rose to 33 °C and to 35 °C. Temperature recordings were taken from one set of animals that produced calling songs with a thermistor implanted in one tymbal muscle. The ambient temperature fluctuated about 35 °C at 10–20 cm above the ground, the height at which the animals were placed, and about 45 °C on the dead clover and short grass on the ground surface. In the animal that gave the clearest results, tymbal muscle temperature equilibrated at 36 °C and this rose to 41 °C when the animal began singing. As singing continued intermittently, muscle temperature ranged from 41 to 45 °C, tending to drop when the animal was quiet and to rise when the animal sang. After a long period of silence the temperature had dropped to 38 °C and the observations were ended. A second animal equilibrated at 35 °C but then it fell to the ground and muscle temperature soon rose to 42 °C. The animal sang intermittently, while still on the ground, and muscle temperature ranged from 43 to 45 °C.

Evidently these cicadas tend to warm up a few degrees above ambient as they rest in their natural position several centimetres above the ground in full sun. Our measurements show that a further rise in temperature is experienced as singing commences, so that with ambient temperatures of 30–35 °C, the normal operating temperature of the tymbal muscle in *O. vanduzeei* is 40–45 °C.

Myogram recordings during calling song

The most direct evidence that the tymbal muscles are synchronous comes from recordings of electrical activity in the tymbal muscles during singing. We found that animals with electrodes implanted in a tymbal muscle would produce their calling song after being returned to the field, provided that this was done during the normal singing period. Over half the operated animals sang, but in some of them the electrodes were not well placed and no useable electrical recording was obtained. Out of a total of six animals that gave successful recordings, five gave clear evidence of synchrony between right and left tymbal muscles; while the sixth gave equally clear evidence of alternation.

Records from an animal with synchrony of electrical activity between right and left tymbal muscles are shown in Fig. 6A,C,D. There was distinct amplitude modulation of the sound and the muscle action potentials were quite regular in frequency and amplitude with a clear 1:1 correlation with the sound pulses (Fig. 6A). Both electrical potentials and sound pulses had a repetition frequency close to 550 Hz. These same features are seen in a record from another animal in which the song is not amplitude-modulated (Fig. 6B). The sound pulses and muscle action potentials were correlated 1:1 at a frequency of over 500 Hz. Interestingly, in this example muscle action potentials continued unabated through a portion of the record when sound production failed.

In those regions of amplitude-modulated song where the sound pulses were unimodal, the concomitant muscle action potentials had a smooth falling phase

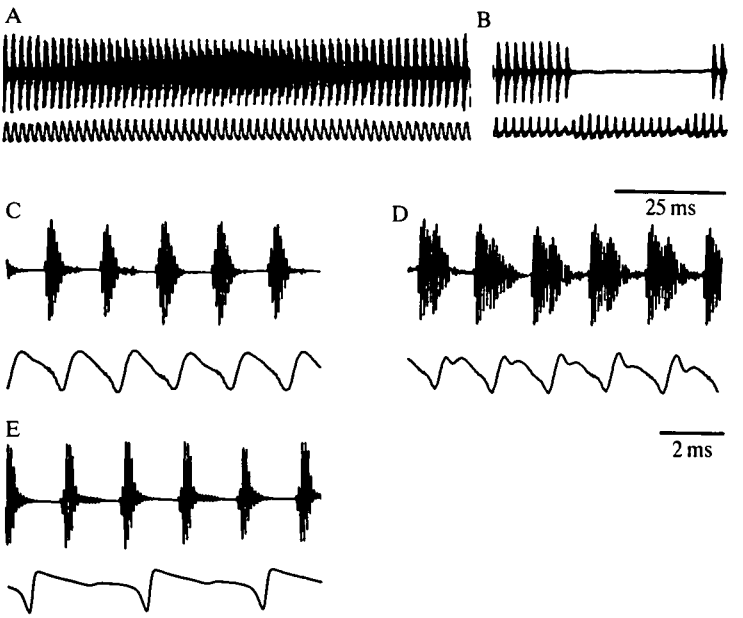


Fig. 6. Recordings from males with implanted electrodes showing, in each case, the sound output from both tymbals (upper trace) and muscle action potentials from one tymbal muscle only (lower trace). (A) Sample of amplitude-modulated song. (B) Sample of uniform-amplitude calling song in which the tymbals fail briefly. (C) High-resolution sample from a region of amplitude-modulated song where the pulses are unimodal. (D) As in C, but showing where the pulses are bimodal. (E) High-resolution sample of calling song from the single male in which muscle action potentials preceded every second sound pulse.

(Fig. 6C). Where the sound pulses were bimodal (Fig. 6D), the muscle action potentials showed a distinct inflection on the falling phase and the latency of this inflection with respect to the main peak was about equal to the latency between the two peaks of the bimodal sound pulses. As the sound pulses shifted from unimodal to bimodal and back over many cycles of the song, the inflection on the muscle action potential remained phase-locked to the second peak of the bimodal sound pulses. This relationship was very clear when the record was examined on an oscilloscope with the sweep triggered off the sound pulses. It seems probable that these inflections in the myogram record represent electrical cross-talk from the contralateral tymbal muscle and that where they occurred, as in Fig. 6D, the right and left tymbal muscles were firing out of phase with each other. Consequently the right and left tymbals were buckled out of phase, thereby producing sound pulses with a bimodal envelope. Where no inflections were apparent, as in Fig. 6C, the right and left tymbal muscles were firing in phase and so the tymbals were buckled in phase, producing unimodal pulses.

These records from the calling songs of animals with implanted electrodes indicate that each tymbal muscle can operate at a frequency of about 550 Hz with a 1:1 correlation between muscle electrical and mechanical activity, and with synchrony between right and left tymbal muscles. Surprisingly and inexplicably, one animal out of the six successful runs yielded a record that indicates equally

clearly that the right and left tymbal muscles can contract alternately (Fig. 6E). In this record the sound pulses still occur at a frequency of about 550 Hz but the muscle action potentials are correlated with every second pulse. Here the right and left tymbal muscles seem to be firing alternately, each operating at a frequency of 275 Hz.

Muscle ultrastructure

Although the contraction frequency of asynchronous muscles may be quite high during oscillatory activity, under isometric conditions asynchronous muscles are basically slow with long twitch rise and decay times (Pringle, 1954*b*; Josephson & Young, 1981), and the ultrastructure of an asynchronous muscle is that of a slow muscle. As compared with fast, synchronous muscles, asynchronous muscles have poorly-developed sarcoplasmic reticulum and T-tubules, and they have myofibrils which are large in diameter (Smith, 1966; Elder, 1975; Josephson & Young, 1981, and in preparation; Young & Josephson, 1984). In addition, for reasons which are not clear, the T-tubules of asynchronous muscles tend to lie half-way between the Z-lines of the sarcomere whereas in fast synchronous muscles of insects the T-tubules lie at 1/4 and 3/4 of the distance between Z-lines (Cullen, 1974; Pringle, 1981).

The ultrastructure of the tymbal muscle from *O. vanduzeei* is clearly that of a very fast, synchronous muscle (Fig. 7). The myofibrils are small (about 0·7 μm diameter, see Table 1), the sarcoplasmic reticulum (SR) is extraordinarily well developed (34 % of fibre volume, Table 1), and the T-tubules lie at the 1/4 and 3/4 positions along the sarcomere. The ratio of thin to thick filaments is 3:1. The myofibrils of *O. vanduzeei* are the thinnest, the volume fraction of fibre as sarcoplasmic reticulum the largest, and the ratio of myofibril volume to SR volume the smallest that we have yet encountered in tymbal muscles of eleven different cicada species that we have so far examined (Young & Josephson, 1984). This is consistent with the very high operating frequency of the tymbal muscle of *O. vanduzeei*, which is by far the highest operating frequency yet reported for any synchronous muscle.

The myofibrils are of uniform diameter through most of the sarcomere, but they become distinctly narrower at the Z-line (Fig. 8). Except at the Z-lines, the SR between adjacent fibrils is a continuous, multi-layered structure. As the fibrils become narrow at the Z-lines the SR divides, part going with each of the adjacent fibrils, leaving a curious region at the Z-lines, oval in cross section, which lacks SR (Fig. 8B,C). The Z-lines themselves are quite broad and sometimes appear split with a central light area flanked by two darker regions (Fig. 8B,C). Among mammalian skeletal muscles there is a tendency for fast muscles to have narrow Z-lines and slow muscles to have broad ones (reviewed in Eisenberg, 1983). The thick Z-lines of the tymbal muscle of *O. vanduzeei* show that the inverse correlation between Z-line thickness and muscle rapidity is not a universal one.

Synapses are frequently encountered in sections at all levels in the muscle, indicating that the fibres are multi-terminally innervated. The synapses are

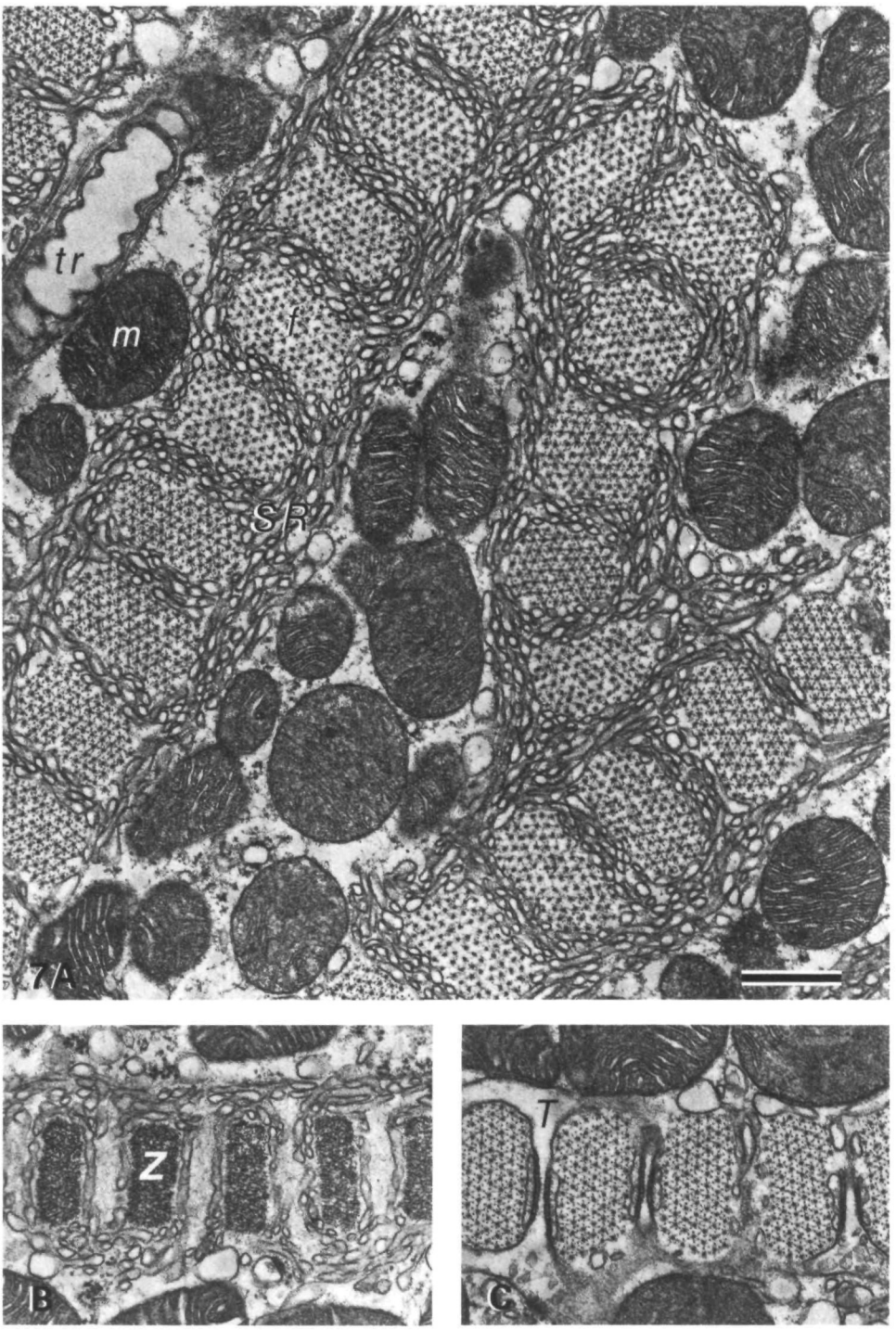

tr
m
f
SR
A
Z
B
T
C

Table 1. *Morphology of the tymbal muscle of* Okanagana vanduzeei

Fibril	
area, μm ($N=3$)	0·261 (S.D. = 0·015, $r=0·244–0·274$)
circumference, μm ($N=3$)	2·11 (S.D. = 0·04, $r=2·08–2·16$)
maximum diameter, μm ($N=3$)	0·725 (S.D. = 0·028, $r=0·700–0·756$)
Sarcomere length, μm ($N=5$)	2·53 (S.D. = 0·36, $r=2·03–2·90$)
Z-line width, μm ($N=5$)	0·38 (S.D. = 0·09, $r=0·26–0·48$)
Percentage of fibre volume as:	
myofibril ($N=3$)	21·9 (S.D. = 1·2, $r=20·5–22·8$)
mitochondria ($N=3$)	33·7 (S.D. = 1·6, $r=32·7–35·6$)
SR and T-tubules ($N=3$)	33·9 (S.D. = 0·3, $r=33·5–34·1$)
other ($N=3$)	10·5 (S.D. = 0·5, $r=10·0–11·0$)
Myofibril volume/volume as SR and T tubules ($N=3$)	0·65 (S.D. = 0·04, $r=0·60–0·66$)

Values given are the mean followed in brackets by the standard deviation and range. N is the number of muscle preparations used, each from a different insect.

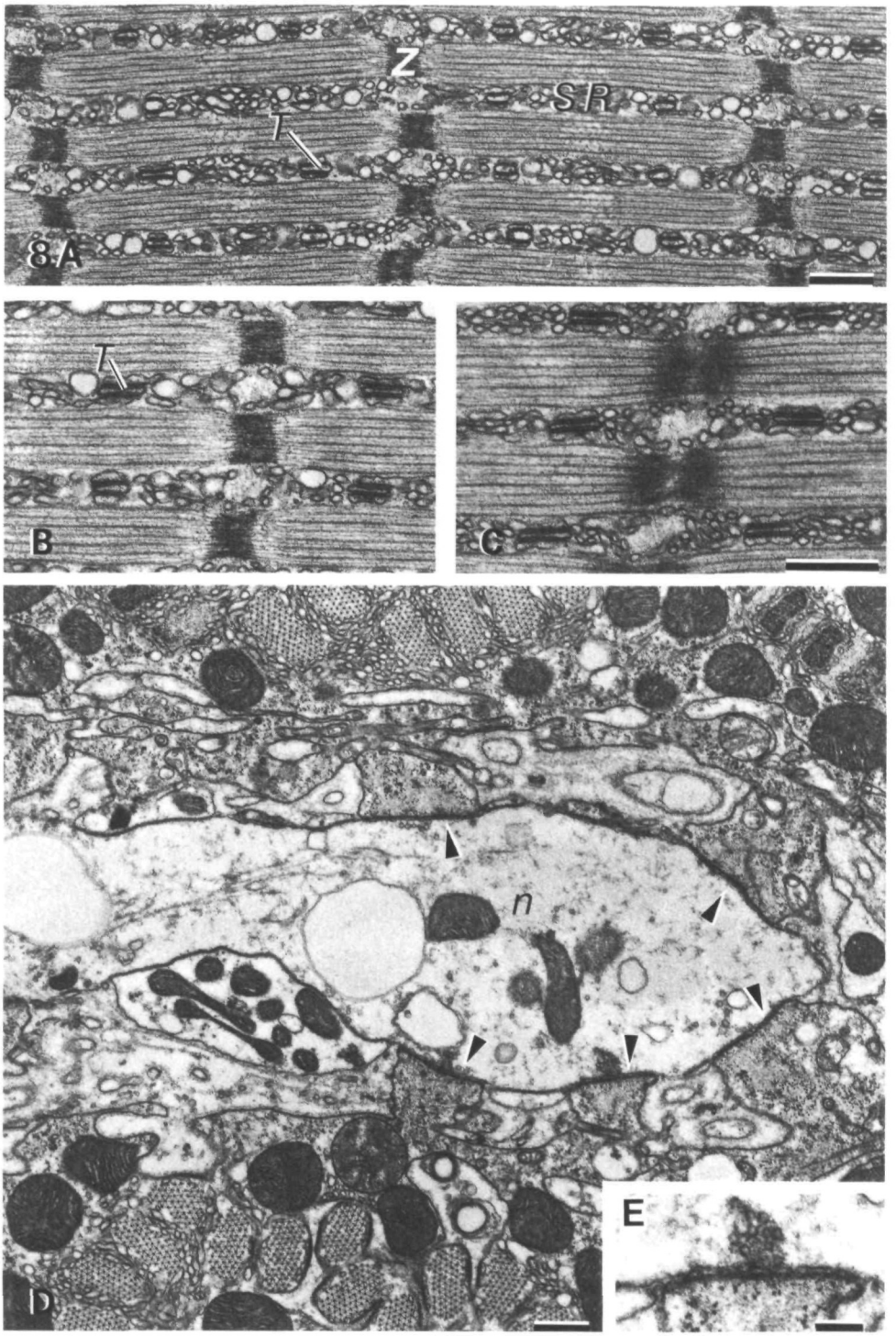
formed on pillar-like extensions of muscle fibres which project towards nerve terminals coursing through the muscle (Fig. 8D). Frequently synapses to adjacent muscle fibres are formed on opposite sides of a single nerve terminal (Fig. 8D).

Contraction kinetics, spontaneous contractions

It proved very difficult to record isometric twitches from tymbal muscles of *O. vanduzeei*. Most preparations were quite unresponsive to electrical stimulation by the time they were mounted for isometric tension recording. Other preparations, when first mounted, produced oscillatory mechanical contractions at a frequency of several hundred Hz. Typically the amplitude of these contractions became progressively smaller until the muscle was inert, at which time it was also unresponsive to electrical stimulation. It seems likely that those muscles that were initially quiet and unresponsive had already passed through a period of spontaneous activity and were exhausted by the time tension recordings were first tried. It should be noted that the nerves from the central ganglia to these muscles had been cut so the oscillatory contractions cannot be ascribed to pattern generators in the central nervous system or to reflex loops. In an attempt to forestall damaging spontaneous activity, some preparations were initially chilled, dissected over ice, and attached to the transducer while still cold. However, when these were allowed to warm up they too became spontaneously active and, eventually, inert.

Isometric twitches were recorded successfully from only two of over 30 preparations tried. In one of these the muscle became spontaneously active

Fig. 7. Transverse sections of tymbal muscle fibres. (A) is from near the middle of the sarcomere, (B) from the Z-line, and (C) from the part of the sarcomere surrounded by a T-tubule. Abbreviations: *f*, myofibril; *m*, mitochondria; *SR*, sarcoplasmic reticulum; *T*, T-tubule; *tr*, intracellular trachea; *Z*, Z-line. Scale bar, 0·5 μm.


Z
SR
T
8A
B
T
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n
D
E

following an evoked twitch (Fig. 9). The spontaneous responses were initially of the same size as the evoked twitches, but became smaller over the next several minutes until they vanished in the recording baseline. The twitches from the successful preparations were quite short in both cases. Values at 30 °C (mean, range) were: rise time, 2·9 ms (2·6–3·2 ms); relaxation time, tension peak to 50 % return, 2·8 ms (2·1–3·4 ms). The twitch duration of 5·7 ms (onset to 50 % relaxation) is the shortest that we have yet recorded from any cicada muscle.

The oscillatory muscle contractions were associated with oscillatory electrical potentials from the muscle which were recorded with extracellular electrodes (Fig. 10). Sometimes the electrical and mechanical events were regular and of a uniform amplitude, but often either the electrical or the mechanical responses showed regular waxing and waning of amplitude (Fig. 10B). The regular patterns in such records suggest that there may have been multiple sites of activity within the muscle that drifted into and out of phase with one another. Although the amplitudes of electrical and mechanical events changed independently, there was always a 1:1 relationship between the occurrence of electrical and mechanical responses.

The frequency of spontaneous oscillations in the tymbal muscle of *O. vanduzeei* was strongly temperature dependent (Fig. 11). At 30 °C the average oscillatory frequency was 266 Hz (S.E. = 21 Hz, $N = 8$ preparations), at 35 °C it was 316 Hz (S.E. = 34, $N = 8$). Frequencies increased still more with further warming but in the laboratory responses became small and the preparation deteriorated rapidly at temperatures of 40 °C or more.

DISCUSSION

The contraction frequency of the tymbal muscle

Our results indicate that the normal operating frequency of the tymbal muscle of *O. vanduzeei* is about 550 Hz. The sound pulse repetition frequency during calling songs was about 550 Hz in every example that we recorded, including songs from animals in which one tymbal was so severely damaged that it could not possibly have participated in sound production. The extreme regularity of the pulse intervals during the calling song suggests that each sound pulse is produced by a single muscle contraction and consequent tymbal buckling. This interpretation is reinforced by the observation that manipulating a tymbal results in one major sound pulse being produced during inward buckling with a shorter and much fainter pulse occurring with the subsequent return of the tymbal to its rest

Fig. 8. (A–C) Longitudinal sections of tymbal muscle fibres. (D) A nerve process (*n*) lying between two muscle fibres, one above and the other below the nerve branch. Processes from the muscle fibres project toward the nerve branch and form synaptic contacts with it (marked by arrows). (E) An enlarged view of the synapse above it and to the left showing a cluster of vesicles in the nerve terminal. Abbreviations are as in Fig. 7. Scale bars are 0·5 μm in (A–D) and 0·2 μm in (E). (B) and (C) are at the same magnification.

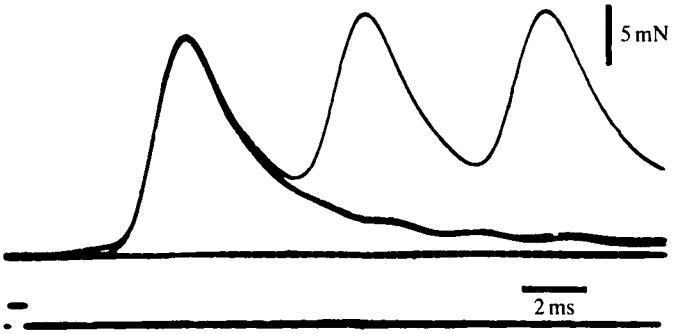

Fig. 9. Isometric twitches from a tymbal muscle, 30°C. The upper set of traces are tension recordings, the lower set mark stimuli. After several twitches had been recorded the muscle suddenly became spontaneously active, producing twitches which were initially similar in shape and time course to the evoked responses.

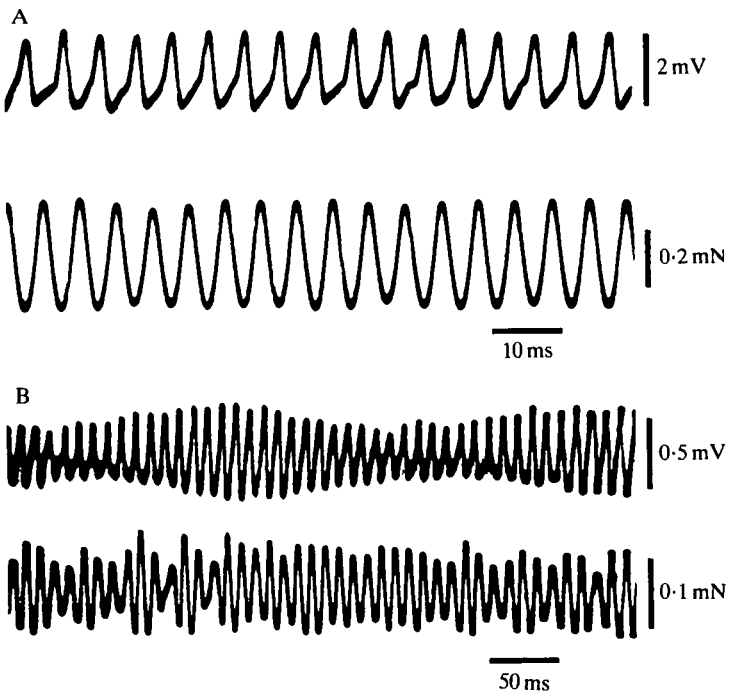

Fig. 10. Spontaneous electrical (upper trace in each set) and mechanical (lower trace) responses from tymbal muscles. (A) was recorded at a muscle temperature of 30°C, (B) at 20°C.

position. There was one electrical potential recorded from a tymbal muscle for each sound pulse in most of the animals from which muscle myograms were obtained during calling song. The correspondence between muscle potentials in one of the two tymbal muscles and sound pulses produced by the pair of tymbals indicates that the muscle was activated with each sound pulse and, further, that the two tymbal muscles must have contracted synchronously rather than on

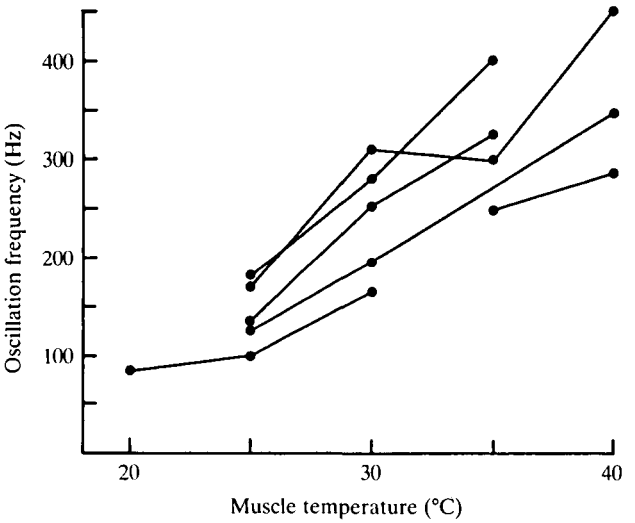

Fig. 11. Spontaneous oscillation frequency, measured from either electrical or mechanical oscillations in tymbal muscles, as a function of muscle temperature. Only records in which the oscillations were of regular amplitude were considered. Lines join separate determinations made from a single muscle.

alternating cycles. Synchronous activity between the two tymbal muscles is also suggested by the occasional biphasic sound pulses and associated myograms, which are most easily explained as being due to the two tymbal muscles being active slightly out of phase with respect to one another.

In one of the animals from which myogram recordings were obtained there was a muscle action potential with only every other sound pulse. Why the records from this animal are so strikingly different from the others is not easily explained. Apparently the tymbal muscle from which the recording was made was being activated on every other sound pulse cycle and the 550 Hz overall sound pulse frequency was a result of the two tymbals operating in antiphase. An alternative hypothesis, namely that one tymbal apparatus was operating at 275 Hz and its partner at 550 Hz, is unlikely since this would result in successive pairs of large and small pulses, whereas the pulses were observed to be quite regular (Fig. 6E). One suspects that had the contralateral tymbal been destroyed, the sound pulse frequency from this animal would have dropped to 275 Hz rather than remaining at 550 Hz as it did in all other animals so treated. We are left with the conclusion that the muscle contraction frequency is usually about 550 Hz but that rarely it may be half this value with alternation between the two tymbals maintaining the pulse frequency at 550 Hz.

The tymbal muscle is a synchronous muscle

Although its normal contraction frequency is very high, the tymbal muscle of *O. vanduzeei* is clearly a synchronous muscle. The *prima facie* evidence for the muscle being a synchronous muscle is the demonstrated 1:1 relationship between

muscle contraction, inferred from the sound pulses produced, and electrical potential recorded from the muscle (Fig. 6, compare Roeder, 1951). Even in the absence of electrical recordings, the muscle would be judged to be a synchronous muscle from its short twitch duration and its ultrastructure. The differences in contraction kinetics and ultrastructure between the tymbal muscle of *O. vanduzeei* and that of *Platypleura capitata*, which has been shown to be an asynchronous muscle (Pringle, 1954*b*; Josephson & Young, 1981), could hardly be more striking. The twitch duration (onset to 50 % relaxation, 30 °C) for the muscle of *P. capitata* is 54 ms, that from *O. vanduzeei* about 6 ms. The tymbal muscle of *P. capitata* has very little sarcoplasmic reticulum and the T-tubules of this muscle encircle the fibrils at the middle of the sarcomeres; the tymbal muscle of *O. vanduzeei* has an unusually abundant sarcoplasmi reticulum and T-tubules at the 1/4 and 3/4 positions in the sarcomere, both features of fast, synchronous, insect muscles.

Spontaneous electrical and mechanical oscillations

The spontaneous, oscillatory contractions and associated electrical potentials frequently encountered in tymbal muscle preparations from *O. vanduzeei* are similar to the oscillatory mechanical and electrical responses seen occasionally or regularly in tymbal muscles of a number of other cicada species (Wakabayashi & Hagiwara, 1953; Hagiwara, 1953; Ikeda, 1959; Wakabayashi & Ikeda, 1961; Josephson & Young, 1981). It should be emphasized that the oscillatory activity occurs in muscles which have been disconnected from the central nervous system; thus the oscillation is inherent to the muscle itself. We presume that the electrical oscillations are primary and that the mechanical oscillations are caused by fluctuating membrane potentials which are the source of the extracellularly recorded electrical responses. In *O. vanduzeei* the frequency of the spontaneous oscillation approaches the sound pulse frequency during calling song as the muscle temperature approaches the normal operating temperature (Fig. 11). It is difficult to believe that the tendency to produce oscillatory electrical potentials is not important in the control of muscle contraction during sound production, perhaps in facilitating high-frequency performance. In this regard it is of interest that fibres from the muscles used in stridulation by the katydid *Neoconocephalus robustus*, which can operate at a high frequency (200 Hz), show damped, oscillatory membrane potential changes to imposed depolarizing current or synaptic input, while fibres from homologous but slower wing muscles not used in stridulation do not (Josephson & Stokes, 1982). Here, as we suspect is also the case in fast cicada muscles, the membranes of the muscle fibres seem to be poised on the edge of oscillatory instability. The role of neural input in sustaining activity in the tymbal muscle of *O. vanduzeei* is uncertain. It is likely that there is an incoming impulse along the motoneurone supplying the muscle for each contraction, but it is also possible that motoneurone impulses occur at a frequency considerably lower than muscle contractions but often enough to sustain oscillatory activity of the fibre membranes.

The amplitudes of the spontaneous electrical and mechanical oscillations recorded from tymbal muscle preparations of *O. vanduzeei* were moderately large and must have been due to the concerted activity of many individual muscle cells. How these individual cells were coordinated to act in synchrony is not known. Several possibilities suggest themselves. (1) The coordination may be achieved by cyclic activity originating in and spread throughout the muscle along the terminal ramifications of the motoneurone which courses through the muscle. (2) The muscle fibres could be electrically coupled to one another, either directly or indirectly through low resistance pathways between muscle cells and nearby nerve terminals or glia. This possibility seems unlikely since no gap junctions which might have provided electrical current pathways were seen between muscle cells or between muscle cells and neighbouring non-muscle cells. (3) The muscle fibres all insert on a common apodeme and changes in the position of this apodeme will alter the length of all the fibres of the muscle and the tension on them. If the membrance potential of the muscle fibres is sensitive to mechanical distortion or changes in tension, coordination between fibres could be achieved mechanically through the changes in length or tension experienced simultaneously by all the fibres of the muscle.

Asynchronous muscles can give sustained mechanical oscillations if attached to a resonant load, but their oscillation frequency is the mechanical resonance frequency of the load (Machin & Pringle, 1959). The oscillatory frequency of the tymbal muscle of *O. vanduzeei* was not determined by the resonant frequency of the load. The mechanical resonance frequency of the transducer and holder used in these experiments was several kHz, which is much higher than the recorded oscillation frequency. Further, changing the temperature, which should not significantly affect the mechanical resonance of the load, greatly altered the oscillation frequency. Finally, electrical oscillations like those recorded from muscle preparations could also be recorded from electrodes inserted into a minimally dissected muscle still attached to its normal origins and insertions and not to a transducer. The determinants of the oscillation frequency are in the physiology of the muscle and not the physics of its load.

Why asynchronous muscle?

It is generally assumed that the asynchronous mode of muscle control evolved from the synchronous mode as a mechanism allowing higher contraction frequencies than were possible with synchronous control (e.g. Cullen, 1974; Pringle, 1981). In recent years a number of surprisingly high contraction frequencies have been found in muscles using synchronous control: about 100 Hz in a lobster remotor muscle at 16–18 °C (Mendelson, 1969); 100 Hz in stridulatory muscles of the tettigoniid *Neoconocephalus triops* at 30 °C (Josephson, 1984); 200 Hz in stridulatory muscles of *N. robustus* at 35 °C (Josephson & Halverson, 1971; Heath & Josephson, 1970); 50–220 Hz in various cicada tymbal muscles at ambient temperatures of 25–35 °C (Young & Josephson, 1983*a*,*b*), and now 550 Hz in the tymbal muscle of *O. vanduzeei* at a muscle temperature of 40–45 °C. Clearly high

operating frequencies are obtainable from synchronous muscle. What then is the advantage of asynchronous control that has led to its adoption in the flight muscles of a number of the most successful insect groups?

Two advantages of the asynchronous mode over the synchronous mode for high frequency operation are economy of muscle construction and economy of operation. In both synchronous and asynchronous muscle, contractile activity is initiated by the release of calcium into the cytoplasm from the sarcoplasmic reticulum and terminated by the resequestration of calcium by the sarcoplasmic reticulum. In synchronous muscles, high frequency operation requires rapid release and re-uptake of calcium and this capacity is associated with hypertrophy of the sarcoplasmic reticulum. In general there is a good correlation in synchronous muscle between the development of the sarcoplasmic reticulum and either twitch brevity or maximum operating frequency which are, of course, related parameters (Josephson, 1975; R. K. Josephson & D. Young, in preparation). Increases in the fibre volume occupied by sarcoplasmic reticulum are necessarily associated with a decrease in the relative volume of other components; an extreme example being the lobster remotor muscle in which about three-quarters of the fibre volume is sarcoplasmic reticulum, leaving but a small part of the fibre for myofibrils and other components (Rosenbluth, 1969).

A trade-off between increasing sarcoplasmic reticulum volume and decreasing myofibril volume is seen quite clearly in cicada tymbal muscles. In a series of tymbal muscles which we have examined from 11 cicada species, mitochondria make up 34–42 % of the muscle fibre volume and this fraction seems to be relatively independent of the normal contraction frequency of the muscle. The fibre volume other than that taken up by mitochondria is principally occupied by myofibrils and by sarcoplasmic reticulum and T-tubules. In these muscles there is a direct relationship between the contraction frequency during calling song and the relative volume of sarcoplasmic reticulum; and therefore there is an inverse relationship between operating frequency and myofibril volume. In the tymbal muscle from the species with the lowest frequency calling song (*Arunta perulata*, see Young & Josephson, 1983*a*, 1984), the myofibrils comprise about 41 % of the fibre volume; in *O. vanduzeei*, which has the highest frequency, myofibrils occupy only 22 % of the fibre. Thus the hypertrophy of sarcoplasmic reticulum required for high frequency performance in synchronous muscle is associated with a reduction in myofibril volume and therefore, presumably, with a reduction in the muscle force per cross-sectional area and the mechanical power output per gram muscle.

In asynchronous muscle, high-frequency performance is achieved without hypertrophy of the sarcoplasmic reticulum and therefore a reduction in myofibril volume is not necessary. The significance of this is seen by comparing the tymbal muscle of *O. vanduzeei* with that of *Platypleura capitata*, which is an asynchronous muscle with a comparable contraction frequency (about 390 Hz, see Pringle, 1954*a*, Josephson & Young, 1981). Tymbal muscle fibres from *P. capitata* are 49 % myofibril and 3 % sarcoplasmic reticulum by volume (Josephson & Young,

1981). In *O. vanduzeei* sarcoplasmic reticulum occupies about 10 times the relative volume and myofibrils less than half the relative volume that they do in the asynchronous muscle of *P. capitata* (Table 1). Thus one expects that the mechanical power output per gram muscle should be of the order of twice as great in the muscle of *P. capitata* as in that of *O. vanduzeei*.

In synchronous muscle calcium is cycled between the sarcoplasmic reticulum and the cytoplasm with each contraction, while in asynchronous muscle calcium movements are presumably more sluggish and calcium levels in the cytoplasm remain high throughout the period of activity. A significant portion of the energy requirements of cyclically-contracting, synchronous muscle are probably related to calcium release and rebinding (Homsher & Kean, 1980). Although it has not been directly demonstrated, one expects that because the costs of calcium cycling are avoided, it should cost less to operate an asynchronous muscle at a given frequency than a synchronous muscle. Thus there are advantages in both operating efficiency and construction costs for asynchronous muscle over synchronous muscle. It is probably these advantages, rather than the potential for high operating frequency, that have been most important in the evolution of the asynchronous mode of muscle control.

This work was supported by grant PCM-8201559 from the National Science Foundation and by a travel grant from the University of Melbourne. We wish to thank J. Malamud and A. Novicki for help with the portions of this study done in the field; M. Kavanagh for help with sound analysis; G. Marsh for aid in identifying the species used; J. Kiethe and S. Fisher for their assistance with the electronmicroscopy; and P. Steinbach for assistance throughout the study.

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