CONTRACTILE PROPERTIES OF A HIGH-FREQUENCY MUSCLE FROM A CRUSTACEAN

I. ACTIVATION PATTERNS *IN VIVO*

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Summary

1. The flagella of crustaceans are small appendages, borne on the maxillipeds, which beat repetitively when active. Flagellar movement is brought about by contraction of a single muscle, the flagellum abductor (FA).

2. The stroke frequency of the flagella of the green crab, *Carcinus maenas*, was about 11 Hz at 15 °C and was relatively independent of animal size [frequency is proportional to (animal mass)$^{-0.07}$], even though scaling considerations suggest that, for constant muscle stress, frequency should be proportional to mass$^{-0.33}$. The coefficient of variation for intervals between successive strokes of a flagellum was about 4 %.

3. The FA is innervated by two excitatory motoneurones. Each of the neurones fired 0–5 times during a stroke. The interspike interval when a neurone fired more than once during a stroke was 3–4 ms.

Introduction

In decapod crustaceans, the distal portion of the exopodite of each of the three paired maxillipeds is an elongate appendage termed the flagellum. The flagella of crustaceans are remarkable for their high-frequency, synchronized beating. Generally, the flagella of only one side beat at any time, and the beating on the active side is metachronal, beginning at the third maxilliped and progressing forward to the first. Activity tends to alternate between sides, and the bout of activity on a single side may last for many minutes (Burrows and Willows, 1969; Charlton, 1971).

Contraction of a single muscle in the basal segment of the exopodite, the flagellum abductor (FA, muscle 87 of Cochran, 1935), produces the rapid, antero-lateral movement of the flagellar stroke. The FA has no muscular antagonist; return of the flagellum to its resting position is due to elasticity of the basal hinge upon which it pivots. In the green

crab *Carcinus maenas*, the FA is a small muscle composed of several hundred fibres between which there is much branching and coalescence. The muscle is composed, functionally, of 8–10 fascicles of fibres. Within each fascicle, but not between fascicles, there is cytoplasmic continuity by way of extensive anastomoses (Stokes and Josephson, 1992).

Two features of the FA make it an unusually attractive preparation in which to study basic properties of crustacean muscles: the high frequency at which it operates, and the simplicity of both its structure and its neuronal innervation. The beat frequency of a flagellum during activity, and thus the contraction frequency of the FA, is 8–16 Hz in several species examined (Burrows and Willows, 1969; Charlton, 1971). This operating frequency, while high, is not the highest known for crustacean muscles. A much higher frequency is achieved by the remotor muscle of the second antenna of the lobster *Homarus americanus*, which can contract repetitively in short bursts at over 100 Hz (Mendelson, 1969). It has been suggested that the stride frequency of the ghost crab *Ocypode ceratophthalma* may be as high as 40–50 Hz during rapid running (Hafemann and Hubbard, 1969), but recent careful measurements on a related species (*O. quadrata*) suggest that the maximum stride frequency is a more modest 8–10 Hz (Blickhan *et al.* 1993). The feeding appendages of copepods reach frequencies of 20–50 Hz (Koehl and Strickler, 1981). While not the fastest, the FA muscle is certainly among the faster of crustacean muscles, and its organization and physiology can be expected to be revealing about muscle modifications associated with high-frequency performance.

The simple geometry of the FA facilitates analysis of its mechanical responses. It is a parallel-fibred muscle, which is a rarity among the muscles of crustacean appendages. Because the fibres are parallel rather than pinnate, force measured from the muscle tendon can be converted into stress in the individual muscle fibres, and muscle shortening can be converted into fibre strain, without having to compensate for angles of fibre pinnation. Furthermore, the FA is innervated by only two excitatory axons and no inhibitory innervation has been detected in the several species examined (Burrows and Willows, 1969; Charlton, 1971). Therefore, the muscle can be activated by stimulating the excitatory axons in the motor nerve without concern that muscle performance might become degraded because of simultaneous activation of inhibitory axons.

The following, which is the first of three papers on the physiology of the FA muscle in the crab *Carcinus maenas*, describes the neural output to the muscle in nearly intact preparations and examines the extent to which animal size is a determinant of the normal operating frequency of the muscle. This information provides a background for evaluating the isometric contractile performance of the FA muscle in response to different patterns of activation (paper II, Stokes and Josephson, 1994), and it identifies appropriate activation patterns to use in evaluating the work and power output of the muscle during its normal operation (paper III, Josephson and Stokes, 1994).

Materials and methods

The experiments were performed at the Marine Biological Laboratory in Woods Hole, MA. Green crabs, *Carcinus maenas* (L.), were collected by the laboratory supply

department and were maintained in running sea water at 15–21 °C. The animals used ranged in mass from 9 to 110 g.

Flagellar frequency

The legs were bound together and fixed to the body with rubber bands, and the animal was mounted, ventral side up, in a dish of sea water. A cooling coil in the dish maintained the water temperature at 15 °C. The third maxillipeds were removed bilaterally, exposing the underlying exopodites and flagella of the second and first maxillipeds. Flagellar movements were recorded with a force transducer made from a pair of semiconductor strain gauges and an attached insect pin as a lever arm. The transducer was mounted in a manipulator and positioned such that the end of the insect pin rested lightly against the lateral edge of a flagellum. Contraction of the FA muscle caused the flagellum to push against the transducer. The force recordings thus produced were collected in 1–2 s blocks and stored in a digital computer. In some of the later experiments, force signals were collected continuously and stored on tape for later display and analysis.

The animal was allowed to rest for 10–30 min before recording was begun. Each sample was a set of 4–6 consecutive beats, collected from periods during which beating was moderately regular. Samples were gathered at 10–20 s intervals. The reported beat frequency for each animal is the average frequency for five separate samples.

Muscle and nerve action potentials

The animal was mounted as for measuring flagellum beating. Electromyograms from the FA of the second maxilliped were recorded between a pair of silver wires, 50 μm in diameter and insulated except at the cut tip. The wires were inserted through holes in the exoskeleton. One wire was inserted into the muscle near its base and the other into the space lateral to the muscle. The electrodes were fixed in place with a small amount of low-melting-point wax. The electrical signals were amplified with a capacitor-coupled amplifier (band pass 10 Hz to 3 kHz).

Nerve action potentials were recorded with a suction electrode placed over the cut end of the nerve to the muscle. The nerve to the FA of the second maxilliped was exposed in the muscle segment, and side branches of the nerve were cut. The distal end of the nerve was then isolated by removing surrounding muscle and cuticle. Potentials recorded with the suction electrode were amplified with a capacitor-coupled amplifier [band pass 100 Hz (sometimes 10 Hz) to 3 kHz]. In some preparations, the two motor axons to the FA produced action potentials of similar size, which made it difficult to identify the source of individual impulses. Two kinds of preparations were particularly suitable for analysis of impulse patterns in the FA nerve: those in which only one unit was active, probably because the other had become damaged during dissection; and those in which potentials from one motor unit were substantially larger than those from the other motor unit so that the two units could be readily distinguished.

The trauma associated with mounting the crab upside down, removing its exopodites, and exposing the nerve seemingly inhibited spontaneous beating of the flagella, and in many preparations there were neither action potentials in the nerve to the flagellum nor beating of the anterior flagellum or of the contralateral flagella. In some preparations, the

flagella of the contralateral side beat apparently normally while those on the side from which the recording was made were annoyingly quiet. Application of clam juice to the mouthparts sometimes initiated regular flagellar beating. Nerve action potentials in patterns which we interpret to be those of normal beating were obtained from fewer than one-third of the preparations attempted. Although the success rate was low, there is no reason to think that those preparations that did produce continuing bursts of activity were responding in other than a normal manner. The nerve action potential patterns recorded were totally consistent with the recordings of electromyograms and of flagellar movements made from more intact preparations.

Flagellum mass

Varying amounts of adhering water, and varying amounts of drying during the weighing process, made it difficult to obtain reliable values for the wet mass of a flagellum. Therefore, dry mass was taken as a measure in order to obtain more precise values for the size of these small objects. The flagella of the second maxillipeds were removed bilaterally, washed briefly in distilled water, and dried overnight in an oven at 60 °C. The flagella were weighed on an electrobalance to the nearest $0.1 \, \mu$g and the weights of the two flagella were averaged to obtain a measure of flagellar size for the animal from which the flagella had been obtained.

Results

Flagellar frequency

The flagellar beating pattern in *C. maenas* was similar to those described for other brachyurans by Burrows and Willows (1969) and by Charlton (1971). A flagellum, when active, typically beats regularly for extended periods. The three flagella on a side beat together but slightly out of phase, with posterior flagella leading more anterior ones. Beating was usually unilateral, with periods of activity in the left set of flagella alternating with periods of activity on the right side.

The stroke frequency of the flagella was about 11 Hz and was nearly independent of animal size (Fig. 1A). The beat frequency did decline as animals got larger (the slope in Fig. 1A is negative and statistically significantly different from zero), but there was only a small change in frequency over a wide size range. The regression line of Fig. 1A predicts that the flagellar frequency would decline by only 15 % as an animal grew from 10 to 100 g. The dry mass of the flagellum, which we assume is proportional to the wet mass of the appendage, does increase as the animal gets larger. The allometric exponent relating flagellar mass and body mass (*b* in Fig. 1B) is somewhat less than 1, indicating that the flagellum grows relatively more slowly than does the body as a whole. Fig. 1C provides information on the mass of the FA muscle in animals of different size obtained in experiments described in detail in the following two papers. The size range in Fig. 1C is somewhat narrow because the animals were chosen to be of convenient size for physiological manipulations rather than to cover a broad size spectrum. The muscle mass, like that of the flagellum, does increase with animal size and with an allometric exponent
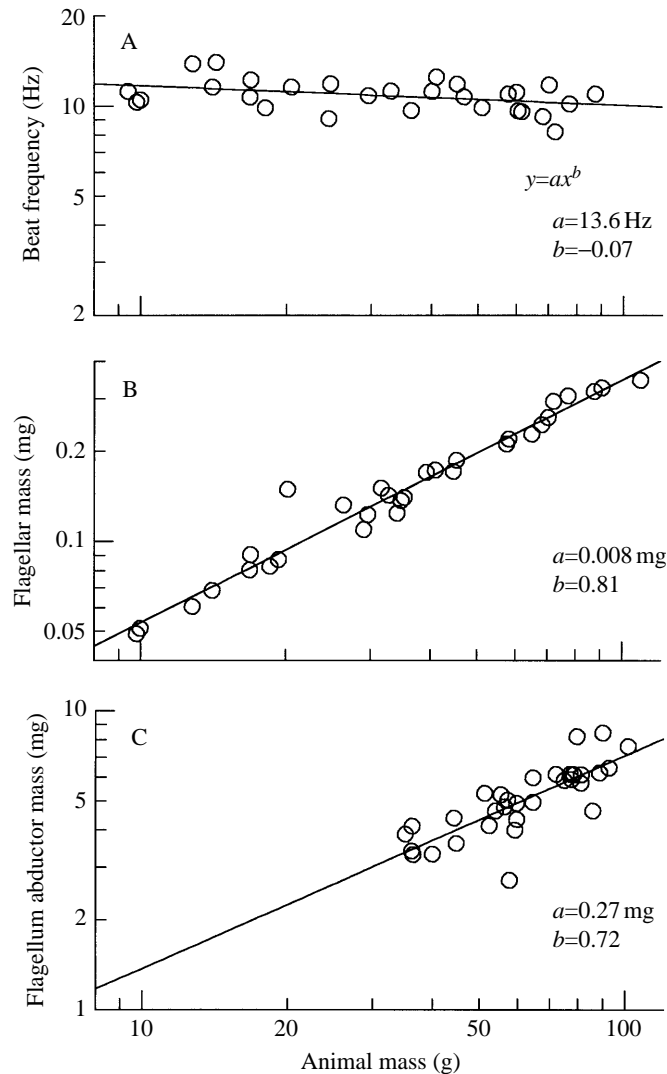
Fig. 1. Relationships between animal size and (A) flagellar stroke frequency, (B) dry mass of the flagellum and (C) the wet mass of the flagellum abductor muscle. The straight lines are least-squares regression lines for the log-transformed data. The slope of the regression line is the allometric coefficient *b*. The 95 % confidence intervals for the slopes of the regression lines were: A, −0.13 to −0.01; B, 0.75–0.87; C, 0.52–0.90. The mean flagellar frequency was 10.9 Hz (s.d.=1.3 Hz) over the range considered.

somewhat less than one, again indicating a relative growth rate slower than that of the body as a whole.

Structure of the stroke

Recordings of the force produced by a restrained flagellum revealed that bouts of activity could begin with full-size strokes from the onset (Fig. 2A) or with obvious facilitation of stroke force early in the bout (Fig. 2B). Once a bout was established, the
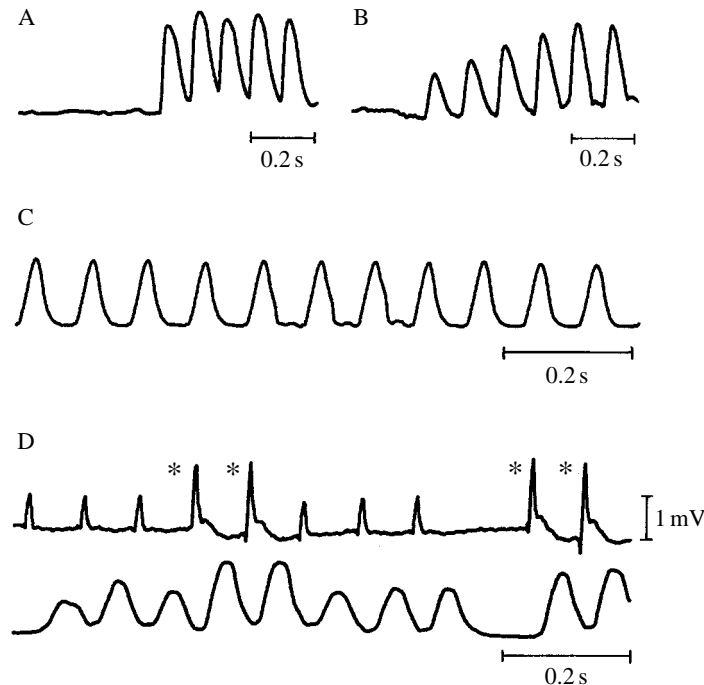
Fig. 2. Force produced by a flagellum during beating. The force transducer was held firmly against the flagellum and the responses were essentially isometric. Muscle contraction produced an upward deflection. Each record is from a different preparation. A and B are bouts that began after 6 and 5 s of rest respectively; C and D are from periods of sustained activity. The upper trace in D shows muscle action potentials recorded from the same flagellum as that generating the force of the lower trace. Asterisks mark instances in which there was an unusually large action potential and an associated large contraction.

force per beat sometimes remained constant for long periods (Fig. 2C), but often the force changed in amplitude in an unpredictable manner from beat to beat, sometimes jumping between discrete force levels (Fig. 2D). Cycle-to-cycle change in muscle force presumably reflects variation in muscle activation, variation due to differences in either the number of motor units active or the number of times per cycle that individual motor units fire, but more about this cannot be said on the basis of the force records alone.

The electromyogram signals recorded from an FA muscle were quite like those recorded from the FA muscles of other crustaceans using a similar approach (Burrows and Willows, 1969; Charlton, 1971). There was a large muscle action potential associated with each stroke. The amplitude of these potentials sometimes showed facilitation at the beginning of bursts and sometimes varied from cycle to cycle within bursts in a manner similar to that of muscle force. There was a strong correlation between the amplitude of the muscle action potential and the flagellum force when the two variables were recorded simultaneously from a single flagellum (Fig. 2D). Muscle action potentials proved little more revealing about neural output patterns to the FA muscle than did force records, and more detailed analysis of the neural events underlying flagellum activity will be based on nerve recordings.
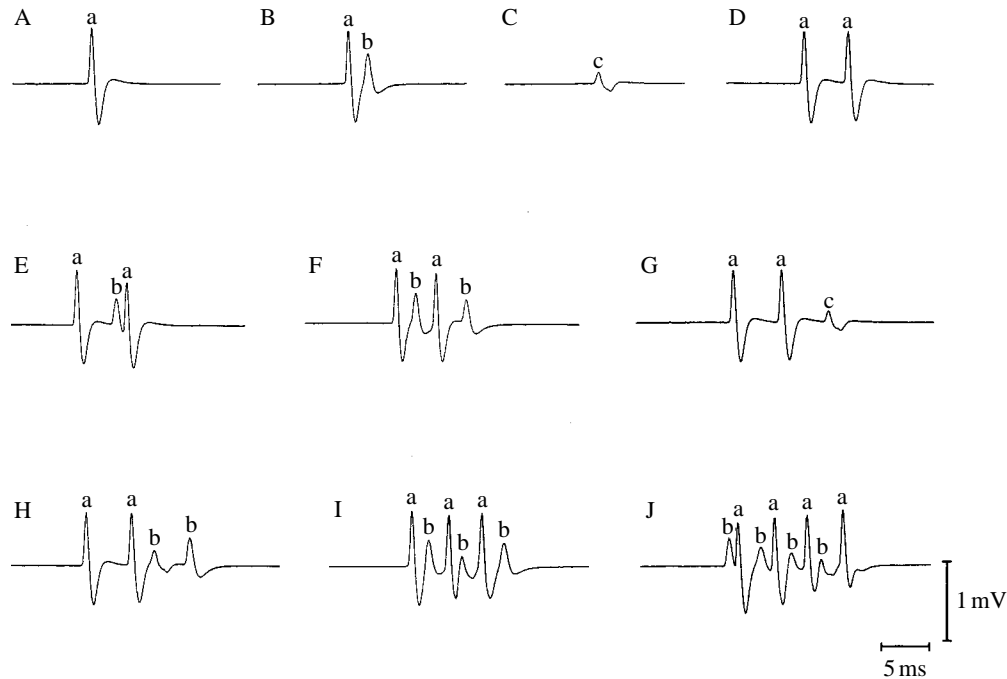
Fig. 3. Variation in neuronal firing patterns in different cycles. Each sample is a single burst with the exception of that in C, which was an occurrence of the smallest unit by itself in a mid-burst interval. Three units, labelled a, b and c, are clearly discernible in the recordings.

Impulse traffic in the nerve to the FA indicated that there are three efferent axons in the nerve (Fig. 3). Two of the units fired in bursts at a burst frequency of about 11 Hz; these units are obviously those associated with the contractions of flagellar beating. The third unit produced action potentials which were generally smaller than those of the other two units. It fired irregularly and there was seemingly no coordination between its activity and that of the other two units. We think it likely that the third unit is not a motor axon to the FA, but rather an axon to the small accessory muscle, described by Charlton (1971), which lies at the base of the flagellum distal to the abductor.

In a few preparations, both axon spikes and muscle action potentials were recorded from a suction electrode over the cut end of the FA nerve (Fig. 4A,B). The muscle action potentials were presumably initiated by activity in branches of the motor axons which had left the main nerve proximal to where it was transected at the electrode site. These combined nerve–muscle recordings demonstrated rather clearly that there could be facilitation of muscle action potentials at the beginning of a bout of activity even when the neural input to the muscle was essentially constant from beat to beat (Fig. 4A). Sudden changes in the amplitude of muscle action potentials were associated with changes in the efferent spike pattern to the muscle (Fig. 4B). Facilitation of muscle action potential amplitude at the onset of a bout of activity was much more pronounced in recordings from exposed muscles (Fig. 4C) than was facilitation of force recorded from an intact flagellum (e.g. Fig. 2B) or facilitation of action potentials recorded from wires
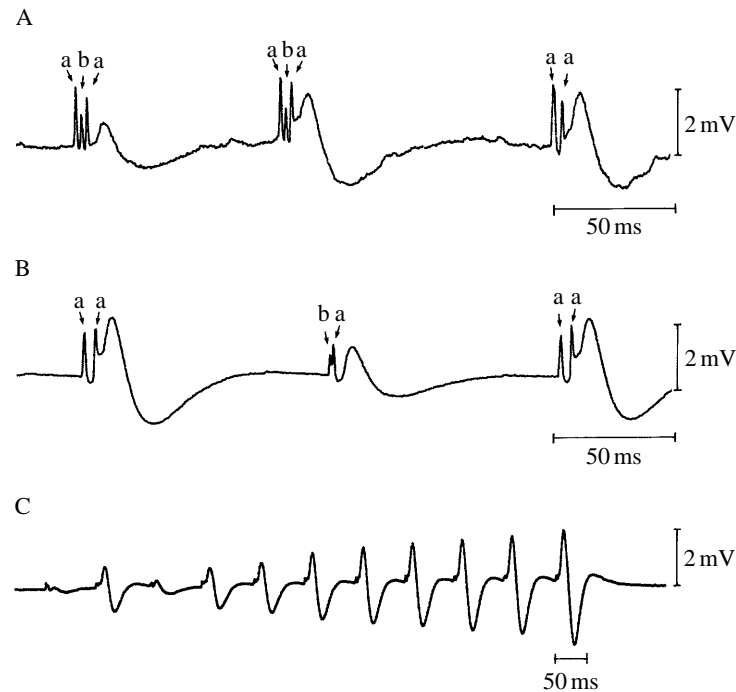
Fig. 4. Motoneurone action potentials and muscle action potentials (slower, biphasic signals) recorded from a suction electrode over the nerve. In A and B, which are from the same preparation, spikes from the two motor units to the flagellum abductor (FA) muscle are identified as a or b. In this preparation, unit b firing by itself did not produce a muscle action potential. (A) Bursts at the beginning of a bout of activity after an 80 s rest period. Note that there was facilitation of the muscle action potentials even though the neuronal bursts were identical or identical except for the absence of a b spike. (B) A segment from the middle of a bout of activity. A decrease in the number of a spikes from two to one (middle burst) resulted in an abrupt drop in the size of the muscle action potential. (C) The beginning of a bout of firing after 56 s of rest showing the pronounced facilitation of muscle action potential amplitude frequently seen in these recordings.

inserted into the muscles of intact segments. This difference in facilitation, we believe, is related to the difference in the solutions bathing the muscle; artificial saline in the exposed preparations and crab blood in the intact ones. This topic is considered further in the following paper.

The motor axons to the FA each fired 0–5 times per cycle (failure of a unit to fire in a cycle could be distinguished from general skipping of a cycle by the presence of potentials in the other unit at the expected time). There were usually two or three spikes per burst in each motor unit. The mean interval between the spikes of a burst when there were two spikes per burst ranged from 3.17 to 4.23 ms in six preparations (16–103 determinations from each preparation; average of preparation means 3.58 ms). When there were three spikes per burst, the interspike intervals within the burst tended to be shorter than when there were two spikes per burst and the second interval tended to be shorter than the first (Fig. 5).
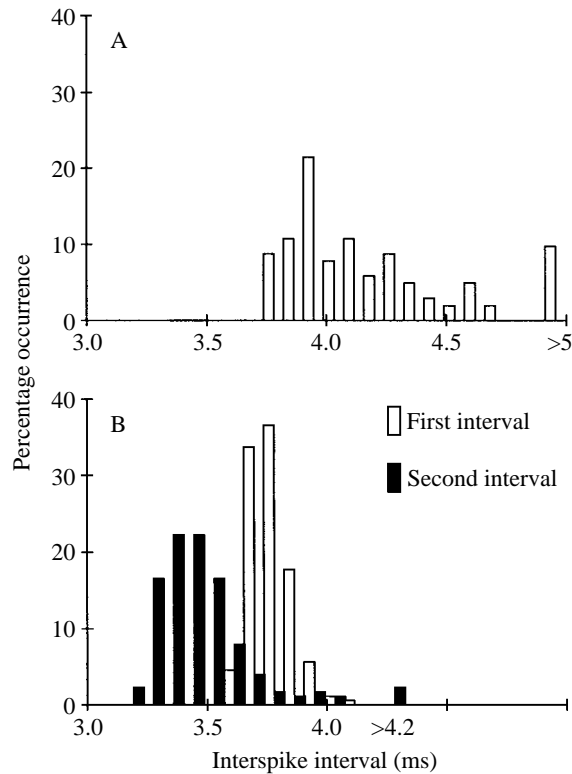
Fig. 5. Distribution of interspike intervals of a single unit (A) in bursts with two spikes per cycle and (B) in bursts with three spikes per cycle.

Regularity of the central pattern generator

The constancy of output from the central pattern generator was evaluated using action potentials from the FA nerve recorded during spontaneous beating and stored on magnetic tape. Segments were selected for analysis in which the output bursts of the motoneurones occurred regularly and without obvious gaps. The preparations used were those in which the spikes of one motor unit were large and unambiguously identifiable. Segments lasting 6.5 s and including about 60 successive bursts were transferred to a computer through an analog-to-digital converter (sample frequency 5 kHz) and stored for detailed examination. Interburst intervals, defined as the time from the start of one burst to the start of the following burst, were measured to the nearest 0.2 ms. Only segments in which bursts occurred continuously without gaps were considered. Five segments from each of five preparations were analyzed. The several segments taken from a single preparation were separated by at least 1 min in the original recordings.

The interburst intervals of the records analyzed varied in a seemingly erratic way, but within rather narrow limits (Fig. 6). The coefficient of variation (=standard deviation/mean) for intervals ranged from 2.0 to 6.1 % with a mean value of 4.1 %. There was a tendency for the coefficient of variation for interburst interval to be directly related to the mean value of the intervals (Fig. 7A); i.e. the output is relatively more regular at
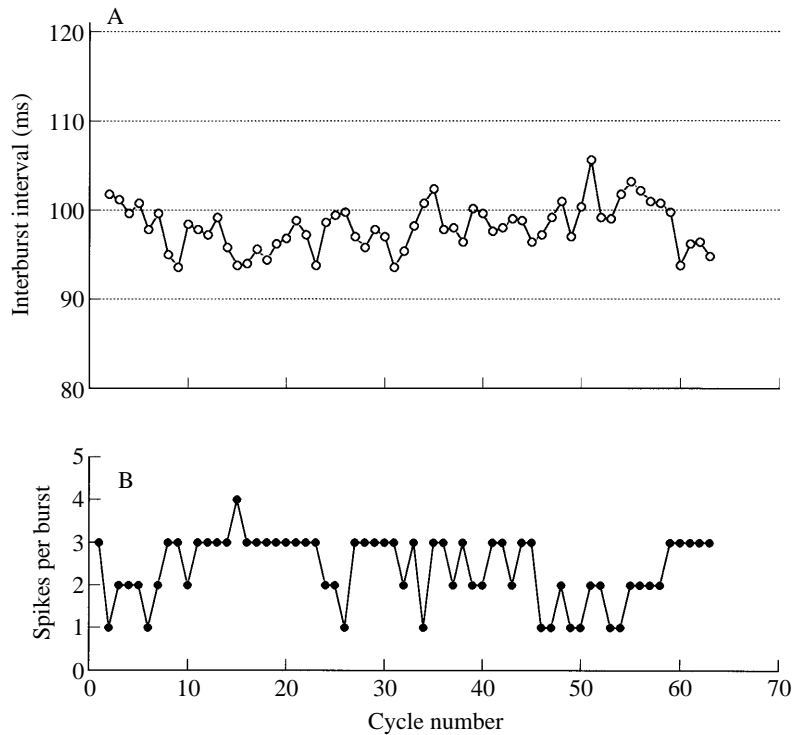
Fig. 6. Interburst intervals (A) and the number of action potentials per burst for one of the two FA motoneurones (B) over a number of successive cycles. This record was chosen as an example because the mean interburst interval (98.1 ms), the coefficient of variation for interburst intervals (2.7 %) and the mean number of action potentials per burst (2.39) were all near the middle of the range for all the preparations examined (see Fig. 7).

higher than at lower frequencies. This trend was statistically significant when each segment analyzed was considered as an independent sample ($N=25$), but not when the multiple values from a single preparation were averaged ($N=5$). The number of action potentials per burst usually varied in no clear pattern, but in one preparation there were stretches of tens to hundreds of successive bursts with exactly two spikes per burst. There was no obvious relationship between the number of action potentials per burst and the burst frequency (Fig. 7B).

Crustacean muscles frequently show facilitation of mechanical responses during repetitive activation (e.g. Hoyle, 1983). However, it was common for flagellar force to be large and nearly constant from the onset of bouts of beating (Fig. 2A). We examined the possibility that changes in the activation pattern to the muscle compensated for the effects of facilitation during bouts of beating. The force of the earliest strokes in a bout might be increased, and flagellar force during a bout made more uniform, if the bursts of neuronal firing evoking early strokes contained a greater than average number of action potentials and if the number of action potentials per burst subsequently declined as facilitation became established. However, the crab does not seem to use this tactic for producing more uniform flagellar strokes. Action potential patterns in a single motor unit were
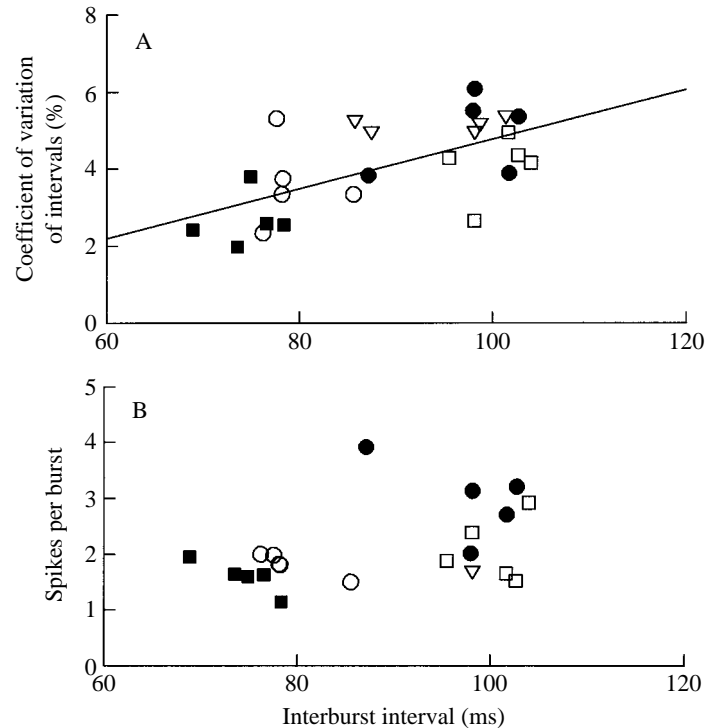
Fig. 7. Coefficient of variation for interburst interval (A) and the mean number of action potentials per burst (B) as a function of mean interburst interval. Each of the points represents a segment of activity made up of 47–93 successive cycles (mean 66 cycles). Values represented by the same symbol are from the same preparation. In one preparation, that indicated by the inverted triangle, spikes could be clearly ascribed to an identified unit in only one of the five segments examined. Note that the multiple points from a single preparation tend to cluster at the same position along the interval axis, showing that the beat frequency is relatively constant in widely separated samples.

examined at the onset of bouts of activity following rest periods lasting 5–21 s. In 16 bouts analyzed from three preparations, the average number of action potentials per burst was 2.0 for the first five bursts of the bouts and 2.5 for bursts 11–15. Thus, the number of spikes per burst does not seem to vary in a way that would diminish the effects of facilitation on stroke amplitude.

Discussion

The accessibility and organizational simplicity of the flagellum and its musculature have made possible the construction of an unusually complete picture of the neuronal control of this appendage. The flagellum beats rhythmically at about 11 Hz. This frequency is rather constant when measured at different times in a single preparation (Figs 6, 7) and it does not vary much between preparations, even for animals that vary considerably in size (Fig. 1A). The beat of the flagellum is caused by contraction of the flagellum abductor muscle. The muscle is innervated by two motoneurones, each of

which fires 0–5 times per cycle. The intervals between the action potentials of a motoneurone, when there are two or more firings per cycle, are 3–4 ms. There can be, but need not be, facilitation of muscle action potentials and contractile force at the beginning of a bout of activity following a rest period. Once beating is fully established, there may be fluctuations in the amplitude of muscle action potentials and of force, presumably reflecting changes in the number of times that each of the two motor units fires per cycle. Central neural circuits for flagellar control that might give appropriate right–left alternation and intersegmental coordination are proposed by Burrows and Willows (1969) and by Charlton (1971).

When beating is established, the coefficient of variation for successive interburst intervals is 2–6 % (Fig. 7). This variability is similar to that between flagellar beats in the crab *Callinectes sapidus* (mean 4 %, calculated from the data in Table 5 of Charlton, 1971) and about the same as the variability of intervals between bursts produced by the central pattern generator controlling heart beat in the leech (about 5 %, Arbas and Calabrese, 1984) or of intervals between spikes in a crayfish stretch receptor (2–3 %, Schulman, 1969, as quoted in Bullock, 1970). The flagellar system is far less regular than the performance achieved by electric organs of some weakly electric fish, for which the coefficient of variation for intervals between successive discharges may be only 0.012 % (Bullock, 1970).

Flagellar frequency and animal size

It is a common observation that the frequency of appendage movements during equivalent activities declines with increasing animal size. This inverse relationship between frequency and size has been found both in comparisons of species of different size and in comparisons of animals of different size within a single species (e.g. Lent, 1971; Heglund *et al.* 1974; Calder, 1984; Marsh, 1988).

In an influential paper, Hill (1950) provided a theoretical basis for the inverse relationship between animal size and operating frequency. Hill noted that inertial stress in structures of geometrically similar animals would increase with animal size if animals of varying size operated on the same time scale and took the same time to complete movements. For inertial stresses to be similar in small and large animals, the time taken for a movement should increase in proportion to a linear dimension of the animal, l. Therefore, operating frequency should decrease in proportion to l^{-1} or, equivalently, in proportion to mass$^{-1/3}$. But the flagellum acts as a paddle, and the principal forces that it faces are probably drag forces rather than inertia. However, considerations similar to those employed by Hill suggest that the frequency of flagellar beating should similarly decline in proportion to l^{-1} if the stress in the FA muscle is to be similar in large and small animals.

The flagellum is a curved structure, concave in the direction of movement. In a 60 g animal, the chord distance from the base to the tip of a flagellum is about 6.6 mm and the width of a flagellum, including the projecting lateral hairs, is about 1.5 mm. The stroke angle of a flagellum is about 96 °. The distance moved during a stroke by a point on the flagellum half-way along its length in a 60 g animal is (6.6 mm) $\times$ π $\times$ (96 °/360 °) = 0.55 cm. At 11 Hz, and assuming that the effective and recovery stroke of the flagellum

are of equal duration, the average velocity of the mid-point of the flagellum during a stroke is about $12\,\mathrm{cm\,s^{-1}}$. The Reynolds number for a flagellum, based on this velocity, the flagellum width and values of the density of sea water and its dynamic viscosity given in Vogel (1981), is about 170. The drag forces acting on a paddle operating at this Reynolds number can be expected to be proportional to the area of the paddle and to the square of its velocity through the water (Vogel, 1981). If animals grow isometrically, the blade area is proportional to l^2 and the velocity of equivalent points along the blade in different animals is proportional to $l\times\Delta t^{-1}$, where Δt is the time taken to complete a cycle. The force on the blade, then, is proportional to the product of the area and the square of the velocity or to $l^4\times\Delta t^{-2}$. The cross-sectional area of the FA muscle is proportional to l^2, so the muscle stress (=force/area) is proportional to $l^2\times\Delta t^{-2}$. For muscle stress to be constant and independent of animal size, the ratio $l\times\Delta t^{-1}$ must remain constant; therefore, the time taken for a beat (Δt) should be proportional to l and the frequency proportional to l^{-1} (=mass$^{-1/3}$). But this, as it turns out, is not the way the flagellum works. The operating frequency is closer to being constant and independent of animal size than it is to being proportional to l^{-1} (Fig. 1A).

Operating at a constant frequency throughout life does avoid the problem, faced by many animals, of matching muscle kinetics to a changing operating frequency. In all muscles that have been appropriately examined, there is an operating frequency at which the mechanical power output during cyclic contraction is maximal (e.g. Stevenson and Josephson, 1990; Altringham and Johnston, 1990; Swoap *et al.* 1993). The optimum frequency for power output is determined by the contraction time course and force–velocity characteristics of the muscle. For movements that operate over different frequency ranges at different life stages, the contraction kinetics of the participating muscles would need to change with life stage if the operating frequency range were to be similarly positioned with respect to the optimum frequency for power output. Appropriate changes in contraction kinetics have been demonstrated in lizard and fish muscles. In the lizard *Dipsosaurus dorsalis*, the maximum stride frequency during running declines and the contraction rise and decay times for a limb muscle become slower as the animal grows larger. The slower contraction kinetics can be expected to lower the optimum frequency for power output, and this has been observed (Johnson *et al.* 1993). Similarly, in cod myotomal muscles, the muscle contraction kinetics become slower and the optimum frequency for muscle power lower as the animal becomes larger (Archer *et al.* 1990; Altringham and Johnston, 1990). Because of the relatively constant operating frequency of the flagellum, the FA muscle can be equally effective throughout adult life with unchanging contraction kinetics.

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