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Two functionally different muscle fibre types in some salps?*

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SUMMARY: This paper describes the structure and operation of the fibres in the locomotor muscle bands of several salp species. In many species, for example *Thalia democratica* or *Pegea confœderata*, all the muscle fibres of the locomotor muscle bands are similar in width and structure. In others, for example *Salpa fusiformis* and *S. maxima*, although fibre structure is similar, the marginal fibres edging the bands may be some 3-4 times the width of those in the centre of the band. In *Ihlea punctata*, not only is there a more striking difference in width between the marginal and central fibres of the bands, but also the two differ in structure. The marginal fibres are up to 10 times the width of the central fibres and the two differ in myofibrillar and mitochondrial content. Intracellular recordings from the fibres show that the normally compound spike potentials do not overshoot resting potentials (up to -70 mV), and are decremental. The two types of fibre may be separately activated. It is suggested that in *Ihlea punctata*, the wide marginal fibres may be involved in slow swimming, the central narrow fibres in 'escape' swimming.

Key words: salps, locomotion, muscle fibres, two muscle fibre types.

INTRODUCTION

Salps swim by rhythmic contractions of the muscle bands which partially or (in some species) completely encircle their tubular bodies; contraction of these muscle bands sends a jet of water out of the posterior or anterior openings and propels the salp forwards or backwards. If undisturbed, they swim forwards.

The contraction of the muscle bands can be graded, and the frequency of the 'cruising' locomotor rhythm may also be altered, for if salps are stimulated, they can increase forward speed or reverse rapidly, according to the site of stimulation. Locomotor behaviour has been examined in most detail in *Salpa fusiformis* (Bone and Trueman, 1983).

The muscle fibres within the bands vary in width and number between species, hence these characters are of taxonomic importance, and have been used to erect subspecies, and to discern clinal variation. In consequence, fibre number in different muscle bands of many species is well known. Structural details of the fibres in several species were described at the light microscope level by Fedele (1932, 1938), whilst more recently their ultrastructure and development were examined by Bone and Ryan (1973) and Toselli and Harbison (1977).

In most salps, the muscle fibres in the bands seem similar to each other, but in *Ihlea punctata*, Streiff (1908) and Metcalf (1918) observed that all of the locomotor muscle bands contained two strik-

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ingly different types of muscle fibre, for the marginal fibres at the edges of the bands were very much wider and more transparent than the other fibres in the band. Foxton (1967) confirmed this pattern in the other two members of the genus (*I. magalhanica* and *I. racovitzai*), pointing out that in the latter they had been figured (though not mentioned in the text) by Van Beneden and De Selys-Longchamps (1913).

We have re-examined the locomotor muscle fibres of *Ihlea punctata*, and were thus led also to examine the muscle fibres of several other species. Although wider marginal fibres have not been reported in other salps, they certainly exist in some species. Our observations suggest the possibility that in *I. punctata* (but not in other species) the two different types of muscle fibre may be involved in different types of swimming.

MATERIAL AND METHODS

Oozooids and blastozooids of *Ihlea punctata* Metcalf and blastozooids of *Pegea confœderata* Forsskål were collected from the Rade de Villefranche (in the Mediterranean near Nice) by dipnetting from the surface and from plankton tows. Fixed specimens of both stages of *Thalia democratica* (Forsskål), *Cyclosalpa affinis* (Chamisso), *I. punctata* and *Salpa fusiformis* (Cuvier) and an oozooid of *P. confœderata* obtained earlier were also examined. By the kindness of Prof. J. Godeaux (Liège) we have been able to examine an oozooid and a blastozooid of *Traustedtia tentaculata* (Metcalf), and Dr. D. Simms (Plymouth University) kindly allowed us to examine a blastozooid of *Thetys vagina* (Tilesius).

Intracellular records from muscle fibres of *P. confœderata* and *I. punctata* pinned-out on Sylgard bases in small dishes were obtained by impaling the fibres from the inner (pharyngeal) surface after removal of small areas of the tough pharyngeal epithelium. Conventional KCl-filled electrodes (resistance 25-50 MW) led to voltage followers and to a Tektronix 5103 oscilloscope, or to a Gould-Brush 220 pen recorder. Pinned-out animals continue their normal rhythmic activity for long periods; for current injection the rhythmic spontaneous activity was abolished either by brain removal, or by the addition of a small amount of MS 222 to the bath. After brain removal the muscle fibres often fibrillate for long periods (presumably because of the damage to their motor nerves), hence most current injections were performed after anaesthesia. Salps are very transparent, and the motor nerve bundles to the muscle bands are readily visible, so that the fibres of the muscle bands could be impaled at varying distances from the regions of motor endings upon them. Other animals were fixed for histological examination in 5% glutaraldehyde in seawater buffered to pH 7.2 with 0.2 M cacodylate, then post-fixed in osmium and processed for embedding in Spurr resin. Semi-thin sections were stained with toluidine blue. Some animals were fixed in unbuffered 5% formalin in seawater, and then stained for acetylcholinesterase by Gomori's variant of the Koelle method (Gomori, 1952), using dithioxamide instead of sulphide to visualise the copper salt at the sites of enzyme activity.

OBSERVATIONS

Structure of the muscle bands

The strap-like muscle fibres of all salps are built essentially to the same design, with a central mitochondrial core in which the fibre nuclei are embedded, sandwiched between the two cortical myofibrillar layers. However, as Streiff (1908) first observed, within this general design, *Ihlea punctata* is unlike other salps. Fig.1a-d shows the remarkable arrangement in *I. punctata*. Fig.1a-c, are from a small blastozooid, and show that whilst in some muscle bands there are only single marginal fibres, up to 40 μm wide, next to central fibres 5-7 μm wide, in others there may be two marginal type fibres, the different margins of a single band differing in this respect. Fig. 1d shows the edge of a muscle band in a small oozooid, where the marginal fibres are up to 50 μm wide. In a large *I. punctata* oozooid, they may be up to 190 μm across, the much more numerous central fibres being around 20 μm across. Next to the outer fibres, there are in some muscle bands one or two fibres of marginal type, which are 2-5 times the width of the central fibres. The wide marginal fibres are present in all except the smallest of the multiple thin bands around the posterior aperture. Very small *I. punctata* blastozooids (12 mm) show the same arrangement. Fibre number is similar in such very small blastozooids to older and much larger blastozooids, though fibre width is much less, hence it seems that increase in size of the muscle bands during growth takes place mainly by increase in width and length of the original fibres.

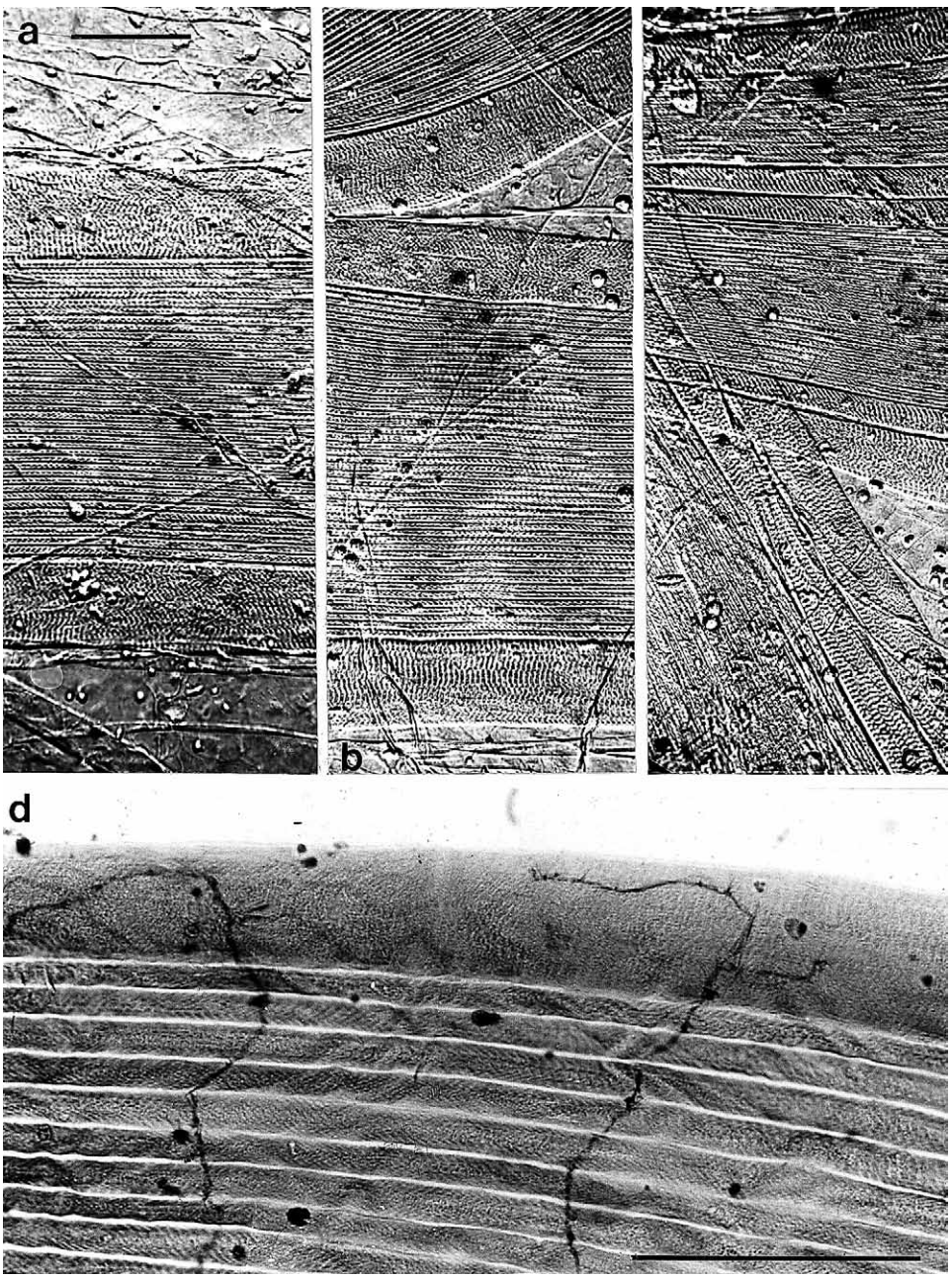

FIG. 1. – a-d, *Ihlea punctata*: marginal and central fibres in muscle bands; a-c, different muscle bands from a young blastozooid, fixed, interference contrast. Scale bar: 100 μm; d, innervation of edge of muscle band. Acetylcholinesterase method. Scale bar: 100 μm.

Fig. 2a shows the pattern of fibre width across various muscle bands in an oozooid of *I. punctata*, with very abrupt change in fibre width between the marginal and central fibres. Fig. 2b-d show for comparison fibre widths across the muscle bands of other salp species. In *Salpa fusiformis* (Fig. 2b), whilst the marginal fibres are wider than the smallest central fibres, the fibres grade gradually across the band, without abrupt change in width. In *S. maxima* (Fig. 2c) there is a similar gradation in fibre width across the band although the outer fibres are not so much wider than the central fibres as they are in *S. fusiformis*. Finally, the different species shown in Fig. 2d, have fibres of more or less uniform width across the bands.

There is therefore some variety in the patterns of fibre width in the muscle bands of different salp species. Some of these different patterns are seen in Fig. 3d-h. However, *I. punctata* is set apart from all other species because not only are the marginal fibres much wider than the other fibres of the band, but they are also much more transparent, (both in

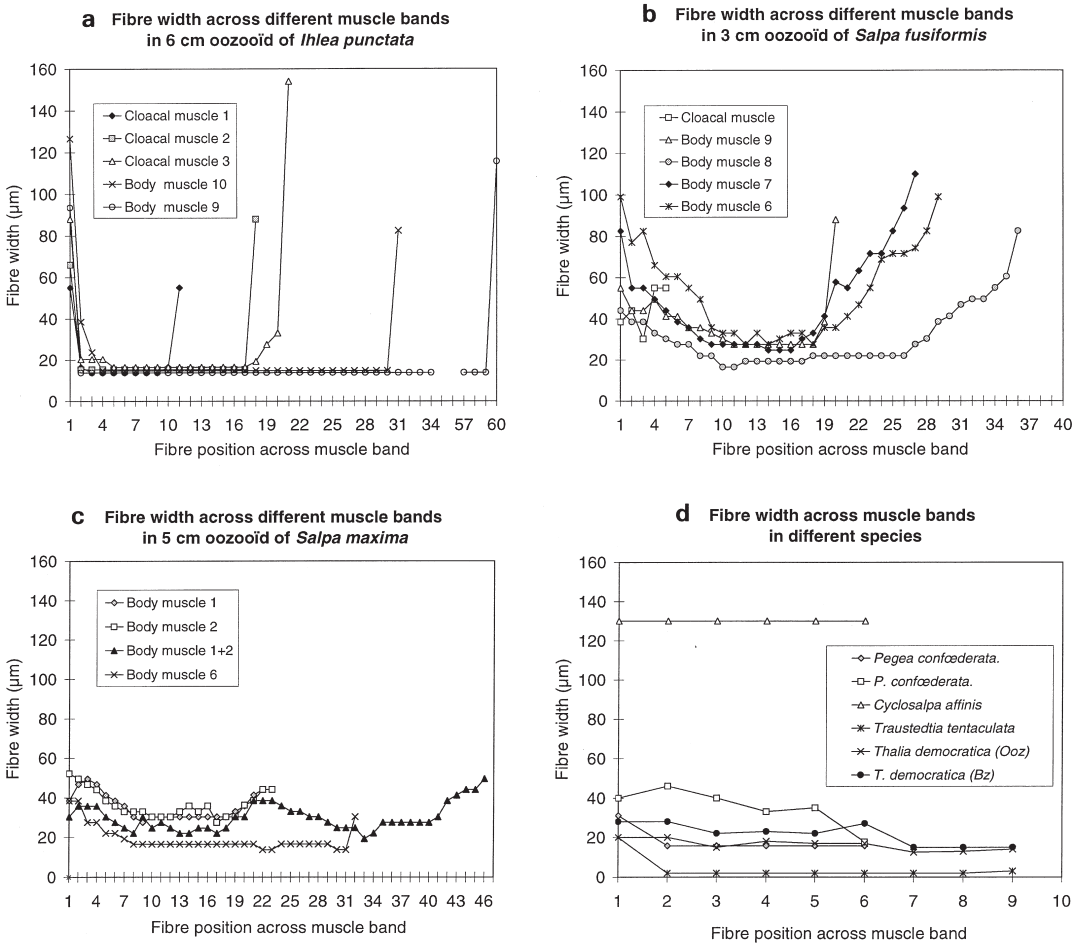

FIG. 2. – a-d, Fibre width across muscle bands. a, *Ihlea punctata*: 6 cm oozooid; b, *Salpa fusiformis*: 3 cm oozooid; c, *Salpa maxima*: 5 cm oozooid; d, *Pegea confœderata*, *Traustedtia tentaculata, Thalia democratica* and *Cyclosalpa affinis*.

preserved material as well as in living animals, as Metcalf (1918) noted and Fedele (1938) confirmed). This is presumably not only because the relative volumes of the mitochondrial and myofibrillar regions are very different in the two fibre types, but also because the central fibres are much thicker than the marginal fibres (Fig. 3a).

In the marginal fibres, the myofibrillar zones are some 2 μm thick, whilst in the much narrower central fibres they are around 8 μm thick. These are significant differences for although the width of the marginal fibres at each edge of a given band may be 35% of the total width of the band, the cross-sectional area of the myofibrillar component in the marginal fibres is only around 3% of the total cross-sectional area of myofibrils within the whole band. The differences between the two types of fibre seem likely to be of functional significance (see discussion). In contrast to the fibres of *I. punctata* seen in Fig. 3a, those of *P. confœderata* and *S. fusiformis* (Figs. 3b and c) are in transverse section of similar structure throughout the band; that is, the relative proportions of myofibrillar and mitochondrial zones are similar in marginal and central fibres. So far as we are aware, it is only in *Ihlea* that there are fibres of different structure in the muscle bands.

Innervation

Mixed nerves radiate from the dorsal brain, passing between the muscle bands and the epithelia covering them, and the motor axons issuing from the nerves run across both inner and outer faces of the band. Motor axons pass across the bands at more or less regular intervals, in a large oozooid of *Ihlea punctata*, crossing the bands at intervals around 2-300 μm apart along the axis of the band. The innervation of the central fibres is mainly intercalary, as Fedele (1925) and Bone and Ryan (1973) previously observed, successive expansions of the axon innervating successive fibres across the band. In some species there are occasional short side branch-

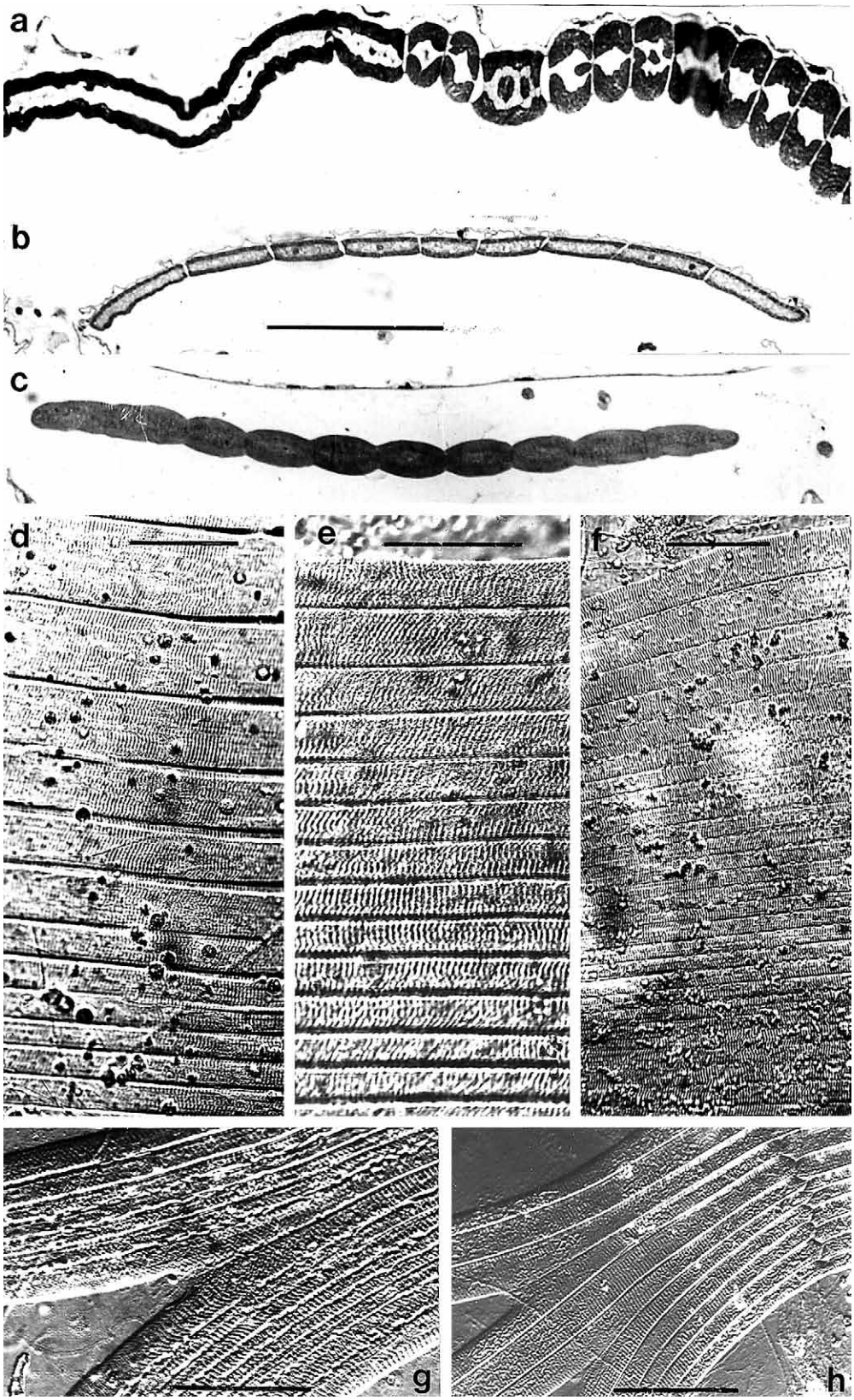

FIG. 3. – a-c, Semi-thin resin transverse sections of muscle bands. Note dark cortical myofibrillar zones. a, *Ihlea punctata*; b, *Pegea confœderata* oozooid; c, *Salpa fusiformis*. d-h, Marginal and central fibres in muscle bands. d, *Salpa fusiformis,* 3 cm oozooid, body muscle 9; e, *Salpa maxima*, 5 cm blastozooid, body muscle 4; f, *Pegea confœderata*, blastozooid; g, *Salpa fusiformis,* young blastozooid; h, *Thalia democratica*, young blastozooid

es which may divide several times before terminating in small expansions (Bone, 1989), but in *I. punctata* these have not been observed. The marginal fibres are also crossed by motor axons passing from one band to the next adjacent, but in some cases appear to be innervated differently, by axons which pass out of the nerve bundle crossing the band and branch several times before reaching the marginal fibre, where they bend to follow the long axis of the fibre (Fig.1d) to terminate or continue to the marginal fibres of the adjacent band. Intracellular records from rhythmically active animals described in the next section, show that the marginal and central fibres may be independently active.

The neuromuscular transmitter is not known, but seems likely to be acetylcholine. After staining for acetylcholinesterase, nerve bundles and single axons are seen, and similar small varicosities to those seen by silver-impregnation techniques are seen along the nerves as they cross the muscle bands. Iontophoretic application of 1.5 M acetylcholine evokes membrane depolarisations up to 25 mV (Fig. 4a) which are lacking or greatly reduced after prior treatment with d-tubocurarine, but spikes were never observed. Possibly this is due to the difficulty of placing the tip of the iontophoretic electrode close enough to the fibre impaled by the recording electrode, but in any event, similar depolarisations are

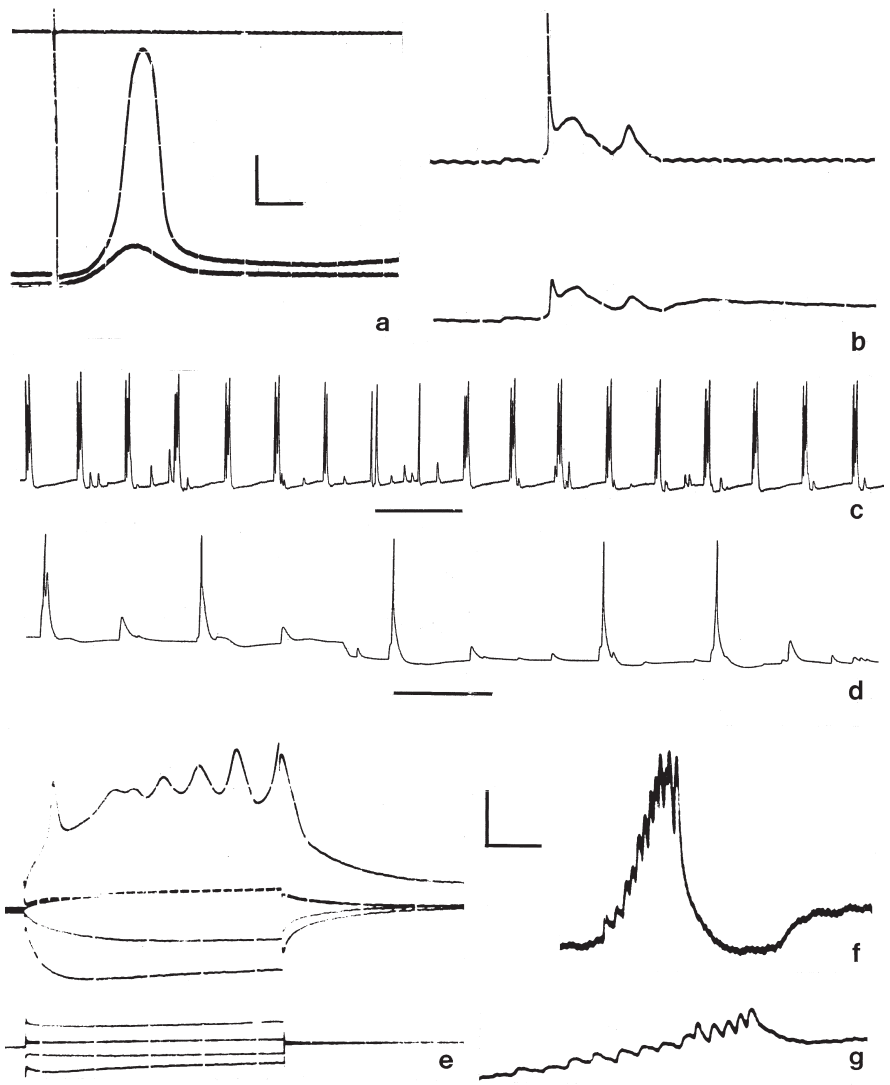

FIG. 4. – a, Iontophoretic application of acetylcholine evokes depolarisation. Scale bars: 5 mV, 100 ms; b, electrical events associated with the same contraction recorded from a muscle fibre close to nerve bundle crossing muscle band (upper), and in a same muscle fibre 120 μm away (lower). Scale bars: 5 mV, 100 ms; c and d, intracellular records of rhythmic activity from muscle bands, note small potentials between larger bursts, and in d, that the single large potentials are in fact summated small potentials. Scale bar: 1s; e, current injection into marginal fibre of *Ihlea punctata* evokes series of potentials. Scale bars: 20 mV, 10 nA, 50 ms; f and g, records from *Pegea confœderata* muscle bands showing summation of successive small potentials. Scale bars: 5 mV, 100 ms.

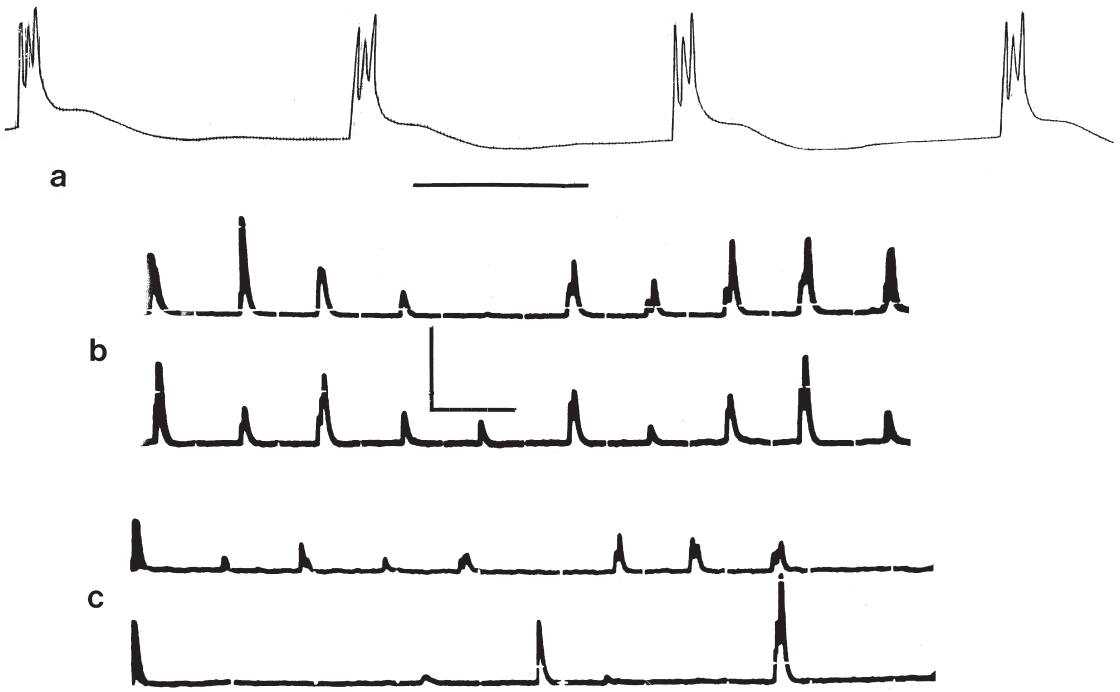

FIG. 5. – a, *Ihlea punctata*. Intracellular record of rhythmic activity showing similarity of successive potential bursts and absence of intermediate or pre-potentials. Scale bar: 1s; b and c, in each, simultaneous intracellular records from marginal fibres (upper line) and central fibres (lower line) from 1.2 cm *I. punctata* blastozooid. Note that in b electrical events in both fibres are largely similar, whereas in c the marginal fibre is more active than the central fibre. Scale bars: 20 mV, 0. 5 s.

seen when acetylcholine is iontophoresed close to muscle fibres of doliolid and appendicularian tunicates, and in appendicularians, Flood (1973) has shown acetylcholinesterase to be present under the neuromuscular junctions.

Electrical activity of the muscle fibres

Resting potentials of the fibres of both *Ihlea punctata* and *Pegea confœderata* ranged up to 70 mV, mean values for *I. punctata* being 60.8 ± 3.7 (n=13) and for *P. confœderata*, 64.4 ± 4.6 (n=9). Lower values of 50-55 mV were previously obtained by Mackie and Bone (1977) in *I. punctata* and *S. fusiformis*. During the rhythmic activity of pinned-out preparations, the amplitude of the potentials associated with contraction varied in different preparations: maximum amplitudes of compound potentials were around 55 mV and hence did not overshoot resting potential. Since it is possible to see nerve bundles in living preparations, it is easy to show that the largest potentials are recorded close to the nerve bundles crossing the fibre, and that they are decremental, declining with distance from the nerve bundle (Fig. 4b). It seems evident that even at the junction itself, the potentials are non-overshooting, since impalement sometimes leads to spike potentials at the site of impalement, which are of the same magnitude as those obtained from sites close to the neuromuscular junctions during spontaneous activity.

In line with this, sometimes when the fibre was impaled close to the nerve crossing the fibre, when the largest potentials were seen, smaller pre-potentials were found (Figs. 4c and d) which presumably represented junction potentials. Single or summating small groups of junction potentials without observable muscle contractions are common between the bursts of spikes associated with contractions. Possibly these are the result of the 'out-of-sequence' potentials seen from brain motoneurons (Anderson *et al.*, 1979).

Depolarising current injection evoked single spikes or a series of spikes (Fig. 4e) around 20 mV amplitude when recorded by a second electrode within 50 μm of the current injection site.

The records of rhythmic activity (Figs. 4c and d, 5a) were all obtained from the large edge fibres of *I. punctata* muscle bands, but very similar results were obtained from the much smaller fibres making up the

main width of the band. Usually, contractions of the large marginal fibres were associated with compound potentials (Figs. 4c, 5a), and these varied rather little both in number and amplitude between one contraction and the next (in contrast to previous records from *S. fusiformis*, Anderson *et al*., 1979). In some preparations (Fig. 4d), contractions were associated with single spikes only. In *Xenopus* embryos (Sillar *et al*., 1992) motoneurons fire single action potentials during the swimming cycle, but in all other vertebrates the neurons involved in locomotor rhythms fire multiple spikes as they usually do in salps. In *P. confœderata* (Figs. 4f and g) where the records were not made close to a neuromuscular junction, and hence are of low amplitude, series of successive small junction potentials summate relatively slowly to reach the amplitude to evoke contraction.

In *I. punctata*, no significant differences in resting potential or activity were seen from the two types of fibre. However, when large and small fibres were impaled within the same muscle band in small blastozooids, although the rhythmic spontaneous activity was similar in both, it was not exactly identical (Fig. 5b), sometimes the large fibres were active and the smaller fibres silent, or *vice versa*. That is, the two types of fibre within the band could be activated independently, although often contracting together.

The ionic basis of muscle potentials

In other tunicates, such as *Ciona intestinalis* (Nevitt and Gilly, 1986), *Doliolum* (Bone, 1989), or *Oikopleura* (Bone, unpublished), the spontaneous muscle potentials are rapidly and reversibly abolished by Ca^{2+}-blockers such as Co^{2+}, hence are largely or entirely carried by Ca^{2+}. The results of bath application of Co^{2+} in salps are less clear cut. Spontaneous rhythmic muscle electrical activity was sometimes abolished after a few minutes by addition of 25 mM Co^{2+}, but in other experiments continued for 30 min or longer in salps which were pinnedout but otherwise intact. However, in anaesthetised animals in normal seawater, in which larger areas of the pharyngeal epithelium were removed to permit dual impalement, after 30 min in 25 mM Co^{2+}, contractions were no longer observed following depolarising current pulses. It seems most probable that the muscle potentials are largely carried by Ca^{2+} as in other tunicates, but that the inner and outer epithelia between which the muscle bands lie, are impermeable so that the absence of an effect of Co^{2+} in some intact preparations resulted from failure of access to the muscle fibre membrane.

These results are similar to those observed in the smaller thaliacean *Doliolum* (Bone, 1989; Bone *et al*., 1997)

DISCUSSION

Structure

Van Beneden and De Selys-Longchamps (1913) and Foxton (1967) observed special wide marginal fibres in *Ihlea racovitsai* and *I. magalhanica*, as did Streiff (1908) but in his taxonomic study of a large number of salp species, Metcalf did not describe a similar peculiarity in the muscle bands of other genera, nor was it noted by other authors, such as Fedele (1938) and Van Soest (1974, 1975). Since the more transparent wide marginal fibres of *Ihlea* are readily visible in fixed material, it seems that similar fibres must be uncommon in salps. We have however found wider marginal fibres in the muscle bands of other salps, such as *Salpa fusiformis,* although they are similar in structure to the central fibres, and hence much less strikingly obvious than in *Ihlea*.

Fedele (1938) made careful light microscope observations both on living and fixed salp muscle fibres. He distinguished five types of muscle fibre in different salp species, according to various details of the disposition of the nuclei, myofibrils and sarcoplasmic regions. Subsequent ultrastructural studies on some of the species which he examined such as *Iasis zonaria* and *Thalia democratica* (Bone and Ryan, 1973) and *Cyclosalpa affinis* (Toselli and Harbison, 1979) have shown that in fact, all salp muscle fibres are of essentially similar design.

In those salps where all muscle fibres seem to be of the same structure, we do not know why some species have all muscle fibres in each band of similar width, whilst in others, marginal fibres are wider. Certainly during growth, spatial constraints might hinder growth in width of the central fibres, permitting the marginal fibres to expand more widely, but if that were the case, it is hard to see why in other species, marginal fibres are of the same width as the central fibres. At present, the three salps in the genus *Ihlea* seem to be the only ones in which the marginal fibres are different in *structure* to the central fibres. However, our specimens of *Thetys vagina* were not well enough fixed to see if they may share this feature.

Innervation and membrane properties

The present observations on the multiply-innervated muscle fibres of salps clearly show that conduction is decremental, and that even very close to neuromuscular junctions, spike potentials do not overshoot resting potential. Motor axons cross the muscle bands at more or less regular intervals of 2-300 μm. This is a sensible design for such extremely elongate muscle fibres (in large species, 12 cm or more long) that contract relatively slowly, and where the spike potentials are probably largely carried by Ca^{2+}. Normalised contraction velocity in the muscles of the active species *S. fusiformis* is around 1.5 L/s (Bone and Trueman, 1983). The neuromuscular transmitter is likely to be acetylcholine, although definite proof of this obviously requires further criteria to be satisfied.

In these respects, unsurprisingly, salp muscle fibres are similar to those of other tunicates. The chief differences between salp muscle fibres and the striated locomotor fibres of other tunicates are that in other tunicates the muscle fibres are electrically coupled to their neighbours, and that in appendicularians and ascidian tadpole larvae, there appears to be a dual innervation of the locomotor system (Bone, 1992). In doliolids, which most closely resemble salps in their design for jet propulsion, coupling between fibres in the muscle bands is not so extensive as in appendicularians and ascidian tadpole larvae (Bone, 1989), and a dual innervation is lacking. Most stages of doliolids are inactive unless stimulated to single escape response contractions, but old nurse stages of *Dolioletta* show similar compound muscle potentials to those of salps in their slow rhythmic activity. Both salps and these old nurse stages can grade their locomotor muscle contractions by varying the frequency and extent of the compound potentials.

Functional role of marginal fibres

Fedele (1938) noted that in *Ihlea punctata* the wide marginal fibres of the bands resembled those in the muscle bands of *Pegea confœderata, Salpa fusiformis* and *S. maxima* whatever the state of contraction when fixed, whereas the narrower central fibres showed a striking variety of appearances. If uncontracted, they were similar to the marginal fibres, whilst at different stages of contraction they appeared zig-zag or spiral (even obliquely-striated rather than cross-striated). We have not observed spiral features in the central fibres, and regard such appearances as artefactual, perhaps due to the fact that although narrow, the central fibres are much deeper dorso-ventrally than the marginal fibres or fibres in others salps hence the myofibrillar cortices of the fibres may be viewed in part obliquely. Certainly the marginal fibres in fixed preparations may be normal in appearance whilst the central fibres are often zig-zag, (as can be seen in several places on Fig. 1), indicating that the marginal fibres are contracted to a greater extent than the central fibres.

Fedele (1938) was much struck by the unusual appearance of the central fibres in *I. punctata*, which he supposed indicated that the operated in a highly peculiar way, unknown in any other animal. He suggested that the central fibres contracted obliquely to their long axis, supposing that by so doing, the muscle bands would produce a more uniform contraction of the pharyngeal cavity as the salp swam. It is not clear how this could be done, and in any case the central fibres do not have the spiral structure he ascribed to them.

Any alternative view of the operation of the two fibre types has to explain (as Fedele realised) why there are two fibre types in both stages of *Ihlea* and not in other salps. Since we have found that the two types of fibre can contract independently, we suggest that gradation of contraction in *Ihlea* (and any other salps where they occur) may result not only from variation in the neural input to all fibres of the band resulting in variations in the compound muscle potentials in both fibre types, but also from joint or independent activity of the two. The marginal fibres have relatively more mitochondria and a smaller cross-sectional area of myofibrils than the central fibres, and we may thus suppose that during normal sustained slow swimming, the marginal fibres alone are active, whilst during short periods of more vigorous swimming the central fibres are also employed. Thus approximately 3 % of the cross-sectional area of myofibrils within the band would be employed during slow cruising swimming.

In this way, by rather slight modification of the design of the muscle fibres in the locomotor muscle bands, *I. punctata* would be able to swim slowly with gentle pulsations at low frequency, whilst retaining the ability to accelerate into more rapid `escape' swimming when disturbed. In other words, if this view of the role of the marginal fibres be correct, the salps in which they occur would resemble fish and cephalopods (Bone *et al.,* 1993) in using different muscle fibre types for slow and fast swimming, an hitherto unsuspected design feature of any tunicate.

Both stages of *I. punctata* are rather inactive and slowly-swimming, with a relatively 'floppy' non-rigid test in contrast to *T. democratica* and *S. fusiformis*. Specialising a few of the many muscle fibres in the locomotor bands for the weak contractions required during normal swimming would be a sensible solution in such a design although it apparently does not occur in other weakly-muscled salps such as *P. confœderata* or *Thetys vagina*. More active salps such as *S. fusiformis* and *T. democratica* have many fewer fibres in the muscle bands, and all are of similar type; with their more rigid and less-deformable test a few specialised fibres in each band would be insufficient for cruise swimming.

Fedele sought to correlate the easily deformable test of *I. punctata*, and special central fibres, whereas it seems more probable to us that the correlation is with the special marginal fibres. Evidently what is now needed to confirm these speculations are simultaneous records from the two fibre types in intact zooids of *I. punctata* during normal slow swimming and during 'escape' swimming.

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