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# CHROMATOPHORE MOTOR FIELDS IN THE SQUID, *LOLLIGUNCULA BREVIS*

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## SUMMARY

Chromatophore motoneurones in *Lolliguncula brevis* are known to originate in the suboesophageal lobes of the brain and to project directly to the mantle and fin through bilateral stellate ganglia and fin nerves. The chromatophore motor fields of stellar and fin nerves were investigated by stimulation of the cut end of individual nerves in a semi-intact preparation. This elicited expansion of yellow and brown chromatophores in distinct motor fields. Brown chromatophores extended over the entire mantle, whereas yellow chromatophores were limited to the dorsal and lateral mantle areas. Combined nerve stimulation and lesions demonstrated substantial overlap between adjacent chromatophore motor fields and innervation of individual chromatophores by different stellar nerves.

## INTRODUCTION

The bay squid *Lolliguncula brevis* has brown and yellow chromatophores and a simple repertoire of nine chromatophore patterns (Dubas, Hanlon, Ferguson & Pinsker, 1986*a*). Using retrograde transport of horseradish peroxidase (HRP), it has been found that the motoneurones which innervate the fin and mantle chromatophores are located mainly in the posterior chromatophore lobes (PCL) of the suboesophageal brain and project directly to the skin without synapsing in the stellate ganglia (Dubas, Leonard & Hanlon, 1986*b*; Dubas *et al.* 1986*a*), as in *Octopus* (Sereni & Young, 1932). These neurones have highly localized motor fields, usually consisting of 6–20 chromatophores, as revealed by extracellular focal threshold stimulation of the PCL (Dubas *et al.* 1986*a*). From the PCL the motor axons of mantle chromatophores travel *via* the ipsilateral pallial nerve and mantle connective to the stellate ganglion. They then enter the stellar nerves, which radiate to innervate the mantle (Fig. 1). However, it is not known which areas of the mantle are

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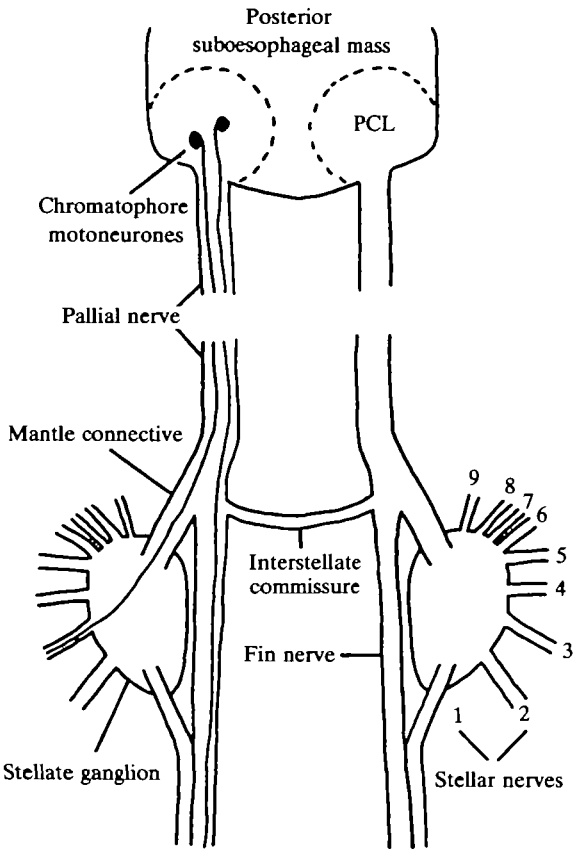



Fig. 1. Diagram of efferent pathways of mantle and fin chromatophore motoneurones. From cell bodies in the posterior chromatophore lobe (PCL) of the suboesophageal mass, axons project into the pallial nerve. One motoneurone projects to the mantle *via* the mantle connective, stellate ganglion and a stellar nerve; the other bypasses the stellate ganglion and projects to the fin by the fin nerve.

innervated by particular stellar nerves. The motor axons of fin chromatophores presumably travel directly to the fin *via* the ipsilateral pallial nerve and the fin nerve, which bypasses the stellate ganglion (Young, 1939). The pallial, fin and stellar nerves also contain motor axons for other superficial and deep muscles in the mantle and fin as well as afferent fibres.

Previous work has described the organization of the stellate ganglion of the squid *Loligo* (Young, 1972) and its innervation of the mantle musculature (Wilson, 1960; Young, 1938). In the cuttlefish *Sepia*, the chromatophore responses evoked by stimulation of single chromatophore motor axons within the stellar nerves have been characterized (Maynard, 1967) but the chromatophore motor fields of the entire stellar nerves have not been mapped. These fields have been mapped for *Octopus*, and no overlap was found between adjacent fields (Buhler, Froesch, Mangold & Marthy, 1975).

Semi-intact squid preparations do not show normal chromatophore patterns, so the underlying motor programmes must be characterized initially by means of chronic recordings in intact, freely behaving animals. Before it is possible to obtain representative whole-nerve recordings during normal chromatophore patterning, we must first understand the distribution of motor axons in the nerves leaving the stellate ganglion. The present study uses semi-intact preparations of *L. brevis* (whole nerve stimulation and lesions) to characterize chromatophore motor fields of individual stellar nerves and quantify the degree of overlap between adjacent fields.

## MATERIALS AND METHODS

Squids were caught (between May and August) in the Gulf of Mexico and maintained (20°C) in large, recirculating artificial sea water systems (ASW; Instant Ocean; Hanlon, Hixon & Hulet, 1983). Male and female adults were used ($N = 27$), but most data are from adult males (mantle lengths 50–80 mm) which appeared to be healthier. The number of stellar nerves varied from 6 to 10 in different individuals (with a median value of 8 in 25 animals). This was not related to the mantle length of the animal. Throughout this study stellar nerves were numbered beginning with the most posterior nerve (see Fig. 1).

### *Semi-intact preparations*

Animals were anaesthetized in 50% isotonic $MgCl_2$ (Messenger, Nixon & Ryan, 1985). The distal segment of the pallial nerve, the stellate ganglion and the stellar nerves (Fig. 1) were exposed by decapitation. A lateral incision along one side of the mantle left the contralateral mantle intact for mapping motor fields. Some preparations were perfused using the procedure of Dubas *et al.* (1986*a*), but with the main artery to the head tied off. The edge of the mantle was pinned with the outside of the skin facing the transparent bottom of the chamber which was then perfused with ASW.

### *Stimulation of chromatophores*

Individual stellar nerves were cut near their exit from the ganglion. The distal segment of the stellar nerve was stimulated (Grass SD9 stimulator) using a suction electrode with removable glass tips of different diameters to ensure a tight fit. In some preparations, the mantle connective (or pallial nerve) was stimulated with a second suction electrode.

### *Experimental protocols*

One series of experiments ($N = 11$) examined dynamic chromatophore responses to different intensities, frequencies and durations of nerve stimulation. These preparations were perfused because it took about 3 h to run through the protocol. Successive stimulus trains were delivered (6 ms pulses, usually negative polarity, 5 s trains, at least 10 s intertrain intervals) first at 2 Hz and then at 20 Hz, with gradually increasing voltages until a maximal response was reached (defined as two stimuli with

no definite change in the motor field when the voltage was increased by 1 V). The size of the 'stimulation motor field' was defined as the area of the mantle that showed at least partial chromatophore expansion when a given nerve was stimulated maximally. Another series of experiments ($N = 16$) obtained an independent estimate of the size of the motor field by stimulating the mantle connective before and after a given stellar nerve was lesioned. These preparations were not perfused because it took less than 1 h to run through the protocol. Stimulation frequency was 20 Hz and voltage was brought directly up to elicit maximal responses. The size of the 'lesion motor field' was defined as the area of the mantle that showed no chromatophore expansion when the mantle connective was stimulated after a given stellar nerve was cut.

### *Quantification of motor fields*

Responses were recorded using a Panasonic colour VCR (Model MV-8410), with the camera (Model WV-3370) located below the transparent chamber. For greater clarity, stimulation and lesion motor fields were photographed with a Nikkormat 35 mm camera and traced from black and white prints (brown chromatophores) or projected colour slides (yellow chromatophores as well). The photographs or tracings were mounted on an acoustic digitizing tablet (Scientific Accessories Corp., Graf-Pen Model GP-3), calibrated using a ruler in the field of view, next to the preparation. The outlines of the mantle (an algorithm calculated the mantle length) and motor fields (an algorithm calculated the area included) were traced. To facilitate comparisons among animals of different sizes, the values for motor field areas (in $mm^2$) were normalized (using mantle length) to a 'typical' animal with a 60 mm mantle. The number and sizes of individual chromatophores at different frequencies of stimulation were measured from tracings of colour slides.

## RESULTS

### *Dynamic chromatophore responses*

#### *Stimulus intensity*

In most cases, the weakest stimulus used (1 V) was above threshold for eliciting chromatophore responses. With weak nerve stimulation, the motor field often consisted of discrete 'blotches' of expanded chromatophores with pale regions in between. With increasing voltages, the intervening pale regions became smaller until the entire motor field became active. Chromatophores in the centre of the stimulation motor field showed a greater degree of expansion than those at the borders of the field. Maximal motor fields were usually obtained with a stimulus of about 3 V. Unlike the other stellar nerves, the first nerve typically had two motor fields separated by a pale region located next to the rostral insertion of the fin (see, for example, Fig. 3). Chromatophores in this region did not expand when other stellar nerves or the fin nerve were stimulated, although stimulation of the entire mantle connective usually produced continuous chromatophore expansion throughout this region. Furthermore, stimulation of the first stellar nerve often produced chromatophore expansion in different regions of the fin. For selective stimulation, it was

necessary to desheath and separate the first stellar and fin nerves (due to their close proximity at the posterior end of the stellate ganglion). The risk of damaging both nerves was high and may account for the frequently observed discontinuities in the motor fields. Also, the short length of the first stellar nerve before it joined with the fin nerve raised the possibility of current spread, which could account for the expansion of fin chromatophores when the first stellar nerve was stimulated.

*Stimulus frequency*

With stimulation at 2 Hz, most chromatophores followed in a one-for-one manner, whereas at 20 Hz the contractions gradually became tetanic. At 20 Hz the expansion of individual chromatophores was usually much greater than at 2 Hz. In two preparations, we examined the effect of stimulating with intermediate frequencies (4–32 Hz) on the number of chromatophores responding and the size of the largest 25 chromatophores (of each colour) in the motor field. Representative results are shown in Fig. 2, where all measurements were made from colour photographs taken 2 s after the onset of the 5 s stimulus train. At suprathreshold voltage for the maximal motor field (see Materials and Methods), the number of both brown and yellow chromatophores increased gradually with increases in stimulus frequency (Fig. 2A). The increases in numbers of both colours of chromatophores were similar between 4 and 16 Hz, but between 16 and 32 Hz there was relatively greater increase in the number of expanded yellow chromatophores. These data indicate that additional chromatophores were recruited by increasing the frequency of stimulation, in keeping with previous observations on the octopus *Eledone* (Dubas, 1982). The degree of expansion of both colours of chromatophores also increased as the stimulus

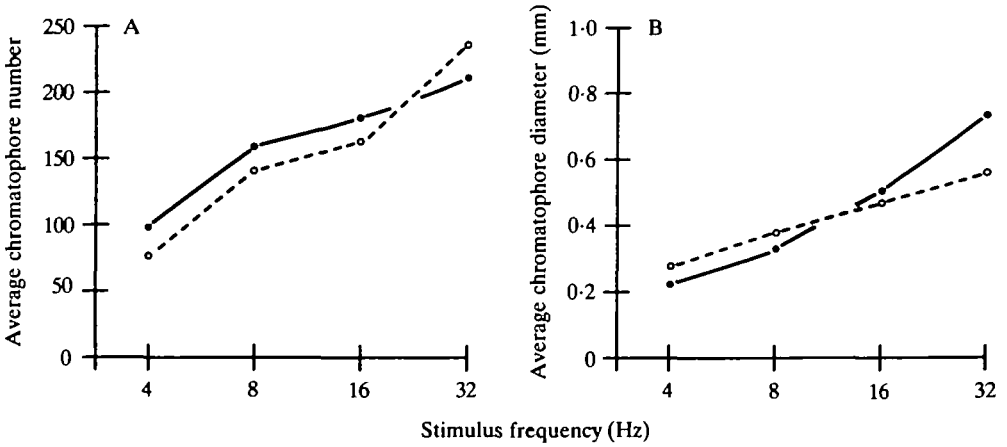
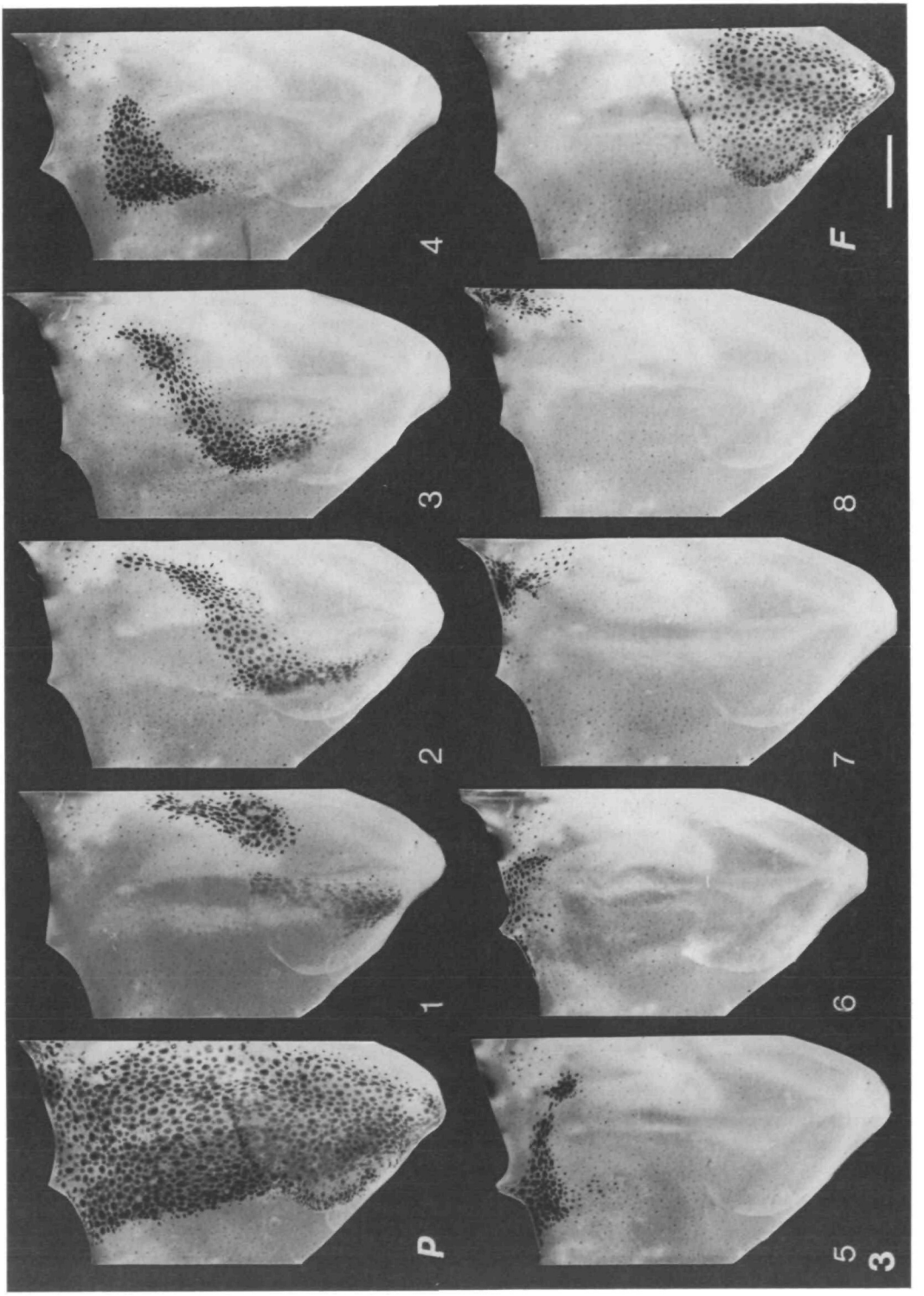



Fig. 2. Effects of stimulus frequency on chromatophore activity. The number (A) and the diameter (B) of the 25 largest chromatophores of each colour are plotted for different frequencies of tonic stimulation. The number of chromatophores expanding and the degree of expansion of individual chromatophores increases with stimulus frequency. Data based on stellar nerves 1, 3, 5, 7 and 8 of a single preparation. ○, yellow chromatophores; ●, brown chromatophores.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frequency was raised (Fig. 2B). Between 4 and 8 Hz brown and yellow chromatophores showed a similar increase in diameter, but from 8 to 32 Hz the brown chromatophores showed a greater diameter increase than the yellow ones.

*Train duration*

At a given frequency and voltage of stimulation, the responses of individual chromatophores often increased within the 5 s train. Thus, at the beginning of the stimulus train, individual chromatophores began to expand slightly. As the duration of the stimulus train increased, the same chromatophores gradually expanded more fully, usually without an overall increase in the size of the motor field. This gradual increase in the response of individual chromatophores occurred more rapidly for stimulation at 20 Hz than at 2 Hz.

### *Elicited contractions of skin and mantle muscle*

We did not make a thorough comparison of the thresholds for chromatophore expansion with the thresholds for contraction of mantle and skin muscle. However, muscle contractions were usually elicited at voltages that elicited chromatophore expansion. The motor fields for muscle contractions overlapped with those for chromatophore expansion and sometimes led to a decrease in the apparent size of the chromatophore stimulation motor fields. In general, muscle contractions followed the 2 Hz stimulus in a one-for-one fashion, whereas at 20 Hz they appeared to fatigue or become tetanic. These contractions, especially those of the skin muscles, often confounded the quantitative measurements of the chromatophore motor fields for individual stellar nerves as well as the estimates of overlap between adjacent motor fields.

### *Chromatophore stimulation motor fields*

Stimulation motor fields were determined for individual stellar, pallial and fin nerves (Fig. 3). The pallial nerve was stimulated before the fin nerve branch, so chromatophore expansion occurred on the entire ipsilateral mantle and fin. Pallial nerve stimulation never produced contralateral activation of chromatophores (or mantle muscle contractions), suggesting that these fibres do not travel in the interstellate commissure (Fig. 1). Because of the radial arrangement of the stellar nerves, the corresponding chromatophore motor fields also extended in a radial

Fig. 3. Representative stimulation motor fields of brown chromatophores in a preparation with eight stellar nerves. Photographs show left hemimantle (anterior margin at top and dorsal midline at right). Stimulation of the pallial nerve (*P*) produces chromatophore expansion of the entire hemimantle and fin. When stellar nerve 1 is stimulated, the motor field is discontinuous (see text) and the left portion is seen through the transparent fin. Motor fields of nerves 2–5 extend continuously to the ventral midline and are located at progressively more anterior mantle regions. Motor fields of nerves 6–8 are restricted to the dorsal surface. Stimulation of the fin nerve (*F*) produces chromatophore expansion over the entire fin. Scale bar, 10 mm.

fashion, centred on the region of the ipsilateral mantle directly above the location of the stellate ganglion (star in Fig. 4). The smaller anterior nerves had smaller motor fields limited to the dorsal surface of the anterior mantle, whereas the larger posterior nerves had correspondingly larger motor fields that also extended onto the ventral surface. The yellow chromatophores typically were not present on the ventral surface of the mantle (confirming a previous observation of F. Dubas, personal communication).

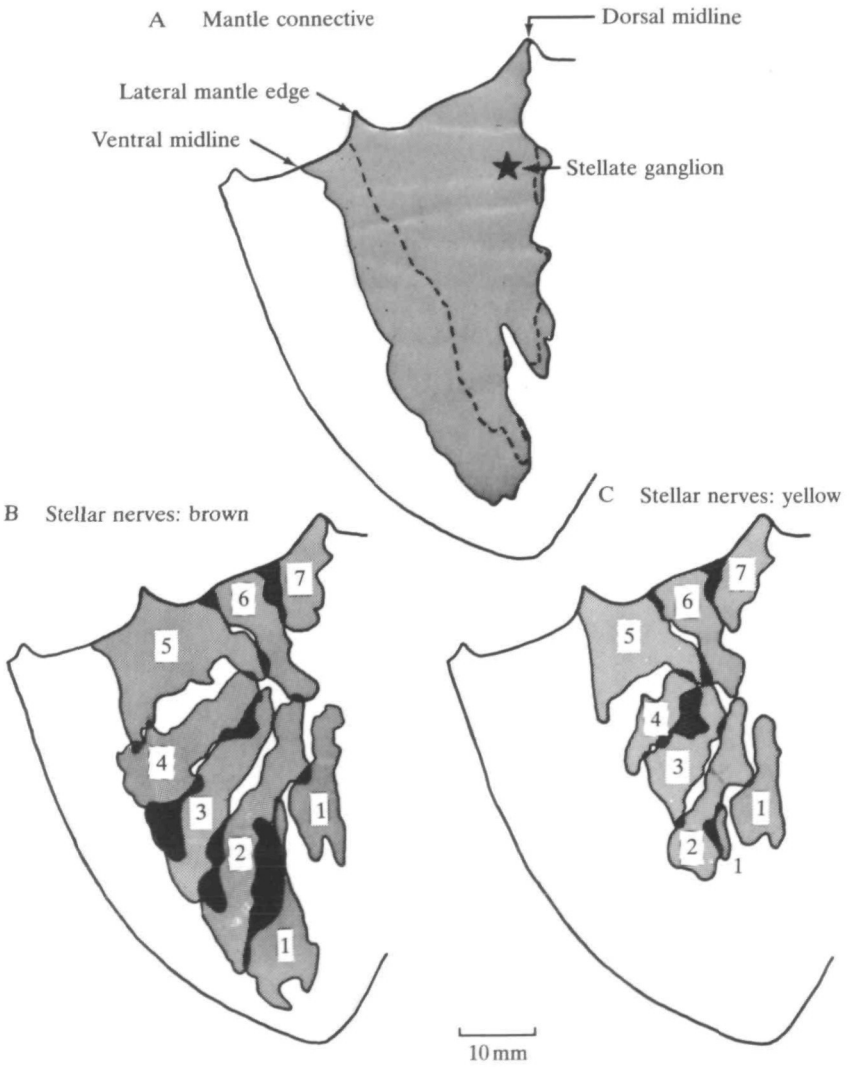


Fig. 4. Composite tracings of brown and yellow chromatophore motor fields in a preparation with seven stellar nerves. Stimulation of mantle connective produces continuous expansion of brown chromatophores on the entire hemimantle, dashed line indicating limit of yellow chromatophores (A). Composite tracings of motor fields of stellar nerves 1–7 show overlap between adjacent fields (darker regions) of brown (B) and yellow (C) chromatophores.

Stimulation of the mantle connective produced continuous chromatophore expansion of both yellow and brown chromatophores throughout the ipsilateral mantle (Fig. 4A), and stimulation of individual stellar nerves showed substantial overlap of the fields for both brown chromatophores (Fig. 4B) and yellow ones (Fig. 4C). The small white areas (Fig. 4B,C) are probably artifacts due to the simultaneously elicited skin and mantle muscle contractions that reduced the apparent area of each chromatophore motor field. However, the lack of yellow chromatophore response in the posterior mantle may be due, in part, to damage to the first and second stellar nerves.

Brown and yellow chromatophore motor fields of individual nerves were compared in six preparations (Fig. 5). The shape and extent of the motor fields for the yellow chromatophores were often different from those for the brown chromatophores: the yellow field of posterior nerves that innervate both the ventral and dorsal surfaces was often smaller than the brown field (because of the absence of yellow chromatophores on the ventral mantle); on the dorsal mantle, the stimulation motor fields of the yellow chromatophores often extended beyond those of the brown chromatophores.

The average size of the normalized stimulation motor fields of brown chromatophores for each stellar nerve was compared for preparations grouped according to the

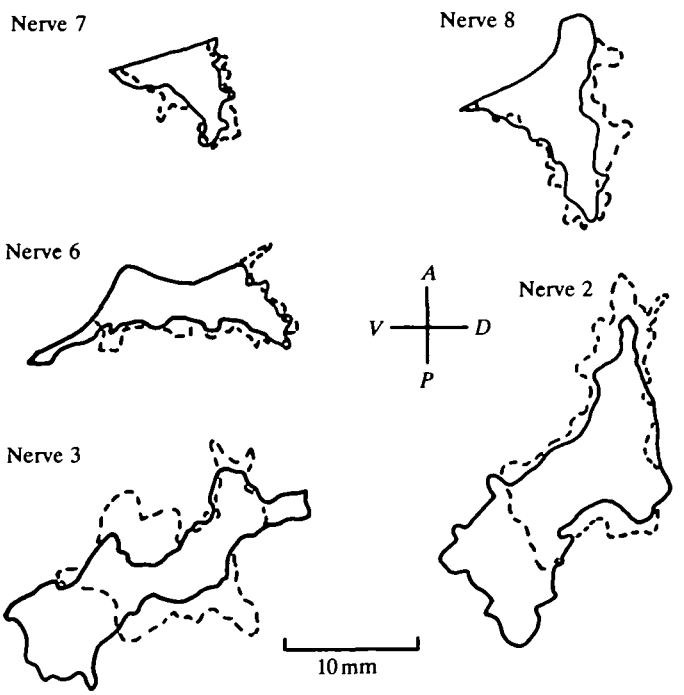


Fig. 5. Stimulation motor fields of yellow (dashed) and brown (solid lines) chromatophores for representative stellar nerves in a single preparation. Motor fields are similar for anterior stellar nerves (7 and 8) which only innervate dorsal mantle surface, but yellow motor fields of posterior nerves (6, 3 and 2) do not extend onto ventral mantle surface. (*A*, anterior; *P*, posterior; *D*, dorsal; *V*, ventral).

Table 1. *Areas of stimulation motor fields for brown chromatophores*

| Nerve | Seven stellar nerves Mean ± S.D. | N | Eight stellar nerves Mean ± S.D. | N | Nine stellar nerves Mean ± S.D. | N |
|---|---|---|---|---|---|---|
| First stellar | 164·0 ± 39·9 | 2 | 220·4 ± 57·0 | 2 | 217·6 ± 71·6 | 3 |
| Second stellar | 258·2 ± 126·3 | 2 | 209·8 ± 21·1 | 2 | 232·6 ± 81·7 | 5 |
| Third stellar | 149·2 | 1 | 164·7 ± 29·1 | 2 | 181·3 ± 33·7 | 5 |
| Fourth stellar | 197·1 ± 77·0 | 3 | 146·1 ± 22·3 | 2 | 186·6 ± 83·2 | 5 |
| Fifth stellar | 126·4 ± 56·3 | 2 | 120·5 ± 3·0 | 2 | 154·4 ± 44·4 | 5 |
| Sixth stellar | 58·5 ± 23·2 | 2 | 69·2 ± 1·0 | 2 | 162·2 ± 66·2 | 5 |
| Seventh stellar | 55·0 ± 6·6 | 2 | 45·0 ± 11·1 | 2 | 89·2 ± 54·5 | 6 |
| Eighth stellar | — | | 65·5 ± 15·3 | 2 | 47·2 ± 16·4 | 5 |
| Ninth stellar | — | | — | | 50·0 ± 10·6 | 5 |
| Fin nerve | 511·4 ± 30·0 | 2 | 460·1 ± 41·8 | 2 | 517·3 | 1 |
| Mantle connective | 909·5 ± 105·8 | 3 | 959·4 ± 225·9 | 2 | 925·3 ± 77·5 | 5 |

The values for the areas are in $mm^2$ (normalized for an animal with a mantle length of 60 mm). Areas were calculated from photographs or tracings of stimulation motor fields that were digitized on an acoustic tablet (see Materials and Methods).

Data based on a total of six preparations with nine stellar nerves, two with eight nerves and two with seven nerves (the *N* values vary because it was not possible to obtain satisfactory data from all nerves in all preparations).

presence of seven, eight or nine stellar nerves (Table 1). In all groups, the posterior nerves had the largest motor fields and the anterior nerves the smallest. There was substantial variability among animals in terms of which individual stellar nerve had the largest or the smallest motor field. For example, in different animals with nine stellar nerves, the first, second, third or fourth nerve might have the largest field (ranging from 23·0 to 33·6 % of the entire mantle connective motor field), whereas the seventh, eighth or ninth nerve might have the smallest field (ranging from 3·8 to 7·4 %). Yellow chromatophore motor fields were quantified for two preparations (Table 2). The posterior nerves again had larger motor fields than the anterior ones, but (because yellow chromatophores are limited to the dorsal surface) the differences between the areas of the posterior and anterior motor fields were not as great as for the brown chromatophores.

### *Chromatophore lesion motor fields*

The lesion motor field for a given stellar nerve was measured by stimulating the mantle connective after cutting the nerve. The area that remained pale when the mantle connective was stimulated after the stellar nerve lesion was substantially smaller than the area that showed chromatophore expansion when the same stellar nerve was stimulated selectively (Fig. 6). Lesion and stimulation motor fields were compared directly by plotting the areas against each other for individual nerves (Fig. 7). For the brown chromatophores (open circles), in 21 out of 22 cases the area of the stimulation motor field was larger than that of the corresponding lesion motor field (ratios of stimulation-to-lesion areas from 0·89 to 6·64, average 2·11 ± 1·3).

Table 2. *Areas of stimulation motor fields for yellow chromatophores*

| Nerve | Seven stellar nerves | Eight stellar nerves |
|---|---|---|
| First stellar | 65·5 | 186·0 |
| Second stellar | 77·0 | 171·9 |
| Third stellar | 80·1 | 145·5 |
| Fourth stellar | 53·9 | 100·0 |
| Fifth stellar | 109·9 | 90·1 |
| Sixth stellar | 65·4 | 78·7 |
| Seventh stellar | 47·7 | 44·7 |
| Eighth stellar | — | 102·1 |
| Fin nerve | 456·7 | 487·0 |
| Mantle connective | 630·4 | 834·3 |

The values for the areas are in $mm^2$ (normalized for an animal with a mantle length of 60 mm). Areas were calculated from tracings of stimulation motor fields that were digitized on an acoustic tablet (see Materials and Methods).

Data based on one preparation with seven stellar nerves and one with eight nerves.

Stimulation and lesion motor fields of the yellow chromatophores were plotted for nine nerves (closed circles) and, in all cases, stimulation motor fields were larger than the corresponding lesion motor fields (ratios from 1·1 to 3·62, average 1·98 ± 0·79).

### *Overlap between chromatophore motor fields*

The above independent estimates of the amount of overlap between adjacent chromatophore motor fields of the stellar nerves both suggest substantial overlap of the adjacent fields for both brown and yellow chromatophores. However, both methods can underestimate the degree of overlap. First, the estimate from comparison of the fields for all stellar nerves (Fig. 4) could underestimate because of simultaneously elicited muscle contractions causing the stimulated areas to shrink. Second, the estimate obtained from comparison of the lesion and stimulation motor fields for the same nerve (Fig. 7) could underestimate if the region of the lesion motor field were not contracted (the motor innervation of the skin and mantle muscles being cut).

Direct evidence for overlap between adjacent motor fields was obtained from observations of individual chromatophores within the area of overlap (Fig. 8). This demonstrated clearly that the same chromatophores were innervated by two different stellar nerves (nerves 3 and 4 in this example).

## DISCUSSION

This is the first study in a squid to map lesion and stimulation motor fields for stellar and fin nerves. Unlike most cephalopods, *L. brevis* has only two chromatophore colours, brown and yellow. Both brown and yellow motor fields elicited with maximal voltage were consistently larger at higher frequencies of nerve stimulation

because more chromatophores were expanded, but it is not clear whether this was due to the recruitment of additional motoneurones or to chromatophore neuromuscular properties. Yellow chromatophores rarely extended onto the ventral surface of the mantle. Presumably, this would alter the visual impact of chromatophore patterns if the animal was viewed from below (as compared to above).

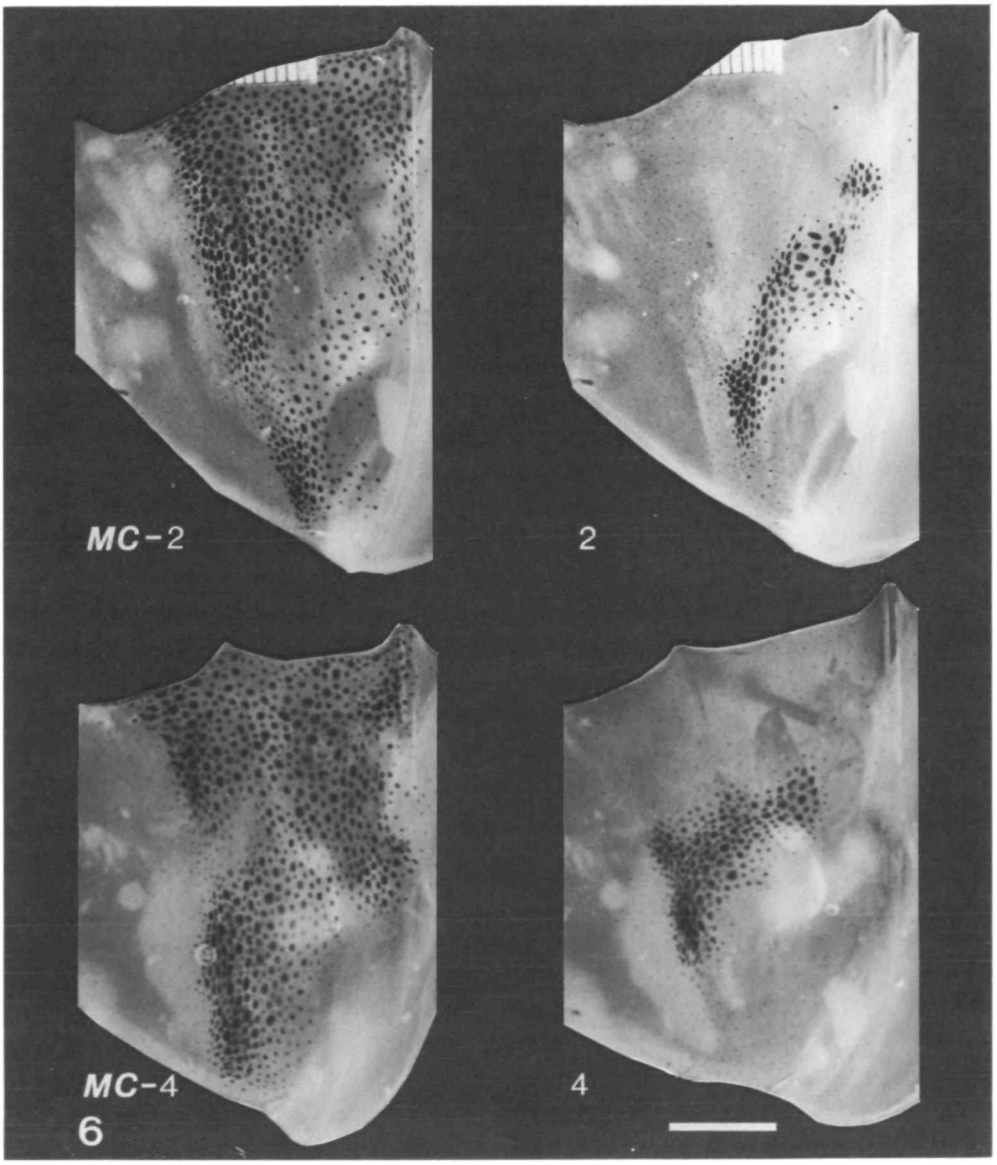


Fig. 6. Corresponding lesion (left) and stimulation (right) motor fields for representative stellar nerves (2, top; 4, bottom) in two different preparations. The lesion motor field is the pale area produced by stimulation of the mantle connective (*MC*) after the nerve is cut. Scale bar, 10 mm.

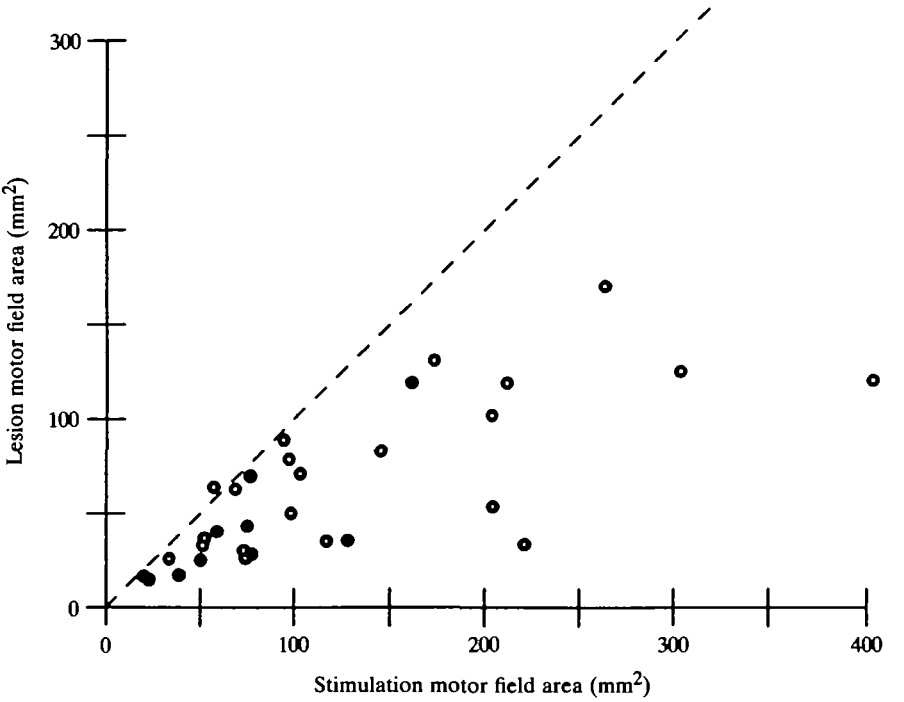


Fig. 7. Areas of stimulation *vs* lesion motor fields. Each point plots area of stimulation motor field (abscissa) against lesion motor field (ordinate) for a given stellar nerve. Data based on 22 nerves (nine preparations) for brown chromatophores (open circles) and nine nerves (four preparations) for yellow chromatophores (closed circles). The areas of lesion motor fields were consistently smaller than those of corresponding stimulation motor field (30 out of 31 points fall below dashed diagonal).

Considerable overlap between chromatophore motor fields of adjacent stellar nerves was indicated by comparing lesion and stimulation fields (Fig. 6). Froesch (1973) used lesions to examine chromatophore motor fields of *Octopus* nerves, but did not compare these with effects of nerve stimulation or with elicited activity in the surrounding motor fields. Buhler *et al.* (1975) observed substantial overlap between the chromatophore motor fields of different stellar nerves in *Octopus*, but attributed this to current spread. It seems unlikely that overlap between mantle motor fields in *L. brevis* can be explained by current spread, except for the special case of cross-talk with the fin nerve when the first stellar nerve was stimulated (see Results). Overlap between motor fields of adjacent stellar nerves is not surprising since in octopods (Dubas, 1982; Dubas & Boyle, 1985) and squids (Dubas *et al.* 1986; Florey, 1969) the muscle fibres of a single chromatophore can be innervated by more than one motoneurone. Also, in octopods (Dubas, 1982; Dubas & Boyle, 1985) small dermal branches of stellar nerves have overlapping motor fields. We have now shown that motoneurones travelling in different stellar nerves can also innervate the same chromatophores (see Fig. 8). In addition to polyneuronal innervation of individual

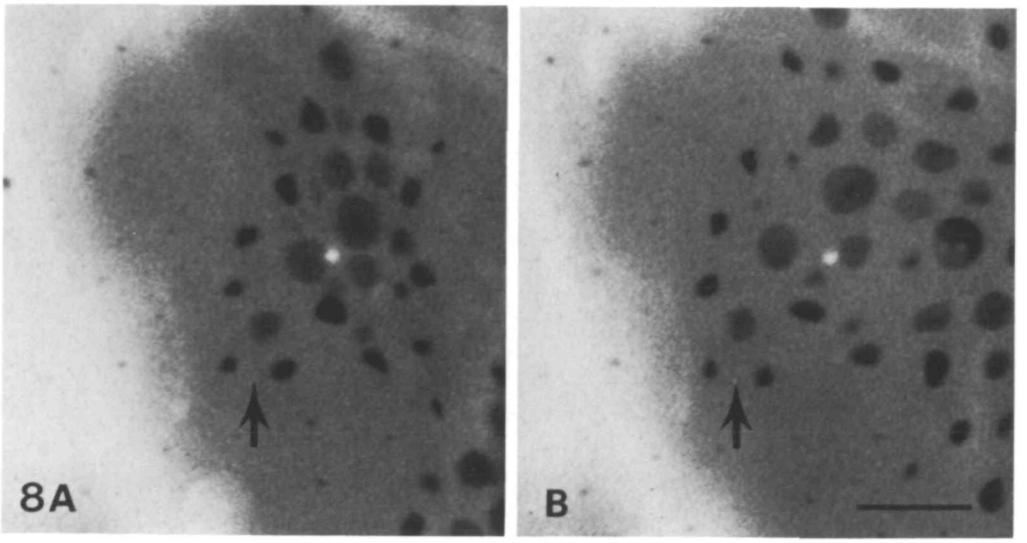


Fig. 8. Innervation of individual chromatophores by two different stellar nerves. Within the overlap area between the motor fields of stellar nerves 3 (A) and 4 (B) the same chromatophores (arrowheads) expanded when either nerve was stimulated. Owing to skin contractions, the positions of the expanded chromatophores relative to each other and to the white air bubble (landmark in centre) differ slightly with stimulation of each nerve. Scale bar, 2 mm.

muscle fibres, electrical or mechanical coupling between neighbouring chromatophores could also produce some overlap (e.g. Froesch-Gaetzi & Froesch, 1977), but such coupling would only account for spread over very short distances and may not play a role in normal chromatophore expansion. Our findings are in keeping with the data of Boyle & Froesch (1979) on sensory receptive fields in *Octopus* which radiate outwards on the mantle from the locus of the underlying stellate ganglion and also show overlap between adjacent stellar nerves.

Despite variations among animals (both number of nerves and motor field areas of a given stellar nerve) the present results allow a reasonably accurate prediction of which nerves carry efferent activity associated with chromatophore patterns on the mantle and fin. A comparison of the positions of the mantle patterns (see fig. 2, Dubas *et al.* 1986*a*) with the present motor fields suggests that subpopulations of chromatophore motoneurones travelling in several specific stellar nerves are involved in the production of each pattern on the mantle.

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