Post-Hatching Development of Motor Innervation of Lateral Muscle in the Seabream *Sparus aurata*

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**Abstract**

In many fish, both production of new muscle fibres and neurogenesis continue into juvenile life. To test the hypothesis that new motoneurons are produced to supply the expanding muscle target we used the seabream (*Sparus aurata*), which shows a many-fold increase in the number of fibres in lateral muscle during posthatching juvenile development. A motor nerve branch innervating a segment of epaxial lateral white muscle was identified, and the type and number of its axons were measured in fish of several larval and post-larval ages.

Contrary to expectation, total axon number was greatest in the larval fish (114.3±22.6); unmyelinated axons were found only in the larval nerves, and the number of myelinated axons increased only modestly over the ages examined, from 58.5±12.4 in larval fish to 77.5±7.3 in post-larval juveniles. We conclude that in seabream the larval nerve still includes axons of motoneurons destined to die during the normal developmental phase of target-dependence in addition to those axons which will survive into juvenile life, and that the definitive number of motoneurons is already present in the larval fish before the main increase in muscle fibre number occurs.

**Key words**: axon, larval, motoneuron, neurogenesis, post-larval.

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and ray are elasmobranchs, the others teleosts), it is difficult to evaluate the significance of the discrepancies between the results obtained.

The main objective of the study described here was to test the hypothesis that a marked expansion in the peripheral target of motoneurons in a teleost fish would be accompanied by a concomitant increase in their number. We used the seabream (*Sparus aurata* L) because this teleost fish does grow to a large size, it is known to show a many-fold increase in the number of fibres in lateral muscle during postlarval development [26] and it is commercially farmed so that appropriate developmental stages raised under well-defined conditions are available. A nerve branch innervating epaxial lateral white muscle was selected for study at a position close to its exit from the spinal cord, where it contained only motor axons (see Discussion), and the number and diameter of its myelinated and unmyelinated axons were measured in fish of several larval and post-larval ages from 40 days post-hatching to 2 years.

**Materials and Methods**

**Fish**

*Sparus aurata* ranging in age from 40 days post-hatching to more than 2 years old were obtained from a commercial fish farm on the Adriatic coast of Italy, anaesthetised by immersion in MS222 (Sandoz) and killed by decapitation. Metamorphosis occurs at about 50 days post-hatching in this species, and the post-larval hyperplastic growth phase of lateral muscle starts shortly afterwards [26]. By 90 days all fry have reached this phase. The number and ages of fish used for axon counts are shown in Table 1, together with body weights for fish of these ages. Acetylcholinesterase staining [23] was carried out on paraformaldehyde-fixed muscle of fish aged 40, 60 and 100 days to check for any change in the distribution of innervation over the transition from larval to juvenile life, but revealed the distributed innervation typical of fish white muscle at all these ages.

**Electronmicroscopy**

Whole larvae and small fry, and small tissue blocks containing the appropriate area of spinal cord and epaxial muscle dissected out of larger fish immediately posterior to the anal vent, were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1M cacodylate buffer, pH 7.4 at 4°C for 3 hours. The samples were then rinsed overnight in cacodylate buffer, treated with 1% osmium tetroxide for 2 hours, and rinsed again in cacodylate buffer before being dehydrated and embedded in epon-araldite resin.

Blocks were sectioned in a plane giving transverse sections of the chosen nerve branch (DRv) shortly after its separation from the ventral root, as shown in figure 1a. Blocks of whole fish (larvae and small fry) were initially trimmed in transverse section to the rostro-caudal axis until the anal vent was reached, and then tilted to the appropriate plane. Semi-thin (1 µm) sections from all blocks were stained with toluidine blue to check that the level and plane of section were correct.
before cutting thin (70-90 nm) sections which were then contrasted with uranyl acetate and lead citrate.

Thin sections were viewed in a Zeiss EM900 electron microscope at magnifications ranging from 3000x to 50000x, and the nerve photographed on Kodak SO 163 film. The entire cross-section of the nerve was then reconstructed from the photographs, and all axons present as myelinated or unmyelinated, and their diameter measured using a Digicad Plus digitising tablet linked to Videoplan software. If the axon was myelinated, the diameter measured included the myelin sheath. In the case of the older fish, once examination of thin sections in the EM had shown that no unmyelinated axons were present, diameter measurements were made with the same system from prints of serial semi-thin sections photographed in the light microscope (with a 100x objective). Direct comparison of myelinated axon diameters made on photographs of thin (EM) and semithin (LM) was made with two samples, and showed no significant difference in values.

Results

The appearance of a 100 day nerve in situ at light microscope level is illustrated in figure 1b. The electron-micrograph in figure 1c shows a portion of this nerve in a 46 day larva. This nerve contained both myelinated and unmyelinated axons; when present, unmyelinated axons usually occurred in 2 or 3 groups, not singly. Unmyelinated axons were found in all the subjects in the age range 46-60 days, but not in any older fish. The numbers of axons of both types in fish at each age examined are shown in Table 1.

Examination of the epaxial muscle in all these subjects confirmed that the post-larval hyperplasia which results in the appearance of new fibres throughout the myotome had not started in the fish aged 60 days or less, but was underway in all fish aged 100 days or more. Axon numbers were therefore compared for these two groups of fish: post-larval (≥100 days) and ‘larval’ (≤60 days; strictly, some of these fish had completed metamorphosis, but the stage of muscle growth was still typical of the late larval stage). If only myelinated axons are taken into consideration, there was a modest but significant increase in their number in the older fish: mean values (±s.d.) were 58.5 ± 12.4 in larval fish and 77.5 ± 7.3 in the postlarval fish. Though small, this difference was significant as evaluated by student’s t-test (P < 0.001, unpaired [30]). However, there was also a substantial, though quite variable, population of unmyelinated axons in the larval nerve (see Table 1), and if these are taken into account, the total number of axons (114.3 ± 22.6) in the nerve was significantly greater in the larval fish (P < 0.001, unpaired and corrected for unequal variances [30]).

Unmyelinated axons were all of very small diameter (< 1 µm). Measurement of myelinated axon diameters revealed a gradual increase with age, as summarised in Table 1. Mean diameter, minimum diameter and the largest diameter were all more than two-fold greater in the 60 day nerve with an ‘adult’ number (77) of myelinated axons than in the 46 day larva. The smallest

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Table 1. Axon numbers, types and diameters by fish age and weight.

<table>
<thead>
<tr>
<th>AGE OF FISH</th>
<th>Larval</th>
<th></th>
<th></th>
<th>Juvenile</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>46d</td>
<td>56d</td>
<td>60d</td>
<td>100d</td>
<td>150d</td>
<td>700d</td>
<td></td>
</tr>
<tr>
<td>body weight</td>
<td>~0.01g</td>
<td>~0.03g</td>
<td>~0.04g</td>
<td>~0.6g</td>
<td>~3g</td>
<td>120-400g</td>
<td></td>
</tr>
<tr>
<td>no. axons M/U</td>
<td>52/30</td>
<td>52/73</td>
<td>44/64</td>
<td>72/0</td>
<td>84/0</td>
<td>82/0</td>
<td></td>
</tr>
<tr>
<td>48/31</td>
<td>71/56</td>
<td>42/66</td>
<td>65/43</td>
<td>68/0</td>
<td>84/0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64/90</td>
<td>70/50</td>
<td>85/0</td>
<td>77/55</td>
<td>70/0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean diam. M</td>
<td>1.14±0.79</td>
<td>n</td>
<td>2.38±1.31</td>
<td>3.3±1.5</td>
<td>3.5±1.8</td>
<td>10.4±3.4</td>
<td></td>
</tr>
<tr>
<td>mean diam. U</td>
<td>0.26±0.08</td>
<td>n</td>
<td>n</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>mean no. M</td>
<td>58.5±12.4</td>
<td>n</td>
<td>2.38±1.31</td>
<td>3.3±1.5</td>
<td>3.5±1.8</td>
<td>10.4±3.4</td>
<td></td>
</tr>
<tr>
<td>mean no. U</td>
<td>55.8±18.6</td>
<td>n</td>
<td>n</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

1: includes data of Berardinelli et al. [4] and additional data for larval ages; 2: typical body weight for age; 3: M = myelinated, individual values for each fish; 4: U = unmyelinated, individual values for each fish; 5: body weight 0.051 g; 6: body weight 0.03 g; 7: body weight 0.02 g; 8: M = mean diameter (±s.d.) in µm for all myelinated axons at that age (except for 60 d, where value is derived from only 2 nerves containing a total of 142 axons); 9: U = mean diameter (±s.d.) in µm for unmyelinated axons at that age; 10: mean number (±s.d.) for all ‘larval’ fish from 46 - 60 days; 11: mean number (±s.d.) for all postlarval fish from 100 days; n = not determined.
myelinated axon diameter was 0.53 \( \mu \text{m} \), found in one of the 46 day larvae. It was also observed that the number of lamellae within myelin sheaths was least in the larval fish, but this was not quantified.

**Discussion**

**Identity of axons**

An important issue is the identification of the axons we counted in the DRv nerve as motor axons. The equivalent branch in other fish is considered to be the main motor nerve branch innervating epaxial muscle (teleosts: [3, 8]; elasmobranch: [20]) and several observations suggest that the nerve contained only motor axons at the point we examined in the seabream. Axon counts were made at the point shown in figure 1a, i.e. very shortly after the point where the DRv nerve branch separates from the ventral root shortly after its exit from the spinal cord, and before the point where the remaining ventral root axons are joined by dorsal root axons to form the (mixed) ventral and medial rami. The size distribution and type of axons in our oldest fish (i.e. all myelinated, and containing large diameter axons) was also typical of motor rami, rather than sensory ones (which contain small diameter myelinated and unmyelinated axons) [8]. Furthermore, because the DRv branch selected supplies only epaxial territory in a segment caudal to the abdomen, the presence of visceral afferents or a substantial autonomic component can also be excluded.

Sakamoto et al. [27] have also shown that in ventral roots of the angelfish at anal vent level the number of axons is equal to the number of motoneurons.

**Developmental changes in axon morphology**

Our observations that the larval nerves contain both unmyelinated axons and axons with only thin myelin sheaths (figure 1c), and that even myelinated axons are very small in diameter (Table 1), indicate that the nerve is very immature at 46 days and that the process of myelination of axons is still at an early stage. In developing nervous systems, myelination starts soon after axon formation once a critical diameter has been exceeded, and is accompanied by a gradual increase in the number of membrane layers as the axon diameter increases further [6, 10, 17]. In mammals, myelination generally occurs at a smaller axon diameter in central axons (about 0.3 \( \mu \text{m} \)) than in peripheral axons (about 1 \( \mu \text{m} \)), but there is little comparable information about myelination in fish axons. The smallest diameter axon in the spinal motor nerve we examined was about 0.5 \( \mu \text{m} \) (and 26% of the axons in the 46d nerves had diameters less than 1 \( \mu \text{m} \)), which is significantly less than for mammalian peripheral axons, but slightly larger than the values of 0.2 \( \mu \text{m} \) [10] and 0.3 \( \mu \text{m} \) [12] observed in two cranial nerves of growing fish. Axon diameter subsequently increased with age (Table 1).

**Numbers of axons**

If only myelinated axons are taken into account, there was a modest increase in motor axon numbers in the postlarval fish in which muscle fibre hyperplasia had occurred. However, the size of the increase (about 30%) is far smaller than the increase in muscle fibre number (several-fold, [26]), suggesting that these changes were not causally linked. Given the immature state of the nerve in the larval fish, it is most likely that the unmyelinated population seen at that age contains axons which will contribute to the final myelinated population in postlarval fish, but which have not yet acquired a myelin sheath. In other words, the combined myelinated and unmyelinated population present in larval fish already included all of the 77 or so motor axons seen in this nerve in post-larval fish.

The population of unmyelinated axons in the larval nerves was very variable, however, and overall (mean number 55.8) exceeded the difference (19 axons) in myelinated axon numbers between larval and postlarval fish. How can this discrepancy be accounted for? Although the myelinated axons were easily identified and counted at all ages, the very small diameter and grouped nature of unmyelinated axons made them more difficult to identify. We cannot exclude the possibility that there was a small error in unmyelinated axon counts, but when the counts were made by two individuals on the same nerve there was quite good agreement, and we estimate that counting errors could account for at most a 10% difference. The use of the anal vent as a marker to identify the equivalent nerve and myotome in each case (see Methods) also ensured that errors due to variable position along the rostro-caudal axis were avoided.

Even in these commercially raised fish there can be large differences in body size and developmental status between fish of the same age. To reduce the effects of this variation we normally take only the larger (fast-growing) fish from any cohort, but an additional comparison of unmyelinated axon counts for some subjects of known body weight at the same age was also made to see if there was a relation between unmyelinated axon number and body size. However, no simple relation between unmyelinated axon number and either age or size was evident within the larval fish population (see Table 1).

Most probably, the variable number of unmyelinated axons in DRv nerve is the result of two overlapping but not synchronous processes which both affect unmyelinated axon numbers: normal developmental motoneuronal death [22] resulting in loss of axons, and gradual myelination of axons of motoneurons which will survive. Work on other vertebrates suggests that there is an initial over-production of motoneurons by a factor of 2 or more [2, 18, 22]. Although most of the developing motoneurons do make functional contact with their target muscle [2, 6, 19], many will fail to make sufficient peripheral
contacts to survive the subsequent period of target dependence and will therefore die [15, 24, 31]. Developmental cell death has also been found in the electromotoneuron pool of the fish *Toledo marmorata*, although it accounted for a smaller proportion of cells [11]. Finally, in the angelfish investigated by Sakamoto et al. [27], there was a roughly 40% fall in motor axons number in the immediate post-hatching period. We therefore suggest that the unmyelinated axon population in the larval DRv nerve includes axons of motoneurons which would not have survived the phase of target-dependence in addition to axons which would have survived but had not yet begun myelination, a situation similar to that described for ventral roots in the embryonic chicken [6].

Given the general context of known persistence of neurogenesis in fish, and the studies which have described motoneuronal hyperplasia linked to muscle growth in fish [1, 9, 16, 20] and in another lower vertebrate (axolotl: [13]), we were surprised to obtain results suggesting that the definitive number of motoneurons is already present before the main increase in muscle fibre number occurs. However, an estimate of the corresponding ratio of motoneurons to muscle fibres, indicates that the number of axons is within the expected range for the adult number of muscle fibres innervated.

Although we do not know the exact area within the myotome supplied by the DRv nerve, we can assume that it supplies a substantial part of the epaxial muscle (as described for cat-fish, [3, 8]), and we do know how epaxial white muscle fibre number increases during development [26]. Supposing that the DRv nerve supplies, say, the apical 50% of epaxial muscle in its myotome, this is equivalent to about 420 fibres at 60 days and 3500 fibres by 150 days, giving ratios of motor axons : muscle fibres of 1:5.4 and 1:45.2, respectively. As muscle fibre hyperplasia continues beyond 150 days, the final ratio could reasonably reach at least double that value i.e. about 1:100. This is well within the range for many mammalian motor units, and reasonably close to the ratio in zebrafish, which is about 1:50 (assuming that half of the 71 motor axons per ventral root [37] supply one epaxial quadrant of the myotome which contains about 1700 fibres [32].

In conclusion, our results suggest that in the seabream *Sparus aurata*, the definitive number of motoneurons is already present and in contact with the lateral muscle in the larval fish, well before the main increase in muscle fibre number occurs. This is contrary to the results of Alfei et al. [1], but both supports and extends the conclusions reached by Smit et al. [28], who found no change in motoneuronal numbers during postlarval growth in the eel (another teleost), but were not able to study larval stages. The possibility remains that motoneuronal hyperplasia may be genuinely prolonged in large elasmobranchs [16, 20], and it would be interest-

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### References


[11] Fox GQ, Richardson GP: The developmental morphology of *Toledo marmorata*: electric lobe elec-


