

Posthatch development of the axial musculature of the common dentex *Dentex dentex*, L (Teleostei)

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Summary. The common dentex is a promising candidate for Mediterranean aquaculture. The present work is aimed at describing the development of the axial musculature from hatching to postlarval life. Transmission electron microscopy, histochemical (NADH-TR and mATPase) and immunohistochemical techniques (S-58 and TUNEL) have been used. At hatching superficial red and deep white muscles can be distinguished. Presumptive dermomyotome (external) cells are initially located over the superficial red muscle but shortly (2 days) tend to concentrate towards the epaxial and hypaxial limits of the myotome. Then, these cells enter the myotome and spread around and within the white muscle thus being apparently responsible for the stratified hyperplasia of the myotome. Mosaic hyperplasia is activated during the second half of the larval period and initially relies on differentiation of a population of atypical premyoblastic cells (APC). APC are mononuclear cells with euchromatic nuclei, cytoplasm full of thin longitudinally projected tubules, occasional mitochondria and scattered ribosomes. By the end of the larval period these cells tend to disappear, partly due to apoptosis, but postlarval mosaic hyperplasia continues by differentiation of presumptive myosatellite cells. APC are an unexpected and singular finding of this study which deserves more research, so as to further characterize their ancestry, developmental programme and fate. In addition to the white and superficial red muscle fibres, intermediate (pink) and tonic fibres appear during larval metamorphosis. Later, during the early postlarval life, a new type of slow twitch red muscle fibre is differentiated (red adult type).

Key words: Fish muscle, Dentex, Development, Myogenesis, Apoptosis

Introduction

The common dentex (Osteichthyes, Sparidae) is a marine species highly appreciated in Mediterranean countries. Its high growth rate and spawning period, not coincident with the gilthead sea bream *Sparus aurata* or sea bass *Dicentrarchus labrax*, makes its culture very interesting in terms of diversification (Abellán, 2000; Rueda and Martínez, 2001). However, the high levels of mortality when larvae are reared in intensive conditions have delayed and even questioned its commercial farming. In this sense, a thorough knowledge of the ontogeny of this species, as well as its feeding requirements is a prerequisite for the future feasibility of its industrial farming. Previous work has been focused on its general biology and larval development (Glamuzina et al., 1989; Jug-Dujakovic et al., 1995; Abellán et al., 1997). More specific studies have been aimed at describing the development of the skeleton (Koumoundouros et al., 1999, 2000, 2001) and the digestive apparatus (Santamaría et al., 2004). However, the development of the axial musculature, which approximately comprises up to 60-70 % of the total body mass of teleosts and is responsible for their nutritional and economic value, has hardly been studied until now. Uniquely, in a previous short work we described the muscle growth dynamics of larvae when reared at different temperatures or fed different diets (Arizcun et al., 2003).

The development of the axial musculature of teleosts begins early in embryonic life. Shortly after segmentation of the paraxial mesoderm the recently

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formed metameric somites become subdivided into three distinct tissues: the sclerotome, the primary (embryonic) myotome and the dermomyotome. Whereas it is well established that the sclerotome forms the axial skeleton (Morin-Kensicki and Eisen, 1997), and the primary myotome the earliest muscle fibres of embryos (superficial slow and deep fast muscle fibres) (Devoto et al., 1996; Stoiber et al., 1998), it is only in recent years that the presence of a dermomyotome has been demonstrated in teleosts (Devoto et al., 2006; Stellabotte and Devoto, 2007). Instead, a population of undifferentiated external cells located on the surface of the developing myotomes was described in different species, including zebrafish *Danio rerio*, sea bass, sea bream, herring *Clupea harengus*, pearlfish *Rutilus meidingeri* (Waterman, 1969; Veggetti et al., 1990; Johnston, 1993; López Albers et al., 1998; Stoiber et al., 1998). Recent studies have demonstrated the external cells are a source of myogenic and dermal precursors, so that this cellular structure is homologous to the amniote dermomyotome (Devoto et al., 2006; Stellabotte et al., 2007). Thus, similarly to amniotes the dermomyotome of teleosts plays a key role in the development of the axial musculature, the dorsal fin muscle, the dermis and the vascular endothelia (Devoto et al., 2006; Stellabotte and Devoto, 2007). However, contrarily to amniotes prior to the dermomyotome there is a primary myotome which consists of an external monolayer of slow muscle fibres over a deeper mass of fast muscle fibres, both of them postmitotic and mononuclear (Scaal and Wiegrefe, 2006). Cells of the primary myotome of teleosts never express detectable levels of dermomyotome markers such as Pax7, Meox or dacD (Stellabotte and Devoto, 2007).

Following the early differentiation of the primary myotome (embryonic myogenesis), muscle fibre recruitment of teleosts continues during the embryonic, larval and even postlarval life. In the second myogenesis, also known as stratified hyperplasia (Rowlerson and Veggetti, 2001) new muscle fibres are added in germinal areas located in the periphery of the myotome, such as the epaxial and hypaxial extremes, the lateral boundary of the fast muscle, and over the slow muscle, next to the horizontal septum. Although the stratified hyperplasia has been traditionally considered a posthatching phenomenon commonly associated to the onset of external feeding (Rowlerson and Veggetti, 2001), recent investigations based on molecular approaches to the expression levels of myogenic regulatory factors (MRFs) in myotome and dermomyotome cells have alternatively reported an embryonic beginning of this myogenic period (Steinbacher et al., 2006, 2007). In embryos of trout (*Onchorynchus mykiss*) and pearl fish, Pax7+, Myogenin- cells of the posterior lip of the dermomyotome enter the subjacent myotome, proliferate and eventually differentiate into myogenic precursors (Pax7-, Myogenin+, MyoD+) of slow and fast muscle fibres.

Similarly, the third myogenesis or mosaic hyperplasia is not only a postlarval phenomenon of large final size teleosts but a distinct process of muscle expansion that may also be initiated in the early stages of development (Stoiber and Sanger, 1996). Mosaic hyperplasia is characterized by the presence of new, small muscle fibres interspersed between older, bigger muscle fibres. The cell source for such hyperplastic process has been a traditional subject of discussion which still remains unclear. Typical myosatellite cells located under the basal lamina of mature muscle fibres are considered to be the cell precursors of the new muscle fibres of the mosaic in juvenile and adult large size species (Koumans and Akster, 1995). However, the specific ancestry of the population of myosatellite cells has not been established yet in teleosts. Whether the dermomyotome of teleosts is involved in the origin of the population of myosatellite cells is a subject that is in present debate, as mechanisms other than those in amniotes might account for the insertion of the fish mosaic precursors (Marschallinger et al., 2009). A precise knowledge of all these events is not only required from an embryological point of view, but also important to understand other related aspects, such as swimming activity, larval survival and postlarval growth trends. All these aspects are of particular importance in candidate species for the farming industry.

Dealing with the potential farming relevance of the common dentex the present work is aimed at describing the major histological events occurring in the axial musculature of this species from hatching to the juvenile period. The main goals of the study are defining the progressive maturation of the presumptive red and white muscles, typifying the different types of muscle fibres and illustrating the cellular basis for the hypertrophic and hyperplastic growth of muscle fibres. Transmission electron microscopy and histochemical and immunohistochemical techniques have been used for this purpose. This work may contribute to a better understanding of the biology of the common dentex and enhances the specificity of the development of the axial musculature of teleosts.

Materials and methods

Common dentex eggs were obtained at the Instituto Español de Oceanografía (Centro Oceanográfico de Murcia, Mazarrón) from spawners adapted to captivity. Several spawns were selected in different years: April and May of 2003 to 2006. The appropriate quality of the eggs was checked before incubation. Tanks of different shapes and sizes were used for egg incubation, larval and postlarval rearing. The phenotypical characters and swimming activity of larvae were carefully surveyed on a daily basis. Larvae were reared under a light:dark regime of 12:12 and temperature regimes were those of the Spanish south-eastern coast. The feeding regimes - the same in all stocks - consisted of enriched rotifers *Brachionus plicatilis*, from mouth opening to 19-20 days

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post hatch (dph) at a concentration of 20 rotifers ml⁻¹ plus green water (*Nannochloropsis gaditana* and *Tetraselmis suecica*). *Artemia* sp. nauplii were supplied from 14 to 21 dph and enriched *Artemia* sp. metanauplii from 21 to 41 dph at a concentration of 5 specimens/ml. Dry pellets of increasing size were supplied from 31 dph onwards.

During the larval period fish were sampled on a daily basis and classified into stages I-III, according to phenotypic characteristics and nutrition events (see results for details). Postlarvae (stage IV) were sampled every 3-5 weeks, until a final age of 232 dph (18±1.3 cm), so none of the individuals had presumably reached reproductive maturity. Larvae and early postlarvae (until 52 dph) were delivered alive to the Veterinary Faculty (University of Murcia) in opaque aerated containers. After a rest of 20-30 min. fish were overanaesthetized with tricaine methanesulphonate (MS222, Sigma). Postlarvae older than 52 days were overanaesthetized with clove oil (Guinama^R) in the Oceanographic Institute and immediately delivered to the Veterinary Faculty in polystyrene boxes with ice pellets. Depending on the specimen size, the whole larvae, the caudal half of the fish or a 1x1x1 cm block of the axial musculature were processed for these three different methods: paraffin embedding, epoxy embedding and tissue freezing. Specimens for paraffin embedding were fixed in 10% buffered formaline or Bouin fixative, whereas for epoxy embedding specimens were fixed in 2.5% glutaraldehyde in cacodylate buffer for 3 h at 4°C. Freezing of samples was carried out in 2-methylbutane cooled over liquid N₂. Frozen samples were then stored at -65°C until cryostat sectioning. After tissue fixation or freezing, the subsequent processing of the material was carried out according to the common light and electron microscopy protocols of the Microscopy Service of the University of Murcia (see López-Albors et al., 1998 for details). 10 randomly selected specimens per processing methodology were used at each sampling stage.

Muscle development was studied by both transmission electron microscopy and light microscopy techniques (bright field). For the transmission electron microscopy study, semithin and ultrathin sections were obtained from epoxy embedded material. Ultrathin sections were viewed in Zeiss M-10 and Philips Tecnai 12 (Megaview II) transmission electron microscopes at

80 kV. Histological (Haematoxyline/Eosine), histochemical (mATPase and NADH-TR reactions) and immunohistochemical (S-58 antibody) techniques were used to define muscle fibre types throughout the larval and postlarval periods. Additionally, the possible apoptosis of muscle progenitors was evaluated by means of the TUNEL reaction method. Further details of the histochemical and immunohistochemical methods have been summarized in Table 1. S-58 antibody was obtained from I.A. Johnston (Gatty Marine Lab, St Andrews), and the apoptosis detection kit (TUNEL reaction) from R&D Systems Inc, Minnesota, Minn.

Results

Stage I (yolk sac period, 1-3 dph, initial size 3.1±0.1 mm)

At hatching (Fig. 1), the axial musculature is arranged in an initial series of 25 to 28 myotomes with a caudally opened V-shape. A cross-section of the body just behind the anal opening shows the notochord and spinal chord, both centrally situated and surrounded by the corresponding right and left myotomes. Two muscle layers -superficial and deep muscles- are observed in the myotomes. Throughout the larval life these layers progressively differentiate into red and white muscles, respectively. Muscle fibres in the superficial layer (superficial red muscle fibres) are subdermally located and consist of a monolayer of squared or rectangular muscle fibres that extend through the entire myotome by attaching to successive myosepta (myocommata). These fibres run in parallel to the longitudinal axis of the body. Superficial red muscle fibres contain abundant mitochondria but scarce myofibrillar content. Myofibrils tend to be located at the deep side of muscle fibres, whereas mitochondria and nuclei are found towards the external side. The mitochondria are big and full of cristae, the nuclei euchromatic and the sarcoplasm highly granular. Beneath the superficial red fibres there are several layers of immature deep (white) muscle fibres, which extend rather obliquely between successive myosepta. Immature white muscle fibres (myotubes) have centrally located nuclei, detached nucleoli and active myofibrillogenesis. Myofibrils initially spread over the sarcoplasm but in a more mature state (end of this period) they assemble into well organized

Table 1. Summary of histochemical and immunohistochemical techniques.

Technique	Target	Reference
NADH-TR	Oxidative metabolism of muscle fibres	López Albors et al., 1998
mATPase after acid (pH 4.6) and alkaline (9.45, 10.1 and 10.3) preincubations	Myosin ATPase isoforms	Mascarello et al., 1986
S-58	Chicken slow muscle myosin	Johnston et al., 2004
TUNEL	Apoptotic nucleus	Morales et al., 2007

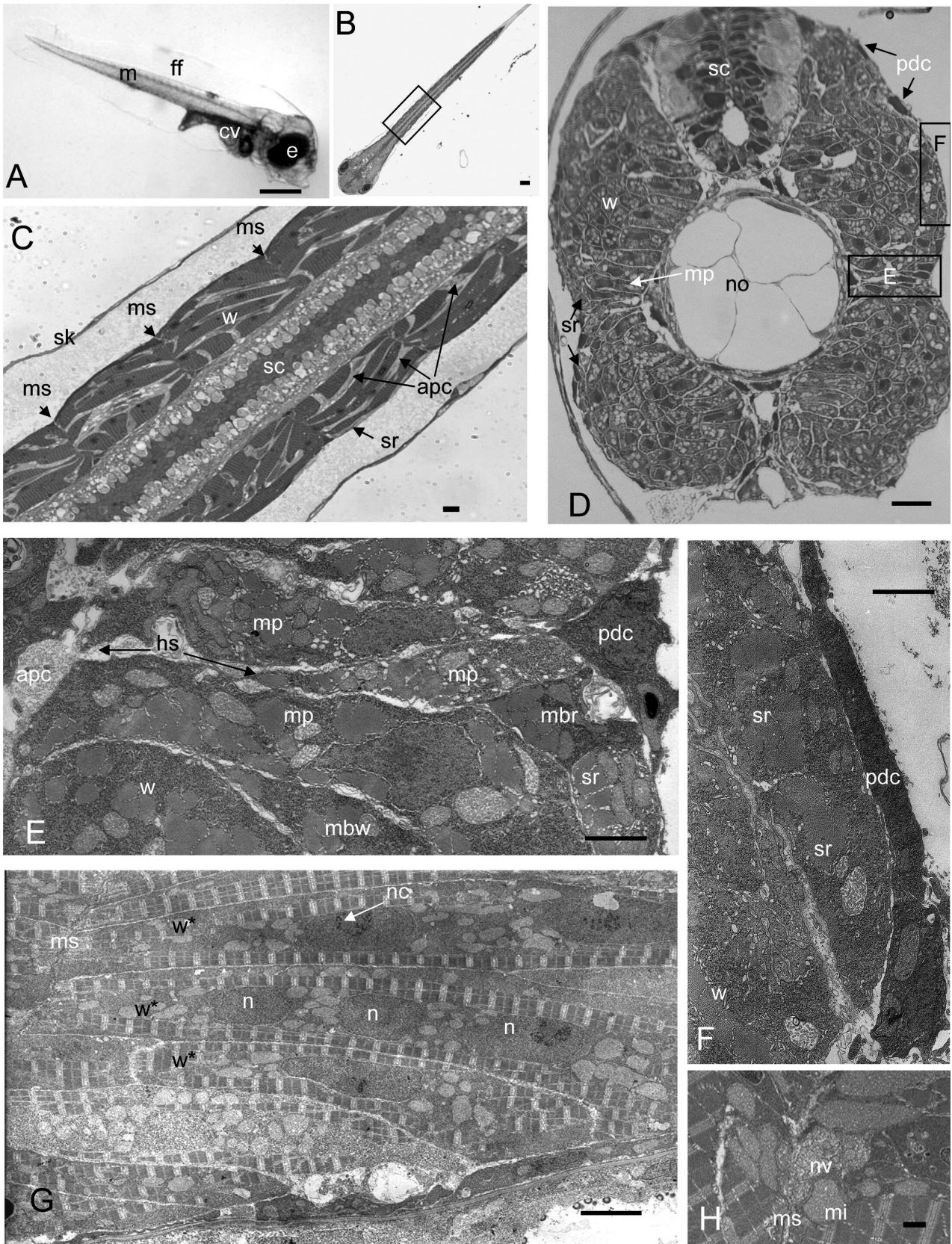


Fig. 1. Stage I: yolk sac period. Except when indicated, all larvae are 1 dph. **A.** External morphology of a 3 dph larvae. **B and C.** Horizontal section of 2 dph larvae. **C.** Is a magnification of the selected area in **B.** **D.** Transversal semithin section of the myotomal musculature stained with Toluidine Blue. **E-H.** Transmission electron micrographs of the myotome. **E and F** are transversal sections from corresponding zones indicated in **D** (different specimen). **G and H** are longitudinal sections from the inner part of the deep white muscle, or at the level of a myoseptum, respectively. apc: atypical premyoblastic cell, cv: celomic viscera, pdc: presumptive dermomyotome cell, e: eye, ff: fin fold, hs: horizontal septum, m: myotomes, mbr: myoblast (red), mbw: myoblast (white), mi: mitochondria, mp: muscle pioneer, ms: myoseptum, n: nucleus, nc: nucleolus, nv: nerve, no: notochord, sr: superficial red muscle fibre, sc: spinal chord, sk: skin, w: white muscle fibre, w*: white myotube. Scale bars: A, 0.6 mm; B, 0.12 mm; C, 7.7 μ m; D, 12 μ m; E, 2.3 μ m; F, 2.1 μ m; G, 1.8 μ m; H, 1.98 μ m.

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sarcomeres. An effective contractile activity of muscle fibres is guaranteed at this stage by the early innervation, which reaches the myotomes via the myosepta or the lateral nerve. A slight septum of connective tissue - horizontal septum- separates the epaxial (dorsal) and hypaxial (ventral) half of the myotomes. Adjacent to this septum a few muscle fibres extend from the inner to the outer part of the myotome. According to their location and ultrastructure these fibres correspond to the muscle pioneers described in other teleosts (Felsenfeld, 1991; Johnston et al. 1997). On the surface of the superficial muscle fibres there is a monolayer of flat cells with long fingerlike projections, heterochromatic nuclei and cytoplasm full of ribosomes. These presumptive dermomyotome cells cover the primary myotome from the mid-lateral line to the epaxial and hypoaxial boundaries.

Two days after hatching the cell populations in the myotome display new structural characteristics, and some new cell types are distinguished (Fig. 2). At the level of the horizontal septum a few typical myoblasts - with scarce but evident contractile material- can be observed (Fig. 2D). In the epaxial and hypoaxial limits of the myotome presumptive dermomyotome cells are abundant (Fig. 2E), and some of them seem to enter the white muscle from the periphery. Cells with a similar ultrastructure to the presumptive dermomyotome cells are also occasionally observed between the superficial red and deep white muscles (Fig. 2F). Also, among the white muscle fibres an unexpected and atypical population of cells is now found. We have tentatively called these cells atypical premyoblastic cells (APC). APC are very rare at hatching but very common in larvae older than two days, and their number increases significantly throughout the larval life. APC are mononuclear cells with a cytoplasm full of thin, longitudinally projected tubules which, in cross-section, appear as round, empty vesicles (Fig. 2). Small mitochondria and scattered ribosomes are also common. Nuclei are euchromatic and nucleoli either detached or absent. Dealing with the chronological sequence observed in this chapter, further structural and histochemical description of these cells is given in the text of the following stages, as well as in Figures 3-5.

Histochemically, only the superficial red and deep

white muscle fibres are differentiated in this period (Table 2, Fig. 2). Both fibre types are identified by their oxidative activity (NADH-TR reaction) and myosin content (S-58 immunostaining). Accordingly, the superficial red muscle fibres are classified as slow-twitch, highly oxidative fibres, and the white fibres as fast twitch, poorly oxidative (glycolytic) muscle fibres. The mATPase reaction is hardly capable of differentiating fibre types at this moment, being slightly positive for the red and white muscle fibres after alkaline preincubations, but negative for white fibres after acid preincubations.

Stage II (free swimming larvae, 4-13 days after hatching, initial size 3.5±0.1 mm)

Once the mouth is open and the yolk sac exhausted larvae exhibit new external features and active swimming aimed at searching for external food. Swimming is supported by the coordinated contraction of the myotomes which, at microscopical level, display higher maturity and a higher number of muscle fibres and myogenic cells (Fig. 3). In this sense, soon after the beginning of the external feeding a considerable proliferation of presumptive myogenic cells and myoblasts occurs at the level of the horizontal septum (Fig. 3A). Similarly, proliferation clusters are observed at both sides of the horizontal septum, between the red and white muscles. From all these germinal zones, red and white muscle fibres are recruited. Later on (8-9 dph), additional active germinal zones are observed at the periphery of the myotomes (epaxial and hypaxial limits) (Fig. 3B-D) Most muscle fibres recruited here join the white muscle, so a clear image of progressive muscle fibre differentiation from the outer to the inner part of the myotome is distinguished. All these typical images of muscle fibre hyperplasia correspond to the 2nd myogenesis or stratified hyperplasia (Rowlerson and Veggetti, 2001).

Despite displaying increased structural maturity, the superficial red and white muscle fibres do not change their histochemical profile (Table 2). Conversely, the population of APC has significantly increased in number and tends to form parallel rows, which are intermingled among the white muscle fibres (Fig. 3D,E).

Table 2. Histochemical properties of muscle fibre types.

Fibre type	Stage	NADH-TR	S-58	mATPase acid	mATPase alkaline
Superficial Red	I to 90-100 days	+++	++	++	++
Adult Red	IV (since 70/80 days)	+++	++	-	-
White	I - onward	-	-	+/-	+
Small White	III - onward	-	-	+/-	+
Intermediate (pink)	End stage III - onward	+	-	++	++
Tonic	III - onward	-	++	+/+	++

-: negative; +: moderate; ++: intense and +++: very intense staining.

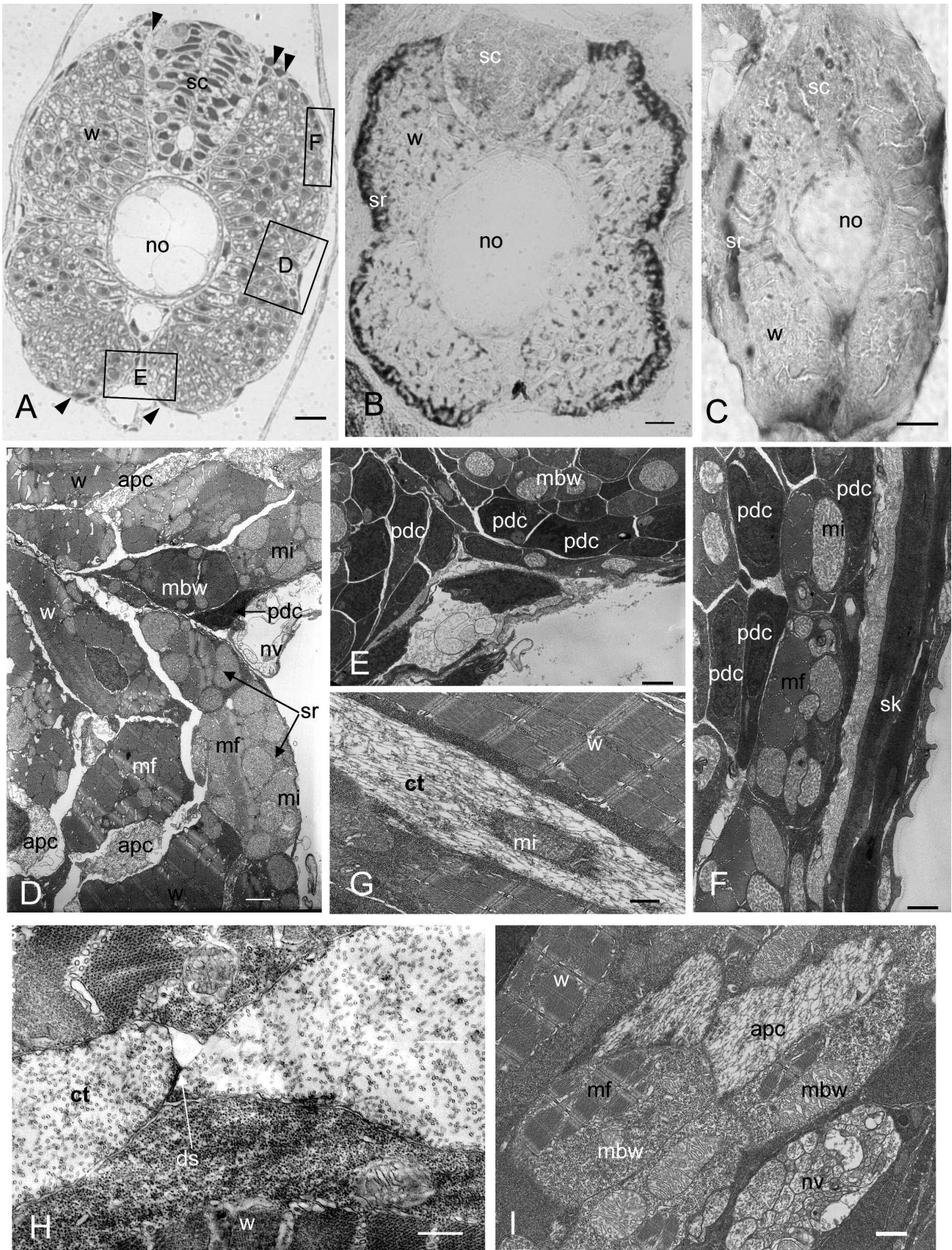


Fig. 2. Stage I: yolk sac period. Larvae 2-3 dph. Transversal sections stained for Toluidine blue (**A**), NADH-TR (**B**) and mATPase, pH 4.6 (20 sec) (**C**). **D-F**. Transversal transmission electron micrographs of the myotome. The corresponding zones are indicated in picture A (different specimen). **G-I**. Transversal and longitudinal transmission electron micrographs of atypical premyoblastic cells. APC show abundant longitudinal cytoplasmic tubules (ct) and occasional desmosome unions (ds). apc: atypical premyoblastic cell, mbw: myoblast (white), mf: myofibrils, mi: mitochondria, no: notochord, nv: nerve; pdc: presumptive dermomyotome cell (arrowheads in A), sc: spinal cord, sk: skin, sr: superficial red muscle fibres, w: white muscle fibre. Scale bars: A, 8 μm ; B, 10 μm ; C, 15 μm ; D, 1.26 μm ; E, 0.95 μm ; F, 0.98 μm ; G, 1.07 μm ; H, 0.35 μm ; I, 1 μm .

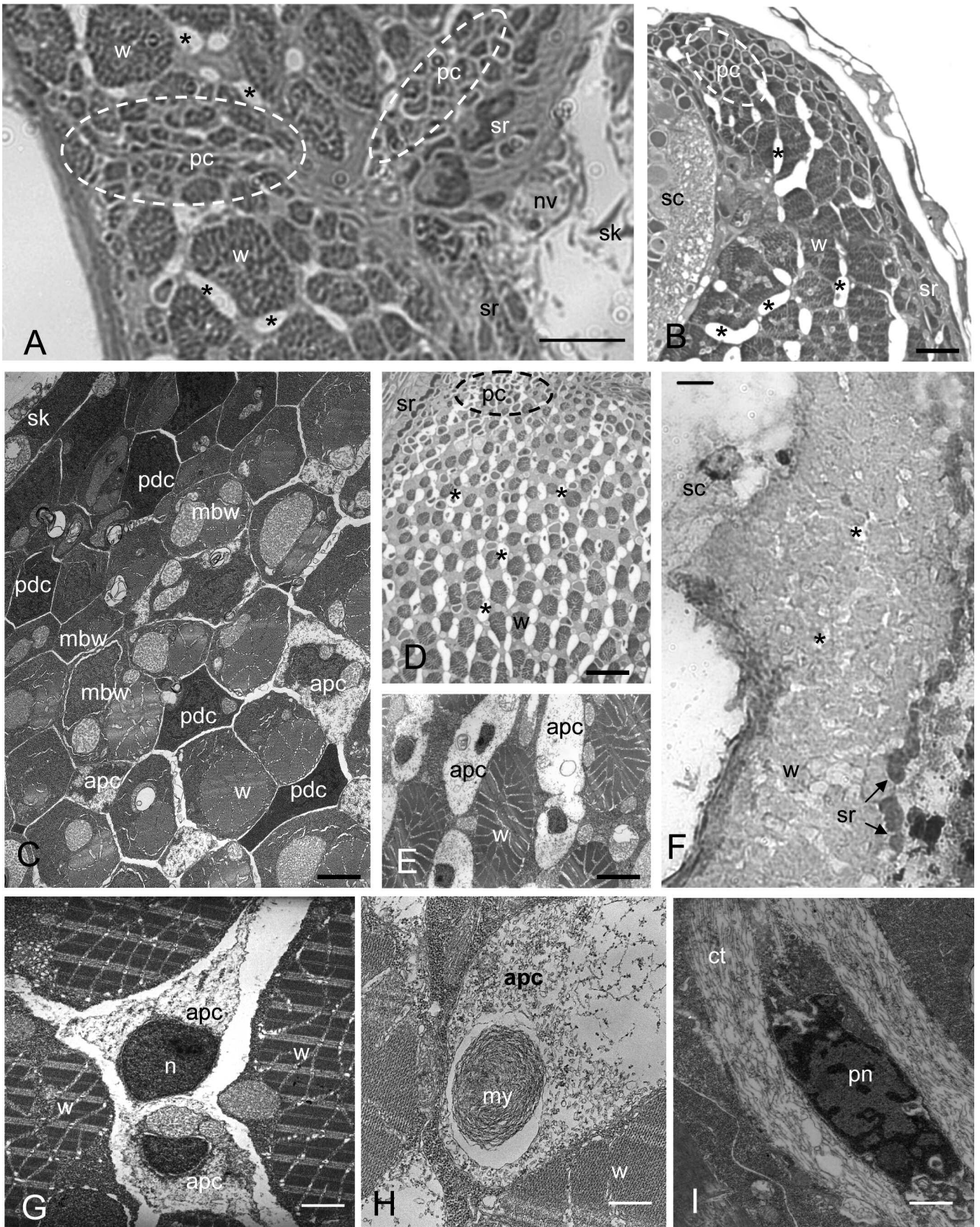


Fig. 3. Stage II: free swimming larvae. Transversal sections of larvae aged 9 (**A-C**), 13 (**D-F**), 8 (**G, H**) and 10 dph (**I**). **A and B.** Semithin sections stained for Toluidine blue at the level of the horizontal septum and the epaxial right quadrant (caudal view), respectively. **C-E.** Electron micrographs and semithin section (**D**) of the white muscle. **F.** Frozen section stained for S-58 antibody. **G-I.** Electron micrographs of atypical premyoblastic cells. Sporadic apoptotic bodies such as myelinic figures (my) and pyknotic nucleus (pn) with clumped chromatine were occasionally found. apc: atypical premyoblastic cells, asterisc (*): atypical premyoblastic cells under light microscopy viewing, ct: cytoplasmic tubules (within apc), mbw: myoblast (white), nv: nerve, pc: proliferation cluster, pdc: presumptive dermomyotome cell, sc: spinal chord, sk: skin, sr: superficial red fibre, w: white muscle fibre. Scale bars: A, B, 10 μm ; C, 2.5 μm ; D, 17 μm ; E, 0.65 μm ; F, 22 μm ; G, 1.74 μm ; H, 0.73 μm ; I, 0.65 μm .

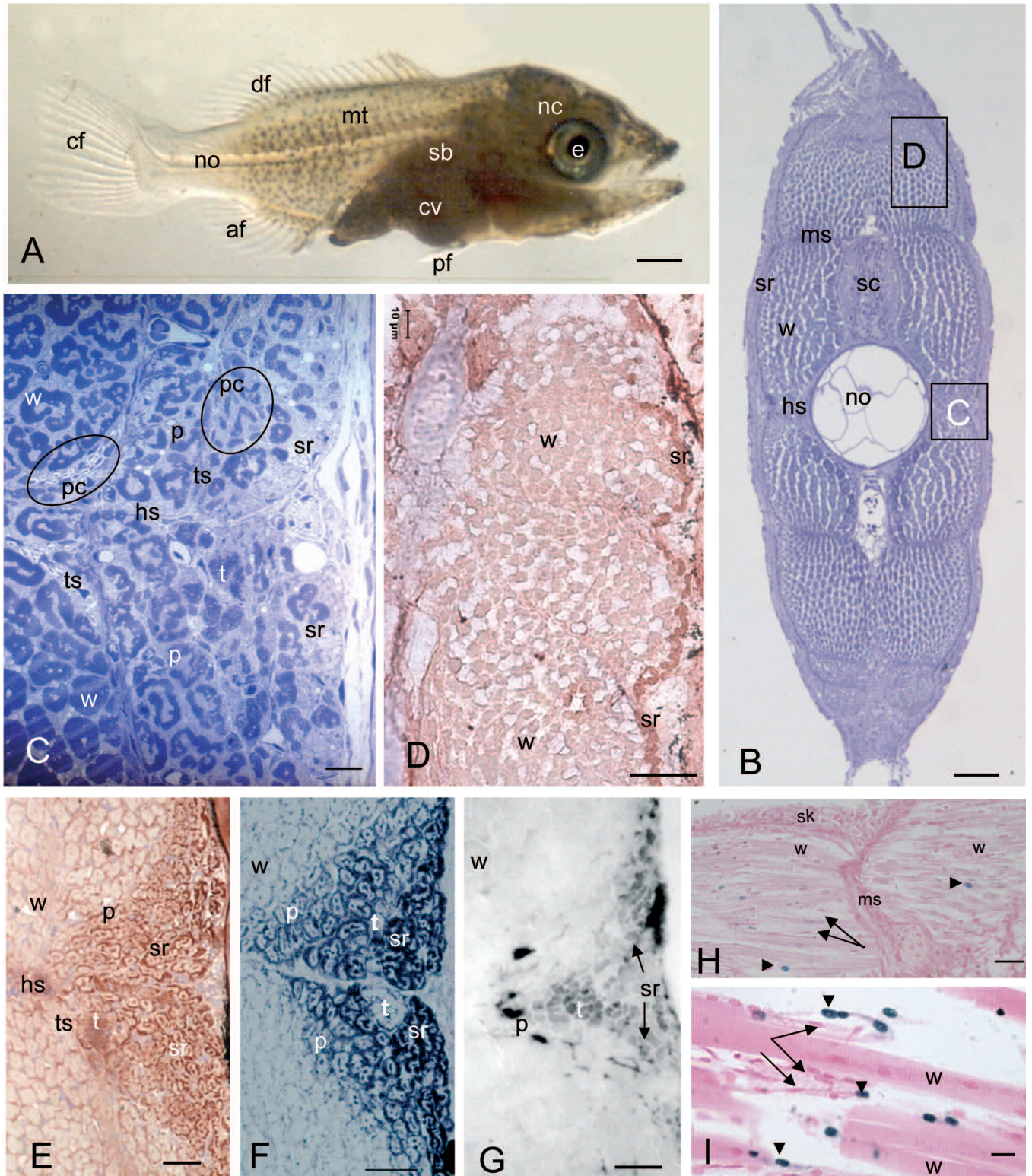


Fig. 4. Stage III: larval metamorphosis. **A.** Free swimming larvae 23 dph. **B.** Transversal section just caudally to the anal opening (15 dph). Squares have been drawn to indicate correspondent zones in pictures **C** and **D** (different specimens). **C.** Semithin section stained for Toluidine Blue (28 dph). **D.** S-58 antibody staining (20 dph). **E.** S-58 antibody staining (34 dph). **F.** NADH-TR staining (27 dph). **G.** Acid mATPase reaction (pH 4,6 20 sec) (32 dph). **H, I.** TUNEL staining, larvae aged 18 and 41 dph, respectively. af: anal fin, sb: swim bladder, cf: caudal fin, cv: celomic viscera, df: dorsal fin, e: eye, hs: horizontal septum, ms: myoseptum, mt: myotomes, nc: neurocranium, no: notochord, p: pink (intermediate) muscle fibre, pc: proliferation cluster, pf: pelvic fin, sb: swim bladder, sc: spinal chord, sr: superficial red muscle fibres, t: tonic fibres, ts: transversal septum, w: white muscle fibre, arrows: TUNEL (-) nuclei of atypical premyoblastic cell (no apoptosis), arrowheads: TUNEL (+) nuclei of atypical premyoblastic cell (apoptosis). Scale bars: A, 0.4 mm; B, 50 μ m; C, 8 μ m; D, 20 μ m; E, 35 μ m; F, 22 μ m; G, 30 μ m; H, I, 10 μ m.

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Stage III (larval metamorphosis, 14-35 days after hatching, initial size 4.9±0.4 mm)

Throughout this stage all the myotomes achieve a typical W-shape. Thus, several myosepta are now observed in a cross-section of the trunk (Fig. 4). The white muscle comprises most of the myotomal mass, whereas the red muscle still consists of a monolayer of superficial fibres which at the level of the lateral line expands to several layers. Dorsally and ventrally to the horizontal septum a thin, vertical fascia –the transversal septum– progressively extends between the red and white muscles.

White muscle growth is very intense in this period. Recruitment of new muscle fibres by stratified hyperplasia (2nd myogenesis) is not only observed in the vicinity of the horizontal septum and the epaxial and hypaxial limits of the myotome, but in close relation with the incipient transversal septum. Additionally, according to ultrastructural evidence new white myoblasts are recruited from the population of APC (Fig. 5). APC increase the amount of ribosomes and mitochondria, differentiate a relevant rough endoplasmic reticulum and Golgi complex, and synthesize some clusters of myofibrils. Nuclei are euchromatic and nucleoli big and round. The broad system of smooth tubules seems to be used as a matrix to develop the sarcoplasmic reticulum and T-tubule system. Myofibrillar synthesis determines the progressive appearance of immature but well organized myofibrils within the sarcoplasm. Myogenesis based upon the differentiation of APC seems to establish the beginning of the so called mosaic hyperplasia (Rowlerson and Veggetti, 2001). However, shortly after the end of the larval life the population of APC becomes apparently exhausted. Typical morphological features of APC death or degeneration, such as myelinic figures and pyknotic nucleus with clumped chromatin have been observed throughout the larval period (Fig. 3). Such structural findings deal with the presence of TUNEL+ cells among the white muscle fibres towards the end of this stage and the beginning of the postlarval life (Fig. 4).

Two new types of muscle fibres appear during the larval metamorphosis: intermediate (pink) and tonic (Fig. 4). Intermediate muscle fibres are located between the red and white muscles, and compared to them exhibit intermediate structural characteristics. These fibres progressively spread through the transversal septum towards the apices of the myotome. The histochemical profile of the intermediate muscle fibres is summarized in Table 2 and shown in Figure 4(E-G). Tonic fibres form a cluster of small, round muscle fibres located deep in the red muscle at the level of the horizontal septum. These fibres are clearly smaller than the superficial red muscle fibres, have packed myofibrils and scarce mitochondria. Histochemically, they have slow contracting myosin (S-58+) and low oxidative capacity (NADH-TR-) (Table 2, Fig. 4E-G). On the other hand, no more presumptive dermomyotome cells are found

around the myotomes during this period.

Stage IV (postlarvae, 35 days onward, initial size 13.3±1.3 mm)

During the postlarval life both the hypertrophy and hyperplasia of the red and white muscle fibres are responsible for an intense and continuous growth of the myotome. In the white muscle the mosaic hyperplasia becomes the main mechanism of muscle growth. As stated before, the mosaic hyperplasia is apparently initiated towards the end of the larval period by a myoblastic differentiation of the APC. However, throughout postlarval life, scattered, typical premyoblastic cells (presumptive satellite cells) are observed among the white muscle fibres (Fig. 6). Presumptive myosatellite cells are surrounded by a thin connective layer or basal lamina, which in most cases is shared with that of the adult fibres. As a result the white muscle displays a typical mosaic image in the cross-section.

The histochemical profile of muscle fibres shows slight changes during the postlarval life (Table 2, Fig. 6). These are mainly referred to the mATPase activity of red and white muscle fibres. From 70-80 dph (7-8 cm), the mATPase activity of the superficial red muscle fibres progressively decreases after both alkaline and acid preincubations. In contrast, at the level of the lateral line new recently recruited red muscle fibres exhibit low mATPase activity (adult red). Thus, according to mATPase staining, two distinct layers of red muscle fibres can be transitionally observed at the level of the lateral line in juveniles of 70-90 days (Fig. 6G). Later on all the red muscle fibres display the same mATPase staining (adult red) (Fig. 6H). On the other hand, all the red muscle fibres show high oxidative capacity and a positive reaction against the S-58 antibody which confirms their slow myosin composition (not shown). Concerning the mATPase activity of the white muscle fibres a plurality of staining grades is progressively established. In contrast to what is a common result in the literature, no correspondence between the size of the fibres and the staining intensity is established. Depending on the sample and zone within the myotome, the highest mATPase activity either corresponds to the smallest (recently formed) or biggest (older) white muscle fibres (Fig. 6I). Thus, no correlation between the developmental stage of the muscle fibres and the mATPase staining is established during the postlarval life of the common dentex. On the other hand, it is interesting to mention that despite becoming fast muscle fibres the new muscle fibres of the white mosaic are initially positive against the S58 antibody (slow myosin content) (Fig. 6D).

Discussion

The development of the axial musculature of the common dentex has been described in detail from

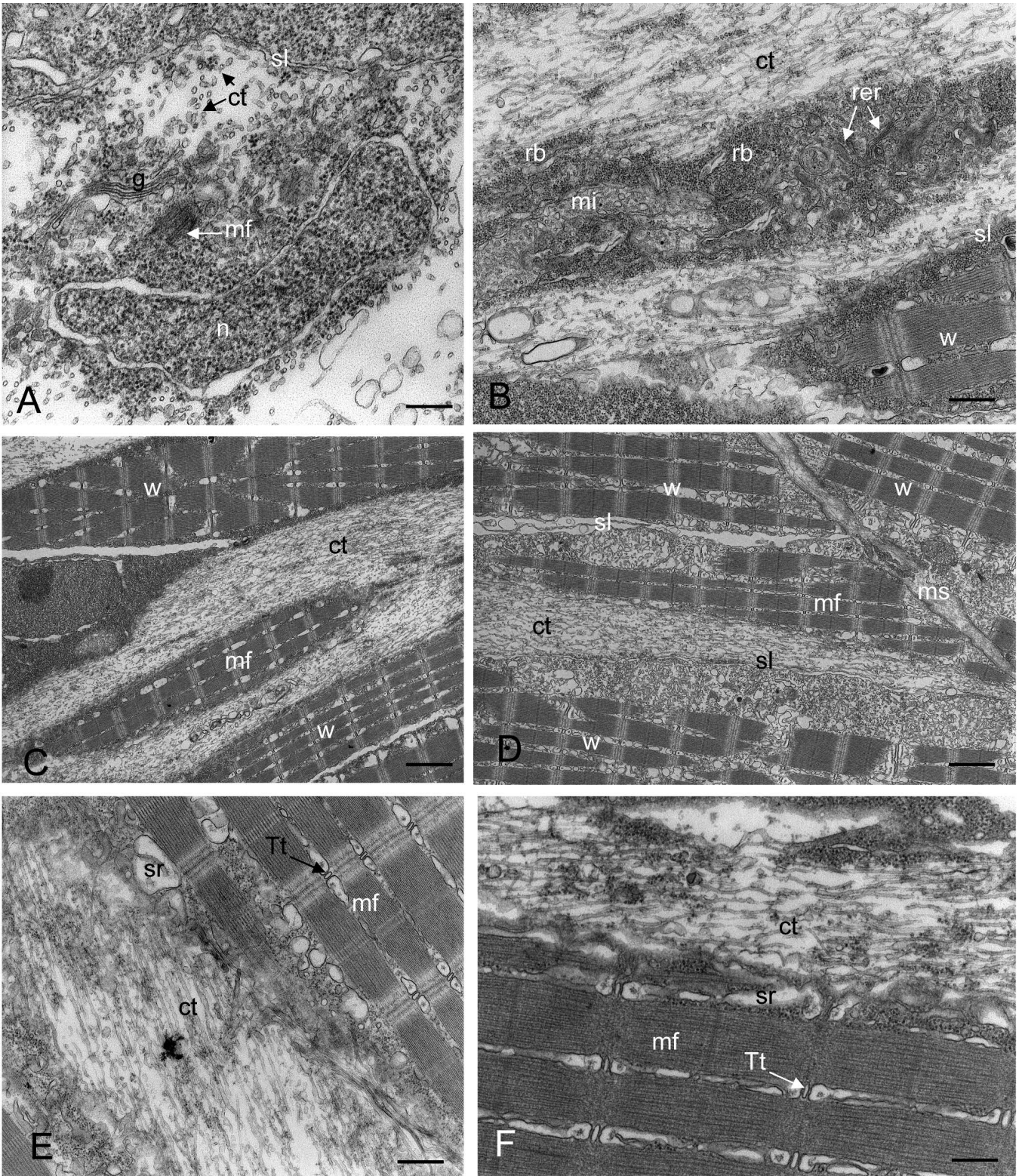


Fig. 5. Stage III: larval metamorphosis. Transmission electron micrographs of APC. **A and B.** Activation of myoblastic differentiation. Poor amount of myofibrils but abundant ribosomes, rough endoplasmic reticulum and Golgi are evident. **C-F.** More advanced stages of myoblastic differentiation: myofibrils have been assembled, cytoplasmic tubules apparently become sarcoplasmic reticulum and some APC attach to the myoseptum. ct: cytoplasmic tubules, g: Golgi complex, mf: myofibrils, mi: mitochondria, ms: myoseptum, n: nucleus, rb: ribosomes, rer: rough endoplasmic reticulum, sl: sarcolemma, sr: sarcoplasmic reticulum, Tt: T-tubule, w: white muscle fibre. Scale bars: A, 0.23 μm ; B, 0.75 μm ; C, 2.15 μm ; D, 1.52 μm ; E, 0.54 μm ; F, 0.42 μm .

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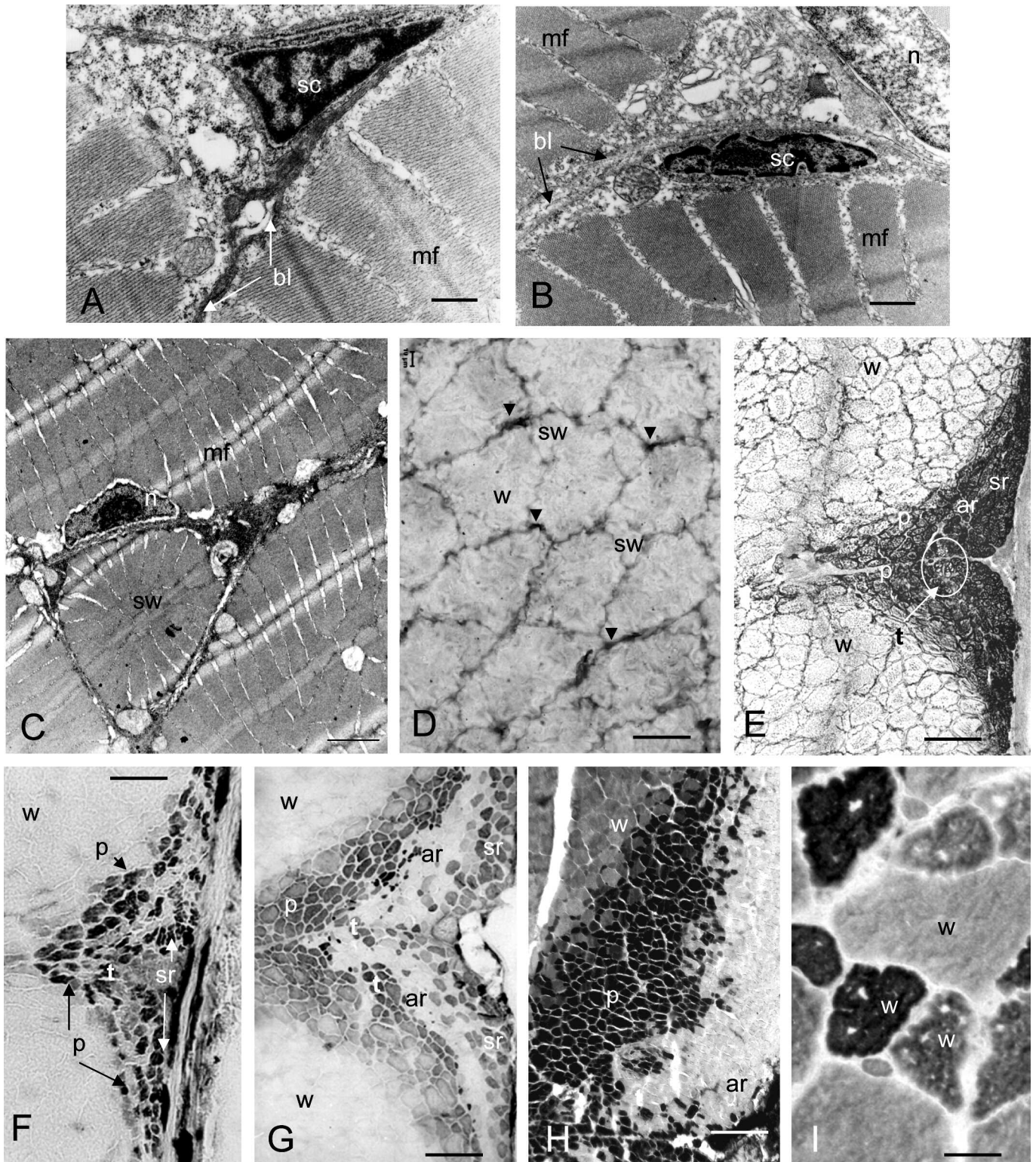


Fig. 6. Stage IV: postlarvae. **A-C.** Transmission electron micrographs of the white muscle in postlarvae aged 70, 250 and 50 dph. **D.** S-58 antibody against white muscle fibres (64 dph). **E.** NADH-TR (63 dph). **F.** mATPase, pH 4.6 20 sec (52 dph). **G.** mATPase, pH 4.6 30 sec (77 dph). **H.** mATPase, pH 4.6 30 sec (195 dph). **I.** mATPase, pH 4.6 20 sec (189 days). ar: adult red muscle fibres, bl: basal lamina, mf: miofibrils, n: nucleus, p: pink (intermediate) muscle fibres, sc: presumptive myosatellite cell, sr: superficial red muscle fibres, sw: small white muscle fibre, t: tonic muscle fibres, w: white muscle fibre, arrowheads: S-58 (+) cells. Scale bars: A, 1.07 μm ; B, 1.15 μm ; C, 2.5 μm ; D, 25 μm ; E, 105 μm ; F, 110 μm ; G, 115 μm ; H, 240 μm ; I, 45 μm .

hatching to the juvenile period. During the larval phase muscle growth is very intense, concomitant with changes in external features and related to swimming demands. The development of muscle fibre types is also fairly rapid as the majority of the adult types are already differentiated by the end of the larval period. Other tissues and organs also undergo intense morphological changes throughout the larval period: the digestive system and its associated glands, the gills, swim bladder, heart (Santamaría et al., 2004; Carrascon et al., 2006) and skeleton (Koumoundouros et al., 1999, 2000, 2001). Organogenesis is most crucial during the early steps of the larval life of teleosts so as to allow the larvae to change from an endogenous to exogenous food supply, to swim actively in search of living preys or escape from predators and, in the end, culminate the metamorphosis with a complete functional and morphological competence. Once the larval metamorphosis is accomplished dentex juveniles display very high body growth rates associated with voracious appetite during the summer period, which is consistent with its predator nature throughout its adult life. Concerning the development of the axial musculature, the consolidation of the histochemical maturity of all the muscle fibre types and the continuous growth of the musculature by both hypertrophy and recruitment of muscle fibres are the two of the most relevant topics throughout the juvenile period. On the whole, the larval and postlarval development and growth dynamics of the axial musculature of the common dentex is hardly different from what has been described in other sparidae, such as the gilthead sea bream, the red sea bream *Pagrus major* and the blackspot seabream *Pagellus bogaraveo* (Matsuoka and Iwai, 1984; Mascarello et al., 1995; López Albors et al., 1998; Patruno et al., 1998; Ayala et al., 1999; Silva et al., 2009).

The rapid development and growth dynamics of the axial musculature of dentex larvae determines relevant structural changes in both the myotomes and muscle fibres. The number of myotomes increases from 25 to 28 at hatching to a final number of 33-34 by the end of the yolk sac period (stage I). Related to that, the muscle pioneers located in the core of the myotomes at the level of the horizontal septum are involved in the morphogenesis of the myotomes, and particularly in determining their ultimate W-like morphology (Hatta et al., 1991). Both the monolayer of superficial muscle fibres and the more immature deep muscle fibres present at hatching progressively differentiate into superficial red and white muscle fibres, respectively. Innervation of muscle fibres is established early after hatching, hence enabling the recently hatched larvae to perform sudden and complete flexions of the tail while floating passively. Such twitching activity of muscle fibres may be essential to adjust the neuromuscular excitatory system and to allow larvae to escape from predators (Patruno et al., 1998). Muscle growth during the larval period is powered by hypertrophy of the existing muscle fibres and the recruitment of new fibres from

undifferentiated muscle precursors (hyperplasia). Regulation of these two mechanisms of muscle growth depends on the production levels of growth factors, such as the insulin-like growth factor I (IGF-I) and myostatin, which in the sea bass are strictly linked to the proliferative activity of the red and white muscle fibres (Patruno et al., 2008). According to the structural features observed in this study two morphologically different populations of muscle precursors have been found in the myotomes of dentex larvae: presumptive dermomyotome cells and atypical premyoblastic cells (APC). Differentiation of these two populations of cells has been observed to give rise to the successive phases of stratified and mosaic hyperplasia, respectively.

It is in recent years that the existence of a dermomyotome has been described in teleosts (Devoto et al., 2006; Stellabotte and Devoto, 2007; Rescan et al., 2008). Previously, and for more than three decades, dermomyotome cells were named as external cells according to their undifferentiated morphology and location over the superficial red muscle (Waterman, 1969; Veggetti et al., 1990; Brooks and Johnston, 1993; Johnston, 1993; López Albors et al., 1998). Further features of the dermomyotome cells are referred to their expression of a large set of genes homologous to those transcribed in the amniote dermomyotome, including both myogenic and dermal molecular markers (Devoto et al., 2006; Dumont et al., 2008). Based on their location and ultrastructure, presumptive dermomyotome cells have been observed in common dentex larvae since hatching. Initially, a single monolayer of presumptive dermomyotome cells with long fingerlike projections, heterochromatic nuclei and narrow cytoplasm full of ribosomes is found over the superficial red muscle fibres. Two days after hatching the number of these cells increases significantly towards the epaxial and hypoaxial limits of the myotome. At this point, some of them seem to enter the myotome and spread around the periphery of the deep white muscle or intermingle among the white muscle fibres (Figs. 2E,F, 3C). Although a precise identification of the presumptive dermomyotome cells by molecular markers has not been carried out in this study, similar morphological characteristics and migration movements have been referred to in other teleosts such as pearlfish and trout (Steinbacher et al., 2008; Marschallinger et al., 2009). In these species the primary myotome receives a number of non-differentiating muscle precursors of a dermomyotome origin, which for a short period maintain their mitotic activity, but eventually differentiate into myogenic cells responsible for the stratified growth of the white muscle. This might also be the case in dentex larvae, as the onset of the stratified hyperplasia, which is very obvious after the conclusion of the yolk sac period, concentrates on a series of proliferative clusters located at the same places where presumptive dermomyotome cells were previously observed to enter the myotome.

Concerning the mosaic hyperplasia two genuine facts have been found in the common dentex. Firstly, it

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is established before the end of the larval period, whereas the common situation is shortly after the end of that period (Rowlerson and Veggetti, 2001). Secondly, it initially relies on differentiation of the atypical premyoblastic cells (APC). The presence of APC in dentex larvae is a relevant finding of this study that deserves further specific research. APC have been mainly characterized by transmission electron microscopy and the TUNEL reaction, but a molecular categorization of their cellular components and differentiation programme is required to gain further specific knowledge about their identity, origin and fate. APC have euchromatic nucleus and the common presence of an evident nucleolus may indicate that these cells are far from being in a quiescent stage. In addition to having some occasional mitochondria and few ribosomes, their cytoplasm is full of longitudinal tubules with a clear content. Ultimately APC synthesize myofibrils and the cytoplasmic tubules are apparently used to assemble the sarcoplasmic reticulum. In order to ascertain the accuracy of this assumption a characterization of the molecular composition of the contractile filaments, as well as the cytoplasmic tubules, is compulsory. For instance, a similar abundance of cytoplasmic tubules has been described in skeletal and cardiac muscles of several neuromuscular diseases (Morgan-Hughes, 1998; North, 2004), and particularly in the muscle fibres of humans suffering from the so called "tubular aggregate myopathy" (Rosemberg et al., 1985). Typical tubular aggregates are also described in the IIB muscle fibres of male inbred mice during aging (Chevessier et al., 2004). Despite their unknown origin and role (Chevessier et al., 2005), a molecular characterization of the presence of proteins involved in calcium uptake, storage and release such as calsequestrin, triadin, SERCAs and sarcalumenin, has established that the tubular aggregates may originate from the whole sarcoplasmic reticulum. A similar demonstration is required in future studies on dentex larvae to support the assumption suggested here that the cytoplasmic tubules are used as a matrix to develop the sarcoplasmic reticulum and T-tubule system of the myoblasts derived from APC. Similarly, further research is needed to characterize topics such as APC's ancestry and their developmental programme; mainly the expression of myogenic regulatory factors (MyoD, Myf5, Myogenin, etc) responsible for their myogenic differentiation. Concerning their ancestry, no direct conclusions may be deduced from our results, but a tentative explanation could be that they are a remnant of the population of pluripotential lateral cells of the somites (Devoto et al., 1996) which extend their proliferative capacity until the end of the larval stage. Besides, the simultaneous presence of presumptive dermomyotome cells and APC in early larvae (Fig. 3C) gives support to a likely independent origin for both myoblastic precursors. Another issue is why APC are specific of the common dentex? We have no answer for this question, but their genuine appearance in this fish is

supported by the fact that we have been sampling dentex larvae for 4 years and have always found them. This was independent from the genetic background and feed supply of progenitors, as well as the rearing temperature and nutrition patterns of larvae (Arizcun et al., 2003, 2007).

The population of APC becomes apparently exhausted shortly after the end of the larval period. The ultimate evidence of their existence as revealed by the TUNEL reaction was dated at 41 days after hatching. Apoptosis is a common phenomenon during development of vertebrates (Sanders and Wride, 1995). In fish embryos, cell apoptosis has been reported for many developing tissues and organs, including the somites and myotomes (Cole and Ross, 2001). Also, apoptosis of muscle fibres, myoblasts and muscle precursors is a common phenomenon in fish muscle cells cultured *in vitro* (Sandri and Carraro, 1999). In contrast, as far as we know apoptosis of myoblasts or muscle precursors has never been reported in developing fish larvae. Hence, our results describe a new situation that, on the other hand is the result of having combined electron microscopy view with the TUNEL reaction. Suppression of cells in the somites may be due to several ongoing developmental processes. These include the normal development of somites and synaptogenesis between motor neurons and muscle cells (Cole and Ross, 2001). In the common dentex apoptosis of APC seems unlikely to be related with developmental processes such as somitogenesis and synaptogenesis, but with the disappearance of a population of embryonic cells responsible for establishing an early period of mosaic hyperplasia.

Considering that no APC are found in dentex postlarvae, the juvenile mosaic hyperplasia of the white muscle relies on other myogenic cells, which, as observed by transmission electron microscopy, correspond to presumptive myosatellite cells, as described in the skeletal muscle of other fish and mammals (Koumans and Akster, 1995). In fish myosatellite cells are pleomorphic and, contrarily to mammals, not always surrounded by the basal lamina of the adult muscle fibres. The origin of the presumptive myosatellite cells in the common dentex is apparently independent of the APC, but most probably derived from a population of myogenic cells which remain quiescent in the myotomes since the earliest stages of development. Whether these myogenic cells are directly related with dermomyotome cells is a possibility that is in debate nowadays (Hollway et al., 2007; Marschallinger et al., 2009). On the other hand, admitting a possible different origin for the APC and the presumptive myosatellite cells, both of them responsible for the mosaic hyperplasia, implicates the recognition of a new mechanism of muscle growth in teleosts and also the potential use of the common dentex as a model for studies of muscle growth in fish.

Dealing with the rapidity of the larval stage of the common dentex (Koumoundouros et al., 2004), the

development of the axial musculature is characterized by a rapid appearance of all muscle fibre types and the early start of the mosaic hyperplasia. The remarkable presence of a population of APC during the myogenesis of dentex larvae emphasizes the existence of species specific developmental events during the muscle development of teleosts.

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