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Muscle development and differentiation in the urodele *Ambystoma mexicanum*

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SUMMARY

Skeletal muscle in vertebrates is the most representative tissue and it is composed by different types of fibers which differ for anatomical and physiological features. These traits permit to distinguish them basing on the speed of twitch and the load resistance. The characteristics of a fiber depend on the amount of glycogen and mitochondria in their cytoplasm, as well as by the presence of glycolitic and oxidative enzymes.

In particular, slow fibers are specific for a long-lasting contraction, they express a MyHC isoform having an ATPase activity at acidic pH (pH 4.0) and an aerobic oxidative metabolism. In contrast, fast fibers are larger in size, with few mitochondria. They are specialized in rapid and short-lasting contractions and express a MyHC isoform, having an ATPase activity at basic pH (pH 10.0) and an anaerobic glycolitic metabolism.

These different types of fibers, both in amniotes and in lower vertebrates such as fish and anuran amphibians s come from distinct populations of myoblasts which appear in the somites during the embryonic development.

Even if substantial data concerning the muscle differentiation and the characterization of the different types of fibers in zebrafish and *Xenopus* are available, nothing is still known about the mechanisms regulating these processes in amphibian urodeles. Since these animals present some anatomical features and life style between fish and anurans, the study of myogenic processes in these animals could be useful to clarify the evolutionary changes which lead to the formation of skeletal muscle in the trunk of land vertebrates.

To shed light on the myogenic processes in urodele amphibians we focused our studies on the axolotl *Ambystoma mexicanum* and we analysed several embryonic stages of this amphibian in order to identify the different types of fibers and their pattern of distribution during the myogenic process. Using morphological analysis, enzymatic hystochemistry and immunohystochemistry we showed that in *A. mexicanum*, as in zebrafish, the first differentiating fibers are the slow ones deriving from myoblasts localized close to the notochord. These fibers then migrate towards the somitic surface and here they give rise to a superficial layer of slow fibers, while the myoblasts forming the medial part of the somite differentiate into fast fibers.

Further, in order to evaluate the possible involvement of muscle-specific transcriptional factors and protein signalling in the regulation of myogenic process of this amphibian, we used molecular biological approaches to identify and clone the muscle-specific transcriptional factor (Myf5) and the Sonic hedgehog signalling protein, known to regulate the muscle development and differentiation both in amniotes and lower vertebrates.

INTRODUCTION

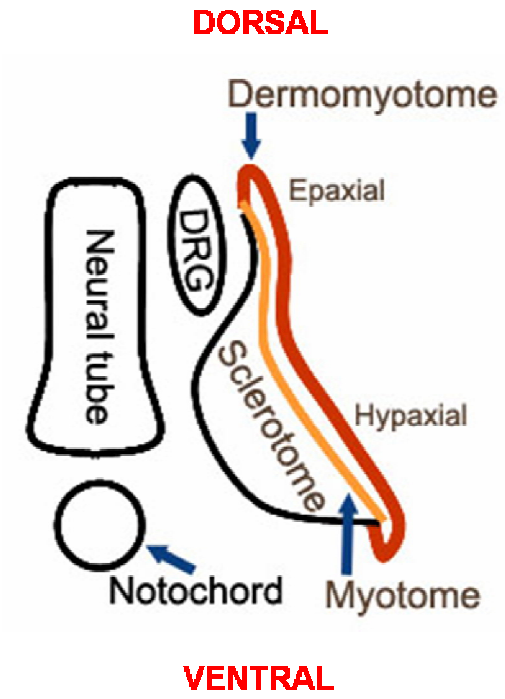
Skeletal muscle in vertebrates is composed by a high number of different types of fibers, the slow fibers (type I) and the fast fibers (type II) (Kelly and Rubinstein, 1980) which differ basing on the speed of twitch, the metabolic activity and the different type of innervation.

In particular, slow fibers are specialized in long-lasting contraction and are characterized by the expression of an isoform of skeletal myosin (MyHC) with an ATPase activity at acid pH (pH 4) and an oxidative aerobic metabolism. Unlike the slow fibers, the fast ones are specialized in short and rapid contractions and they are characterized by the expression of a MyHC with ATPase activity at basic pH (pH 10) and a glycolitic anaerobic metabolism (Pette and Staron, 1990; Schiaffino and Reggiani, 1996). Therefore these different types of fibers are specialized for phasic or tonic contraction relating on the functional needs of the various body parts or of the different areas of the same district.

These fibers come from several populations of myoblasts which appear during the embryonic development into the somites.

VERTEBRATE SOMITOGENESIS

Somitogenesis is an important developmental process that is involved in establishing the segmental arrangements of various structures of the body wall (reviews – Bellairs et al., 1986; Keynes and Stern, 1988; Smith and Malacinski, 1983). Somites are mesodermal structures that derive from paraxial somitogenic mesenchyme on either side of the neural tube into pairs of epithelial masses, a process that reflects the vertebrate segmentation (Epperlein et al., 2007a-b). During the developmental process, somites result subdivided in three distinct portions (Kaestner, 1892; Buckingham et al., 2003): the sclerotome, which forms the axial skeleton; the myotome, which forms the axial musculature and the dermomyotome, which give rise to the axial dermis, to angiogenic cells of the trunk and to limb vascular lymphatic vessels both in amniotes and lower vertebrates (reviewed in Kusakabe and Kuratani, 2005; Devoto et al., 2006).



Sclerotome
Mesenchymal cells
Location: Ventral somite
Derivatives: Ribs & Vertebrae

Dermomyotome
Epithelial structure
Forms from somites
Location: Dorsal somite
Derivatives: Skin & Skeletal muscles
Epaxial: Back muscles
Hypaxial: Trunk & Limb muscles

Myotome
Origin: Dermomyotome
Derivative: Trunk muscle

DRG: Dorsal root ganglion

MYOTOME FORMATION AND MUSCLE DIFFERENTIATION

Myogenesis occurs in different ways depending on the species (Sabourin and Rudnicki, 2000; review-Bryson-Richardson et al., 2008).

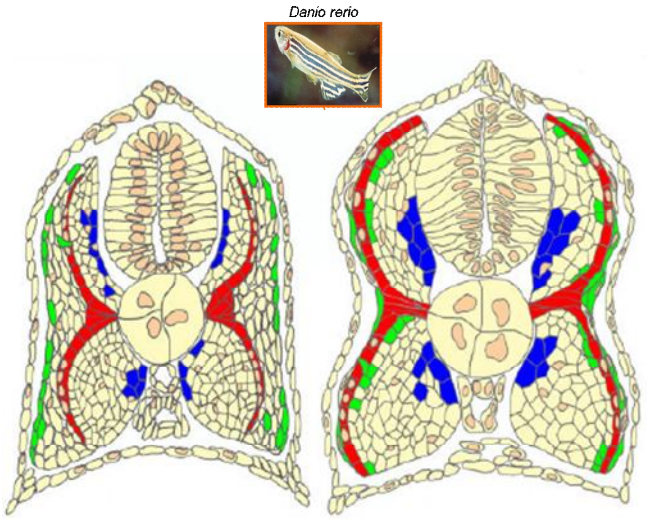
In **amniotes** the dermomyotome is the 'engine room' of myogenesis. It is nominally divided into epaxial and hypaxial domains that relate to the nature of innervation of the distinct muscle populations deriving from these regions (Bryson-Richardson et al., 2008). The dermomyotome is constituted by proliferative, multipotent precursors that give rise to many different cell types such as dermis and angiogenic cells, and axial and appendicular muscle (for review, see Brand-Saberi and Christ, 2000; Scaal and Christ, 2004).

Cells, located in the dermomyotome, delaminate to lie between the dermomyotome and the sclerotome, forming the primary myotome, which is exclusively composed of post-mitotic myocytes aligned such that they span the somite length along the embryo axis (Denetclaw et al., 1997; Kahane et al., 1998; Cinnamon et al., 1999; Denetclaw et al., 2000; Ordahl et al., 2001; Kahane et al., 2002; Gros et al., 2004; Ben-Yair and Kalcheim, 2005).

In **anamniotes** (fish and amphibians), muscle fibers derive from two different regions: the primary myotome and the dermomyotome-like structure, which appears later during development.

Fish Myogenesis

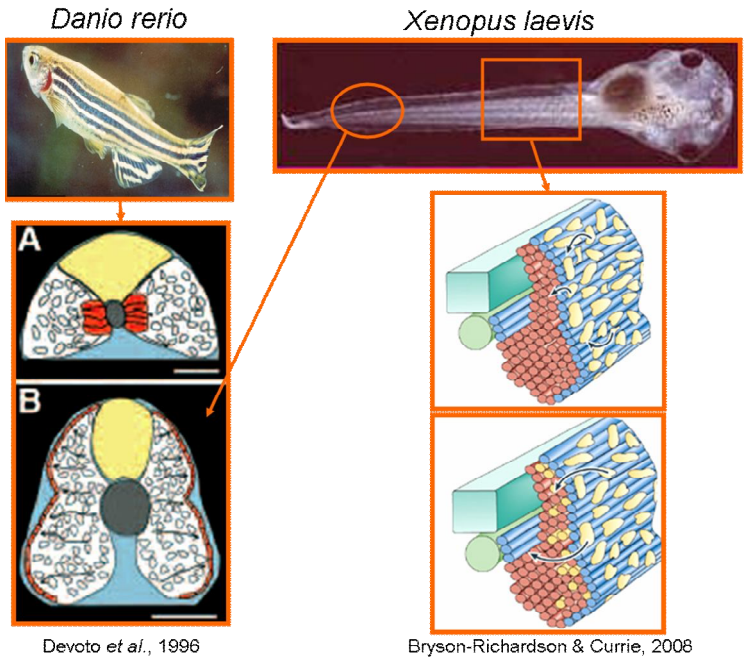
Myogenesis in fish has been widely studied in teleosts and in particular in zebrafish (*Danio rerio*) model. Unlike amniotes, the teleost dermomyotome is evident only after the formation of a primary myotome, early developed and composed by a superficial layer of slow muscle fibers lying over a mass of fast muscle fibers (Stickney et al., 2000; Scaal and Wiegreffe, 2006). The first fibers to differentiate are the slow ones, which develop from cells adjacent to the notochord (Devoto et al., 1996). In all examined teleosts, including zebrafish (Thisse et al., 1993), slow muscle precursors, also called adaxial cells, are the most medial paraxial mesoderm cells and have been identified morphologically or by gene expression. Thus the slow fibers do not arise from the dermomyotomal-like compartment of the somite but, all slow-MyHC-expressing adaxial myocytes undergo a remarkable cell-sorting behaviour, moving from their origin, next to the notochord, to become elongated and striated cells on the lateral-most surface of the myotome (Devoto et al., 1996; Blagden et al., 1997; Stellabotte and Devoto, 2007 drawing 1). Coincident with the migration of the slow-muscle cells, the medial part of the somite differentiates to form fast-muscle fibers. Thus the deep fast muscle fibers form the second component of the primary myotome. Shortly after primary myotome formation, an epithelial layer of proliferative cells can be distinguished on the external surface of the embryonic myotome: the dermomyotome. By the end of the paraxial somitogenic mesenchyme segmentation, muscle precursors cells, deriving from dermomyotome give rise to new lateral fast fibers (Stellabotte and Devoto, 2007 drawing 1). Thus, in fish, the dermomyotome layer contributes to the growth of the primary myotome.



Drawing 1

Amphibian Myogenesis

Myotome differentiation in amphibians proceeds in a complex, highly coordinated manner. The most data available in literature refer to muscle development in anurans and in particular in *Xenopus laevis***.** In this amphibian, two distinct kinds of muscle cells differentiate during development. Although these two types of fibers are analogous to the superficial slow and medial fast fibers of zebrafish, there are temporal and regional differences to this pattern along the rostral–caudal axis. The formation of slow fibers in the most posterior somites, from which the tail derives, occurs from the primary myotome, as described in the zebrafish model, while in the anterior somites, from which the body trunk derives, slow fibers arise from the dermomyotome (Grimaldi et al., 2004), as observed in amniotes. The fast fibers, like in fish, derive by the medial deeper region of each somite (drawing 2).



Drawing 2

Thus, in *X. laevis* there are two co-existing mechanisms giving rise to slow muscle fibers: the first one that, occurring in the somites of the tail, corresponds to an ancestral situation appeared in a common progenitor for fish and amphibians and maintained also in the primitive agnatha vertebrates (Flood et al., 1977), while the second one is typical of adult land vertebrates, including amniotes, which need robust trunk and limbs to support a body out of water.

MUSCLE REGULATORY FACTORS AND SECRETED SIGNALLING MOLECULES REQUIRED FOR MUSCLE DEVELOPMENT

Myogenesis, in different regions of the embryo, is controlled by a series of complex transcriptional regulatory networks that ultimately result in the expression of members of the basic helix-loop-helix domain-containing myogenic regulatory factors (MRFs*).* MRFs are responsible, in concert with a myriad of cofactors, for directing the expression of genes that are required to generate the contractile properties of a mature skeletal-muscle cell. These genes act in different phases of myogenesis in the embryo and adult (Bryson-Richardson et al., 2008).

Four members of the MRF family (MyoD, Myf5, myogenin and MRF4) have been found in vertebrates. The different time-patterns of expression of these MRFs also show a complex spatial expression pattern in the somite (Ontell et al., 1995; Sassoon, 1993). They therefore play a distinct role in myogenesis, acting on different myoblast populations (Braun and Arnold, 1996). All together they control gene expression during myoblast specification and myofiber differentiation, maintenance, hypertrophy, repair, and regeneration.

It is well known that the early slow and fast myogenesis both in amniotes and lower vertebrates (zebrafish and *Xenopus*) depend on Myf5 and MyoD and in particular Myf5 is required for superficial slow muscle differentiation (Rudnicki et al., 1993; Blagden at al. 1997, Grimaldi et al, 2004).

During the last decades, many studies have been done to investigate how muscle fibers diversification is achieved during the myogenic process. A key point has been the discovery that notochord and neurotube, which lie close to the somite, can induce myogenesis (Buffinger and Stockdale, 1994). Further studies showed that the myogenic effect of the axial structures is mediated by a pool of secreted proteins (Münsterberg and Lassar, 1995; Münsterberg et al., 1995; see review Lassar and Münsterberg, 1996). Sonic hedgehog (Shh) is one of these proteins, and several studies in zebrafish and *Xenopus* have demonstrated that it is able to induce the formation of slow fibers (Blagden et al., 1997; Cann et al., 1999; Barresi et al., 2000, Grimaldi et al., 2004) by committing a specific population of myoblasts and by controlling the fate choice between fast and slow-twitch muscle within early differentiating myocytes (Blagden et al., 1997; Du et al., 1997; Feng et al., 2006). Shh activates myogenic process by inducing, in muscle

precursors, the expression of specific muscle regulatory transcriptional factors. In particular in zebrafish and in *Xenopus* tail, Shh signalling is required for the muscle regulatory factor Myf5 expression and generation of superficial slow muscle fibers (Martin et al., 2007).

Differently from fish and anurans, very few data are available concerning both the adult muscle structure and the embryonic myogenic process in urodele amphibians. In the adult, different types of fibers (slow and fast) have been described (Totland 1976). Though the myotome (Neff et al., 1989) and the dermomyotome (Epperlein et al., 2007b) have been characterized in the *A. mexicanum* embryos, nothing is still known about the time of appearance of the different types of fibers during the embryonic development and the mechanisms regulating the muscle differentiation process in these animals.

WHY USING THE ANIMAL MODEL *Ambystoma mexicanum*?

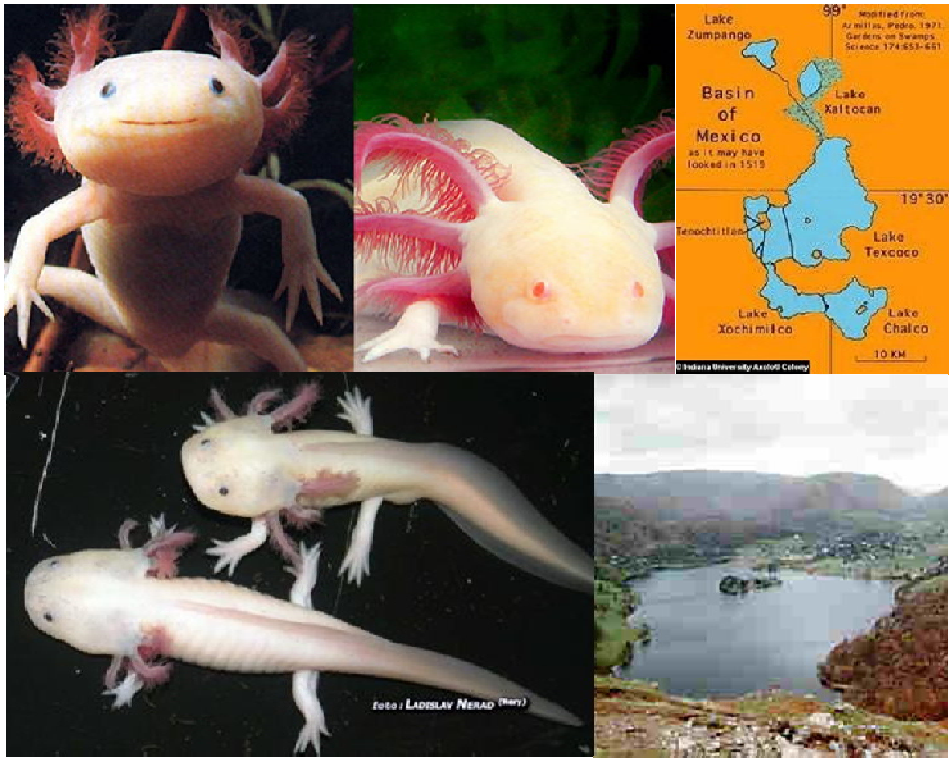
We chose the urodele *A. mexicanum*, as lower vertebrate animal model, to further investigate the myogenic process because it presents some anatomical features and a life style between fish and anurans and it could be useful to clarify the evolutionary changes which lead to the formation of skeletal muscle in the trunk of land vertebrates.

A. mexicanum DESCRIPTION:

Salamanders of the genus *Ambystoma*, one of which is *A. mexicanum*, commonly called “mexican walking fish” or “axolotl”, are a complex monophyletic group that lives in North America from northern Mexico to southern Canada (Shaffer, 1993,1996). They belong to the family of the Ambistomidi, order Caudata (Urodela), class Amphibia, phylum Cordata.

In particular, the axolotl appears to be indigenous to the spring-fed Lakes Xochimilco and Chalco along the southern edge of the Basin of Mexico (Armstrong and Malacinski, 1989). The optimal habitat for the axolotl is characterized by dew pond with slimy bottoms and lots of plants. The adult can

be 28-30 cm long and it presents a round face from which depart the outer red gills; it has a stocky and compact body and two pairs of legs with 4/5 fingers and there's behind a big and laterally compressed tail utilized for locomotion.



This urodele is a neotenic (ability to reproduce conserving larval character) animal. Natural populations of *Ambystoma* can be purely neotenic, transforming, or polymorphic (i.e. some specimens undergo metamorphosis, whereas others reproduce as larvae), and it has been suggested that this represents an ecological adaptation useful for a better exploitation of the available resources (Hanken, 1999). The reproductive period of these animals is at the end of the winter or at the beginning of the spring and the female, after fertilization, can release around 300 eggs in a gelatinous substance which protects them. Structures such as plants are needed to lay eggs.

GOAL OF THE RESEARCH

This work focuses on muscle development and differentiation in *A. mexicanum* embryos using different approaches to obtain:

1) spatial distribution, morphological, hystochemical and immunohystochemical characterization of slow and fast muscle fibers in the different districts of the myotome;

2) timing of appearance of the different types of fibers during the embryonic development;

3) identification and cloning of Shh and Myf5 (muscle-specific transcriptional factor) gene involved in the regulation of muscle development and differentiation.

MATERIALS AND METHODS

ANIMALS

To describe the observations on the muscle fibers differentiation during the embryonic development of *A. mexicanum* (Amphibia, Urodela), we selected different stages of development: 24/25, 28/29, 33/34, 36/37, 40/41 and 44 (hatching animals).

Adults of *A. mexicanum* were maintained in aquaria at a temperature between 14 and 20 degree Celsius and pH between 6.5 and 8.5. These standards were constantly monitored. After the mating and the deposition of the fertilized eggs, embryos were divided basing on the stage of development (Bordzilovskaya N.P. and Dettlaff T.A., 1979).

LIGHT MICROSCOPY AND TRANSMISSION ELECTRON MICROSCOPY

For routine transmission electron microscopy (TEM), fertilized eggs were dechorionated and embryos were removed and fixed overnight in 4% glutaraldehyde in amphibian Ringer buffer (Source: Humason, *Animal Tissue Techniques*: 6.5 g NaCl, 0.42 g KCl, 0.25 g $CaCl_2$, 1 liter dH_2O). Specimens were washed in Ringer buffer and then post-fixed for 1h with 1% osmium tetroxide in Ringer solution at room temperature. After standard dehydration in ethanol series, specimens were pre-stained for 30 minutes with a solution of uranyl acetate in ethanol 90%.

Specimens were embedded in a Epon-Araldite 812 mixture and sectioned (semithin and thin sections) with a Reichert Ultracut S ultratome (Leica, Nussloch, Germany).

Semithin sections (0.75 µm) were stained by conventional methods (crystal violet and basic fuchsin, Moore *et al.*, 1960) and were observed with a light microscope (Olympus BH12, Tokyo, Japan).

Thin sections (80-90 nm) were stained by uranyl acetate and lead citrate and observed with a TEM Jeol 1010 electron microscope (Jeol, Tokyo, Japan).

PREPARATION OF SAMPLES FOR IMMUNOHISTOCHEMISTRY AND ENZYMATIC HISTOCHEMISTRY

Embryos were embedded in the cryoprotector OCT (polyfreeze tissue freezing medium, Tebu-Bio, Italy), rapidly frozen in liquid nitrogen and subsequently sectioned with the cryostate Leica CM 1850.

After the reactions of immunohistochemistry and enzymatic hystochemistry, the cryosections (7 µm) were collected on polylysinated slides and observed with the light microscope Olympus BH12.

ENZYMATIC HISTOCHEMISTRY

Cryosections were rehydratated with PBS for 5 minutes and stained with purple-blue formazan utilizing histoenzymatic kits (Bio-Optica) for NADH-diaphorase to detect the mitochondrial activity, and with Schiff's Reagent utilizing hystoenzymatic kits (Bio-Optica) for periodic acid Schiff (PAS) to highlight the presence of glycogen. The slides were mounted with the balsam Eukitt, observed and photographed with the light microscope Olympus BH12 and the digital camera Nikon D5-5M.

IMMUNOHISTOCHEMISTRY

Cryosections were incubated for 30 minutes in a blocking solution with 2% BSA (Bovine Serum Albumin) and 0.1% Tween20 in PBS 0.1M. Subsequently sections were incubated with the primary antibody for 1h at 37°C and then washed with PBS. After washing, sections were incubated for 45 minutes at room temperature with a secondary antibody conjugated with a fluorocrome and then washed again with PBS. Cryosections were incubated for 10 minutes with the nuclear marker DAPI (4',6-diamidino-2-phenylindole). Then, slides were mounted with Cytifluor (Cytifluor, USA) and subsequently observed with the light microscope Olympus BH12. Images were captured with the digital camera Nikon D5-5M. Negative controls were performed without incubation in primary antibody. Images were combined with Adobe Photoshop.

All the antibodies were diluted at different concentrations in the blocking solution (2% BSA, 0.1% Tween20 in PBS):

- Primary: chicken anti-mouse IgG1 **EB-165** (DSHB 1:100)
- Primary: human anti-mouse IgG2a **A4.1025** (DSHB 1:100)
- Primary: bovin anti-mouse IgG2b **BAF-8** (DSHB 1:10)
- Primary: chicken anti-mouse IgG **B103** (DSBH 1:10)
- Primary: chicken anti-mouse IgG1 **S35**, kappa light chain (DSHB 1:10)
- Secondary: mouse IgG TRITC-conjugated (Jackson Immuno Research Laboratories 1:100)

WHOLE MOUNT *in situ* IMMUNOHISTOCHEMISTRY

Embryos were fixed in DENT (1 part DMSO + 4 parts MetOH) for 2 hours at 4°C and stored in methanol at -20°C until use. Before immunostaining embryos were bleached for 10 minutes with the bleaching solution: 0.5x SSC, 10% H_2O, 5% formamide.

Embryos were incubated for 1 hour rocking at room temperature with the blocking solution [PBT (PBS + 0.1% Triton X100) + 5% BSA (Bovine Serum Albumin)] and then with the following primary antibodies, diluted in the same solution, overnight at 4°C:

- Primary: human anti-mouse IgG2a **A4.1025** (DSHB 1:10)
- Primary: chicken anti-mouse IgG **F59** (DSHB 1:10)
- Primary: bovin anti-mouse IgG2b **BAF-8** (DSHB 1:25)

After washing embryos were incubated (overnight at 4°C) with the secondary antibody mouse IgG HRP-conjugated (diluted 1:200).

The enzymatic reaction was developed using the substrate DAB (3,3'-diaminobenzidine).

BIOCHEMICAL PROCEDURES

Embryos (at different stages of development), stored at -80°C, were homogenized in liquid nitrogen with a mortar and solubilised with 900 µl of denaturating Laemmli Buffer (0.02M Tris HCl pH6.8, 2% SDS, 5% β-mercaptoethanol, 0.001% bromophenol blue, 10% glycerol) and 100 µl of protease inhibitor (Sigma) at 100°C for 10 minutes. Particulate material was

removed by centrifugation at 13000 rpm for 3 minutes and the supernatant was analyzed by SDS-PAGE.

SDS-PAGE

Analytical SDS-PAGE using 7,5% and 5% acrylamide minigels were made according to Laemmli (1970). Molecular weights were determined by concurrently running broad range standards from Bio-Rad (Bio-Rad, Richmond, CA, USA). Electrophoresis was made at 200 V for 45 minutes. Gels were stained with Coomassie Brilliant Blue R-250 (2.5g/l) in acetic acid 10% and ethanol 25%.

WESTERN BLOT

Proteins separated by SDS-PAGE were transferred onto Bio-Rad nitrocellulose filters according to Towbin et al. (1979). After the transfer (at 350 mA per hour) the membranes were incubated with a blocking solution (2% BSA, 0.1% Tween20 in PBS) for 1 h rocking. After washing the nitrocellulose membranes were incubated overnight at 4°C with primary antibodies:

- Primary: chicken anti-mouse IgG1 **EB-165** (DSHB 1:100)
- Primary: human anti-mouse IgG2a **A4.1025** (DSHB 1:100)
- Primary: bovin anti-mouse IgG2b **BAF-8** (DSHB 1:10)
- Primary: chicken anti-mouse IgG **B103** (DSBH 1:10)
- Primary: chicken anti-mouse IgG1 **S35** (DSHB 1:10)
- Primary: chicken anti-mouse IgG **F59** (DSHB 1:50)

After three washes of the membrane with the blocking solution (15 minutes each), antigens were revealed with an appropriate secondary antibody (mouse IgG HRP-conjugated 1:5000) coupled with peroxidase incubating for 45 minutes at room temperature rocking.

The secondary antibody was revealed using LUMINOL (5-Amino-2,3-dihydro-1,4-phthalazinedione; Pierce, prod #34080).

SHH, Myf-5 CLONING

Total RNA from Ambystoma mexicanum embryos at different stages was extracted with "Tri Reagent" (SIGMA) following the manufacturer's instructions.
RNA was reverse transcribed with random primers with the "high capacity cDNA Reverse Transcription Kit" (Applied Biosystems).
In order to amplify Shh sequence, two specific primers were designed on the annotated sequence of A. mexicanum Shh cDNA:
Amb.mex. SHH fw – BamHI: 5'CGGGATCCTTGCCTCCTGATCGCGGCCT 3' ;
Amb.mex. SHH rev – EcoRI: 5'CGGAATTCGCACCCTGTCACCCGGCCTC 3' .
A 865 bp fragment was obtain from 36/37 stage embryos, cloned in a T-vector and sequenced.
To obtain Myf5 sequence, cDNA from 36/37 stage embryo was PCR amplified with a couple of primers specific for the annotated Notophtalmus viridescens Myf5 sequence:
N. viridescens Myf5 fw: 5' TCCAACTGTTCCGACGG 3' ;
N. viridescens Myf5 rev: 5' ACAACACGTGGTAGATGGG 3'.
A 289 bp fragment was cloned and subsequently extended with the following 5' degenerated primer:
Degenerated Myf5 fw 5'ARIWYTTCTAYGACRGCICYTG 3'.
PCR conditions: initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 sec, X °C for 30 sec (depending on melting temperature), 72°C for 30 sec.; final extension at 72°C for 5 min.

QUANTITATIVE RT-PCR FOR Myf5 and SHH

Real time RT-PCR was performed on ABI PRISM 7000 with "Fluocycle II Sybr ROX" (Euroclone) following the manufacturer's instructions. The sequence of the primers used for the amplification is the following:
Amb. mex. Myf5 fw: 5'CCCTGCCCGGCCAGCACTGC 3'
Amb. mex. Myf5 rev: 5'GGGTGTTGATTTTGTCTGTGGGGTAAA 3'
Amb. mex. SHH fw: 5'CCAAGGCCCACATTCATTGCTCTGTG 3'
Amb. mex. SHH rev: 5'CAGGTCTTTCACCGGTCTGGTCACT 3'
Beta actin fw: 5'CGCGAGAAGATGACCCAGAT 3'
Beta actin rev: 5'ACAGCCTGGATAGCAACGTACA 3'

The reactions were performed in triplicate in a 25-ul volume containing target cDNA, 40 nM primers, 12.5 ul of master mix and water to the final volume.
Following a polymerase activation step for 10 min at 95°C, samples were denatured at 95°C for 15 sec and annealed/extended for 1 min at 60°C, for 40 cycles.
Comparison of the amount of each gene transcript revealed by the fluorescence was normalized with the housekeeping gene beta actin using the ΔCt method.

WHOLE MOUNT mRNA *IN SITU* HYBRIDIZATION

Embryos were fixed in 1XMEMFA (0.1M MOPS pH7.4, 2 mM EGTA, 1 mM MgSO4), 3.7% formaldehyde and DEPC treated H2O for 2 hours (as described in Hutchinson C., 2007) and stored in methanol at -20°C until use. Whole mount in situ hybridization was performed as described in the Core protocol for both mouse and chick embryos of Cepko/Tabin lab with some modifications basing on the methods of S. Hughes lab.
Digoxigenin (DIG)-labeled antisense RNA probe was obtained using T7 RNA polymerase (Promega) and DIG RNA labelling mix (Roche Diagnostics, Laval, Quebec, Canada). Sense probe was generated with SP6 RNA polymerase (Ambion). For probe synthesis, a 634bp Myf-5 fragment was cloned into the pGEM-T-easy plasmid which was then linearized using the appropriate restriction enzymes.
Prehybridization was performed for at least 6 hours, instead probe hybridization was overnight. Prehybridization and hybridization temperature was 60°C. Antisense probe was used at a concentration of 762 ng/ml; sense probe was used at 725 ng/ml.
Finally, NBT/BCIP (Nitro-Blue Tetrazolium Chloride/ 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt, Sigma) was used as the enzyme substrate for the colorimetric reaction for the alkaline phosphatase reaction. Then embryos were bleached (3% H_2O_2, 0,5% Formamide and water) to obtain signal without background.
Control experiments were performed using the “sense” probe instead of the antisense.

RESULTS

MORPHOLOGICAL ANALYSIS OF THE DIFFERENT TYPES OF FIBERS

Morphological analysis were carried out on embryos of *A. mexicanum* at different stages of development starting from stage 24 up to 44 (Figures 1, 2). For each stage of development we analyzed three somitic regions: anterior (Fig. 1a,d,g; Fig. 2j,m,p), medial (Fig. 1b, e, h; Fig. 2 k, n, q) and posterior (Fig. 1c, f, i; Fig. 2 l,o,r).

At early stage of development (**stage 23-25**, corresponding to embryos with around 10 pairs of somites) all the somitic regions (anterior, medial and posterior) were surrounded by two epidermal cell layers and were formed by a compact mass of undifferentiated cells showing a cytoplasm filled by a large amount of yolk droplets and a large nucleus (Fig. 1 a-c). Starting from **stage 28-29** up to **stage 33-34** the somitic mesoderm of the three regions appeared to be divided in two masses constituted by: the sclerotome, localized just close to the notochord and the myotome, visible just underneath the two epidermal cell layers (Fig. 1 d-i). At these stages the myotome was still formed by myoblasts with large nuclei and scarce cytoplasm rich in yolk droplets. The first differentiated muscle fibers in the myotome were detected at **stage 36-37.** At this stage, embryos have about 28 somites, and yet the younger posterior (corresponding to the tail region, somites 20-28) and medial somites (10-19) were still formed by a compact mass of undifferentiated myoblasts lined by epithelium (Fig. 2k, l), in the older anterior somites (1-9) newly differentiated mononucleated myocytes were evident (Fig. 2j-r). As shown also by ultrastructural analysis at TEM, these differentiating cells were characterized by a cytoplasm filled by lipids droplets and electron-dense granules surrounded by a thin discontinuous ring of contractile material. By **stage 40-41** most of the myotome is formed by differentiated muscle fibers well recognizable in all the somitic regions (Fig. 2m-o).

At the time of hatching (**stage 44**), the myotomes of the somitic regions were formed by completely differentiated muscle fibers subdivided in three separate groups similar to those previously described in adult animals by Totland 1976. In centripetal direction, starting from the epithelium, it was possible to distinguish i)

superficial small diameter fibers, characterized by irregular shaped fibrils, containing a large amount of mitochondria (Fig. 3a, a', b); ii) medium diameter fibers with lower contents of mitochondria, but copious glycogen (Fig. 3a', c); iii) large diameter fibers, localized just close to the axial structures of the embryo, characterized by polygonal shaped fibrils, low density of mitochondria and high content of glycogen (Fig.3 a', d).

METABOLIC CHARACTERIZATION OF THE DIFFERENT TYPES OF FIBERS

The variable amount of mitochondria and glycogen in the different types of muscle fibers had been revealed also by using two enzymatic histochemical reactions: NADH-diaphorase (specific for mitochondrial activity labelling) and PAS reaction (Periodic Acid Schiff) used to reveal glycogen (Fig. 4).

The analysis were performed on embryos at stage of development 44.

The superficial small diameter fibers were strongly positive for the NADH-diaphorase activity (Fig4. a,b,d,e,g,h) and presumably correspond to the red slow fibers type in other lower vertebrates (fish and frog), while the medium and the large diameters fibers, localized deeper in the myotome, showed a weak positivity for NADH-diaphorase, while strongly react with PAS reaction (Fig. 4c-i) These muscle fibers probably correspond to the white and fast fiber type in the lower vertebrate.

EXPRESSION OF THE DIFFERENT SKELETAL MYOSIN (MyHC) ISOFORMS

In order to detect the two major types of muscle fibers (slow and fast) in the developing myotome of axolotl, we screened over 10 monoclonal antibodies known to recognize specific isoforms of mammalian, avian, fish and amphibian MyHC. A Western blot analysis was first performed to select the best antibodies to be used for further immunohistochemical analysis on sections.

We found that the antibodies **A4.1025, BA-F8, F59, EB165, B103** and **S35** recognized, in the proteic extract from *A. mexicanum* embryos at stage 44,

bands of about 200 kDa, corresponding to the molecular weight of the skeletal myosin (Fig. 5).

Basing on the results of the Western blot we used the same antibodies to perform immunostainings on cryosections of embryos at developmental stage 44 (Fig. 6). Our data showed that A4.1025 (Fig. 6a) reacts with the vast majority of the muscle fibers, while the two antibodies BA-F8 and F59 (Fig. 6 b,c), specifically stained the superficial layer of oxidative small diameter fibers. From these results we concluded that the superficial muscle fibers were of slow type, while the inner larger diameter fibers were of fast type.

In spite of the presence of the bands detected by western blot analysis, the antibodies B103 (Fig. 6d), and S35 (Fig. 6e) did not stain any type of fibers in sections, while the antibody EB165 (Fig. 6f) was expressed only in the cephalic muscles (as already seen in *Xenopus*, Grimaldi et al. 2004) as shown by the whole mount staining (Fig. 6g).

DIFFERENTIATION OF THE DIFFERENT TYPES OF MUSCLE FIBERS DURING THE EMBRYONIC DEVELOPMENT

Using the selected antibodies A4.1025, BA-F8 and F59 we investigated the timing of appearance of the differentiated slow and fast muscle fibers populations in axolotl. The whole mount immunostainings on different developmental stages of axolotl embryos, show that muscle differentiation occurs from anterior to posterior direction (Fig. 7).

Frozen whole mount stained embryos in cross section, showed that the earliest-formed muscle fibers appeared in posterior somites at stage 24. As in zebrafish the first differentiated fibers were detected close to the notochord (Fig. 7a, b). Starting from stage 31-33, in the posterior and medial half of the embryos (including somites 10-20) groups of cells appeared to span the somite from the adjacent notochord towards the lateral somite surface (Fig. 7 d, e; k-m). These cells formed a superficial layer of slow BA-F8^{+} and F59^{+} cells outlining the lateral border of each anterior somite (Fig. 7 c, j).

As in zebrafish, in *A. mexicanum* as well, fast cells appear later during development, being detected firstly by stage 35. These medial/deep somitic muscle fibers were stained only by the antibody anti A4.1025 and were BA-F8^{-} and F59^{-} (Fig. 7 f- i).

EXPRESSION PATTERN OF THE MUSCLE REGULATORY FACTOR (MRF) Myf5 AND THE SONIC HEDGEHOG SIGNALLING DURING MUSCLE DEVELOPMENT

In order to evaluate a possible role of MRFs in the differentiation of axolotl striated muscle fibers, we cloned a fragment of Myf5 mRNA (289 bp) from *A. mexicanum* (Accession number FJ481985). A Real Time PCR using the cloned fragment was then performed to determine the expression levels of Myf5 mRNA during muscle development. The graph in Figure 8 showed that Myf5 mRNA expression levels was low at early stage of development (22-23), gradually increased in the next stages (28-29), peaked at stage 31 and by stage 33 dramatically decreased. These expression levels were then compared with the results obtained in the experiment of mRNA whole mount *in situ* hybridization, performed with an antisense RNA probe created on the cloned fragment (Figures 9 and 10). The mRNA *in situ* whole mount hybridization showed that at early stages of development Myf5 mRNA is expressed in the middle portion of all the somites (Fig. 9 a, b, b'). From stages 33-37 up to the stages 41/42 the transcript's expression level decreases appreciably (Fig. 9 c, d) and the sense probe, used as negative control, gaves no label (Fig. 9 e).

The whole mount *in situ* stained embryos were then cryosectioned to precisely identify the cells expressing the Myf5 transcript (Fig. 10). In the most anterior somites the transcript was expressed in the superficial layer of cells lining the external region of the somite (Fig. 10 a', b, c), while in the posterior somites the Myf5 mRNA was expressed in cells next to the notochord (Fig. 10 a'', d), resembling the slow muscle fibers distribution over described.

Subsequently we identified and cloned a 865 bp fragment of Shh from *A. mexicanum* and basing on the Shh mRNA cloned fragment we performed a quantitative RT-PCR in order to evaluate the expression pattern of this gene during different developmental stages (Figure 11). We observed that Shh was highly expressed at early stages (by stage 15 up to stage 20), it dramatically decreased around stage 22/23, became highly expressed by stage 28/29 until stage 31 and then totally disappeared in the hatching stage and in the adult animal.

DISCUSSION

In the present study, we chose the urodeles amphibian *A. mexicanum* (known also as axolotl) as a lower vertebrate animal model to further investigate the extent of evolutionary conservation and diversification of muscle development and the role of MRFs and signalling proteins, like Shh, involved in this process.

The rationale for choosing the axolotl for our studies is due to the fact that, among amphibian species, urodeles offer several advantages. Eggs/embryos of *A. mexicanum* are usually larger than typical anuran eggs, so surgical manipulation is easier (Smith and Malacinski, 1983). Myotome development, including segmentation and differentiation, spans several morphogenetic stages, so relatively convenient and precise estimations of the timing of key events can be obtained (Bordzilovskaya and Dettlaff, 1979). Moreover axolotl presents phenotypic and life style features intermediate between fishes and anurans, thus the study of myogenic processes in this animal could be useful to clarify the evolutionary changes which lead to the dramatic modifications of the muscle trunk of land vertebrates.

Even if the muscular organization in adult *A. mexicanum* has been described (Totland 1976a,b), neither the increase in size of myotome nor the final spatial organization that it achieves during development have been comprehensively described. Moreover, although MRFs and Shh have been cloned in urodeles (Torok et al., 1999; Imokawa et al., 2004), their expression has been analyzed only during regeneration processes, while nothing is known about their role during muscle development and differentiation.

To obtain information on the mechanisms involved in muscle patterning during development of this urodeles amphibian we have employed morphological, immuno-histochemical, histoenzymatic, and in situ hybridization assays. Sequential events involving a complex network of cell growth, differentiation and migration during *A. mexicanum* muscle development are the topics of this study.

MUSCLE FIBERS DIFFERENTIATION AND CHARACTERIZATION OF MUSCLE FIBER TYPES IN *A. mexicanum* EMBRYOS

We first delineated morphologically the sequential events during somites development of axolotl.

In *A. mexicanum*, as in the others vertebrates, somitogenesis and muscle differentiation occur following anterior-posterior direction (i.e. embryonic growth) (Buckingham et al., 2003) and the myogenic cells first arise within the most anterior and oldest somites. At early stage (24-25) of development, the somites are composed by a compact mass of blast cells enveloped by two epidermal cell layers. As development proceeds (stage 28-29), the somitic mesoderm is divided into symmetric masses: the sclerotome, just closed to the notochord, and the myotome localized under the epithelium. Differentiating muscle fibers become morphologically visible for the first time in the anterior somites of embryos at stage 36-37. This differentiating process progressively proceed in caudal direction and by stage 44 all the somitic cells differentiate in mature muscle fibers, showing different dimension and morphological features. These fibers can be subdivided in three separate groups: small diameter fibers forming a superficial layer on the external surface of the somite, large diameter fibers forming the deeper region of the somite and medium diameter fibers localized in the middle region of the somite. The histochemical enzymatic and immunostaing experiments demonstrate that the superficial small diameter fibers are oxidative (NADH positive) and express slow MyHC, while the central and the deeper fibres are glycolitic (PAS positive) and fast MyHC positive.

To understand which kind of skeletal myosin (MyHC) isoforms were expressed in axolotl embryos, we performed a Western blot analysis using several monoclonal antibodies known to recognize specific MyHC isoforms of other vertebrates. We screened a wide range of antibodies and we obtained results with A4.1025, BA-F8, F59, EB165, B103, S35. They recognize, in the proteic extract from embryos of *A. mexicanum* at stage 42, a band of about 200 kDa, corresponding to the molecular weight of the skeletal myosin. Once identified the reacting antibodies in *Ambystoma* we used them to perform immunolocalizations on embryos cryosections. We find that A4.1025 reacts with all the muscle fibers, while the two antibodies BA-F8 and F59 react strongly with the single layer of superficial slow fibers. In spite of the presence of the band in the western blot, the antibodies B103, and S35 don't show any positive signal on the cryosections.

EB165 is absent in the somites while it is expressed in the cefalic muscles as shown by the whole mount staining. The same staining for EB165 was found also in *Xenopus* (Grimaldi et al., 2004). Such tripartition of the muscular mass and different mitochondrial enzyme activity of differentiating muscle fibers during axolotl development is in accordance with the muscle organization already described in adult animals by Totland (1976 a,b).

PATTERN DISTRIBUTION OF SLOW AND FAST FIBERS DURING MYOGENIC PROCESS

Immunohistochemical analysis performed on embryos of *A. mexicaum* at different developmental stages demonstrate that the distribution pattern of slow and fast fibers is obtained by the same developmental mechanism already described in the zebrafish body axis (Blagden et al., 1997; Devoto et al., 1996) and in the *Xenopus* tail.

Analyzing cross cryosections of whole mount stained embryos we find that the antibody A4.1025, which recognizes all the MyHC isoforms, at early stages of development (stages 24-28), stains the first differentiating fibers localized next to the notochord. As the development goes on (stages 31-33) these fibers migrate laterally and in the anterior somites, more mature, they form a superficial subepithelial layer. Muscle fibers, forming the middle region of the somites, differentiate in the later stages of development. By stage 35 up to stage 42 we can clearly distinguish two populations of muscle fibers: the superficial oxidative fibers, expressing the slow MyHC isoforms F59 and BA-F8 and the inner fibers, forming the bulk of the somite, expressing A4.1025, which will become the fast glycolitic fibres describe in the pre-hatching stage.

Summarizing, the muscle differentiation proceeds in an antero-caudal direction; the fibres start to differentiate during the early stage of development (stage 24) even if they can be morphologically classified only starting from the stage 36-37. With regard to the different types of myosins, the first isoform expressed is the slow one. As a matter of fact the generic skeletal myosin and the slow type co-localize at early stages of development, starting to be expressed in the most anterior somites and in the cells close to the axial structures of the embryo.

Only starting from the stage of development 33-34, the expression of the generic isoform is localized in the most caudal somites, where the signal for the slow

isoform is absent. It is possible to speculate that at this stage of development in the tail bud starts the expression of a fast type isoform.

Our results lead to the assumption that in *A. mexicanum* the muscle development in the trunk region is comparable with zebrafish one (Blagden et al., 1997), where the slow fibers are the first to appear and derive from cells differentiating close to the notochord and then migrating towards the most superficial region of the somites. The fast fibers appear later and differentiate starting from the most central cells of the myotome.

In conclusion these data demonstrate that in *Ambystoma*, slow musculature of the body axis derive from the ancestral primary myotome like in zebrafish, and not from the dermomyotome, as has been described for Xenopus body trunk. This fact is probably due to the type of locomotion which, in the urodeles, is obtained by lateral waves as in fish.

Myf5 AND Shh ARE EXPRESSED DURING AXOLOTL MUSCLE DEVELOPMENT

Since the muscle regulatory factor Myf5 and the Shh signalling pathway in vertebrates regulate differentiation and positioning of muscle fibers (Blagden et al., 1997; Duprez et al., 1999; Barresi et al., 2000; Grimaldi et al., 2004a), we have considered them as a good candidates for regulating muscle patterning in the axolotl as well.

After cloning the fragment of Myf5 and Shh mRNA from *A. mexicanum*, using degenerate primers, a couple of specific primers have been designed for an experiment of real time PCR. We found that the expression levels of mRNA Myf5 and mRNA Shh reach the higher level of expression at stages 28-29 and 31 when slow muscle fibers star to differentiate. Moreover mRNA Myf5 expression levels are perfectly overlapping the ones observed in the mRNA whole mount *in situ* hybridization. Using an anti-sense probe, created starting from the cloned fragment, we see that at early stages of development (stages 26-29) Myf5 mRNA is expressed in the middle portion of all the somites. Proceeding in the development (from stages 33-37 up to the stages 41/42) the transcript's expression level decreases appreciably: in the anterior somites (more mature) the transcript is expressed in a superficial layer of cells; in the tail, where the

somites are more immature, the Myf5 mRNA is expressed in cells next to the notochord.

The expression pattern of the transcriptional factor Myf5 in *A. mexicanum* is very similar to that already observed in zebrafish.

In the more immature somites Myf5 is expressed in the cells next to the notochord, then they migrate laterally and, at the same time, they differentiate in the superficial layer of slow fibers. So Myf5 and Shh, as in fish and *Xenopus* tail, seem to be regulatory factors involved in the differentiation of the slow muscle fibers.

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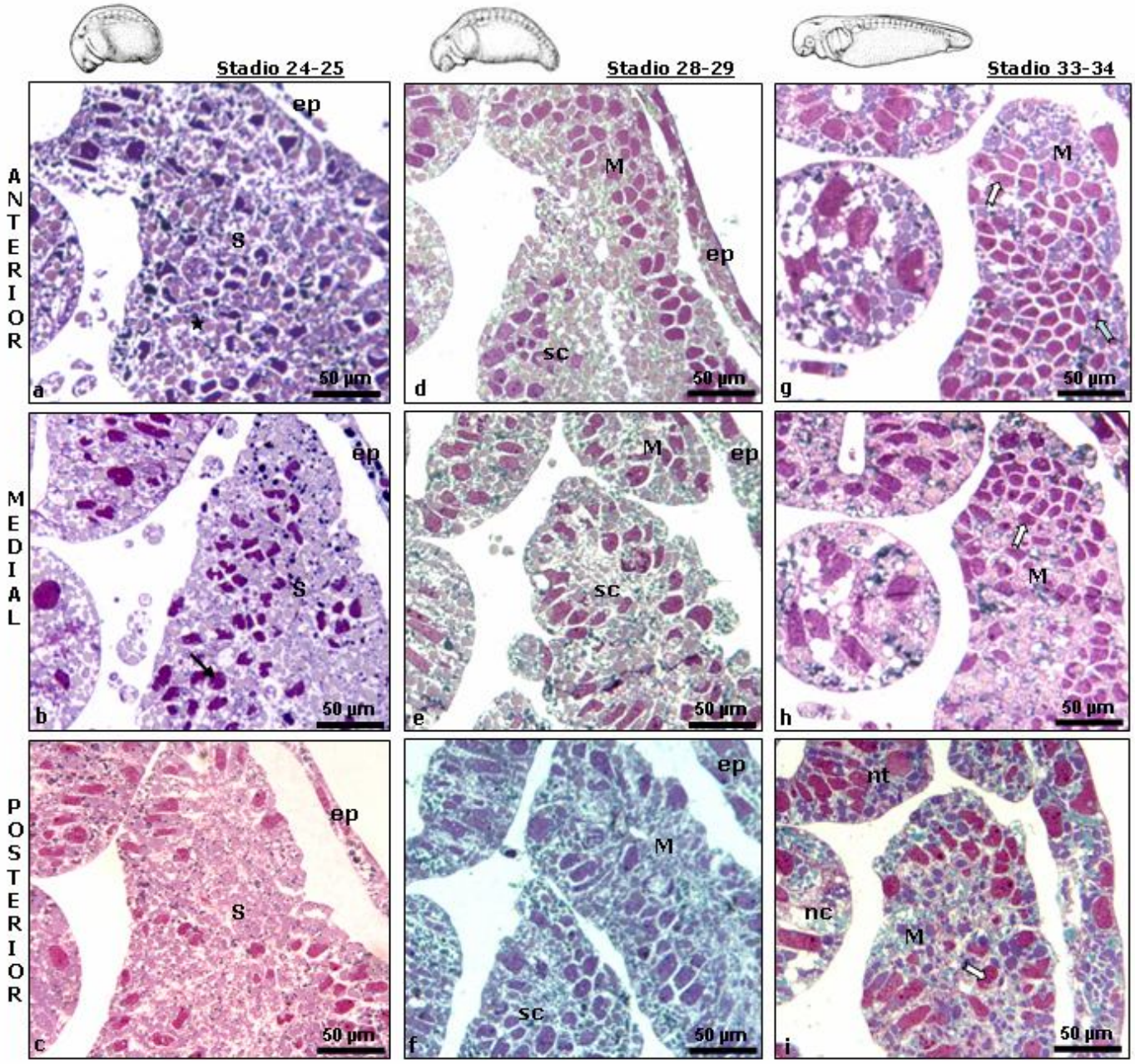
FIGURES

Figure 1



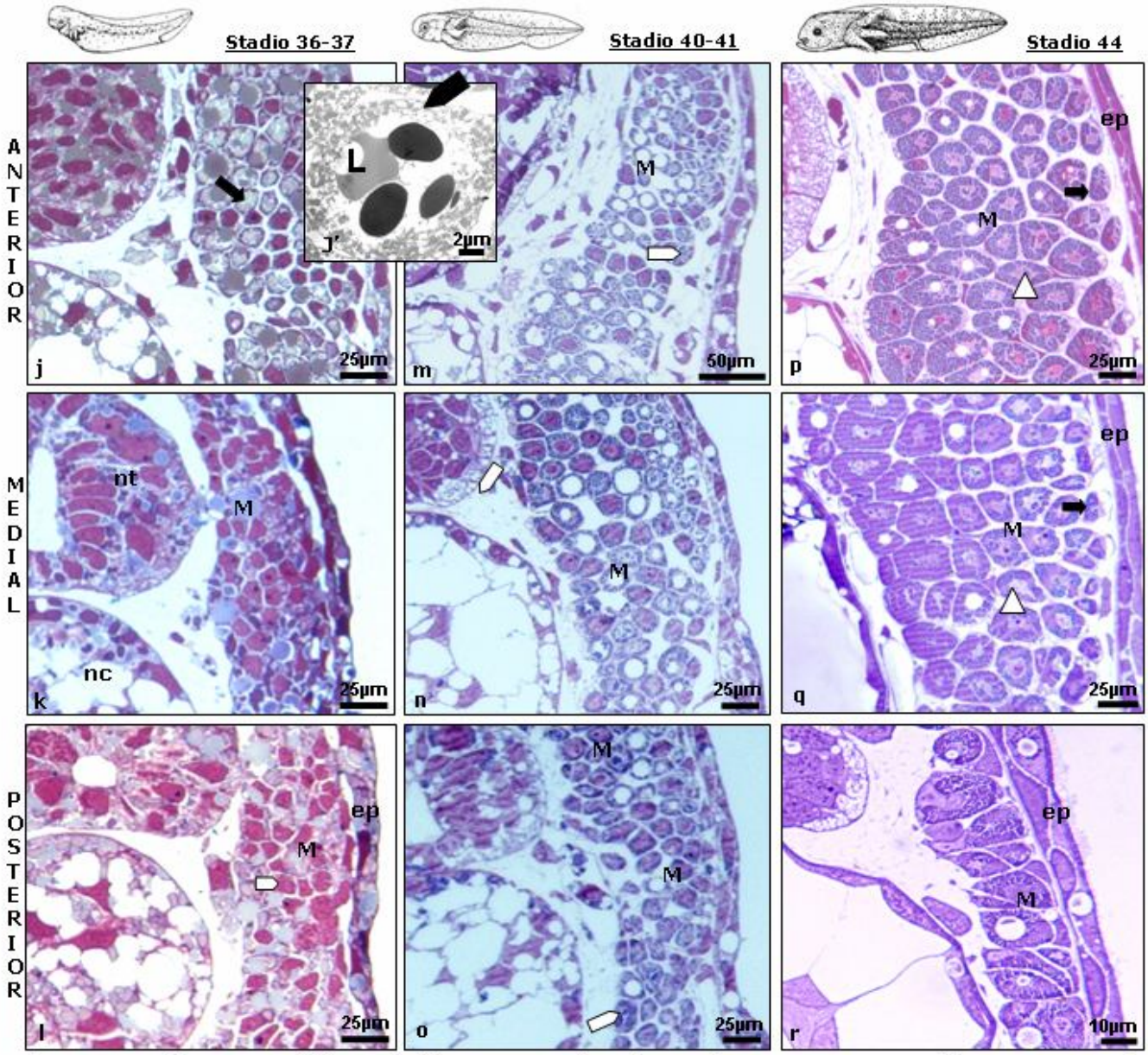
a-i L.M. Semithin transversal sections of *A. mexicanum* at different stages of development.
Sections were analyzed in different regions of each embryo: anterior (a,d,g) central (b,e,h) and posterior (c,f,i).

Stage 24-25 (a-c): somite (**S**) is surrounded by two epidermal cell layers (**E**) and is formed by cells closely associated one to each other with a cytoplasm filled by yolk droplets (★) and a large nucleus (→ in b).

Stage 28-29 (d-f) and **Stage 33-34** (g-i): somitic mesoderm is divided into symmetric masses constituted by: the sclerotome (**sc**), just closed to the notochord (**nc**) and the myotome (**M**), under the epithelium (**ep**). The myotome is still formed by myoblasts with large nuclei (⇦) and cytoplasm rich in yolk droplets.

nc notochord; **nt** neurotube

Figure 2



j-r **L.M. and TEM** Semithin and thin transversal sections of *A. mexicanum* at different stages of development.

Sections were analyzed in different regions of each embryo: anterior (j,m,p) central (k,n,q) and posterior (l,o,r).

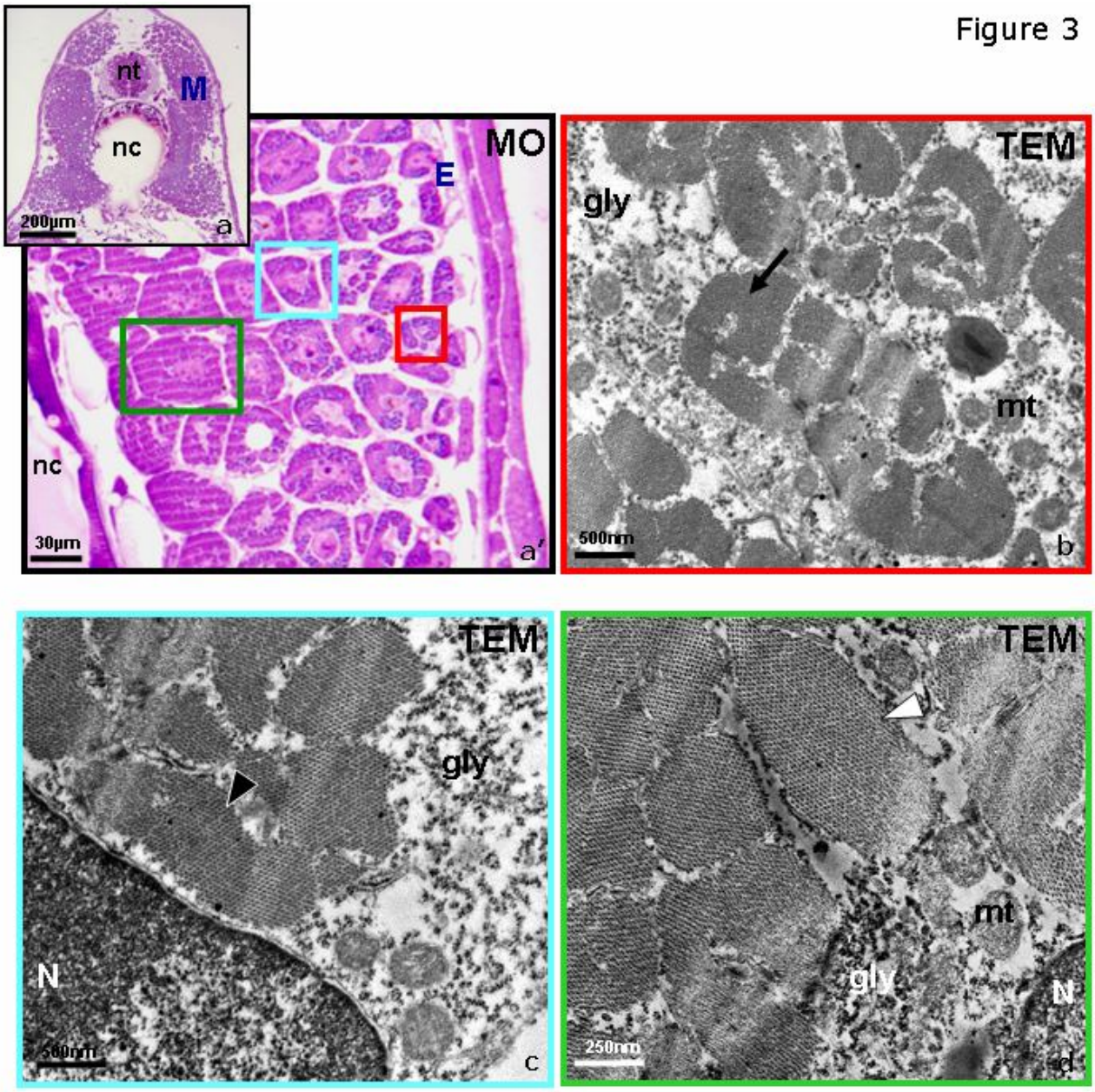
Stage 36-37 (j-l, j'): the myotome (**M**) starts to be differentiated in the anterior somites where newly differentiated mononucleated myocytes are evident (➡ in j). Detail at TEM of differentiating muscle fibers, showing lipids droplets (L) and electron-dense granules surrounded by a thin discontinuous ring of contractile material (➡ in j'). Medial and posterior somites (k,l) are formed by a compact mass of undifferentiated myoblasts (⇨ in l) lined by epithelium (**ep**).

Stage 40-41 (m-o): the myotome (M) is predominantly constituted by muscle fibers completely differentiated (⇨).

Stage 44 (p-r) In the medial and anterior somites (p, q). the myotome (**M**) is formed by three different fibers type; the small diameter fibers (➡), the medium diameter fibers (△) and the large diameter fibers close to the axial structures (**nc** and **nt**)

nc notochord **nt** neurotube

Figure 3



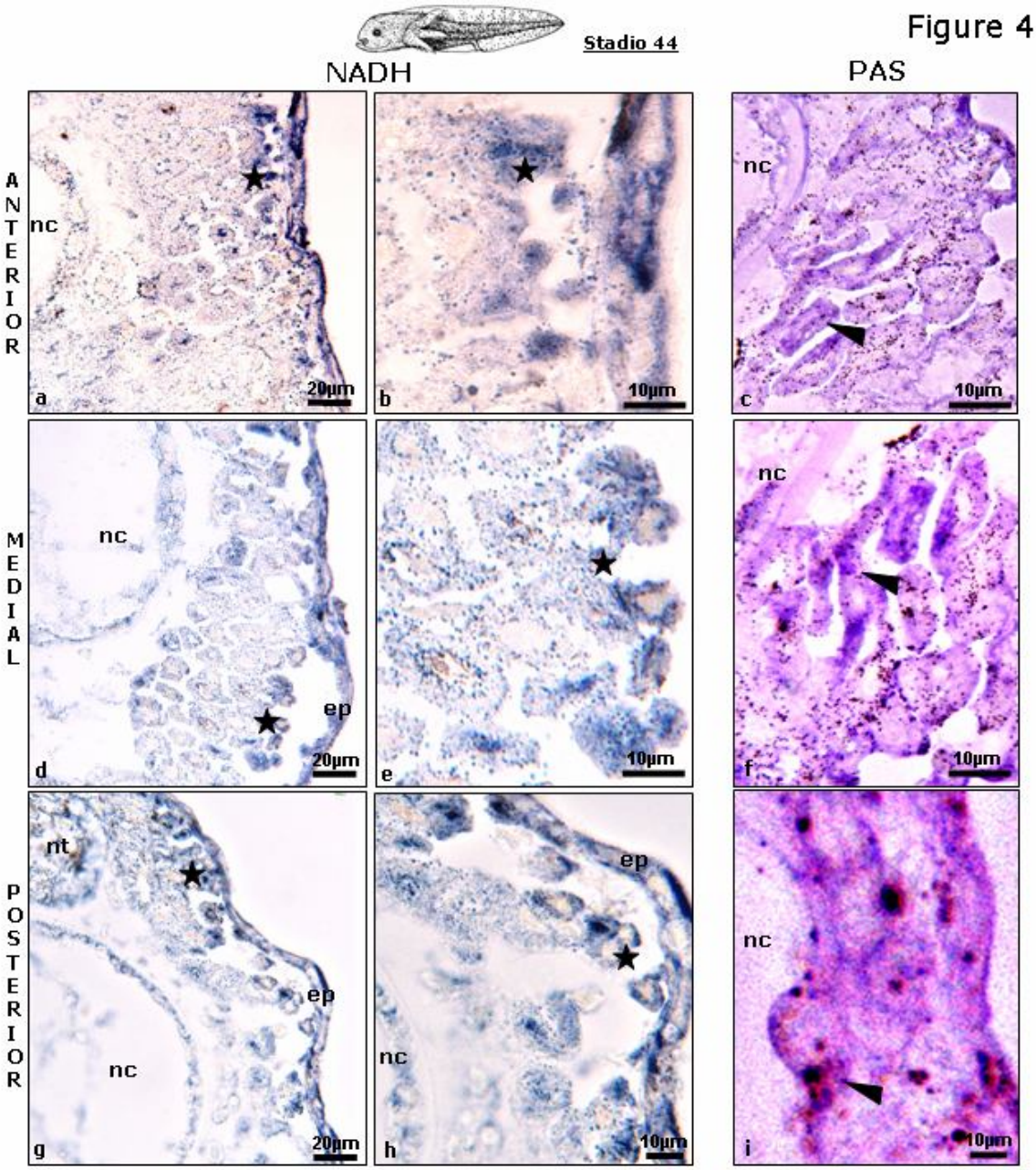
a, a', b-d. Cross sections at light (LM) (a,a') and transmission electron microscope (TEM) (b-d) of *A. mexicanum* embryo at stage 44.

Myotome (M) is formed by different types of muscle fibers:

- small fibers in a superficial subepithelial layer (□ in a') under the epithelium: present a cytoplasm with irregular shaped fibrils (←), lots of mitochondria (**mt**) and a little amount of glycogen (**gly**) (b);
- middle fibers (□ in a') characterized by a cytoplasm with irregular shaped fibrils (▲) and copious glycogen (**gly**) (c);
- large fibers located in the inner part of the myotome (□ in a') characterized by a cytoplasm with regular shaped fibrils (△), copious amount of glycogen (**gly**) and few mitochondria (**mt**) (d).

N nucleus
E epithelium
nc notochord

Figure 4



a-i **L.M.** NADH-diaphorase and PAS reactions on cross cryosections of *A. mexicanum* at stage 44 of development.

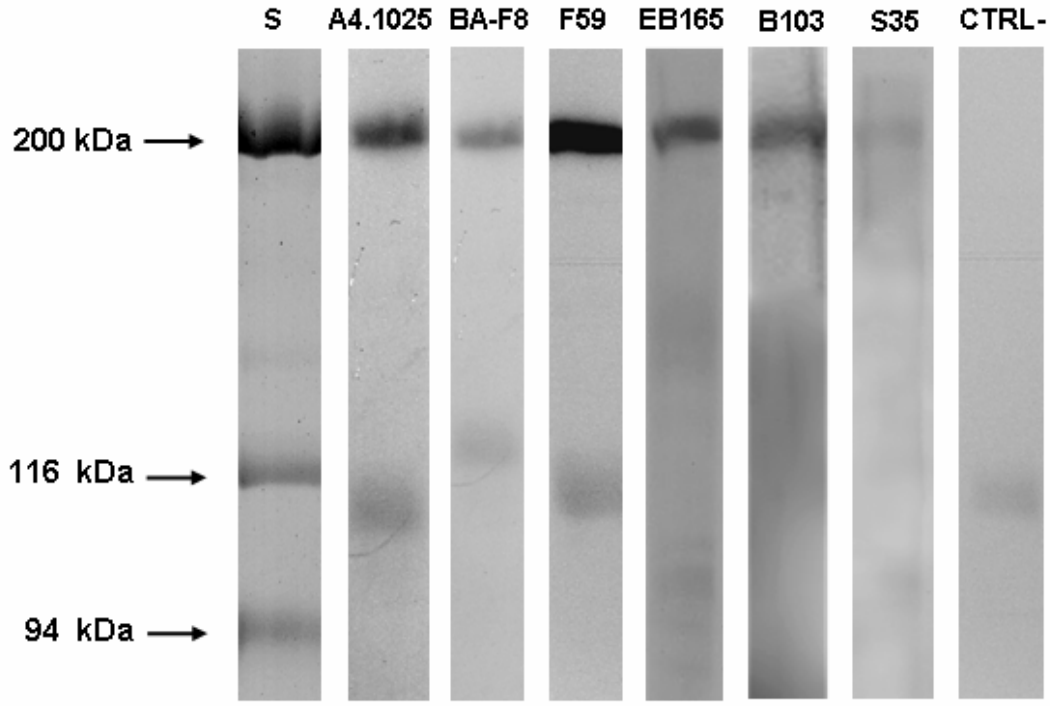
Superficial fibers localized on the outer surface of the somite, just under the epithelium (**ep**) are strongly positive for NADH-diaphorase (a,b,d,e,g,h,j,k; ★).

Medial and inner fibers (▲) close to the embryonic axial structures (**nc** and **nt**), are positive for PAS reaction (c,f,i,).

nc notochord
nt neurotube

Figure 5

Western blot

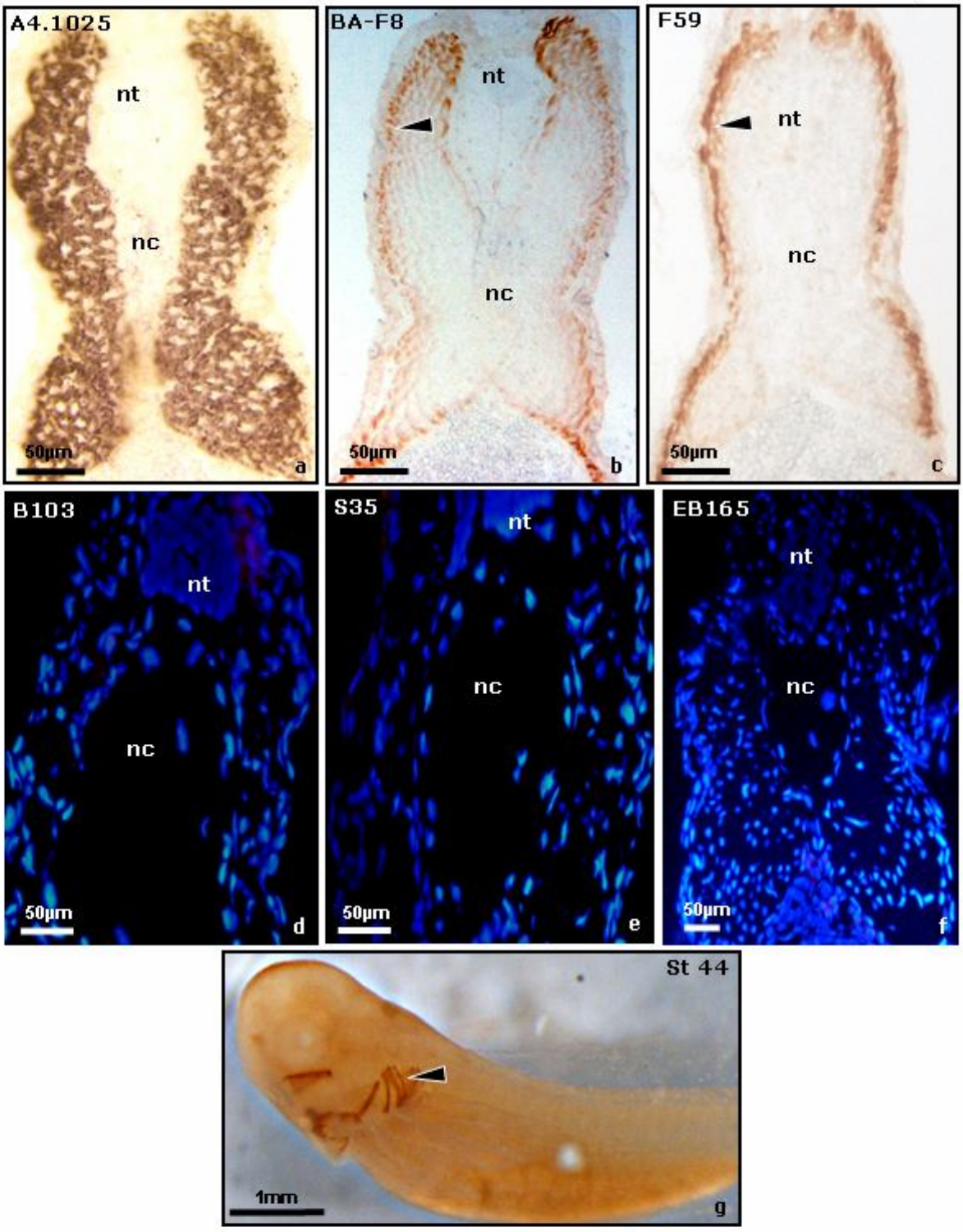


Western blot on embryos at stage of development 42

Antibodies **A4.1025, BA-F8, F59, EB165, B103, S35** recognize a band of about 200 kDa, corresponding to the molecular weight of the skeletal myosin.

S standard

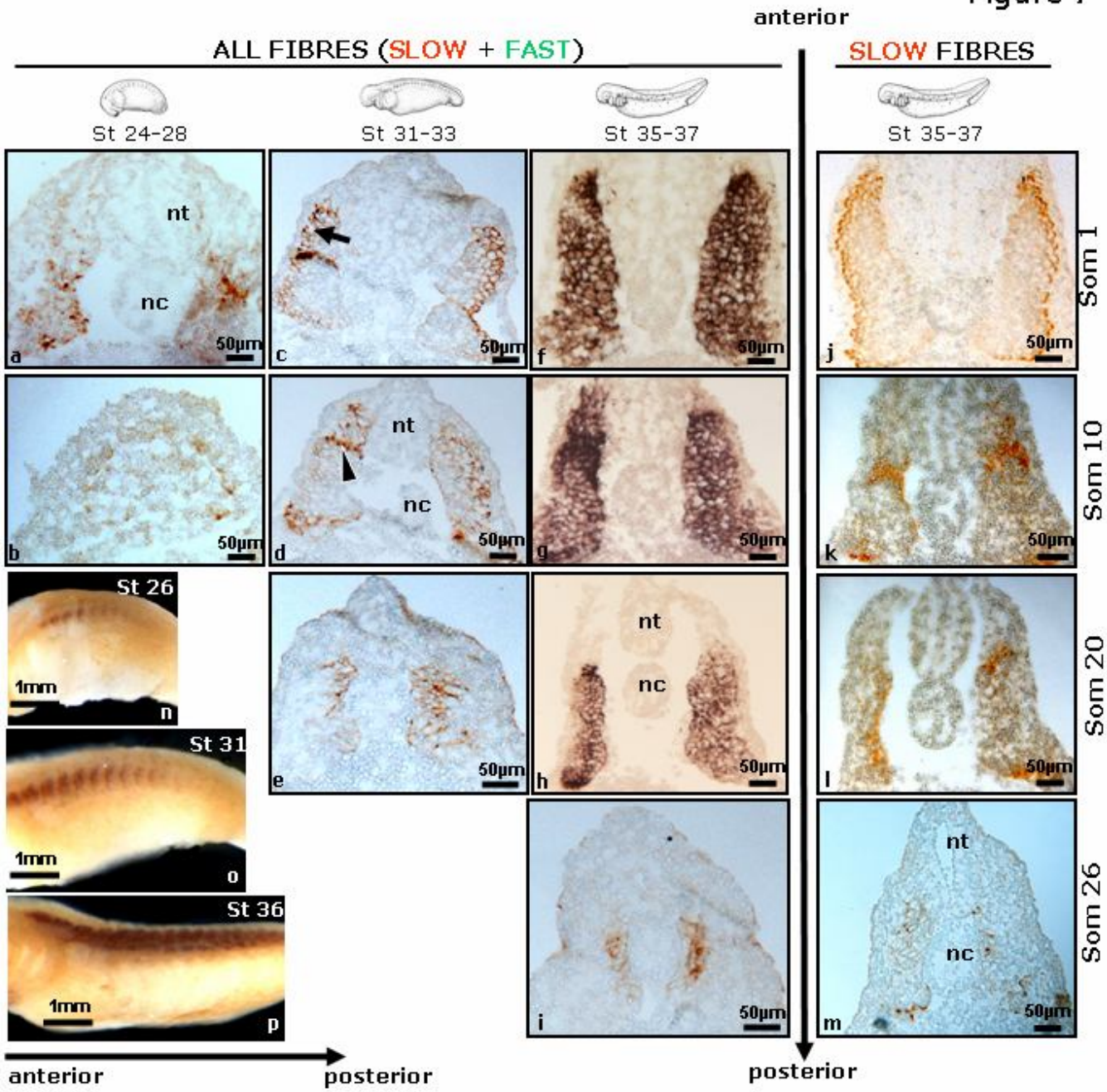
Figure 6



a-g **L.M.** Cross cryosections (a-f) and whole-mount staining (g) of *A. mexicanum* at stage 44.
A4.1025 (a) reacts with the vast majority of the muscle fibers.
BA-F8 and **F59** (▲ in b, c), react strongly with the single layer of superficial slow fibers.
B103 (d), and **S35** (e) don't show any positive signal on the cryosections.
EB165 (f) is absent in the somites while it is expressed in the cefalic muscles as shown by the whole mount staining (▲ in g).

nc notochord **nt** neurotube

Figure 7



a-m. Cross cryosections of whole mount stained embryos with A4.1025 (all fibers marker) and BA-F8/F59 (slow fibers marker)

n-p Whole mount staining with A4.1025 on embryos at different stages of development

A4.1025 recognizes all the MyHC isoforms and shows that the first differentiating fibers are the ones next to the notochord (stages 24-28, a,b).

Later (stages 31-33) these fibers migrate laterally (c-e; ▲in d) and, in the more mature anterior somites, form a superficial subepithelial layer (← in c).

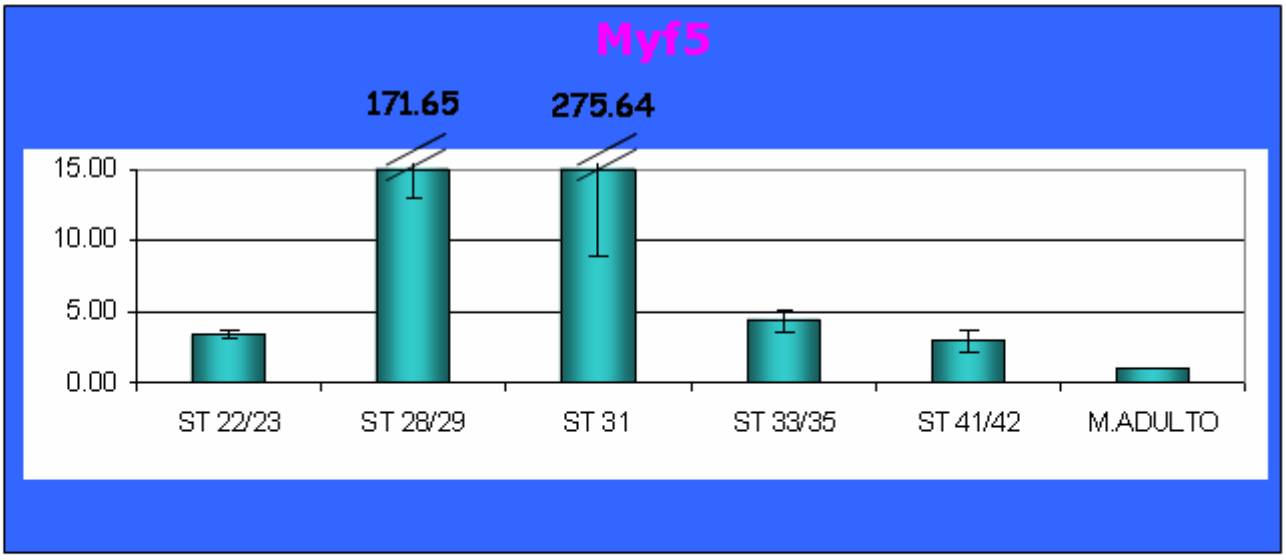
Starting from the stage 35 (f-m):

- F59 and BA-F8 stain the superficial slow fibers (j-m)

- A4.1025 stains the inner fibers that form the most of the myotome (f-i) and shows that differentiation occurs in an anterior to posterior direction (n-p). **nc** notochord **nt** neurotube

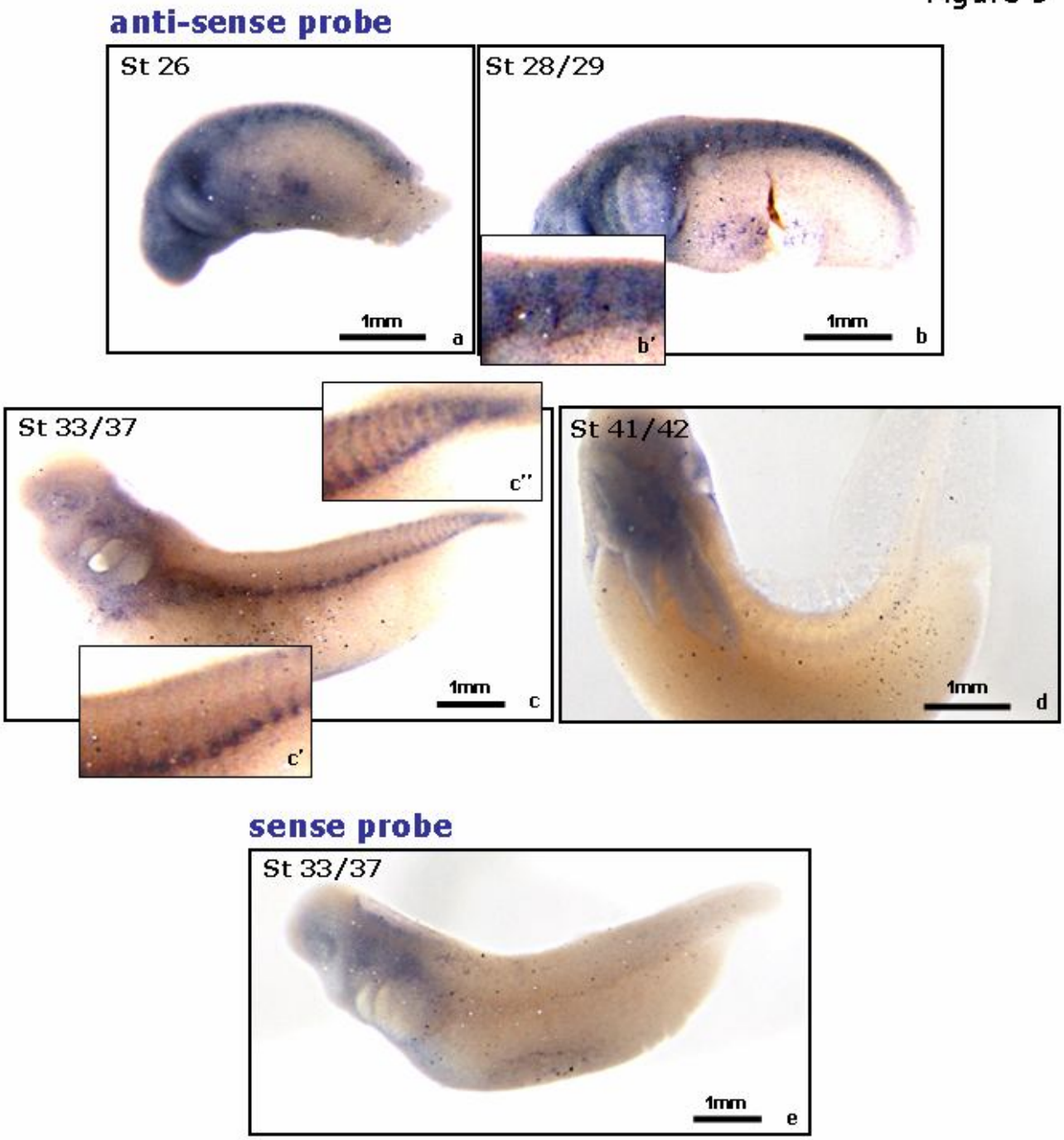
Figure 8

Quantitative RT PCR



Myf5 mRNA expression levels in some embryonic stages, normalized in respect to the beta actin housekeeping gene.
The expression is low at early stage of development (22-23), gradually increases in the next stages (28-29), peaked at stage 31 and by stage 33 dramatically decreases.

Figure 9



a-e. mRNA *in situ* whole mount hybridization on embryo of *A. mexicanum* at different stages of development

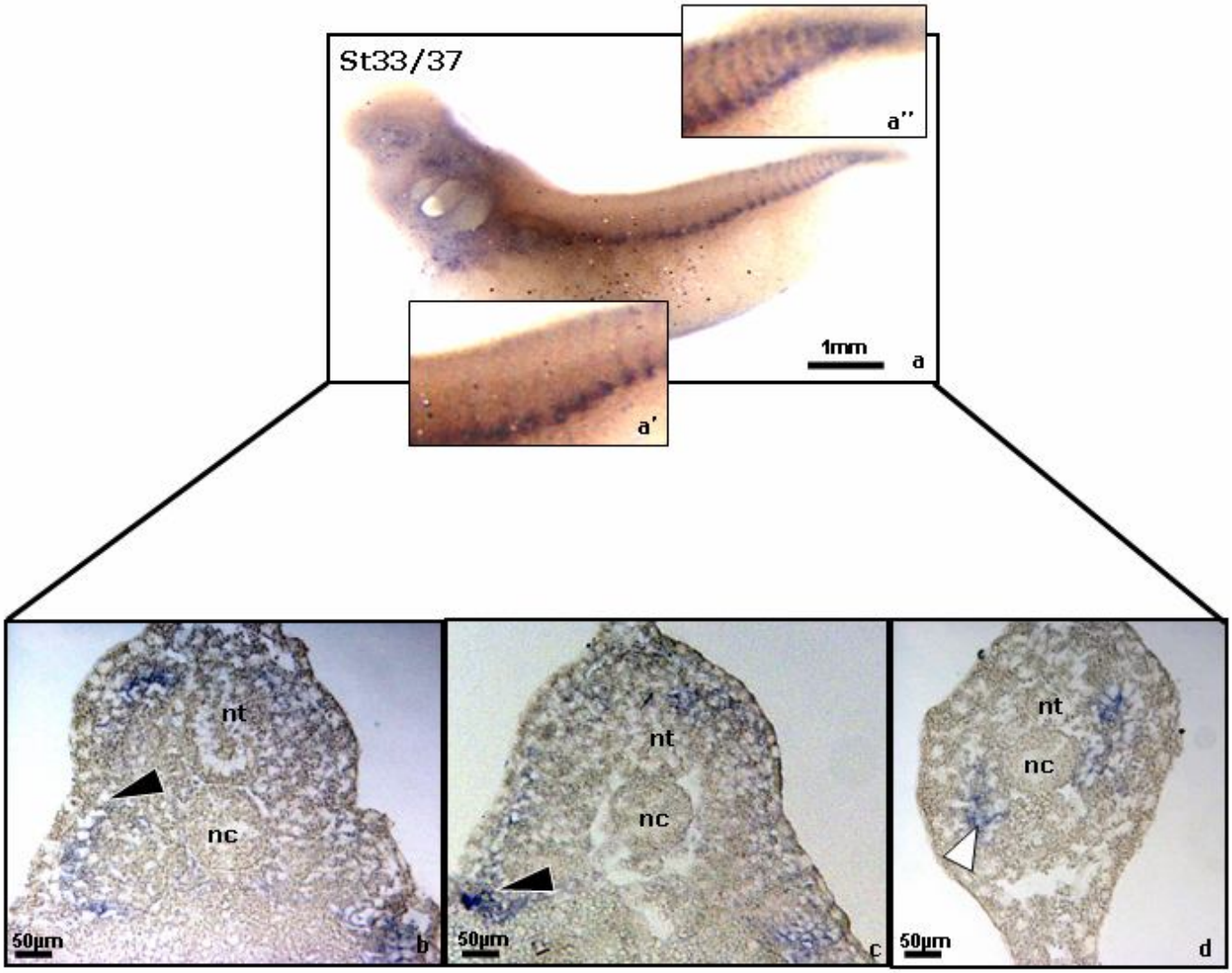
At early stages of development (stages 26, 28/29): Myf5 is expressed in the middle portion of all the somites (a, b, b').

Starting from stages 33-37 the transcript expression level decreases appreciably (b, b',b'',c).

e: *in situ* hybridization with no label, using a sense probe.

nc notochord **nt** neurotube

Figure 10



a-d. mRNA *in situ* whole mount hybridization and cross cryosections of *A. mexicanum* at stage of development 37

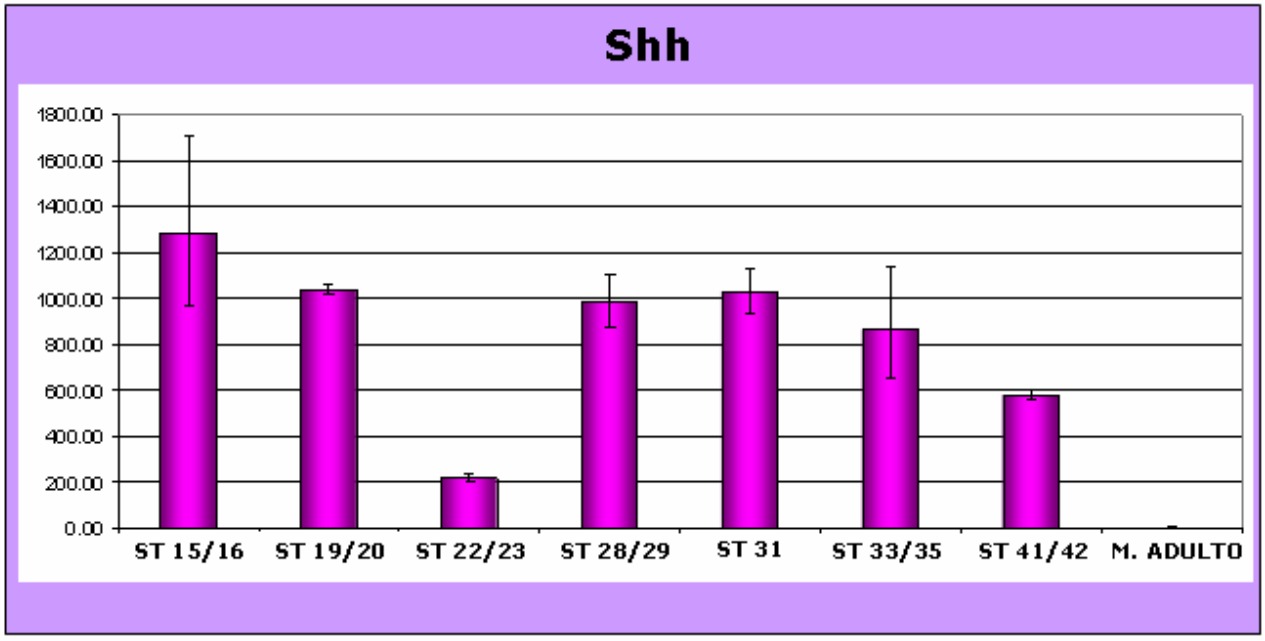
In the anterior somites the transcript is expressed in a superficial layer of cells (a',b,c).

In the tail Myf5 mRNA is expressed in cells localized next to the notochord (a'',d).

nc notochord **nt** neurotube

Figure 11

Quantitative RT PCR



SHH mRNA expression levels in some embryonic stages, normalized in respect to the beta actin housekeeping gene.
Shh is highly expressed at early stages (by stage 15 up to stage 20), it dramatically decreases around stage 22/23, becomes highly expressed by stage 28/29 until stage 31 and then totally disappears in the hatching stage and in the adult animal.