Scanning electron microscopy and histological studies on the skeletal muscles in post hatching Nile tilapia (oreochromis niloticus)

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A B S T R A C T

The aim of the present study is to investigate the histomorphological changes of the skeletal muscle during early larval stages of Nile tilapia with special concern to the hyperplasia, occurring in the skeletal muscles. A total number of 80 Nile tilapia fish *Oreochromis niloticus* divided into six different age-dependent groups were fixed in neutral buffered formalin and examined by light and scanning electron microscope(SEM) .It was found that, the myotomal tissues differentiated and divided into epimeric (epaxial) and hypomeric (hypaxial) part by distinct horizontal septum at 2 days post hatching larvae .Two types of skeletal muscle appear red muscle fiber and white muscle fiber at 3 days post hatching larvae .These findings indicated that hyperplastic growth was increased gradually with significant difference between all stages during early larval stage of Nile tilapia.

Key words: Nile tilapia, Skeletal muscles, larval stage, SEM

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1. INTRODUCTION

The Nile Tilapia has a great economic value. Tilapia species are the major protein in many of developing countries. They are a good fish having a white flesh with no intramuscular bones and it is considered as a good source of animal protein all over the world (Tayel, 2003; Tayel, 2007). Fish axial muscles are organized into a series of segmentally arranged myotomes. The individual myotomes are separated by collagenous sheets called myosepta. Distinct layers of slow and fast muscles are formed during the embryo stage (Devoto et al., 1996). Additional fibres may be added from discrete germinal zones (stratified hyperplasia) during the larval stage to complete the main muscle layers, which are retained throughout life (Rowlerson and Veggetti, 2001). The phenotype of the embryonic slow and fast muscle fibres gradually changes to resemble the adult fibre types (Chanoine et al., 1992 and Mascarello et al., 1995). The major part of the myotome, usually more than 90% by volume, is composed of fast twitch muscle fibres (Johnston, 1981) .The aim of the present study is to investigate the histomorphological changes of the skeletal muscle during early larval stages of Nile tilapia with special concern to the hyperplasia, occurring in skeletal muscles.

2. MATERIALS AND METHODS

2.1. Fish source and sampling

This study was carried out on a total of 80 Nile Tilapia fish (*Oreochromis niloticus*) divided into six different age-dependent groups as the following: 12hr, 2day, 3day, 4day, 6day, 8day post hatching. .All fish were obtained from a private fish farm in Kafr elsheikh governorate. The fish were transported in oxygenated cellophane bags containing water (1/4) and air (3/4) and transported to the laboratory. Once received in the lab, the samples were washed with tap water to remove slime and mud then a skin

flap on the back above the lateral line was incised and the underlying muscles were dissected with the aid of a sterilized scalpel and scissor. Muscle samples were removed immediately to avoid autolysis.

2.2. Materials

10% Neutral Buffered Formalin

- 50 ml 37% Formaldehyde
- 450 ml Distilled Water
- 3.25 gm Sodium Phosphate, dibasic (Na_2HPO_4)
- 2 gm Sodium Phosphate, monobasic (NaH_2PO_4)

2.3. Methods

2.3.1. Histological examination

The fixed samples were extensively washed in 70 % alcohol (3 x 24 hour) to get rid of the fixative before the subsequent step of tissue processing. The tissue samples were then dehydrated in graded series of ethanol (80%, 95% and absolute), cleared in xylene and impregnated and embedded in paraffin wax. Sections of 5-7 μm were cut using Leica rotatory microtome (RM 2035) and mounted on glass slides. Paraffin sections were kept in incubator at 40°C until used for conventional staining (H&E). Staining techniques employed were performed according to (Bancroft and Stevens, 1990).

2.3.2. Scanning Electron Microscope examination

The samples were fixed in a mixture of paraformaldehyde 2.5 % and glutaraldehydehyde 2.5 % solution in 0.1 M phosphate buffer for 4 hours at 4°C. After washing in the same buffer, the specimens were post-fixed in osmium tetroxide 1% in phosphate buffer for two hours followed by washing in the same buffer. The samples were then dehydrated in ascending grades of ethanol followed by critical point drying in carbon dioxide, then sputter-coated with gold and examined with Jeol JSM 5300 scanning electron microscope, Faculty of Science, Alexandria University

2.3.3. Measurement of cross section area

Image J software was used as the cross section area of 100 fibers from each muscle was measured directly from electron micrograph. All of the fibers within each randomly selected fasciculus were measured and the mean myofibre cross sectional area and the mean standard error are calculated for each muscle provided that using fixed magnification power and surface area for all examined slides.

3. RESULTS

3.1. 12 hour post hatching larvae

Analysis of transverse sections of this stage of Nile tilapia larvae at the level of the trunk using scanning electron microscope revealed presence of undifferentiated myotomal tissue on both side of the notochord and developing spinal cord (Fig. 1B). However, transverse section at the level of tail peduncle, which lacks spinal cord, showed undifferentiated myotomal tissue surrounding the centrally located notochord (Fig. 1C). Histological structure of sagittal section reveals appearance of bipinnate segmented myotomal bundles arranged bilaterally and symmetrically on both sides of the notochord and developing spinal cord forming a clear V-shape (Fig. 1D). In all examined samples, the notochord was similar to adipose tissue with notochordal cells resemble fat cells. The notochord bounded dorsally by the developing spinal cord which was enclosed by vertebral canal formed by neural arches (Fig. 1B-D).

3.2. 2 days post hatching larvae

Histological structure of cross section of this stage of at level of trunk showing that myotomal tissues became slightly differentiated and divided into epimeric (epaxial) and hypomeric (hypaxial) part by less distinct horizontal septum (Fig. 2B). The differentiated muscle fibers run obliquely through the entire length of the myotome. The bundles of developing muscles became clearly separated by myoseptal sheet (white arrowheads, Fig.

2B). In addition, the dorsal myotomal tissues (epaxial myotome) were equally separated by median vertical septum into right and left halves (Fig 2B). Comparing to the earlier stages the muscle bundles at tail peduncle became slightly differentiated at this stage as shown by cross section examined by scanning electron microscope (Fig. 2C).

3.3. 3 days post hatching larvae

Histological structure of cross section of this stage of post hatching Nile Tilapia larvae at level of trunk reveals the horizontal skeletogenous septum became evident (line between the two blue arrowheads, Fig.3B, C). This line seemed slightly oblique and extended from the level of the notochord base to the external kink separating the epaxial myotomal part from the hypaxial part. This septum represented externally by a longitudinal groove. The muscle fibers were differentiated into red and white types. The red muscle appeared as a very thin embryonic superficial red layer at the periphery between the white muscle fiber zone (medially) and the skin (laterally) (yellow outlines areas, Fig. 3B, C). The red muscle fibers were densely packed, while the white fibers were loosely packed but with larger diameters. The layer of red muscle formed a wedge shape along the horizontal line, whereas white muscle forms the greatest volume of body tissue near the center. Another less distinct horizontal septum, run from the proximal part of the spinal cord toward the skin and met with the horizontal septum at the external kink, subdivided the epaxial myotome into two parts; the most dorsal part with fibers run obliquely and irregularly (EP1, Fig. 3B), and the ventral part with longitudinally arranged fibers similar to that in the hypaxial myotome (EP2, Fig. 3B).

3.4. 4 days post hatching larvae

Scanning electron microscope of cross section of this stage of post hatching Nile Tilapia larvae at level of trunk reveals that developmental process during this stage was similar to the previous stage but with increase in height of the dorsal part of the epaxial myotome (EP1, Fig.4B) and increase in numbers of the red muscle fibers (yellow outlined area, Fig.4B) as well as well distinct horizontal septum which became straight (area between the two arrowheads, Fig.4B). Unlike in trunk, no distinct horizontal septum, no red muscle layer and no clear division for the myotome was observed in the tail peduncle (Fig. 4C).

3.5. 6 days post hatching larvae

Histological structure of transverse section of this stage of post hatching Nile Tilapia larvae at level of trunk showed noticeable increase in number of the muscle fibers but the diameter seemed to be constant (Fig.5A, B). This means that the increase in the musculature during this stage and other previous examined stages is mainly due to hyperplasia, rather than hypertrophy. This causes a clear increase in the height, rather than the width, of the muscle bundles of both white and red muscle fibers (Fig.5A, B). During this stage, the majority of the muscle fibers became longitudinally orientated in parallel with the larvae long axis, especially in the hypaxial and ventral part of epaxial myotome. Superficial red muscle layers expanded proximally and distally resulting in a continuous increase in the number of slow fibres per myotomal cross-section (Fig. 5A, B). Higher magnification of this section showed each fiber has peripherally located nuclei which appear as flattened dense dark dot. Muscle interstitium in this section appears as empty spaces (Fig. 5C). Cross section at the level of tail peduncle examined by scanning electron microscope showed similar picture as the previous examined stages but with clear longitudinally arranged skeletal muscle fibers surrounding the notochord (Fig. 5D).

3.6. 8 days post hatching larvae

Histological structure of cross section of this stage of post hatching Nile Tilapia

larvae at level of trunk showing that the muscle bundles and fibers increased in numbers. Both types of skeletal muscle (red/white and epaxial/hypaxial) were well differentiated and can be easily noticed (Fig. 6 A-E). Scanning electron micrograph showing myofilaments of skeletal muscle fiber with three –dimensional effect of Scanning electron microscope image and high resolving power permit identification of the myofibers with their clear transverse banding (striations) transverse tubules are tubular extensions of sarcolemma into the fibers and they traverse myofilaments at regular intervals.(Fig. 6 C, D).

3.7. Measurement of muscle fiber number per the same muscular area in different examined stages

Image j software was used to measure the surface area of the myofibers in the same muscular region in all examined stages. The results of this calculation were detailed in Table (1) and were graphed in Fig.7. In general, hyperplastic growth was increased gradually from stage 19 to stage 25 with significant difference ($p< 0.05$) between all stages except between stage 20 (D3) and stage 21 (D5) which showed insignificant difference ($p> 0.05$).

Table (1): Fold changes in means of muscle fiber number per the same muscular area in different examined stages.

Age in day	Range of muscle fiber number		Mean ± Standard error of mean
	Minmum	Maximum	
Day 2	58.067	90.078	74.067±7.45^{d}
Day 3	78.088	110.033	94.023±9.60^{c}
Day 4	88.020	120.047	96.037±9.75^{c}
Day 6	115.876	148.543	129.393±10.50^{b}
Day 8	134.780	168.334	159.305±14.90^{a}

LSD 5% = 0.354. LSD 1%= 0.427

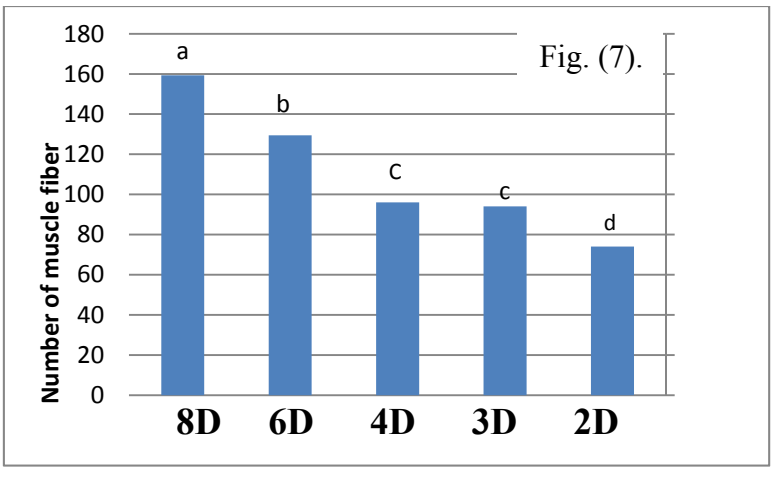

Fig. (7).

Fold changes in means of muscle fiber number per the same muscular area in different examined stages.

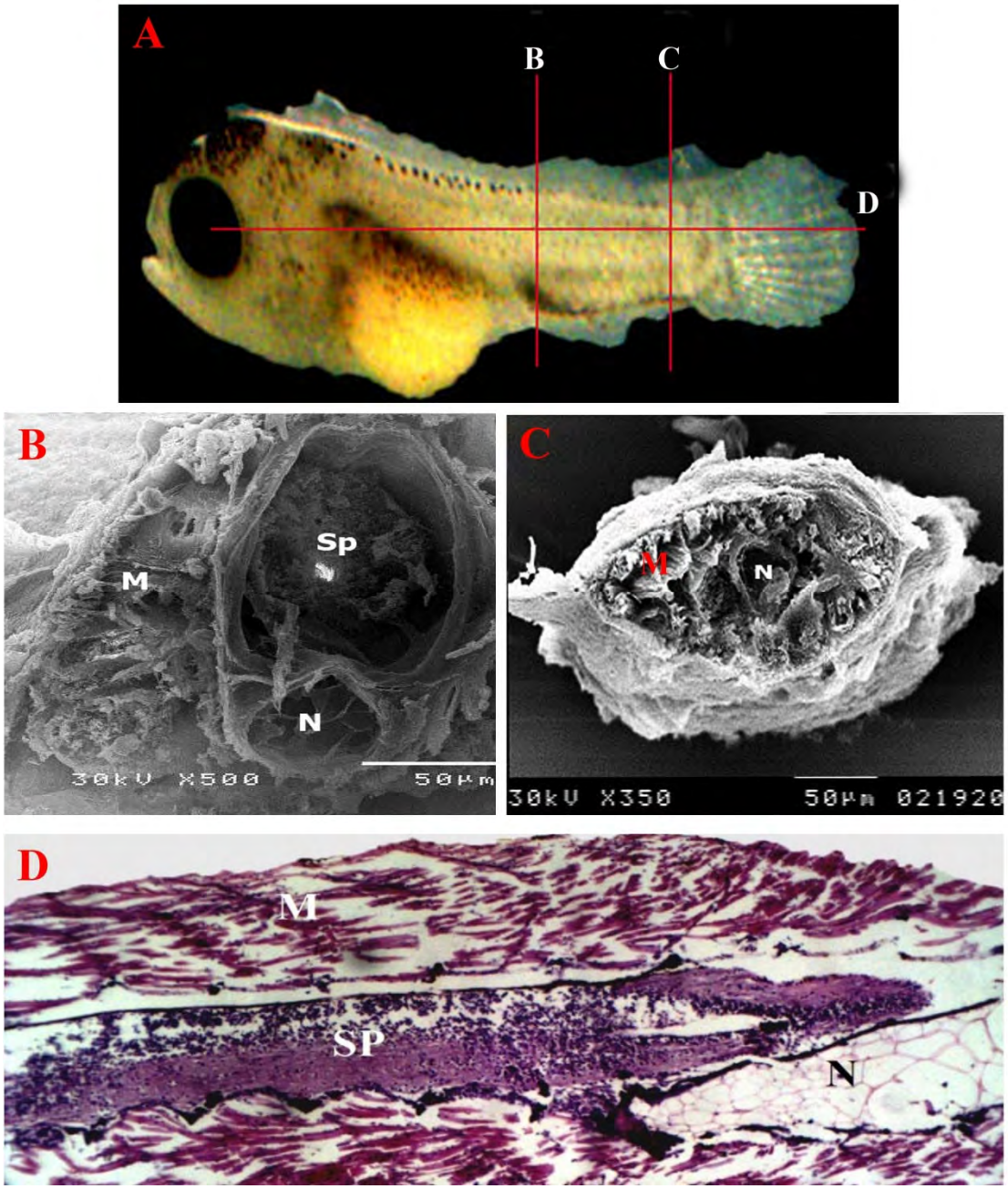

Fig.1. A. Whole mount of (12 hours post hatching) Nile Tlipia larva showing the levels of transverse (B and C) and sagittal (D) sections. B, C. Scanning electronic micrograph showing cross section at the level of trunk (B) and tail peduncle (C). D. Photomicrograph showing sagittal section (H&E) X40: Abbreviations: M=myotome; N= notochord; SP= spinal cord.

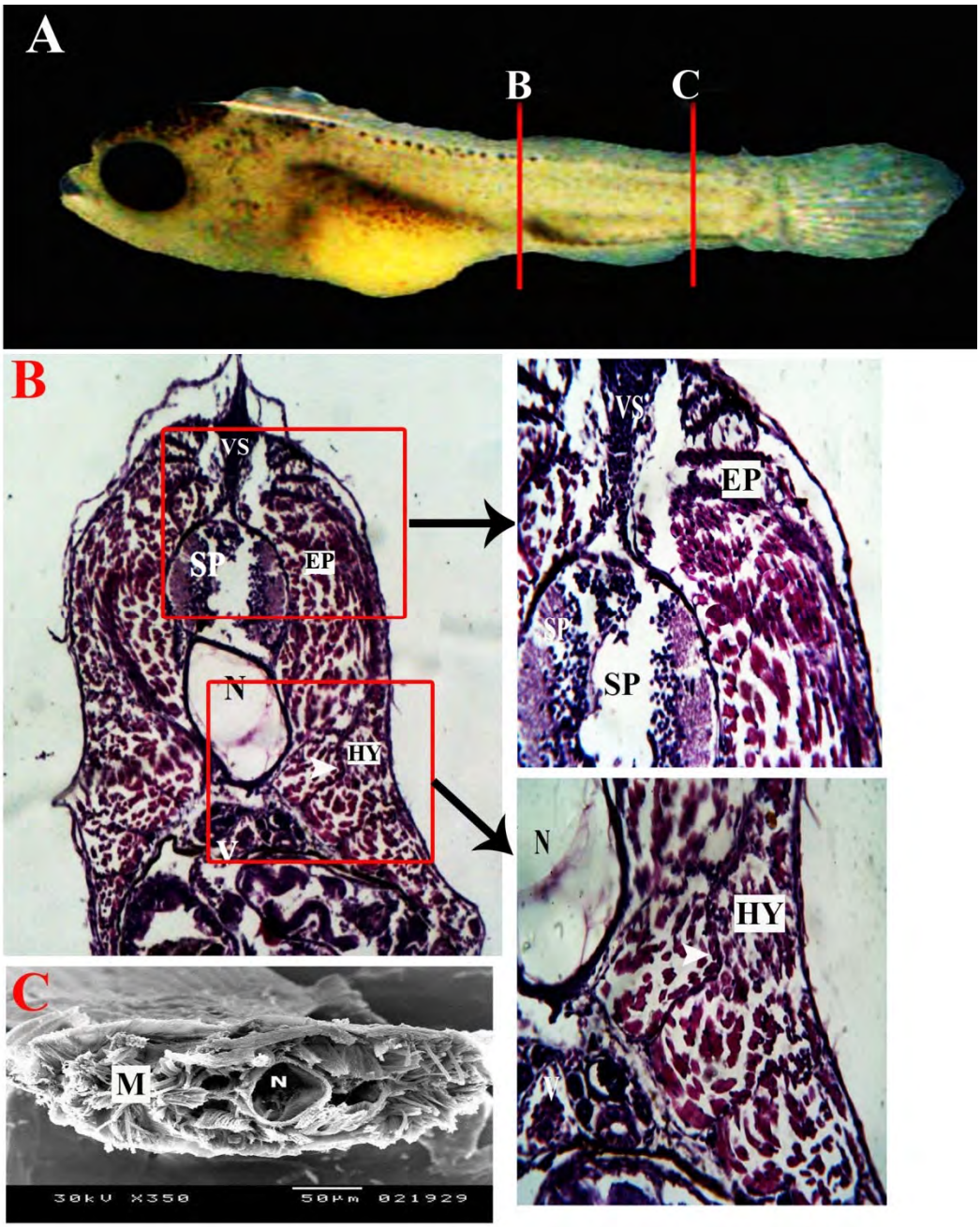

Fig.2. A. Whole mount of (2 days post hatching) Nile Tilapia larva showing the levels of transverse sections (B and C). B. Photomicrograph showing transverse section (H&E) X10 and the levels of the higher magnification (X40) at epaxial and hypaxial levels. C. Scanning electronic micrograph showing cross section at the level of tail peduncle. Abbreviations: EP=epimeric part of the mytome; HP =hypomeric part of the mytome; M = myotome; N = notochord; SP = spinal cord; V = viscera; VS = vertical septum

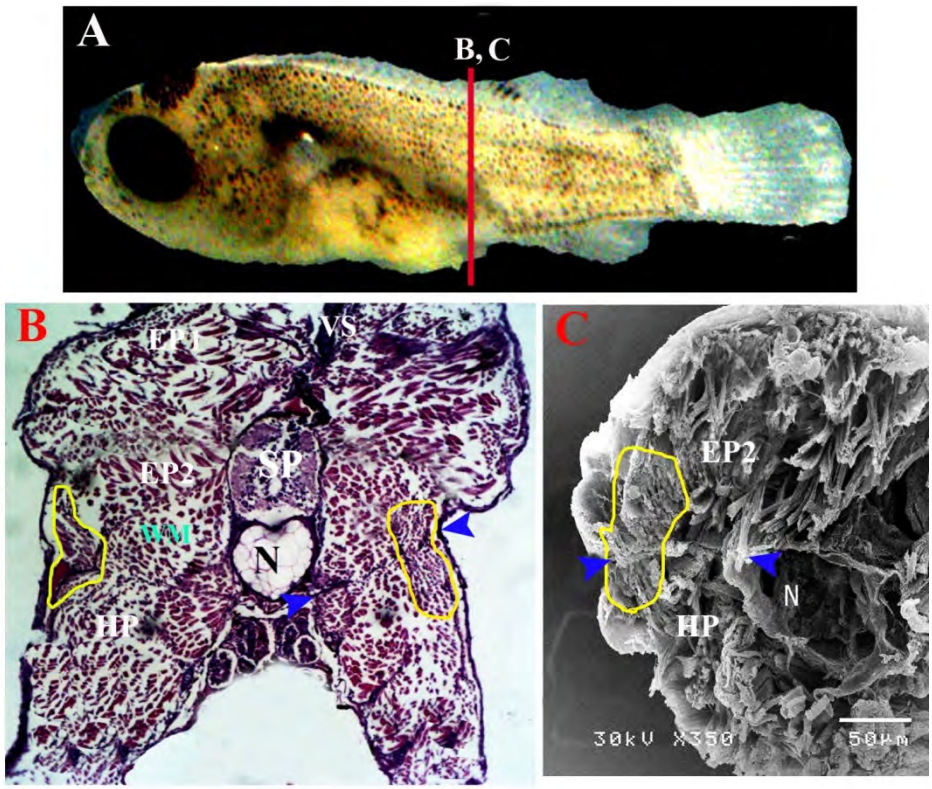

Fig.3. A. Whole mount of (3 days post hatching) Nile Tilapia larva showing the levels of transverse sections (B and C). B. Photomicrograph showing transverse section (H&E) X10. C. Scanning electronic micrograph showing cross section at the same level. The yellow outlined areas indicate the red muscle layer, while the areas between the two arrowheads refer to the horizontal septum. Abbreviations: EP=epimeric part of the mytome; EP1= dorsal part of EP; EP2= ventral part of EP; HP =hypomeric part of the mytome; M = myotome; N = notochord; SP = spinal cord; V = viscera; VS = vertical septum; WM= White muscle

4. DISCUSSION

The present study, showed the first evidence for muscle differentiation during stage 19 (2 days after hatching) of larval development with the early differentiated muscle fibers run obliquely through the entire length of the myotome. The present study comes in agreement with Ramesh and Nagarajan, (2013) who observed that in adult Clarias batarachus The developing myotome is divided into an upper (epaxial) and a lower (hypaxial) half by a horizontal septum (internally) which is externally represented by a groove running along the side of the fish. This septum starts ill distinct and then becomes clearer at stage 20. Similarly,Thisse et al.(1993) found that horizontal septum became more notable during the early larval stages of the fish. The orientation of the horizontal septum was different according to the stage of the development. During early stages, at stage 20, the septum is oblique and later on in the successive developmental stages it becomes straight. This disagrees with results obtained from other fish which show straight horizontal septum along the whole body of other fish during similar larval stages (Ramesh and Nagarajan, 2013). This difference may be due to species variations. In this study a further subdivision of the epaxial muscles are subdivided into two parts, according to fiber orientations. This subdivision was not observed in any other fish according to the published data. Therefore, the Nile tilapia may have a unique distribution pattern of muscle fibers. Our results in consistence with Greer-Walker and Pull (1975) in adult marine fish , Hoyle (1983), Hoyle et al.(1986) in adult grass pickerel, and Luther et al. (1995) in adult teleost fish who found that red (slow) muscle fibers are segregated into a wedge

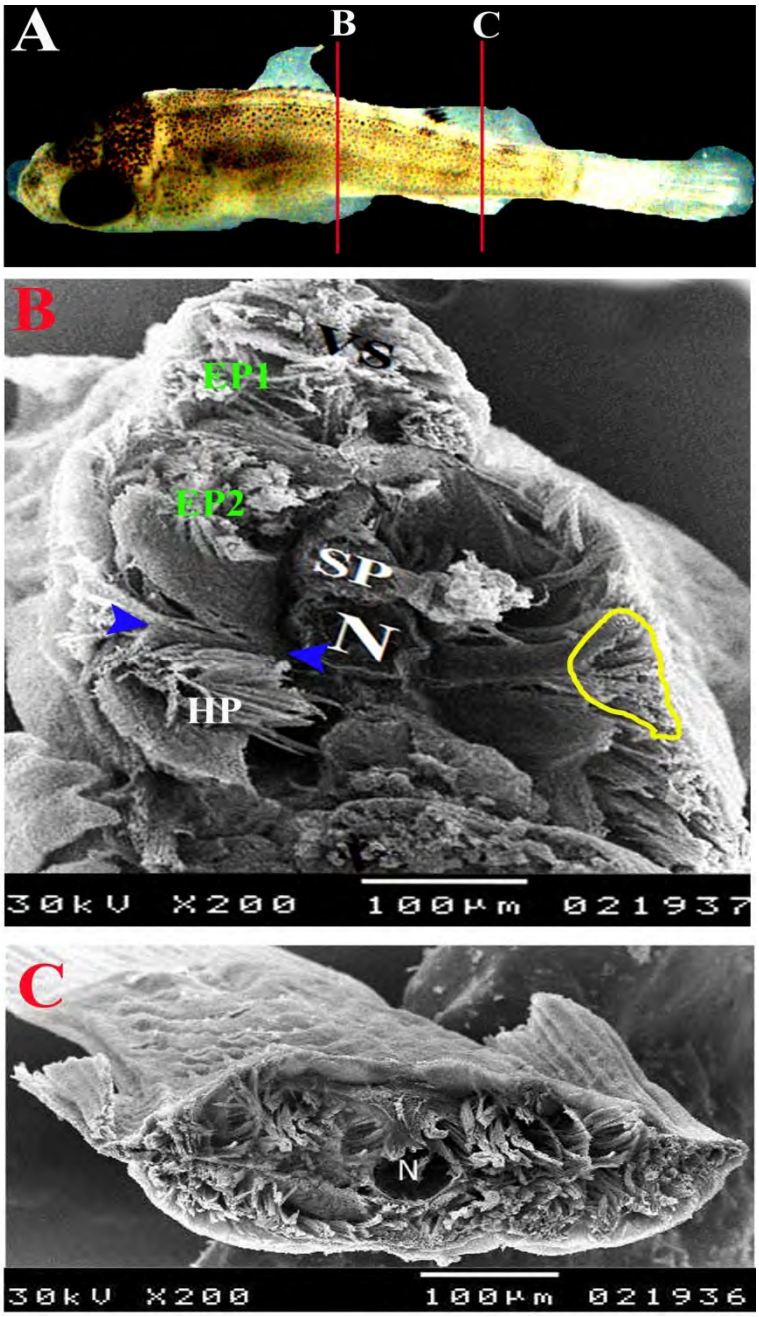

Fig.4. A. Whole mount of (4 days post hatching) Nile Tilapia larva showing the levels of transverse sections (B and C). B and C. Scanning electronic micrograph showing cross section at trunk level (B) and tail peduncle (C). The yellow outlined areas indicate the red muscle layer, while the areas between the two arrowheads refer to the horizontal septum. Abbreviations: EP1= dorsal part of EP; EP2= ventral part of EP; HP =hypomeric part of the mytome; N = notochord; SP = spinal cord; VS = vertical septum.

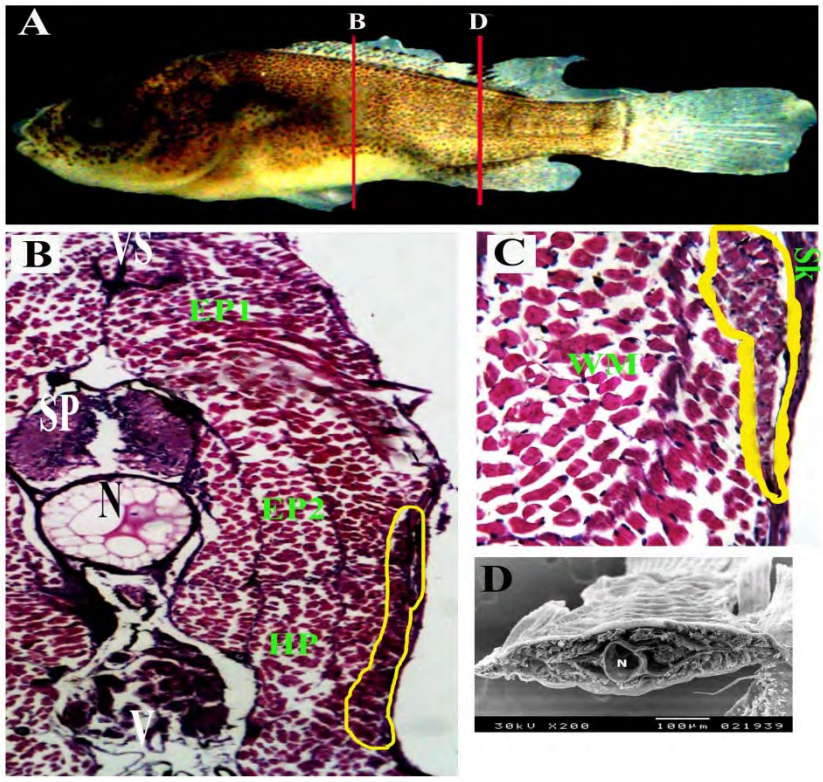

Fig.5. A. Whole mount of (6 days post hatching) Nile Tilapia larva showing the levels of transverse sections (B and D). B and C. Photomicrographs showing transverse section (H&E) X10 (B) and X40 (C). D. Scanning electronic micrograph showing cross section at tail peduncle. The yellow outlined areas indicate the red muscle layer. Abbreviations: EP1= dorsal part of EP; EP2= ventral part of EP; HP =hypomeric part of the mytome; N = notochord; Sk= skin; SP = spinal cord; VS = vertical septum; WM= white muscle.

shaped region of the myotome at the lateral end of the horizontal septum. Red (slow) muscle fibers are small, darkly colored, more heavily vascularized, and contain more lipid and mitochondria than the large, pale white (fast) muscle fibers. In contrast to our study and the above mentioned studies, (Johnston et al., 1977), (Bone ,1978) and (Sa¨nger and Stoiber, 2001) reported the presence of a third muscular layer, which is the intermediate (pink) muscle fibers located between the slow and fast muscle fibers. These three main skeletal muscle types are usually found in the trunk musculature of adult teleosts, and classified according to colour and contraction speed as: red/slow, pink/intermediate, and white/fast. Rowlerson et al. (1997) stated that in fish, as in birds and mammals, hyperplasia plays a larger role in growth during the larval/fetal period, whereas hypertrophy dominates during juvenile and post-juvenile growth. In addition, Rowlerson et al.(1995) noticed that the rate of hypertrophic growth will vary with somatic growth rate and at different stages in life. In agreement, we also found a significant gradual increase of muscle fibers by hyperplasia as revealed by measuring of the muscle fiber area.

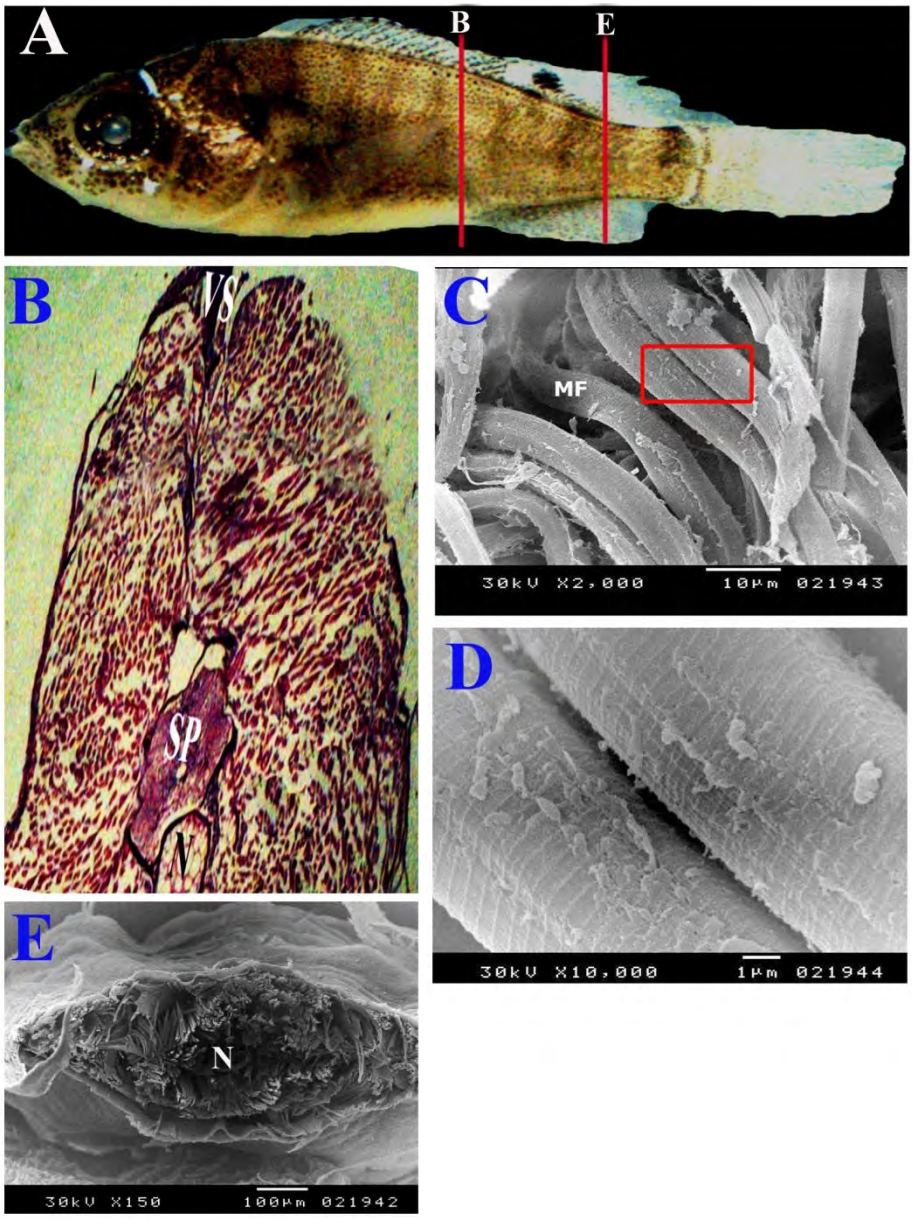

Fig.6. A. Whole mount of (8 days post hatching) Nile Tilapia larva showing the levels of transverse sections (B and E). B. Photomicrograph showing transverse section (H&E) X10. D. Scanning electronic micrograph showing myofiber of trunk. E. Higher magnification of the red boxed region in D showing the striation of myofibers. E. Scanning electronic micrograph showing transverse section at the tail peduncle level. Abbreviations: MF= myofiber, N = notochord; SP = spinal cord; VS = vertical septum.

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