The effect of incubation temperature on embryonic development and muscle growth in yolk-sac larvae of the European eel (*Anguilla anguilla* L., 1758)

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Abstract

Reproduction of European eel (*Anguilla anguilla*) in aquaculture systems has become a focus research area due to severe decline of natural stocks, and increasing interest to breed eels for a self-sustained aquaculture. Despite over hundred years of research there are still large parts of its life cycle and biology that are unknown. Viable embryos and larvae are some of the key issues for rearing European eel in captivity where scarce knowledge of abiotic factors and adequate culture conditions remain a problem. Knowledge on the effect of water temperature is important to improve larval rearing and aquaculture systems, since thermal tolerance and optimal temperature is closely related to growth rate, development and survival.

The aim of the present study was to investigate muscle growth and development in yolk-sac larvae of European eel, and how embryonic incubation temperatures affected survival rate, morphological development and muscle growth. European eel eggs and larvae were therefore incubated at 16, 20 and 24° C.

The previous small scale incubation experiment showed that time until hatching was clearly related to temperature, and provide information about the actual time and day degrees required for embryonic development from fertilization to hatching, similar to those temperatures found in the spawning-area. Incubation at 24° C had a negative effect on survival rates of newly hatched larvae, indicating that this temperature is suboptimal for egg incubation. The present study demonstrated that muscle morphology, rate of development and onset of muscle differentiation follow a unique pattern, indicating that yolk-sac larvae of eels prepare for a somewhat different developmental strategy, i.e. leptocephalus phase. Larval muscle growth occurred by hyperplasia and hypertrophy, and the somatic length growth was poorly correlated to muscle growth in the initial yolk-sac stage.

Contents

Introduction

The catadromous European eel (*Anguilla anguilla*) exhibit one of the more remarkable life histories in the vertebrate kingdom, and its complex and largely unknown lifecycle has been subject to speculations for centuries. It is generally believed that spawning takes place in the Sargasso Sea, based on catches of the smallest larval stages (Schmidt, 1923 ; Munk et al., 2010). Following hatching, the planktonic larvae (leptocephali) are transported along the North-Atlantic currents to the shores of Europe, for an extended but poorly documented period (McCleave, 2008). The larvae metamorphose into glass eel in coastal waters, and become pigmented elvers when entering estuaries. The elvers ascend into brackish and freshwater feeding habitats, change coloration and metamorphose into the yellow eel stage. They feed and grow till partial maturity from 6 to 50 years later, depending on sex and latitudinal positions (Poole & Reynolds, 1996). The eels then transform into the migratory silver eel stage that undertake the spawning migration back to the Sargasso Sea, where they complete the maturation and are presumed to die after reproduction (van Ginneken & Maes, 2005). Other anguillids in the northern hemisphere are represented by the American eel *A. rostrata* and, in the North Pacific, by the Japanese eel *A. japonica* (McCleave, 2008).

At the beginning of the previous century Johannes Schmidt located the spawning ground for European eel by catching small eel larvae near the Sargasso Sea (Schmidt, 1923). He also caught bigger leptocephali near the European coast. These findings have since been confirmed by several other studies (Schoth & Tesch, 1982 ; Munk et al., 2010), and seems to be the only evidence that places the spawning ground in the Sargasso Sea, despite over hundred years of research. Neither eggs nor mature adults have ever been found in this area, so the location is solely based on catches of pre-leptocephalus larvae nearby (Schmidt, 1923). This means that adult eels must cover a distance of 6000 km during the spawning migration, which also occurs against the current. The theory is tested in laboratory with eels in swim tunnels, showing that their energy reserves are sufficient for the migration. Eels are highly effective cruisers with very low energy consumption rates that seem to be 4-6 times more efficient than non-eel-like fish (van Ginneken et al., 2005a ; Palstra et al., 2008).

The ecology and biology of the European eel in the early development stages are still not clear. Recently their spawning site have been revealed in more detail, but the depth and water temperature for spawning and development are not well documented (Bonhommeau et al., 2009). Information about depth of spawning is proposed by data from released hormone treated females, tagged with radio transmitters. The tagged females demonstrated a preference for the upper zone of the ocean at depths of 250-270 m with temperatures at 18.7 – 18.8° C (Fricke & Kaese, 1995). Earlier studies reveal maximum swimming depths of hormone treated females by nearly 700 m (Tesch, 1989). However, the number of radio transmitter tagged females is low. The European eel spawns up to 4 million eggs which rise to the water surface with a speed of over 2/m h (van Ginneken et al., 2005b). The eggs are non-sticky and typically pelagic. Previous studies report egg diameters ranging from 700 μm to 950 μm (Pedersen, 2003 ; Palstra et al., 2005). The leptocephali of European eel and American eel are widely distributed in overlapping areas in the southern Sargasso Sea. European eel leptocephali ≤ 5 mm long were present primarily above 150 m both night and day, within temperature fluctuations of 20 to 25° C (Castonguay & McCleave, 1987 ; Munk et al., 2010).

The feeding biology of eel larvae remains a mystery. Experiments with European eel indicates that larvae may start feeding approximately 12 days after hatching, based on eye pigmentation, digestive enzymes in the pancreas, developed teeth and complete absorption of the yolk-sac, at 19.5° C in captivity (Tomkiewicz & Jarlbæk, 2008). In comparison, Japanese eel are suggested to start feeding 7 days after hatching, reared at 22° C (Tanaka et al., 2003). The Sargasso Sea is oligotrophic, with a generally low plankton biomass (Arenovski et al., 1995). Previous studies based on visual identification of gut contents proposed dissolved and particular organic matter such as marine snow or zooplankton fecal pellets, as source of eel larval nutrition for several species (Otake et al., 1993 ; Mochioka & Iwamizu, 1996). However, these observations were based on larger leptocephalus eel larvae beyond the first feeding stage. More recent DNA studies of gut contents from genetically identified European eel caught in the Sargasso Sea, revealed that even the smallest larvae contained a striking variety of planktonic organisms, suggesting that gelatinous zooplankton are of fundamental dietary importance for newly hatched larvae (Riemann et al., 2010).

The eel populations have been declining worldwide over the last decade, and abundance of the European glass eel has dropped significantly since the early eighties, by 90-98 % (Aprahamian & Walker, 2008). For the last 5 years, *A. anguilla* elver-based catches averaged between 1 % (continental North Sea) and < 5 % elsewhere in Europe, compared to the mean for 1960-1979 levels (WGEEL, 2011). Overall recruitment since records began remains at an all-time low, where the stock continues to decline (Gollock et al., 2011 ; Van Liefferinge et al., 2012). The Japanese eel and the American eel are suffering steep drop-offs as well (Castonguay et al., 1994 ; Dekker, 2003). The actual cause of this decline is unknown, but possible causes include; contamination, over-fishing, migration obstructions, climate changes, parasites, changes in ocean currents, habitat loss and virus infections (Castonguay et al., 1994 ; Cowx et al., 1998 ; Dekker, 2003 ; Macgregor et al., 2010).

The eel has long been an important and traditional food fish, especially in some European countries and Japan. Cultivation of eel for consumption is based entirely on wild catches of glass eels, as reproduction in culture is not possible at a commercial scale. The European eel is generally farmed in land based systems, and currently supplies approximately 50 000 t/y (Nielsen & Prouzet, 2008). The interest in eel aquaculture has fluctuated over the past few years, as a consequence of the population collapse. The public awareness of the situation has led to a restraint in the human consumption marked, due to low marked prices and a growing demand to stop consumption of eel and eel products (Aprahamian et al., 2007 ; Nielsen & Prouzet, 2008). European eel is currently listed as 'critically endangered' on the IUCN red list of threatened species (Freyhof et al., 2011). The long-term goal is therefore to produce glass eels for a self-sustainable farming industry, for restocking rivers and to satisfy the human consumption marked demand.

Challenges regarding eel as aquaculture species
Successful reproduction of European eel has still not been achieved in captivity. In contrast to the Japanese eel, where the first leptocephali larvae were obtained by hormone treated females in 2001 (Tanaka et al., 2001), the success in maturation of European eel has been very limited until recently. Primary bottlenecks in reproduction of eel in captivity concern lack of knowledge and methodology to induce gametogenesis

and produce viable eggs, embryos and larvae. European eel females seem to have a much slower and variable response to hormonal stimulation, compared to e.g. Japanese eel and Australian eel *A. australi* (Palstra et al., 2005).

Fontaine succeeded in 1936 with maturing male eels, *A. anguilla* (Fontaine, 1936). He also reported maturation and egg release of a single female in 1964, but fertilization was not attempted (Fontaine et al., 1964). Several other attempts have been made in order to succeed with breeding of European eels from the middle of the 1950s, until today. The first successfully fertilized eggs from European eels were reported and obtained by a Russian research group in 1983, and in 1987 postembryonic development was described up till 3.5 days after hatching, at which time the larvae died (Bezdenezhnykh et al., 1983 ; Prokhorchik, 1987). Reproduction of Japanese eel became successful in 1996 with a protocol based on 17.20 β-dihydroxy-4-pregnen-3-ones (DHP), a hormonal injection for final maturation and ovulation. Eggs from three out of eleven ovulated females (*A. japonica)* showed a relatively high fertility and hatching percent (Ohta et al., 1996).

Since 2001, there have been several projects with artificial reproduction of European eels, led by the University of Copenhagen. During the project "reproduction of eel" (ROE I), fertilized eggs and embryonic development on European eel was again reported, by using a protocol similar to that successful for Japanese eel (Pedersen, 2003 ; Pedersen, 2004). Successful fertilization took place in three out of twelve females, followed by hatching eggs from one female. However, hatching time was delayed and resulted in deformities and insufficient embryonic development (Pedersen, 2003). Two subsequent projects led by DTU Aqua, University of Copenhagen and the eel aquaculture industry (ROE II & ROE IIB) on reproduction of eels continued, which also succeeded in producing viable embryos and larvae a number of times. They managed to keep larvae alive for 5 days after hatching (Tomkiewicz & Jarlbæk, 2008).

The reports from the most recent project (ROE III) describes great improvements in egg quality and embryonic survival, where the longest living larvae reached 18 days of age (Tomkiewicz & Jarlbæk, 2008). However, there is still a high mortality among European eel larvae, and most die within a few days. Further challenges include identification of

initial first feeding, in order to obtain continued growth and survival during the transfer from endogenous to exogenous feeding.

The establishment of first feeding is generally a difficult step in aquaculture. For eels it is a particular challenge because little knowledge exists about their feeding biology or oceanic larvae phase, including the leptocephalus stage that may last one year or more. Recent intensive research and new technology has made notable progress by improving methods for hormonal treatment, fertilization and larval culture techniques (Tomkiewicz et al., 2012). However, important issues such as high quality eggs, improvement of survival rate in early life stages and development of suitable diets for larvae need to be further examined. The complicated and comprehensive life cycle of the European eel involves major environmental variations, and methods used for larval rearing are still suboptimal in relation to biological and ecological requirements. Japanese researchers have made major progress in terms of start feeding on newly hatched larvae, where the first successful production of glass eel in captivity happened in 2003. A slurry-diet consisting of shark egg yolk and krill hydrolysate, enriched with vitamins and minerals was found to be the most successful feed for the Japanese eel. Larvae fed on this diet reached a total length of 60 mm and started to metamorphose into glass eels approximately 250 days after hatching (Tanaka et al., 2003).

Functional larval development

Marine fish larvae are the smallest free living and actively feeding vertebrates. Most of their functional systems are incomplete at hatching, and fish larvae prioritize to develop organs associated with swimming movements and feed intake, in order to increase their chance of survival (Wieser, 1995 ; Kjørsvik et al., 2011). Previous studies suggest that muscle growth in early life may have an effect on the ultimate size and growth rate in later life stages of several fish species (Weatherley, 1990 ; Johnston & Cole, 1998). Early environmental factors may therefore influence later growth capacity of the European eel larvae. Temperature is one of the most critical and controlling factors to influence muscle development during the ontogeny of teleost fish. It affects dominant processes such as growth, tissue differentiation and physiological changes (Kamler, 2002). In addition to temperature, several other abiotic factors may influence muscle development and growth, e.g. oxygen concentration, salinity, pH, light availability and

water flow (Johnston, 1999 ; Sänger & Stoiber, 2001 ; Johnston, 2006). Maternal investments, hormones, diet composition, density and feed availability are also important factors that may have an effect on the axial muscle growth (Shelton et al., 1981 ; Alami-Durante et al., 1997 ; Gjerde et al., 2004 ; Johnston, 2006). Resulting variations in muscle texture and connective tissue are of importance to produce European eel of desirable quality, since the musculature contributes mainly to the final food product.

Muscle growth and development

Axial muscle fibre types
Axial swimming musculature comprises 40 to 60 % of the total body mass in adult fishes, arranged in a longitudinal series of myomeres separated by collagen sheets called myosepta (Bone, 1979 ; Fauconneau et al., 1995 ; Van Leeuwen, 1999). The axial muscles are organized in layers of red and white fibers. The red fibres form a lateral sheet at the level of the horizontal septum, whereas the white layer constitutes the bulk of the muscle mass (see Fig. 1.1) (Blaxter, 1987). Between these two compartments, there is a pink or intermediate layer of muscle fibres (Dal Pai-Silva et al., 1996). The red fibres are small in diameter and have an aerobic (oxidative) metabolism, abundant in myoglobin and mitochondria, are well vascularized, and function in slow continuous swimming movements. The white fibres are large in diameter and have an anaerobic (glycogenic) metabolism, low myoglobin and mitochondria content, are poorly vascularized and are involved in bursts of high swimming speeds, however. This burst activity can only be maintained for a short period (Walesby & Johnston, 1980 ; Johnston, 1982 ; Jayne & Lauder, 1994). The intermediate pink layer seems to show an intermediate speed of contraction, and contains a high level of glycogen (Johnston et al., 1977 ; Scapolo et al., 1988).

At hatching most teleost larvae have one layer of superficial fibres that surrounds the inner mass (Hubbs & Blaxter, 1986). The superficial fibres are the precursor of the red muscle fibres, and are believed to function in respiration and slow continuous movements. The inner mass is responsible for rapid bursts movements, and will develop into white fibres (El-Fiky et al., 1987). Both fibre types have an aerobic metabolism

during early stages, but the myosin isoforms differ from each other (Scapolo et al., 1988). The timing of red and white fibre differentiation varies between different fish species. It seems to be related to the transition from nutritional dependence on the yolk-sac, to exogenous feeding. Simultaneous development of both layers allows the ability to search for food at low swimming velocity and to avoid predators by escape, in high velocity (Hubbs & Blaxter, 1986 ; Koumans & Akster, 1995). Muscle development and growth has previously been described only in glass eels and adult eels (Willemse & van den Berg, 1978 ; Willemse & De Ruiter, 1979 ; Egginton & Johnston, 1982 ; Romanello et al., 1987). These studies suggested muscle morphology in elvers and adult eels that apparently resembles the pattern of muscle morphology and orientation of the muscle fibre arrangement found in e.g. salmonids and other teleosts fishes (Fig 1.1).
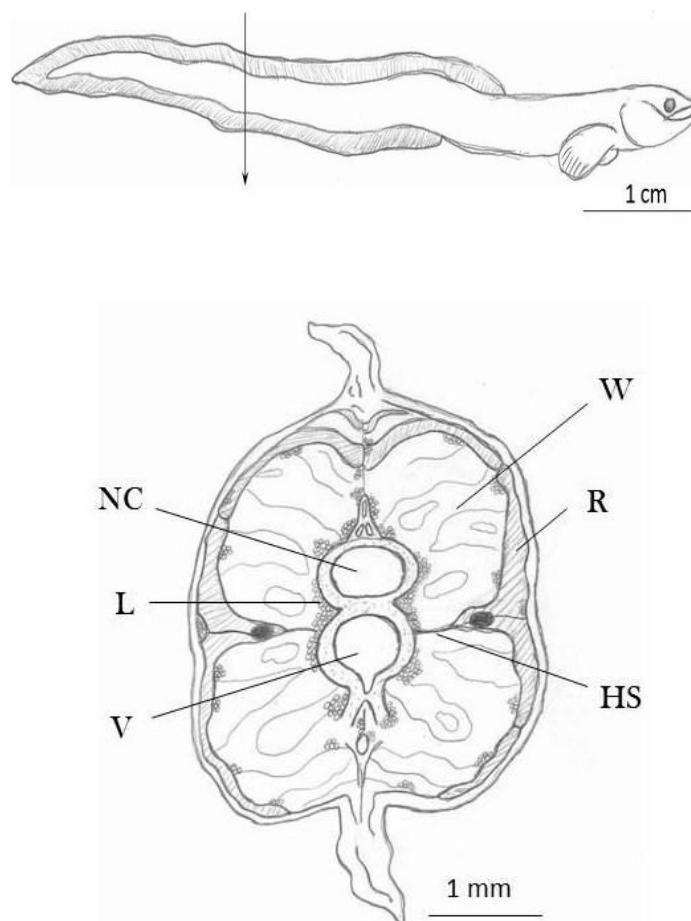
Figure 1.1: Schematic drawings showing the position of different muscle fibre types in the tail of (a) an eel at elver stage (6-7 cm SL) W, white fibres; R, red fibres; HS, horizontal septum; V, vertebra; L, lipids; NC, nerve chord. Outline from elver muscle based on previous studies (Egginton & Johnston, 1982).

Growth and development of musculature

Muscle growth involves a combination of continued enlargement of fibers (hypertrophy), in addition to recruitment of new muscle fibers (hyperplasia). Development and growth of fish muscle differs from that in mammals and birds. In fast growing fish species, hyperplasia plays an important role in muscle growth, even after the juvenile stage, whereas in mammals and birds the increase in number of muscle fibres stops at, or shortly after birth (Goldspink, 1974 ; Weatherley & Gill, 1985 ; Weatherley et al., 1988). Further growth is mainly the result of hypertrophy in mammals and birds. The difference in muscle growth between these animals may be reflected by the availability of muscle progenitor cells (MPC's), or myosatellite cells (Mauro, 1961 ; Koumans et al., 1990). These cells are the source of additional myonuclei, during growth and regeneration of muscle fibres (Nag & Nursall, 1972 ; Koumans et al., 1993 ; Koumans & Akster, 1995). Multiplication by simple mitotic divisions of the fibres is therefore highly improbable (Koumans & Akster, 1995). Previous studies during ontogeny of common carp *Cyprinus carpio* revealed that total percentage of positive MPC's seems to be constant to a certain fish size, but the concentration and percentage of these cells appear to decrease by further growth (Koumans et al., 1990 ; Koumans et al., 1993).

During embryogenesis, muscle fibres arise from the fusion of several myoblasts to form multinucleated muscle cells (Okazaki & Holtzer, 1966). They further differentiate and produce contractile proteins, the myofibrils, which constitute the bulk of the fibre volume. The formation and growth of muscle fibres in fish larvae requires activation of myogenic cells, which are situated between the sarcolemma and basal lamina of the muscle fibres (Koumans & Akster, 1995). During the initial growth, the amount of cytoplasm per nucleus increases until a critical nucleus:cytoplasm ratio is reached. Further growth of the fibres depends on additional myonuclei being added from MPC's. Such cells also contribute to the formation of the new muscle fibres (Watkins & Culien, 1988).

The first phase of hyperplasia occurs during embryonic life, when new fibres originate from the fusion of myogenic cells. A second phase of fibre number increase is called stratified hyperplasia, because it generates new fibres along discrete germinal zones for

both slow and fast muscle fibres, during the larval stages (Rowlerson et al., 1995). The hyperplastic growth pattern seems to be an important contributor to muscle growth in species capable of attaining large sizes (Weatherley et al., 1988).

A third phase of hyperplasia is called mosaic hyperplasia, when the MPC's are activated throughout the myotome, and new white fibres give rise to a mosaic of fibre sizes during late larval and early juvenile stages (Johnston, 2004). Mosaic hyperplasia is of great interest in commercial aquaculture because it brings the fish to marked size and has therefore been extensively studied and described for several teleost species, which reach a large final size, e.g. common carp (Koumans et al., 1990), rainbow trout *Oncorhynchus mykiss* (Weatherley et al., 1980), Atlantic cod *Gadus morhua* (Johnston & Andersen, 2008) and white seabass *Atractoscion nobilis* (Zimmerman & Lowery, 1999). In species of small final body sizes, the mosaic growth pattern is generally reduced or entirely lacking, where most growth after hatching is attributed to hypertrophy, e.g. guppies *Poecilia reticulata* (Veggetti et al., 1993) and zebrafish *Danio rerio* (Raamsdonk et al., 1983).

Identification of factors that might regulate the interaction between the hypertrophy and hyperplasia process in European eel muscle is not only an interesting problem in terms of biological mechanisms, but also of particular importance in aquaculture, to optimize muscle growth, quality and survival rate (Alami-Durante et al., 2010). Formation of new fibres has also been suggested to occur by splitting of existing muscle fibres in some species, including the European eel (Patterson & Goldspink, 1976 ; Willemse & van den Berg, 1978). The process of muscle fibre generation in elvers are described by the term "budding", and seems to be a unique phenomenon, related to the fibre splitting theory (Willemse & Lieuwma-Noordanus, 1984). Previous studies on *A. anguilla* glass eels described a sharp rise in the largest fibre diameters in eels > 10 cm, followed by a complete plateau, while the largest eels < 35 cm, had a fibre diameter decline (Willemse & van den Berg, 1978). These results give rise to the question of whether small fibres may arise from the larger muscle fibres.

Temperature may affect several factors of importance for axial muscle growth and survival in early life stages, such as size at hatching, development rate, rate of yolk-

absorption, behavior and the number and size of axial muscle fibres (Itzkowitz et al., 1983 ; Blaxter, 1988 ; Johnston, 1993). Optimal temperatures for embryonic development are not necessarily the same as for larval growth. Temperature differences of a few degrees during egg incubation and early larval stages have a profound influence on the muscle differentiation in some species. Chang et al. (2004) described Japanese eel embryos physiological capacities for temperature adaption in detail, where the presumed optimal temperatures are 25 ± 1° C for the embryonic stage, and 27 ± 1° C for the yolk sac stage, with respect to survival and normal development.

The onset of hyperplasic and hypertrophic growth patterns in eel is unknown, as previous histological studies on the lateral musculature in early life stage have not yet been reported. An essential step in the successful culture of any fish species is to understand the optimal environmental conditions for egg incubation. Viable embryos and larvae are some of the key issues for rearing European eel in captivity where scarce knowledge of abiotic factors and adequate culture conditions remain a problem. Optimal incubation temperature is therefore a natural first step to investigate, in order to solve problems associated with larval rearing and survival.

Aim of study

The aim of the present study was to investigate muscle growth and development in European eel yolk-sac larvae, and how embryonic incubation temperatures affected survival rate, morphological development and muscle growth. European eel eggs and larvae were therefore incubated at 16, 20 and 24° C.

Morphological development of the axial swimming musculature is described in the early yolk-sac stage, and growth is quantified in terms of red and white muscle fibre cross-sectional area in one bilateral half of the myotome, number and size of red and white fibres, and total contribution of red and white fibres.

Information about growth patterns, structure and optimal temperature might help in the understanding of critical periods throughout the early development processes that are important for eel larval rearing, and thus, improve rearing protocols which ultimately may replace the unsustainable use of wild-caught glass eels.

Materials and methods

The study was carried out as part of the EU-project "Reproduction of European Eel: Towards a Sustainable Aquaculture" (PRO-EEL). Egg and larval culture experiments were conducted in collaboration with Sune Riis Sørensen, at the DTU Research Station Lyksvad, Denmark. Further analytical work on muscle development took place in the histological laboratory of the Norwegian University of Science and Technology (NTNU) at Center of Fisheries and Aquaculture (Sealab) in Trondheim. Transmission electron microscopy was made at St. Olavs Hospital.

A temperature experiment was conducted in order to determine effects on muscle development and survival of the three different incubation temperatures. Unfortunately, most of the fixed samples were accidently frozen during the termination of the experiment and became unavailable for muscle analysis.

Samples from the temperature experiment were therefore replaced by new fixed samples of yolk-sac larvae from three different females for muscle analysis, conducted as part of full scale project experiments a month earlier in the same spawning season, based on DTU standard protocols (Tomkiewicz et al., 2012) at Lyksvad research facility. The thesis is therefore based on two separate studies, one concerning incubation temperature effects on developmental rate and survival (2.1 temperature experiment), and one concerning a general description of muscle morphology and development in newly hatched yolk-sac larvae (2.2 muscle development).

2.1 The temperature experiment

Female broodstock fish were wild silver eels originating from the Danish lake Vandet Sø, while male eels were obtained from a commercial eel farm. Maturation and fertilization procedures were conducted as part of PRO-EEL full scale experiments using hormonally induced maturation (salmon pituitary extract) and dry fertilization based on standard protocols for European eel developed at DTU (Tomkiewicz et al., 2012). Fertilization was attempted for egg batches from three spawning females. Unfortunately, most fertilized eggs from two of these females had abnormal development after 3 hours, and the percentage of surviving eggs was rather low. The experiment may, however, contribute to limited information of the proposed temperatures, as one of the egg batches reached larval stage. Seawater used during the experiment was transported directly from the North Sea, coast of Hirtshals, Denmark and added Tropic Marine™ sea salt to a salinity of 36 ppt, treated with antibiotics based on Japanese protocols, consisting of 60 mg penicillin and 100 mg streptomycin per liter (Okamura et al., 2007).

The fertilized eggs used in the temperature experiment were obtained from one female eel (tagged as C45F, body weight 700 g), which were fertilized by sperm from three males. After fertilization, eggs were transferred to a cylindrical 20 liters holding tank with seawater at 20° C.

2.1.1 Experimental setup

The fertilized eggs were collected and transferred after 3 hours into an incubation setup made by Sune Riis Sørensen, DTU (see Fig. 2.1). The experimental setup was disinfected with Virkon®S and flushed with water, before loading. The egg incubation setup consisted of three 100 liter water baths, adjusted to different temperatures of 16, 20 and 24° C, where each bath contained. three replicate experimental containers These containers were designed as mini incubator systems with separated conical shaped bottom, and contained 4 liters treated seawater and approximately 5 ml eggs (corresponds to around 3000 eggs). Water temperatures were gradually changed during 1.5 hours after loading to avoid thermal shock for the eggs, and measured 8 times within 3 hours immediately after loading into the incubation system. Plastic covers were

provided for shelter and to avoid light on the tanks. Gentle aeration was supplied to each incubator.

The 20 and 24° C egg incubation tanks were immersed in water baths thermostatically controlled by 200 W Eheim electric heaters, while the 16° C incubation tanks were maintained at this temperature by adding cold water in the water bath. Aqua medic Titan 2000 (550 W) provided constant cold water below 15.5° C in a cooling tank and the water was transferred from the cooling tank into the 16° C water bath, by using a pump. A plastic tube on the other side of the tank was connected to the cooling tank, to provide sufficient water flow through the system. Heavy aeration in each water bath prevented temperature gradients throughout the tank. The electric heaters were automatically regulated by sensors connected to a power supply in order to obtain correct temperatures. The system also had constant mild aeration directly in the incubation tanks to distribute eggs and temperatures. Dead eggs were removed by turning off the aeration for 5 minutes. The water temperature was regularly measured minimum 4 times per day with a mercury thermometer. Temperature fluctuations were within the range of ± 0.7° C during the experiment.
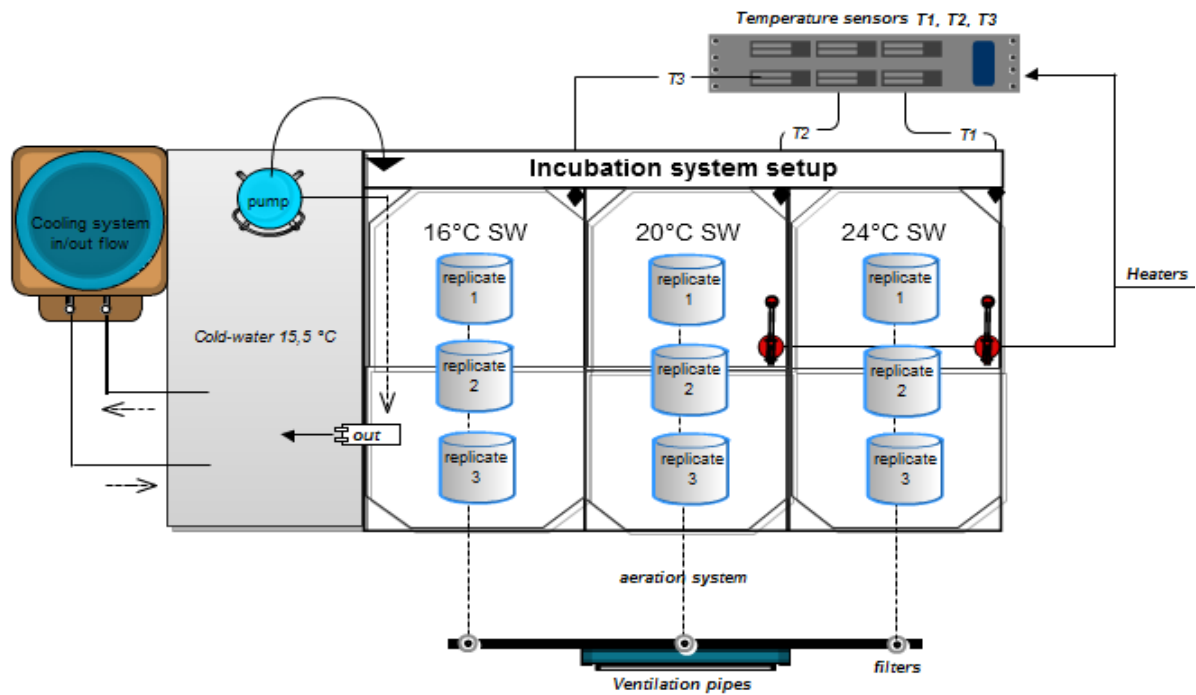
Figure 2.1: The incubator setup built by Sune Riis Sørensen, DTU, consisted of nine incubation tanks, three different temperature regimes separated in plastic containers (16° C, 20° C and 24° C) over three replicates. The water baths were provided by untreated tap water. Plastic tubes were connected from ventilation pipes to the bottom into each incubation tanks, to provide mild aeration. Each tank contained 4 liters seawater. The 20° C and 24° C tanks were thermostatically controlled by electric heaters. The 16° C setup was provided by cold water from the cooling tank in a flow through system. Each replicate were provided by plastic covers to avoid light.

2.1.2 Incubation of yolk-sac larvae

Larvae from all replicates that hatched and survived were gently collected into separated 4 liters glass bottles with stagnant seawater for each temperature treatment, by using a wide glass pipette. The three replicates from each temperature group were pooled because of generally low hatching rates. The 20° C and 24° C treatments were stored in separated water baths regulated by heaters with ± 0.2° C precision. The 16° C treatment was placed inside a temperature regulated cupboard with temperature at 16 ± 0.5° C. All larvae were kept in the dark without aeration. Water exchange occurred once a day, where approximately 90 % of the seawater was gently siphoned out and replaced with new. A 180 μm mesh cup was used to prevent flush of larvae. Dead larvae and eggshells were sorted out. Hatching rate was calculated by following equation;

$$hatching\ rate\ (\%) = \frac{numbers\ of\ hatched\ larvae}{numbers\ of\ incubated\ eggs\ x\ 100\%}$$

2.1.3 Sampling and processing

Samples from the incubator system were randomly selected from each temperature with a wide pipette and transferred to well trays with 15-20 ml ppt seawater. Eggs were collected at regular intervals for light microscopy, and photographed through a microscope Nikon Eclipse 55i connected to 5.0 MP color Digital Camera Head DS-Fi1. Lenses used were CFI Plan Achromat UW (2x = 20x), and CFI Plan Flour (4x = 40x, 10x = 100x). Developmental stage was determined by first observations of embryonic discs (more than one cell layer), early gastrulation, first myotome formation, complete gastrulation and hatching (defined by Kimmel et al. 1995). Three to five larvae from each temperature group were collected twice a day in well trays, anesthetized in metacaine (MS-222) mixed with 15-20 ml seawater for light microscope and photo documentation. Samples were fixed in a mixture of 2.5 % buffered glutaraldehyde, and 4 % phosphate buffered formalin.

2.2 Muscle development in yolk-sac larvae

The collected larvae for the muscle analysis were reared by a PRO-EEL incubation protocol (Tomkiewicz et al., 2012) at an aimed temperature at 19.5 ± 0.5° C and a salinity of 36 ppt. The larvae originated from three females; C881, 5492 and C452, with fertilization rates of 6, 59 and 91 %, respectively. Larvae from the different females were sampled at day 0, 3 and 6 (n=3 larvae) after hatching and used for the muscle analysis. The sampled larvae were fixed in 4 % phosphate buffered formalin (pH 7.4, Apotekproduksjon AS; Norway) and stored at 5° C until further processing. The sampled larvae were sent by air to Trondheim.

2.2.1 Standard length (SL) and myotome height (MH)

SL and MH were measured individually in all fixed larvae by using the stereomicroscope (Leica MZ 7.5, Leica Microsystems, Germany), with an ocular micrometer calibrated at 1.25x, 2.00x and 4.00x, depending on the size of the larvae. SL was measured from the tip of the snout, to the end of the notochord (Fig. 2.2). MH was measured perpendicular to the axial skeleton behind the anus. Measurements were conducted by using stereological analysis software CAST 2 (Olympus inc., Denmark).
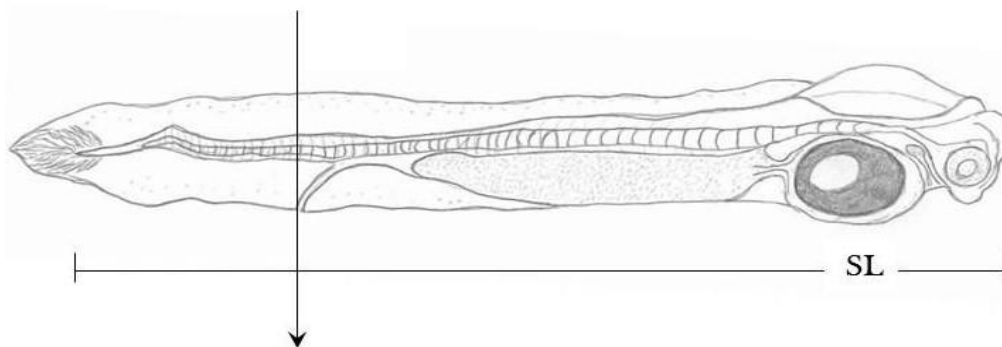
Figure 2.2: Schematic drawing of a eel *A. anguilla* larvae at 3 dph, showing where SL were measured, and the transverse sections were cut.

Larvae were then staged individually, cut posterior to the anus by using a scalpel blade under the light microscope and kept separately throughout the embedding process; post fixation in 2% OsO_4 in 1.5% potassium hexacyanoferrate II (sigma P9386), bulk contrasting in 1.5 % uranyl acetate and dehydration in ethanol before embedding in Epon (Appendix 1). Transverse semi-thin (1 μm) sections were cut for light microscopy and ultrathin sections (70 nm) were cut for transmission electron microscopy, by using a Leica Richert Ultracut microtome (Leica Microsystems, Germany). Several sections were cut per larva to ensure the correct reference point, posterior to the anus. Two serial semi-thin sections were stained with Nile blue (McGee-Russell & Smale, 1963) and Toluidine blue (Mugnaini et al., 1967), respectively. The results are solely based on the Nile blue staining as these sections had better definition of cell borders in the tissue, compared to those obtained with Toluidine staining.

Ultrathin sections were transferred to copper grids and contrasted with 2 % lead citrate for electron micrographs. The ultrathin sections were observed in a Jeol JEM-10011 transmission electron microscope (Jeol LTD, Japan) at an acceleration voltage at 80 kV. Three to five muscle areas at 0, 3 and 6 dph from six different larvae were randomly chosen. The electron micrographs were photographed and observed at magnifications of 5000, 10 000 and 20 000x.

2.2.2 Muscle analysis

The semi-thin sections were photographed with a Zeiss Axioskop 2 Plus microscope (Zeiss INC., Germany) fitted with a JVC TK-1381 color camera (JVC; Japan). The outlines of all muscle fibres in each section were photographed (100.4x magnification) and processed in Adobe Photoshop CS3 (Adobe Systems Inc., USA). The total cross-sectional area of muscle mass in one bilateral half of the myotome was measured directly on the photographs, by using a Wacom Cintiq 24HD drawing board (Wacom Inc., Japan) linked to the image processing software, Image J. The total number and individual cross-sectional area of muscle fibres was counted and measured. All muscle data were presented in relation to larval age, SL and female. An attempt was made in order to count total number of myotomes in each larva, but myotomes in the posterior part of the larvae were too hard to distinguish due to low magnification, and therefore excluded.

Electron micrographs were not initially planned in the muscle analysis, since the larvae were fixated in a mixture of 4 % formalin in phosphate buffered saline (not optimal for EM studies, (Bozzola & Russell, 1992), but became necessary in order to identify the cell structure in the relatively immature muscle appearance in newly hatched larvae. The morphology, however, was considered as good enough in order to distinguish myofibrils, mitochondria, nuclei and other organelles in the muscle fibres from the observed electron micrographs.

2.2.3 Statistics

Normality of data was tested with a Shapiro-Wilk-test (n < 100). The data were further tested for homogeneity of variance by using a Levene test. Different means for normally distributed data were compared by using one-way ANOVA followed by the Post-hoc-tests for comparison of the different groups. The Student-Newman-Keuls test was used for homogenous data, and the Dunnett T3 test for non-homogenous data. Correlations for linear regression were tested by using the Pearson-correlation test, with the level of correlation set to $R^2 > 0.85$. A Kruskal-Wallis test was used for non-normal distributed data. Standard error (SE) was calculated for all data that had replicates. All statistical comparisons are made with a significant level at $P < 0.05$. All statistical analyses were performed by using the program SPSS for Windows (SPSS Inc., USA). Regression lines were performed in the statistical program SigmaPlot 11.0 (Systad Software Inc., USA 2010).

Results

3.1 Temperature experiment

3.1.1 Hatching and survival

The buoyant egg fraction had a fertilization rate of 86 %, observed three hours after dry fertilization. Most of the eggs died within 24 hours. Low hatching rate was observed in all treatments with no significant differences (table 3.1). Larvae incubated in 24° C showed a rapid mortality immediately after hatching, and all larvae died during the first day. The survival rates in 20 and 16° C decreased gradually, and all died by day 3 and 4 after hatching.

Table 3.1: Hatching rate % for each treatment at 16, 20 and 24° C (n = approx. 3000 eggs per replicate, pooled at hatching). No particular difference between the incubation treatments.

treatment	16° C	20° C	24° C
amount of eggs	9000	9000	9000
total hatched larvae	65	71	54
hatching rate	0.72	0.78	0.6

3.1.2 Embryonic development

The time until the different developmental stages and hatching decreased with increased temperature, as presented in figure 3.1. Hatching of eggs occurred at 34, 48 and 58 hours after fertilization, when incubated at 24, 20 and 16° C, respectively. Other developmental events such as gastrulation and myotome formation also appeared later at lower temperatures. Development of the first axial musculature started in the anterior part of the body with the segregation of the first myotomes, observed on both sides of the spinal cord from mid-gastrulation at 18, 25 and 31 hours after fertilization at 24, 20 and 16° C, respectively. There were no particular differences between temperature groups in developmental stage at a given number of day degree, besides hatching at 24° C. (d°; the number of days multiplied by temperature in °C) (Fig. 3.2).
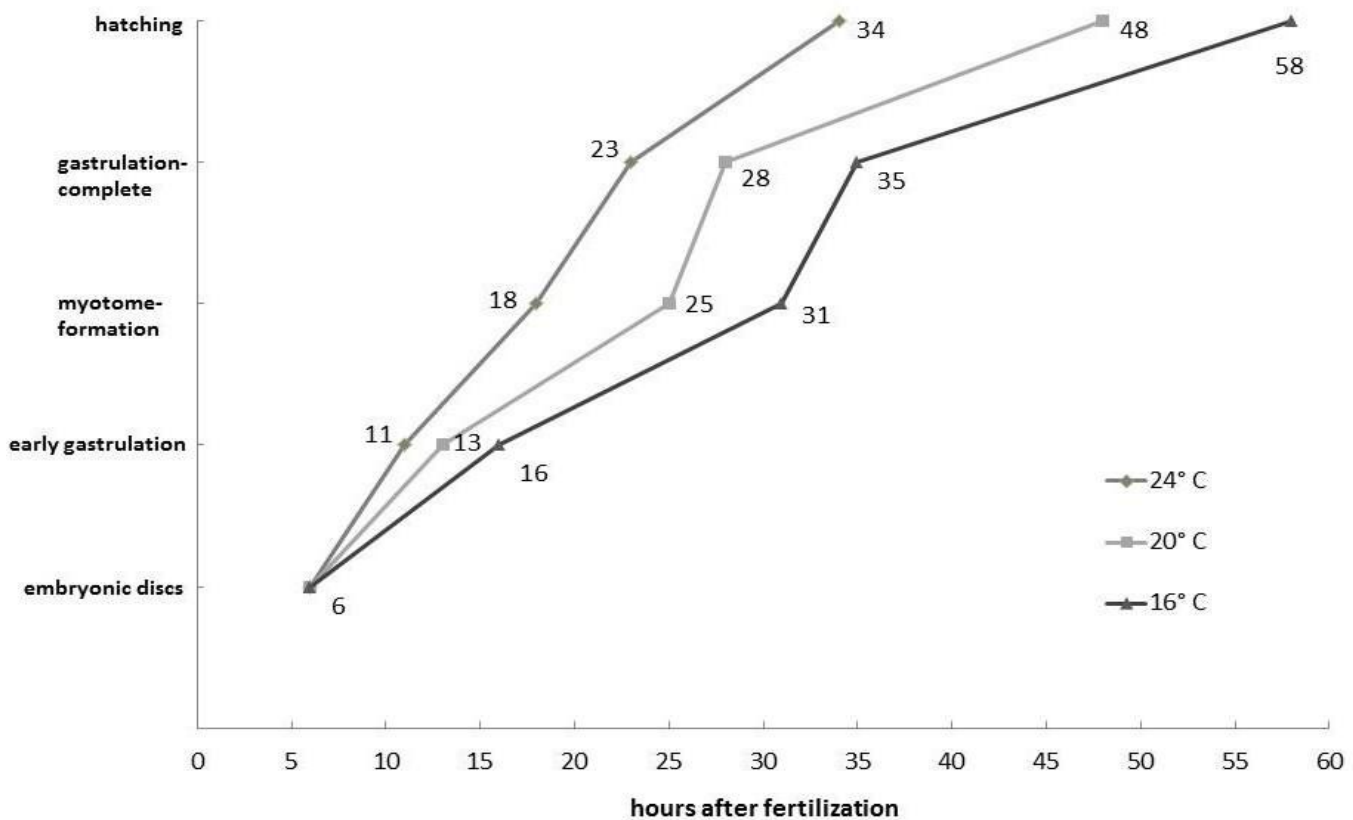
Figure 3.1: Relationship between egg incubation temperature and main embryonic development events such as; embryonic discs (adhering cells), early gastrulation, first myotome formation, gastrulation complete and hatching at 16, 20 and 24° C. The numbers denote actual time of development stage (hours).
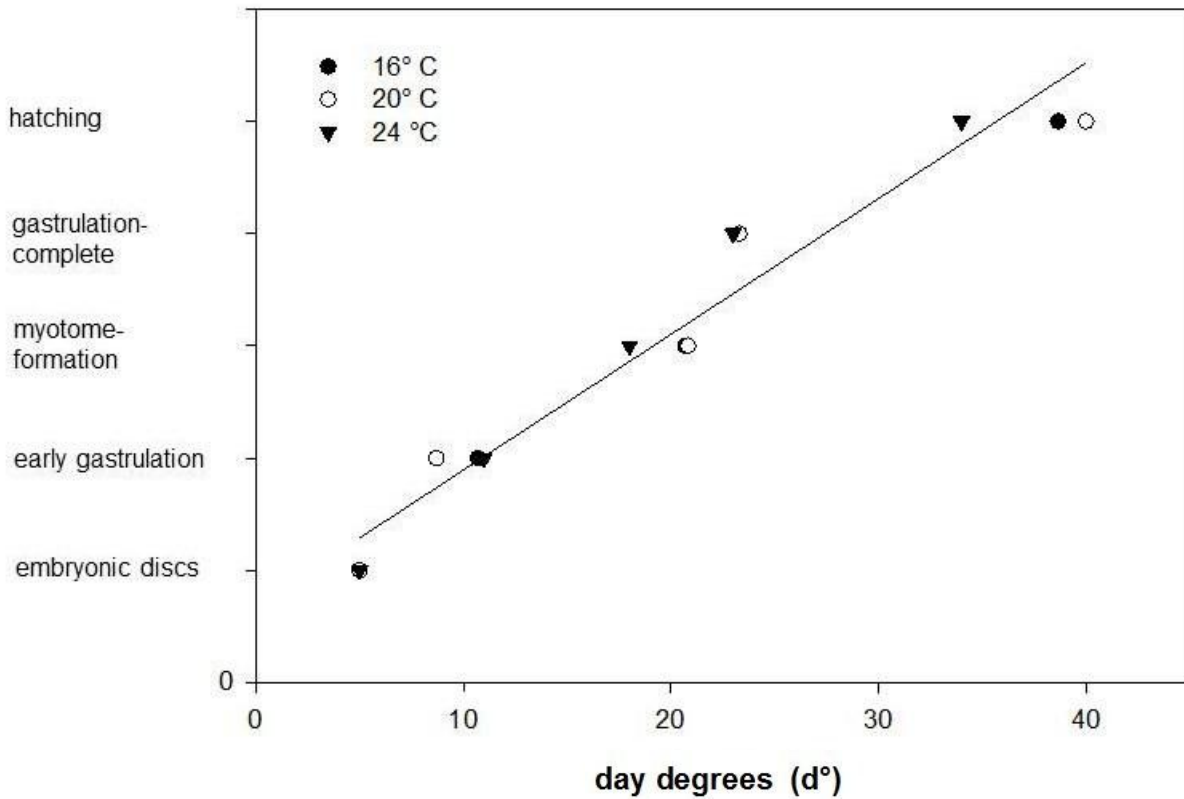
Figure 3.2: Developmental stage as a function of day degrees. The developmental stages are described in figure 3.1. Each point represents 6 to 8 eggs from each incubation temperature at 16, 20, and 24° C. Regression line correlation, $R^2 = 0.95$

At 3 hours post fertilization, the 16-cell stage was observed, and 2 hours later, the embryonic discs started to appear simultaneous with fusion of several fat droplets. At 6 hpf, all eggs had embryonic discs and formation of one major fat droplet. The embryonic body encircled nearly 3/4 of the yolk-sphere at 32 hpf in eggs incubated at 20° C, and approximately 18-20 visible somites were observed along the body (Fig. 3.3).

3.1.3 Larval development and behavior

Most larvae hatched after 48 hours at 20° C, and newly hatched larvae had a non-functional mouth, non-functional eyes, no pigments and had a large yolk-sac and oil droplet. After hatching, the yolk-sac larvae were evenly distributed and positioned upright in the water column, probably due to the position of the yolk and oil droplet at the anterior end of the yolk sac (Fig. 3.3). The first larval movements occurred immediately after hatching, and the newly hatched larvae responded to water movements by swimming in a spiral fashion. The larvae were mostly immobile during the experiment, except when disturbed by light or vibrations in the water, which caused rapid and short horizontal movements. During sampling procedures, 2 day old larvae avoided suction from the pipette by swimming in the opposite direction. Lateral neuromast cells on the flanks were observed from 1 dph (not shown). No visual differences in behavior or development were observed between larvae from the different temperature regimes.
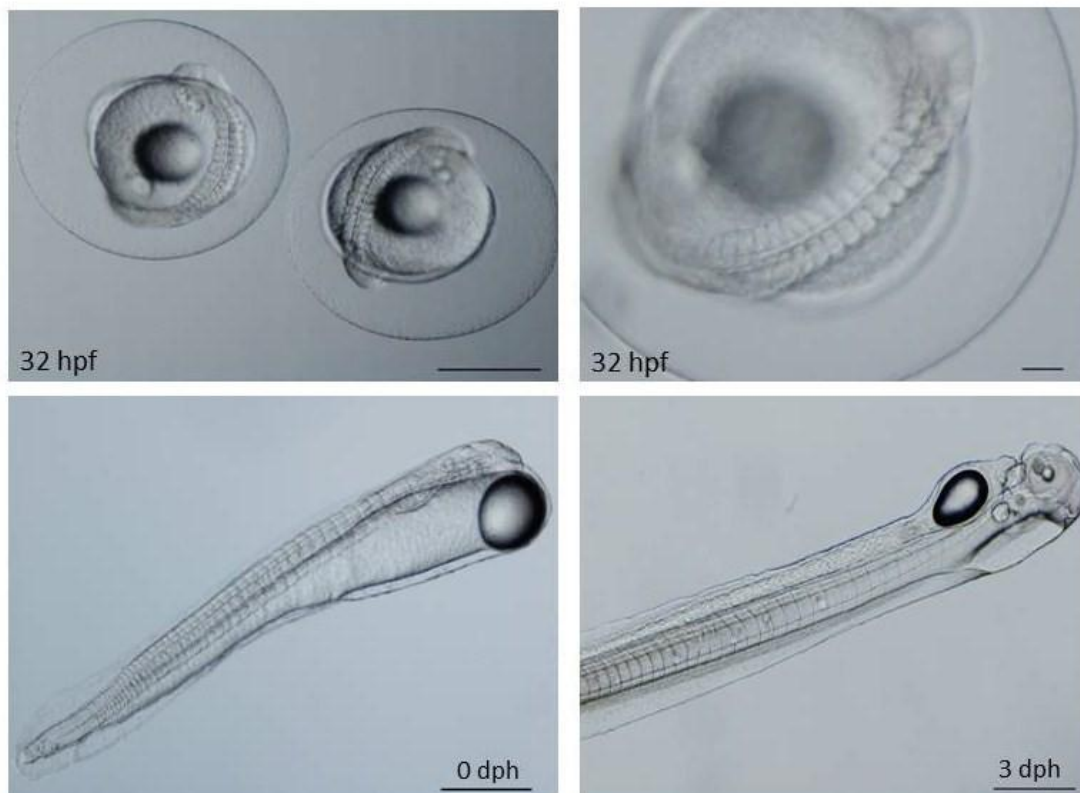
Figure 3.3: Eggs and larvae at 32 hpf, 0 dph and 3 dph from the 20° C treatment in the temperature experiment. Magnification x4 and x10. Scalebar 500 μm and 100 μm. Photo: Sune Riis Sørensen, DTU aqua.

3.2 Muscle development in yolk-sac larvae (standard rearing protocol at 20° C)

3.2.1 Standard length (SL) and myotome height (MH)

Standard length increased from 3.4±0.2 mm, 2.9±0.2 and 2.9±0.1 mm, at hatching to 6.3±0.3 mm, 6.1±0.1 mm and 5.9±0.2 mm for 6 day old larvae from the 5492, C452 and C881 females, respectively. Larvae from the 5492 female had a significantly larger SL than larvae from the other females at hatching (fig 3.4). Larvae from the C452 female had a significantly smaller SL at 3 dph, but no significant differences in SL between larval groups were seen on day 6. There was a significant increase in SL between 0, 3 and 6 dph and the average SL approximately doubled in 6 days. However, the most rapid growth in SL occurred between hatching and day 3.
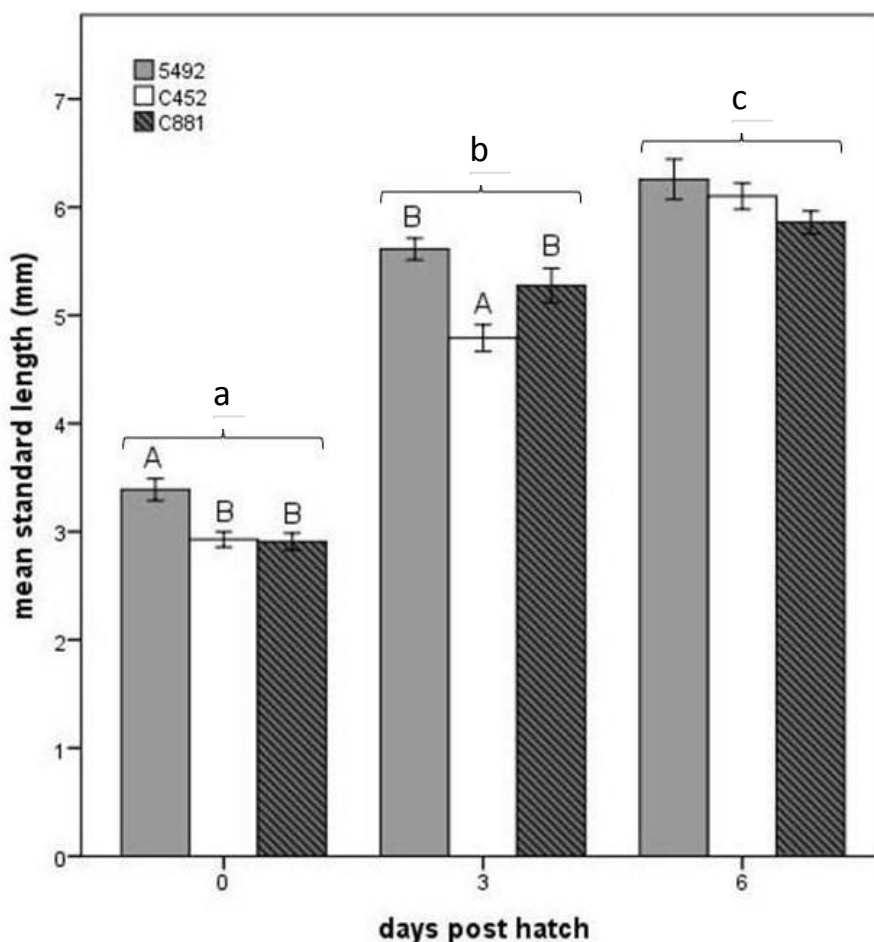
Figure 3.4: Mean standard length (mm) of larvae from each female at 0, 3 and 6 dph, n = 3. Different letters denote significant differences between the different females, and days post hatch. Error bars indicate ± 1 standard error.

Myotome height increased from 0.13±0.01 mm, at hatching to 0.23±0.03mm, 0.23±0.01 mm and 0.21±0.01 mm for larvae from the 5492, C452 and C881 females, respectively, at 6 dph (fig 3.5). There were no significant differences in MH between the larval groups within each sampling day. The average MH increased by approximately 57 % in 6 days. Total number of myotomes in 3 and 6 days old larvae were estimated to around 75-85 in the anterior part of the anus (not shown).
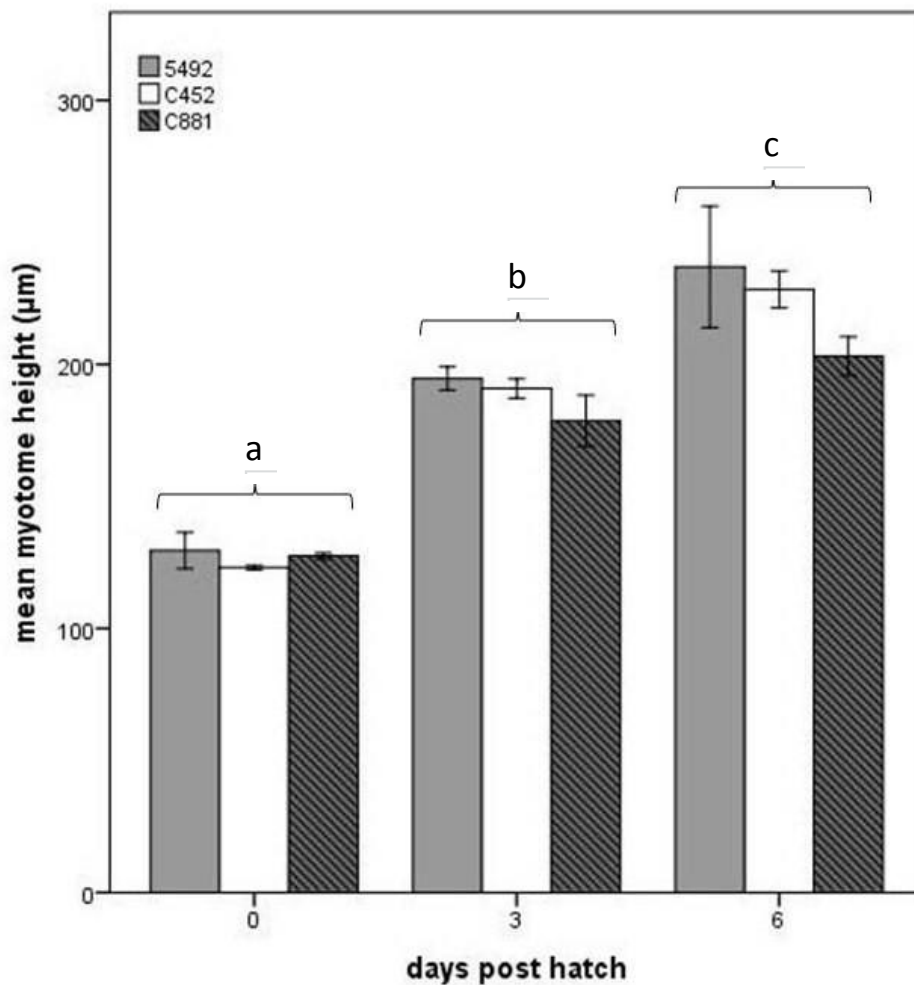
Figure 3.5: Mean myotome height (µm) measured on larvae from each female at 0, 3 and 6 dph, n = 3. No significant differences between the different females. Error bars indicate ± 1 standard error.

3.2.2 Muscle morphology

The muscle fibre morphology and orientation were similar in larvae from the different females. On the first day after hatching, when the larvae were approximately 3.1 mm long, three to four layers of immature muscle cells covered the total cross-sectional area in one bilateral half of the myotome at the level of the anus. Some presumptive myoblasts, with a heterochromatic nucleus were observed (Fig. 3.6b). The cells varied in shape and size, and occasionally contained large central nuclei and numerous mitochondria, but bundles of myofibrils were not observed (Fig. 3.7a), so muscle differentiation was not advanced.

In 3 days old larvae (approximately 5.2 mm), cross-sections of the myotomes showed two muscular layers. A superficial monolayer of fibers, presumed to be red fibres, was distinguishable from a deeper layer of elongated cells with larger diameter, presumed to be deep white fibres. Small recruitment white fibres occurred in the ventral and dorsal germinal zones, at the tip of the myotome (Fig. 3.8a). The transverse-section of white fibres was elongated, and occasionally conically shaped with several peripherally positioned nuclei. Both fibre types had an immature appearance and contained several bundles of myofibrils and scattered mitochondria (Fig. 3.9a).

Six days after hatching the larvae were approximately 6.1 mm. There was still a single superficial layer of laterally flattened red fibres surrounding the deep white muscle mass, but the red fibres did not extend as far ventrally and dorsally as at 3 dph. The larvae had an increased population of large white fibres and a greater proportion of small fibres in dorsal and ventral germinal zones at 6 dph, than at 3 dph (see section 3.2.3). Small fibres could also be seen close to the notochord (Fig. 3.10b).

At 6 dph several distinct myosepts were seen (Fig. 3.10a, c). Fibres involved in these oblique myoseptal edges were omitted from the measurement of individual fibre size in order to prevent underestimated sizes. The small white fibres, those presumed to be recruitment fibres, were irregularly shaped, with a high mitochondrial content and scattered nuclei, but bundles of myofibrils were not observed (Fig. 3.11b).
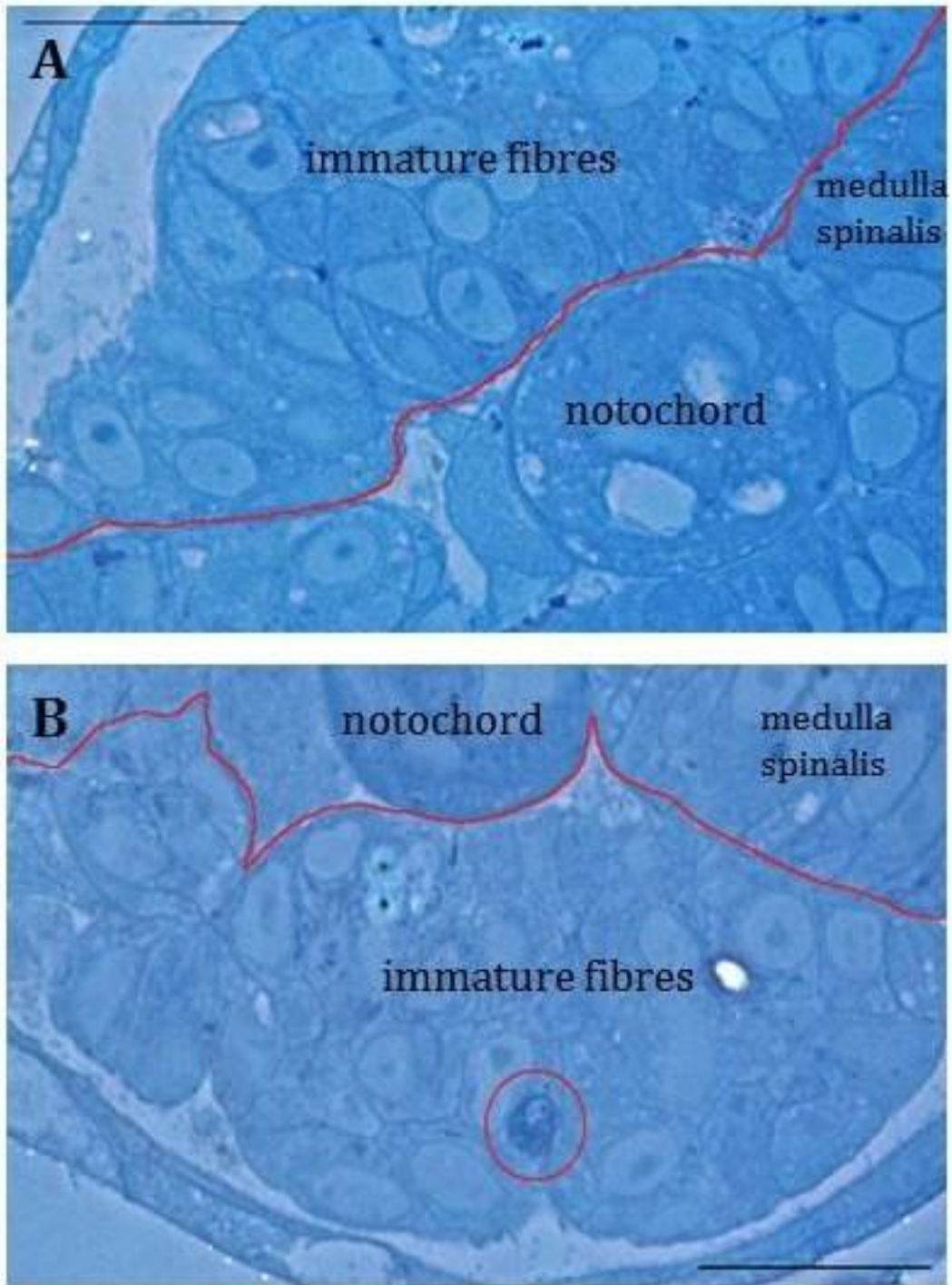
Figure 3.6: Light micrographs showing transverse sections of axial muscle fibres immediately posterior to the anus in two larvae at 0 dph from female 5492. Red circle shows a presumptive myoblast at the apex of the myotome. Red lines show immature muscle fibre area. Magnification 160x. Scale bar 20 μm.
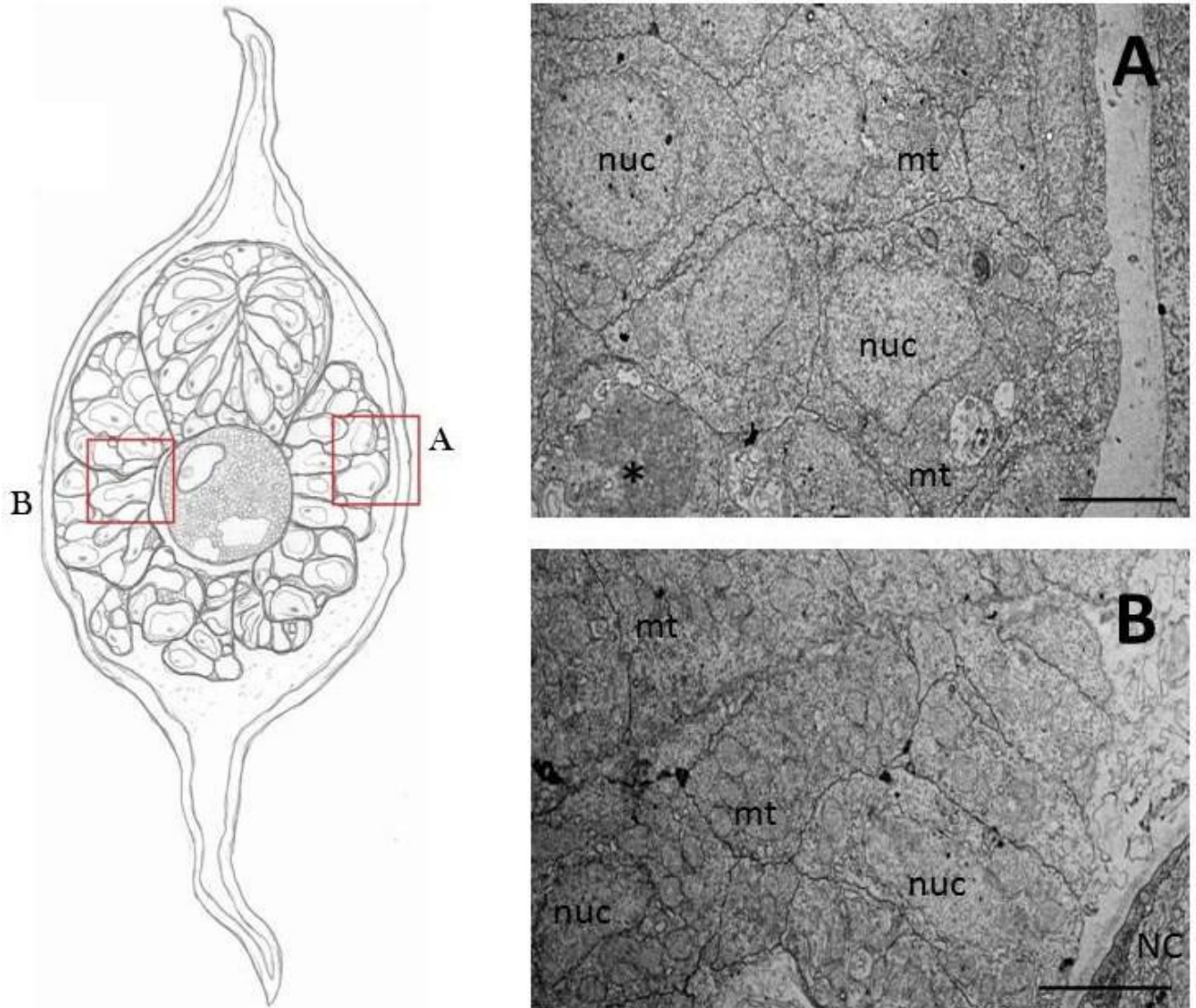
Figure 3.7: Electron micrographs showing transverse sections of axial muscle fibres immediately posterior to the anus in European eel larva at 0 dph. (A) Presumptive white fibres. mt, mitochondria; nuc, nucleus; *, presumptive myoblast; ms, medulla spinalis. The red boxes in the schematic drawing of the presumptive muscle mass mark the approximate location of the electron micrographs. Magnification x5000, scale bar 5 μm.

Figure 3.8: Light micrographs showing transverse sections of axial muscle fibres immediately posterior to the anus in one eel larvae at 3 dph female 5492 (66.5 d°). Magnification 160x. Scale bar 20 µm. Red lines shows the germinal zone at the apex of the myotome, superficial red and individual white muscle fibres in one bilateral half of the myotome.

Figure 3.9: Electron micrographs showing transverse sections of axial muscle fibres immediately posterior to the anus in European eel larva at 3 dph. (A) Deep white fibres. (B) Red fibres. nuc, nucleus; myo, myofibrils; SR, superficial red fibres; DW, deep white fibres. The red boxes in the drawing of the muscle mass mark the approximate location of the electron micrographs. Shaded fibres in the schematic drawing represent the superficial red muscle fibres. Magnification x5000, scale bar 5 μm.
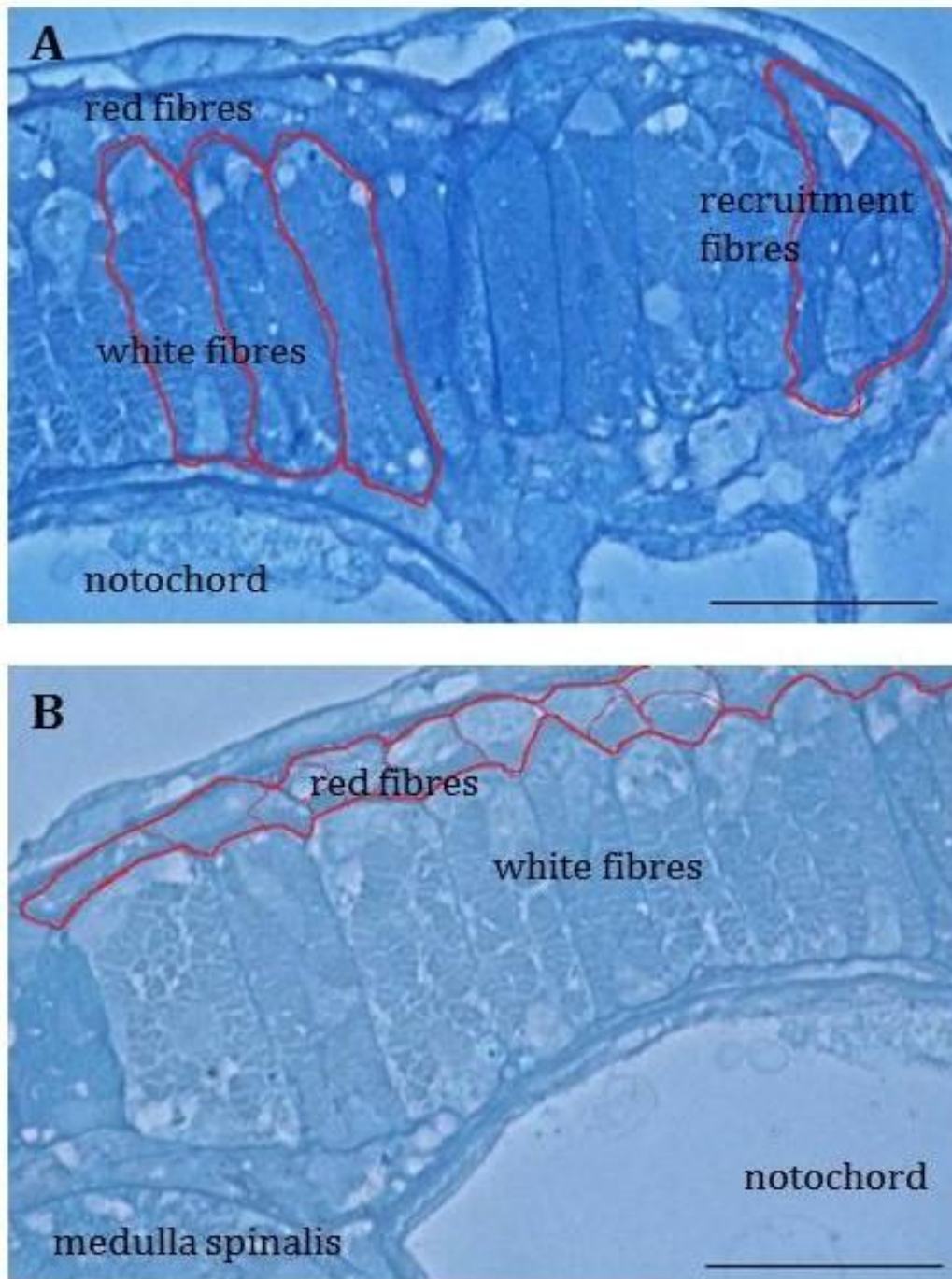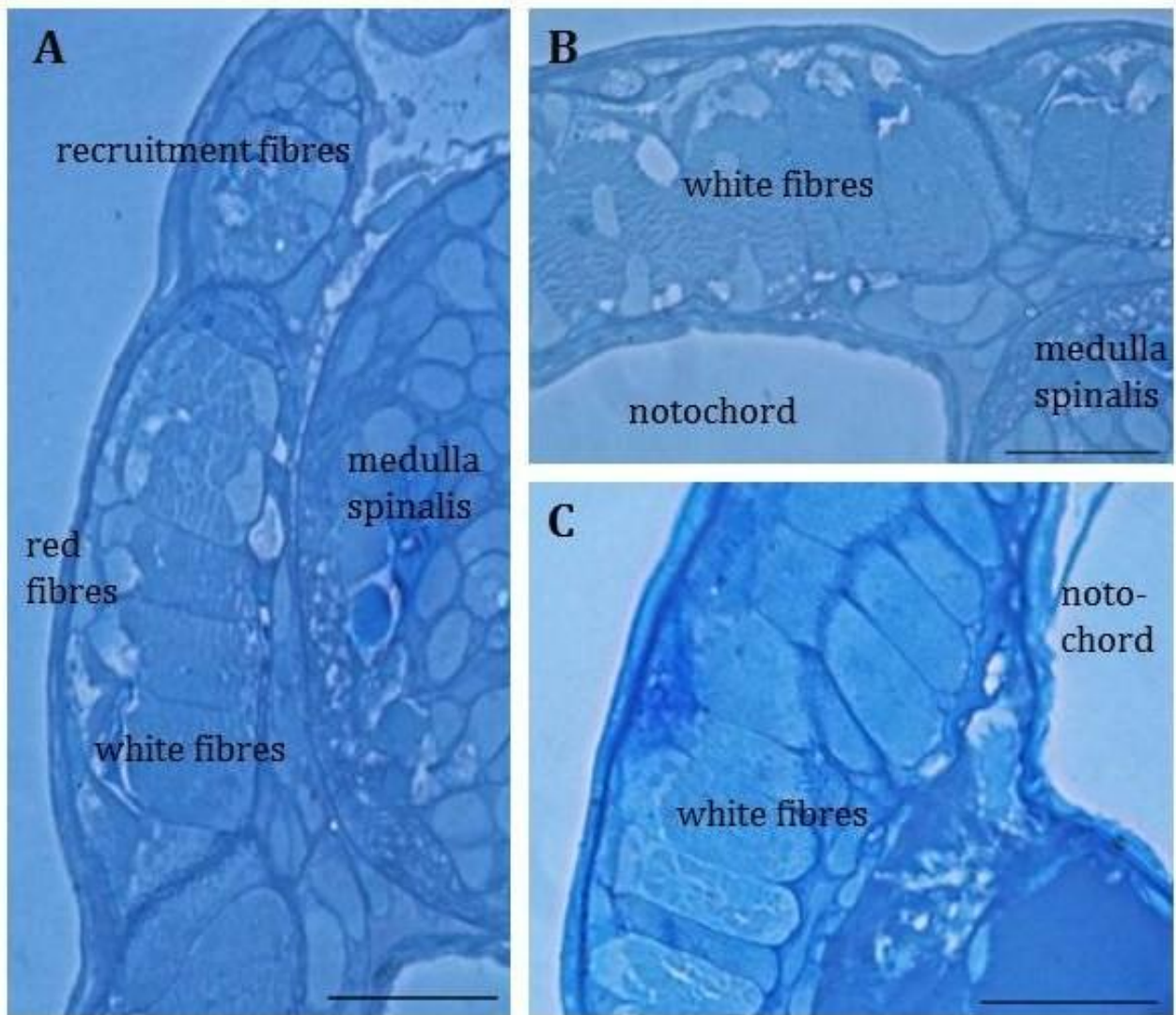
Figure 3.10: Light micrographs showing transverse sections of axial muscle fibres immediately posterior to the anus in one eel larvae at 6 dph female 5492 (122 d°). Magnification 160x. Scale bar 20 μm.
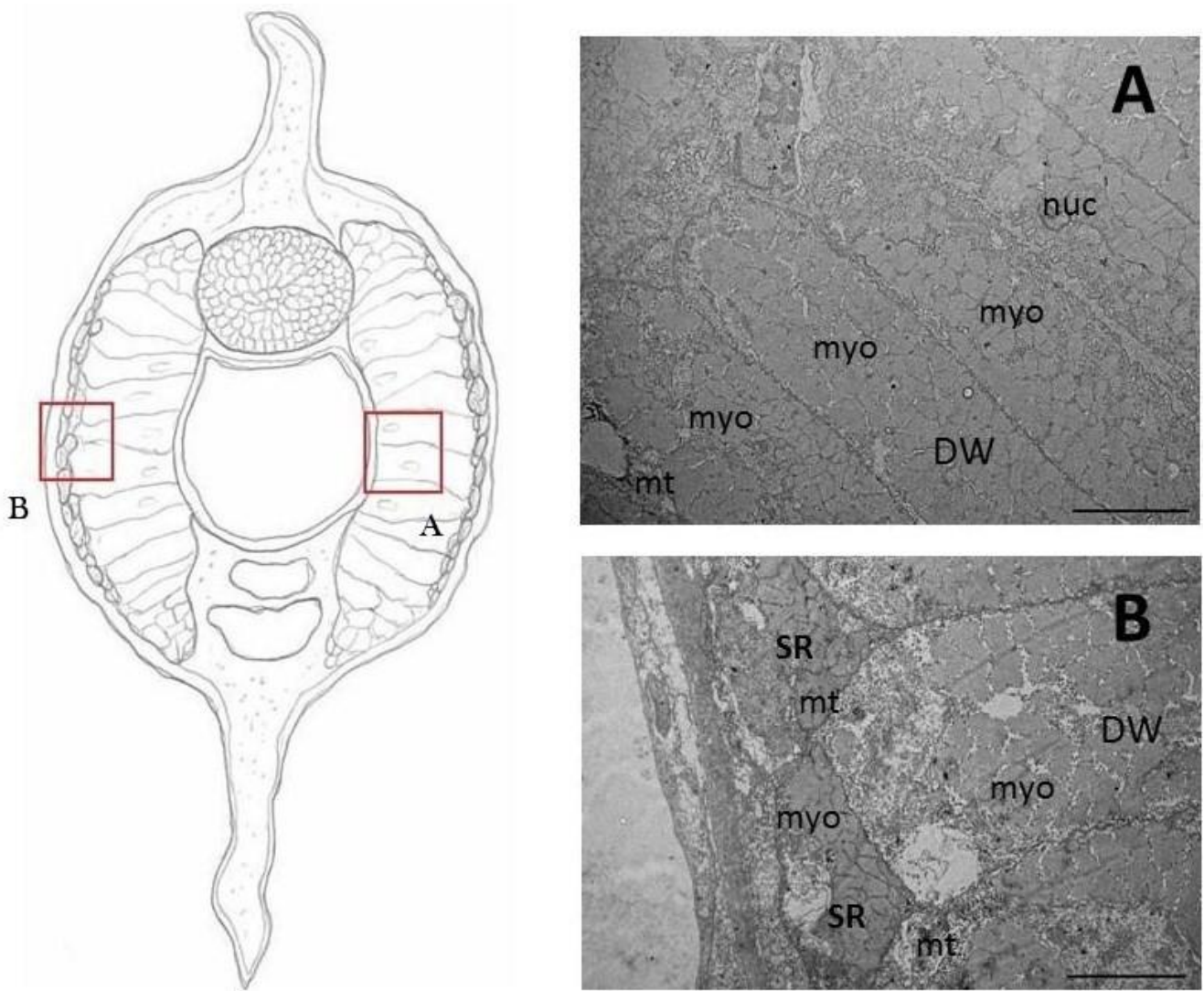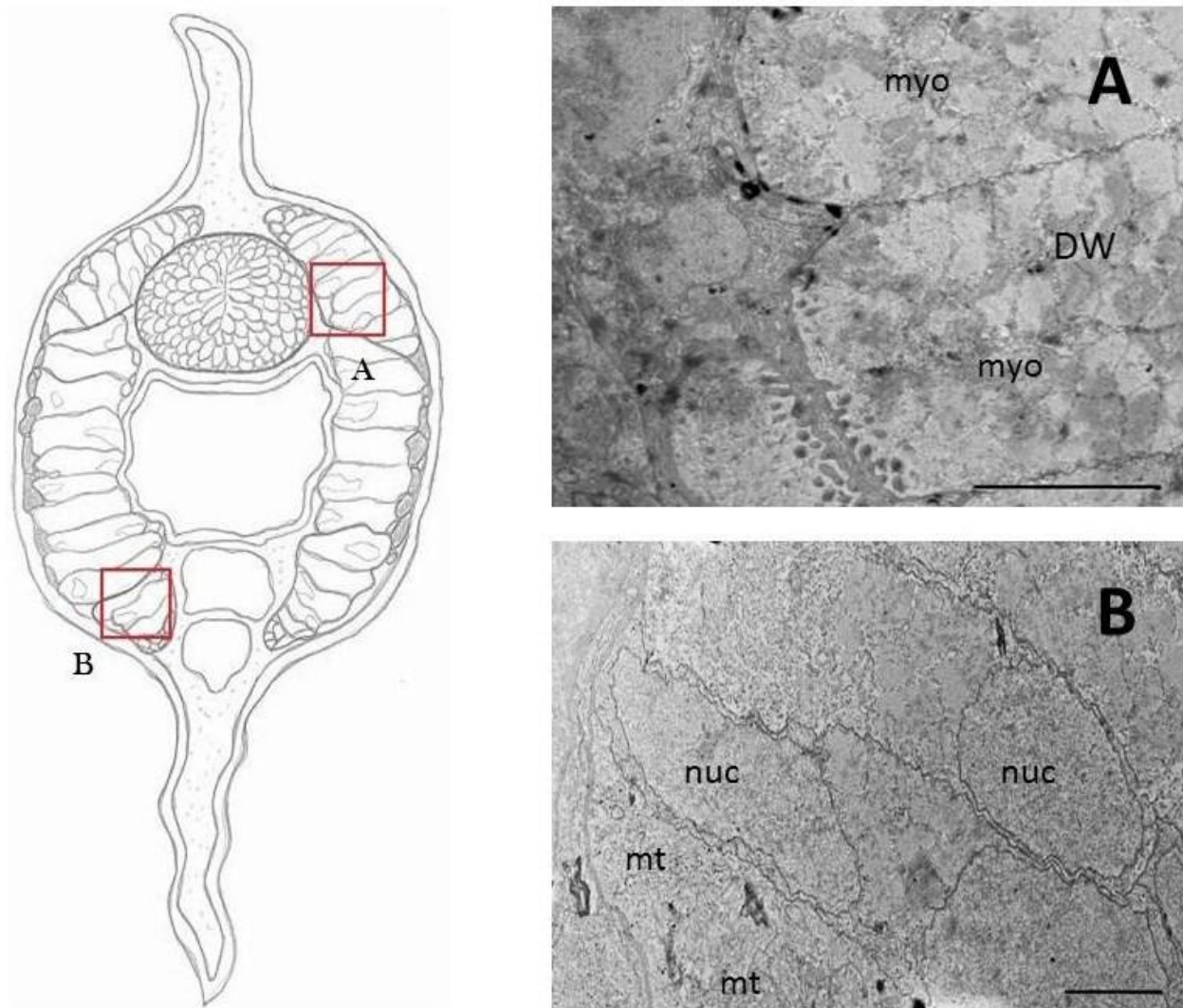
Figure 3.11: Electron micrographs showing transverse sections of axial muscle fibres immediately posterior to the anus in European eel larva at 6 dph. (A) Deep white fibres. (B) Presumptive recruitment fibres. mt, mitochondria; nuc, nucleus; myo, myofibrils; DW, deep white fibres. The red boxes in the drawing of the muscle mass mark the approximate location of the electron micrographs. Shaded fibres in the schematic drawing represent the superficial red muscle fibres. (A) Magnification x5000, scale bar 5 μm (B) x10 000, scale bar 2 μm.

3.2.3 Muscle fibre number and cross-sectional area

One larva at 0 and 6 dph deviated totally from the original muscle orientation (probably dead before sampling) and was therefore excluded from further analysis. Few larvae and missing data (2 larvae from female C452) made it difficult to run quantitative analysis within each sampling day, between the females. The muscle cross-sectional area and distribution of red and white muscle fibres are therefore calculated by mean numbers (all larvae) from each sampling day.

The total cross-sectional area of muscle mass per cross sectional area increased from 3686 μm^2 at hatching, to 4197 μm^2 at 3 dph and 5873 μm^2, at 6 dph, respectively. The total cross-sectional area was significantly larger at 6 dph, than at hatching and 3 dph (Fig. 3.12). Total cross-sectional area was poorly correlated with larval standard length (Fig. 3.13). In 6 mm long larvae, the total cross sectional area of muscle mass was approximately 65 % more than that for the 3 mm long larvae, immediately after hatching.

Immature muscle cells constituted 100 % of the muscle cross sectional area immediately after hatching. By day 6 the white fibres constituted approximately 89 % of the total muscle mass (not shown). There was almost a 100 % increase in the number of muscle cells per cross-sectional area, from 27 at hatching, to 53 white fibres at 6 dph (Fig. 3.14). Larvae at 6 dph had significantly more white fibres than at previous sampling days, but no significant difference was seen in number of immature and white fibres between hatching and 3 dph. At hatching, when the larvae were approximately 3 mm long, the number of immature and white fibres per cross-sectional area ranged from 14 to 55 (Fig. 3.15). By day 6, when the larvae were approximately 6 mm long, the number of white fibres ranged from 17 to 65 (Fig. 3.15). The number of white fibres was poorly correlated with larval standard length, but there was a clear increase (Fig. 3.15).

Histograms, shown in (Fig. 3.16), clearly indicate that the average distribution of white fibre area varies considerably between the different sampling days. In the smallest larvae immediately after hatching, fibres were initially confined to a small range between 5 and 150 μm^2 with a high percentage exhibiting areas under 100 μm^2. As the larvae grew older, this range became progressively broader. The cross-sectional area

ranged from approximately 300 to 600 μm^2 in the largest white fibres, close to the notochord (not shown). The fibre size gradually decreased close towards the germinal growth zones, where the smallest cross sectional area of white fibres ranged from 5 to 50 μm^2. These small fibres became the most dominating population at 6 dph (Fig 3.16). The mean individual immature and white fibre cross sectional area had a slight increase from approximately 69 μm^2 at hatching, to 95 μm^2 and 97 μm^2 at 3 and 6 dph, respectively (Fig. 3.17).

Superficial red fibres were not observed at hatching, and constituted approximately 11 % of the total muscle cross-sectional area at 3 and 6 dph (not shown). The red fibres showed no significant differences between 3 and 6 dph in mean total number of fibres (Fig. 3.18), although it was slightly greater at 6 dph. The red fibre numbers were poorly correlated with larval SL (Fig. 3.19). The mean individual cross-sectional area of red fibres ranged from 25 to 30 μm^2. There was no significant difference in mean individual cross-sectional area between 3 and 6 dph (Fig. 3.20). There was no particular difference or correlation with larval standard length and individual cross-sectional area between 3 and 6 dph (Fig. 3.21).
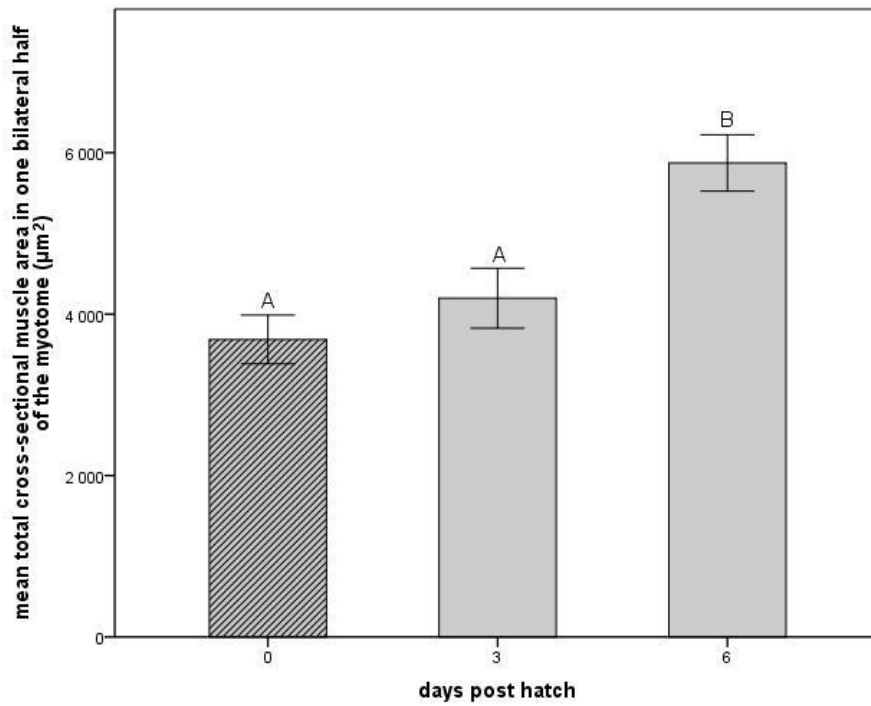
Figure 3.12: Mean total cross-sectional area of one half of the myotome at 0, 3 and 6 dph. (n = 8 at 0 dph, n = 9 at 3 dph, n = 8 at 6 dph). Shaded bar represent immature muscle cells. Different letters denote significant differences at each sampling point. Error bars indicate ± 1 standard error.
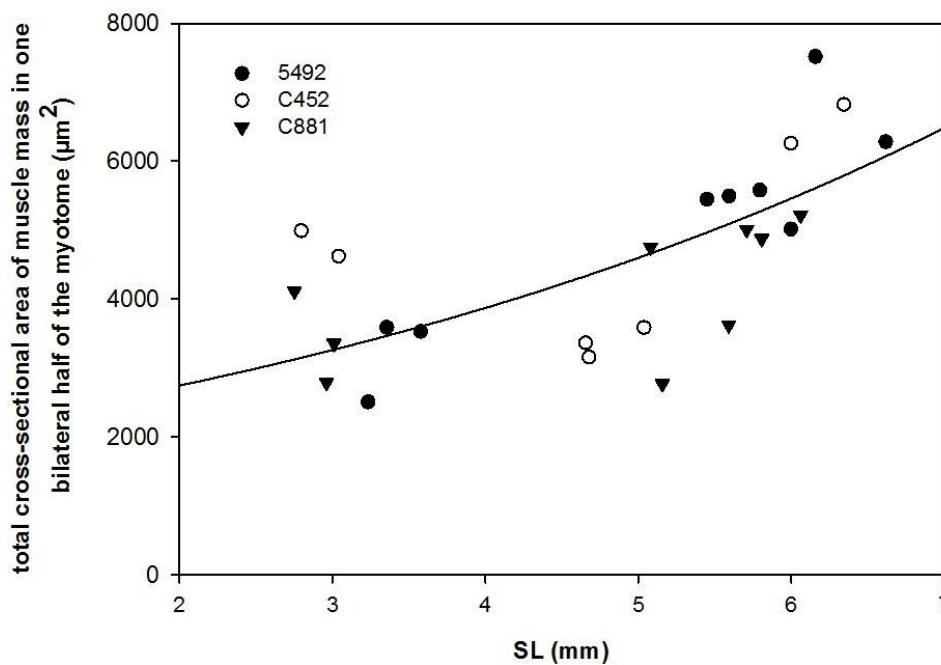
Figure 3.13: Total cross-sectional muscle area one half of the myotome at 0, 3 and 6 dph. (n= 8, 9, 8). Each point represents each larva. Exponential growth line added by following equation; (f = a*exp(b*x)). Regression line correlation, R^2 = 0.420.
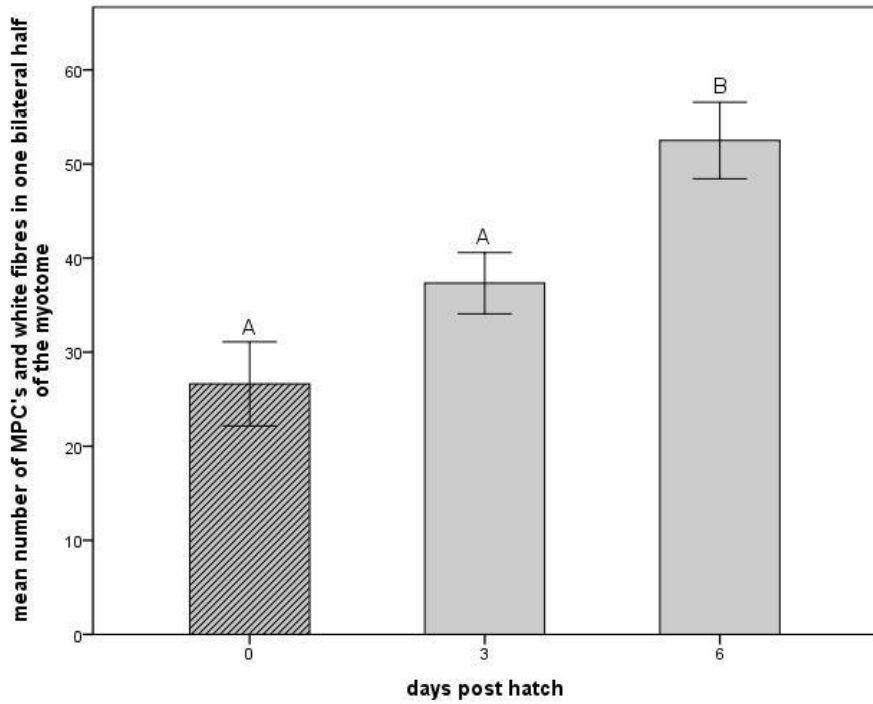
Figure 3.14: Mean number from all groups of immature and white fibres in one bilateral half of the myotome at 0, 3 and 6 dph. (n = 8, 9, 8). Shaded bar represent immature muscle fibres. Different letters denote significant differences at each sampling point. Error bars indicate ± 1 standard error.

Figure 3.15: Total number of immature and white fibres in one bilateral half of the myotome as a function of larval SL. Each point represents one larva. Polynomial regression line is added at total, $R^2 = 0.428$.
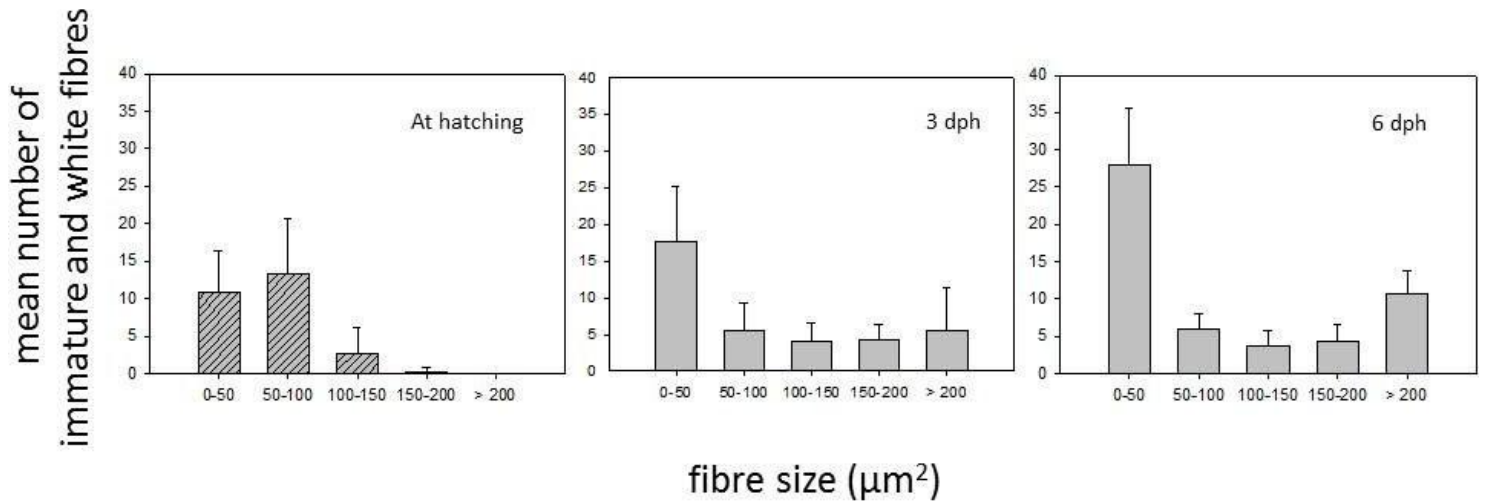
Figure 3.16: Frequency distribution histograms of immature and deep white fibres area (μm^2) in one bilateral half of the myotome. n = 8, 9, 8. Shaded bars represent immature fibres. Each bar represents the mean + S.D of total number of larvae from each sampling point. Sampling point is given at the top of each panel.
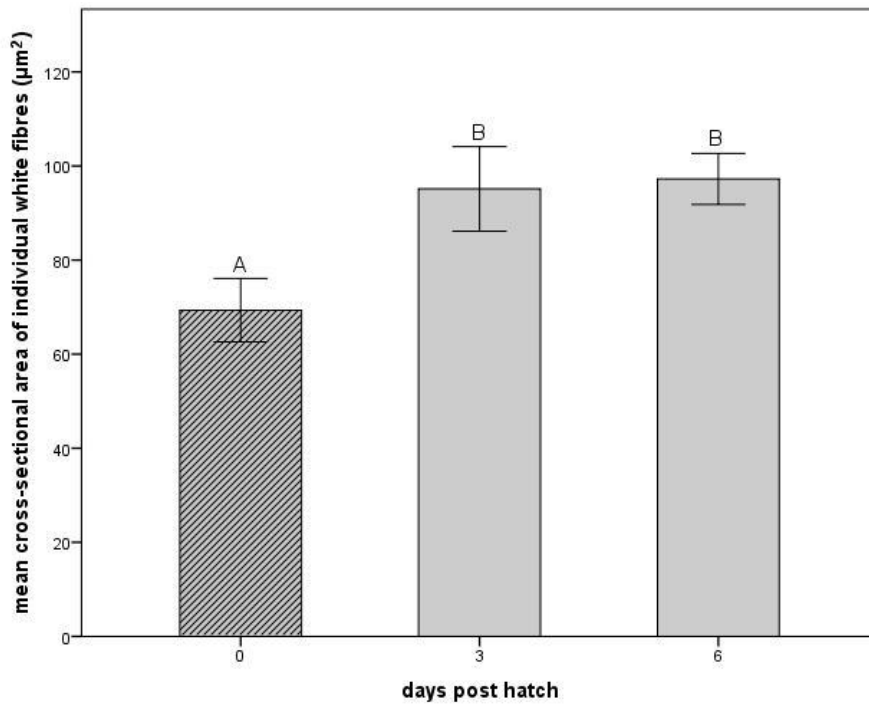
Figure 3.17: Mean cross-sectional area of immature and white fibres in one bilateral half of the myotome in larvae at 0, 3 and 6 dph. (n=8, 9, 8). Shaded bar represent immature muscle fibres. Different letters denote significant differences at each sampling point. Error bars indicate ± 1 standard error.
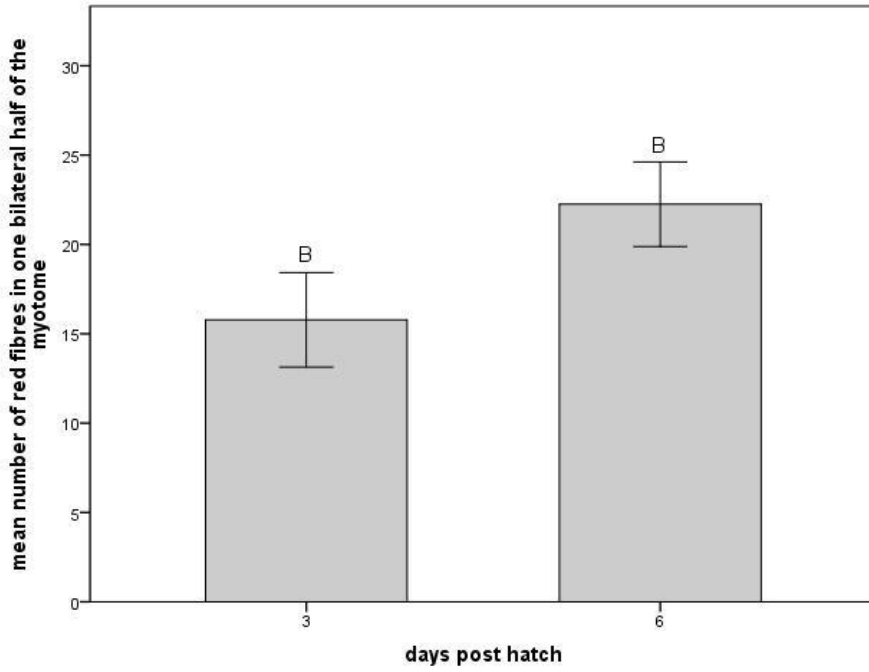
Figure 3.18: Mean number of red fibres in one bilateral half of the myotome in larvae at 3 and 6 dph. (n = 9, 8). Different letters denote significant differences at each sampling point. Error bars indicate ± 1 standard error.
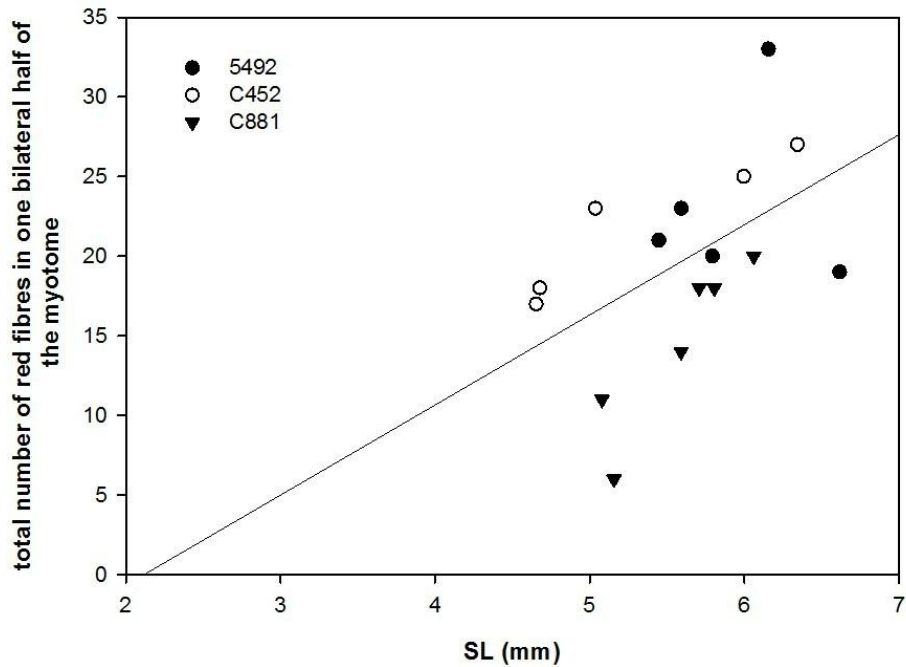
Figure 3.19: Numbers of red fibres in one bilateral half of the myotome as a function of larval SL. Each point represents one larva. Polynomial regression line is added at total, $R^2 = 0.260$.
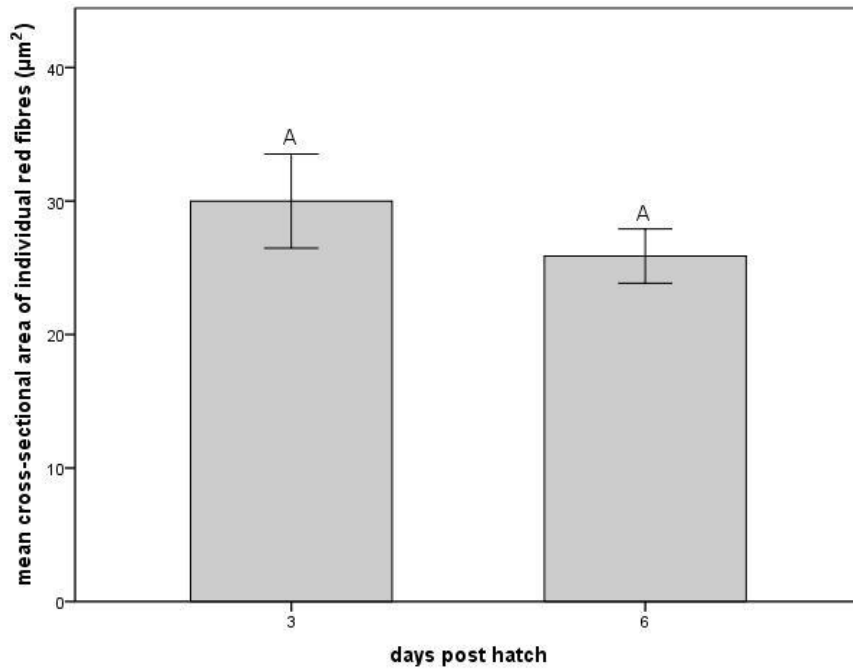
Figure 3.20: Mean cross-sectional area of individual red fibres in one bilateral half of the myotome in larvae at 3 and 6 dph. (n= 9, 8). Different letters denote significant differences at each sampling point. Error bars indicate ± 1 standard error
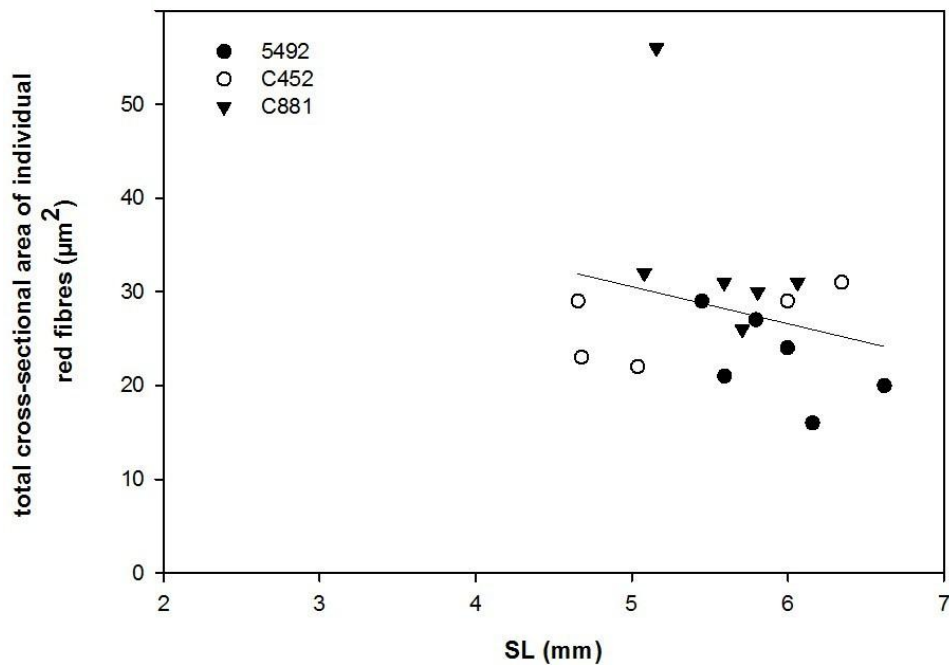
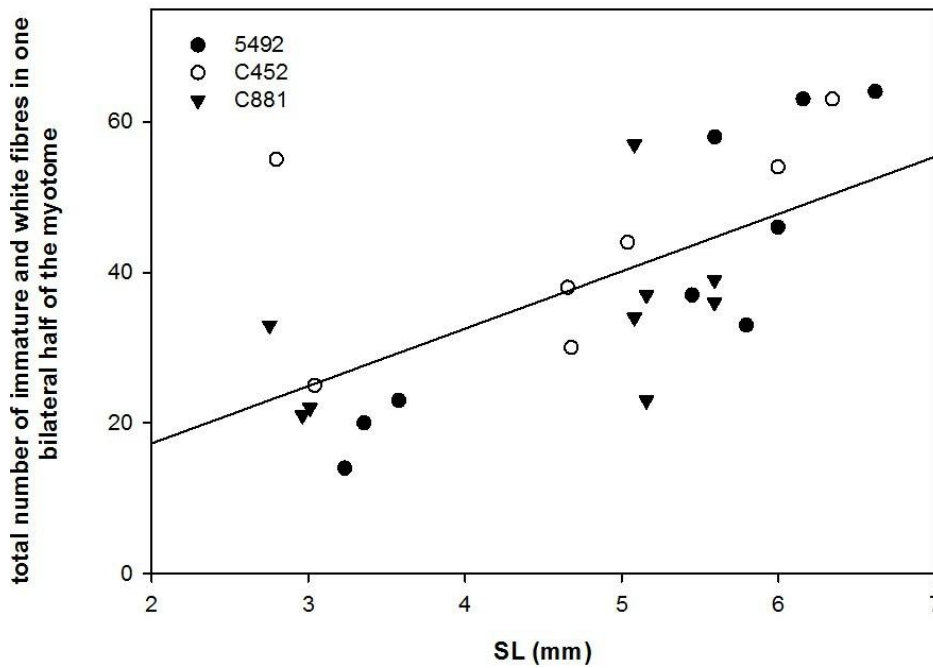**Figure 3.21:** Mean cross-sectional area of individual red fibres in one bilateral half of the myotome as a function of larval SL (n= 8, 9). Linear regression line is added at total, R2 = 0,067.

Discussion

4.1 Temperature effects on developmental rate and survival

Comparing the effects within the observed developmental stages reveals that the embryos developed synchronously, but the time until different ontogenetic events increased with increasing temperature (Fig 3.1). There was a strong correlation between day degrees and the observed developmental stages, although number of day degree was reduced by 15 % at 24° C compared to the embryos at 16 and 20° C (Fig 3.2). The metabolic responses to changing temperature are expected to be linear, and deviations of this pattern generally appear at the extremes of the incubation temperature ranges (Neuheimer & Taggart, 2007). The embryos hatched at the same developmental stage in all incubation temperatures, but incubation at 24° C had an immediate negative effect on larval survival after hatching. All the larvae were dead, overgrown with bacterial films and fungi during the first day. This temperature is therefore believed to be suboptimal for the European eel embryos. At the end of the experiment, larvae from the 16 and 20° C holding tank showed no particular differences in survival, indicating that further incubation experiments should focus on a temperature range close to or below 24° C.

Warmer temperature generally gives an increased developmental rate, within viable thermal range, in marine embryos such as cod (Pepin et al., 1997) and mackerel *Scober scombrus* (Mendiola et al., 2006), which is in agreement with the results of the present study. The European eel larval distribution in nature is suggested to be associated within subtropical convergence zones characterized by steep temperature and salinity gradients (Kleckner & McCleave, 1988). Knowledge about the specific natural biotic and hydrographic conditions during the larval phase is crucial to enhance the production of eel larvae at the first feeding stage. Variations in temperature influence metabolism, physiological processes, growth, and early development and the larval phase is especially susceptible to temperature changes (Johnston et al., 1975 ; Blaxter, 1988 ; Kamler, 2002). Water temperatures have been shown to affect time to hatching in Japanese eel (Okamura et al., 2007 ; Ahn et al., 2012) and European eel (present study), but further temperature effects on development of yolk-sac larvae remain unclear.

The present study demonstrated extremely low hatching rates, irrespective of temperature regimes, where most eggs died after 24 hours when incubated at 16, 20 and 24° C. Incubation at these temperatures induced no particular difference in hatching rate, a parameter that has previously been shown to be affected by differences in egg incubation temperature during fish embryonic development (Blaxter et al., 1983 ; Stickland et al., 1988 ; Baynes & Howell, 1996 ; Lein et al., 1997 ; Ahn et al., 2012). However, the hatching rate from all groups was too low to conclude from. The rate of embryonic development indicates that hatching for European eel occurs approximately 48 hpf, at 20° C, which is in agreement with the findings of (Pedersen, 2004).

In contrast to other eel species such as Japanese eel and Australian eel, European eels show wide individual variability and much slower response to hormonal stimulation (Burgerhout et al., 2011). The egg quality in eel is generally unstable, and survival rates are usually very low (Tanaka et al., 2003 ; Pedersen, 2004 ; Palstra et al., 2005 ; Tomkiewicz et al., 2012). This may be partly due to inadequate maturation induction procedures, or other environmental factors that differ from their natural habitat. Chang et al. (2004) showed that both embryos and yolk-sac larvae of the Japanese eel were able to adapt to wide ranges of water temperatures. Based on egg buoyancy, European eel eggs might float up to the warm surface water of the Sargasso Sea (> 22° C) which is above the presumed spawning depth, *c*. 250 m of depth (Fricke & Kaese, 1995 ; van Ginneken et al., 2005b). On the other hand, hatched yolk-sac larvae might sink to the colder water layer based on observations of negative photo tactic behavior and tolerance of low temperatures (Chang et al., 2004). Therefore, it must be considered that endogenous factors like hormonally induced fertilization, egg quality and sperm affected the results of survival, rather than incubation temperatures.

4.2 Recommendations and further perspectives for eel larval rearing
Rearing of fish larvae under stagnant water conditions has a great potential for microbial growth. In the present experiment antibiotics were therefore added, according to the dosage suggested by (Kagawa et al., 1995). However, the use of antibiotics during rearing of fish larvae is generally not advisable, since it may increase the risk of antibiotic resistance and adversely affect indigenous microflora of the larvae (Hansen & Olafsen, 1999). Suitable rearing conditions for European eel larvae have not been

sufficiently developed, and previous reports describe larval rearing in stagnant seawater treated with antibiotics (Pedersen, 2004 ; Tomkiewicz & Jarlbæk, 2008). The establishment of sustainable rearing technology for European eel larvae is necessary in order to produce on a commercial scale, and techniques for establishing water circulation should be given priority.

The yolk-sac larvae were evenly distributed, vertically suspended and mostly non-motile during the experiment. The onset of early swimming after 1 dph was characterized by rapid undulatory bursts, as an escape behavior, when exposed to light or movements in the water. Some lateral neuromast cells, which are parts of the mechanosensory system, were first observed on the flanks at 1 dph larvae. The larvae were very sensitive to vibrations, indicating that mechanoreception is well developed at this stage (Blaxter, 1987). Similar results were also described for Australian eel and Japanese eel *(Lokman & Young, 2000 ; Okamura et al., 2002).* Sampling with a pipette may therefore cause stress or damage to the larvae.

Water temperature during early development has previously been shown to affect number of somites, vertebrae and axial muscle fibres in several marine fish species, as well as area content of myofibrils and mitochondria (Brander, 1979 ; Stickland et al., 1988 ; Brooks & Johnston, 1994). Atlantic cod embryos incubated at lower temperatures gave smaller larvae at hatching, with more and smaller deep muscle fibres than the warmer incubation temperatures (Galloway et al., 1999a). The low temperature thus had a positive effect on hyperplasia and a negative effect on hypertrophy in deep muscle fibres. A similar pattern was described for newly hatched Atlantic salmon *Salmo salar* (Stickland et al., 1988). Incubation temperature prior to hatching may therefore influence number of muscle cells and MPC's in eels, and hence future fibre recruitment and muscle growth. More research is needed for further temperature investigations, in order to optimize rearing protocols for production of viable embryos and feeding larvae of European eel.

4.3 Preparation for the leptocephalus transition?

The arrangements of stacked, elongated muscle fibres, as well as the lack of a horizontal septum and stratified orientation, seem to be unique for eel larvae, as a similar fibre orientation, to my knowledge, has not yet been reported in other fish species. This unique muscle fibre orientation probably prepares the eel yolk-sac larvae for the ontogenetic transformation to leptocephalus larvae, a larval form with a somewhat different developmental strategy, in eels, bonefish, tarpon and lady fish (Nelson, 1994). Leptocephalus larvae grow at high rates, but unlike most other fish larvae, leptocephali remain planktonic for several months before metamorphosing into juveniles, by accumulating energy reserves along the body in the form of glycosaminoglycan (GAG) (Bishop et al., 2000). The Japanese leptocephalus is characterized by the presence of large spinal nerves which enter the axial muscles, as well as large distributions of the gelatinous material, GAG (Fig. 4.1), but information on somatic growth and muscle morphology in leptocephalus larvae remain unclear (Yoshida et al., 1995 ; Kawakami et al., 2009).
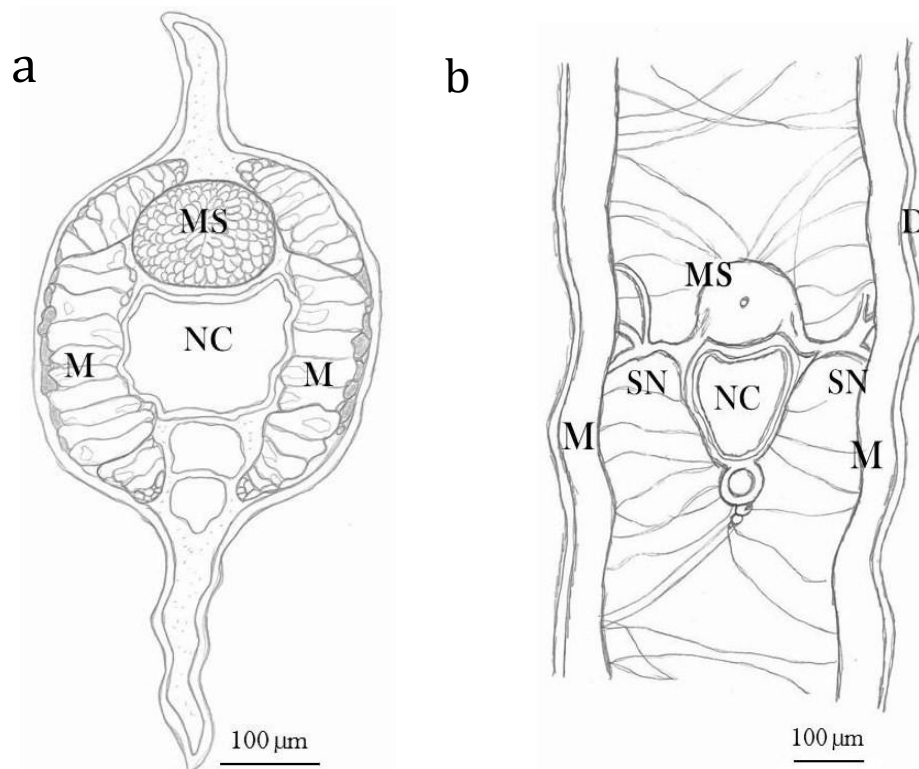
Figure 4.1: Schematic drawings showing transverse section of (a) an 6 day old European eel larva at yolk-sac stage (6 mm SL) and (b) a Japanese eel leptocephalus (20-24 mm SL). M, axial muscle; NC, notochord; MS, medulla spinalis; SN, spinal nerve; D, dermis. Outline from leptocephalus transverse section based on previous studies (Yoshida et al., 1995).

The transverse section of Japanese eel leptocephalus gives rise to the question of whether deep elongated muscle cells next to the notochord, observed at 6 dph, may migrate and develop the prospective superficial muscle layer shown in figure 4.1b. However, more research on leptocephalus larvae should be given priority in order to improve our understanding of further somatic growth and morphological development in yolk-sac larvae of eels.

European eel larvae hatched at approximately 3 mm SL with several layers of undifferentiated precursor cells. The muscle mass had an immature appearance where bundles of myofibrils were absent or totally indistinct (definitions by Veggetti et al. 1990). Electron micrographs revealed that the precursor cells contained large nuclei and high abundance of mitochondria, which indicates high metabolic activity. Some presumptive myoblasts characterized by a heterochromatic nucleus, were observed between the muscle cells at the apices of the myotome at hatching. Similar presumptive myoblast have been observed in newly hatched cod larvae (Galloway et al., 1999a) and other species such as herring *Clupea harengus* (Johnston et al., 1995), seabass *Dicentrarchus labrax* (Veggetti et al., 1990) and guppies (Veggetti et al., 1993).

As the larva grew older, the muscle mass posterior to the anus became more mature with multinucleated muscle cells containing myofilaments organized into bundles of myofibrils. The precursor cells had undergone large rearrangements, into more complex and regularly arranged muscle cells, where red and white muscle fibres were distinguishable in 3 days old larvae. The musculature consisted of a single layer of superficial red muscle fibres that covered the inner white muscle mass, similar to that found in other teleost fish larvae e.g. seabass (Veggetti et al., 1990), cod (Galloway et al., 1999a) zebrafish (Raamsdonk et al., 1982), gilthead sea bream *Sparus aurata* (Rowlerson et al., 1995) and Atlantic halibut *Hippoglossus hippoglossus* (Galloway et al., 1999b).

The inner muscle mass grew by hypertrophy of the existing fibres, in addition of new fibres. The accumulation of these new fibres was only present in the dorsal and ventral tip of the myotomes, and the larvae did not have an observed appearance of myoblasts situated between the superficial layer and inner-muscle mass, as observed in other

teleosts species at the initial yolk-sac stage (Raamsdonk et al., 1982 ; Veggetti et al., 1990 ; Galloway et al., 1999a). In 6 days old larvae, myosepta were observed dorsally and occasionally, close to the notochord, probably due to folding of the myotome.

Timing of muscle differentiation, maturity and myogenesis varies between fish species. The inner white and superficial red muscle fibres appear before hatching in most rapidly developing fish species, whereas the maturity of these fibres at hatching, varies (Raamsdonk et al., 1982 ; Veggetti et al., 1993 ; Johnston & Horne, 1994). In some salmonids and in the red sea bream *Pagrus major,* the inner white muscle mass is well developed at hatching, while the superficial red layer still consist of undifferentiated muscle cells (Nag & Nursall, 1972 ; Proctor et al., 1980 ; Matsuoka, 1984). In sea bass, myofibrillogenesis is still incomplete in both fibre types at hatching (Veggetti et al., 1990), similar to that found in the present study of eel. Superficial red fibres are believed to be involved in slow swimming movements, and development of this superficial red layer corresponds with the onset of a more sustained swimming behavior (Nag & Nursall, 1972 ; Proctor et al., 1980 ; Matsuoka, 1984). Thus, differentiation level of muscle fibres matches the observed passive behavior in eel larvae at hatching from the present study.

However, care should be taken when comparing muscle fibre maturity posterior to the anus in European eel larvae and other non-anguillar species, since growth in eel is characterized more by increased length than increased muscle mass. The transverse sections in the present study were cut posterior to the anus, somewhere between myotome number 75 and 85 in 6 day old larvae. The number of myomeres in European eel leptocephali has previously been suggested to be around 114±1 (Kleckner & McCleave, 1985), whereas the total number of myomeres in for example a cod larvae is around 50 (Lear & Green, 1984). The myogenesis and muscle fibre maturation generally starts in the anterior somites and proceeds towards the posterior somites (Lindsey, 1988 ; Devoto et al., 1996), presumably forming more mature muscle fibres in the anterior part of the body in eel larvae at a given time.

4.4 Origin of new fibres in eel larvae

It is generally accepted that newly recruited small fibres originate from the differentiation of muscle progenitor cells, or myosatellite cells (Nag & Nursall, 1972 ; Koumans et al., 1990 ; Veggetti et al., 1990). It has been suggested, however, that increasing fibre numbers may result from the splitting of existing muscle fibres. The process of fibre splitting is previously suggested in European glass eels *(Willemse & van den Berg, 1978)*, although this could not provide a source of additional nuclei. Furthermore, small fibres have in some species been shown to contain a unique embryonic myosin isoforms, supporting a new origin of recruitment fibres, rather than formation by fibre splitting of the existing fibres (Scapolo et al., 1988 ; Rowlerson et al., 1997). Small recruited fibres in the present study are therefore suggested to originate from MPC's, and not by fibre splitting. However, more studies should be undertaken for further investigations of the origin of new muscle fibres in eel, involving for example immunohistochemistry or *in situ* hybridization techniques, which can identify MPC's.

4.5 Growth and development in yolk-sac larvae of eel

The dynamic changes in fibre size between sampling days (Fig. 3.16) indicate that both hyperplastic development and hypertrophic growth occurs during the yolk-sac stage of European eel. Hypertrophic growth was the most important contribution for increased muscle mass, whereas hyperplasia was the most dominant muscle growth mechanism. In 3 and 6 days old larvae, approximately 55 % of the muscle fibers were in the 0-50 μm^2 size category, and are hence considered to be new recruited fibres (hyperplasia). A similar pattern is found in several other investigated fish species (Matsuoka, 1984 ; Stickland et al., 1988 ; Veggetti et al., 1990 ; Veggetti et al., 1993 ; Galloway et al., 1999b). An exception to the pattern is in herring, where the number of fibres remain constant, such that increases in muscle mass are entirely due to fibre hypertrophy, until the end of the yolk-sac stage (Johnston, 1993).

The present study demonstrated a positive correlation between muscle growth parameters and standard length, although not a linear relationship. An approximate doubling in SL occurred between hatching and 3 days post hatch. The high priority in length growth is probably energetically beneficial for eel larvae because the ratio

between inertial and viscous forces acting on swimming fish larvae (Reynolds number), is length dependent (Osse & Boogaart, 2004). The individual muscle cells increased significantly in size, whereas the number during the same period remained constant. Thus, the newly hatched larvae seem to spend most of their energy resources on differentiating and maturing existing fibres, rather than recruitment of new muscle fibres before 3 dph.

An opposite growth pattern was observed between 3 and 6 days old larvae, where SL and MH had a slight increase. The white muscle fibres increased significantly in number, whereas the fibre size remained constant from 3 to 6 dph. The number of these small recruitment fibres affects mean individual fibre size, indicating that existing fibres continue to grow by hypertrophy. The larvae seem to spend more energy resources on recruiting new fibres, rather than further length growth of the axial musculature after 3 dph. The distribution of red muscle fibres remained constant at approximately 11 % of the total muscle area from 3 dph. The present study shows a rapid growth and muscle development with a slight correlation between muscle growth parameters and standard length. Previous studies demonstrate stronger correlation between larval muscle growth and standard length in e.g. cod (Galloway et al., 1999a) and carp (Alami-Durante et al., 1997). However, older larvae are needed for a complete comparison between muscle growth parameters and standard length.

This rapid larval growth is not unexpected, since the axial musculature is the most rapidly growing tissue in fish larvae bodies. It is an important developmental strategy to reduce vulnerability to predation, catch larger prey and search larger volumes of water for food, by maximizing growth (Blaxter, 1988 ; Alami-Durante, 1990). However, more samples and older larvae are needed in order to improve our understanding of somatic growth and further morphological development during the larval stage in European eel. Information about growth patterns, structure and optimal temperature might help in the understanding of critical periods throughout the early development, processes that are important for larval rearing in aquaculture.

4.5 Conclusions and future perspectives

The described temperature experiment showed that time until hatching was clearly related to temperature, and provided information about the actual time and day degrees required for embryonic development from fertilization to hatching. Incubation at 24° C had a negative effect on survival rates of newly hatched larvae, indicating that this temperature is suboptimal for egg incubation.

The present study is the first know study of axial muscle development in yolk-sac larvae of eels. The muscle morphology, rate of development and onset of muscle differentiation follow a unique pattern in European eel larvae compared to that reported in other teleost fish larvae in the literature. These observations indicate that yolk-sac larvae of eel prepare for a somewhat different developmental strategy, i.e. leptocephalus phase.

Larval muscle growth occurred by hyperplasia and hypertrophy, where hypertrophy seems to be the most important mechanism for increased muscle mass by compare total area contribution of both growth mechanisms. The somatic length growth was poorly correlated to the rate of hyperplasia and hypertrophy in the initial yolk-sac stage.

More research is needed, in order to optimize rearing protocols for production of viable embryos and feeding larvae of European eel. The estimated effect of water temperature on embryonic development and larval survival, in combination with larval morphology based on DTU standard protocols, can be used to compare temperature effects related to muscle, and predict the time required to reach different developmental stages. It should be noted that, the use of antibiotics must be severely reduced in commercial scale production of eel. Sustainable rearing techniques should therefore be further investigated. In relation to muscle growth, older larvae and more long-term studies with more advanced histological techniques should be undertaken to further investigate the origin and development of new fibres in European eel.

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APPENDIX 1 - Embedding eel larvae in EPON 812

The plastic medium that the larvae were embedded in consisted of EPON 812 (25.5 g), DDSA (12.25 g), NMA (12.25 g) and DMP 30 (0.5 g). The solution had been stored in 20 mL disposable plastic syringes at - 20° C. The larvae were rinsed in 0.08 M cacodylic buffer 3 x 10 minutes and post fixated in 4 % osmium: 3 % potassium hexacyanoferrate (II) 1:1 (final 2 % OsO_4: 1.5 % potassium) and kept dark for one hour at room temperature.

The larvae were bulk colored for 1.5 hours with 1.5 % uranylacetat in distilled water, then washed 5x2 minutes in distilled water at room temperature. The next step was gradually dehydrating the larvae in ethanol. 10 min in 70 % ethanol, 10 min in 90 % ethanol, 2x10 minutes in 96 % ethanol, and finally 3x15 minutes in absolute ethanol.

The embedding in EPON was done by mixing absolute ethanol and EPON with gradually increased concentrations. The larvae were first exposed to a mixture 3:1 Ethanol / EPON in 30 minutes, then 1:1 mixture in 30 minutes, then 1:3 mixture in 30 minutes, and finally, only EPON. The embedded larvae were put to stir over night, and the EPON was replaced by new EPON next morning, before the samples were set to polymerize in 60° C for 24 hours.

APPENDIX 2 - Staining of histological sections for light microscopy

The sections used for light microscopy were stained with Nile blue and Toluidine blue.

Toluidine blue:

Stock solution was made from 1.0 g Toluidine blue and 1.0 g sodium borate buffer in 100 mL distilled water. The mixture (1:1) was filtrated two times and stored at room temperature (pH 8 - 8.5). Sections were transferred on an object glass and pre-heated at 60° C till the water evaporated. The sections were covered with Toluidine blue for 30 seconds at 75° C, and rinsed with distilled water. The object glasses were dried before a cover glass was mounted on each object glass with Cytoseal™XYL (Richard-Allan Scientific, USA).

Nile blue:

Stock solution was made from 1.0 g Nile blue and 0.5 mL Sulphuric acid pure in 100 mL distilled water. The Nile blue solution was filtered before use, and transferred sections were pre-heated on object glasses at 60° C. The object glasses were mounted directly into the solution of Nile blue in a Coplin jar, and placed in the oven at 60° C for 2 hours. The sections were rinsed with a gentle stream of distilled water. Object glasses were drained before cover glass was mounted with Cytoseal™XYL.

APPENDIX 3 - Histological sections for transmission electron microscopy

The sections used for electron microscope were contrasted with lead citrate to get better image contrast.

Lead citrate:

The lead citrate solution was made beforehand by dissolving 0.1 g of lead citrate in 50 mL 0.1 sodium hydroxide (0.2 g hydroxide to 50 mL distilled water), stored in a plastic tube over night at room temperature. Each section was placed on copper grids covered with a carbon film, with mesh sizes 75 (Jed Pela Inc., USA) and 100 (EMS Inc., USA). The sections were bulk colored with uranyl acetate in the EPON solution. Three beakers were filled with distilled water and one with lead citrate. The copper grids were mounted directly into the lead citrate for 30 seconds and rinsed consecutive in all three beakers of distilled water. Remaining water droplets on the grids were removed by gently pushing with a filter paper.

APPENDIX 4 - Frequency distribution histograms from each larva.
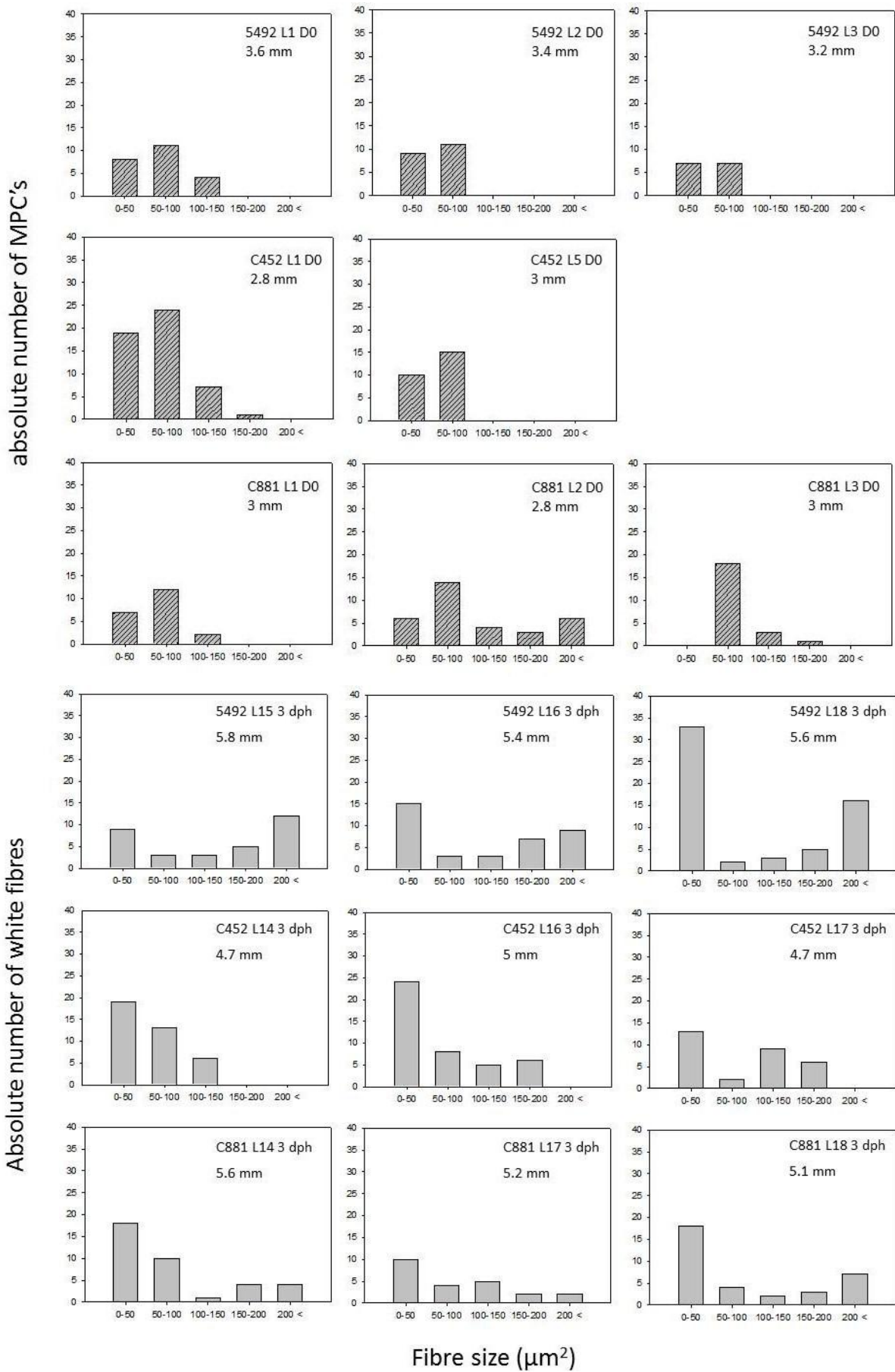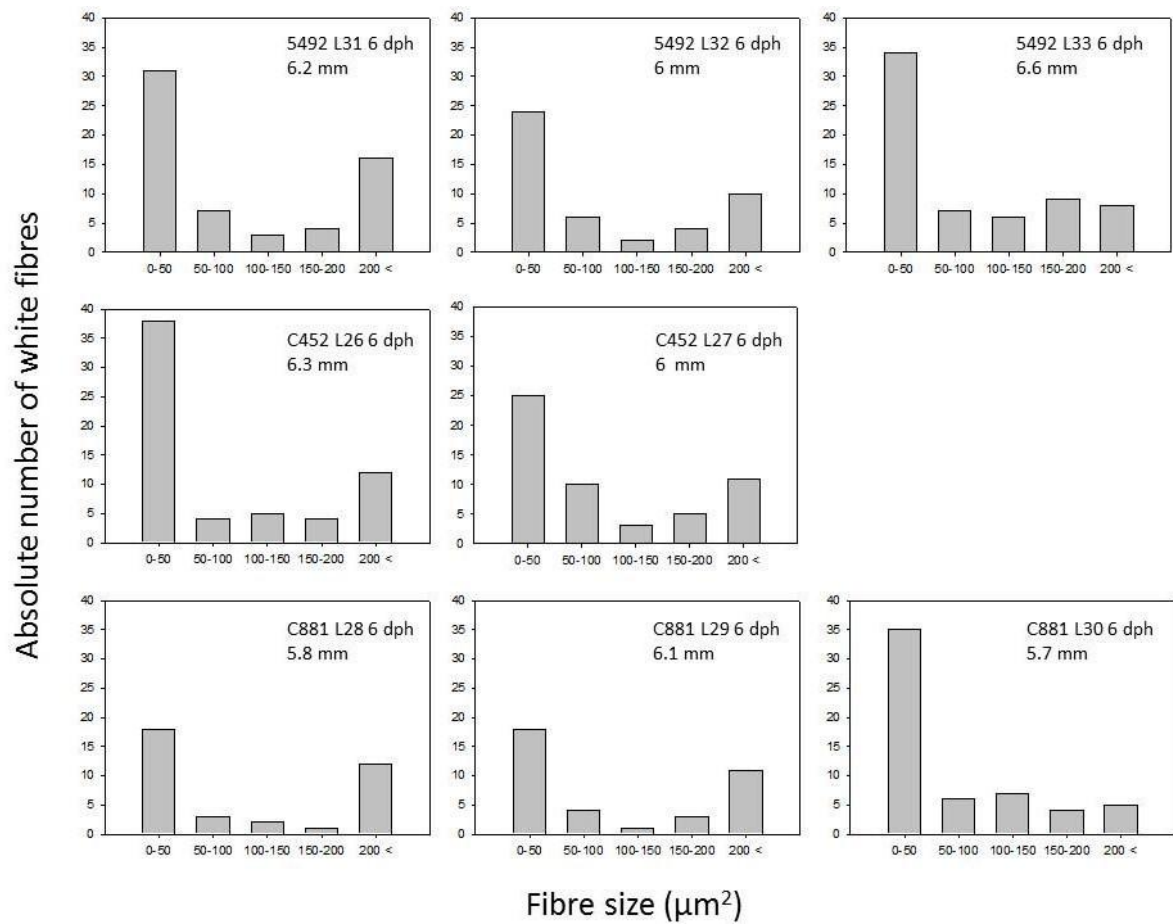
Figure A.1: Frequency distribution histograms of immature and deep white fibres area (μm²) in one bilateral half of the myotome. Shaded bars represent immature fibres. Female, sampling point and SL of the sectioned larvae is given at the top of each panel. D0, D3 and D6, day 0, 3 and 6 after hatching.

APPENDIX 5 - Muscle analysis

DPH	Female	SL (µm)	MH (µm)	TotA white (µm²)	TotA red (µm²)	distribution of total muscle area		# White	# Red	mean W size (µm²)	mean R size (µm²)
						White %	Red %				
0	5492	3577	125	3527	0	100	0	23	0	70	0
	5492	3356	121	3588	0	100	0	20	0	55	0
	5492	3233	143	2507	0	100	0	14	0	55	0
	5492	2796	122	4988	0	100	0	55	0	63	0
	C452	2948	125	x	x	x	x	x	x	x	x
	C452	3041	123	4621	0	100	0	25	0	57	0
	C881	2962	129	2788	0	100	0	21	0	60	0
	C881	2752	125	4115	0	100	0	33	0	109	0
	C881	3010	129	3354	0	100	0	22	0	85	0
3	5492	5794	198	5031	548	90,2	9,8	53	20	138	27
	5492	5448	186	4836	611	88,8	11,2	58	21	127	29
	5492	5593	200	5030	463	91,6	8,4	81	23	108	21
	C452	4654	197	2822	542	83,9	16,1	55	17	62	29
	C452	5037	184	3081	506	85,9	14,1	67	23	65	22
	C452	4678	192	2723	436	86,2	13,8	48	18	83	23
	C881	5591	191	3145	479	86,8	13,2	53	14	77	31
	C881	5157	159	2391	380	86,3	13,7	29	6	85	56
	C881	5080	185	4258	494	89,6	10,4	45	11	112	32
6	5492	6157	270	6880	635	91,6	8,4	63	33	107	16
	5492	5997	193	4323	690	86,2	13,8	46	25	88	24
	5492	6617	248	5913	368	94,1	5,9	64	19	89	20
	C452	5997	227	6035	787	88,5	11,5	63	27	92	31
	C452	6344	241	5457	800	87,2	12,8	54	25	96	29
	C452	5964	217	x	x	x	x	x	x	x	x
	C881	5807	217	4372	502	89,7	10,3	36	18	115	30
	C881	6062	193	4569	647	87,6	12,4	37	20	118	31
	C881	5708	199	4453	553	89	11	57	18	72	26