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Morphological development of wild leptocephalus larvae of the European eel (Anguilla anguilla)

With special emphasis on muscle and
digestive system

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Marine Coastal Development
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Abstract

The larval stage of the European eel (*Anguilla anguilla*) is largely undescribed. Increased knowledge about the ontogeny of the eel is be of interest both in an ecological perspective, but also in relation to a conservation, as the eel stocks in Europe has experienced major declines over a very short period of time. Eel is also a potential candidate for production in aquaculture, where the early life stages are most challenging, mainly in relation to start-feeding the larvae. In the present study, the functional development and morphology of wild captured leptocephalus larvae of the European eel was described. The study had special emphasize on the digestive system and muscle, as this is considered especially important for the survival of fish larvae. Descriptions and measurements were made of the sensory organs (eyes, olfactory organ, taste buds, neuromasts, ear), brain and neurons, circulatory system, respiratory organs, skin and skeleton. Growth and development of the larvae in relation to standard length was also investigated. External and internal descriptions and measurements were performed, and different histological methods and techniques were used.

The digestive system appeared to be very similar to the Japanese eel, apart from the muscular anterior esophagus with the rough surface, which is hypothesized to have a food-crushing function. This might explain the long and fragile teeth of the leptocephalus larvae that are thought have a grasping function, rather than being used for biting or chewing. The muscle tissue of the larvae had a unique morphology with a thin, almost stacked layer covering the gelatinous matrix in the middle. This may be unique to the leptocephalus larvae, and has previously only been described for yolk-sac larvae. The muscle of the larvae was found to grow mainly by hypertrophy. The main growth both in number and size of muscle happened in the middle of the larvae, indicating that they grow in length both from the tail area and head area. The skin was covered in a cell layer, probably consisting of a large proportion of the lectin-secreting club cells based on the findings for the Japanese and American eel. This thought to be of importance in relation to protection of the larvae. It might seem that the leptocephalus larvae have a unique development of the eye, based on the observation of golden retinal pigmentation in all larvae and a highly developed eye in the largest larva. It seems to be something important happening both with respect to growth and organ development between the size 18 and 23 mm, as there for almost all organ systems that were viewed could be observed a major difference between the largest specimen and the other larvae, especially in relation to the development of the eye, finrays and in the muscle tissue.

Sammendrag

Larvestadiet til den Europeiske ålen (*Anguilla anguilla*) er svært ubeskrevet. Økt kunnskap om ålens livssyklus er både av interesse i et økologisk perspektiv, men også i forhold til bestandsforvaltning, ettersom ålebestandene i Europa har gjennomgått store nedganger over svært kort tid. Ål er også en potensiell kandidat for produksjon i akvakultur, der de tidligste stadiene er de mest utfordrende, særlig i forhold til startfôring av larvene. I denne studien har morfologi og funksjonell utvikling for villfangede ålelarver av Europeisk ål blitt beskrevet. Studien har hatt særlig fokus på fordøyelsessystem og muskel, ettersom disse er regnet som særlig viktige for overlevelsen til fiskelarver. Beskrivelser ble også gjort av sanseorganene (øyne, lukteorgan, smaksløker, neuromaster, øre), hjerne, og nevroner, sirkulasjonssystemet, respirasjonsorganer, hud og skjelett. Vekst og utvikling av larvene i forhold til standard lengde ble også undersøkt. Utvendige og innvendige målinger ble gjort, og ulike histologiske metoder og teknikker ble benyttet.

Fordøyelsessystemet var veldig likt det som er observert for den Japanske ålen, med unntak av det muskuløse fremre esophagus med en røff overflate, som er antatt å ha en funksjon i forhold til knusing av matpartikler. Dette kan forklare de lange og skjøre tennene til leptocephalus larvene, som heller er antatt å ha en gripefuksjon, heller å bli benyttet til biting eller tygging. Muskelvevet til larvene hadde en unik morfologi som et tynt, nesten stablet lag som dekket gelé-laget i midten av larven. Dette kan være unikt for leptocephalus larver, og har tidligere kun blitt beskrevet for plommesekklarver. Muskelen til larvene ble funnet å vokse hovedsakelig vis hypertrofi. Hovedvekten av vekst både i forhold til antall og størrelse på muskelen foregikk i midten av larven, noe som indikerer at vokser i lengde både fra hodeområdet og fra haleområdet. Huden var dekket av et cellelag som trolig består av en stor andel av lectin-sekreterende club-cells, basert på tidligere funn for den Japanske og Amerikanske ålen. Dette laget har trolig en viktig funksjon i forhold til beskyttelse av larven. Det kan også se ut til at leptocephalus larvene har en unik utvikling av øyet, basert på observasjonen av gyldne pigmenter i netthinnen, og et svært utviklet øye hos den største larven. Det virker også som at en viktig del av vekst og organutvikling skjer mellom størrelsen 18 og 23 mm, ettersom det for nesten alle organsystemer som ble undersøk ble observert en stor forskjell mellom den største larven og alle de andre larvene, særlig i forhold til utvikling av øyet, finnestråler og muskelvev.

Contents

Abbreviations

At Atrium (heart)

Ba Branchial arch (gills)

Bv Blood vessel

CC Club cell

ChlC Chloride cell

DOM Dissolved organic matter

DTU Technical University of Denmark

Ed Epidermis

EH Eye height

EL Esophagus Length

%EL Esophagus Length expressed as % of TT

Epi Epithelium

Eso Esophagus

EW Eye width

Fa Fat deposits

FT Front tooth

G Goblet cell

GAG Glycosaminoglycan

GB Gall bladder

Gg Glycogen granules

H Heart

HC Hair cell (ear/neuromast)

HK Head kidney

IL Intestine Length

In Intestine

INL Inner nuclear layer (retina)

JL_L Jaw length lower

JL_U Jaw length upper

K Kidney

Ku Kupffer cells (liver)

L (in eye) Lens

L Liver (digestive system)

La Lamella (gills)

Lu Lumen

M Muscle/muscularis

MC Mucus cell

MHA Myotome Height at Anus

MHM Myotome Height at the middle of the larva

MS Medulla spinalis

MTS Main transverse section

Mu Mucosa

N Notochord

NF New fiber (muscle)

NM Neuromast

Noto Notochord

NTNU Norwegian University of Technology and Science

OC Opercular cavity

ON Optical nerve

ONL Outer nuclear layer (retina)

Oth Otholit

Pa Pancreas

PE Pigmented epithelium (retina)

PFA Paraformaldehyde

PL Plexiform layer

POM Particulate organic matter

PRL Photoreceptor layer (retina)

RM Red muscle

Se Serosa

SL Standard Length

TT Total digestive tract

%TT Total digestive tract length, expressed as % of SL

UB Urine bladder

VB Vitreous body (eye)

Vt Ventricle (heart)

WM White muscle

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1. Introduction

1.1 – The European eel (*Anguilla anguilla*)

The European eel (*Anguilla anguilla* Linnaeus 1758) is a catadromous fish that is found in fresh, brackish and coastal water along the coast of Europe and parts of northern and western Africa (Moriarty and Dekker, 1997). Its complex life cycle has been a mystery that has fascinated scientists for more than a century, and parts of it still remains to be uncovered, especially in relation to its reproductive biology and larval development. The European eel is also an attractive product, especially in the European and Asian markets. It is exploited throughout its ontogeny, from the young glass eels migrating back to the coast of Europe from their spawning grounds in the Sargasso Sea, to mature silver eels migrating in the opposite direction to spawn (Kirkegaard et al, 2010, Moriarty and Dekker, 1997).

The European eel is today categorized as critically endangered (CR) on the IUCN redlist of threatened species, with a population at a historically low of only 1-5 % of the pre-1980 levels (IUCN, 2013). The reason for this decline is still uncertain, but it is probably a result of several factors, that combined creates a negative impact on the recruitment to the population of European eel. Overfishing, parasites (like *Anguillicola crassus*), pollution, habitat loss and shifts in the Gulf Stream and are some examples of proposed mechanisms for this decline (Bonhommeau et al, 2008, Friedland et al, 2007, Kirk, 2003, Feunteum, 2002). The European eel is also considered a potential candidate for production in aquaculture, both because of the possibility of restocking the natural population, and to reduce the pressure on the natural stocks. Aquaculture of European eel is performed today, but not in a sustainable and profitable way, mainly because of the use of wild-caught glass eel in the production (Kirkegaard et al, 2010). Glass eels are a limited resource, and the use of these for aquaculture production impacts the recruitment to the natural stocks of eel in Europe. The use of wild-caught glass eels is also making it difficult to achieve a profitable production, as glass eels are very expensive to purchase (Kirkegaard et al, 2010). However, the ability to produce the whole eel life cycle in captivity would eliminate this dependency on glass eels, and hence make a self-sustained production, which is both more economically feasible and sustainable.

Successful artificial maturation of eels in captivity, with following embryonic development resulting in viable hatched larvae was first accomplished for the Japanese eel (*Anguilla japonica*) (Yamamoto and Yamauchi, 1974), which is very similar to the European eel.

Artificial maturation of the European eel, with following egg and larval development, was achieved for the first time in 1983 (Bezdenezhnykh et al, 1983). In this experiment, the larvae only lived for a few days. Through improvements of the fertilization protocols, the fertilization rates are today highly improved (Butts et al, 2014, Pedersen, 2003). New research has given much insight on the reproductive biology and early life stages of the eel. For example has the rearing environment been shown to be of great importance for the production of viable eggs and larvae. The microbial activity of the rearing environment has been shown to affect the hatching success, and the microbial cover of the eggs to have an effect on the larval survival (Sørensen et al, 2014). The light conditions have also been shown to have an effect on the survival of the larvae, as larvae reared in red light and low light intensity have been shown to increase the survival (Politis et al, 2014). By the use of these protocols for artificial reproduction, the production of eel larvae in captivity has been possible, although the inability to make them feed has made it difficult to get the larvae to survive past the yolk-sac stage (Kirkegaard et al, 2010). Part of the step forward towards the production of glass-eels in captivity will also be to ensure that the eggs and larvae produced are of the best quality possible. Ultimately, part of the solution, is to increase the understanding of the eels life cycle, and especially the earliest stages.

1.2 – Life cycle and developmental stages

The life cycle of the European eel can be divided into several developmental stages (Figure 1.1), each with their own specific characteristics. It is now quite certain that the life of the eel starts and ends with the spawning in the Sargasso Sea, after undertaking an extensive migration from the coast of Europe (Munk, 2010, Schmidt, 1923). During the spawning migration, the eels will go through a process known as silvering, and the migrating eels are called silver eel (van Ginneken et al, 2007). The underlying mechanisms for the silvering of the European eel are yet to be described, but it is a complex process involving both morphological, physiological, biochemical and behavioral changes (Van Ginneken et al, 2007). The silvering is sometimes described as a second (partial) metamorphosis, and is both an adaption to the oceanic migration route and the onset of the sexual maturation of the eels (van Ginneken et al, 2007). The triggering factors for the onset of the spawning migration and sexual maturation is not completely understood, but it has been shown that for example temperature variations and swimming affects oocyte maturation of the European eel (Pérez et al, 2011, Palstra et al, 2007).

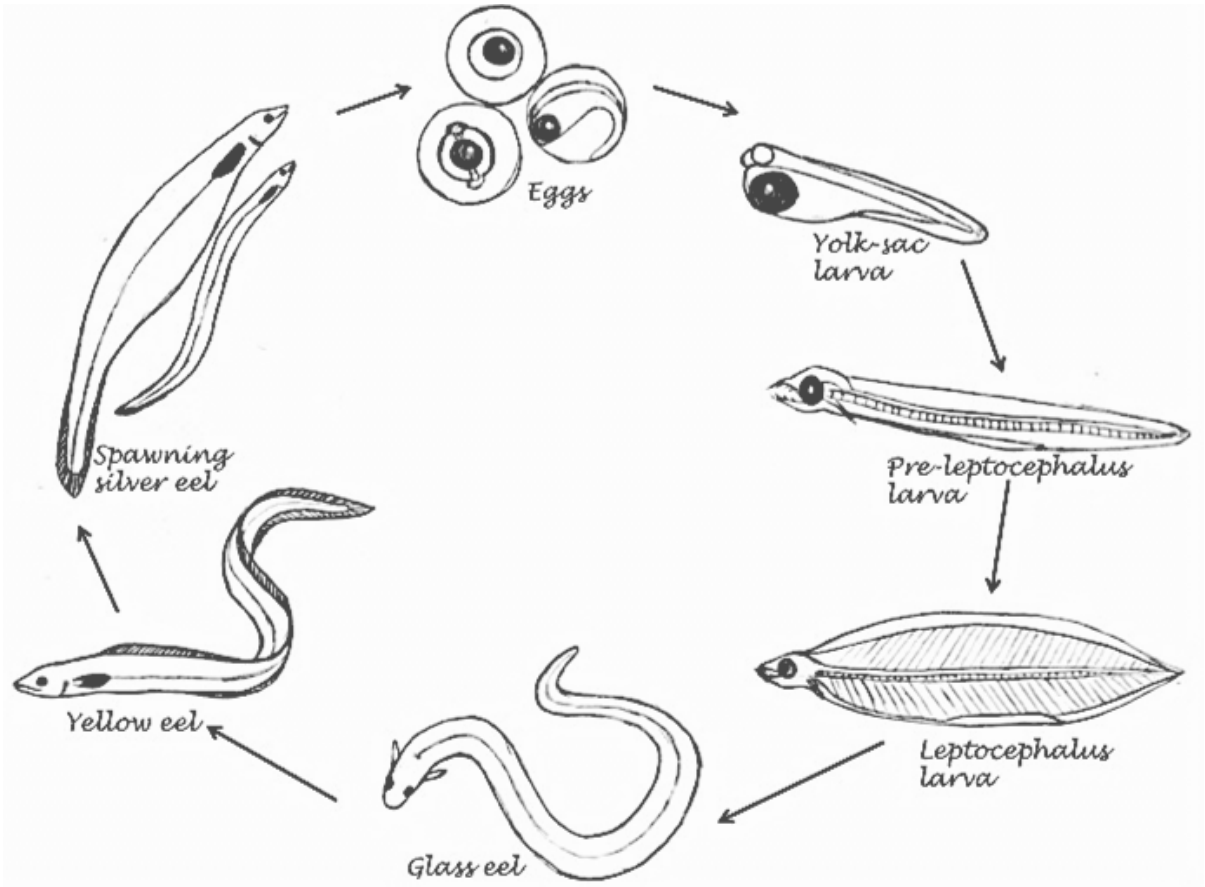

Figure 1.1: Sketch of the life cycle of the European eel (*Anguilla anguilla*). The size of the different stages are not to scale.

Very little is known about the eels after they leave the coast of Europe. It has been possible to track them parts of the way using satellite technology (Aarestrup et al, 2009), but today the estimation of the spawning grounds in the Sargasso Sea is mainly based on the findings of the smallest larval stages (Munk, 2010, Schoth and Tesch, 1982, Schmidt, 1923), and one has yet to find both spawning eels and eggs in nature. During the spawning migration, the eels will cease feeding, and rely solely on their stored energy reserves during the 6000 km long migration to the spawning grounds in the Sargasso Sea. Due to the eels highly energy efficient swimming, it has been proven that the stored energy is actually sufficient for both migration and for the maturation of gonads (Van Ginneken et al, 2005a). As no eels have been found returning from the Sargasso Sea, it is presumed that they die after spawning (van Ginneken et al, 2007).

Most of the knowledge today regarding the eels reproductive biology and egg and larval development is based on studies of artificially matured eels spawned in captivity, and the development of their eggs and larvae. Under experimental conditions, The European eel has produced up to 4 million transparent and non-sticky pelagic eggs, which rise to the surface

with a speed of more than 2 m/h (van Ginneken et al, 2005b). The data on the diameter of the eggs are quite variable, but unfertilized eggs may seem to measure around 0.8 mm in diameter (Palstra et al, 2005). For the European eel, the eggs hatches about 48 h post fertilization (Pedersen, 2004), and the eel emerges from the egg as a 4-5 mm long yolk-sac larvae, which is the free-living embryonic stage. In the beginning of its free-living life, the eel is dependent on the yolk-sac reserves. This is period before the onset of exogenous feeding, is known as the pre-leptocephalus stage (Miller, 2009). The pre-leptocephalus stage is followed by the leptocephalus larval stage, i.e. from exogenous feeding.

It is common to find that the larval stage of the life of a fish differs a lot from the adult, both in morphology, behavior and also even habitat use. The larvae of the European eel were for a long period even classified as a separate species of fish called *Leptocephalus brevirostris* (Kaup, 1856). The evolutionary adaption of leptocephalus larvae is unique to the fishes of the superorder Elopomorpha, which consists of 25 families of fishes, some with a typical fish-like and some with a more eel-like morphology (Chen et al, 2014, Greenwood et al, 1996). Even though only found within this order, the species which have a leptocephalus larval stage counts more than 1000 species, and they are found all over the world. Despite their wide distribution and abundance, little is known about the leptocephalii larvae, including the larvae of the European eel. Leptocephalus larvae differ from other marine fish larvae in many aspects, the most apparent being their large size, transparency and laterally compressed bodies, which gives them an almost leaf-like appearance (Miller, 2009). The transparency of the larvae is due to the content of a gelatinous matrix, mainly consisting of glycosaminoglycans (GAG), and hence often referred to as the GAG-layer (Pfeiler, 1991). The GAG-layer makes up most of the body of the larvae, and the external layer of the larvae is only a few cell layers thick (Miller, 2009). It is thought to be an energy storage material, used as an energy source during the transformation into the juvenile glass eel, possibly after being converted to glucose (Kawakami et al, 2009). The gelatinous matrix also gives the larvae structural support and helps the larva maintain neutral buoyancy (Smith, 2005). This layer also gives the larva its unique ability to achieve a large body size without the costs of increasing the cell number, as the GAGs are non-metabolizing compounds (Bishop and Torres, 1999). This enables the larvae to rapidly increase in size, without the same energy expense as for other fish larvae (Bishop and Torres, 1999). The metabolism of several species of leptocephalus larvae has in fact been shown to be very low (Bishop and Torres, 1999, Pfeiler and Govoni, 1993).

The fact that their external layer is only a few cell-layers thick also makes them very fragile, which makes them difficult to capture without damaging them (Miller, 2009). In addition, the leptocephalus larvae, and especially the larger larvae, probably actively avoid the capture gear (Castonguay and McCleave, 1986). It may also be quite challenging to determine the species they belong to, without the use of molecular and genetic tools. Many other Elopomorphs spawns in the Sargasso Sea, where it has been observed a variety of leptocephalus larvae (e.g. McCleave and Miller, 1994, Castonguay and McCleave, 1986, Castonguay and McCleave, 1979). This has also complicated the studies of the European eel, as it closely shares spawning grounds with the very similar American eel (*Anguilla rostrata*). These two species are even able to produce viable hybrids (Albert et al, 2006). Their leptocephalus larva can however be separated from each others based on the number of vertebrae/myomeres (Kleckner and McCleave, 1985).

From the Sargasso Sea, the leptocephalus larvae are transported back to the coast of Europe and North-Africa, following the eastern path of the Gulf Stream (Munk et al, 2010). This part of the European eel's life is not well described, but it is assumed that the drifting stage has a duration of about 1-2 years (Munk, 2010, Bonhommeau et al, 2009, van Ginneken and Maes, 2005). When the eel reaches the coastal areas, it will metamorphose into the juvenile form, the glass eel. Glass eels are small and transparent, but they have lost the leptocephalus morphology and have an eel like body shape. Their length is also reduced during the transformation. After developing its pigmentation, it is known as a yellow eel, and it is ready for a life in brackish water or freshwater habitats (Tesch, 2003). However, as it has been shown that some eels never leave the ocean, the European eel is sometimes also classified as a semi-catadromous fish (Tzeng et al, 2000). The yellow eel has the final morphology of an eel, but is not yet sexually mature. The following growth-period normally last 3-8 years for males and 8-15 years for females (Feunteum, 2002). Probably triggered by both internal and external cues, the eels will begin the transformation into silver eels (van Ginneken & Maes, 2005), and start the extensive spawning migration back to the Sargasso Sea where it was born.

1.3 – Functional development of leptocephalus larvae

During the earliest stages of a fish's life, the most critical organ systems to develop are those that are related to feeding and escaping from predators (Osse et al, 1997). Because of this, the development of the digestive system and muscle may possibly be the most important parts of the development of the larvae. Still, many other aspects of the functional development are both of importance and interest, such as the sensory organs, the skeleton, nervous system,

respiratory organs (e.g. the gills), circulation system etc. The functional development of leptocephalus larvae of the European eel is today quite undescribed, although several studies exist for other species, including the very similar Japanese eel.

1.3.1 – Leptocephalus larval feeding and development of the digestive system

Increased knowledge is especially needed in relation to the feeding apparatus and digestive system, for example in relation to the inability to find a suitable feed for captive reared larvae. Research on the Japanese eel has come a lot further than the European eel, and in 2001 the first leptocephalus larva of *A. japonia* were produced in captivity (Tanaka et al, 2001), and in 2005 the first glass eels were produced (Kagawa et al, 2005). There are no reports on feeding European eel larvae in captivity, but the Japanese eel have been shown to ingest rotifers, and has been possible to rear on a diet of egg yolk and krill (Okamura et al, 2013, Tanaka et al, 1985). Actually, the feeding habits of leptocephalus larvae are considered one of their greatest mysteries and it is still not certain what they eat in nature, other that that it is assumed that the larvae feed on planktonic material, as the leptocephalus stage is planktonic. For many temperate marine species with pelagic larvae, spawning is often correlated to the primary production, so that the larvae hatch when the abundance of food is high (e.g. Beaugrand et al, 2003). However, the Sargasso Sea is an oligotrophic ocean, and the primary production is for that reason low. This has lead to several theories of alternative food sources for the leptocephalus larvae, most of which are on a low trophic level. This includes among others discarded larvacean houses, feces, gelatinous zooplankton, marine snow (Miller et al. 2012, Riemann et al, 2010, Mochioka and Iwamizu, 1996).

One of the most discussed theories is probably the dissolved organic matter (DOM) vs. particulate organic matter (POM) theories. The high surface area, thin external surface, and the poorly differentiated digestive tract has given rise to the theory that the leptocephalus larvae do not feed as normal larvae do, but rather absorb dissolved nutrients over their skin and digestive tract (Otake et al, 1993, Pfeiler, 1986). Hulet (1978) even described microvilli-like structures on the surface of the skin of the larvae *Arisoma balearicum*, which further supported this theory. However, most studies investigating this theory concludes that the main proportion of the nutrient uptake has to come from particulate feed, although the dissolved nutrients may still play an important part. It is still not certain what it is they actually eat, as there has not been observed feeding larvae in nature, or identified any of the food items in their gut (Miller 2009).

At hatch, the digestive system of most marine pelagic fish larvae is usually quite undeveloped and appears as a simple, undifferentiated straight tube, often closed in both ends (Zambino Infante et al., 2008). During the yolk sack stage, the mouth and anus is formed, in addition to an elongation and differentiation of the gut into the forgut, midgut and hindgut (Kjørsvik et al, 2004). During the growth and development of the larvae, the gut will be further elongated and strengthened, the surface absorption increases and the nutrient-uptake gets more efficient. However, many parts do not become fully functional until after the metamorphosis (Lazo et al., 2011). The stomach is usually not functional until the juvenile stage (Lazo et al, 2011). Still, at the onset of exogenous feeding, the larvae are certainly able to perform digestion, although not of all food components. Both the liver, gallbladder and pancreas are generally developed in marine fish larvae at the time when the yolk sack is absorbed, and the larva begins its exogenous feeding (Lazo et al., 2011).

For the European eel, some studies have been performed on the digestive ability of the larvae. European eel larvae have been observed to have a very high activity in lipase-like enzymes immediately after hatching, which is an indication of a high requirement for lipids. This activity also increased as the larvae developed. This enzymatic pattern has not been observed in any other marine fish larvae (Mazurais et al., in press). In the same project it was also shown that the eel larvae had functional digestive enzymes (lipase, amylase, trypsin and aminopeptidase N) already the first day after hatching (Mazuaris et al. in press). However, at 1 day post hatching (dph), the digestive tract was only poorly developed. It appeared as a more developed, straight tube at 6 dph, and at 12 dph they had an immature liver, pancreatic tissue and a gall bladder (Mazuaris et al., in press). They had numerous cilia in the intestinal tract anterior to the liver, and numerous microvilli posterior to the liver (Mazuaris et al., in press). However, no further studies exists in relation to the digestive system of European eel larvae, neither for older larvae nor for wild caught leptocephalus larvae.

1.3.2 – Muscle development and growth

There is also hardly any studies performed purely on the muscle development both of the European eel and for leptocephalus larvae in general, including the highly studied Japanese eel. The morphology of the muscle of the larvae is usually only mentioned as a side note, where it is described as a thin external cover outside the GAG-layer (e.g. Hulet, 1978). The only study, to my knowledge, is the master-thesis of Davidsen (2012), on muscle development and growth of captive reared European eel larvae up 6 dph. No literature exists for older or wild caught leptocephalus larvae. For most fish larvae, the axial muscle is the

most rapid growing organ, and it also makes up most of their body mass (Bone, 1978). Successful and rapid development of muscle is also important for the larvae's survival, as it is crucial both in order to capture food particles or prey, but also to escape predators in order to not end up as food themselves (Osse et al, 1997). Although not seen immediately, the consequence of poor muscle development can have negative effects on later life stages, and this is hence an important aspect of the larval development. Increased knowledge about the muscle development of the European eel is for that reason of interest.

The axial muscle of fish is organized in layers of red and white muscle fibers. The red muscle fibers form a lateral sheet outside the white muscle fibers. Red muscle is responsible for normal, slow movements, is high in mitochondria and myoglobin and have aerobic metabolism (Bone, 1978). The white muscle function during rapid bursts of movement, e.g. during prey capture or escaping a predator. White muscle has low content of mitochondria and myoglobin, and have anaerobic metabolism (Bone, 1978). White muscle fibers make up the bulk of the muscle mass of the fish. Between the red and muscle fibers, the intermediate pink fibers, used for intermediate swimming speeds, can be found (Bone, 1978).

At hatch most fish larvae have one layer of superficial fibers, that later develops into the red muscle, surrounding an inner muscle mass, which develops into the white fibers (Johnston et al, 2011). The muscle growth mainly happens as myogenic precursor cells (MPC) goes through determination, where stem cells are determined as either red, white or pink MPC, migration of cells to their predeterminated location in the red, white or pink layer and then either fusion or differentiation (Johnston et al, 2011). Fish musculature grows in two main ways: hyperplasia, which is an increase in the number of fibers; and hypertrophy, which is an increase in the fiber size (Weatherly et al, 2006). In other words, the MPC may either end up as new myotubes or they are absorbed into the existing growing multi-nucleated fibers (Johnston et al, 2011). Because of this, a cross-section of muscle in fish will show muscle cells of many different sizes, where the largest cells are the oldest and the smallest cells are the newest. In the beginning of the life of the fish larva, the muscle will appear as one cell later (Johnston et al., 2011). The development will further advance as the myotubes are produced in several discrete layers, known as stratified hyperplasia (Rescan, 2005). Mosaic hyperplasia, where new fibers are added throughout the whole myotome, creating a mosaic pattern of muscle cells of different sizes, are also found in some species as the muscle development advances (Rescan, 2005).

In the master thesis of M. Davidsen (2012) it was reported that the muscle morphology of the larval European eel was arranged as elongated, stacked muscle fibers lacking a horizontal septum and stratified orientation. It was also registered that the muscle growth happened both by hypertrophy and hyperplasia (Davidsen, 2012). From the existing studies, it may appear that the muscle development and growth of leptocephalus larvae differs from other fish larvae, probably due to the developmental strategy of leptocephalus larvae, but further investigations are needed in order to confirm this.

1.4 – Aims of the study

The present study aims to increase the knowledge on wild-captured larvae of the European eel. This will be done by analyzing the functional organ development and growth of wild leptocephalus larvae collected in the Sargasso Sea during the winter of 2014 through the Technical University of Denmark (DTU) expedition "Danish eel expedition 2014 – Sargasso eel". Growth and development will be evaluated by comparing larvae of different standard length. The study will have special emphasize on (i) the morphological development of the digestive system and (ii) growth and development of muscle, as both of these are considered especially important for the survival of marine fish larva. Growth and development of the muscle will be evaluated in relation to hyperplasia and hypertrophy, by measuring both the number and size (area) of the muscle cells. The study also aims to (iii) describe other organs, such as the sensory system, skeleton, the glycosaminoglycan layer, nervous system and circulatory system. There will also (iv) be made an attempt to see if it is possible to see any indications of patterns or trends in the growth of the larvae.

The analysis will be done by light microscopy using histological techniques as a tool to describe organ development, on leptocephalus larva from 10-23 mm standard length. The main focus will be on transverse sections, where EPON embedding will be used. Longitudinal sections in Technovit will also be performed, as well as staining techniques of whole larvae in order to evaluate development of bone and cartilage.

2. Materials and methods

2.1 – Sampling and preservation of larvae

European eel larvae analyzed in this study were collected in spring 2014 from the Sargasso Sea during the "Danish Eel Expedition 2014 – Sargasso Eel". These wild-caught leptocephalus larvae were sampled (see Appendix 1 and 2) at different locations in the Sargasso Sea (Figure 2.1). Larvae were identified as the species *Anguilla anguilla* by counting the number of myomers following the guidelines by Smith (1979). After identification and digital imaging, the larvae were fixated in a mixture of paraformaldehyde (PFA), glutaraldehyde (GA), and 0.11 M hepes buffer (pH 7.4) (Appendix 3), and then stored cold (+4^{o}C). Fixed larvae were later transported to the collaborators in the project, including some (n = 21) to the Norwegian University of Science and Technology (NTNU).

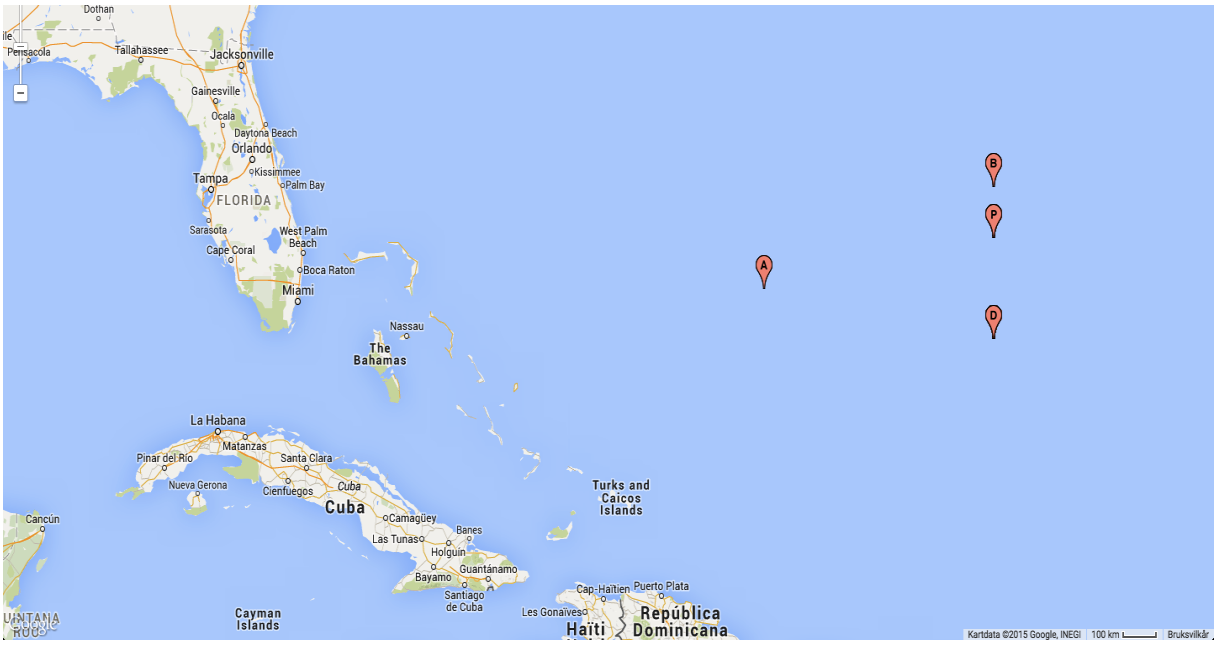

Figure 2.1: Sampling locations of European eel (*Anguilla anguilla*) larvae in the Sargasso Sea. This map created in BatchGeo (07.04.2015, https://batchgeo.com/). A (west), B (north east), C (mid east), D (south east).

2.2 – External measurements and morphology

Larvae were rinsed and transferred to hepes buffer (0.11 M) before being digitally imaged using a stereo microscope (Leica MZ75, Leica Microsystems, Germany) and camera (Carl Zeiss Microscopy Axiocam ERc5s, Zeiss Inc., Germany). Measurements of standard length (SL), myotome height (not including finfold), esophagus length (EL), intestine length (IL), total digestive tract (TT), eye height (EH) and width (EW), jaw length (JL) and length of the front tooth (FT) (Figure 2.2 and 2.3) were performed on images using ImageJ software (NIH,

USA). SL was measured from the tip of the upper jaw to the tip of the tail, not including the finfold. Measurements were used both for comparing the changes in organ growth with size, and in order to make a rough estimate of the age of the larvae. Myotome height was measured perpendicular to the spinal chord, right behind the anus (MHA), and at the middle of the larvae (MHM). For the measurement of MHM, the transition area between the esophagus and intestine was used as a reference point. The measurements of myotome height was used to compare the difference between larvae of different size, and if there was any difference between MHM and MHA with increasing SL.

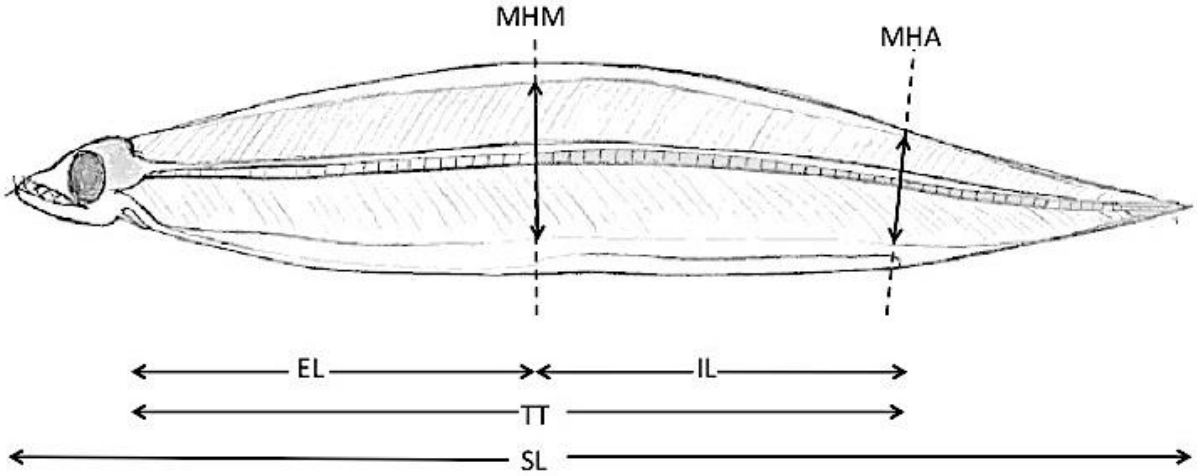

Figure 2.2: Schematic drawing of leptocephalus larvae of the European eel (*Anguilla anguilla*), used as a template for the external measurements of the whole larva. Finfold is not included in any measurements. EL = esophagus length, IL = intestilne length, MHA = myotome height at anus, MHM = myotome height at the middle of the larva, SL = standard length, TT = total digestive tract.

The morphology of the leptocephalus was also described, with special emphasize on the teeth, presence of fins and potentially also finrays, presence of renal arteries, muscle tissue, appearance of the digestive system, potential presence of food particles in the esophagus or intestine, the eyes and the shape of the larvae. It was also investigated whether or not there could be observed any visual difference between the morphology of fresh and fixated specimens, based on the pictures of fresh larvae that were received from the Technical University of Denmark (DTU). After describing and imaging the larvae, the larvae were transferred back into the fixative and stored cold (4^{o}C) until further processing.

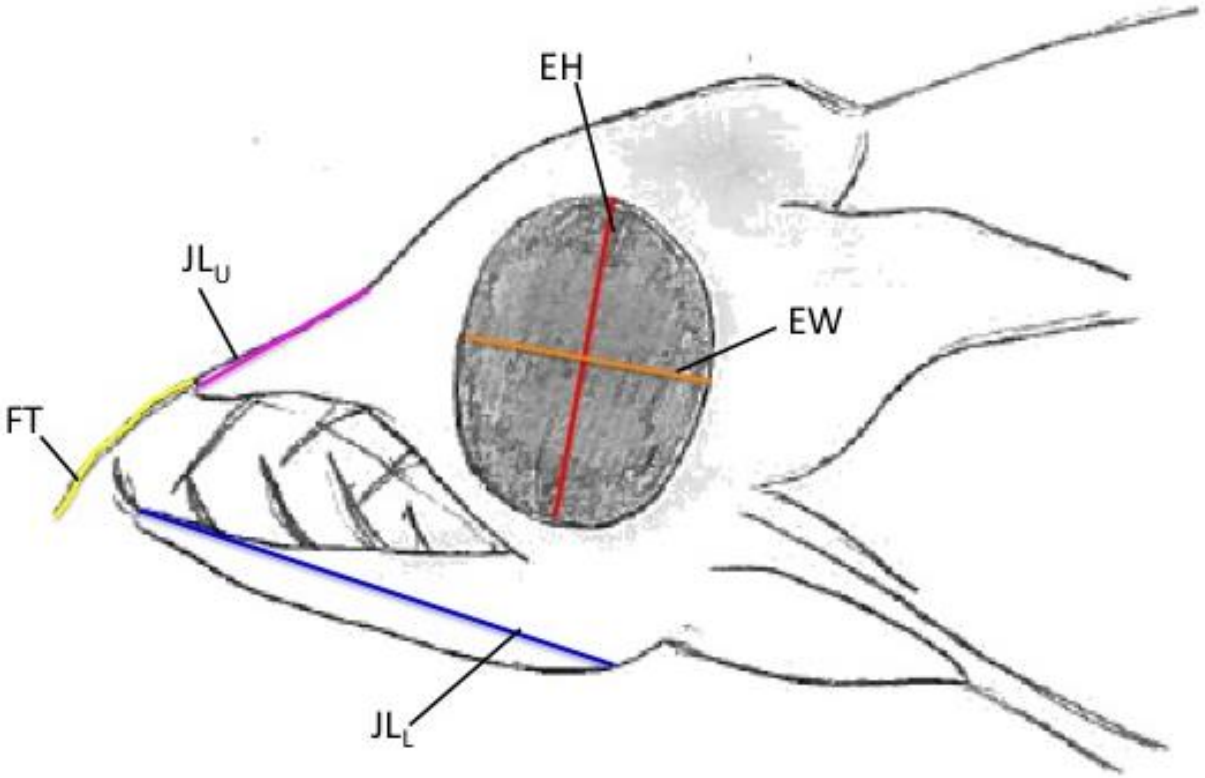

Figure 2.3: Schematic drawing of head of leptocephalus larvae of the European eel (*Anguilla anguilla*), used as a template for the external measurements of the whole larva. EH = eye height, EW = eye width, FT = front tooth, JL_L = jaw length lower, JL_U = jaw length upper.

2.3 – Some comments regarding the state of the material

After the first leptocephalus larvae had been sectioned, it became apparent that the condition of the fixed larvae was suboptimal. Because of this, some changes had to be made in relation to both the content of the thesis and in relation to how the measurements were done. First of all, it was decided that histological sectioning would only be performed on the best-preserved specimens. Secondly, it became apparent that some parts of the body of the leptocephalus larvae were better preserved than others. Especially the intestine was almost completely degraded, except from in the largest larva. The esophagus and liver/pancreas was in most larvae adequately preserved, so it was decided to have more focus on the internal morphology of the esophagus than on the intestine. The muscle layer was also damaged in some larvae, and in some areas muscle cells could be displaced or even missing. Because of this, the area of the muscle cells sometimes had to be estimated based using the size of the gap of the missing cells and the morphology of the muscle of the opposite side. The head was usually very well preserved. Further comments about the state of the material, including examples can be found in Appendix 8.

2.4 – Light microscopy

Of the 21 available larvae, 13 were used for analysis by light microscopy and the rest was used for external measurements only (Table 2.2). The selection of larva was based both on the evaluation of the external morphology and the SL, with the intent of both choosing the best-preserved larvae and at the same time covering a broad range of sizes. To simplify the description of the different sizes, it was defined size groups from A-H to categorize the larvae (Table 2.1). All size categories except A are represented in the light microscopy analysis. In order to study organ development, 10 larvae were selected for embedding in EPON to use for transverse sections, one larva was selected for embedding in Technovit, for longitudinal sections, and two larvae of different sizes were selected for bone and cartilage staining.

As the ID-number of the sampled larvae was quite long, the larvae were given a shortened ID for use in this thesis. The larvae were named L1-L21, with the numbers indicating increasing SL (Table 2.2).

Table 2.1: Definitions of size groups and number of larvae in each group.

Group	Size range (mm)	Number of larva in group
A	7.0-8.9	1
B	9.0-10.9	8
C	11.0-12.9	5
D	13.0-14.9	2
E	15.0-16.9	1
F	17.0-18.9	3
G	19.0-20.9	0
H	≥21	1

Table 2.2: Larval ID (full and shortened), standard length (measured on fixated specimens), size group, area of usage and location where they were sampled (see Figure 2.1 for place).

Full ID	Short ID	SL (mm)	Size group	Usage	Location in Figure 2.1
s-4a-140403 L5	**L1**	6.97	A	External measurements	C
s-4a-140401 L2	**L2**	9.68	B	External measurements	D
s-4a-140402 (390) L3	**L3**	9.93	B	External measurements	C
s-4a-140326 L1	**L4**	10.01	B	Technovit	B
s-4a-140330 L7	**L5**	10.08	B	External measurements	D
s-4a-140402 L4	**L6**	10.13	B	EPON	C
s-4a-140330 L10	**L7**	10.46	B	External measurements	D
s-4a-140401 L3	**L8**	10.67	B	EPON	D
s-4a-140330 L6	**L9**	10.95	B	External measurements	D
s-4a-140330 L2	**L10**	11.17	C	EPON	D
s-4a-140326 L2	**L11**	11.69	C	EPON	B
s-4a-140401 L9	**L12**	11.77	C	EPON	D
s-4a-140402 L2	**L13**	12.26	C	External measurements	C
s-4a-140402 (389) L3	**L14**	12.76	C	External measurements	C
s-4a-140330 L12	**L15**	13.78	D	Bone and cartilage	D
s-4a-140402 L1	**L16**	13.78	D	EPON	C
s-4a-140330 L4	**L17**	15.77	E	EPON	D
s-4a-140330 L11	**L18**	17.03	F	EPON	D
s-4a-140330 L13	**L19**	17.11	F	EPON	D
s-4a-140320 L4	**L20**	18.85	F	Bone and cartilage	A
s-4a-140329 L1	**L21**	23.33	H	EPON	D

Leptocephalus larvae were embedded in EPON using the standard protocol for EPON-embedding at the NTNU Sealab (Appendix 4). The embedded larvae were then sectioned using a microtome (Leica Reichter Ultracut, Leica Microsystems, Germany) into 1 µm thick transverse sections. Glass knives produced with a knife maker (Leica Reichter Knifemaker, Leica Microsystems, Germant) were used for rough trimming of the EPON blocks, and a diamond knife (Diatome Histo 45^{o}, size 6, Diatome AG, Switzerland) was used to produce the histological sections.

Three main transverse sections (MTS) were made for each larva (solid line in Figure 2.4). The MTS were made in the beginning of the alimentary canal (1), in the transition area between esophagus and intestine (2) and right after the anus (3), and they were used for both morphological descriptions and for measurements. Additional sections were also made throughout the larvae and mainly in the areas that are indicated with a dotted line in Figure 2.4. This is, from left to right; through the snout, the lens of the eye, several sections in the beginning of the esophagus, the middle of the esophagus, the middle of the intestine, right before the anus, in the middle of the tail and at the very tip of the tail.

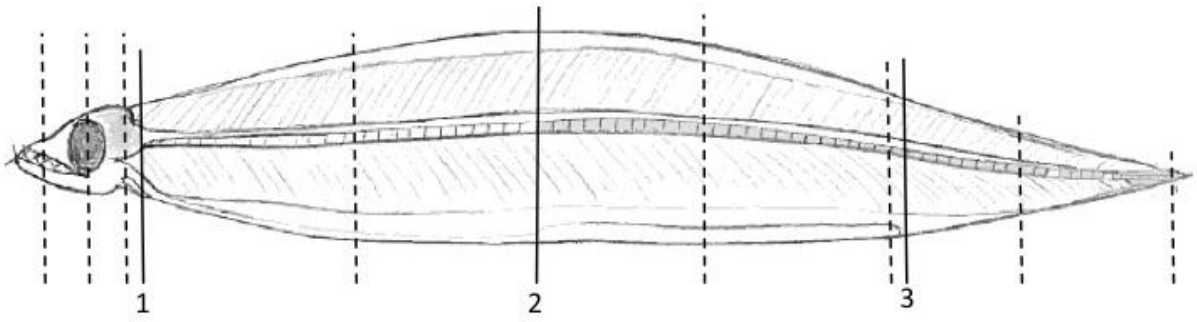

Figure 2.4: Schematic drawing of leptocephalus larva of the European eel (*Anguilla anguilla*), showing the three main areas for measurements (solid line), and other important areas that was sectioned in order to study organ development (dotted line). The three main sections are (1) the beginning of the esophagus, (2) the transition area between the esophagus and intestine and (3) right behind the anus.

Sections were stained with Toluidine blue (Sigma-Aldrich, USA) (Appendix 5), and mounted on a glass slide with NeoMount (Merck Chemicals, Germany). The sections could then be viewed under a light microscope and photographed using a Zeiss Axioskop 2 plus (Zeiss Inc., Germany). The pictures were analyzed and measurements were done using a Wacom Cintiq 24HD drawing board (Wacom Technology Corporation, USA) and ImageJ.

Morphological descriptions of the transverse sections were mainly focused on the muscle and digestive system. The morphology of both red and white muscle cells and the presence of

growth zones were described, both in relation to size and potential variation in the three MTS. For the digestive system, morphological descriptions were focused on investigating the change in morphology from the mouth to the termination at the anus as a function of size. In addition, morphological descriptions were also made of the eye, brain and nervous system, skeleton, gills, nostrils, neuromasts, ears, skin and glycosaminoglycan (GAG)-layer, and how this changed with increasing SL.

Measurements on transverse sections (Figure 2.5) were focused mainly on the muscle, and how it changed both with increasing SL and for the three MTS. All measurements and counting were performed on in one bilateral half of the transverse section. The total number of white muscle (WM) cells was counted in order to evaluate hyperplasia. The average area of the 25 largest white muscle cells was measured in order to evaluate hypertrophy. The total area of white muscle was also measured. Total area was calculated by adding together the area of each individual muscle cell. Red muscle (RM) was not measured, as the cells were too small and damaged. Measurements of the muscle layer were performed on one half of the section, under the assumption that the two lateral muscle layers were identical. In some cases the muscle layer could be damaged, due to the state of preservation of the material (see Appendix 8). In these cases, measurements/counting was always done on the half-side that was in best condition. In cases where muscle cells were missing, the area of the missing cells was estimated based on the gap in the muscle layer and the appearance of the other half.

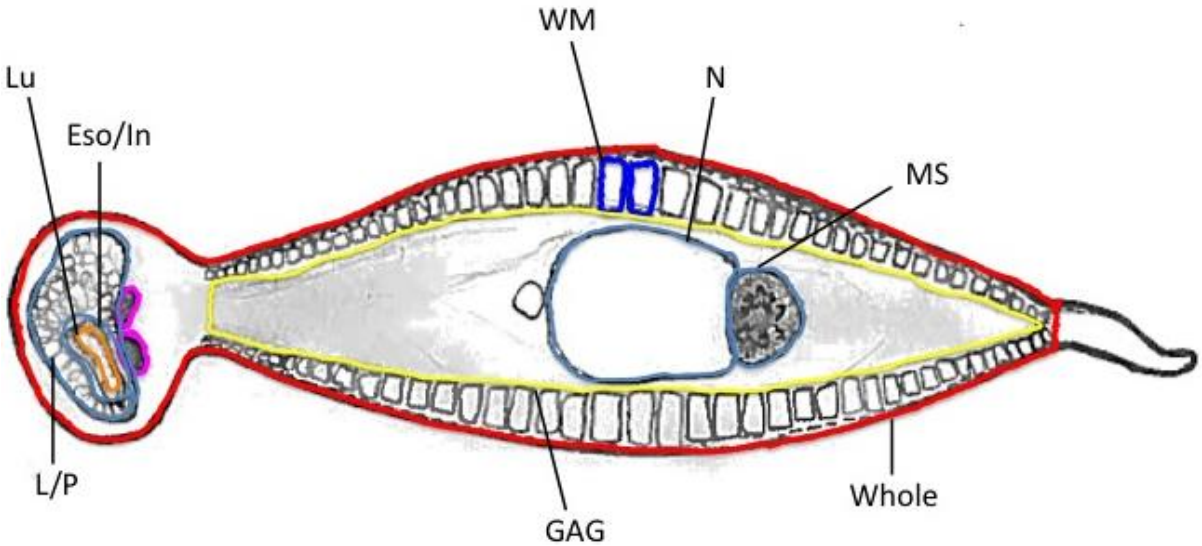

Figure 2.5: Schematic drawing of transverse section leptocephalus larva of the European eel (*Anguilla anguilla*) based on section approximately in the middle of the esophagus and used as a template for measurements of area of esophagus/intestine (Eso/In), glycosaminoglycan layer (GAG), liver/pancreas (L/P), lumen (Lu), medulla spinalis (MS) and notochord (N), white muscle (WM) and the whole section (Whole).

Area measurements were also made at the three MTS of the area of the medulla spinalis (MS) and notochord (N). Measurements of the digestive tract and its accessory organs had to be excluded due to poor preservation that made the results too variable. So the results of the digestive system will be restricted to external measurements and morphological descriptions. Measurements of the total area of the transverse section and GAG-layer in the section had to be excluded for the same reason (see Appendix 8).

One larva (L4) was embedded in Technovit 7100 (Heraeus Kulzer, Germany), following the standard protocol for Technovit-embedding at NTNU Sealab (Appendix 6). The Technovit-embedded larva could then be sectioned using (Leica Jung Ultracut 2055, Leica Microsystems, Germany). The larva that was embedded in Technovit was sectioned into 2 µm thick longitudinal sections. The sections were stained with Toluidine blue (Sigma-Aldrich, USA), mounted on an glass slide and could then be viewed under a light microscope and photographed Zeiss Axioskop 2 plus (Zeiss Inc., Germany) for analysis. The longitudinal sections were used for morphological descriptions, mainly of the head. It was also used as an aid for the analysis of the transverse sections.

L15 and L20 were stained for bone and cartilage, and were prepared following the standard protocol for bone and cartilage staining at NTNU Sealab (Appendix 7). These larvae were used to evaluate the presence of cartilage and ossified structures in the leptocephalus larvae.

2.5 – Processing of data and statistical analysis

The program SigmaPlot 12.0 (Systad Software Inc., USA) was used for statistical analysis and for creation of graphs. Tables were created in Microsoft Excel for Mac 2011. The initial data processing and transformation was also performed in Microsoft Excel.

The results of most of the measurements of different organs were all presented as a scatter plot correlated to SL, in order to see if there were any differences in growth with increasing size. Based on this scatter plot, a regression analysis was performed on the data. Different types of regression lines are used for the different data. The choice of regression curve was based on the best-fit solution, using the least square and model reduction methods, based on the R^2 and p-values. Based on this, the regression models with the lowest p-value and highest R^2 were selected.

For the external measurements of myotome height, it was decided to log-transform the data, as this was shown to enable the use of a linear regression model on both the data for MHA

and MHM. When using non-transformed data it was not possible to use the same regression model on both MHA and MHM.

A Shapiro-Wilk-test was used to test for normality of data ($P<0.05$), and all data were shown to be normally distributed.

The measurements of the TT investigate how this length, expressed as percentage of the total length of the larvae (from now %TT), changed with increasing SL. The measurement of EL was used to investigate how the EL, expressed as percentage of the TT (from now %EL, changed with increasing SL. As both of these values were found to be quite constant, the average value was calculated along with the standard error (SE).

For the external measurements of the front tooth, the data was expressed in two different ways. The first was the actual length of the tooth, and the second was as the percentage of the length of the jaw. The average value±SE was also calculated for the tooth length, and the number of teeth pairs were counted.

The relative organ growth was also estimated, by calculating the difference in size of the organ compared to the smallest larvae. This was calculated by subtracting the organ size of the smallest larvae from all the other measurements. The results were plotted as a scatterplot in one combined graph. Two graphs were made, one with and one without the length of the total digestive tract and esophagus, in order to make it easier to look for patterns in growth for the other organs.

For the measurements of the muscle tissue, the total area of white muscle fibers was calculated by adding together the area of individual muscle cells. The average area of the 25 largest fibers was calculated by sorting the data, selecting the 25 largest values and calculating the average. As it was not possible to fit any statistical significant regression curves to the data of the average area of the 25 largest fibers, it was added trend-lines using the automatic regression function in SigmaPlot. Relative growth was also calculated for the internal measurements of the muscle tissue, the same way as for the external measurements.

3. Results

3.1 External morphology and quantitative analysis

3.1.1 – External morphology

The leptocephalus larvae all had long, slim and laterally compressed leaf-like bodies (Figure 3.1). They were almost transparent, with the exception of the eyes and some slight pigmentation spots along the notochord that was observed in some specimens. The muscle layer, brain, notochord, medulla spinalis, heart, otoliths and gastrointestinal tract were visible through the transparent body (Figure 3.1 and 3.2). The larvae were surrounded by a finfold from the back of the head to the tail, as the uppermost structure on the dorsal side and on the ventral side placed between the muscle layer and the digestive tract (Figure 3.1 and 3.3). The teeth of the larvae were long, thin and protruding from the jaw. The larvae were found to have between 8-10 pairs of teeth (Table 3.1), where the pair of front teeth was the longest and the remaining teeth being successively shorter further into the mouth. The pair of front teeth in the lower jaw also had a hooked shape.

Pectoral fins were present in all larvae, but no other defined fins were observed. Finrays were present in the pectoral fins of all larvae (Figure 3.3-a), and for the largest larva (L21, SL = 23.33 mm) there was also observed finrays protruding into the finfold in the tail area (Figure 3.3-b). The renal arteries, characteristic for leptocephalus larvae, could also be observed from the outside, stretching from the digestive tract and up to the notochord (Figure 3.3-c). The notochord was straight and separated into several disks (1-2 disks per myotome).

The fixated specimens were stained slightly brown, and was also a less transparent, compared to the fresh larvae (Figure 3.4). Another apparent difference between the fixated and fresh larvae was the pigmentation of the eyes. The fixated larvae had very dark, almost black eyes, while the fresh larvae had a yellow color on the reflective surface of the eye, with a black lens in the middle. Other than this, it did not appear to be any difference in the condition of the larvae before and after fixation (for more details, see Appendix 8).

From the outside, the axial muscle tissue of the leptocephalus larvae had a transparent appearance. The myomeres were for larvae L1-L20 arranged in V-shaped stacks of myocytes (Figure 3.4). The myomeres in L21 were arranged in W-shaped stacks of myocytes (Figure 3.1). The stacked appearance was more visible in the smaller larvae (Figure 3.2), but was still present in the larger specimens.

The digestive tract of most larvae had yellow/brown coloration, as most other internal organs of the larvae. The color appeared uniform across the digestive tract of all larvae, with the exception of a few individuals, that appeared to have food particles in the intestine. The larvae that had food-like particles in the intestine were L1 (SL 6.97 mm), L10 (SL 11.17 mm) and L9 (SL 10.95 mm) (Figure 3.5). Of these three, only L10 was embedded in EPON and used for histological sections, and for this larva, it was not seen any food particles in the histological sections. The particles were dark in color, oval in shape and some also had a slightly greenish hue surrounding them. The intestine also appeared to be filled with an unidentifiable mass that gave it an opaque appearance.

From the outside, the digestive tract appeared as a long, straight tube with no folding and very little bulkiness. It was observed that it appeared to be parted into two morphologically different areas. The front part of the digestive tract was completely straight in appearance and very narrow, and the backmost part was thicker and slightly more bulky in appearance. These two regions were separated by a transition zone, where there could be seen an increase in thickness. In some larvae, the gall bladder could also be seen from the outside in this transition zone. This distinction between the two different areas is shown in Figure 3.6, and as the figure shows, it is actually easy to see the transition even at low magnification. Later, from the histological sections, the front area was identified as the esophagus and the backmost area as the intestine.

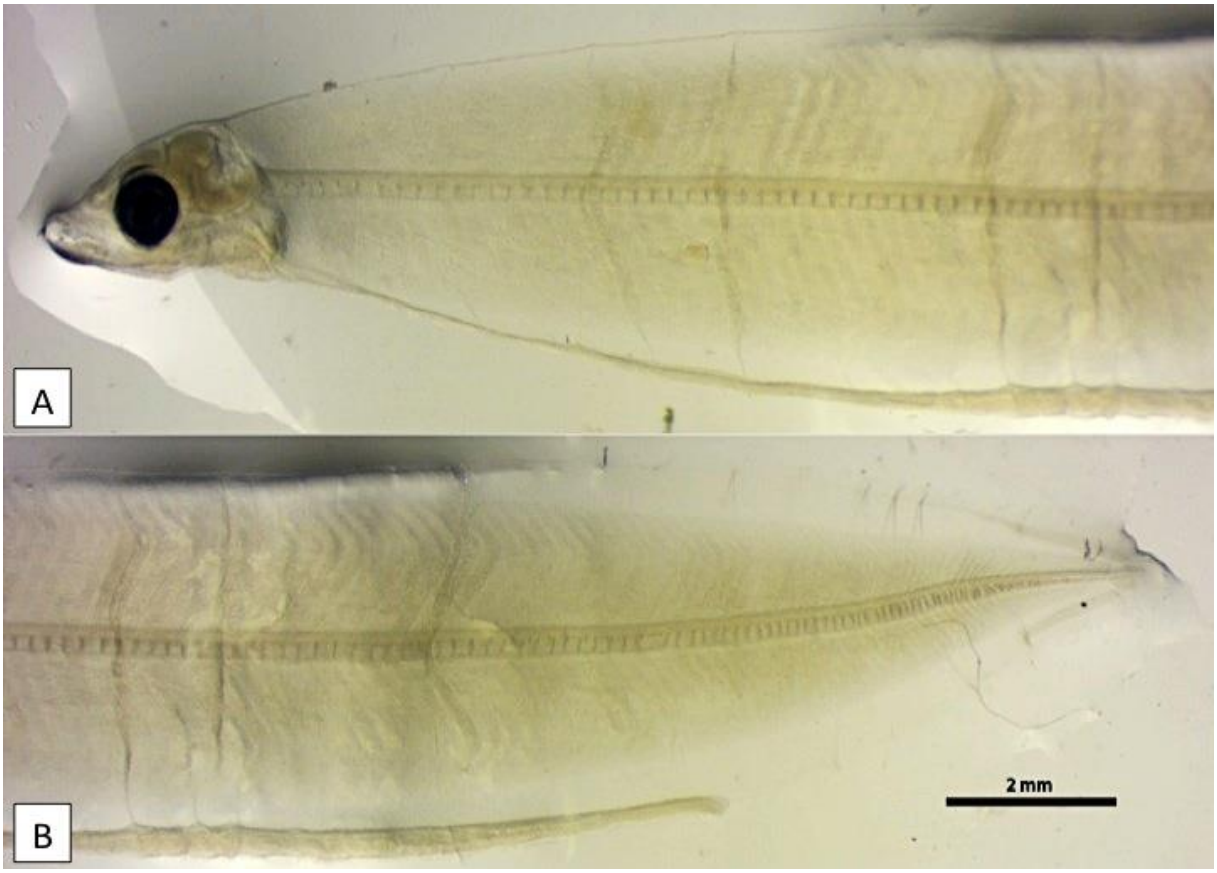

Figure 3.1: External morphology of leptocephalus larvae of the European eel (Anguilla anguilla), after fixation (L21, 23.33 mm). Picture taken from the side, at 0.63X magnification. A: Front-part of the body. B: Back-part of the body.

Figure 3.2: Head of leptocephalus larvae of the European eel (*Anguilla anguilla*), after fixation (L16, 13.78 mm). Picture taken from the side at 4.0x magnification. The picture shows the transparent morphology of the head, long, protruding teeth and large eye.

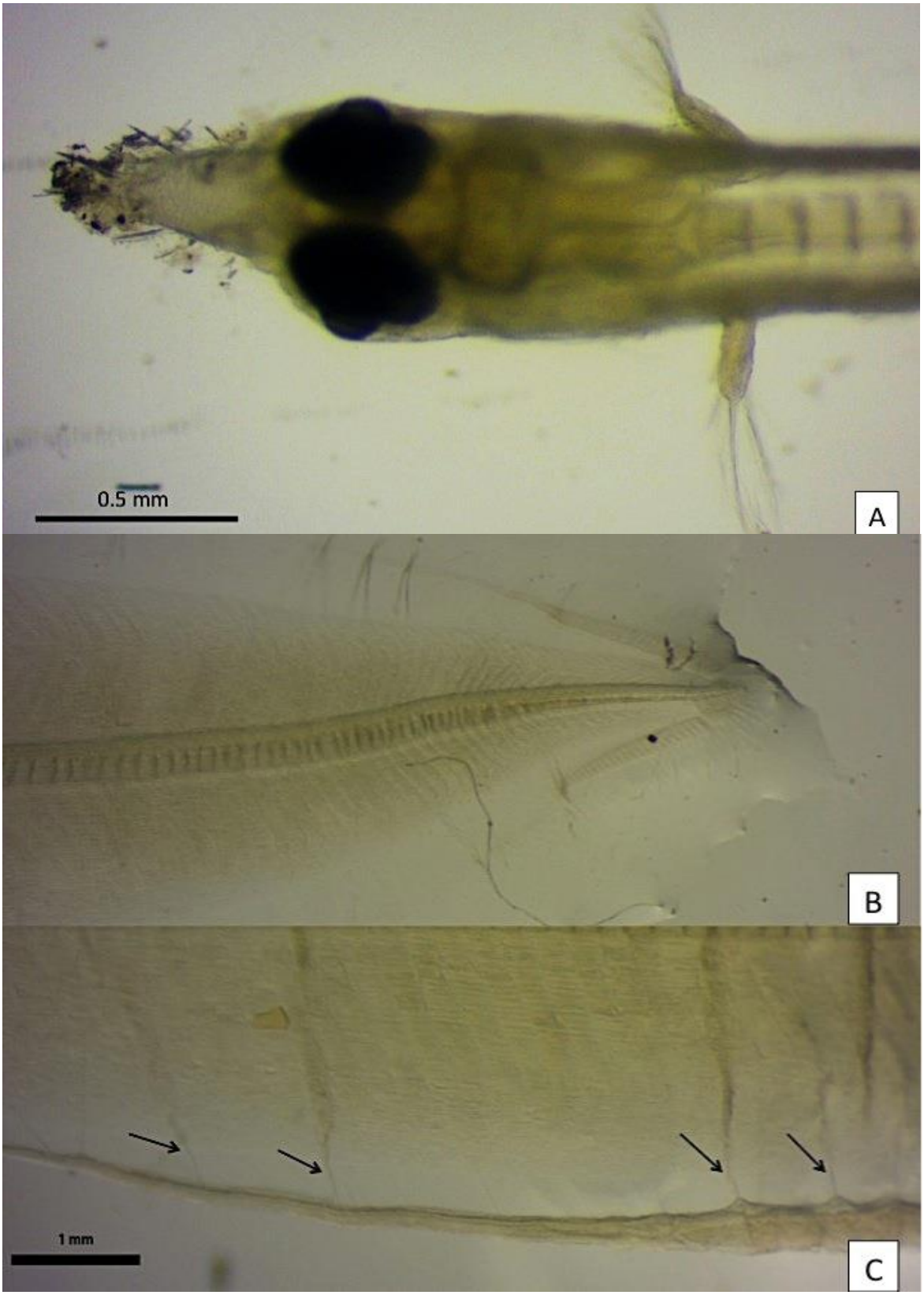

Figure 3.3: External morphology of leptocephalus larvae of the European eel (*Anguilla anguilla*), after fixation. (a) Picture taken from above showing pectoral fins with finrays (L16, 13.78 mm), 4.0x magnification. (b) Picture taken from the side showing tail with finrays (L21, 23.33 mm), 1.25x magnification. (c) Picture from the side with arrows showing the renal arteries (L21, 23.33 mm), 1.25x magnification.

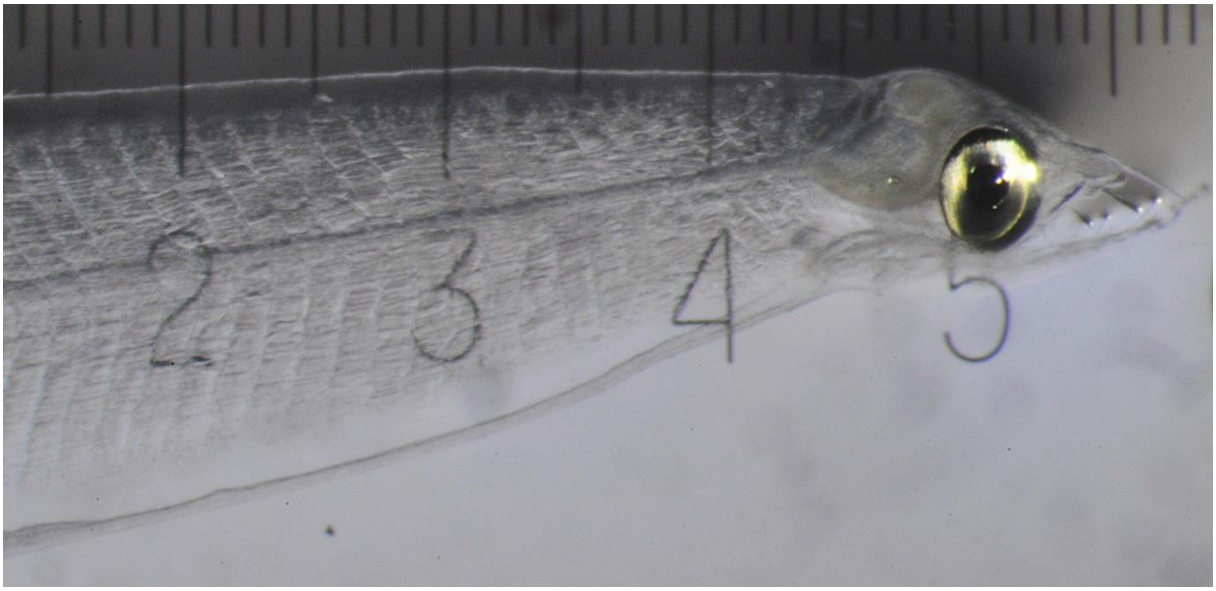

Figure 3.4: External morphology of leptocephalus larvae of the European eel (*Anguilla anguilla*), before fixation, showing the transparent morphology, the V-shaped muscle layer and the yellow reflection in the eye.

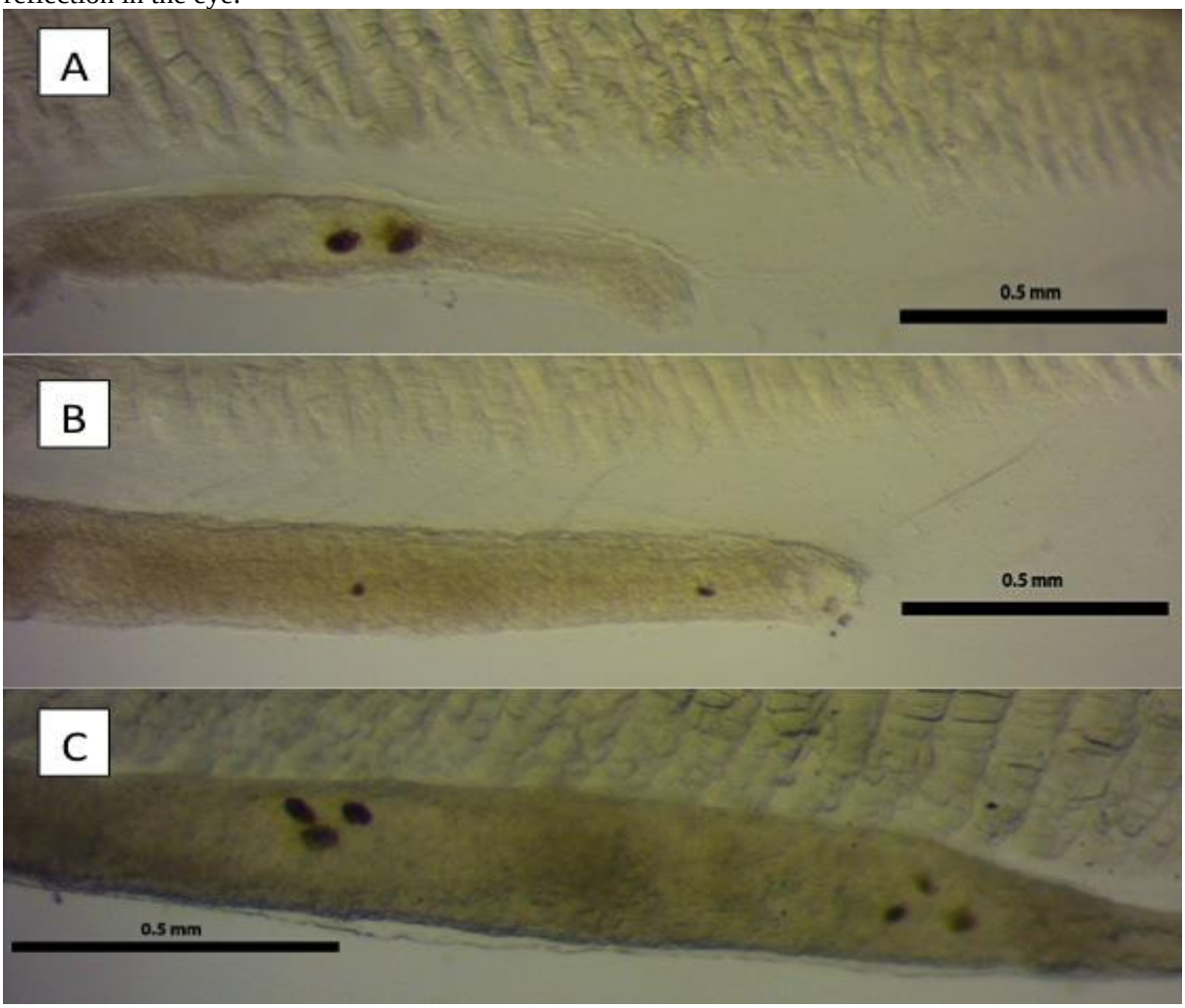

Figure 3.5: Presence of food-like particles in the intestine of three leptocephalus larvae of the European eel (*Anguilla anguilla*). A: L9 (SL 10.95 mm), 4.0x. B: L10 (SL 11.17 mm), 4.0x. C: L1 (SL 6.97 mm), 5.0X.

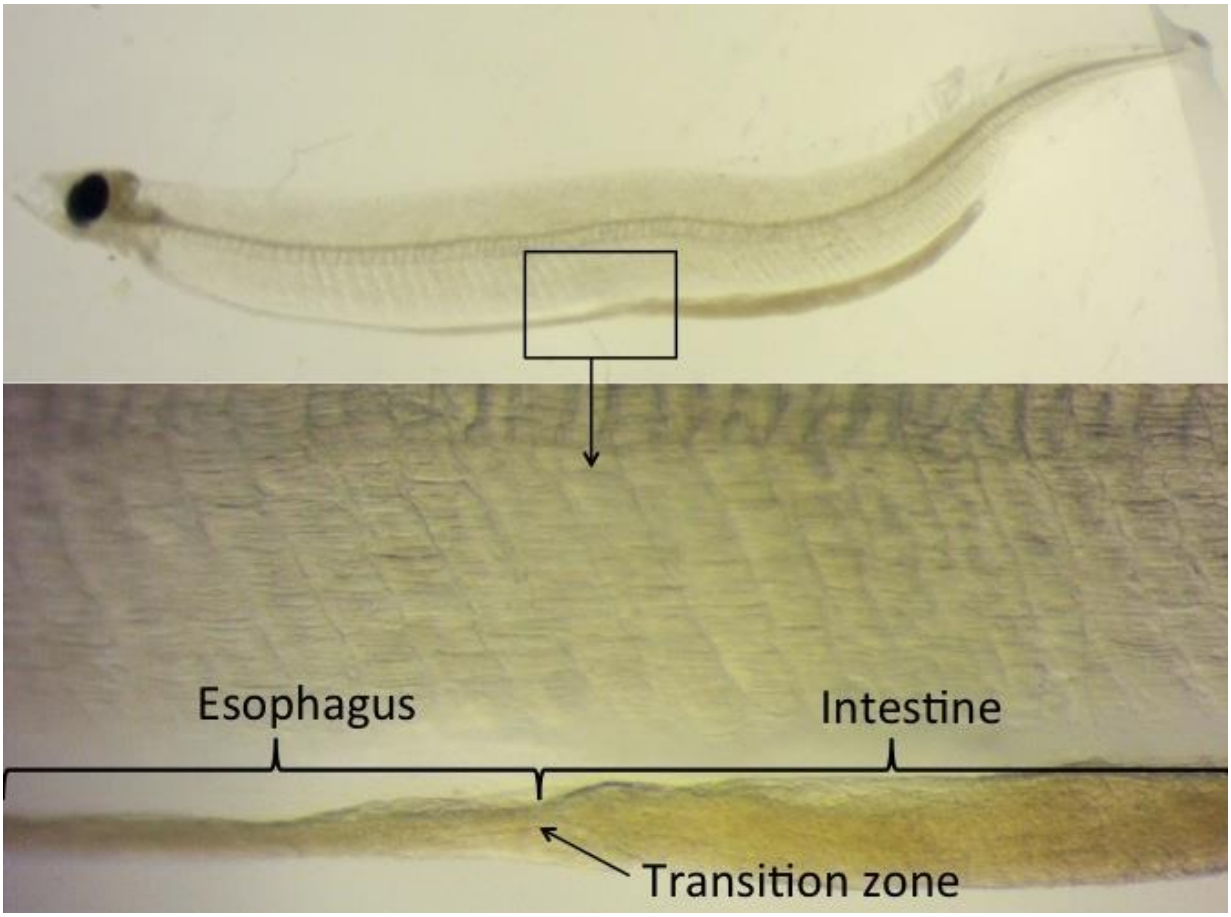

Figure 3.6: External appearance of the digestive tract of leptocephalus larvae of the European eel (Anguilla anguilla) (L10, SL 11.17) showing the difference between the two areas of the digestive tract; the esophagus and the intestine, and the transition zone between these.

3.1.2 – External measurements

All external measurements of the larvae are summarized in Table 3.1. The lengths of the specimens were distributed from the smallest larva of 6.7 mm and the largest larva of 23.3 mm (Table 3.1). The myotome height increased following a quadratic regression curve. However, the regression was not significant for the constant a in the quadratic equation ($y=y_0+ax+bx^2$). Although the myotome height at anus (MHA) was smaller than the myotome height at the mid point of the larvae (MHM), they were both increasing equally fast, based on the slope of their regression curves.

Both the length of the digestive tract, expressed as percentage of standard length (%TT), and the length of esophagus expressed, as percentage of the length of the digestive tract (%EL), was quite constant for all sizes. The digestive tract covered on average 69±0.94% (±SE) of the length of the leptocephalus larva body, and of this 37±1.59% (±SE) was esophagus, meaning that the remaining 63% was intestine.

The length of both the upper and lower jaw increased in a linear fashion with increasing SL (Figure 3.8), and from these data it could seem like the lower jaw is increasing in length faster than the upper jaw. The length of the front tooth appeared to be quite constant, with an average±SE of 0.30±0.01 mm. However, when the length of the tooth was expressed as percentage of the length of the jaw, the tooth length decreased linearly with increasing jaw length (Figure 3.9).

The height of the eye was initially larger than the width of the eye (Figure 3.10). As the size of the larvae increased, the width of the eye increased faster than the height of the eye, causing the eye width and height to become more similar (Figure 3.10), meaning that the eye changed from oval shape to a round shape (equal height and width).

From the relative growth of the different organs, expressed as change in size compared to the smallest larva, it appeared that the most rapid growth happened in the digestive tract (Figure 3.11 and 3.12). The growth of the total digestive tract is also more rapid than the growth of the esophagus. For the growth of the other organs, the steepest growth is found for the myotome height and for lower jaw (Figure 3.11 and 3.12). The growth appears to be quite constant at first, before starting to increase at about 18 mm SL (Figure 3.12).

Table 3.1: External measurements of leptocephalus larvae of the European eel (*Anguilla anguilla*). SL = standard length, MHA = myotome height at anus, MHM = myotome height at thickest point, TT = total digestive tract, Eso = esophagus. All measurements are in mm, except from the number of tooth pairs.

		Myotome height		**Digestive tract**		**Jaw**		**Eye**			**Number of tooth pairs**	
ID	SL	MHA	MHM	TT	Eso	Upper	Lower	Width	Height	Length front tooth	Upper	Lower
L1	6,97	0,54	0,88	5,46	1,70	0,34	0,57	0,24	0,38	0,31	5	4
L2	9,68	0,84	1,23	6,44	2,41	0,39	0,83	0,37	0,48	0,36	5	4
L3	9,93	0,48	0,75	6,38	2,21	0,32	0,66	0,30	0,47	0,31	4	-
L4	10,01	0,75	1,13	6,91	2,56	0,29	0,69	0,37	0,44	0,33	4	4
L5	10,08	0,50	0,76	7,55	2,69	0,28	0,62	0,31	0,30	0,24	4	-
L6	10,13	0,61	0,96	7,59	2,68	0,36	0,73	9,32	0,54	-	-	-
L7	10,46	0,67	0,97	6,85	2,31	0,34	0,69	0,32	0,51	0,27	4	-
L8	10,67	0,60	0,97	7,14	2,59	0,26	0,91	0,28	0,41	0,25	4	4
L9	10,95	0,66	-	-	-	0,34	0,77	0,29	0,43	0,30	4	4
L10	11,17	0,72	1,18	8,16	2,45	0,32	0,74	0,35	0,45	0,33	4	4
L11	11,69	0,90	1,50	7,63	2,38	0,40	0,71	0,28	0,43	0,17	4	5
L12	11,77	0,89	1,30	8,12	2,65	0,46	0,78	0,37	0,48	0,30	5	4
L13	12,26	1,10	1,72	8,85	3,34	0,49	0,88	0,39	0,58	0,34	5	-
L14	12,76	0,83	1,50	8,38	4,24	0,48	0,84	0,42	0,56	0,38	4	4
L15	13,78	0,96	1,42	8,81	2,59	0,37	0,90	0,37	0,53	0,26	5	-
L16	13,78	0,89	1,52	9,01	4,55	0,50	0,82	0,35	0,51	0,29	4	4
L17	15,77	1,07	1,53	11,20	3,10	0,52	0,92	0,41	0,53	0,31	5	5
L18	17,03	1,01	1,26	11,11	5,75	0,43	0,94	0,38	0,45	0,23	5	5
L19	17,11	0,62	1,00	11,10	3,71	0,45	1,08	0,39	0,49	0,34	4	5
L20	18,85	1,11	2,03	13,57	4,84	0,69	1,32	0,55	0,68	-	4	4
L21	23,33	2,49	3,42	15,96	7,09	0,78	1,41	0,77	0,76	0,32	5	-

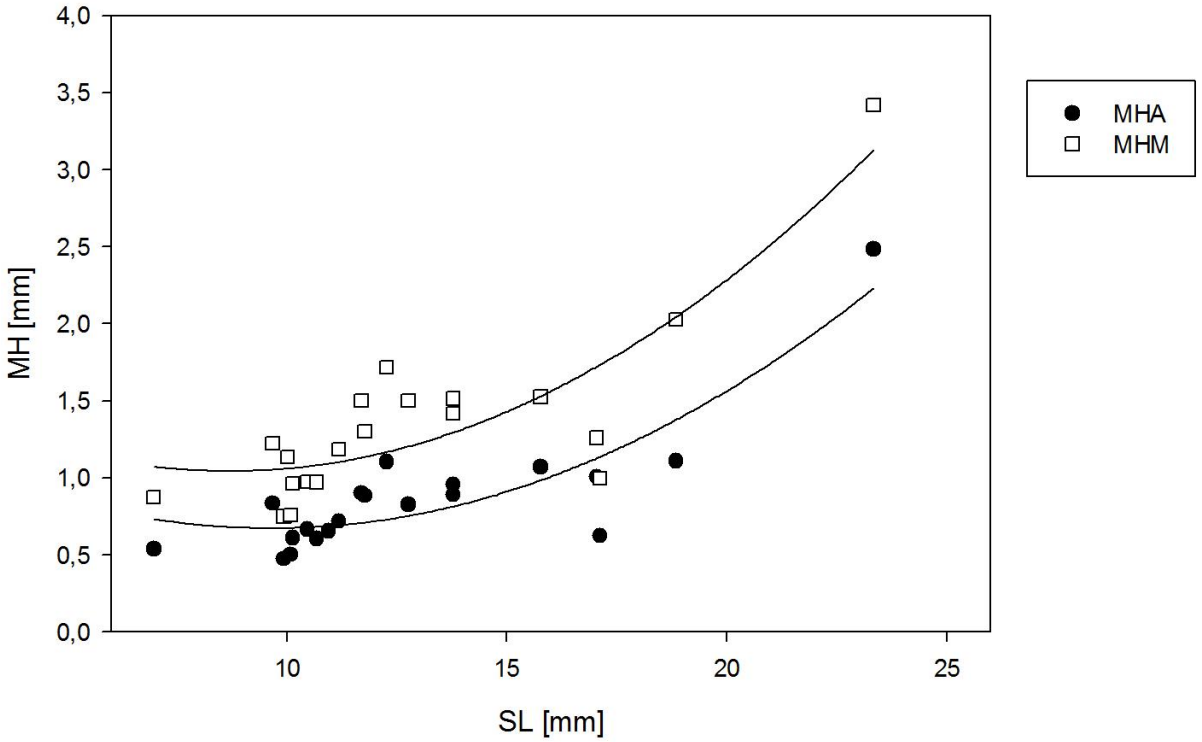

Figure 3.7: Measurements of myotome height (MH) of leptocephalus larvae of the European eel (Anguilla anguilla) in relation to standard length (SL), and for two different areas of the larvae. MHA = myotome height at anus. MHM = myotome height at thickest point. Quadratic regression curves ($y=y0+ax+bx^2$) are shown for MHA (R^2 = 0.5907) and MHM (R^2 = 0.5634). The regression curves were however not significant ($p<0.05$) for a, neither for MHA (pa=0.1726) or MHM (pa = 0.0558).

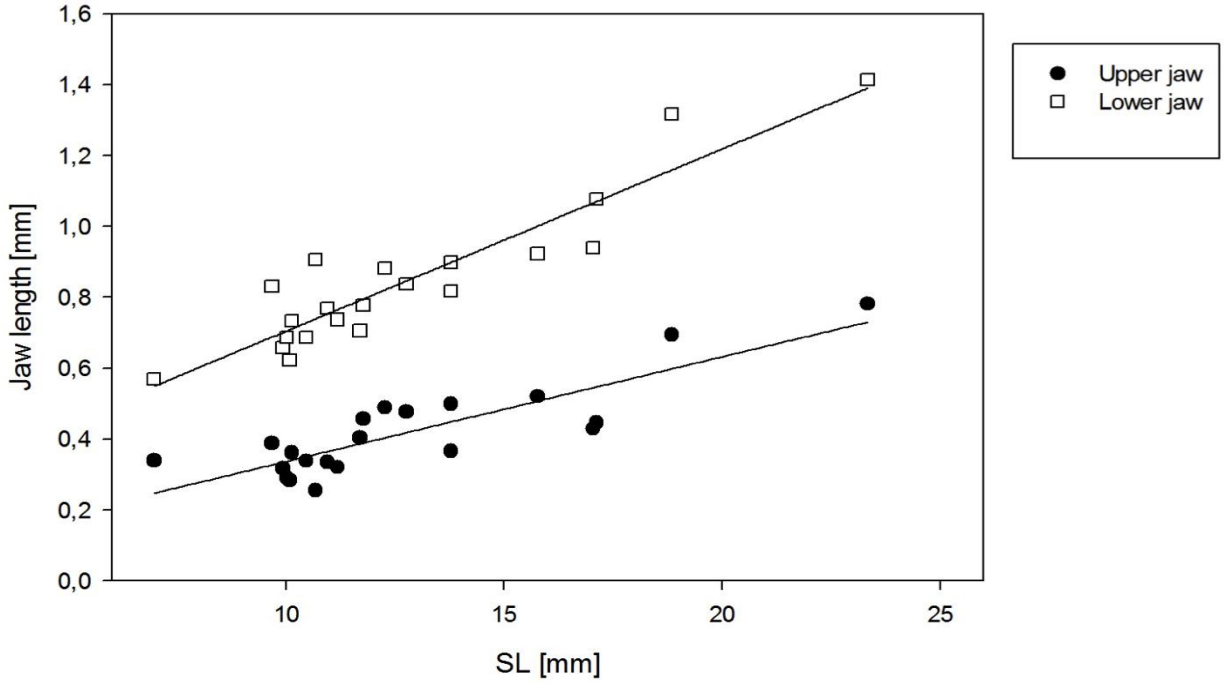

Figure 3.8: Measurements of jaw length of leptocephalus larvae of the European eel (*Anguilla anguilla*) in relation to standard length (SL). Linear regression curve (y=ax+b) fitted to data for both of the upper (R^2 = 0.7306) and lower jaw (R^2 = 0.8565).

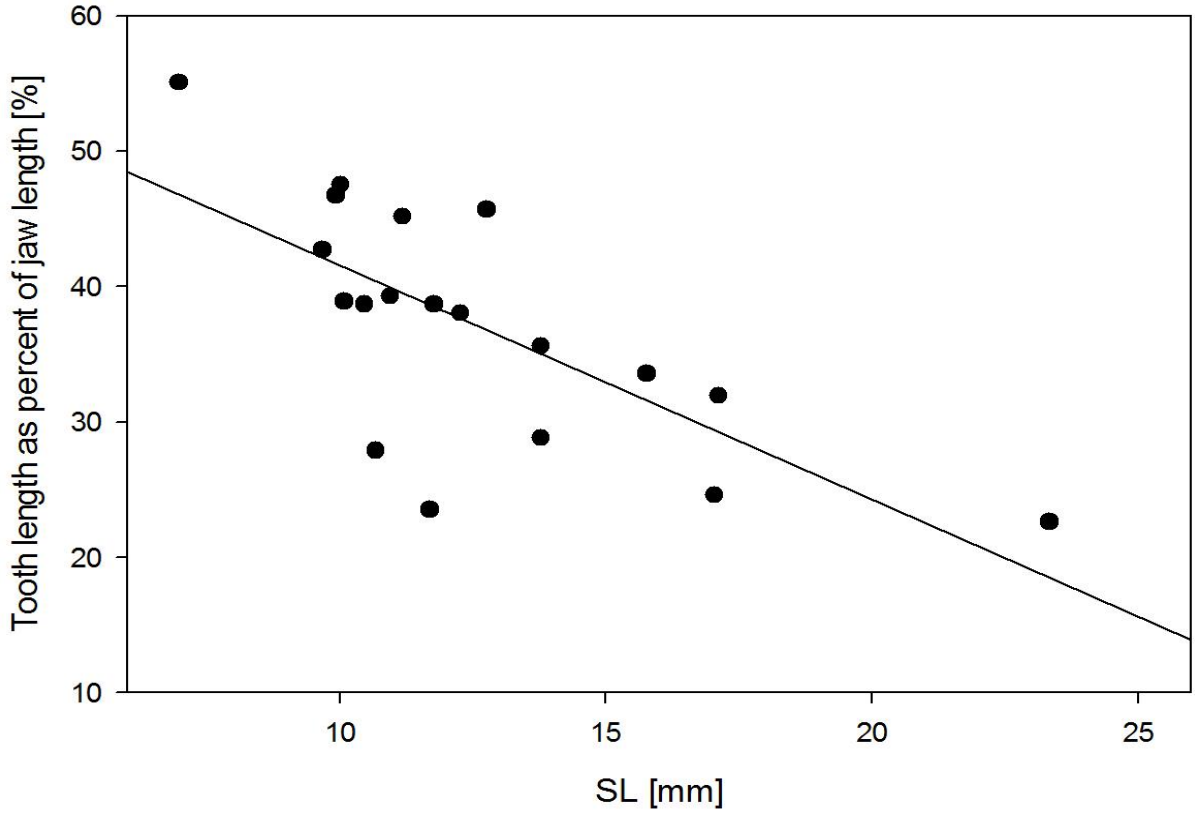

Figure 3.9: Measurements of length of front tooth of leptocephalus larvae of the European eel (Anguilla anguilla) in relation to standard length (SL), expressed as percentage of the length of the jaw. Linear regression curve (y=ax+b) is fitted to the data (R_2 = 0.4996).

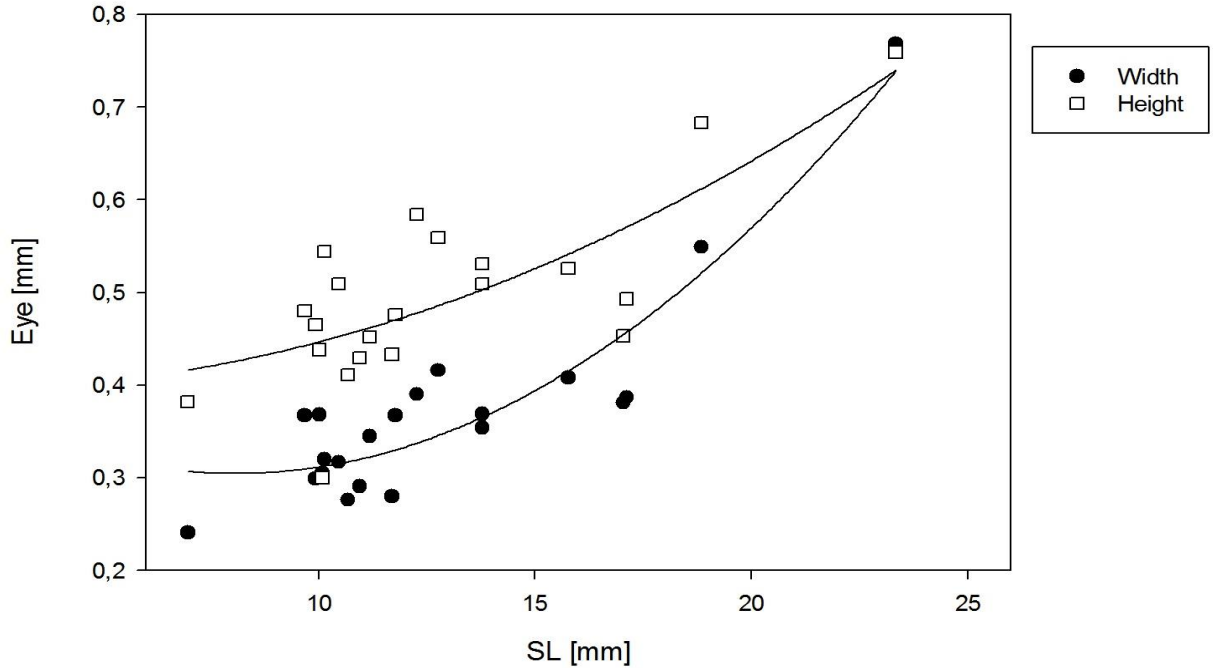

Figure 3.10: Measurements of eye width and height of leptocephalus larvae of the European eel (*Anguilla anguilla*) in relation to standard length (SL). A quadratic regression curve ($y=y0+ax+bx^2$) was fitted to both the data of the height (R^2 =0.5729) and width (R^2 = 0.8491) of the eye.

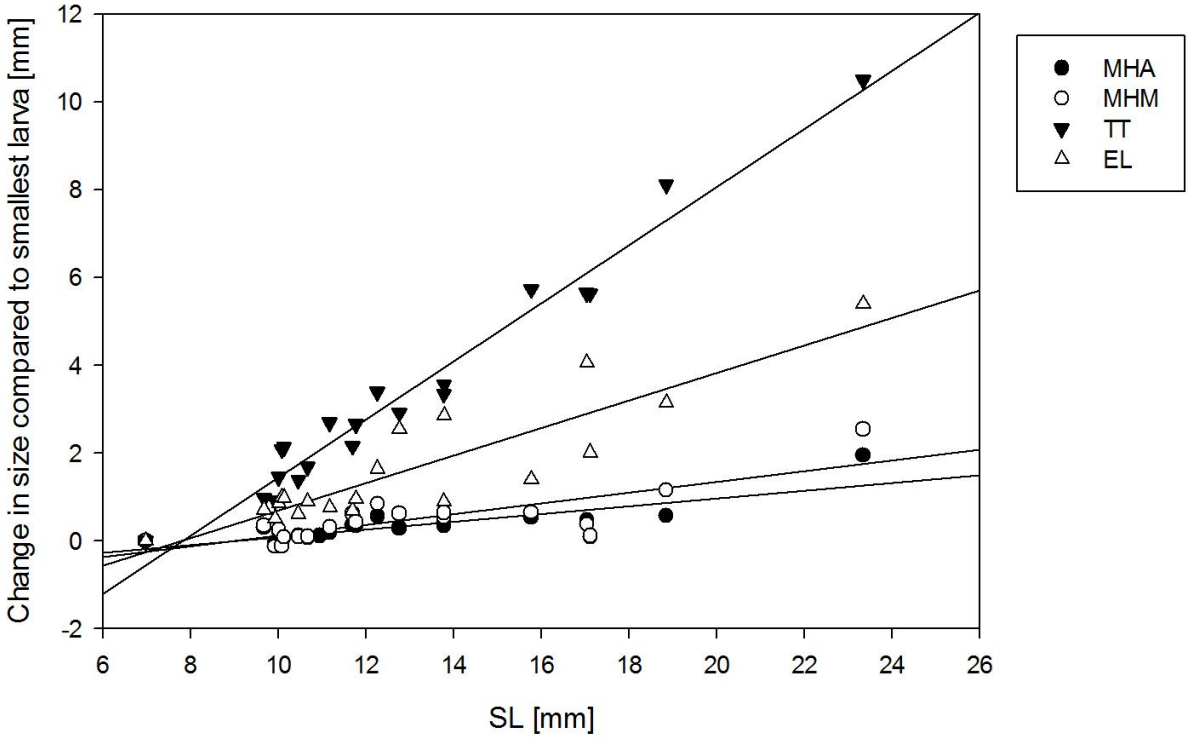

Figure 3.11: Relative growth (compared to smallest larva) of organs of European eel (*Anguilla anguilla*) in relation to standard length (SL). Linear regression lines are added (y=ax+b). MHA = myotome height at anus (R^2 = 0.6379), MHM = myotome heigh at thickest point (R^2 = 0.6363), TT = length total digestive tract (R^2 = 0.9680) , EL= esophagus length (R^2 = 0.7858).

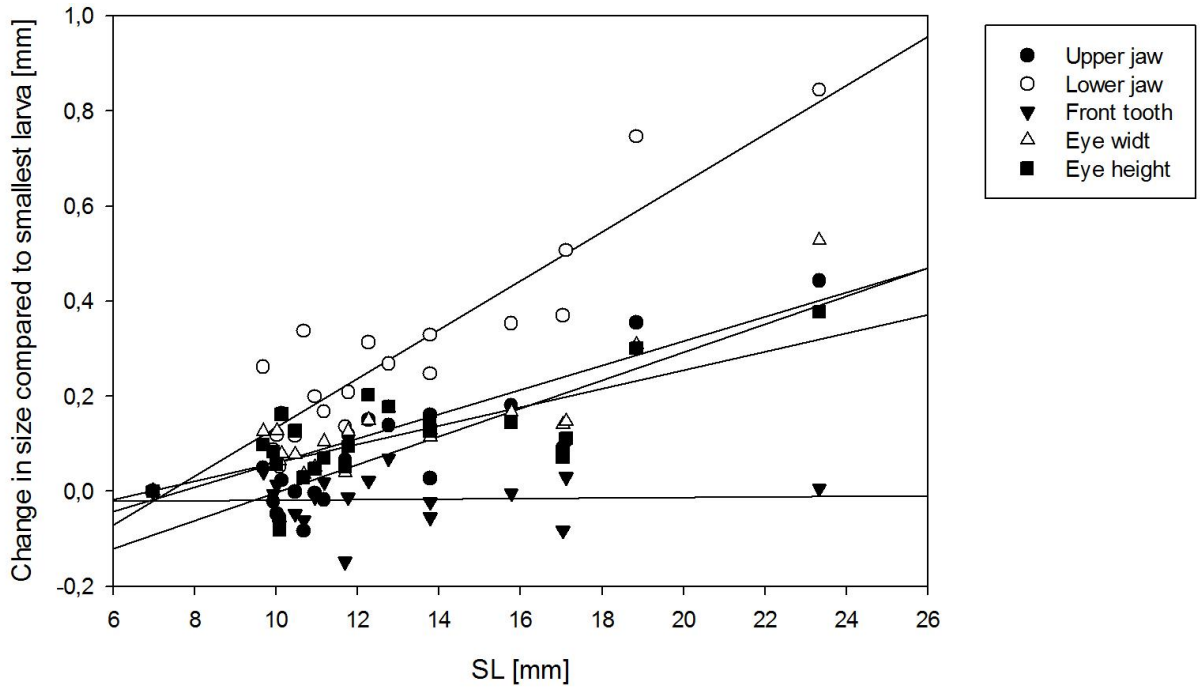

Figure 3.12: Relative growth (compared to smallest larva) of organs of European eel (*Anguilla anguilla*) in relation to standard length (SL). Linear regression lines are added (y=ax+b). Upper jaw (R^2 =,0.7306) Lower jaw (R^2 =0.8565), eye width (R^2 =0.7306), eye height (R^2 = 0.5546) and front tooth (R^2 = 0.0015).

3.2 The digestive system - internal morphology

The internal morphology of the digestive system of the leptocephalus larvae is described starting from the mouth, continuing with the esophagus, the transition zone, intestine and ending with the anus. The accessory organs (liver/pancreas, kidneys, gallbladder, urinary bladder) are treated as they appear in the digestive tract. In this section, the morphological descriptions will mainly be focused on the largest larvae (L21, SL = 23.33), as the other larvae were too damaged, especially in the intestine (see Appendix 8).

3.2.1 Mouth

The leptocephalus larvae had a mouth cavity that gradually became narrower further into the head. The epithelium of the mouth had a rough texture, with a keratinized-like appearance (Figure 3.13). This rough epithelium was found for all size groups, and in all areas of the mouth, but it was most prominent in the area around the heart and opercular cavity. Surrounding the mouth was a layer of muscle tissue, which started to appear around the mouth cavity starting above the heart.

3.2.2 Esophagus

The anterior part of the esophagus for size groups A-F appears as an oval shaped tract appearing right behind the heart (Figure 3.14). In all larvae, a thick muscularis surrounding the lumen of the esophagus was observed. A thin mucosa and the same rough-textured, possibly keratinized, epithelium that also was observed in the mouth could be seen (Figure 3.14). For the larvae of size group F it was possible to see what may be the serosa underneath the muscularis.

The anterior part of the esophagus of L21 (size group H), appeared to be more variable in morphology. It first appeared as a triangular shaped structure, with a large lumen and only slight folding (Figure 3.15-A). The epithelium had a keratinized-like rough appearance, and goblet cells were present in the epithelium. The mucosa was a thicker on the dorsal side of the lumen than on the ventral side. The muscularis was present, as a double layer between the mucosa and serosa (Figure 3.15-A). 200 µm further into the esophagus, the wall of the esophagus became much more folded and the lumen more narrow (Figure 3.15-B). The esophagus was here oval in shape. The keratinized epidermis was still present, and more goblet cells could also be seen in the epithelium. The mucosa was equally thick on the ventral and dorsal side. The thickest muscularis to be observed in the esophagus could be seen in this area. The serosa was thin (Figure 3.15-B).

600 µm into the esophagus, the most distinct change was the rounded shape of the esophagus, disappearance of the keratinized epithelium and what appears to be cilia. The wall was highly folded, and the muscularis was thick (Figure 3.15-C). The kidneys could be seen dorsally to the esophagus. They had a distinct lumen that appeared to be ciliated. However, for all other larvae except L21, there was no distinct lumen in the kidneys.

About 800 µm into the digestive tract, the esophagus had a more triangular shape (Figure 3.16). The kidneys could be seen, and also the renal arteries stretching from the kidneys and up to the blood vessels at the notochord. The basic layers with a thin serosa, muscularis and mucosa were present in the esophagus. Both the mucosa and muscularis was thinner than observed before. The lumen appeared larger than before, and no folding of the wall could was observed. Goblet cells containing mucus with a granular appearance was observed, especially on the ventral side of the esophagus. The epithelium was covered in a layer that appear to be cilia (Figure 3.16). This was similar for all larvae.

The liver appeared ventrally to the esophagus approximately in the middle of the esophagus (Figure 3.17). In the middle part, the esophagus had an oval flattened shape, still with the same basic layers, but now a much thinner muscularis and mucosa compared to before, and what possibly are goblet cells could be seen in the epithelium. This morphology was found throughout remains of the esophagus, all the way into the transition area.

A clearly distinct submucosa was not observed in any parts of the esophagus. The serosa was always thin, and the muscularis and mucosa was usually the most dominant basic histological layers present for all larvae.

3.2.3 Transition area

The transition area between the esophagus and intestine was defined both from the external morphology of the sections, and by the appearance of the gall bladder in the histological sections (Figure 3.18). The esophagus was here located dorsally to the liver, with the kidneys located next to it rather than dorsally to it as before. The esophagus was similar in morphology as before, with a thin serosa, muscularis, mucosa and a thick epithelium with goblet cells containing granular mucus (Figure 3.19-B). The liver appeared as a large structure surrounding the ventral side of the digestive organs. The structure of the liver appeared to consist of basic lobules of hepatocytes, with numerous nuclei and fat deposits in-between the lobules (Figure 3.19-A). Structures that resemble granules of glycogen could also be seen, but fat deposits appeared to be more dominating (Figure 3.19-A). The gall bladder

was located approximately in the middle of the digestive area (Figure 3.18). The tree basic histological layers of the gall bladder was observed, with a thin serous membrane, a layer of smooth muscle and the innermost epithelium (Figure 3.19-A). The wall of the gall bladder was thin, with a large lumen in comparison. A blood vessel, that may be the hepatic vein, was observed dorsally to the gall bladder Figure 3.19-B). The pancreas was possibly observed laterally to the liver. Pancreas was identified based on the location of the nuclei, and the organized structure of the tissue, that resembles the rosette-like organization that is characteristic for the pancreas.

3.2.4 Intestine

The intestine was completely or nearly completely degraded in all larvae except L21. The intestine was uniform in appearance throughout its whole length, apart from the anus. Dorsally to the intestine, the kidneys could be observed (Figure 3.20-A), but these started to disappear at around the middle of the intestine. The intestinal tract had no folding and appeared as a straight tube. The wall of the intestine had numerous foldings (Figure 3.20-A). The intestinal wall consisted of a serous membrane, a thick mucosa with numerous foldings and a thick columnar absorptive epithelium. The columnar epithelium had large nuclei with an even organization. In-between the columnar cells in the epithelium, there could sometimes be seen lighter structures that are thought to be goblet cells, and some structures that may be vacuoles (Figure 3.20-C). Covering the columnar epithelium was a layer of microvilli. The wall of the intestine was thick all around the tube, except in the dorsal side, where it was much thinner than the rest of the tube (Figure 3.20-B). No distinct muscularis was observed in the intestine.

3.2.5 Anus

The most distinct morphological features at the anus was the narrowing of the lumen, a more even thickness of the mucosa all around the tube and the appearance of the urine bladder dorsally to the intestine (Figure 3.21). The area around the anus also appeared to be vascularized, as at least three blood vessels could be observed in the section.

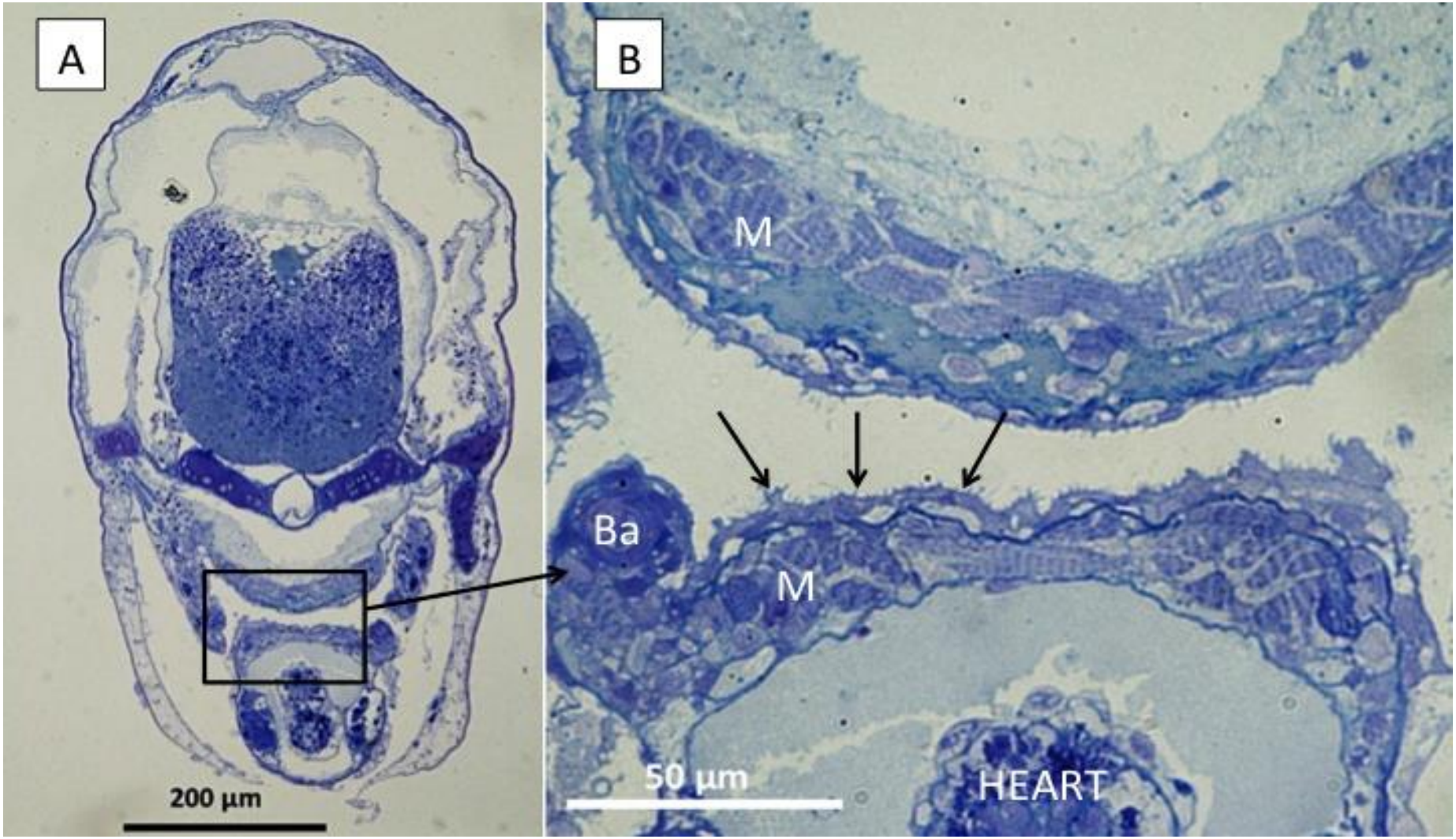

Figure 3.13: Figure showing the mouth of a leptocephalus larvae of the European eel (Anguilla anguilla) (L10, SL = 11.17 mm). Section is taken through the heart. A: Overview of sectional area. 16x magnification. B: Close up of mouth, showing the rough epithelium (arrows) and muscle (M). Ba = branchial arch.

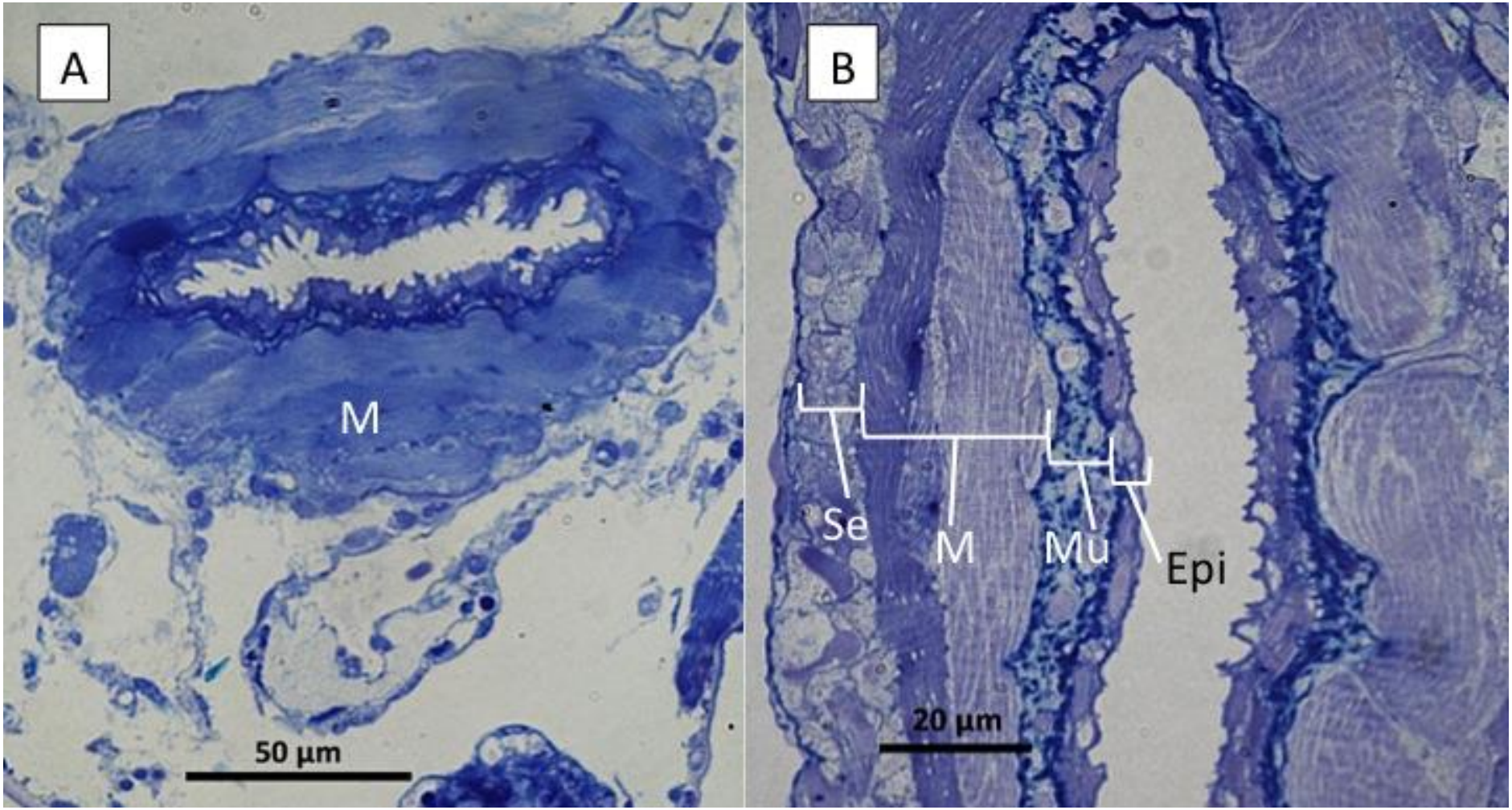

Figure 3.14: The anterior part of the esophagus of leptocephalus larvae of the European eel (Anguilla anguilla) from size group B and F. A: L8 (SL = 10.67) showing the thick muscularis, thinner mucosa and rough epithelium, 63x magnification. B: L19 (SL = 17.11) showing rough epithelium (Epi), mucosa (Mu), double layered muscularis (M) and serosa (Se), 100x magnification.

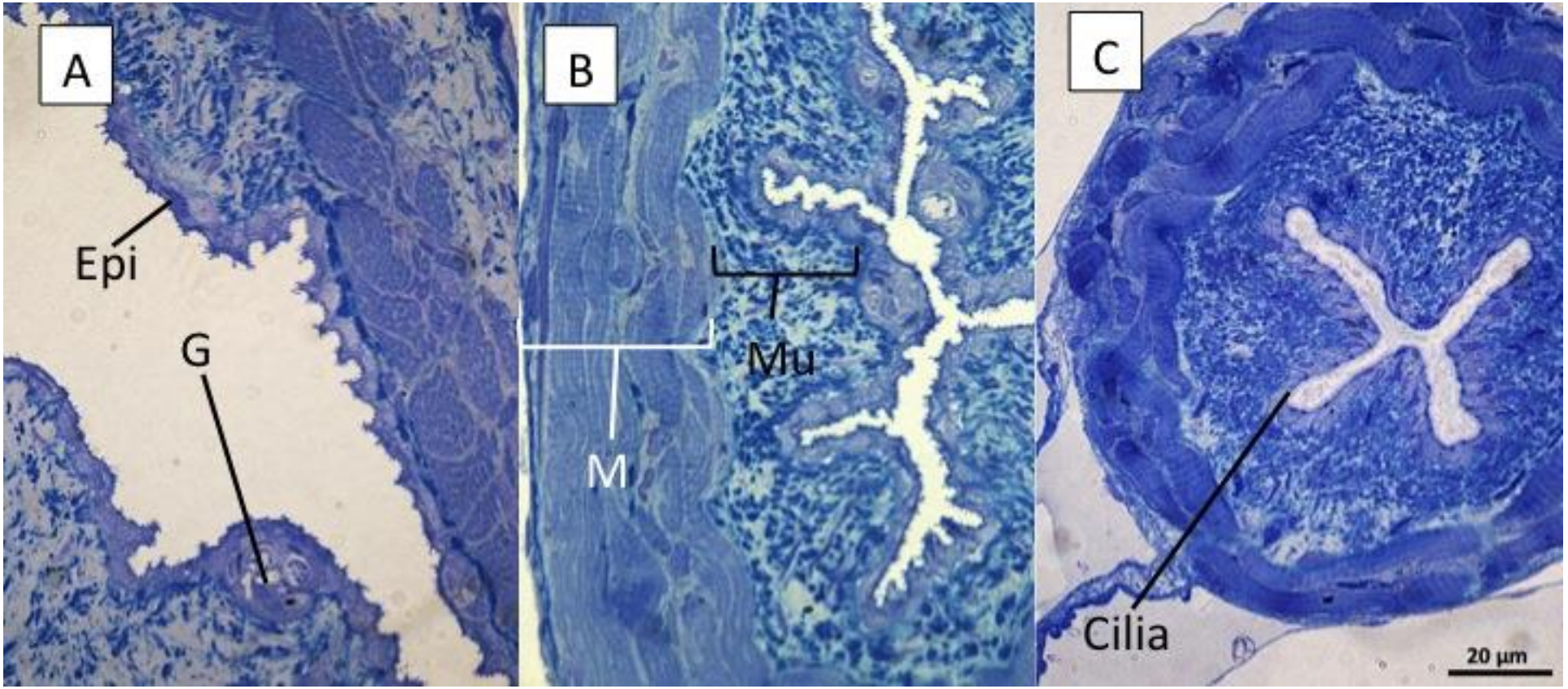

Figure 3.15: Three parts of the anterior esophagus of leptocephalus larvae of the European eel (Anguilla anguilla) (L21, SL = 23.33), 100x magnification, showing the presence of basic histological layers and the rapid change in morphology that happens in the anterior esophagus. A: Beginning of esophagus. B: 200 µm behind (A). C: 600 µm behind (A). Epi = epidermis, G = goblet cells, M = muscularis, Mu = mucosa.

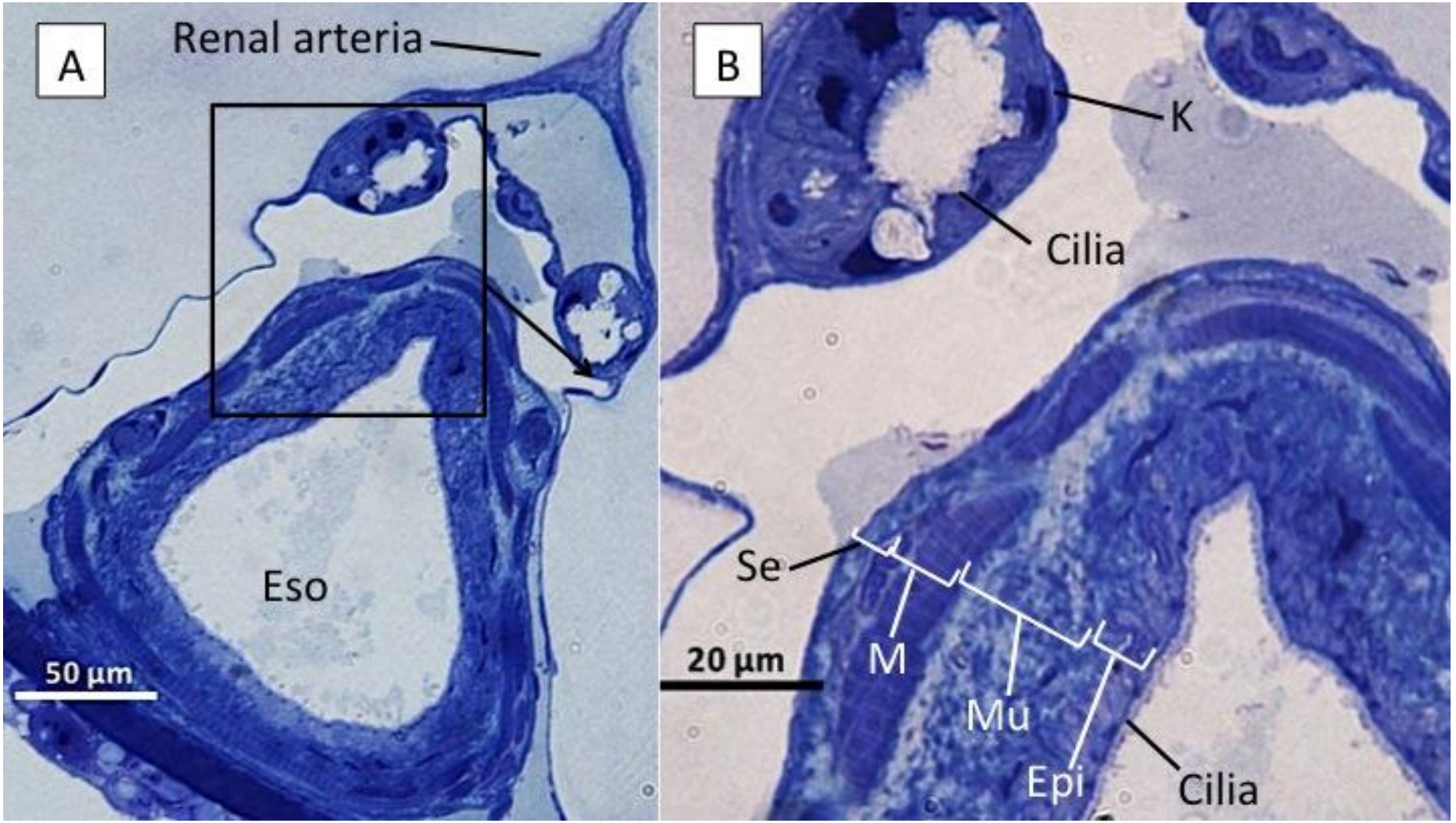

Figure 3.16: Further into the anterior esophagus (200 µm behind Figure 3.15-C) of leptocephalus larvae of the European eel (Anguilla anguilla) (L21, SL = 23.33). A: 40x magnification, showing the triangular shape, lack of folding, large lumen presence of kidneys and renal arteria. B: 100x magnification, showing the cilia in the kidneys and lumen, and the basic histological layers; epidermis (Epi), mucosa (Mu), muscularis (M) and serosa (Se).

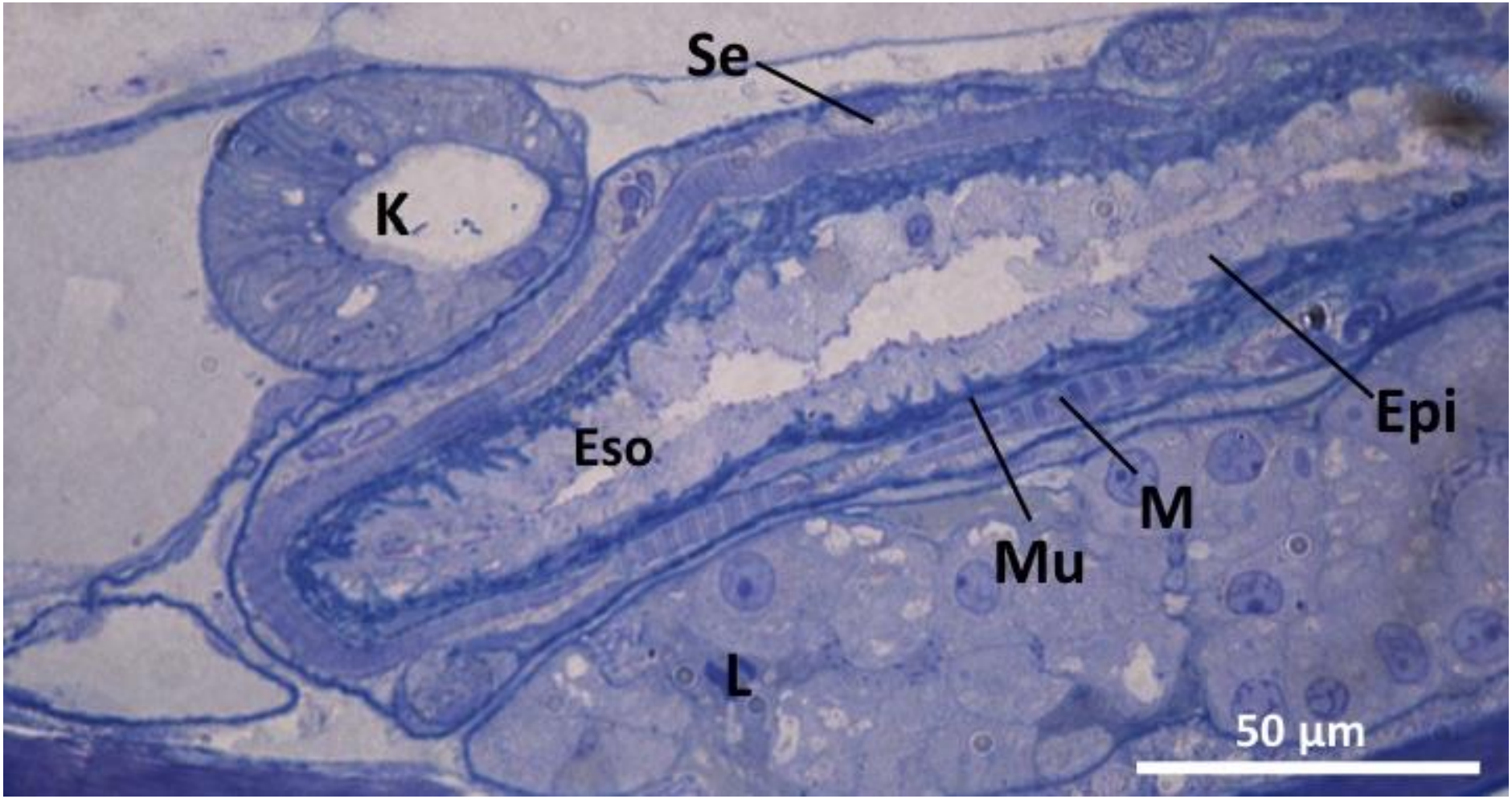

Figure 3.17: Middle part of esophagus of leptocephalus larvae of the European eel (Anguilla anguilla) (L21, SL = 23.33), 100x magnification. Showing one of the kidneys, the esophagus with the basic histological layers of serosa (Se), mucosa (Mu), muscularis (M) and a thick epidermis (Epi). The liver (L) is also present. K = Kidney.

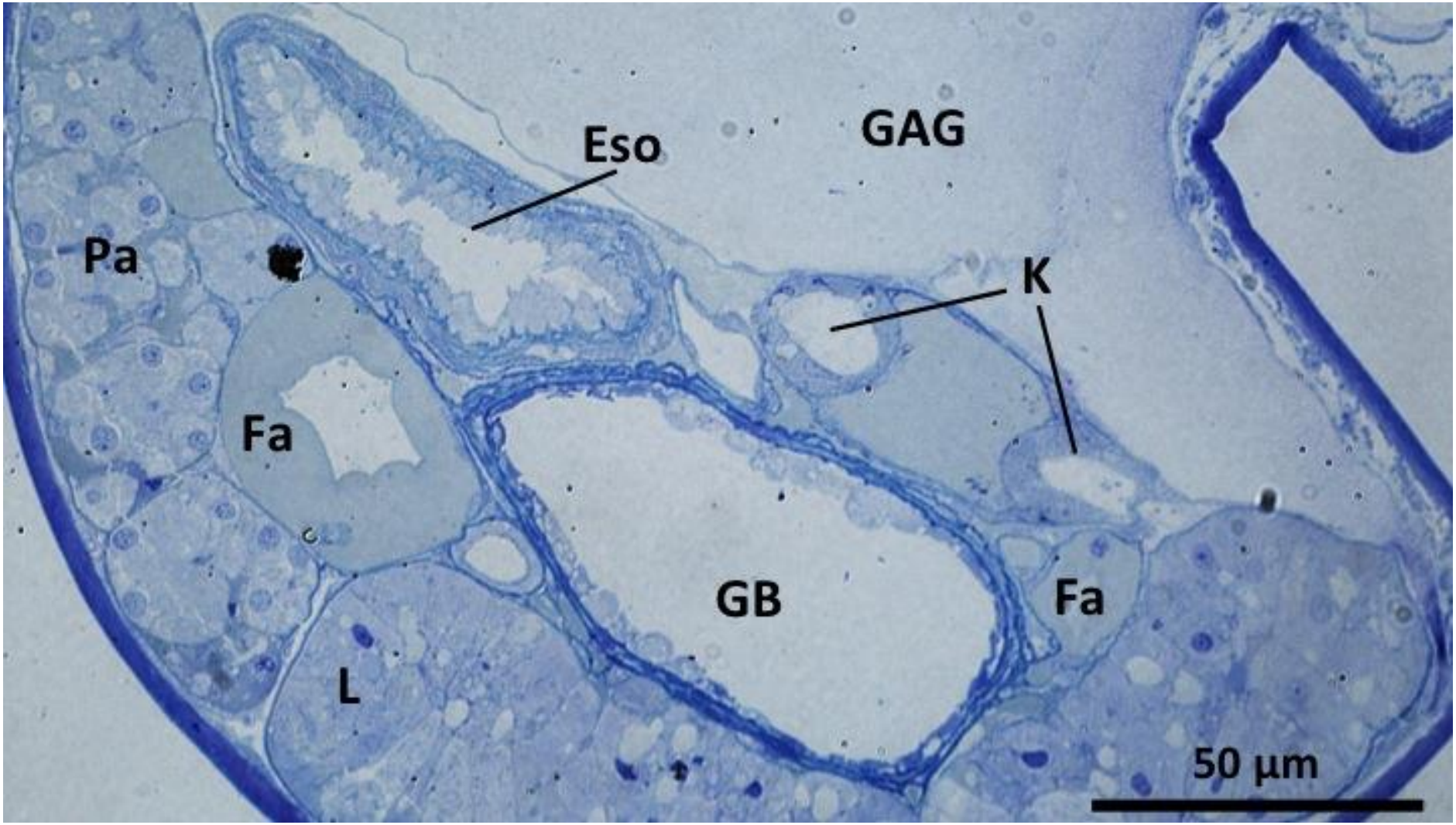

Figure 3.18: Transition area between esophagus and intestine in leptocephalus larvae of the European eel (Anguilla anguilla) (L21, SL = 23.33), 40x magnification. A large liver (L) is present with fatty deposits (Fa), esophagus (Eso), kidneys (K), the gall bladder (GB) and possibly pancreas (Pa). The glycosaminoglycan layer (GAG) can be seen above the digestive system.

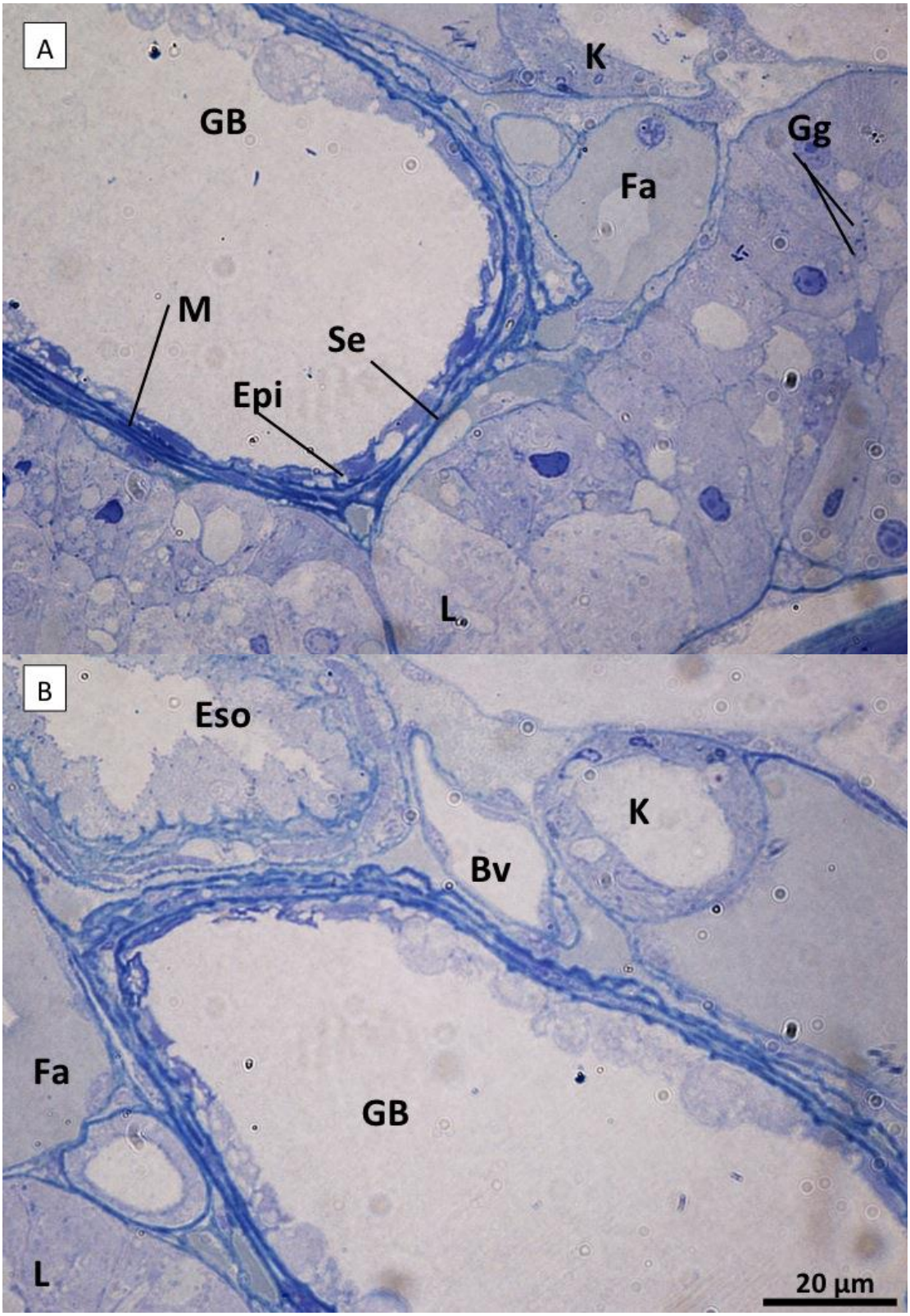

Figure 3.19: Liver and gall bladder at the transition area between esophagus and intestine in leptocephalus larvae of the European eel (Anguilla anguilla) (L21, SL = 23.33), 100x magnification. A: Structure of the liver (L), with hepatocytes arranged in lobuli, Kupffer cells (Ku), fat deposits (Fa), glycogen granules (Gg). Upper part of gall bladder (GB), with smooth muscle (M), serous membrane (Se) and epidermis. B: Lower part of gall bladder, esophagus (Eso), blood vessel (BV) and kidney (K).

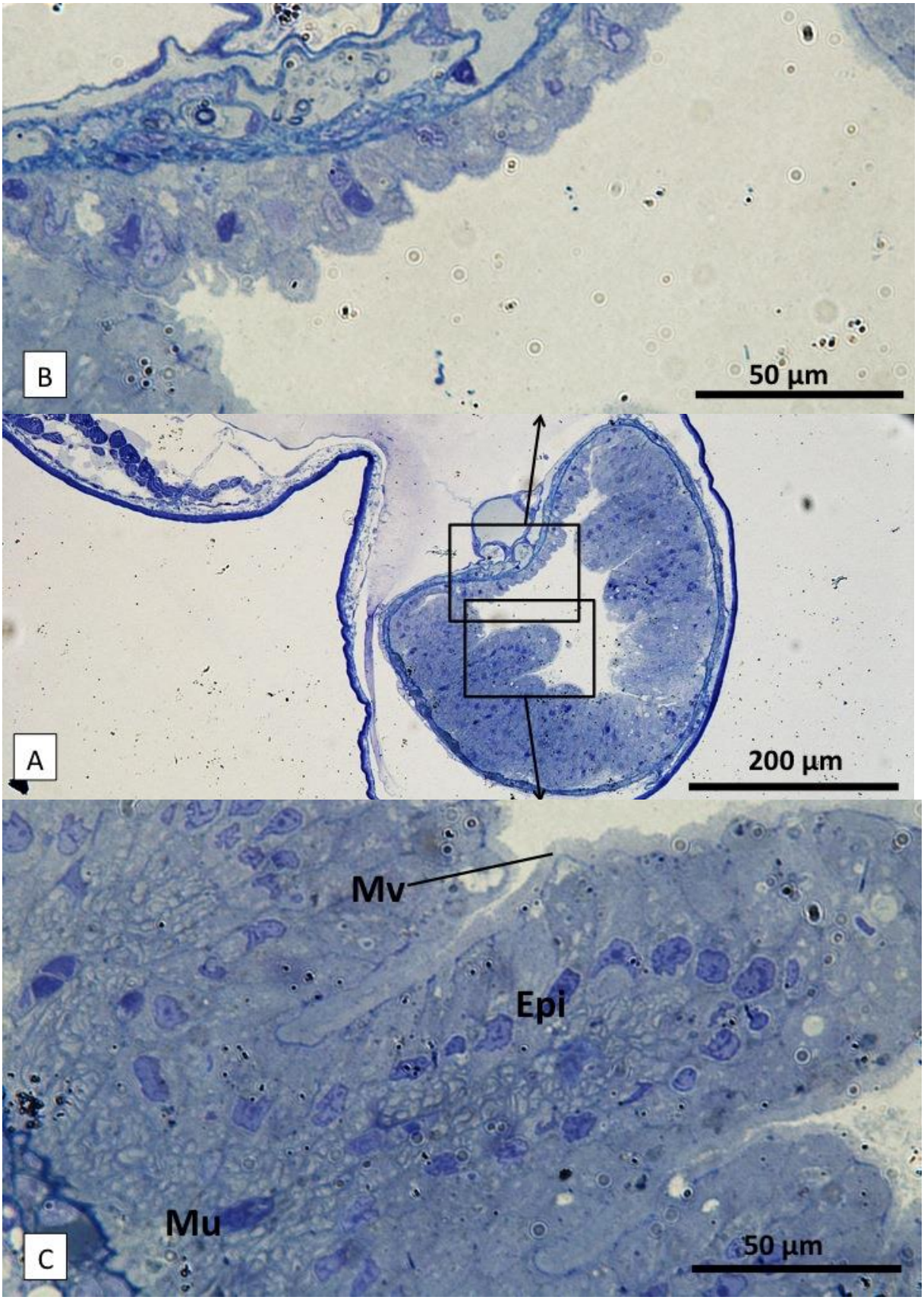

Figure 3.20: Intestine of larvae of the European eel (Anguilla anguilla) (L21, SL = 23.33). A: 40x magnification. B: 63x magnification, showing the thinner intestinal wall at the dorsal side. C: 63x magnification, showing the thick intestinal wall, columnar epithelium and the microvilli brush border.

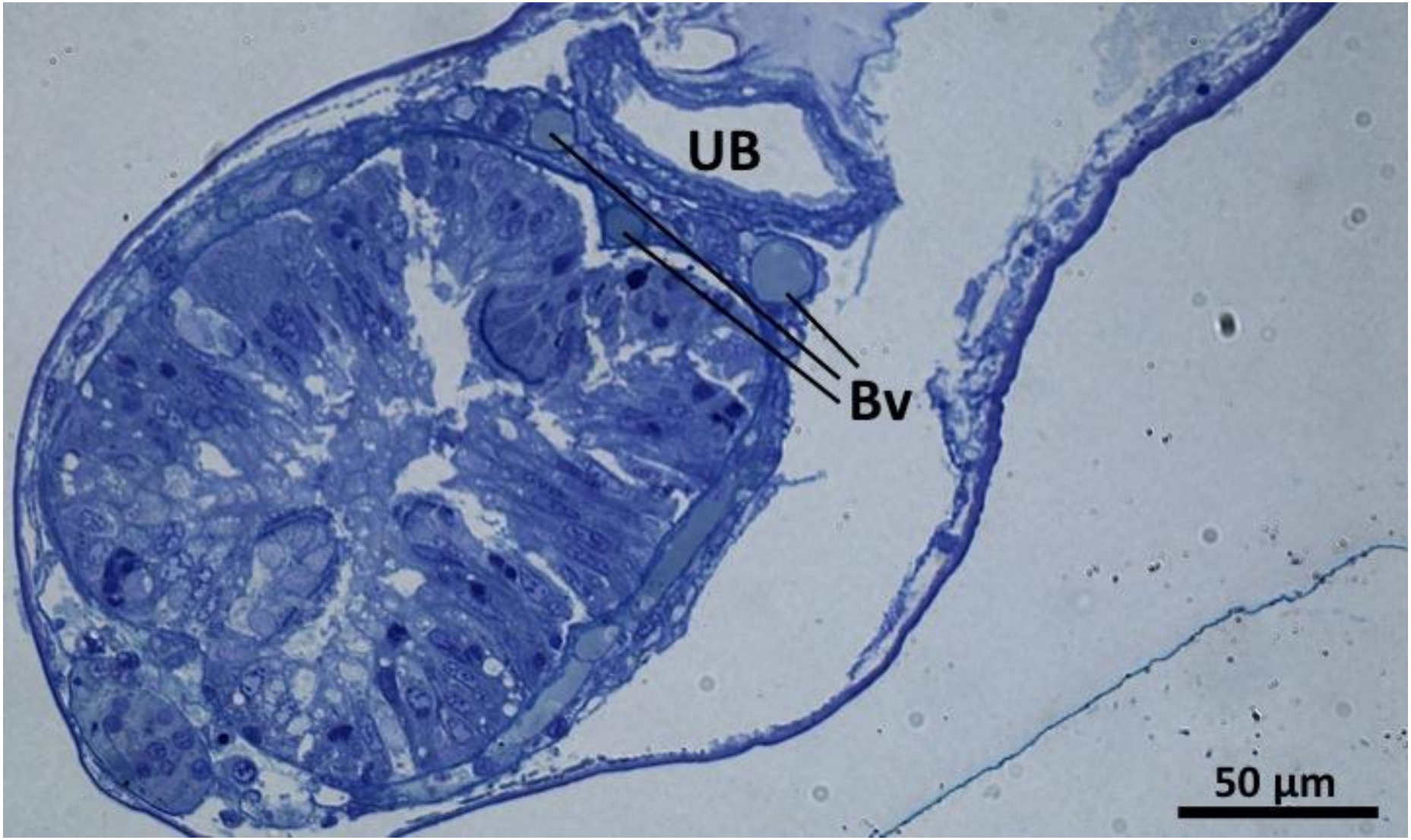

Figure 3.21: Termination of the intestine in the anus of larvae of the European eel (Anguilla anguilla) (L21, SL = 23.33), 40x magnification, showing the thick intestinal wall and the urine bladder (UB) dorsally to the intestine. Blood vessels (Bv) can also be seen.

3.3 Muscle

3.3.1 Histology of muscle tissue

The overall internal morphology was quite similar for all main transverse sections (MTS) throughout the larva, with some exceptions. The muscle layer generally appeared as one-cell layer thick stacks of white muscle (WM) cells (e.g. Figure 3.22-C), as was also observed during the external examination. Outside the layer of WM, a one-cell thick layer of red muscle (RM) cells was observed, which also appeared to have a stacked formation. The cells of WM appeared to be thicker along the lateral midline and decrease in transverse-section area towards the dorsal and ventral side of the larvae. The area of smaller cells on the ventral and dorsal side of the larvae was hence thought to be growth zones (e.g. Figure 3.22-A).

There were some exceptions to these general findings. In the smallest larvae (size group B and C), the muscle tissue was mostly combined two-cell layers thick, consisting of one cell layer of small RM fibers and one cell layer of WM fibers within the RM layer (Figure 3.22 and 3.26). In the white muscle layer, there was also observed some new fibers, mainly on the outside of the larger old fibers. In the smallest larvae, this was mainly observed at MTS-2 (Figure 3.34), and only occasionally in other areas.

In the largest larvae (L21, SL = 23.33 mm), the WM still appeared somehow as stacks of individual cells, but the muscle layer was here several cell-layers thick. The thickest cell layers were found in MTS-2 where the WM layer appeared to be 4-5 cell layers thick at the lateral midline of the section (Figure 3.25-A). In the dorsal and lateral growth zone, the WM was 1-2 cell layers thick (Figure 3.25-B). Toluidine blue also stained the muscle tissue differently, as the larger cells were stained stronger than the smaller cells. This was observed both in the growth zones and in the middle of the section (e.g. Figure 3.25). The only exception to this was the smallest cells in the innermost layer, which were stained equally strong as the large cells (Figure 3.25-A). This appearance of smaller cells in the innermost layer was only observed in the largest larva (L21).

The muscle layer was very similar in MTS-1 and MTS-3. In the smaller larvae (size group B and C), the WM generally appeared as one-cell layer thick stacks all over (Figure 3.22 and 3.26). In the largest larvae (L21), the WM appeared as an about three-cells thick layer in the section-middle, and one to two-cell layers thick in the growth zone (Figure 3.23 and 3.27). The RM always appeared as stacks one cell layer thick, both for all areas in the larvae and in all size groups.

Larvae in size group E-F were observed to be somewhere in-between the findings for group B-C and H, with more new fibers than observed in group B-C but less than in group H.

Based on this observation, growth by hyperplasia appeared to be present in all larvae in the dorsal and lateral growth zones, in each bilateral muscle layer. Growth by stratified hyperplasia was present mainly in the largest larvae and also to the largest extent in MTS-2. Mosaic hyperplasia was not observed in any larvae.

3.3.1 Measurements of muscle tissue

The total area of white muscle tissue in one bilateral half of the transverse section increased in a linear fashion with increasing SL (Figure 3.28). In addition, the muscle area in MTS-2 appear to be both generally higher than at MTS-1 and MTS-3, but also to increase faster with increasing SL (Figure 3.28). The muscle area in MTS-1 and MTS-3 appear both to increase in a similar pattern, and to be about equal in size. It may also seem like the total area of muscle cells is growing slowly at first, but then increasing rapidly from SL 18 to 23, although there is only one larva in the largest size group.

The same pattern can also be observed for the total number of white muscle cells in the three main transverse sections. The number of muscle cells is about equal for MTS-1 and MTS-3, and higher for MTS-2 (Figure 3.29). The growth pattern also appears to be similar for all three sections, with a quite slow growth in the beginning, before a rapid increase between SL 18 and 23. The increase does however, based on the slope of the regression curve, appear to be faster in MTS-2 (Figure 3.29).

The average area of the 25 largest muscle cells appeared to increase linearly with increasing SL (Figure 3.30). However, do note that the regression for these data is not significant for b. The largest fibers were found at MTS-2.

From the estimated relative growth, it may appear that the growth in both the 25 largest muscle cells and in the total area of WM happens faster than the growth in number of WM (Figure 3.31). It may also seem that major growth happens in the leap from SL 18 to 23, except from the growth in both the 25L (Figure 3.31). Do however note the low R^2 value for the 25 largest muscle cells, which indicates a weak adaption of the data points to the curve.

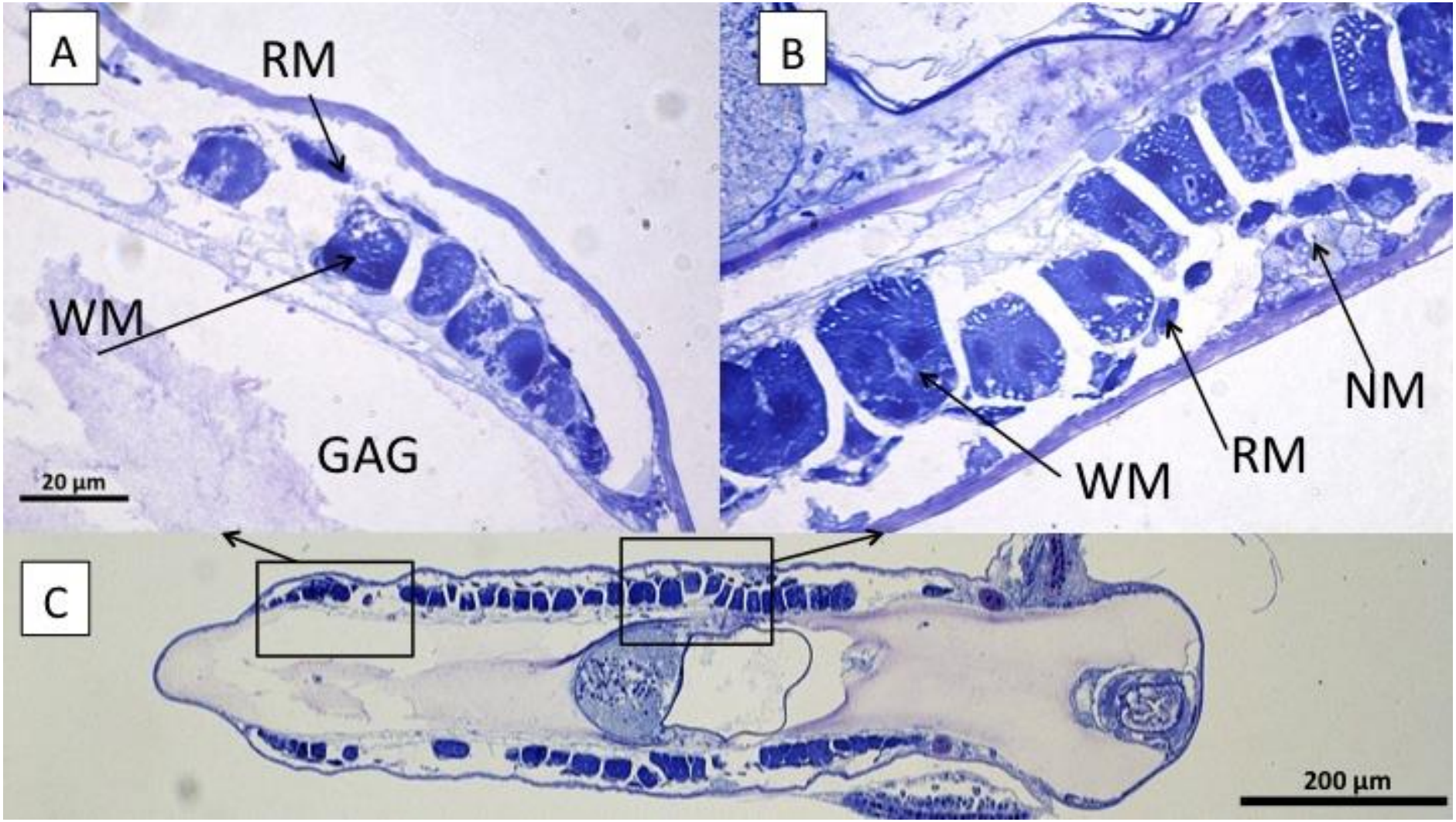

Figure 3.22: Muscle tissue at MTS-1 of larvae of the European eel (*Anguilla anguilla*) (L10, SL = 11.17 mm). One layer of large white muscle (WM) cells with a layer of smaller red muscle (RM) cells on the outside can be seen A: Growth zone, showing gradual increase in size of WM, 100x magnification. B: Muscle tissue in the middle of the section, the base of a neuromast (NM) can also be seen. 100x magnification. C: overview of section, 10x magnification.

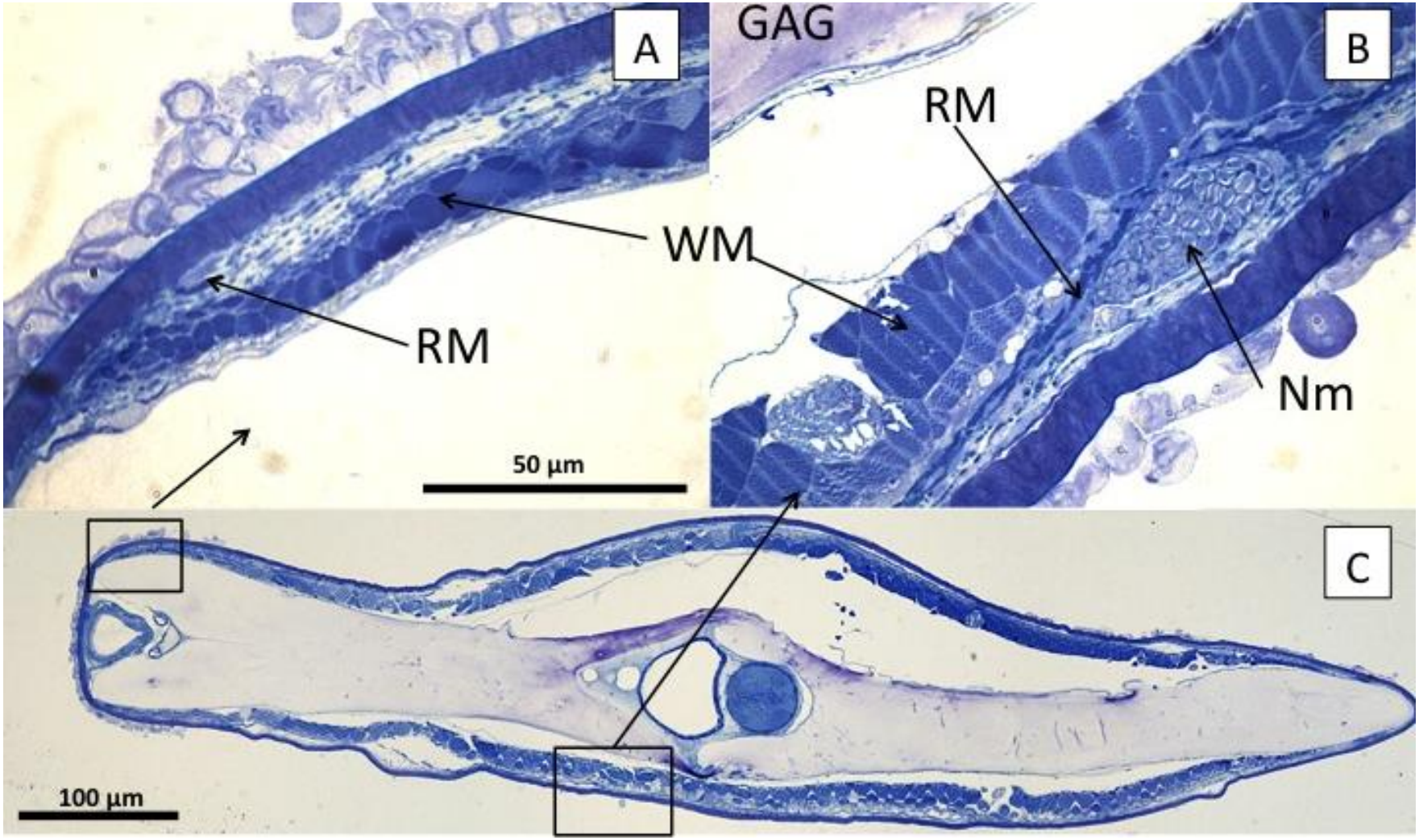

Figure 3.23: Muscle tissue at MTS-1 of larvae of the European eel (*Anguilla anguilla*) (L21, SL = 23.33). Several layers of large white muscle (WM) cells with one layer of smaller red muscle (RM) cells on the outside can be seen A: Growth zone, showing gradual increase in size of WM, 100x magnification. B: Muscle tissue in the middle of the section, the base of a neuromast (Nm) can also be seen. 100x magnification. C: overview of section, 10x2 magnification.. GAG = glycosaminoglycan layer.

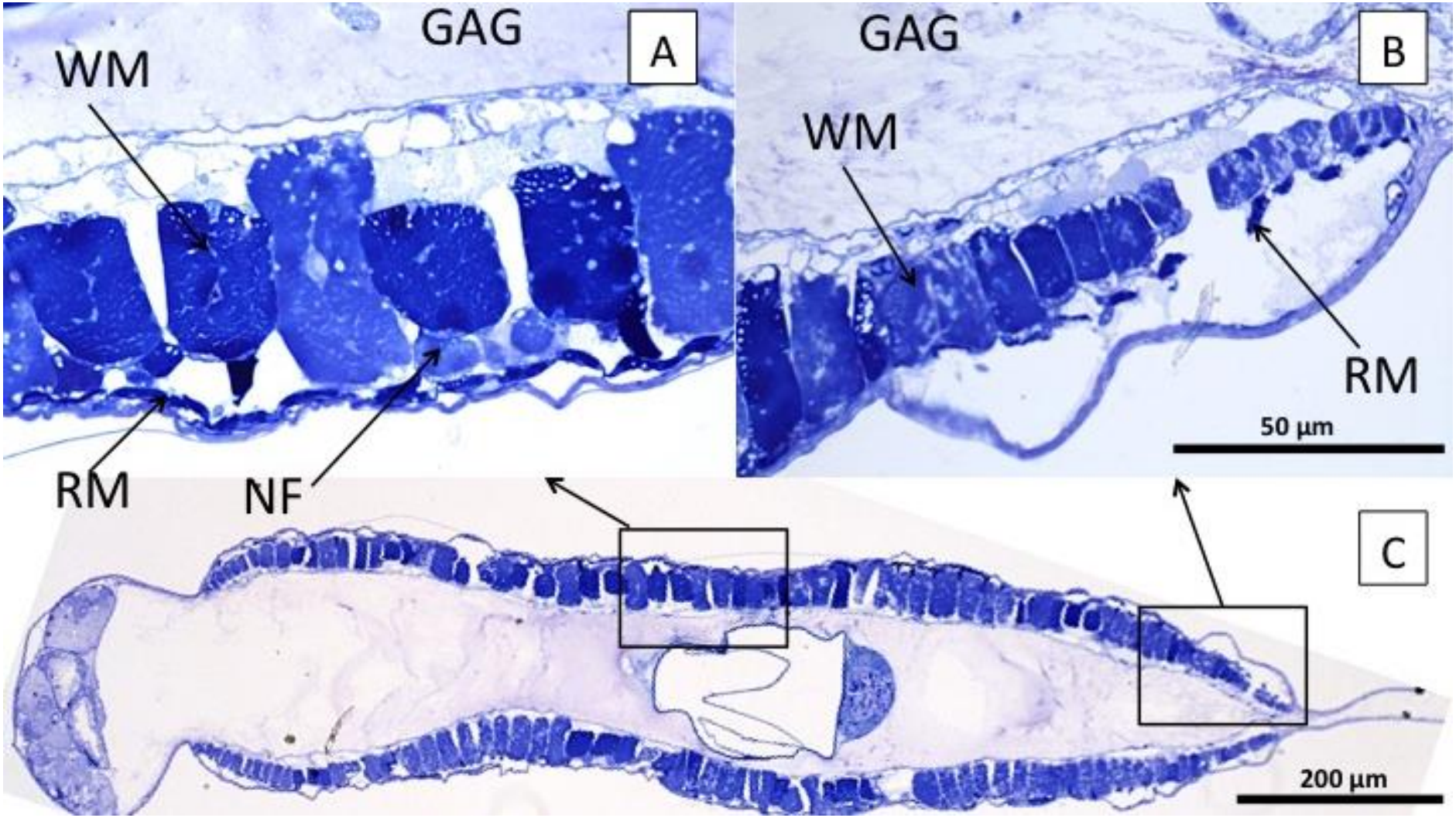

Figure 3.24: Muscle tissue at MTS-2 of larvae of the European eel (*Anguilla anguilla*) (L10, SL = 11.17). One layer of large white muscle (WM) cells with a layer of smaller red muscle (RM) cells on the outside can be seen A: Muscle tissue in the middle of the section, new fibers can be seen (NF), 100x magnification. B: Growth zone, showing gradual increase in size of WM, 100x magnification. C: overview of section, 10x magnification. GAG = glycosaminoglycan layer.

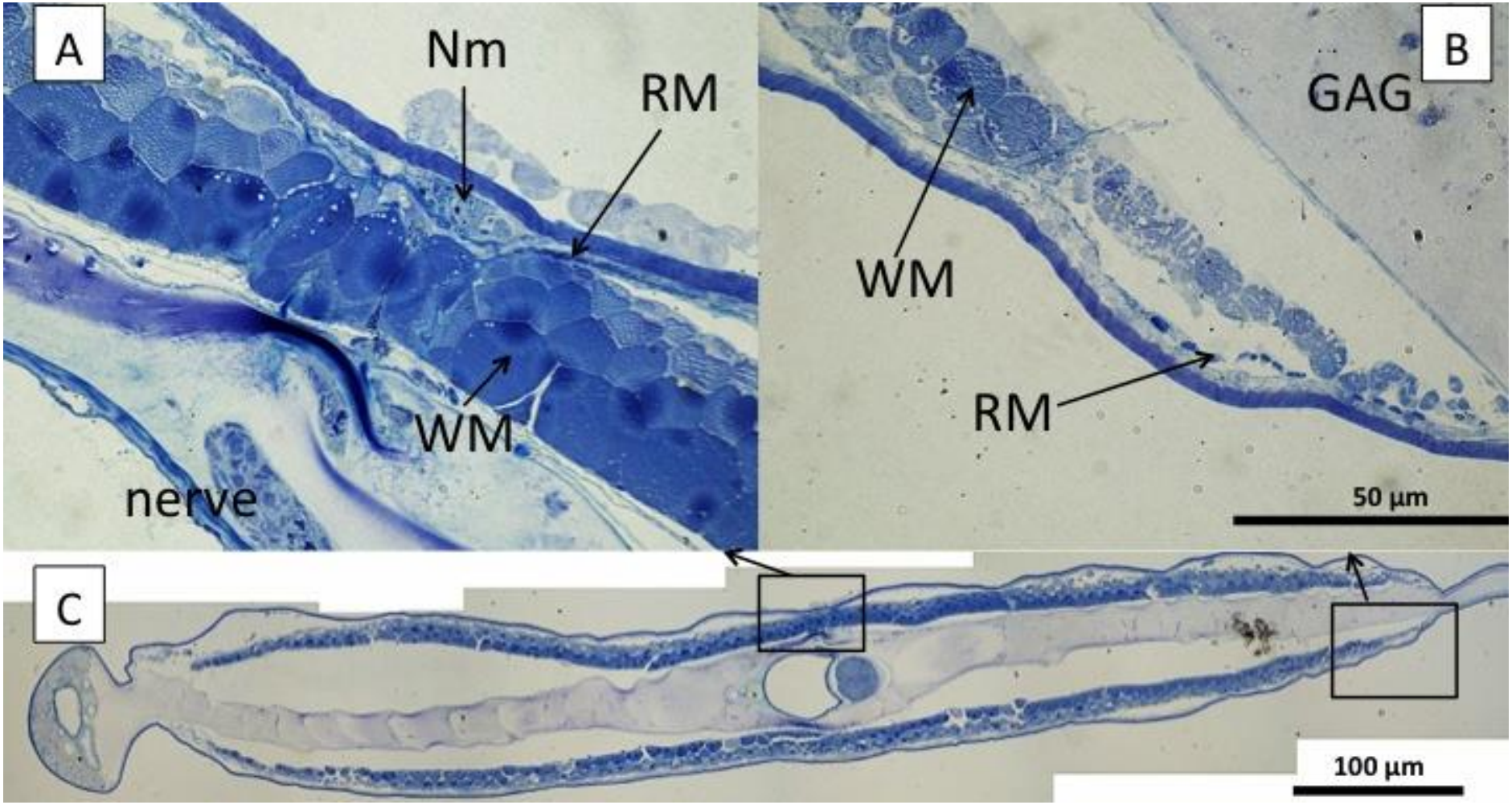

Figure 3.25: Muscle tissue at MTS-2 of larvae of the European eel (Anguilla anguilla) (L21, SL = 23.33). Several layers of large white muscle (WM) cells with one layer of smaller red muscle (RM) cells on the outside can be seen A: Muscle tissue in the middle of the section, the base of a neuromast (Nm) and a nerve can also be seen. 100x magnification B: Growth zone, showing gradual increase in size of WM, 100x magnification.. C: overview of section, 10x2 magnification.. GAG = glycosaminoglycan layer.

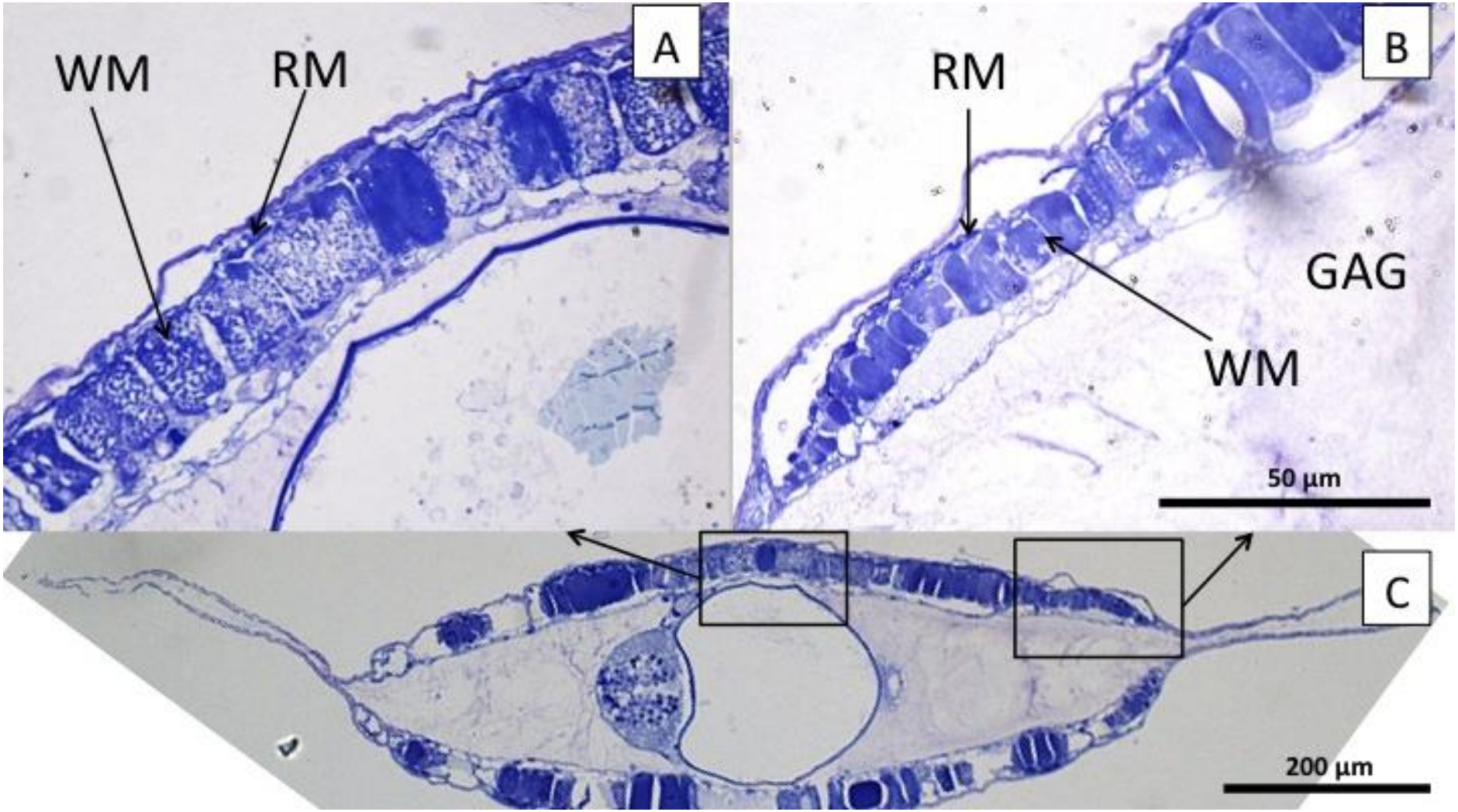

Figure 3.26: Muscle tissue at MTS-3 of larvae of the European eel (*Anguilla anguilla*) (L10, SL = 11.17). One layer of large white muscle (WM) cells with a layer of smaller red muscle (RM) cells on the outside can be seen A: Muscle tissue in the middle of the section, 100x magnification. B: Growth zone, showing gradual increase in size of WM, 100x magnification. C: overview of section, 10x magnification. GAG = glycosaminoglycan layer.

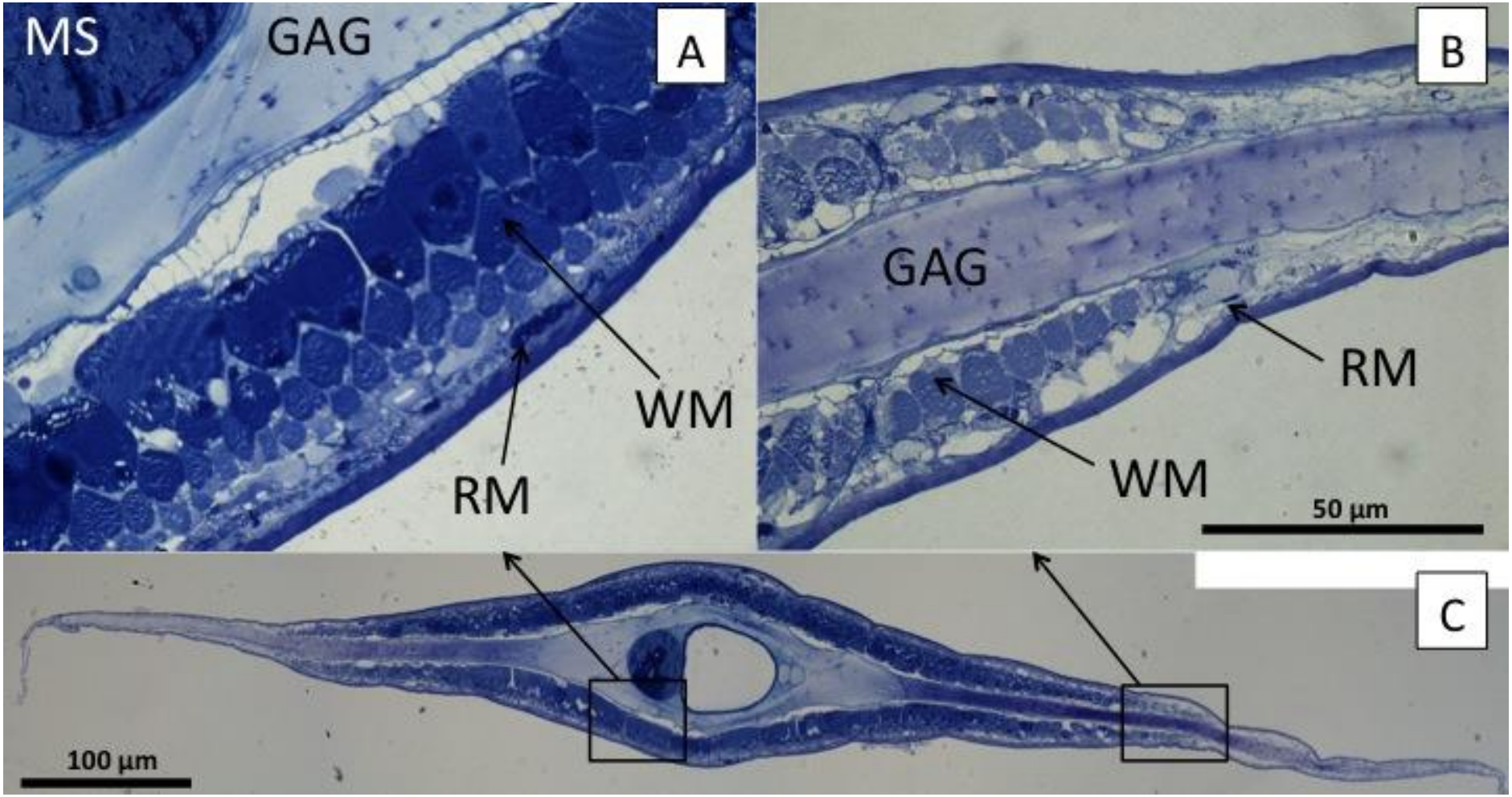

Figure 3.27: Muscle tissue at MTS-3 of larvae of the European eel (*Anguilla anguilla*) (L21, SL = 23.33). Several layers of large white muscle (WM) cells with one layer of smaller red muscle (RM) cells on the outside can be seen A: Muscle tissue in the middle of the section, a neuromast (Nm) and a nerve can also be seen. 100x magnification B: Growth zone, showing gradual increase in size of WM, 100x magnification.. C: overview of section, 10x2 magnification.. GAG = glycosaminoglycan layer, MS = medulla spinalis.

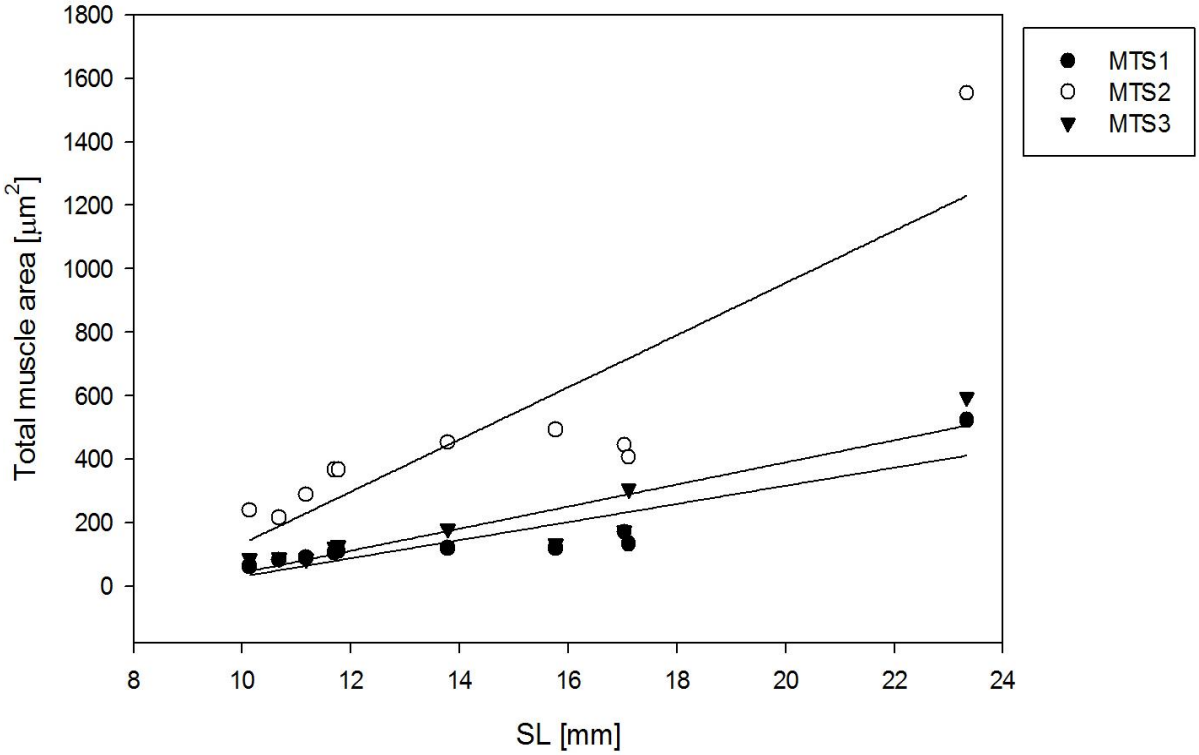

Figure 3.28: Total area of white muscle in one bilateral half of three main transverse sections (MTS) of larvae of the European eel (*Anguilla anguilla*) in relation to standard length (SL). Linear regression lines (y=ax+b) are added for each dataset. MTS1: R^2 = 0.7754. MTS2: R^2 = 0.7660. MTS3: R^2 = 0.8369.

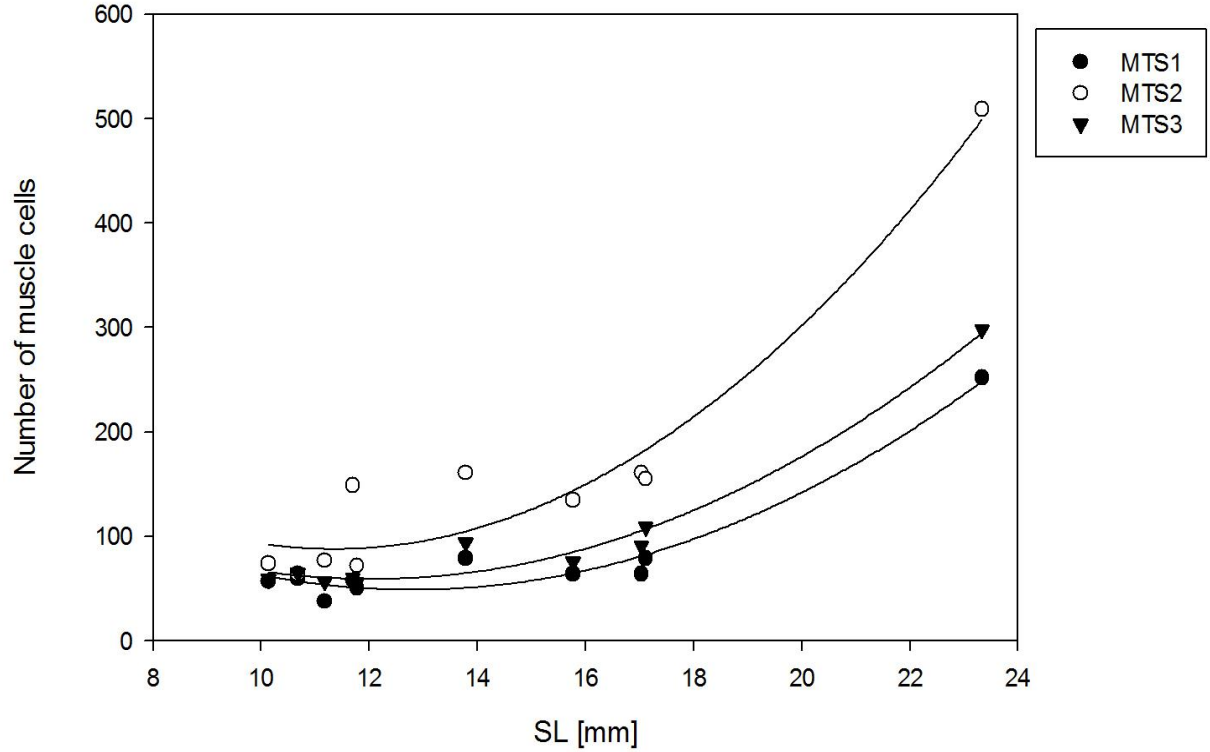

Figure 3.29: Total number of white muscle in one bilateral half of three main transverse sections (MTS), of larvae of the European eel (*Anguilla anguilla*) in relation to standard length (SL). Quadratic regression curved ($y=y0+ax+bx^2$) are added for each dataset. MTS1: R^2 = 0.9576. MTS2: R^2 = 0.9379. MTS3: R^2 = 0.9745. Be aware of missing data-points between SL 18 and 23.

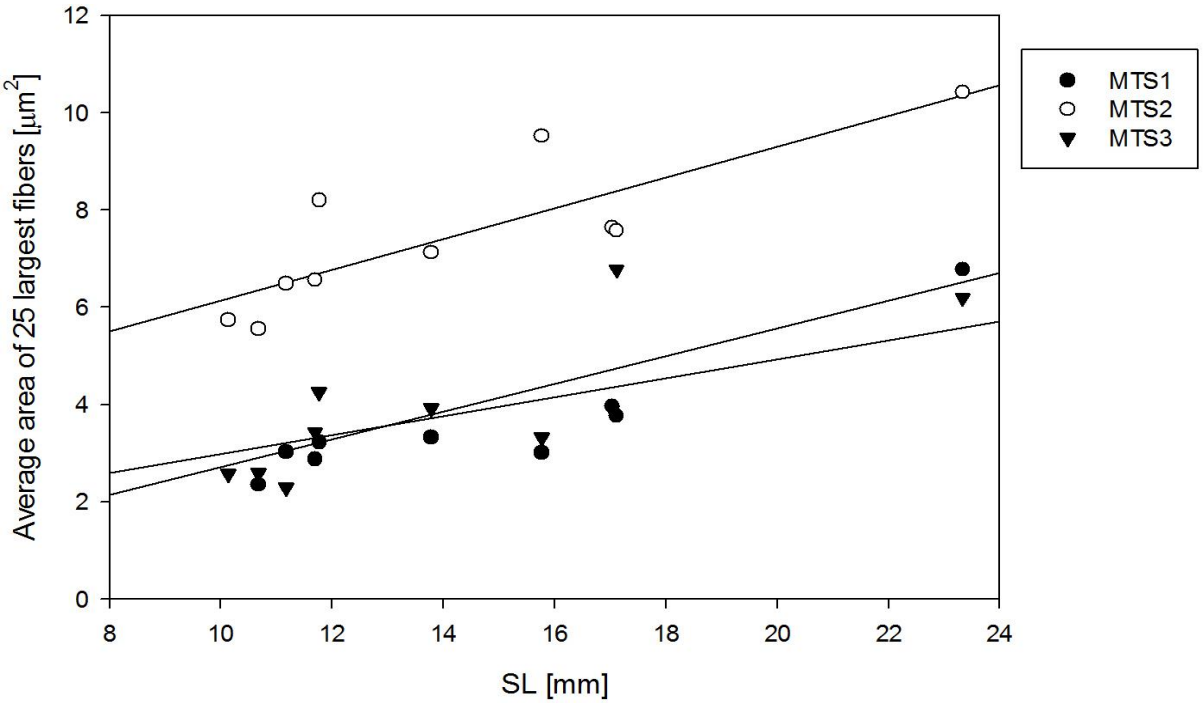

Figure 3.30: Average area of the 25 largest white muscle cells in one bilateral half of three main transverse sections (MTS), of larvae of the European eel (*Anguilla anguilla*) in relation to standard length (SL). Linear regression lines are added to the data, but note that these are not significant for b (y=ax+b).

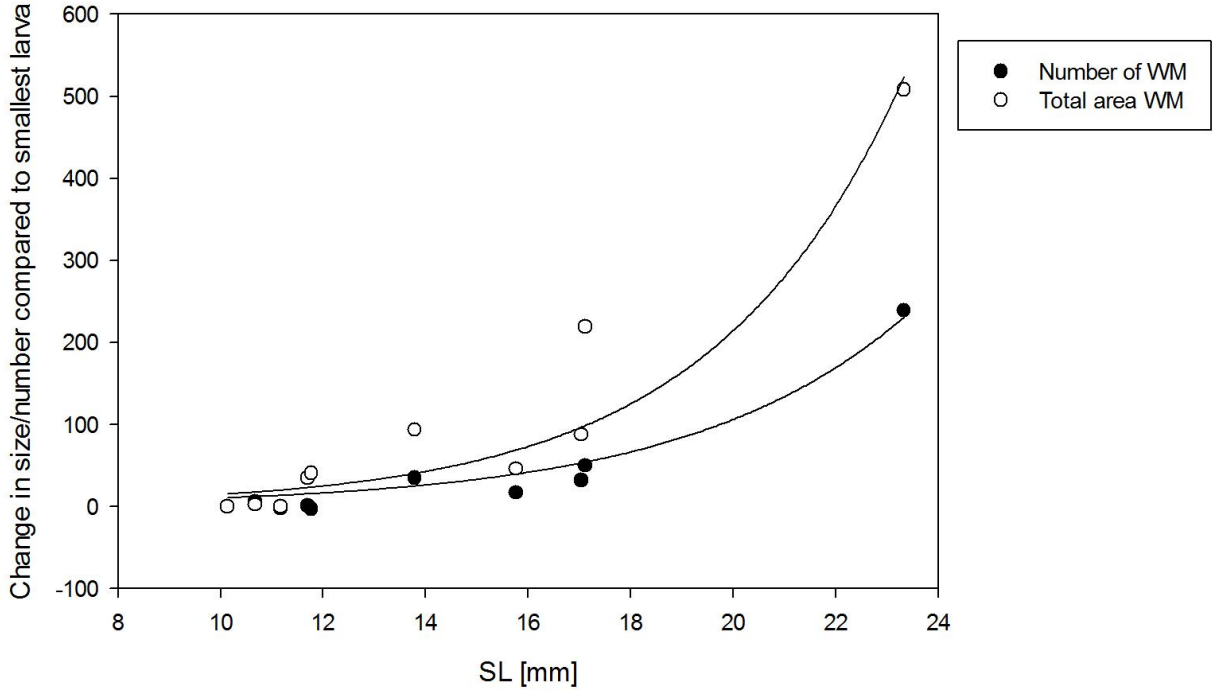

Figure 3.31: Relative growth (compared to smallest larva) of muscle of larvae of the European eel (*Anguilla anguilla*) in relation to standard length (SL). WM = white muscle. Exponential growth regression curves (y=exp(ax)) are fitted to the data. Number of WM (R^2 = 0.9569), total area of WM (R^2 = 0.9119).

3.4 Other organs

3.4.1 Head

Most of the major organs, except from the digestive system and the axial muscle are found in the head region. From the longitudinal section, it can be observed that the jaws consist of a major cartilage proportion (Figure 3.32), The mouth has a large opening, and a tongue like structure consisting of connective tissue can be seen in the base of the mouth, right beneath the eye. There is an opening to the nostrils on the side of the eye, and the brain has numerous foldings and lobules. The branchial arches can be seen a bit behind and below the eye. The esophagus is surrounded by a thick muscle layer, and it is even possible to see the rough-textured epithelium on the longitudinal section. The GAG can also be observed, and right behind it is a section of the beginning of the axial muscle layer (Figure 3.32).

3.4.2 Eyes

In all larvae, the retina was still making up most of the area of the eye (Figure 3.33 and 3.34). All larvae had developed pigmented epithelium (PE) in the retina. This pigmented epithelium was also very visible before the staining with toluidine blue, and appeared to have a dark coloration with a slightly brown or yellowish hue. This was too observed in the fresh specimens, as mentioned in 3.1. A thick photoreceptor layer (PRL) was present in all larvae, but it was not possible to distinguish between rods and cones, so it is uncertain whether or not both cones and rods were present in the leptocephalus larval retina (Figure 3.33 and 3.34) Covering the photoreceptor layer was the outer limiting membrane (OLM) (Figure 3.34), followed by the outer nuclear layer (ONL), plexiform layer (PL) and finally the inner nuclear layer (INL) (Figure 3.33 and 3.34).

There was only one plexiform layer present in the retina, so there is not differentiated between outer and inner plexiform layer in the figures. In the center of the eye was a large lens (Figure 3.33 and 3.34). For all size groups, except from the larges specimen (L21, SL 23.33 mm), the lens was still connected to the retina. Lens separation, and formation of the vitreous body was only found in L21 (Figure 3.34).

Patches of cartilage surrounded the eyes of all larvae. And in some sections it was also possible to see the optical nerves, as a thick structure stretching from the lens and out of the eye towards the brain (Figure 3.33). It was not observed any defined eye muscles.

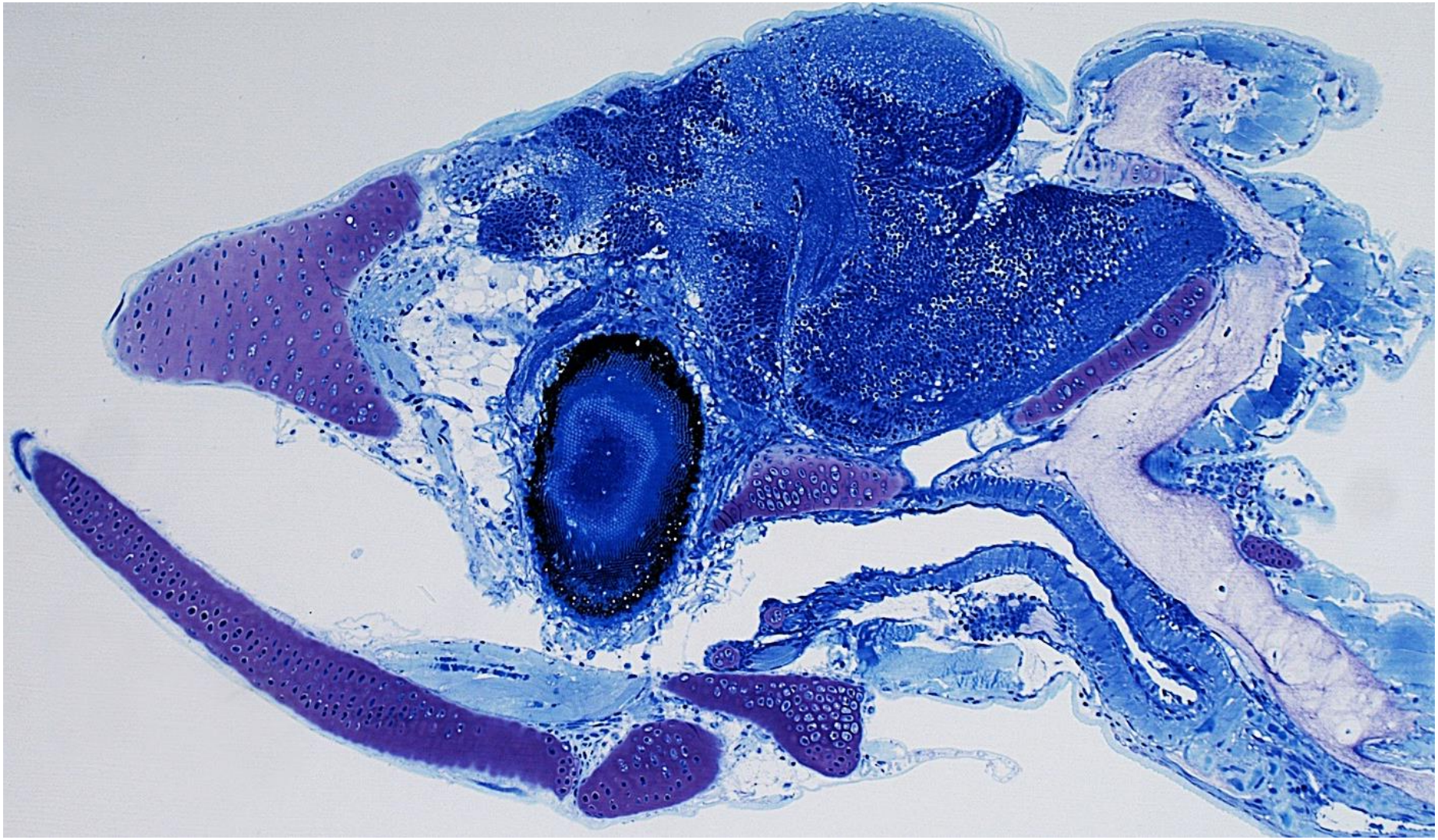

Figure 3.32: Longitudinal section of the head of a leptocephalus larva of the European eel (*Anguilla anguilla*) embedded in Technovit.

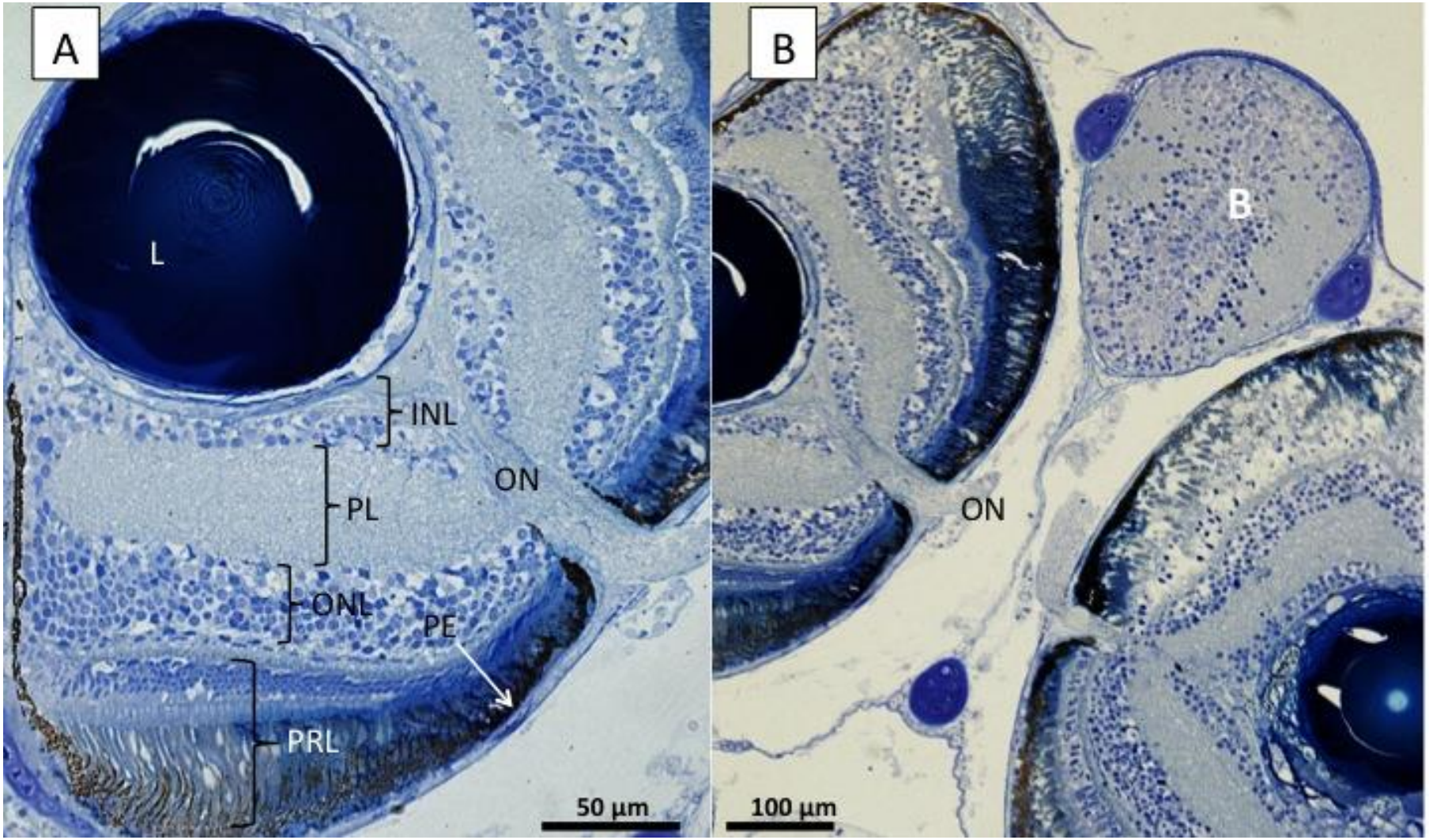

Figure 3.33: Transverse section through the lens of the eye of larva of the European eel (*Anguilla anguilla*) (L12 SL 11.77). A: Details of the retina of the eye, showing the lens (L), inner nuclear layer (INL), plexiform layer (PL), outer nuclear layer (ONL), photoreceptor layer (PRL), pigmented epithelium (PE) and the optical nerve (ON) going into the eye. 40x magnification. B: Overviwew of the eye, showing how the optical nerve leaves the eye, 16 x magnification.

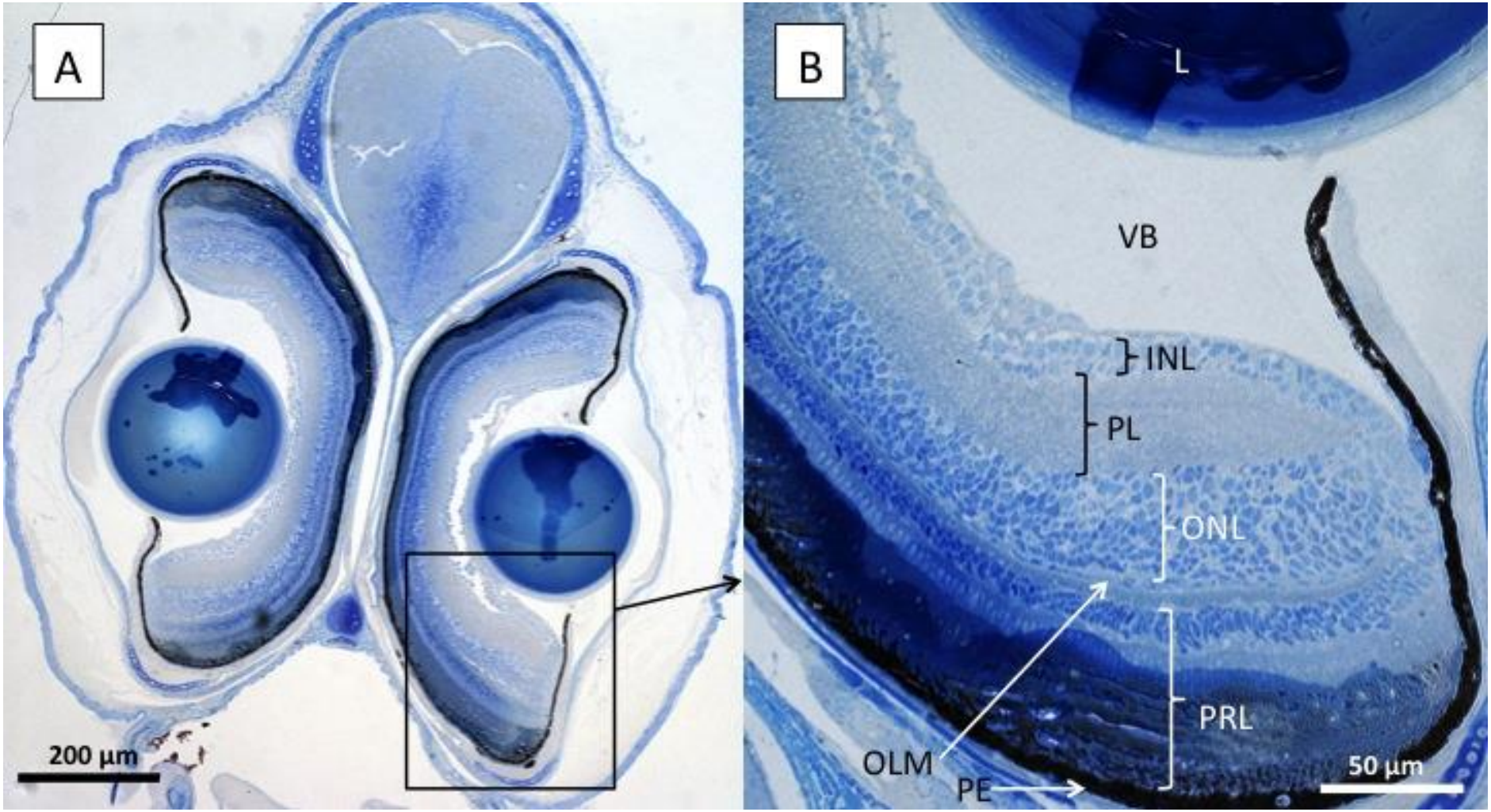

Figure 3.34: Transverse section through the lens of the eye of a leptocephalus larva of the European eel (*Anguilla anguilla*) (L121 SL 23.33). A: Overviwew of the section, showing the separation of the lens. 10x magnification. B: Retina of the eye, showing the lens (L), inner nuclear layer (INL), plexiform layer (PL), outer nuclear layer (ONL), photoreceptor layer (PRL), pigmented epithelium (PE), outer limiting membrane (OLM) and the vitreous body (VB) 40x magnification. 40x magnification.

3.4.2 Chemical receptors: Smell and taste

The nostrils were only investigated for the largest larva, so it is not possible to say anything about the olfactory organ of the smaller larvae. The nostrils of the larvae were lined with a well-developed olfactory epithelium Figure 3.35). The olfactory epithelium appeared to consist of columnar epithelium, with a layer of cilia/microvili covering the external surface. It is not possible to determine if there are cilia or microvilli with this magnification. The olfactory bulbs was also well defined in the brain (see Figure 3.42)

In the mouth cavity there was observed a structure that may be the tongue of the larva (Figure 3.36). Dispersed especially in this tissue were a series of bud-like structures that resembled taste buds. It is however difficult to say without higher magnification. These bud-like structures were found all around in the epithelium in the mouth, but especially in the tongue-like tissue. This was found for all size groups.

3.4.3 Mechanoreceptors: Ears and neuromasts

The ear of the leptocephalus larvae appeared as two cavities located laterally on the head, starting about right behind the eye and ending just before the base of the pectoral fins (Figure 3.37). The ears of all larvae, except the largest specimen, had an oblong shape. The ear cavity of the largest specimen had a more round shape. Nerve-endings were found underneath the external surface of the ear (Figure 3.37-B). Hair cell was observed in the ear of all larvae, especially at the lower base of the enclosed ear. Otoliths were observed in some of the larvae, appearing as strongly colored, layered and round structures inside the ear (Figure 3.37-C).

Neuromasts were observed scattered along the lateral sides of the body, approximately next to the medulla spinalis and notochord (Figure 3.22, 3.23 and 3.25). The base of the neuromasts appeared as small bundles, right below the skin and above both the layer of red and white muscle. The neuromasts were often quite damaged, and was clearly best preserved in the largest specimen (Figure 3.23 and 3.25)-

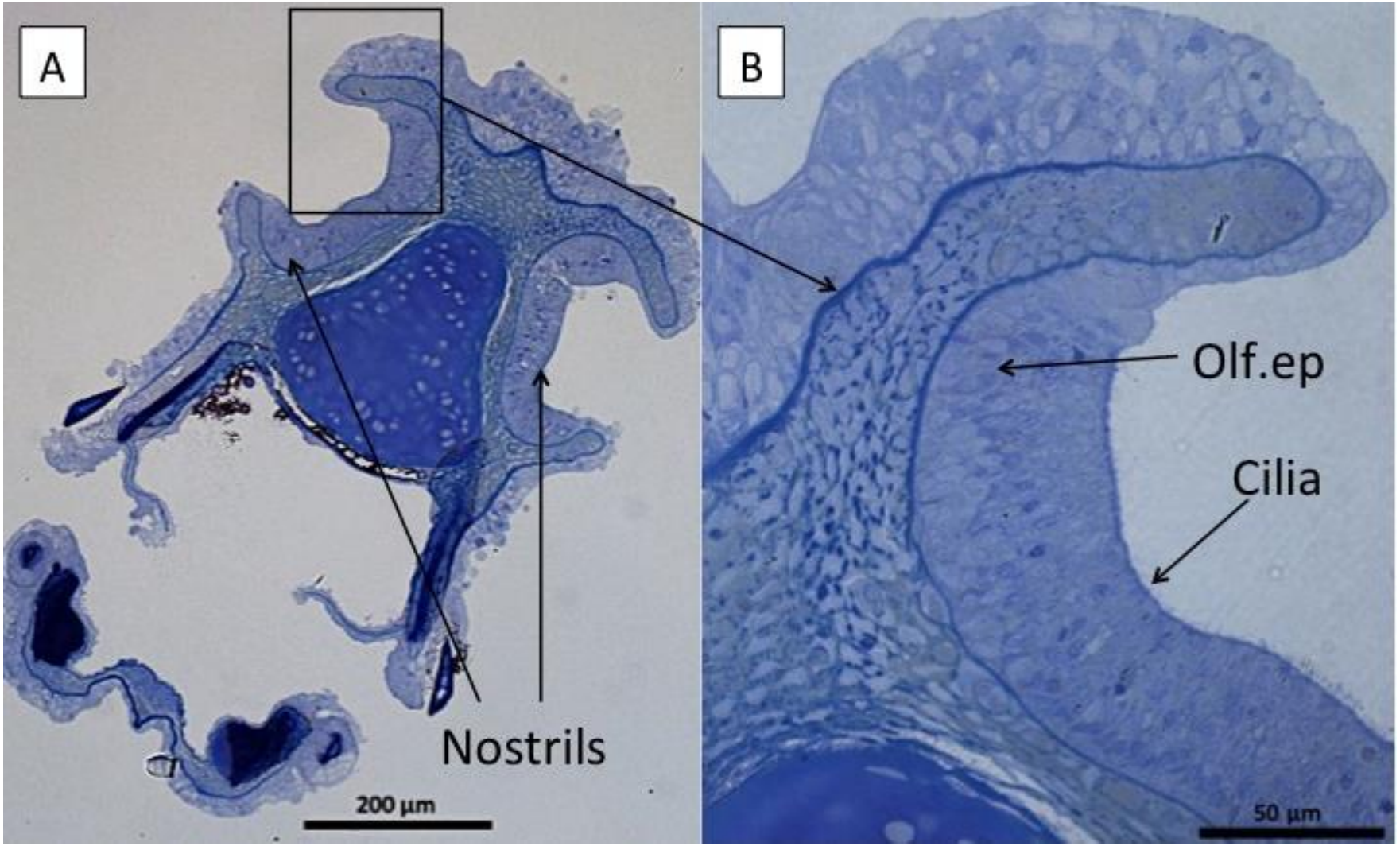

Figure 3.35: Transverse section through the snout larva of the European eel (*Anguilla anguilla*) (L21 SL 23.33). A: Nostrils of larva, 10x magnification. B: Magnified nostril showing the columnar olfactory epithelium (Olf.ep) with a cilia like surface and continuum with the cell layers on the skin, 40x magnification.

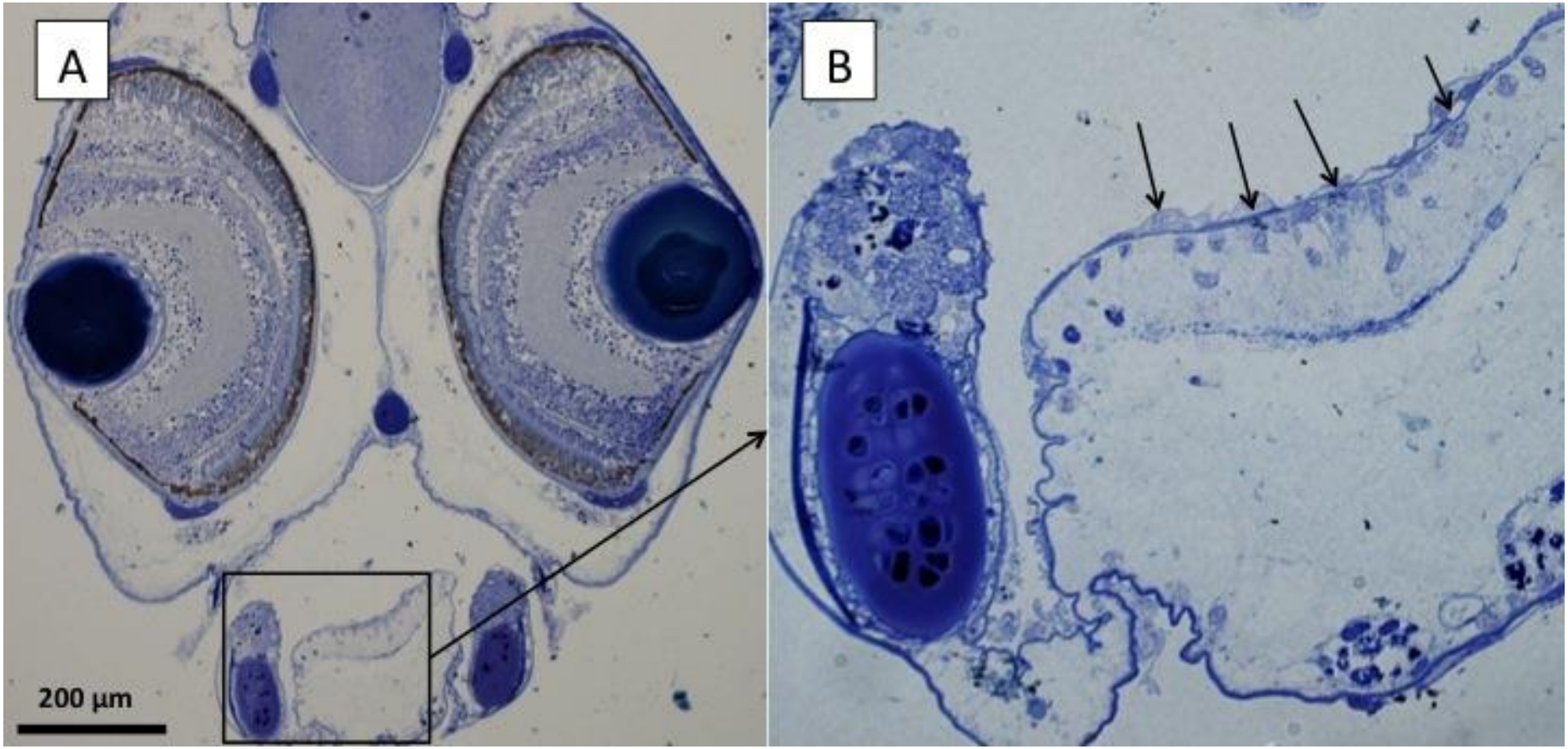

Figure 3.36: Transverse section through the head larva of the European eel (*Anguilla anguilla*) (L16 SL 13.78), showing the mouth and what appears to be the tounge with taste-bud-like structures. A: Overview of section, showing the mouth of the larvae, 10x magnification. B: Tongue-like structure found in larvae, with what appears to be taste buds indicated with arrows. 40x magnification

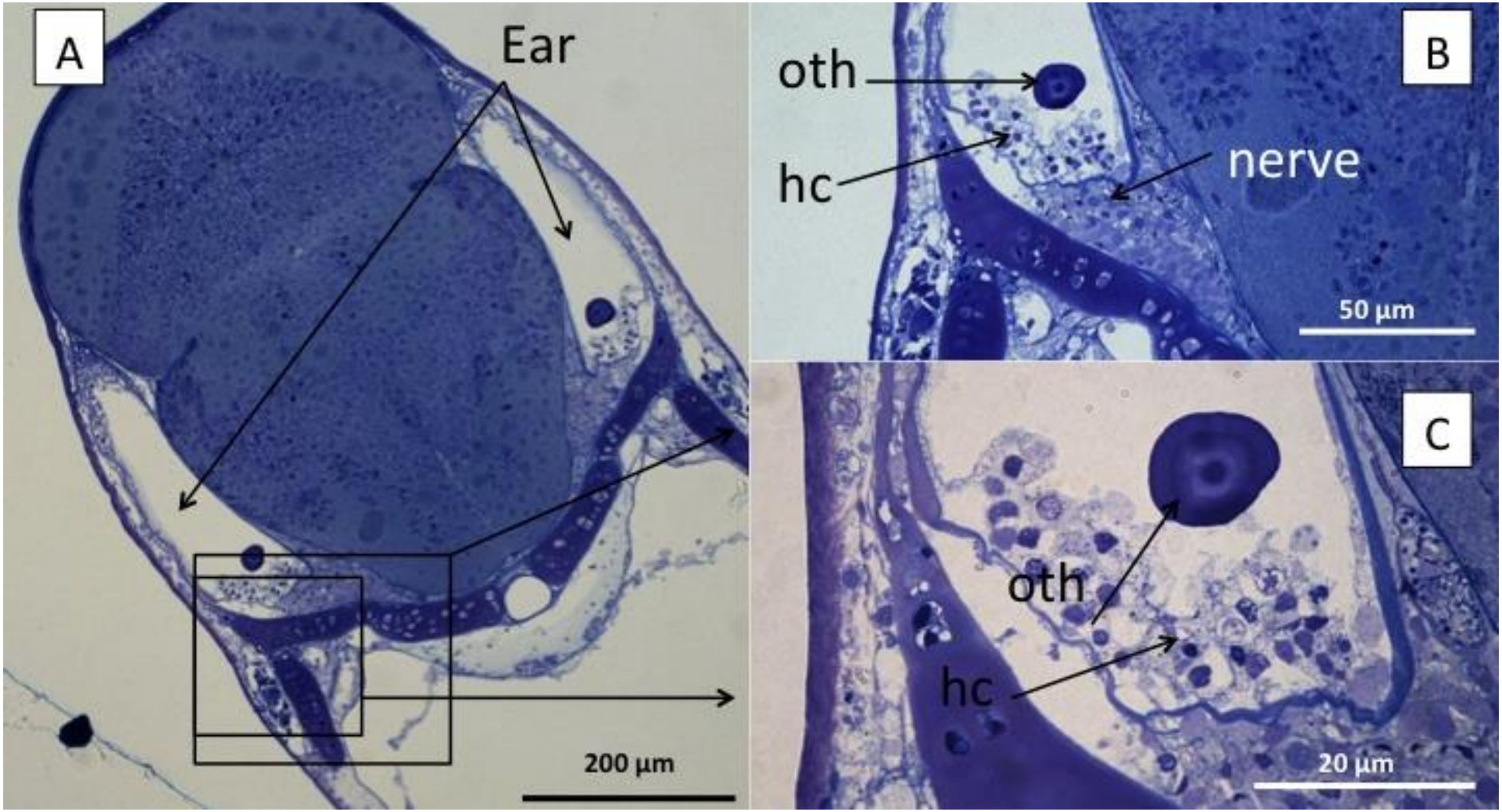

Figure 3.37: Transverse section through the head larva of the European eel (*Anguilla anguilla*) (L19 SL 17.11), showing the ear. A: Overview of section, showing the ears of the larvae on each side of the brain, 10x magnification. B: 40x magnification of the ear, showing the otholith, hair cells and nerve ending connected to the ear. C: 64x magnification, showing the hair cells and layered structure of the otoliths.

3.4.5 Gill structures

In all larvae, there were observed branchial arches in the head, with the main proportion in close proximity to the heart (Figure 3.38-A). The branchial arches could be seen as purple stained structures, indicating that they consist of cartilage, and with more or less undefined soft tissue around. In the area where the brachial arches were found, there was also an extension of the mouth cavity into the opercular cavity, which was extended both ventrally and dorsally (Figure 3.38-A).

The branchial arches appeared quite similar for all size groups, with the exception of the largest larvae (L21, SL 23.33 mm). In the smaller size groups, the gill structures that were found appeared to consist mainly of the branchial arches, located laterally on each side of the mouth (Figure 3.38-A). The branchial arches and the surrounding tissue, opened up from the mouth and into the opercular cavity. The opercular cavity opened up through a narrow slit in the on the ventral side of the leptocephalus larvae (Figure 3.38-A). The tissue around the heart and opercular cavity was largely vascularized, and several large blood vessels could be observed. In the largest specimen, the branchial arches were located on the ventral side of the mouth and the tissue also appeared to be more organized. Structures with a lamellae-like appearance, although with a primitive appearance were also observed. These structures were located at the dorsal side of the opercular cavity (Figure 3.38-B and 3.39). The area around the heart and opercular cavity of the largest specimen was also highly vascularized, with several large blood vessels both in the lamellae-like structures, and around the opercular cavity itself. No functional gills were observed in any larvae.

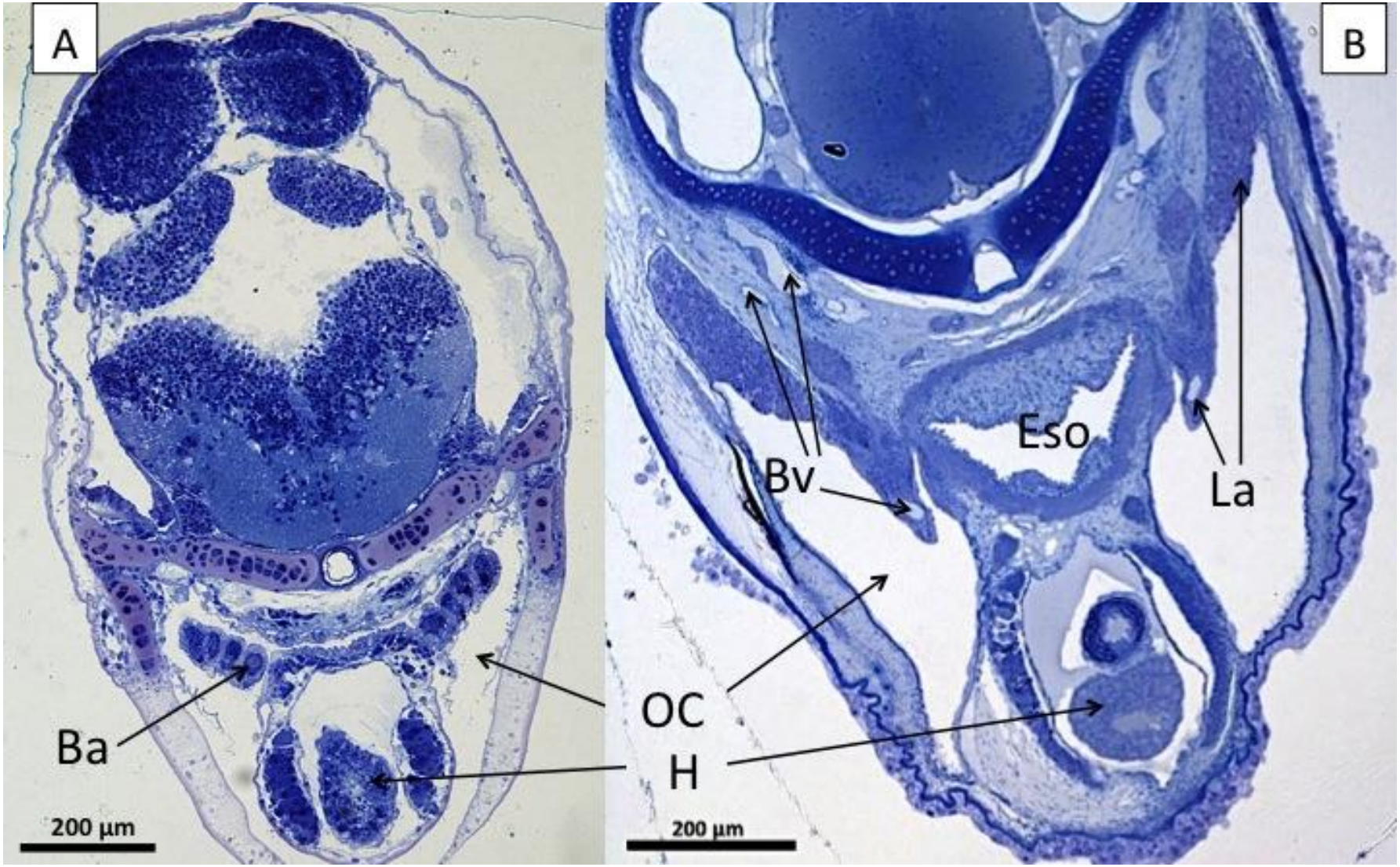

Figure 3.38: Transverse section through the opercular cavity (OC) of larva of the European eel (Anguilla anguilla). A: L11 (SL 11.69), 10x magnification, showing the OC with eight branchial arches (Ba). Section before esophagus (Eso). B: L21 (SL 23.33), 10x magnification, showing the OC with lamellae (La) like structures. Section at Eso. H = heart. Bv = blood vessel.

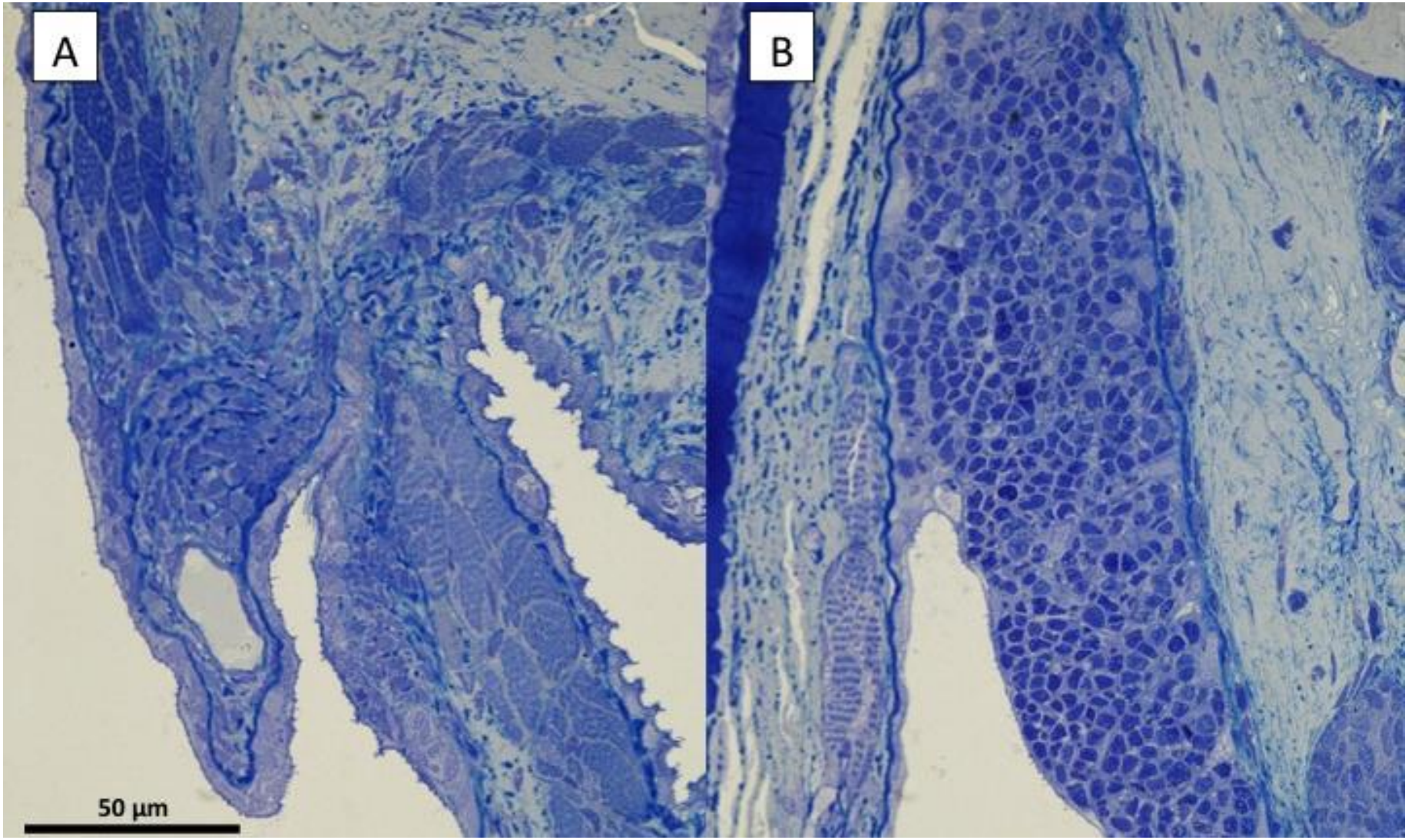

Figure 3.39: Transverse section through the opercular cavity (OC) showing details of lamellae-like structures of larva of the European eel (Anguilla anguilla) (L21, SL 23.33), 63x magnification. A: Lower lamellae (see lower arrow for La in Figure 3.38). A blood vessel can be seen in the middle of the structure. B: Upper lamellae (see upper arrow for La in Figure 3.38).

3.4.5 Circulation system

The heart of L21 was sectioned most thoroughly, and will for that reason be highlighted in this section. The heart appeared right below the opercular cavity, and was located ventrally to the anterior end of the esophagus. The frontmost part of the heart consisted of the bulbous aorta and the ventricle, enclosed in a liquid filled chamber surrounded by a thin layer of white muscle (Figure 3.40-B). It is not certain whether or not the layer of white muscle is part of the heart. The ventricle consisted of a thick layer of myocardium. The middle part of the heart was parted into the atrium and the ventricle, in other words. The myocardium in the atrium was thinner than in the ventricle (Figure 3.41). The heart ended in the sinus venosus (not seen in figure). In the smaller larvae, a bulbous aorta was located dorsally to an enclosed chamber surrounded by white muscle (Figure 3.40-A). It was not observed any definable myocardium in the smaller larvae, but they were also not sectioned as thoroughly as L21.

No spleen was observed in any of the larvae.

The blood had a colorless appearance and no pigmented blood cells could be observed in the heart of any of the vessels. Blood vessels were observed several places in the larvae. Located ventrally to the notochord, there could be seen two major vessels, with one smaller in the middle, following the notochord along the length of the larval body (Figure 3.23-C). The renal vessels were also connected to several renal arteries, which lead from the kidneys and up the notochord (Figure 3.16). The renal vessels could also be seen from the outside (Figure 3.3). The area around the opercular cavities and the anus appeared to be especially well vascularized (Figure 3.38-B and 3.21).

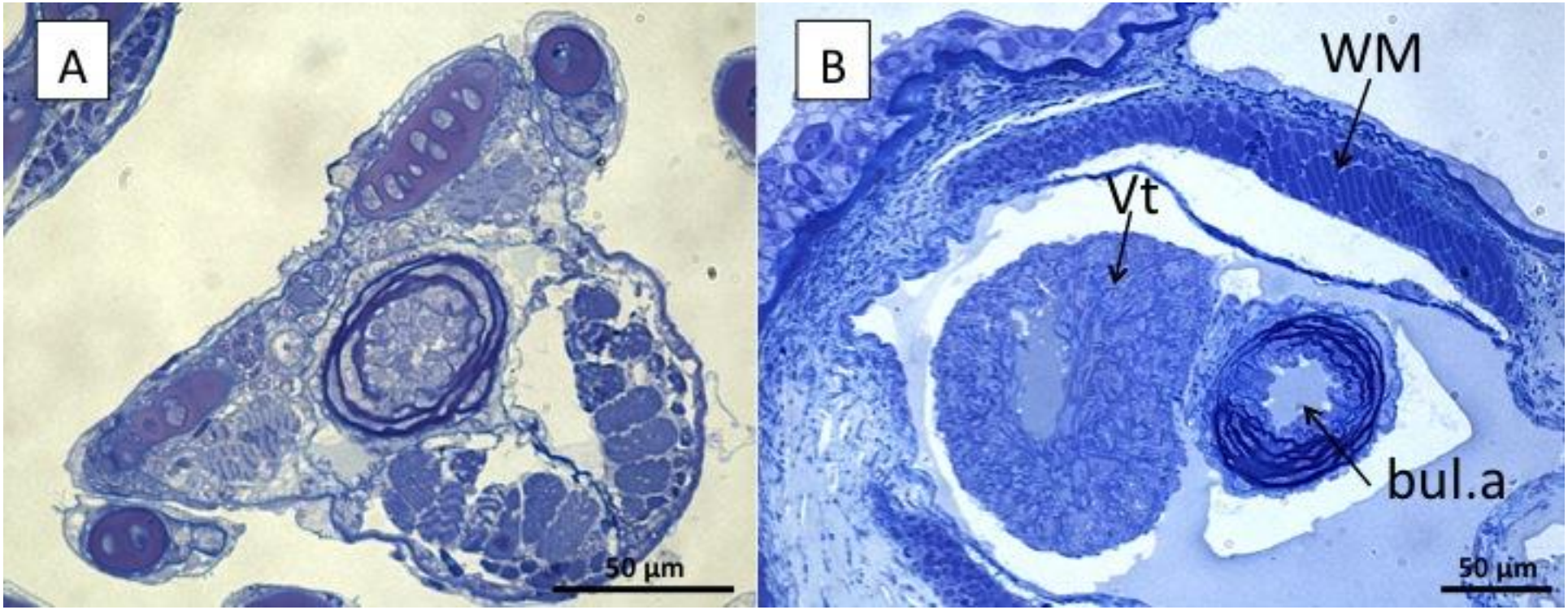

Figure 3.40: Transverse section through the heart of larva of the European eel (*Anguilla anguilla*) A: L12 (SL 11.77) showing the front part of the heart, with the bulbous aorta (bul.a) and structures of white muscle (WM) around. The cartilage structures (purple) are the branchial arches. 63x magnification. B: L21 (SL 23.33), showing the front part of the heart with a defined ventricle (Vt), bulbous aorta and surrounding white muscle tissue.

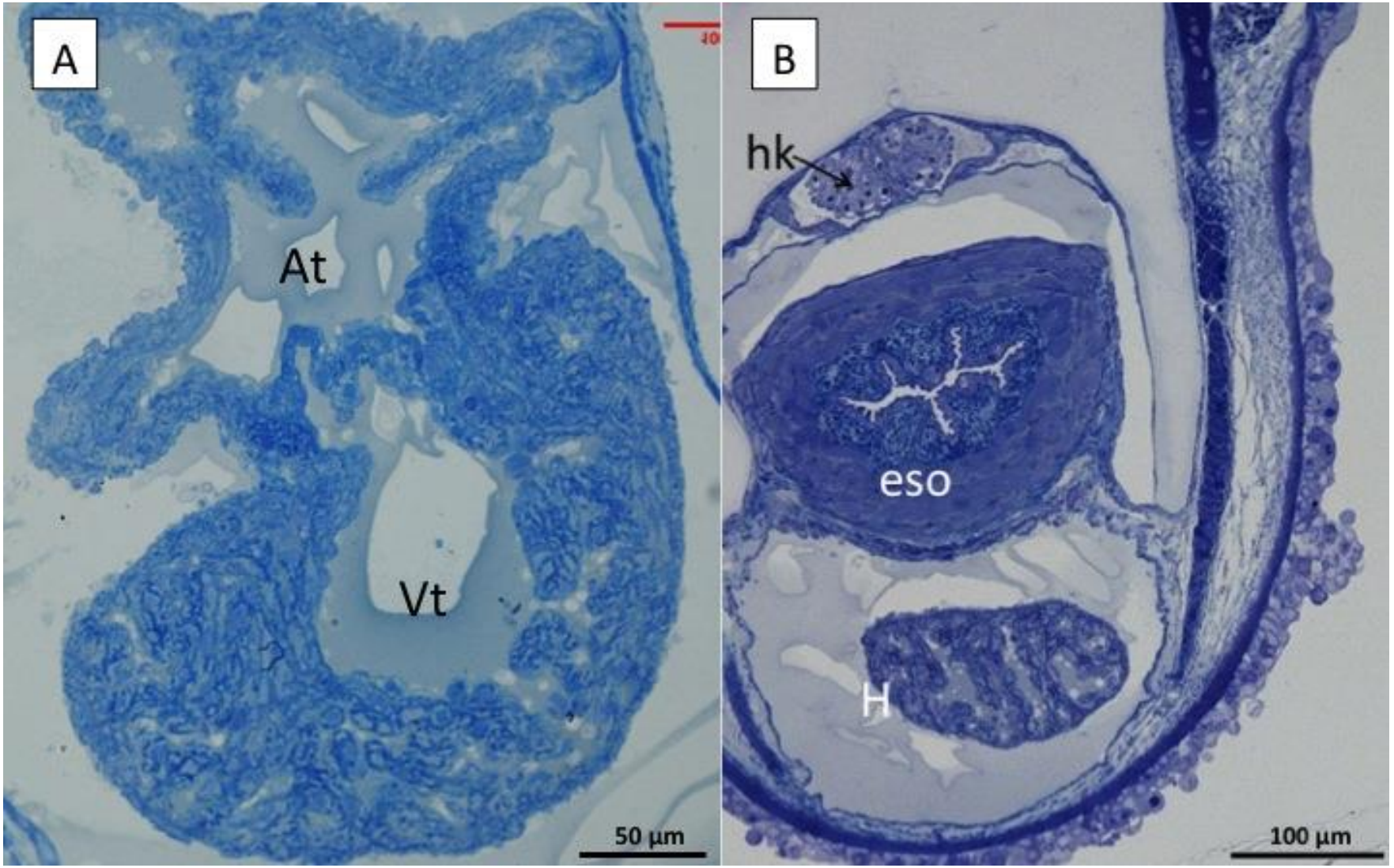

Figure 3.41: Transverse section through the heart of larva of the European eel (*Anguilla anguilla*) L21 (SL 23.33). A: Middle part of heart, with two chambers; the atrium (At) and the ventricle (Vt), 40x magnification. B: Back part of the heart. The esophagus (eso) and head kidney (hk) is also shown here. 16x magnification.

3.4.7 Nervous system

The brain of the leptocephalus larvae was highly folded, and could be parted into the main lobes of the fish brain. In the front-most part of the brain section, the olfactory bulbs were observed, enclosed in a protective cartilage cover (Figure 3.42). Below the optical lobes was a bundle of nerves, that is assumed to be the optical chiasm, based on the morphology and location (Figure 3.42).

Further backwards, the brain increased in size, and was parted into several lobes. The cerebrum (Figure 3.43), followed by the optical lobe, thalamus, hypothalamus and the hypophysis (Figure 3.44), then the cerebellum and finally the medulla oblongata which extended into the medulla spinalis (Figure 3.45). The medulla spinalis continued along the body, all the way to the tip of the tail.

Several neurons were observed in the leptocephalus larvae. In the head, the most prominent ones were the thick optical nerves extending from the eye, through the optical chiasma and to the optical lobe of the brain (Figures 3.42-C). The bundle of neurons leading from the ear to the thalamus (Figure 3.44-C) was also very visible in the head. In the rest of the body, the most visible neurons were those leading from the neuromasts to the medulla spinalis. The neuromasts and their nerve connections were scattered pairwise along the body of the leptocephalus larvae (see e.g. Figure 3.25).

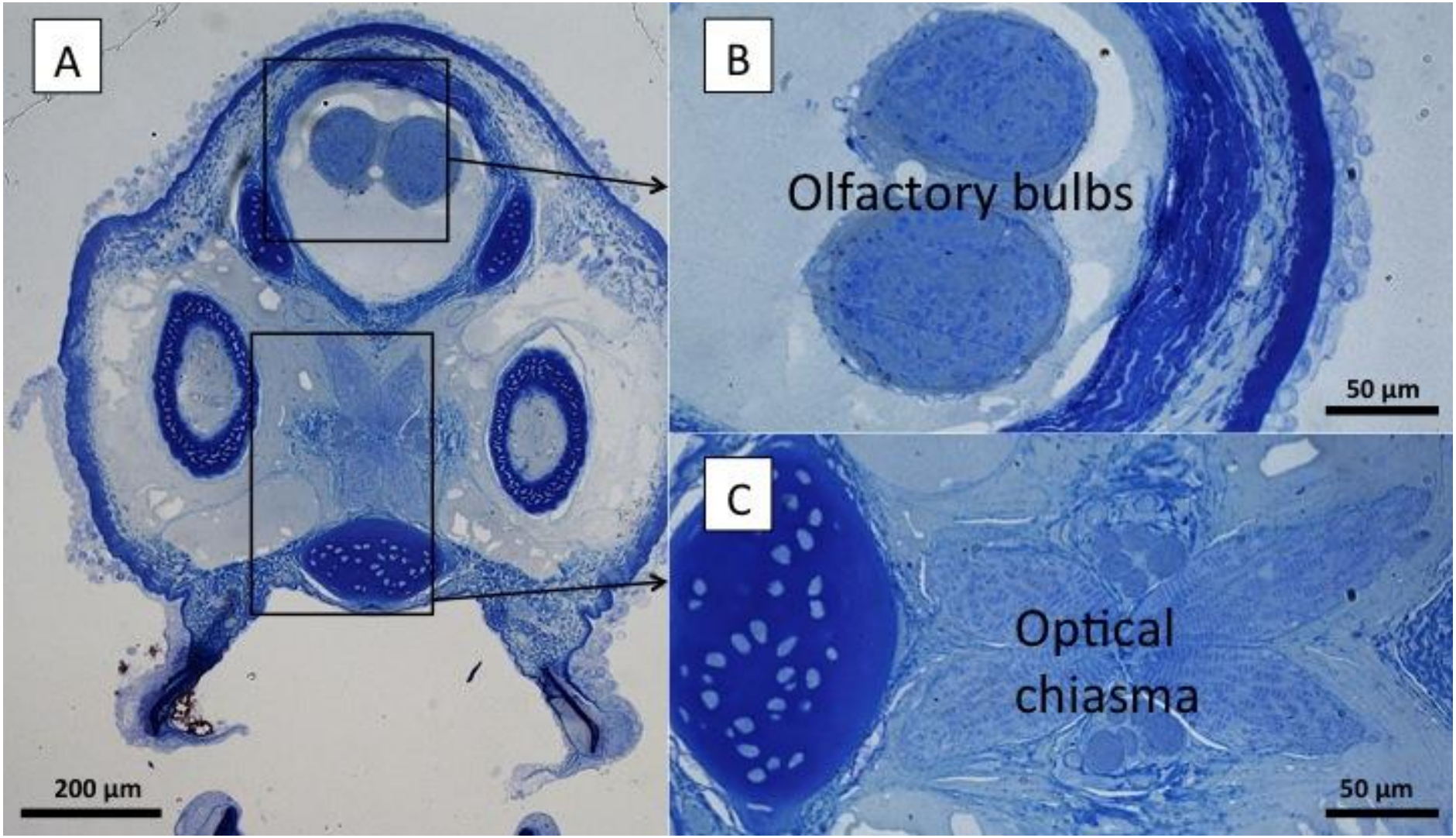

Figure 3.42: Frontmost part of brain of larva of the European eel (*Anguilla anguilla*) L21 (SL 23.33), showing the olfactory lobes and the optical chiasma. A: Overview of section, 10x magnification. B: Olfactory lobes, 40x magnification. C: Optical chiasma, 40x magnification.

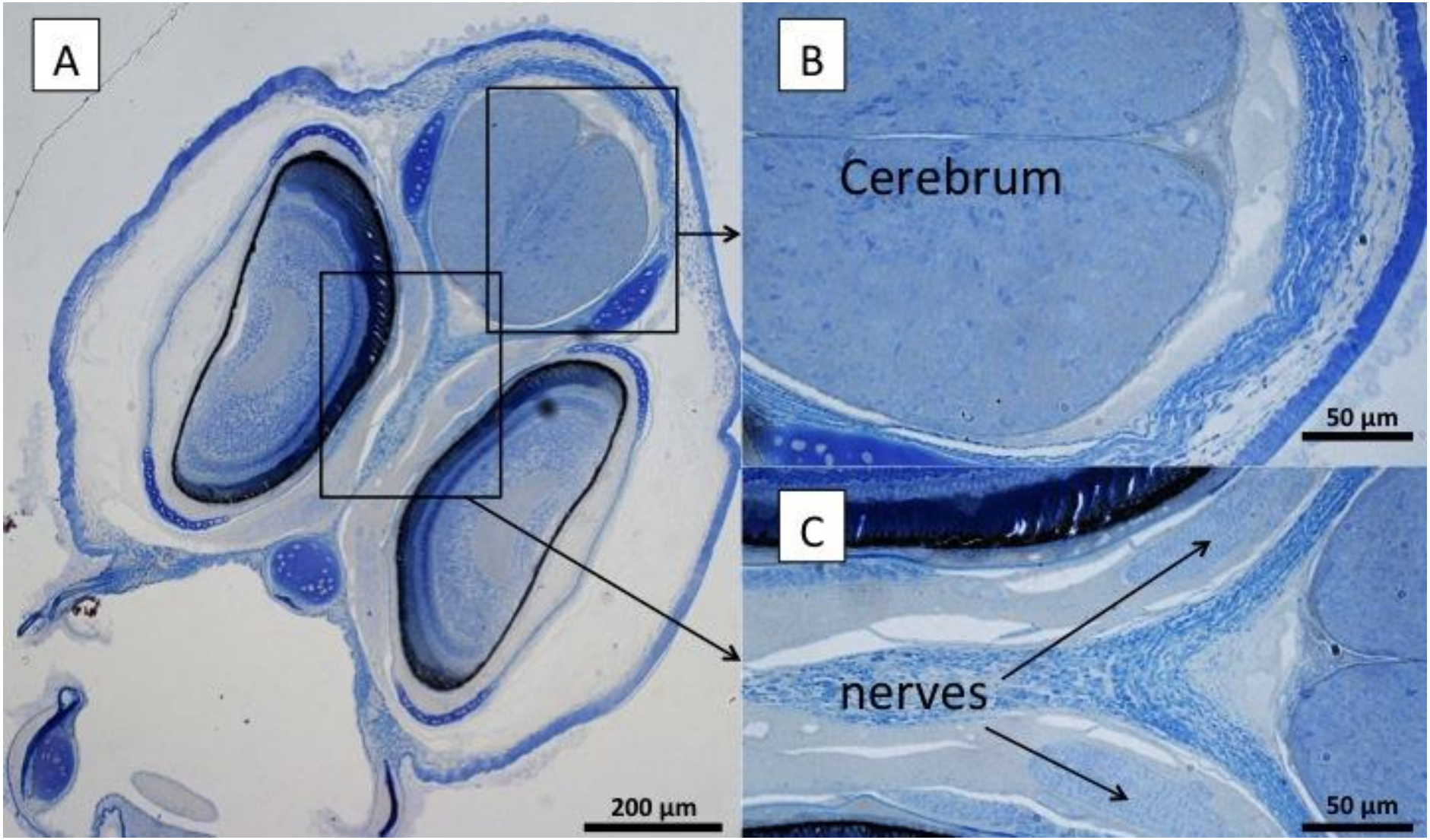

Figure 3.43: Brain of larva of the European eel (*Anguilla anguilla*) L21 (SL 23.33), at the beginning of the eye. A: Overview of section, 10x magnification. B: Cerebrum, 40x magnification. C: Nerves, 40x magnification.

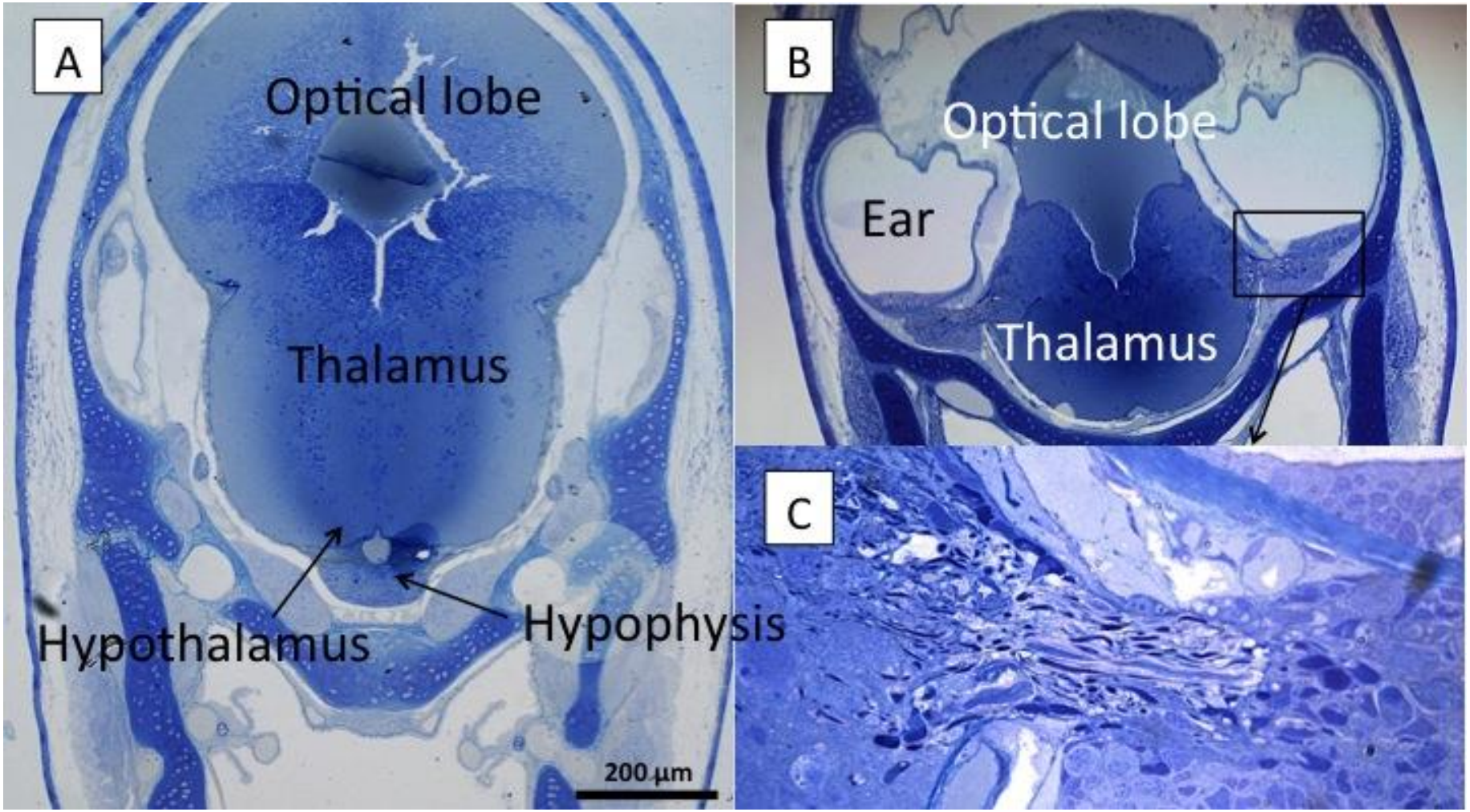

Figure 3.44: Middle part of brain of larva of the European eel (*Anguilla anguilla*) L21 (SL 23.33)A: 10x magnification, showing the large optical lobes, thalamus, hypothalamus and hypophysis. B: 16x magnification, showing the back of the optical lobe, thalamus and ear. C: Close up of nerve going from the ear and into the thalamus.

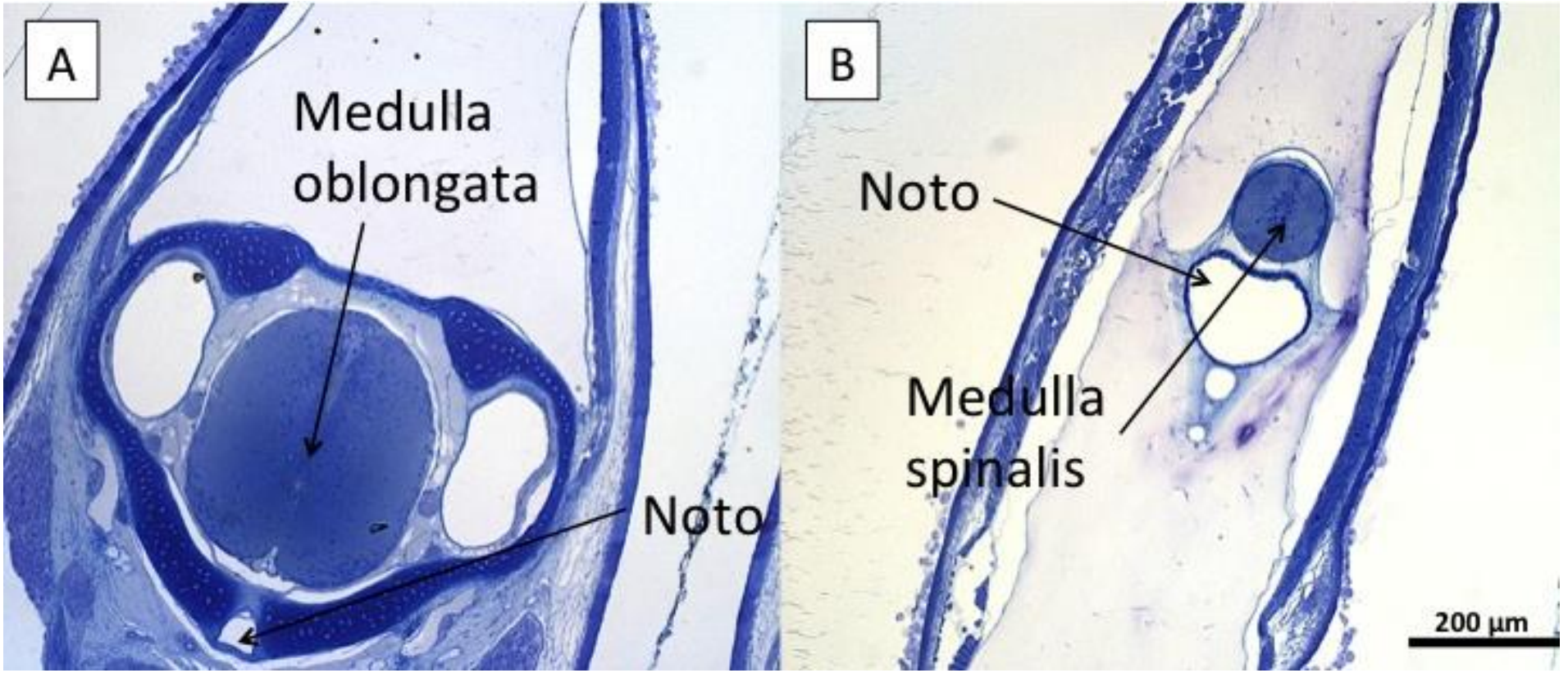

Figure 3.45: Back part of brain and medulla spinalis of larva of the European eel (*Anguilla anguilla*) L21 (SL 23.33)A: 10x magnification, showing the medulla oblongata and the beginning of the notochord (noto). B: 10x magnification, showing the medulla spinalis located dorsally to the notochord. Blood vessels can be seen located ventrally to the notochord.

3.4.8 Skin

The leptocephalus larvae were covered by a single layer of epithelium with a stratified appearance all over the body (Figure 3.46-A). Beneath the epidermis was a layer of what appears to be connective tissue, followed by the layers of muscle tissue. The only exception was some areas of the body where there was observed a layer of oval shaped cells on the outer surface of the epidermis. This can be seen e.g. in Figure 3.35, 3.38, 3.40, 3.41 and 3.42. The layer of oval shaped cells was between one to several cell-layers thick. It was only observed on the front part of the larval body, and most of it on the head. The presence of this layer in other areas than the head appeared patch-wise and discontinuously. The thickest layer of oval shaped cells was found on the snout (3.46-B), where it also was continuous with the olfactory epithelium in the nostrils (see Figure 3.35).

The oval shapes cells appeared to consist of at least three different types of cells. The first was large cells, with intermediate color, and a large nucleus and vacuole. These cells were assumed to be club cells. The second type were smaller cells with a lighter coloration. These cells were thought to be mucus cells. The third type were darker cells, with no distinct nucleus or vacuole. These were thought to be chloride cells. However, it is not possible to determine exactly which cell types that are present in this layer at this level of magnification.

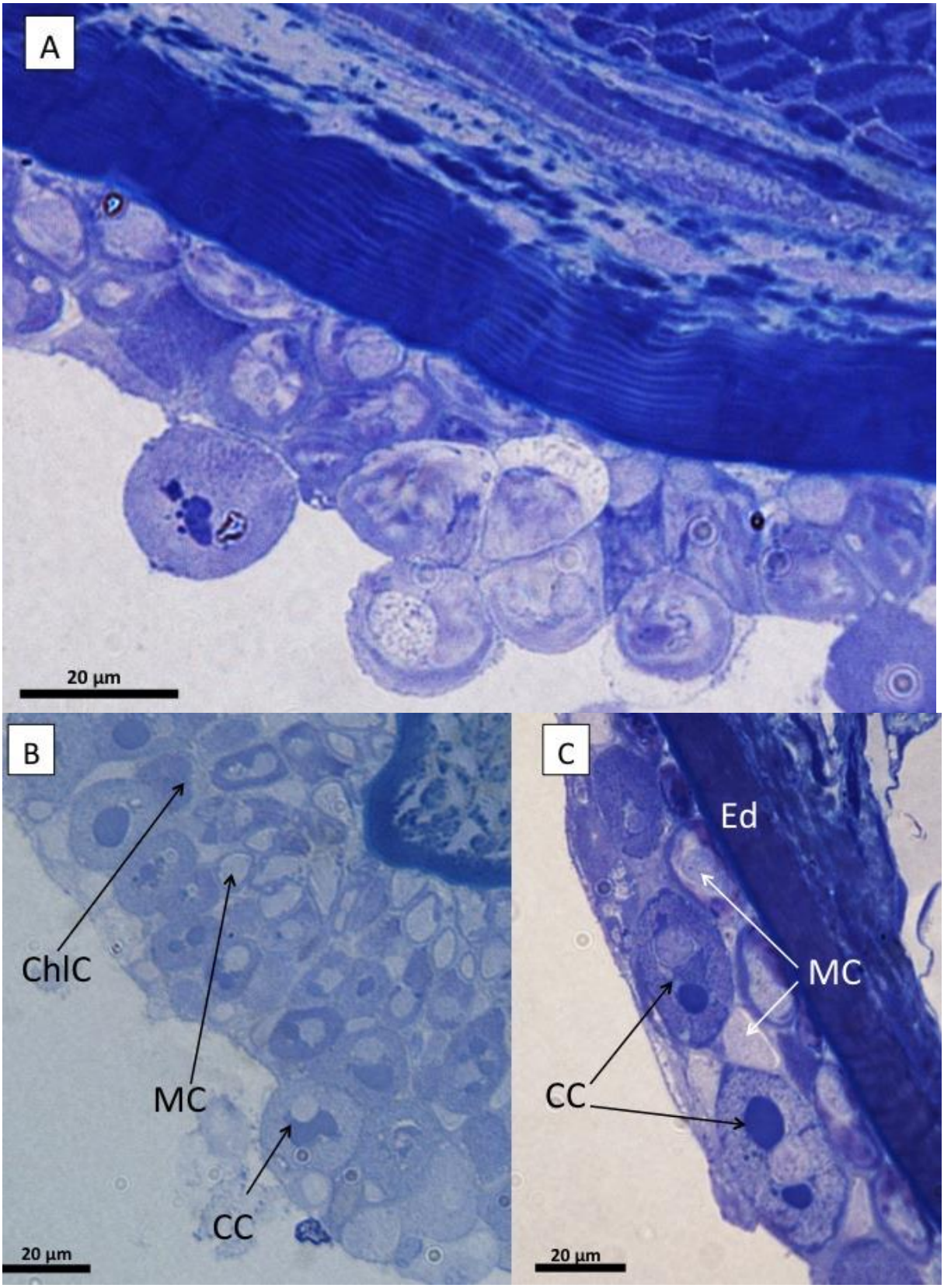

Figure 3.46: The skin of larva of the European eel (*Anguilla anguilla*) L21 (SL 23.33). A: 100x magnification, showing the epidermis and the external layer of oval cells. B: 63x magnification, showing the thick layer of oval shaped cells on the snout. The different cell types are thought to be club cells (CC), chloride cells (ChlC) and mucus cells (MC). C: Thinner layer of CC, ChlC and MC found on the head. Ed = epidermis.

3.4.9 Skeleton

Only limited parts of the larvae consisted of cartilage, and the main cartilage staining was found in the head; in the jaws, around the eyes, at the base of the pectoral fins and surrounding what may possibly be the opercular cavities (Figure 3.47 and 3.48). The largest of the bone and cartilage stained larvae (L20, SL = 18.85 mm) had some cartilage formation in the tail area that was not seen for the smaller larvae (L15, SL = 13.78 mm) (Figure 3.46-C). The smallest larvae had however some staining of cartilage diffusely around the body, that was not seen for the largest larva (Figure 3.47-A).

Bone staining was mostly found in the largest larva and only in the mouth (Figure 3.46-B). The two upper front teeth of the largest larva were calcified, as well as the tissue in the upper jaw where the teeth appear to emerge from (Figure 3.48-B). In the smallest larva there was only a tiny area in the upper jaw that was stained pink for bone (Figure 3.47-B).

The formation of finrays was also possible to see from the internal transverse sections. The finrays in the tail area of the largest larvae (L21) could be seen as very dark blue-stained structures in the tail area (Figure 3.49-A). This was only observed in the largest specimen. The finrays in the pectoral fins was stained dark blue, and had a porous appearance (Figure 3.49-C). This was observed for larvae of all size groups.

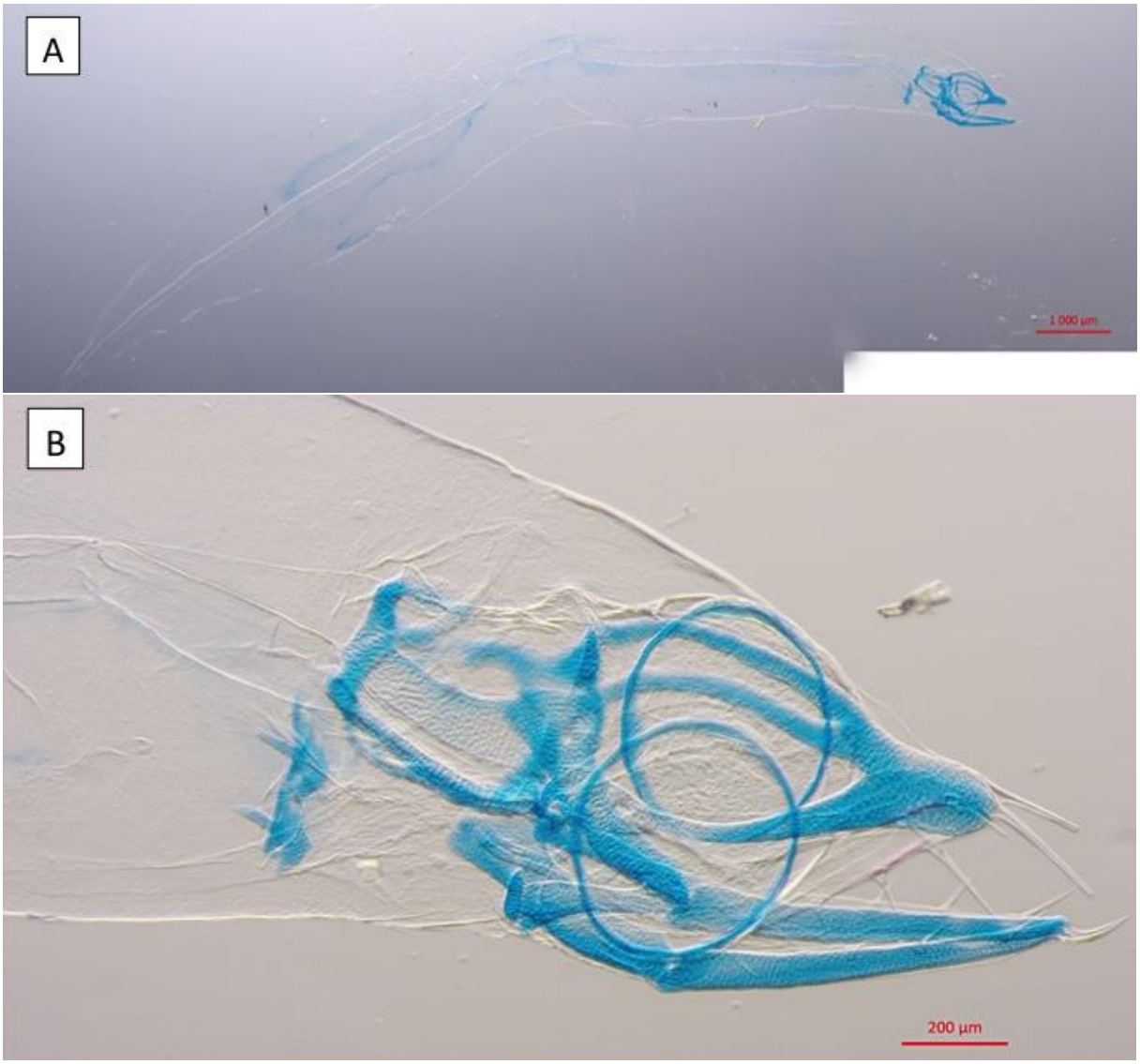

Figure 3.47: Presence of bone (pink) and cartilage (blue) in larvae of the European eel (*Anguilla anguilla*) (L15, SL = 13.78). A: Overview of whole larvae, showing main presence of cartilage in the head area, and some slight staining in the rest of the body, including in the intestine at the anus. B: Head of larvae, showing main staining of cartilage structures in the jaws, around the mouth, in the opercular cavity, around the brain and at the base of the pectoral fins. Slight staining of bone at the base of the teeth in the upper jaw can also be seen.

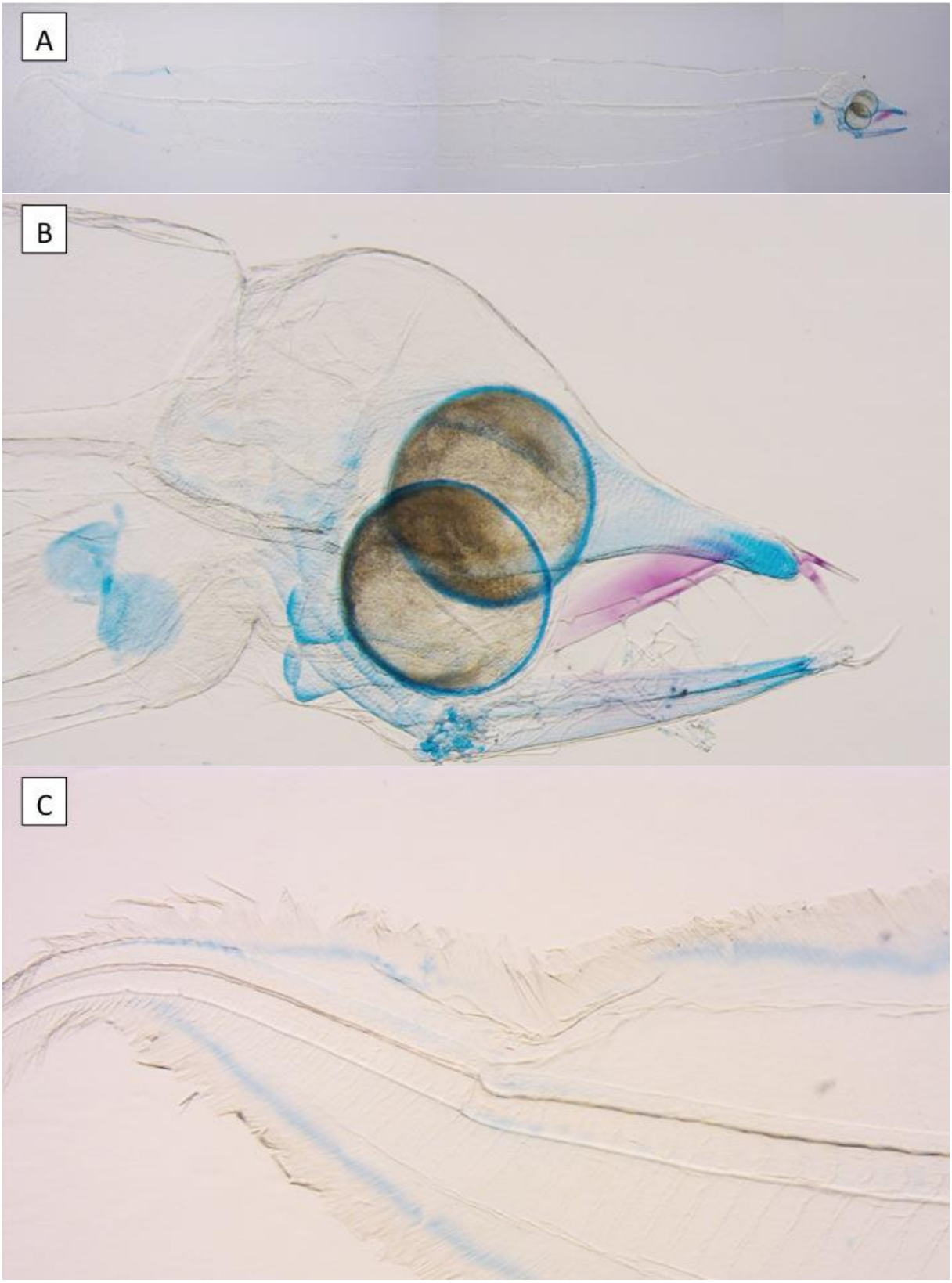

Figure 3.48: Presence of bone (pink) and cartilage (blue) in larvae of the European eel (*Anguilla anguilla*) (L20, SL = 18.85 mm). A: Overview of whole larvae, showing main presence of cartilage in the head area. B: Head of larvae, showing main staining of cartilage structures in the jaws, around the mouth, in the opercular cavity, around the brain and at the base of the pectoral fins. Staining of bone at the base of the teeth and in the front teeth in the upper jaw can also be seen. C: Cartilage staining in the tail area.

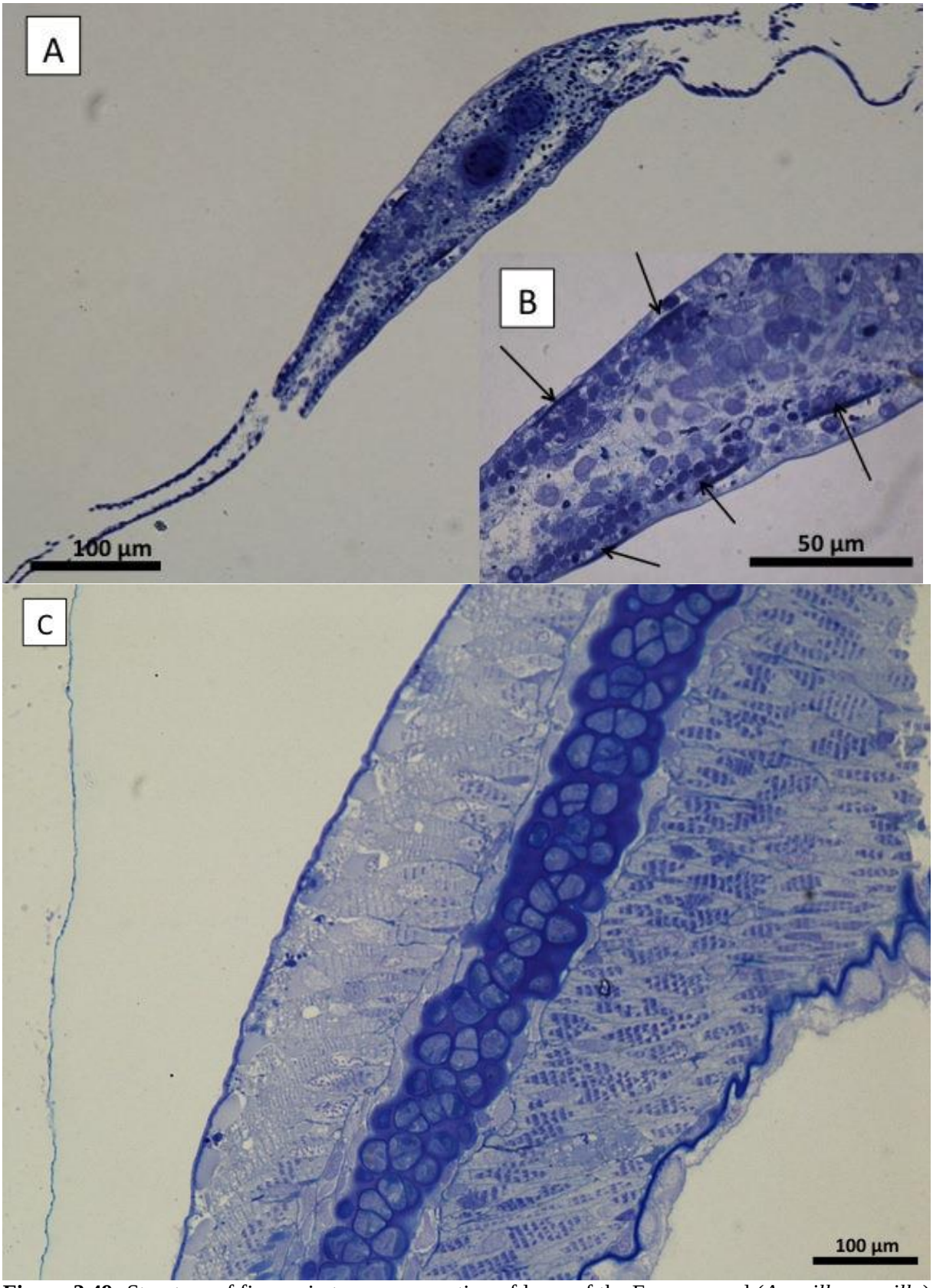

Figure 3.49: Structure of finrays in transverse section of larva of the European eel (*Anguilla anguilla*) L21 (SL 23.33). A: Tail of larva, 16x magnification. B: Finrays indicated with arrows, 100x magnification. C: Fin-ray in pectoral fin, 63x magnification.

3.4.10 Glycosaminoglycan layer

The glycosaminoglycan (GAG) layer can be seen in several of the previous figures, but is maybe best presented in Figure 3.22-27, in the muscle section. The GAG layer consisted of most of the internal area of the body of the leptocephalus larvae. It was stained slightly purple, compared to the blue staining of most of the other tissue. GAG was found all over the body of the leptocephalus larvae, and the organs were dispersed in the layer. In many of the sections, the GAG was however badly preserved, and appeared to have been subjected to significant shrinkage in most of the larvae (see Appendix 8).

The GAG could first be seen at the dorsal side of the head approximately right behind the eye of the larvae (see e.g. Figure 3.32). Protuding backwards, the GAG area increased until it made up most of the area of the leptocephalus larval body. In some areas, the GAG appeared to have an almost crystalline appearance (Figure 3.27-C), and in others a network of thin lines could be observed in the layer (see e.g. Figure 3.24-C). Other than this, the GAG was uniform in appearance, except from the areas with significant shrinkage where the shrunken area sometimes was stained darker than the rest (see e.g. Figure 3.23-C).

4. Discussion

4.1 – Size based estimation of age

The size of the leptocephalus larvae ranged from 6.97-23.33 mm standard length (SL) (Table 3.1). In their review of the life cycle, evolution and reproduction of the European eel, van Ginneken and Mayes (2005), estimated that larvae of the European eel <7 mm were spawned about 3 weeks earlier, based on findings from laboratory studies of the Japanese eel (Yamamoto and Yamauchi, 1974; Yamauchi et al, 1976). Based on this information, in addition to the estimated locations of the spawning grounds through the elaborate study by Schmidt (1923), the larvae that were used for this study are assumed to be from one to a few months old. The smallest larvae of 6.97 mm should be excluded from these estimations, as it has a deformity or damage in the tail that makes the measurement uncertain. The smallest larvae, of about 10 mm, are hence probably the youngest in the sample, and are estimated to be at least 1 month old. The largest larva (L21) of 23.33 mm is the oldest, and is estimated to be about 2-3 months old. The larvae are probably from the same spawning season, but spawned in different batches. Based on this, it is assumed that the majority of the specimens in this study were in the earliest to early-mid phase of their life as leptocephalus larvae. They still had several more months to grow to the full leptocephalus size of an average of 75 mm (Schmidt, 1923).

4.2 – Morphology of the digestive system

The feeding habits of leptocephalus larvae, including the larvae of the European eel, are considered one of the largest mysteries in relation to their ontogeny. It is also one of the main constrains in relation to the production in aquaculture. Today, only limited literature exists for the digestive system of the larval stages of the European eel. The digestive tract of the European eel larvae was a long straight tube, with no coiling. This corresponds to findings for other leptocephalus larvae (Miller, 2009). The digestive tract of the adult European eel is also very little folded, with the only foldings being the Y-shaped stomach and slight twisting of the tube right before the rectum (Clarke and Witcomb, 1980). The rapid increased length of the digestive tract during the larval stage and numerous villi in the in the intestine contributes to increasing the absorptive area. The digestive tract of most fish larvae will coil at some point in the development, but it is not uncommon that it doesn't. This is for example also seen in Zebrafish (*Danio reiro*) and also for herring (*Clupea harengus*) (Wallace et al, 2005, Hansen et al, 1992). Herring also have larvae with long, slim bodies.

The division into esophagus, intestine and anus corresponds to similar findings for the Japanese eel, just that it was here referred to as foregut, midgut and hindgut, respectively (Otake, 1996). The morphology of the digestive tract of the European eel appears to be very similar to the Japanese eel. For example, goblet cells were found in highest numbers in the esophagus for Japanese eel, and less in the intestine and anus (Otake, 1996). Although it was difficult to identify at the level of magnification, it appeared that the leptocephalus larvae of the European eel had numerous goblet cells in the esophagus, and fewer in the intestine. In the Japanese eel larvae, there was not observed any muscularis in the intestine (Otake, 1996). This was also observed for the European eel larvae in this study. The well-developed muscle tissue around the esophagus in the European eel probably contributes to pushing the food particles through the tube, lubricated by the mucus from the goblet cells. The esophagus was also found to be very long, covering about 35% of the length of the digestive tract. In other marine fish larva, such as e.g. cod, the esophagus is short compared to the rest of the digestive tract (e.g. Kjørsvik et al, 1991)

The thick muscular layer and rough/keratinized epithelium in anterior esophagus that was observed in the European eel larvae has, to my knowledge, not been described for the Japanese eel or in any other fish larvae. Still, this does not mean that it is not present, it may simply not have been investigated. Because of this, the function of this area can only be speculated about for now. A hypothesis may be that the rough textured epithelium combined with the strong double muscle layer contributes to a crushing function of ingested food particles. The teeth of the European eel larvae have been suggested to have a grasping function, rather than biting and chewing prey, and/or a filtering function (Bouilliart et al, 2013, Appelbaum and Riehl, 1993). A crushing function in the anterior esophagus may contribute to sort of a “chewing” of the ingested food, if the teeth are rather used to filter and/or grasp prey. The function of the teeth of the leptocephalus larvae has been widely discussed, as they are very long but also very fragile. The yolk-sac larvae have been found to have both low theoretical bite force and limited ability to move the jaws, which has contributed to the assumption that the larvae feed on small and soft food particles (Bouilliart et al., 2013). The function of the teeth has for that reason been suggested to be a grasping function, rather than biting and chewing prey (Bouilliart et al., 2013). This may also be supported by the observation of the hooked shape of the lower front teeth in the present study, which may contribute to holding food particles in place in the mouth. This may also explain why it has been difficult to identify the food particles in the gut of the leptocephalus larvae.

The food particles may be crushed in the anterior esophagus and then transported through the long esophagus where they are lubricated by the mucosa from the goblet cells and mixed by the muscle tissue surrounding the esophagus. When entering the intestine, the food particles are also exposed to high levels of digestive enzymes. When they then are detected in the intestine, the food particles may be a partly degraded mix of crushed food items, mucus and digestive enzymes, which would be difficult to identify. This can still only be considered speculations from my side. Content of the gut of leptocephalus larvae have been described to consist of an unidentifiable mass, often flowing out of the gut, and occasionally also with oval shaped objects dispersed in the intestine (Miller 2009). The unidentifiable flowing mass that Miller (2009) describes, was also observed in the larvae in this study, and may be this hypothesized crushed and mixed food. The oval shaped, dark colored particles have by previous studies been identified as zooplankton fecal pellets (Miller, 2009, Mochioka and Iwamizu, 1996). The oval shaped objects observed in the European eel larvae are probably also zooplankton fecal pellets.

The thick epithelium and mucosa in the intestine had a typical structure for absorption in the digestive tract. Submucosa and muscularis was not observed in the intestine and anus, only in the esophagus. This was also observed for the Japanese eel (Otake, 1996). The absence of submucosa in the intestine is also found for Zebrafish (Wallace et al, 2005). Fish larvae do usually have muscularis in the intestine and anus, in order to churn and mix the food particles in order to aid absorption, and push the food through the tube (Lazo et al, 2011, Govoni et al, 1986). The muscularis is present in the adult eel intestine (Clarke and Witcomb, 1980). It is not clear why the muscularis has not been observed in European and Japanese eel larvae, but maybe the muscular esophagus with cilia is enough to push the food through the tube. A theory may be that it is beneficial with a long retention time in the gut, in order to increase the absorption of the available food, but will naturally only be speculation.

A final important observation regarding the digestive tract is the presence of a large liver, with fat-like and glycogen-like deposits dispersed in it. It has previously been observed that yolk-sac larvae of the European eel have an especially high level of lipase and lipase-like enzymes, even from very early stages (Mazurais et al, in press). These levels are much higher than what is found in other pelagic fish larvae, and lead to the hypothesis that lipids probably are very important for the earliest life stages of the European eel (Mazurais et al, in press). The larvae do possibly store excess energy both in the liver (fat and glycogen) and in the glycosaminoglycan layer. Although it was difficult to identify the pancreas, it must be

present, as both a pancreas like-structure and pancreatic enzymes (lipase, lipase-like enzymes, amylase, trypsin and aminopeptidase) was reported in 12 days old eel larvae (Mazurais et al, in press). Most marine pelagic fish larvae also have a pancreas present even from the early stages (Kjørsvik et al, 2004). The adult European eel also has a defined pancreatic organ (Clarke and Witcomb, 1980), while it in adult fish usually is mainly present as diffuse tissue (Kjørsvik et al, 2004).

In relation to the question concerning dissolved organic matter (DOM) vs. particulate organic matter (POM) hypothesis, it is probably safe to hypothesize that the larvae of the European eel do not survive solely on dissolved nutrients. The observed food particles in the gut, is alone enough to support the claim that leptocephalus larvae do eat, just as any other fish larvae. The presence of digestive enzymes from the study by Mazurais et al (in press) also further supports this. The presence of a muscular esophagus with cilia, normal absorptive tissue in the intestine also supports the claim that European eel leptocephalus larvae do eat and have a functioning digestive system. However, it may still be possible that the nutrient uptake of the larvae is enhanced by absorption of DOM. Some species of leptocephalus larvae has been shown to have an active uptake of seawater in the intestine, which has supported the theory about a combined energy source (Bishop and Torres, 2001, Otake et al, 1993).

4.3 – Morphology and growth of muscle

The muscle layer of the leptocephalus larvae was very thin, for the smallest larvae only two cell layers thick and even for the largest larva only a few cell layers thick. Davidsen (2012) also observed this, although for younger eel larvae. In the earliest stages, the muscle tissue of marine pelagic fish larvae usually consist of two layers, one that develops into the red muscle (RM) and one that develops into the white muscle (WM) (Johnston et al., 2011). However, a surprising finding is that the muscle layers of the larvae still are only two cell layers thick (combined for RM and WM) at the earliest leptocephalus stages (size group B and C). In the smallest larvae (size group B and C) hyperplasia mostly occurred in the ventral and dorsal area of each bilateral half of the myotome. The WM layer was also only slightly starting to grow by stratified hyperplasia, i.e. forming more cell layers, in the larvae of intermediate size. Even for the largest larvae, the WM layer was only 5 cell layers at its thickest. The RM layer was one cell layers thick for all sizes. Mosaic hyperplasia was not observed in any larvae, but in studies of older eels, this has not been registered until the yellow and silver stages (Romanello et al., 1987).

This thin layer of muscle, with almost a stacked appearance in the beginning, is to my knowledge only found for species with the leptocephalus developmental strategy. It is however not well described, and is usually only mentioned briefly in the literature (e.g. Hulet, 1978). The thin muscle layer does however not mean that the leptocephalus larvae are not capable swimmers. Several observations mention that leptocephalus larvae are highly capable swimmers (Miller, 2009, Bishop and Torres, 1999). They are also thought to actively avoid the capture gear, which is part of the reason why they are difficult to catch (Castonguay and McCleave, 1986). The reason for having only a thin layer of muscle may be related to the energy advantage of the leptocephalus developmental strategy. The growth of the larvae is achieved by production of the metabolically inactive GAG´s, which allows the larvae to grow very large, without the same energy expense as other larvae (Bishop and Torres, 1999). The metabolic rate of leptocephalus larvae decrease with increasing size, which shows that the leptocephalus larvae have very low metabolic rates, even though they grow to achieve large sizes (Bishop and Torres, 1999).

As muscle growth was observed to increase in both the number of muscle cells and in the area of muscle cells, it is concluded that muscle growth in European eel larvae happens both by hyperplasia and hypertrophy. However, the growth in the area of the muscle appeared to happen more rapid than the increase in number of cells, indicating that the growth happens faster in relation to hyperplasia than hypertrophy. Growth by hyperplasia and hypertrophy vary for fish throughout its developmental stages. Hypertrophy dominates the growth of white muscle in the earliest larval stages of cod, before switching to about equal growth by hyperplasia and hypertrophy for the late larval stages (Vo et al., in press). The white muscle growth in juvenile and adult cod mainly happens by hypertrophy (Vo et al., in press). Juvenile eels have shown a substantial increase in new fibers, while hypertrophy dominates for the silver eel (Romanello et al, 1987).

Another theory may also be that increased hypertrophy may be related to leptocephalus larval strategy. When there is a need to conserve energy, it may seem that it is more advantageous to increase the size of the muscle fibers rather than the number of cells. It has e.g. been shown that coho salmon (*Onchorynchus kisutch*) on a calorie-restricted diet had a decrease in number of muscle cells produced (hyperplasia) and a 20% increase in size of the fibers (hypertrophy) (Johnston et al, 2014). The leptocephalus developmental strategy is related to grow large without increasing metabolically active tissue, such as muscle cells, so this may also apply for the European eel larvae. However, salmon and eels have very different

developmental strategies, so this relationship might not be directly comparable between the species.

The size of the muscle cells was largest in the middle of the larvae, and decreased towards the anterior and posterior end. Based on this, the oldest muscle cells in the larvae are the ones about in the middle of the larvae, in both the longitudinal and transverse dimension. From the observations in this study, it may hence seem that the larvae hence grows in length both from the tail area and head area.

4.4 – Other relevant findings

One of the most apparent observations was the difference between the eyes of L21 and the eyes of all the other larvae. The largest larva was the only one to have formed a vitreous body and lens separation, and hence had much more mature and adult-like eyes. The eye was also advanced in development compared to other marine pelagic fish larva, where lens separation usually do not occur until the metamorphosis (Kjørsvik et al, 2004). The eyes of all the smaller larvae had a more embryonic appearance, with the retina dominating the volume of the eyes. The external measurements of the eye was also relatively constant for the smaller larvae, before there could be seen a rapid increase in size for the two largest specimens. The eye of the largest larvae also had a more equal width and height, which gave rise to a more rounded shape. This may also be related to a more advanced eye. Vision has also been shown to be very important in relation to feeding in the Japanese eel (Pedersen et al, 2003). Another interesting observation is the yellow pigmentation of the eye of the larvae, which could be seen on the fresh leptocephalus larvae, and also to some extent in the pigmented epithelium in the histological sections. Yellow or golden retinal pigmentation is often found in deep-sea fish (Carlisle and Denton, 1959). In addition, the European eel has been shown to change pigmentation of the eye when it goes through the silvering in relation to the spawning migration back to the Sargasso Sea. During the yellow-eel stage of its life, it has purple retinal pigmentation, while during the silvering the eyes both becomes larger and the retina gets a golden color, like the photosensitive chryopsin pigments that are found in deep-sea fish (Carlisle and Denton, 1959). In addition, leptocephalus larvae of the European eel of 50 mm standard length have also been found to have a retina consisting of a single rod-like photoreceptor (Pankhurst, 1984). Usually, marine fish larvae do only have cones in the retina before the metamorphosis (Kjørsvik et al., 2004). This may be an adaption to the diel vertical migrations of the leptocephalus larvae. Leptocephalus larvae of the European and American eel >5mm have been found to have diel vertical migrations. Larvae in the range 5.0-19.9 mm

were found to have a preference for depths from 100-150 m during the day, and 50-100 m during the night (Castonguay and McCleave, 1986). Larvae >20 mm were found to have a preference of depths of 125-275 m during the day and 30-70 m during the day (Castonguay and McCleave, 1986). In other words, they seem to avoid the light, which would make sense in relation to the golden retinal pigmentation and rod-dominated retina that normally is characteristic for deep-sea fish. Another interesting aspect of this observation, is that it might seem like the retinal pigmentation of the eye of the eel changes two times during its ontogeny, as the larval and sexually mature eels appear to have a different pigmentation of the retina than the yellow eel.

Another noticable observation was the presence of the thick olfactory epithelium in the largest larva. This may indicate that both vision and smell is important for the leptocephalus larvae, as these senses appeared to be well developed. This and the observation of the structures resembling taste buds may indicate that the larvae may actively search for the preferred food, based on vision, taste and smell. This may also explain the difficulties with feeding the larvae in captivity, as they may have a preference for a certain visual of chemical stimuli in order to start feeding. The Sargasso Sea is also considered one of the most biologically dead or inert Seas in the world. The level of advancement in relation to vision and smell may also be due to the difficulty of even locating food sources. It may also not be related to feeding at all, but be due to predator escape or something completely different.

The only ossified structures that were observed in the larvae was a slight staining in the teeth, and most of the cartilage was present in the head area. No cartilage or bone was observed in the notochord of the larvae. Hardly any studies exist on the development of the skeleton of leptocephalus larvae. However, one study has been performed of the development of the skeleton of the leptocephalus larvae of the speckled worm eel (*Myrophis punctatus*). In this study, it was found that the development of fin rays happened from the posterior and towards the anterior with a completion at 41.7 mm length, and they metamorphose to glass eels at a length of on average 53 mm (Leiby, 1979). The observation of finrays in the tail of L21 indicates that this developmental pattern may be the case for the European eel as well. The speckled worm eel did not develop fin rays in the pectoral fins until the glass eel stage (Leiby, 1979), but this was observed in the European eel, even for the smallest size group. Ossification of the skeleton in this species do not happen until the metamorphosis into glass eels, so this may also be the case for the European eel (Leiby, 1979). The lack of a skeleton may also be related to the eels swimming pattern. Eels, and also their larval stages, swim in an

anguilliform pattern, which means an undulating movement of the whole body of the fish (Sfakiotakis et al., 1999). It may be hypothesized that the combination of a relatively soft mucinous pouch (GAG-layer) and flexible notochord may be advantageous in relation to an undulating swimming pattern. It has been hypothesized that the GAG-layer can act as a firm, gelatinous skeleton for the muscle to work against, making the larvae able to swim without the energetic cost of a skeleton (Bishop and Torres, 1999). A skeleton is both energetically costly to produce, and to maintain as the larvae has to spend more energy to maintain buoyancy.

The fact that no functional gills were observed in the larvae may also be consistent with the leptocephalus larval strategy. The lavas relatively small size and laterally compressed bodies gives them a high surface area. This means that they probably can suffice with cutaneous respiration (through the skin), through diffusion. The thin outer layers of the leptocephalus larvae, only a few cell layers thick, further supports this claim. Even though no functional gills were observed, it is also probable that a large part of the respiration happens in the opercular cavities, as these provide an increased surface area where the water is in close contact with highly vascularized tissue. For fish larvae the critical size for the transition from cutaneous and opercular (gill) respiration has been shown to be highly dependent on metabolism (Rombough, 2004). Because of this, the eel larvae probably also dont have a very high requirement for oxygen, due to the low metabolic rates of leptocephalus larvae (Bishop and Torres, 1999). The lack of pigmentation of the blood is hence not necessary for the larvae in relation to respiration, but it is also an advantage in relation to predator escape. The leptocephalus larvaes transparent appearance is probably also a predator defense strategy, and they are almost invisible in the water column, with the exception of the large pigmented eyes. Leptocephalus larvae are probably effective at escaping predators, as they are rarely observed in the stomach of predatory fish (Miller et al., 2015).

The skin of the leptocephalus larvae was found to be thin and uniform at most of the larval body, with the most prominent exception being the presence of the layer that in this study was named the “oval cell layer”. The identification of the composition of this layer was largely based on similar findings in other species of eel. This layer has also been observed in the American eel (Leonard and Summers, 1976). In the American eel, the bulk of this layer was composed of club cells, although other cell types, such as mucus cells, chloride cells, taste buds and filament containing cells also was present (Leonard and Summers, 1976). Club cells are also found in the Japanese eel, where they are described as lectin-secreting cells (Suzuki

and Otake, 2000). Lectin-secreting cells are also found in adult eels, and although the function of the lectin is not entirely certain, it is thought to have some protective function (Suzuki and Otake, 2000). The presence of club cells in the skin of the European eel is quite certain, based on the descriptions for the American and Japanese eel. However, further studies would be necessary to confirm this, and also to determine the other cell types that seem to be present in the oval cell layer.

4.5 – Some thoughts concerning the relative growth and maturity of organs

Both growth and morphology-based maturity of organs was very similar for all the analyzed larvae, with the exception being the largest larvae (L21). For the measurements of relative growth of all organs, the growth appeared slow or stable in the beginning, before suddenly leaping between 18-23 mm SL. The only exception was the digestive tract and the teeth, where the first was found to grow in a linear relationship with increasing SL and the second was stabile all the time. This was also observed for the muscle tissue, where the relative growth appeared to happen in an exponential pattern. Both the area of muscle cells and the number of muscle cells increased rapidly between 18-23 mm standard length. For the morphological descriptions, the largest larva also differed from the rest, especially in relation to the development of the eye and for the several cell layers thick muscle layers, being the only larva with finrays in the tail, and the only larva with lamellae-like structures in the opercular cavity (although no functional gills). The anterior esophagus of the largest larva also appeared to be more differentiated, and the oval cell layer was mainly found in the largest specimen. The presence of the oval cell layer might be due to preservation rather than development, as the largest larva also was best preserved. Because of this, there may be some indications that there is an important increase in development, possibly starting somewhere between 18-23 mm SL. However, as there is only one larvae in the largest size class, it may be a bit difficult to say something absolute in relation to the relative growth, but it is probably safe to say that there are signs of a possible trend in the development.

5. Conclusions and further prospects

In this study, a description of the morphology and an estimation of growth related trends of leptocephalus larva of the European eel were performed. Although the available larvae were few and many of the specimens were partly degraded, it may still be possible to draw some conclusions from the study.

The digestive system appears to be very similar to the Japanese eel, and it was also described some differences in relation to the general patterns for marine fish larvae. The most apparent difference was the muscular anterior esophagus with the rough surface, which is hypothesized to have a food-crushing function. This has not been described for the Japanese eel, or for any other fish larvae to my knowledge. This potential crushing function of the esophagus might explain the long and fragile teeth of the leptocephalus larvae that are thought to possibly have a grasping function, rather than being used for biting or chewing. The thick epithelium and mucosa in the intestine had a typical structure for absorption in the digestive tract, but no muscularis was observed in the intestine. The muscular esophagus might be enough to push the food through the digestive tract. The digestive system is clearly adapted for feeding at this stage, which is further supported by the observations of food particles in the intestine of three of the larvae. The observation of well developed eyes in the largest larva, as well as a thick olfactory epithelium and structures resembling taste buds, might suggest that the larvae are actively searching for their preferred food source. The muscle tissue of the larvae also had a unique morphology with a thin, almost stacked layer covering the GAG in the middle. This may be unique to the leptocephalus larvae, and has only previously been described for yolk-sac larvae in the master-thesis of Martin Davidsen. It was also very surprising that the larvae still had this thin muscle layer at this stage. Differentiation of the muscle into several layers was mainly found in the largest larva. The muscle of the larvae was also found to grow mainly by hypertrophy. The main growth both in number and size of muscle happened in the middle of the larvae, indicating that they grow in length both from the tail area and head area.

The skin was covered in a cell layer, probably consisting of a large proportion of the lectin-secreting club cells based on the findings for the Japanese and American eel. This thought to be of importance in relation to protection of the larvae. It also appeared to be other cell types present in this layer that may be of importance for the larvae. It also seems to be something important happening in relation to growth and organ development between the size 18 and 23 mm, as there for almost all organ systems that were viewed could be observed a major

difference between the largest specimen and the other larvae, especially in relation to the development of the eye, finrays and in the muscle tissue. The larvae also appeared to have golden pigments in their pigmented retina, which most often is found in deep-sea fishes, but also in the silver-eels. The yellow-eels have purple pigmentation in their retina, indicating that the eel might go through two changes in retinal pigmentation throughout its life. It is hypothesized that this observation of golden pigmentation of the eye might be an adaption to the vertical distribution of the larvae. It might also seem that the leptocephalus larvae have a unique development of the eye, compared to other marine pelagic fish larvae.

Many interesting observations were done in this study, but still, it is necessary with more research both in relation to the European eel, but also for leptocephalus larvae in general. It would be especially interesting to do for example immunuhistochemical and ultrastructural studies, in order to be able to identify the different cell types, e.g. the pancreas, the cells in the oval cell layer, the epithelium in the esophagus and the pigmentation of the retina. A study with a larger sample size, and also more larvae of larger sizes would also be interesting. Still, there is a lot of information lacking about the larger leptocephalus larvae, although this study does say something about the earliest parts of the leptocephalus larval life. However, these are estimated to be between 1-3 months old, meaning that they still have a long way to go until they become the fully-grown 75 mm long giants among the marine fish larvae. It would be especially interesting to see how the muscle development changes during the transformation from the latest leptocephalus stages and into the glass eel stage. A detailed muscle study, possibly also including gene expression, measurements of mitochondria and maybe also related to swimming behavior would be especially interesting, as there is hardly any studies today covering the muscle development of leptocephalus larvae.

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Appendix 1

Sampling of lepocephali larvae in the Sargasso Sea

By Sune Riis Sørensen (Post Doc, DTU).

Larvae were collected during the first leg of the Danish Sargasso-Eel cruise between the 20th of March to the 4th of April 2014. The sampling gear was a ring-net of 3.5 m diameter, equipped with a conical net of 25 m length. The net had a mesh size of 500 µm and the cod end had a mesh size 350 µm. The gear was towed in oblique hauls from 0 to 250 m using a cruising speed of 2 knots. Average duration was 55 min. Sampling stations were positioned between 26.41.752N, 068.29.370W and 27.45.134N, 062.44.868W. Within 45 min from catch, all *Anguilla sp.* candidate larvae were manually sorted and digitally imaged prior to fixation. A solution of cold, sterile filtered physiological seawater (0.9%) was used when photographing and handling larvae. Larvae were photographed on a stage micrometer slide in full size and in detail to focus specifically on the posterior and anterior body parts. To identify *Anguilla anguilla* from other leptocephali larvae, the number of myomeres were counted and other characteristics evaluated following the guidelines by Smith, D.G. (1979). The fixative was prepared immediately prior to sampling, mixing newly thawed glutaraldehyde 25% (Grade I, Sigma-Aldrich, Missouri, USA) buffered with 0.15 M HEPES buffered to pH 7.4. Single larva were transferred to a vial containing the fixative and stored at 4°C.

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Appendix 2

Sampled European eel leptocephalus larvae from the Sargasso Sea

Table A.1: Additional data from leptocephalus larvae of *Anguilla anguilla* collected during the "Danish Eel Expedition 2014 – Sargasso Eel".

ID	MIK	LARVAE #	LEG	STATION	ACTIVITY	TRANSECT	YEAR	MONTH	DAY	HOUR	MIN	LATITUDE	LONGITUDE	LATDEC	LONGDEC
s-4a-140320	3f	L4	1	10	122	1	2014	3	20	20	4	26.41.752 N	068.29.370 W	26,69587	-68,4895
s-4a-140326	3f	L2	1	24	251	3a	2014	3	26	21	25	28.30.437 N	062.29.796 W	28,50728	-62,4966
s-4a-140326	3f	L1	1	24	251	3a	2014	3	26	21	25	28.30.437 N	062.29.796 W	28,50728	-62,4966
s-4a-140329	3f	L1	1	28	299	3	2014	3	29	18	5	25.00.015 N	062.29.827 W	25,00025	-62,4971
s-4a-140330	3f	L2	1	29	311	3	2014	3	30	12	57	25.20.059 N	062.45.018 W	25,33432	-62,7503
s-4a-140330	3f	L10	1	29	310	3	2014	3	30	12	57	25.20.059 N	062.45.018 W	25,33432	-62,7503
s-4a-140330	3f	L12	1	29	310	3	2014	3	30	12	57	25.20.059 N	062.45.018 W	25,33432	-62,7503
s-4a-140330	3f	L4	1	29	311	3	2014	3	30	12	57	25.20.059 N	062.45.018 W	25,33432	-62,7503
s-4a-140330	3f	L13	1	30	324	3	2014	3	30	19	49	25.40.034 N	062.44.781 W	25,66723	-62,7464
s-4a-140330	3f	L7	1	30	324	3	2014	3	30	19	49	25.40.034 N	062.44.781 W	25,66723	-62,7464
s-4a-140330	3f	L6	1	30	324	3	2014	3	30	19	49	25.40.034 N	062.44.781 W	25,66723	-62,7464
s-4a-140330	3f	L11	1	30	324	3	2014	3	30	19	49	25.40.034 N	062.44.781 W	25,66723	-62,7464
s-4a-140401	3f	L2	1	30	341	3	2014	3	30	19	49	25.40.034 N	062.44.781 W	25,66723	-62,7464
s-4a-140401	3f	L9	1	30	341	3	2014	3	30	19	49	25.40.034 N	062.44.781 W	25,66723	-62,7464
s-4a-140401	3f	L3	1	30	341	3	2014	3	30	19	49	25.40.034 N	062.44.781 W	25,66723	-62,7464
s-4a-140402	3f	L2	1	34	389	3	2014	4	2	20	27	27.04.946 N	062.44.818 W	27,08243	-62,747
s-4a-140402	3f	L3	1	34	389	3	2014	4	2	20	27	27.04.946 N	062.44.818 W	27,08243	-62,747
s-4a-140402	3f	L4	1	34	389	3	2014	4	2	20	27	27.04.946 N	062.44.818 W	27,08243	-62,747
s-4a-140402	3f	L1	1	34	390	3	2014	4	2	20	27	27.04.946 N	062.44.818 W	27,08243	-62,747
s-4a-140402	3f	L3	1	34	390	3	2014	4	2	20	27	27.04.946 N	062.44.818 W	27,08243	-62,747
s-4a-140403	3f	L5	1	36	410	3	2014	4	3	11	35	27.45.134 N	062.44.868 W	27,75223	-62,7478

Appendix 3

Preparation of fixative and fixation of larva

Larvae were fixated in EM-fixation medium, which was a mixture of paraformaldehyde (PFA, 2.5%, Carl Roth, Germany), glutaraldehyde (GA, 2.5%, Carl Roth, Germany), sucrose (0.5%, Sigma-Aldrich, USA) and hepes buffer (0.11 M, Carl Roth, Germany) (final concentrations). Stock solution of hepes buffer, sucrose and PFA was prepared and stored beforehand. Hepesbuffer (0.15 M) was prepared by mixing distilled water and hepesbuffer (molecular weight 238.3) in a ratio of 100 mL : 3.5745 g. Solution of 5% sucrose was prepared by mixing 2.5 g sucrose to 50 mL distilled water. Solution of paraformaldehyde (25%) was prepared by dissolving 50 g paraformaldehyde in 50 mL distilled water by heating to 60^{o}C. A few drops of concentrated sodium hydroxide (NaOH) were then added, until the solution switched from opaque to transparent. The solution was then cooled and filtered before use.

For preparation of 100 mL EM-fixation medium, 70 mL stock solution of hepesbuffer, 10 mL sucrose, 10 mL of PFA and 44.3 mg calcium chloride ($CaCl_2$) was mixed and adjusted to pH 7.4. Just before use, 10 mL of freshly thawed GA (25%) was added. The fixation medium was first mixed well in the Eppendorf vials, in a ratio of 9 parts stock solution and 1 part GA (25%), before a single eel leptocephalus larvae was added to each vial and then stored cold (4^{o}C).

Appendix 4

Embedding larvae in EPON

Leptocephalus larvae were embedded in a plastic medium consisting of EPON (epoxy resin embedding kit, Sigma-Aldrich, USA). The EPON solution was stored in 20 mL disposable plastic syringes at -20^{o}C, and thawed 15-20 min before use. The day before embedding, the larvae were cut in three pieces using a small scalpel, transferred to hepes buffer (0.11 M) and stored cold overnight (4^{o}C).

The embedding of eel larvae in EPON was performed in a fume hood. Each larva, separated into three pieces, was transferred to a separate chamber in a cell cultivation tray. The larvae were then post-fixated in 3% potassium ferrocyanide (KFC, Sigma-Aldrich, USA) : 4% osmium (Os, stored at -20^{o}C and thawed before use, Carl Roth, Germany) (final 1.5% : 2%) for 1 h in room temperature and protected from light. The larvae were then washed in distilled water for 5 x 2 min rinses.

The larvae were then bulk-colored for 1.5 h with 1.5% uranylacetate (VWR Chemicals, USA) in distilled water at room temperature and protected by light. The larvae were then washed in distilled water for 5 x 2 min rinses.

The larvae were dehydrated in ethanol (VWR Chemicals, USA). First in 70% ethanol for 10 min, then in 90% ethanol for 10 min, in 96% ethanol for 2 x 10 min and in 100% ethanol for 2 x 15 min. After the first dehydration (70%) the larvae were transferred to polypropylene vials. The next step was to transfer the larvae to propylene oxide (Acros Organics, Belgium) for 2 x 15 min. Then the larvae were transferred to a mixture of propylene oxide : EPON of 3:1 for 30 min, and then in a mixture of propylene oxide : EPON of 1:1 overnight. The next day the larvae were transferred to a mixture of propylene oxide : EPON of 1:3 for 5 to 6 h and then to pure EPON and placed on a rotator overnight. The next day the larvae were embedded in fresh EPON in embedding molds and set to polymerize for 60^{o}C for 24 h.

Appendix 5

Staining of sections for light microscopy

Toluidine blue

A stock solution of Toluidine blue was made by mixing 1 g Toluidine blue (Sigma-Aldrich, USA) and 1 g sodium borate (Sigma-Aldrich, USA) in 100 mL distilled water. The sections of larvae were first transferred to a water droplet on an object glass, and then dried on a heating plate at 80^{o}C until the water was entirely evaporated, and then a bit longer to ensure that the sections were properly fastened to the object glass. The sections were then covered by a mixture of Toluidine blue and borate buffer of about 1:1 for 1 min, before they were rinsed with distilled water and dried completely on the heating plate.

After staining the sections were mounted on the object glass with Neomount (VWR Chemicals, USA), covered with a coverslip, and dried at room temperature for 30 min.

Appendix 6

Embedding larvae in Technovit

Before embedding, the larvae were washed in and transferred to HEPES buffer (0.11 M, Carl Roth, Germany). The specimens were then first dehydrated in a mixture of water/buffer : basic resin (hydroxyethylmethacrylate, HEMA, Heraeus Kulzer, Germany) of 3 : 1 for 3 hours at 4^{o}C. The specimens were then transferred to a mixture of water/buffer : HEMA of 2 : 2 for 2 hours at room temperature.

An infiltration fluid consisting of 50 mL HEMA and 0.5 g activator (benzol peroxide, Heraeus Kulzer, Germany) was then prepared by stirring until dissolved. The larvae were then transferred to the infiltration fluid and left over night on a rotator.

The next day an embedding fluid consisting of 30 mL new infiltration fluid and 1.5 mL hardener II (Heraeus Kulzer, Germany) was mixed for 3-5 min, and then used immediately. The embedding molds were filled with some of the embedding fluid before transfer of larva. A holder was placed above, and the mold was filled completely with embedding fluid through the hole in the holder. The molds were set to polymerize for 40-120 min at room-temperature.

Appendix 7

Staining of bone and cartilage using Alizarian Red and Alcian Blue

Fixed larvae were first rinsed in distilled water (2x10 min), and then stained for cartilage with Alcian blue solution until moderately blue. Alcian blue staining solution was prepared by mixing stock solution of Alcian blue (10 mg, Sigma-Aldrich, USA), ethanol (abs., 70 mL, VWR Chemicals, USA) glacial acetic acid (100%, 20 mL, Merck Millipore, USA). Neutralization was then performed by soaking larvae in absolute ethanol bath containing freshly added KOH (1%, Merck Millipore, USA) for a few minutes.

Larvae were then put in 96% ethanol for 2 x 1 hour, then transferred to 50% ethanol for 1 hour, 15% ethanol for another hour and finally distilled water for 1 hour, or to the sample sunk. Bleaching was then performed for about 5 hours in 1:9 H_2O_2 (3%, Merck Millipore, USA) : KOH (1%) under strong light. During bleaching, the samples was watched very carefully and stopped when they appeared to be ok. Larvae were then cleared in trypsin buffer for up to 2 days, until they were almost transparent. Trypsin buffer (100 mL) was prepared from trypsin (0.5 g, Sigma-Aldrich, USA) and KOH (1%, 10 g to 1 L distilled water).

Bones were then stained in Alizarin solution for 2 days. Alizarin staining solution was prepared from Alizarin red S (Sigma-Aldrich, USA) in ethanol (15%) and then filtered.

Larvae were then rinsed in distilled water for 10 minutes, before being transferred to rinse in KOH (1%). KOH rinse was performed 2x, or until surplus color was gone. Larvae were then transferred to glycerol (40%, Sigma-Aldrich, USA) in KOH (1%) for two days. After two days the larvae could be photographed and evaluated for bone (pink) and cartilage (blue) staining. Larvae were then transferred to glycerol (70%) in KOH (1%) for 1 day, before they could be stored in glycerol (100%) with a few crystals of thymol (Sigma-Aldrich, USA).

Appendix 8

Concerning the state of the material

After the sectioning of the first larva, it became apparent that the state of the material was not optimal. The digestive tract, and especially the intestine, appeared to be most damaged, but the muscle tissue was also strongly affected. In this section, some examples of the state of the material will be given, in order to demonstrate the difficulties that appeared with the analysis, and also why the study ended up as it is presented here. The original study was intended to only describe the digestive system of the wild leptocephalus larvae, but this was later changed to include also the muscle tissue. However, when it became apparent that also the muscle tissue was damaged, it was decided to include other organs in the study as well.

The digestive system was best preserved in the largest larva (L21, SL = 23.33 mm). For most of the other larvae, the sections of the intestine was not worth including in the report due to the state of degradation (Figure A.1). Many of the sections had the appearance that is illustrated in Figure A.1-C. Measurements of the digestive system and the accessory organs were performed, but it was decided to exclude them from the report due to the high variability and poor reliability due to the state of the material. It was in stead focused on the morphology of the digestive system.

The muscle tissue was also quite damaged, and often muscle cells were found to be missing or moved from their original location. Sometimes the sections GAG layer also appeared to be disconnected from the muscle tissue, causing the muscle tissue to dislocate and produce sections with almost a balloon like appearance. This made it difficult sometimes to measure the muscle tissue, and impossible to measure the area of the transverse sections. Measurements of the area of the whole section and the GAG layer was performed, but excluded from the report.

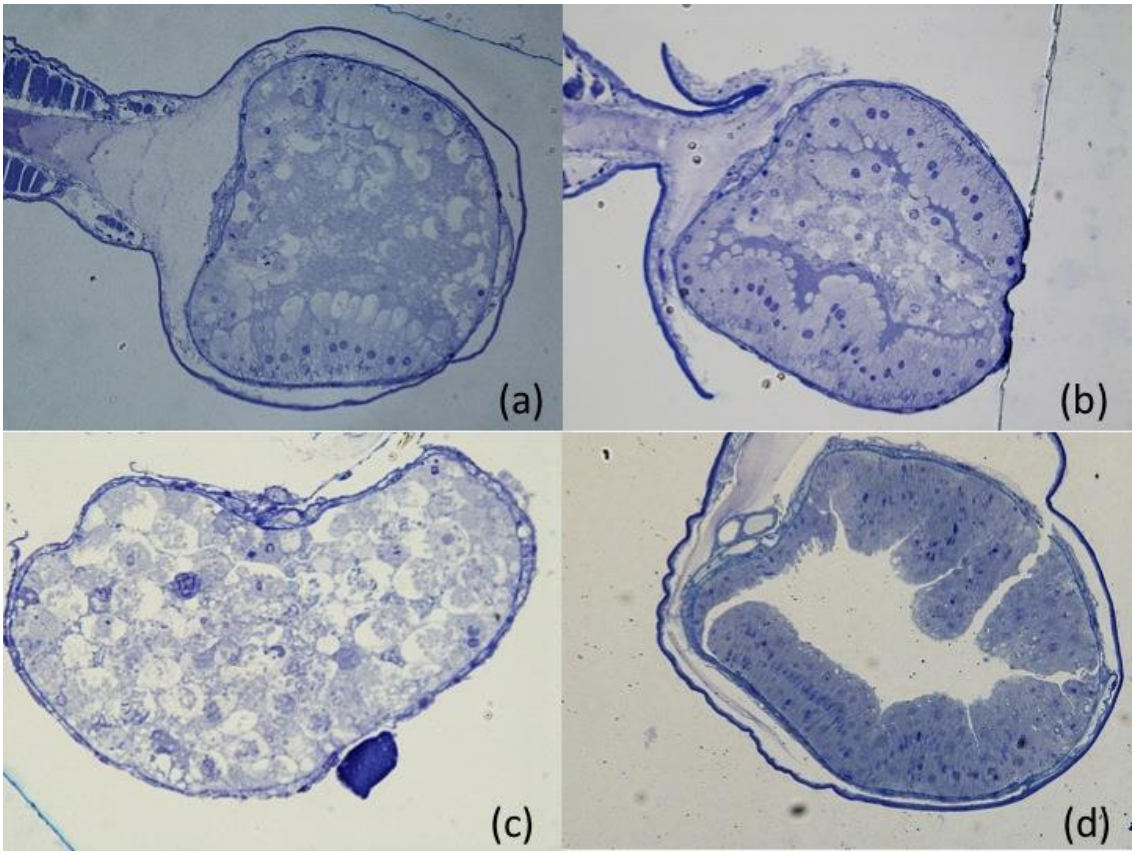

Figure A.1: Morphology of the best preserved intestinal tissue (middle of intestine) for four different size classed of larvae. A: L10, SL = 11.17. B: L16, SL = 13.78 mm. C: L19, SL = 17.11 mm. D: L21, SL = 23.33 mm.

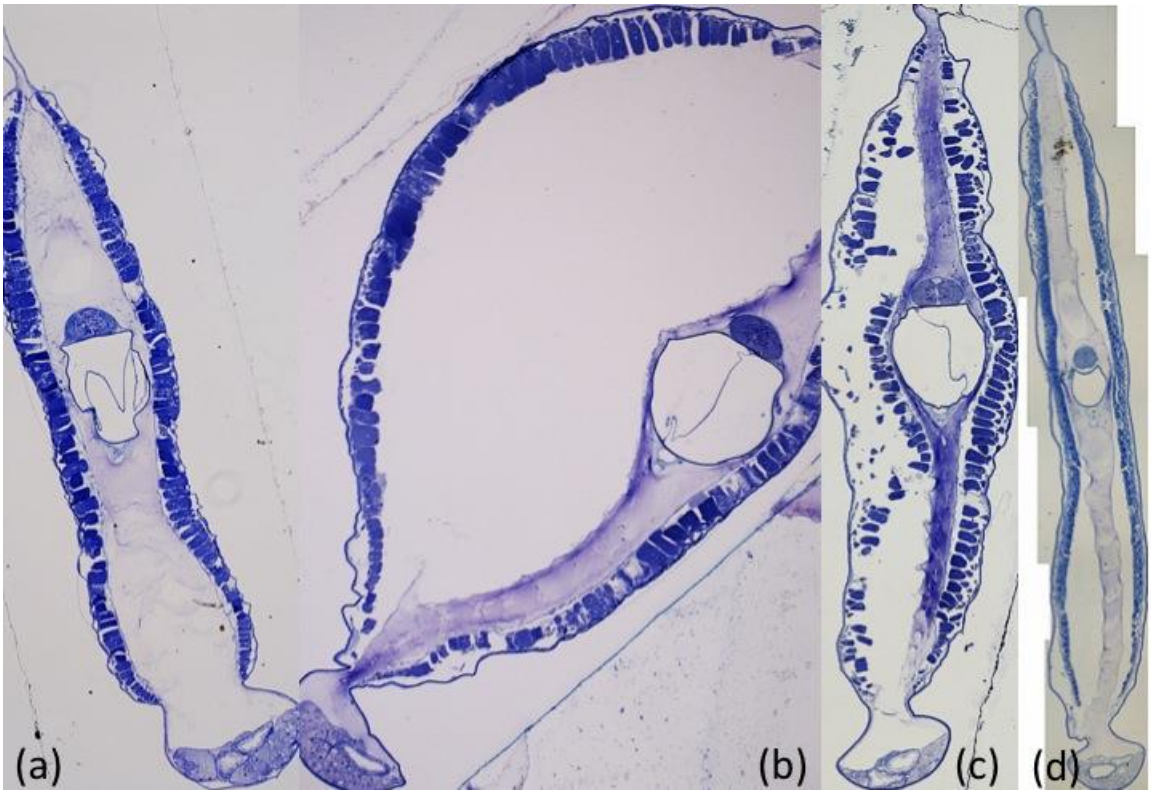

Figure A.2: Morphology of the sections tissue in the transition area for four different size classed of larvae. A: L10, SL = 11.17. B: L16, SL = 13.78 mm. C: L19, SL = 17.11 mm. D: L21, SL = 23.33 mm.