Examining IgM in ballan wrasse (*Labrus berggylta*) larvae

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ABSTRACT

Aquaculture has grown to become one of the most important industries in Norway, but its growth is currently limited by infections such as sea lice. Cleaner fish such as ballan wrasse are used to remove sea lice, but these cleaner fish are also subject to diseases, and their mortality is high. In order to reduce ballan wrasse mortalities, it's important to understand their unique immune system. This study examines the immune system of two groups of ballan wrasse larvae, fed with two different diets: a standard diet and a plankton-based diet. This was done by observing the IgM immunoglobulin, which is a key immune component. The larvae were assessed at seven different developmental stages, and two methods were used to study their IgM: immunohistochemistry and transcriptome data analysis. Immunohistochemistry works by attaching antibodies and chromogens to IgM in larvae sections, thereby staining it with color. The sections can then be examined with a microscope. Transcriptome data analysis works by examining all the mRNA expressed in the larvae's cells. mRNA coding for IgM and other related immune components was studied at all seven developmental stages, and ANOVA tests were used to analyze the data. This study did not find a clear difference in IgM development between the two diets.

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1 INTRODUCTION

1.1 AQUACULTURE IN NORWAY

Food production is a serious global issue, and the growing aquaculture industry is becoming a valuable contributor to global food supply. In Norway, aquaculture is one of the country's most profitable industries. The jagged Norwegian coastline has many sheltered water areas with depth and currents suitable for open net pens (Hersoug et al., 2021). These are excellent locations for the aquaculture industry, which in Norway grew massively from the 1990s to the early 2010s. The cost of producing salmon on the Norwegian coast was consistently decreasing up until 2005 (Iversen et al., 2017). In a report from 2012, the Royal Norwegian Society of Sciences and Letters and the Norwegian Academy of Technological Sciences claimed that the Norwegian aquaculture industry could double in size by 2030, and grow fivefold by 2050 (DKNVS and NTVA, 2012). The report inspired strong optimism, and quickly led to keen interest and large investments from both government and businesses.

Norwegian aquaculture's biggest asset is by far the Atlantic salmon (*Salmo salar*). These large predatory fish from the Salmonidae family are found throughout the North Atlantic Ocean and the rivers surrounding it (Klementsen, 2003), where they've been fished by humans for centuries. Salmon were always the cornerstone in the industry, and they've only grown more important over time. Norwegian fish farmers went from getting 89% of their income from salmon in 2007 to getting 95% of their income from it in 2019 (Directorate of Fisheries, 2021).

Despite its strong economic success, the Norwegian aquaculture industry may not be sustainable. Today, developers struggle to find new areas for open pen facilities (Hersoug et al., 2021). This area problem was already identified back in 2011, and has only grown more serious since then (Hersoug et al., 2020). The cost of producing salmon in Norwegian aquaculture approximately doubled from 2005 until 2016 (Iversen et al., 2017), and this could make the goals from 2012 unachievable. The area problem exists because facilities must be placed far apart to reduce the prevent of infections. Infection level controls industry growth or reduction an area, and they are the main reason why production hasn't increased over the last years (Hordvik and Mortensen, 2019). This means these infections are the biggest challenge Norwegian aquaculture currently faces, and the most important infection is salmon lice (Lepeophtheirus salmonis).

1.2 SALMON LICE

Salmon lice (*Lepeophtheirus salmonis*) are small parasitic copepods that attack salmonids. They can survive in most marine environments, and are found throughout the northern hemisphere. They cause damage by attaching to the outside of the fish, and eating its skin, mucus, and blood. Salmon lice harm the fish directly by eroding the fish's epidermis, creating sores, and damaging its mucus (Skiftesvik et al., 2014), but they also weaken the fish, thereby hurting its immune system, its swimming ability, and its ability to feed (Torrissen et al., 2013 and Skiftesvik et al., 2014). Even

subclinical levels of salmon lice can change the fish's physiology, biochemistry, and immunology (Johnson and Fast, 2004). The lice are especially dangerous to juvenile salmon (Fast et al., 2008). The high salmon population density of open sea pens provides a perfect habitat for salmon lice, and the Norwegian louse population has therefore increased drastically after the growth in the aquaculture industry (Torrissen et al., 2013). This is not only an issue for the industry, but also for wild salmon populations. The lice spread freely between wild and domesticated salmon, and infections have become much more common among wild salmon over the last decades (Torrissen et al., 2013).

A salmon louse becomes increasingly dangerous as it grows into adulthood. It starts its life as a copepodid, which will try to attach to a host and feed for 3-4 days (Pike and Wadsworth, 1999). After it has grown enough, it molts into a chalimus louse, which can use its frontal filament to attach to hosts and adhere to hard structures. It eventually develops into a pre-adult, and finally an adult louse. Pre-adults and adults can use their cephalothorax as a suction cup, and they can move freely around the host body (Pike and Wadsworth, 1999). This mobility allows the louse to escape inflammation, which is the salmon's main method of fighting it (Wagner et al., 2008). Adults and pre-adults have a significantly stronger effect than copepods and chalimus lice (Bowers et al., 2000), and salmon infected with many juvenile lice will typically die when they develop into pre-adults (Ross et al., 2000).

Many methods have been attempted to combat the salmon lice issue, both mechanical, chemical, and immunological. Bath treatments, additions to fish feed, and other pharmaceutical approaches have shown some promise in the past, but large-scale use could induce resistance in the lice (Skiftesvik et al., 2014). Meanwhile, non-pharmaceutical countermeasures have their own drawbacks. Lice skirts around the sea cages can help keep lice out, but they don't block them out completely. Thermal treatment effectively harms lice, but it also stresses the salmon (Dalvin et al., 2020 and Nilsson et al., 2019). Freshwater treatment has shown more promise. When wild salmon ascend rivers, lice detach and let the stream carry them back to the ocean, since they can't osmoregulate in freshwater. Similarly, submerging salmon in freshwater can cause lice to detach, but some lice have been observed to develop resistance to this (Wagner et al., 2008). Out of all the countermeasures that have been discovered, the most reliable method so far has been cleaner fish.

1.3 CLEANER FISH

The best anti-lice measure yet discovered is to deploy lice eating fish together with the salmon in the sea cage. These lice eating fish are wrasse and lumpfish (*Cyclopterus lumpus*). Inside a sea cage, they will eat salmon lice directly off the salmon's skin as long as they're sufficiently hungry. Compared to other delousing methods, these cleaner fish are efficient and inflict little stress on the salmon (de Leaniz et al., 2017). Although wrasse and lumpfish can eat lice, they aren't specialized to do so; they are both generalists with a varied diet in nature (Overton et al., 2020). Today, Norwegian aquaculture deploys between 40 and 60 million cleaner fish annually (Directorate of Fisheries, 2021). These used to be primarily caught in the wild, but most cleaner fish today are bred in facilities **(**Bilal et al., 2016). Cleaner fish suffer from and high mortality both in farming facilities and in sea cages. The losses are particularly high in cages, where cleaner fish can succumb to infections, starve to death, be eaten by the salmon, escape, or die of old age (Hordvik and Mortensen, 2019).

Wrasse's ability to delouse has been known about since the 1980s (Bjordal, 1988), whereas lumpfish's delousing abilities weren't discovered until 2014 (Imsland, 2014A and Imsland, 2014B). Although wrasse's delousing abilities have been known for a long time, their use increased drastically in response to lice infections around 2010, and has been increasing since then (de Leaniz et al., 2017 and Directorate of Fisheries, 2022). There are many differences between wrasse and lumpfish. Wrasse need over a year to grow up, whereas lumpfish can be deployed in sea cages when they're only four months old (de Leaniz et al., 2017). Lumpfish are typically bred, whereas wrasse are typically wild caught (Directorate of fisheries, 2022). Wrasse prefer temperatures above 10°C, and enter a state of torpor below 6°C (Morel et al., 2013). By comparison, lumpfish can feed at temperatures down to 4°C (Nytrø et al., 2014). Wrasse can go dormant during winter, whereas lumpfish stay active throughout the year (Imsland et al., 2014B). During early adoption of wrasse delousing, low winter temperatures led to high mortalities (Sayer et al., 1996).

1.4 WRASSE

The wrasse family (*Labridae*) makes up 33% of Norwegian cleaner fish (Directorate of Fisheries, 2022). Wrasses are the second biggest family of marine fish, encompassing 453 species found throughout the world (Sayer et al., 1996). In the wild, these fish are typically generalists that feed on gastropods, bivalves, crustaceans, fishes, coral mucous, zooplankton, ectoparasites, and algae (Westneat and Alfaro, 2005). Most of the species live in tropical waters (Parenti and Randall, 2011), and only six wrasse species are found in Norway. These are ballan wrasse (*Labrus bergylta*), corkwing wrasse (*Symphodus melops*), goldsinny wrasse (*Ctenolabrus rupestris*), rock cook (*Centrolabrus exoletus*), cuckoo wrasse (*Labrus mixtus*), and scale-rayed wrasse (Acantholabrus palloni) (Espeland, 2010 and Hordvik and Mortensen, 2019). Ballan wrasse, gold-sinny wrasse, corkwing wrasse, and rock cook are used to delouse (Directorate of Fisheries, 2022). With about 5% of the fish in a sea cage being wrasse, they can delouse efficiently, on average leaving less than one adult/pre-adult louse per fish at any point (Skiftesvik et al., 2013A).

One of the wrasse species used for delousing is ballan wrasse (*Labrus berggylta*). These are found throughout the Atlantic European coast, typically living around rocks, reefs, and kelp forests at a depth of 20-30 meters (Sayer et al., 1996), where they prefer to feed on crustaceans and bivalves (Westneat and Alfaro, 2005 and Hordvik and Mortensen, 2019). Although most ballan wrasse only grow to a length of about 30 cm, the biggest grow up to 60 cm, which makes them the largest wrasse species in Norway (Sayer et al., 1996). They can become 25 years old, although very few actually live that long (Sayer et al., 1996). Ballan wrasse is a hermaphrodite species, where all individuals are born female, and some fish later transform to male (Sayer et al., 1996). During cold winter temperatures, ballan wrasse will move to deeper waters, where it hides on the sea floor and moves as little as possible (Treasurer, 2002).

Despite being the least abundant wrasse species in Norwegian waters (Skiftesvik et al., 2013A), ballan wrasse are a valuable asset to the aquaculture industry. Out of the four wrasse species used for lice removal in Norway, ballan wrasse is the third most common, making up 6.4% of Norwegian cleaner

fish (Directorate of Fisheries, 2022). It has proven to be an effective and robust delouser (Hordvik and Mortensen, 2019), and it's the only wrasse species that is being farmed in facilities (Directorate of Fisheries, 2022). Along with corkwing wrasse, it's one of the few species that can be used for delousing for more than one year (Skiftesvik et al., 2013A). They were exclusively wild caught until 2009, when they were first farmed in a facility (Skiftesvik et al., 2013A). However, there are still few ballan wrasse farming facilities, and the overwhelming majority of ballan wrasse are still wild caught (Directorate of Fisheries, 2022).

1.5 BALLAN WRASSE DISEASES

Domesticated ballan wrasse are subjected to many welfare issues in sea cages, such as low temperatures, starvation, and diseases. Cleaner fish have high mortalities, and their deaths often happen after stress factors weaken their immune system, and pathogens kill them. These pathogens are often opportunistic, and they come in many forms: bacteria, virus, parasites, and fungi (Norwegian Scientific Committee for Food and Environment, 2017). Bacteria are the most threatening form of pathogen, and some of the most dangerous species *are Aeromonas salmonicida*, *Vibrio splendidus,* and *Vibrio tapetis* (Gulla, 2015, Gulla, 2016, and Papadopoulou et al., 2019). *A. salmonicida* can cause bloody boils, rashes, and internal bleeding, whereas *Vibrio* bacteria typically cause weariness and skin necrosis, which in turn leads to various deadly symptoms (Ina-Salwany, 2019). Other bacteria that attack ballan wrasse include *Vibrio anguillarum, Vibrio ordalii*, and *Pseudomonas anguilliseptica* (Gulla, 2015).

Bacteria aren't the only pathogen that attack ballan wrasse. Despite being deployed to remove parasites, ballan wrasse suffer from parasites of their own. One of the worst is *Paramoeba perurans*, which causes Amoebic Gill Disease (Karlsbakk et al., 2013). Many other parasites have been observed to attack them too: various forms of copepods, nematodes, cestodes, trematodes, and ciliates (McMurtrie et al., 2019). Less commonly, they are also subjected to virus, such as viral haemorrhagic septicaemia (Karlsbakk et al., 2013 and Erkinharju et al., 2020). Fungal infections are also uncommon in ballan wrasse (Karlsbakk et al., 2013), despite being very frequent in lumpfish (Erkinharju et al., 2020).

1.6 THE TELEOST IMMUNE SYSTEM

Since diseases are the biggest cause of ballan wrasse mortality in sea cages, it's important to understand the fish's immune system. Even though humans and fish lie far apart phylogenetically, our immune systems share a lot of similarities. As with other vertebrates, the teleost immune system can be loosely split into two parts: the innate immune system and the adaptive/acquired immune system.

The innate part mounts a non-specific but immediate response whenever a pathogen is detected. It is developed very early in a teleost's ontogeny, which is exemplified by the fast-growing zebrafish (*Danio rerio*). Zebrafish granulocytes, which are central to the organism's innate immune system, are

first found about 34 hours after fertilization (Willet et al., 1999). This is important because fish hatch at the embryonic stage, and have to defend themselves early (Rombout et al., 2005). Against a serious threat, the innate immune system's response can take the form of an acute inflammation, where immune cells such as neutrophiles destroy foreign microorganisms through phagocytosis (Magnadóttir, 2006 and Schmid-Schönbein, 2006). There are many components in a teleost's innate immune system. Phagocytes and nonspecific cytotoxic cells can destroy obvious foreign targets (Uribe et al., 2011). Phagocytosis is a very reliable part of any poikilothermic organism's immune system, since the process isn't heavily influenced by temperature (Magnadóttir et al., 2005 and Blazer, 1991). Neutrophils and macrophages are the main phagocytic cells in fish. Natural antibodies exist in the fish's serum, and are an important part of the nonspecific protection against bacteria and viruses (Whyte, 2007). Teleosts also have many types of signal proteins called cytokines that play various important roles to the non-specific immune system (Uribe et al., 2011). The first line of defense against infection is outer physical barriers, such as skin, mucus, and gills. It's important that the non-specific immune system has a strong presence in these parts of the fish (Uribe et al., 2011). Since fish are free-living from an earlier embryonic stage than other vertebrates, they rely on their innate immune system for survival until their adaptive immune system is fully developed (Uribe et al., 2011). Even later in life, fish remain more reliant on their innate immune system than other vertebrates. This is because they are poikilothermic, and they have a limited adaptive immune system (Uribe et al., 2011).

The adaptive immune system provides a better response to any specific pathogen than the innate system, but it takes longer to activate after first encountering it. This is because the adaptive system can recognize and remember specific pathogens. This system is a vertebrate adaptation. Some of the lowest vertebrates, such as early jawed fishes, have a very similar adaptive immune system to some of the highest vertebrates, such as humans (Wilson, 2017). Scientists used to believe the adaptive immune system hadn't changed much since it first developed, but newer studies have revealed that there's still much we don't know about its evolution (Wilson, 2017).

The fish's immune system encompasses a lot of organs and tissue. The process of creating blood cells such as T cells and B cells is called hematopoiesis, and the first hematopoietic organ to develop in a typical teleost is the intermediate cell mass (Uribe et al., 2011). This intermediate cell mass then further develops into various other lymphoid organs, some of which retain the ability to produce blood cells. In order, these are the kidney, the spleen, and the thymus (Mulero et al., 2007). The different lymphoid organs have different functions. The kidney produces the most blood cells in juvenile fish, and is the equivalent of the bone marrow in higher vertebrates (Zapata et al., 2006). The thymus mostly produces T cells (Davis et al., 2002). The spleen mostly does erythropoiesis rather than lymphopoiesis during larval stages, meaning it produces more red blood cells than it does B cells and T cells. Later in a fish's life, the spleen detains antigens for longer periods of time (Uribe et al., 2011). The lymphoid organs aren't the only parts of the fish that are important to the immune system. Its mucosa acts as a physical barrier with immunological function (Salinas et al., 2011). There is mucosa associated lymphoid tissue (MALT) in the fish's skin, gills, gut, olfactory epithelium, pharyngeal epithelium, and buccal epithelium (Salinas et al., 2022). The MALT is crucial for combating infections, and it has all types of leukocytes necessary for immune defense (Salinas et al., 2011). Some teleost species can mount a local specific immune response from their MALT without triggering an immune response in the entire organism. Research on trout has shown that their skin, gills, and

gut can make their own antibodies when needed (Lumsden et al., 1993, Jones et al., 1999, and Cain et al., 2000).

The teleost immune system is influenced by various external factors, like temperature, light, salinity, water quality, and stress inducers such as high population density (Magnadóttir, 2010). Low temperatures or oxygen levels can slow down body functions, and the immune system is no exception (Uribe et al., 2011). Higher salinity or longer photoperiod can lead to increased IgM levels in the blood (Bowden, 2008).

1.7 B CELLS AND T CELLS

The adaptive immune system is split into humoral and cell-mediated immunity. Humoral immunity is mediated through proteins called antibodies, whereas cellular immunity is mediated through cells. The cell-mediated system typically handles intracellular threats. Both parts of the adaptive immune system are activated by a type of white blood cells called lymphocytes. The two types of lymphocytes relevant for the adaptive immune system are B cells and T cells. B cells produce antibodies for the humoral immune system, whereas T cells perform the functions of the cell-mediated immune system. In fish, T cells are produced in the thymus, whereas B cells are produced in the head kidney (Rombout et al., 2005 and Geven and Klaren, 2017).

T cells come in three types: cytotoxic T cells, which kill other cells, helper T cells, which enable other lymphocytes to do their job, and regulatory T cells, which prevent autoimmune responses. As with the non-specific immune system, the specific one also relies on cytokines, which play an important role as signal molecules (Uribe et al., 2011). They are released by helper T cells to summon killer T cells and macrophages. T cells can't recognize invaders on their own. Instead, major histocompatibility complex (MHC) proteins embed themselves in the membrane of foreign cells, where they present antigens to the T cells (Wieczorek et al., 2017). By marking pathogens and activating T cells, these proteins are essential for the adaptive immune system. They come in two types: class I and class II: MCH class I proteins consist of three α-helices and one β-sheet, and they activate cytotoxic CD8+ T cells. MCH class II proteins consist of two α-helices and two β-sheets, and they activate CD4+ T cells, which in turn coordinate effector B cells (Wieczorek et al., 2017 and Uribe et al., 2011).

Fish only create one type of B cell, but these cells later develop into different distinct types. Any B cell's purpose is to produce immunoglobulins, which are proteins that play a central role in the adaptive immune system. Teleosts create three types: IgM, IgD, and IgT/IgZ. These are characterized by the heavy chains of the molecules μ, δ, and τ, respectively (Bilal et al., 2016). Immunoglobulins' main function is to attach to pathogens that enter the body, which they do by binding to a compatible antigen on the pathogen's surface. Immunoglobulins consist of two heavy chains and two light chains (Schroeder and Cavacini, 2010), and they come in countless different shapes, so that the body always has an immunoglobulin that's compatible with whatever pathogen might infect it. Immunoglobulins exist in two forms: soluble and membrane-bound. Membrane-bound immunoglobulins are attached to the outside of a B cell membrane, and are called B cell receptors.

Any given B cell only has one single type of B cell receptor in its membrane. If a B cell receptor binds to an antigen, the B cell is activated, and transforms into a plasma cell, also called effector B cell. The plasma cell then starts producing soluble immunoglobulins, called antibodies, and releasing them into the blood. These antibodies bind to the same antigen as the original B cell receptors. The difference is that antibodies are released freely in the blood, and can therefore disperse quickly throughout the body, attaching to antigens wherever they encounter them. In adult teleosts, antibodies are typically found in the skin, intestine, gill mucus, bile, and blood plasma (Uribe et al., 2011). Antibodies are found in the fish body even before the immune system has encountered any antigens (Boes, 2000). Not all B cells transform into plasma cells upon activation. A small portion of them instead transform into memory B cells, which stay in the fish's body long-term. If the same pathogen infects the fish again in the future, memory B cells will enable the specific immune system to respond quicker (Suan et al., 2017). The three types of antibodies that B cells produce have different functions. IgM is the main antibody in teleosts. IgT/IgZ is specialized for mucosal immune responses (Salinas, 2015). IgDs function in teleosts appears to vary between species, and isn't yet fully understood (Bilal et al., 2016 and Bilal et al., 2019). IgM and IgT are most often tetramers in teleosts, whereas IgD appears in many forms (Bilal et al., 2016 and Bilal et al., 2019).

1.8 BALLAN WRASSE ADAPTATIONS

The ballan wrasse's immune system is quite unusual. Ballan wrasse have a remarkably high concentration of antibodies in the blood plasma (Bilal et al., 2019), and an adult wild-caught ballan wrasse of 800 grams has a serum IgM concentration of around 13 mg/mL (Bilal et al., 2016). This is ten times higher than in a typical wild Atlantic salmon (Magnadóttir, 1998). Ballan wrasse also have an unusually high amount of antibody producing cells specifically in the gut (Bilal et al., 2018), which in turn leads to a high local antibody concentration (Bilal et al., 2019). Most teleost species have all the cells necessary for a local immune response present in the gut mucosa (Rombout et al., 2011), but ballan wrasse is an extreme example. This adaptation likely came about because the species has no stomach, which makes the gut an important barrier against pathogens (Hordvik and Mortensen, 2019 and Bilal, 2019). In other teleost species, the stomach's acidic environment kills many bacteria.

As a stomach-less fish, ballan wrasse's digestive system is also unusual. The species lacks stomach (Bilal et al., 2018), pyloric caeca (Hordvik and Mortensen, 2019), and a functional colon (Lie et al., 2018). Instead, digestion happens in a short gut that runs about 2/3 of its body length (Bilal et al., 2019). The anterior segment of the ballan wrasse gut does not function as food storage. Its main function is digestion and absorption of macronutrients, which it does to a higher degree than any other section of the fish's gut (Hoang et al., 2019 and Zhou et al., 2021). Through convergent evolution, different teleosts have lost their stomach 15 times. Today, 20-27% of teleosts are stomach-less (Wilson and Castro, 2010). In addition to its regular teeth, strong pharyngeal teeth line the ballan wrasse's throat, and helps it crush the tough shells of its prey (Hordvik and Mortensen, 2019).

1.9 IgM

IgM is the most common antibody in most teleosts' serum and mucus (Bengtén et al., 2006 and Solem and Stenvik, 2006), and ballan wrasse is no exception (Hordvik and Mortensen, 2019). This means it can tell a lot about ballan wrasse's immune system.

IgM antibodies are paramount to the specific immune response. They are the first antibodies produced in response to an infection (Boes, 2000), and IgM memory B cells are the first to respond when a previously encountered pathogen returns (Capolunghi et al., 2013). In teleost species, serum IgM concentration varies with age, size, sex, season, environment, and vaccination/infection status (Bilal et al., 2016). Teleost IgM usually comes in the form of a tetramer (fig. 1), although some teleost species also produce an IgM monomer. (Uribe et al., 2011 and Ye et al., 2013). An IgM tetramer is made of four monomers: two heavy chains and two light chains. An IgM heavy chain has four constant heavy chain domains, called μ1, μ2, μ3, and μ4. Various things can bind to these chains, such as cytotoxic cells, effector cells, or complement proteins (Ye et al., 2013). IgM produced by different B cells will have different shapes. This shape determines its antibody affinity to any given antigen, which is its ability to bind to that antigen. An IgM antibody's affinity to different antibodies is what gives it its function. As the adaptive immune system responds to an infection, it will deploy antibodies with increasing affinity to the threat (Ye et al., 2013). IgM is primarily produced in the lymphoid organs, but as with other antigens, it can also be produced locally in the fish's mucous membranes in response to an antigen (Zhao et al., 2008). It can be transferred across the mucosal epithelia via the polymeric Ig Receptor (pIgR) protein (Hamuro et al., 2007). It takes some time from a fish larva is hatched until it can produce its own IgM. In species such as ballan wrasse, this is compensated for by maternal IgM transferred from the parent fish (Mulero et. al., 2007).

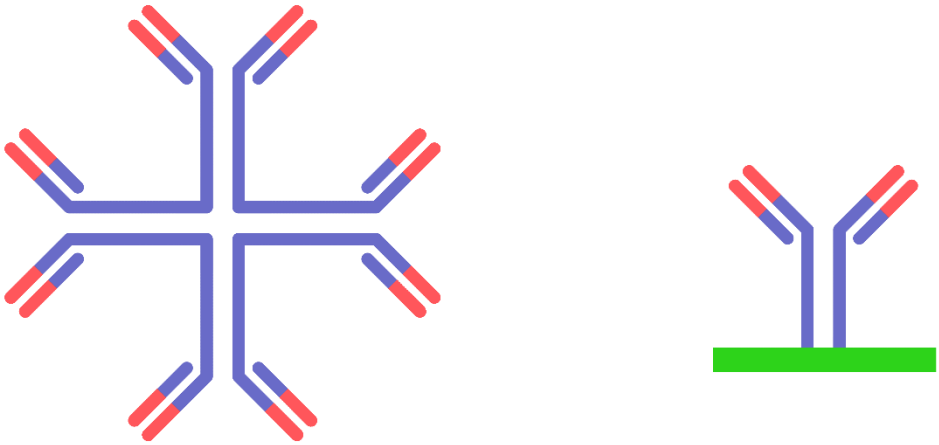

Fig. 1: A secreted IgM tetramer (left) and a membrane bound IgM monomer (right). The blue parts of the IgM are the common parts, whereas the red parts vary between IgM molecules. The green line is the cell membrane.

Although IgM is primarily a part of the adaptive immune system, it helps the innate immune system too. Its main job is to mark pathogens for destruction, e.g. by phagocytosis, but it also has many

other functions. It fixates complement, agglutinates foreign objects together, binds mannose binding lectin, and mediates cellular cytotoxicity. Agglutination means clumping together bacteria and other foreign objects in the body. Once they're gathered, they're more easily marked and destroyed with phagocytosis or cellular cytotoxicity. Mannose binding lectin are proteins that's part of the innate immune system. Complement are proteins in the blood. They are primarily a part of the innate immune system, but some parts of the adaptive immune system use it too. They can be activated by antibodies, such as IgM (Ye et al., 2013).

As previously mentioned, ballan wrasse have a unique immune system. Most teleosts have more IgM in their kidney and spleen, but ballan wrasse have their highest IgM concentration in the hindgut (Bilal et al., 2019). This isn't entirely unprecedented. Common carp, which are also stomach-less, have also been shown to have high gut IgM concentration. The effect is however less extreme in carp, which still have more IgM expression in their spleen and kidney (Savan et al., 2005). Ballan wrasse have higher IgM secretion in the body and higher immune activity in the gut compared to other teleosts, and an overwhelming majority of their IgM is in the form of free antibodies (Bilal et al., 2019). More antibodies in the gut likely means a stronger first line of defense (Bilal et al., 2019).

1.10 STUDYING IgM

Even though ballan wrasse have been used for delousing for decades, it wasn't widely used until recently. This means there are relatively few studies on the ballan wrasse immune system, and it's still poorly understood (Bilal et al., 2018 and Yuen et al., 2019). This is especially true for early life stages, in which ballan wrasse display high mortality. Further studies on the species can not only improve both their health, welfare, and efficiency, but also provide valuable insight into related fields of study. Studying teleosts with unusual adaptive immune systems can help understand the adaptive immune system better (Wilson, 2017). Some of the most interesting discoveries within immunology come from studies on teleosts, where many species have lost key components of the adaptive immune system, and evolved alternate mechanisms of protection (Star and Jentoft, 2012 and Haase et al., 2013).

Since IgM is the most important antibody in ballan wrasse, it can tell a lot about how strong the fish's immune system is. Antibodies are important to both the adaptive and the non-specific components of its immune system. Studying IgM in ballan wrasse larvae could provide valuable insight into their high mortality, and how to reduce it. There are several steps between an IgM coding section of DNA and a functioning IgM protein. As all proteins, immunoglobulins are translated from mRNA in a cell's ribosome. The translation process constructs the protein from amino acids. Before this point, the mRNA needs to be transcribed from DNA in the cell's nucleus.

In order to examine IgM concentration in ballan wrasse larvae, it is best to study IgM proteins directly. This can be done with immunohistochemistry (IHC). IHC works by attaching colorful chromogens to antibodies, which themselves are attached to receptors in cells. In this case, the receptor is transmembrane IgM attached to B cells. The antibodies used in ICH are just an intermediary, and are selected based on which chromogen is used and which cells are being searched

for. Since IHC is an old and well-developed method, there are a lot of chromogens and antibodies known to work for a wide array of purposes (Duraiyan et al., 2012).

If it's not possible to study IgM proteins directly, studying mRNA that codes for IgM will also offer valuable insight. The difference is that not all mRNA ends up being translated, meaning IgM concentration and IgM mRNA concentration don't correlate perfectly. mRNA concentrations can be examined with PCR, which can be used to gather transcriptome data. A transcriptome consists of all the mRNA expressed by a single organism, and can therefore show how much IgM coding mRNA is being transcribed.

1.11 THE AIM OF THE THESIS

The aim of this thesis is to examine ballan wrasse larvae under two different feed regimes: one experimental plankton diet and one standard diet. The goal is then to figure out when the larvae start producing their own IgM antibodies, and what effect the experimental diet had. There are two ways this could be achieved:

1. Using immunohistochemistry to examine transmembrane IgM proteins in B cells

2. Using transcriptome data to examine mRNA coding for IgM proteins

2 MATERIAL AND METHOD

2.1 SAMPLING

The sampling for this study took place at a research facility at Austevoll run by The Institute of Marine Research. It was carried out by supervisor Angela Etayo. At the facility, newly hatched ballan wrasse larvae were reared in six tanks, each containing between 31.000 and 35.000 larvae (table 1). They were subjected to two different feeding regimes. Three tanks were fed with planktonic nauplius larvae from common barnacles (*Semibalanus balanoides*), while the other three were fed with a control diet consisting of rotifers and artemia. The larvae were fed with autofeeders three times per day and once per night.

Table 1: Number of ballan wrasse larvae in each tank, and their diets

Tank	Diet	Number of larvae
A	Plankton	35.000
B	Control	32.300
C	Plankton	33.000
D	Control	33.400
E	Plankton	34.750
F	Control	31.210

Larvae from both feeding regimes were sampled at seven different stages, as shown in table 2. The first sampling (stage 1) was performed in the incubator just after the larvae had hatched, and before they were subjected to the two different feeding regimes. From stage 5 and onwards (40 days post hatch), all the ballan wrasse larvae were fed with regular pellets. Stages 2 to 7 in the study were based on the six developmental stages described by Norland, Sæle, and Rønnestad in the paper "*Developmental stages of the ballan wrasse from first feeding through metamorphosis: Cranial ossification and the digestive system*" (Norland et al., 2022). In the paper, the six subdivisions were based on cranial ossification, maturation of the digestive tract, and growth-correlated stages.

Table 2: Larvae sampling stages

Stage	Days post hatch
1	0 dph
2	9 dph
3	18 dph
4	25 dph
5	40 dph
6	58 dph
7	78 dph

The sampled larvae were embedded in paraffin blocks, and these blocks were stored at 4°C.

2.2 SECTIONING

The paraffin blocks were cut into 3 µm sections with a Leica RM 2165 epitome. Sectioning started at a dorsal side, moving towards the opposite dorsal side as sections were cut out. These sections were then picked up with a small paintbrush and dropped onto the surface of a water bath. The water was double distilled and heated to 37°C using a heating plate. After the sections were put on the hot water surface, they were immediately placed on microscope slides. The slides used were Thermo Scientific's Superfrost Plus. These slides were then left to dry on the 37°C heating plate. 1-3 sections on were put on one slide, depending on the size of each sample, and the sections stayed on these microscope slides for the rest of the process. After sectioning, the slides were stored long-term in plastic boxes at 4°C.

To prepare the cold slides for immunohistochemistry (IHC), they were incubated on a heating plate. The plate would be set to 37°C, and the slides would be heated for one day and night. During incubation, the slides were covered with aluminum foil to protect them from dust and keep them hot.

2.3 IMMUNOHISTOCHEMISTY

Five sessions of IHC were carried out, with various parameters. Two antibodies were used. The primary antibody would bind to transmembrane IgM, the secondary antibody would bind to the primary antibody, and the chromogen would bind to the secondary antibody. During IHC, the slides were placed in a metal rack and immersed in glass boxes filled with various liquids, as described further down. For the rest of the process, they would only be briefly exposed to air as they were transferred from one liquid to another. This way, they'd never dry out. The five different IHC sessions used various antibody concentrations, timings, and chromogens (details in the *results* section).

The first step of IHC was to deparaffinize the slides in xylene. They are initially immersed in a box of xylene for 5 minutes, and then immersed in a different box of xylene for another 3 minutes. Two different boxes were used, so that the second xylene box stayed somewhat free of paraffin residue. After the xylene, the slides are hydrated in graded alcohol solutions. The slides were immersed in each box for 3 minutes, one after another, in the following order: 100% alcohol, 90% alcohol, 80% alcohol, 70% alcohol, and finally 50% alcohol. To finish the hydration process, the slides are immersed in double distilled water for 5 minutes.

The next step was heat-induced epitome retrieval. The slides were immersed in a heated sodium citrate buffer, and left for one hour. The buffer was heated to 84°C with a water bath. This buffer was made by mixing 2.94 grams of tri-sodium citrate (dehydrated) with 1 liter of twice distilled water. The pH was then adjusted to 6. Finally, 0.5 mL of tween was added to the mix.

After the epitome retrieval, the box with the buffer and the slides was taken out of the heater. The lid was removed from the box, and it was left to cool in room temperature for 20-30 minutes. The slides were then washed, by immersing them in 0.01M PBS. They were washed for five minutes twice, and the box was lightly shaken during the process.

To avoid non-specific binding, the sections were covered in blocking solution. The sections were circled with hydrophobic marker, and the solution was dropped onto the slides. This blocking solution was made by mixing 25 mL of 0.05M tris-buffered saline (TBS), 0.5 grams of bovine serum albumin (BSA), and 500 µL of goat serum. Typically, 200 µL of blocking solution was used to cover each section. The slides were then kept in a plastic box at room temperature for 1 hour. In order to keep the air inside the box moist, the box was closed, and the bottom was filled with wet tissue paper. After blocking, the slides were drained, and tissue paper was used to wipe around the sections without touching the organic tissue.

After blocking, the primary antibody was applied to the sections. This was done very similarly to the blocking; the solution was put inside the hydrophobic circles, and the slides were placed in a plastic box with high humidity. Polyclonal ballan wrasse IgM was used as the primary antibody. This antibody was dissolved in a solution made from 20 mL of 0.05M TBS and 0.2 grams of BSA. The ratio between antibody and antibody dilution varied in different experiments, as described for each experiment under the “results” section. The sections used as control were not treated with the primary antibody. After the primary antibody was applied, the slides were incubated at 4°C overnight, usually about twenty hours. The slides were then washed in 0.01M PBS for five minutes twice, while the box was lightly shaken.

The secondary antibody, polymer HRP anti-rabbit, was applied in a similar manner to the blocking solution and the primary antibody; the solution was put inside the hydrophobic circles, and the slides were placed in a plastic box with high humidity. The slides were then incubated at room temperature for one hour, before being immersed in 0.05M TBS to be cleaned. They were immersed three times, each time for five minutes.

After being treated with both antibodies, the samples were colored with two chromogens. The slides were drained, and tissue paper was used to wipe around the sections without touching the organic tissue. Immediately after each slide dried, the first chromogen was applied to them. This was 3-amino-9-ethyl carbazole (AEC). The AEC was kept on the slides for 10 minutes, staining them with a red color. Afterwards, they were immersed in TBS for five minutes thrice. Finally, they were immersed in twice distilled water and drained. Before the sections dried out, the slides were immediately immersed in Mayer’s haemalum solution for 10 minutes. This served as the counterstain, giving the sections a blue/purple color. Finally, the slides were placed in a box and continually rinsed in tap water until the water in the box was colorless.

Finally, to prepare the sections for examination with microscope, two or three drops of mounted aqueous media were dropped on them and put cover slips on top. The cover slips were attached with

blank nail polish, and the finished slides were stored in plastic boxes at 4°C. Pictures were then taken digitally with a microscope.

2.4 STATISTICS

The transcriptome data for 14 genes from the ballan wrasse genome (table 3) was examined with box plots and anova tables. All statistical methods used in this thesis were performed in R version 3.5.3, with the help of Rstudio version 1.3.1073. The packages used were readxl, datasets, and tidyverse.

Table 3: Genes examined in this thesis, and the proteins that they code for

Gene	Protein/region	Function
TM-IgM	Transmembrane IgM	Membrane-bound version of IgM, which is the most important immunoglobulin in the system
TM-IgT	Transmembrane IgT	Membrane-bound version of IgT, which is mainly responsible for mucosal immune responses
IgM123	Regions 1-3 of IgM	Main antibody
sIgM4	4th region of IgM	(same as above)
492pIgR	Polymeric immunoglobulin receptor	Supports immunoglobulins, mainly with transport
pIgRlike	pIgR-like	Related to polymeric immunoglobulin receptor
IgD1234	Regions 1-4 of IgD	Not fully understood
Tcra	T cell receptor alpha	Bound to T cell membranes, recognizes antigens bound to MHC molecules (Marrack and Kappler, 1987)
Tcrd	T cell receptor delta	(same as above)
sIgT	Secreted IgT	Antibody mostly responsible for mucosal immune responses
IgT1234	Regions 1-4 of IgT	(same as above)
sIgD	Secreted IgD	Not fully understood antibody
IgD6	6th region of IgD	(same as above)
TM-IgD	Transmembrane IgD	Membrane-bound version of IgD, which is not fully understood

The following line was used for importing the transcriptome data from an excel sheet. “DataTMIGM” and “A4:C88” were swapped out to import data from 14 different places in the excel sheet and give them 14 different names.

```
DataTMIGM <- read_excel("~/master stuff/GeneData2.xlsx",
                                range = "A4:C88")
```

The following lines were used to generate and display a boxplot for one of the data sets. For other data sets, “DataTMIGM” was swapped out for the name of the other data sets and “Transmembrane IgM expression” was swapped out for a different title.

```
ggplot(DataTMIGM, aes(x = factor(DevelopmentalStage), y =
GeneExpression,
                              fill = Diet)) +
  geom_boxplot(outlier.size = 3, outlier.color = "black") +
theme_bw() +
  labs(x = 'Developmental stage', y = 'Gene expression', title =
'Transmembrane IgM expression')
```

The following lines were used to generate and display an anova table. For other datasets, “DataTMIGM” was swapped out for the names of the other data sets.

```
AnovaTMIGM <- aov(GeneExpression ~ Diet * DevelopmentalStage, data
= DataTMIGM)

anova(AnovaTMIGM)
```

3 RESULTS

3.1 IHC: CONCENTRATION TEST OF BOTH ANTIBODIES

The first immunohistochemistry session tested two different concentrations of both of the two antibodies. It had two control samples, which only received the second antibody. It used larvae of the fifth developmental stage, taken from the tank C (plankton diet).

Table 4: Parameters and results from the first IHC test.

	1st antibody concentration	**2nd antibody concentration**	**Result**
Sample 1A	1:5000	1:500	No coloration outside of the eye
Sample 1B	1:5000	1:1000	No coloration outside of the eye
Sample 1C	1:10000	1:500	No coloration outside of the eye
Sample 1D	1:10000	1:1000	No coloration outside of the eye
Sample 1E	-	1:500	No coloration outside of the eye
Sample 1F	-	1:1000	No coloration outside of the eye

All the five samples offered very similar results. They were uniformly colored by the blue counterstain, except for an area around the eye, which was stained with red. Some of the samples were damaged.

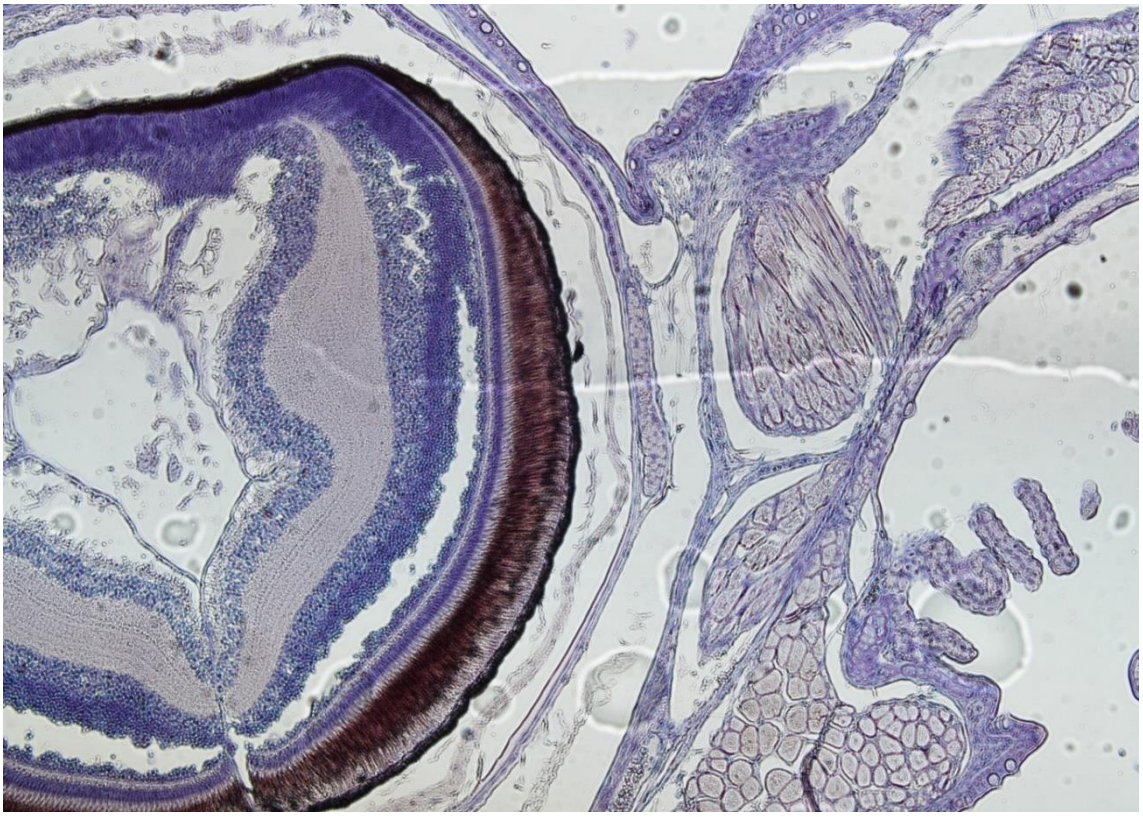

Fig. 2: The sample 1A shows barely any red color. The only colored area is around the eye.

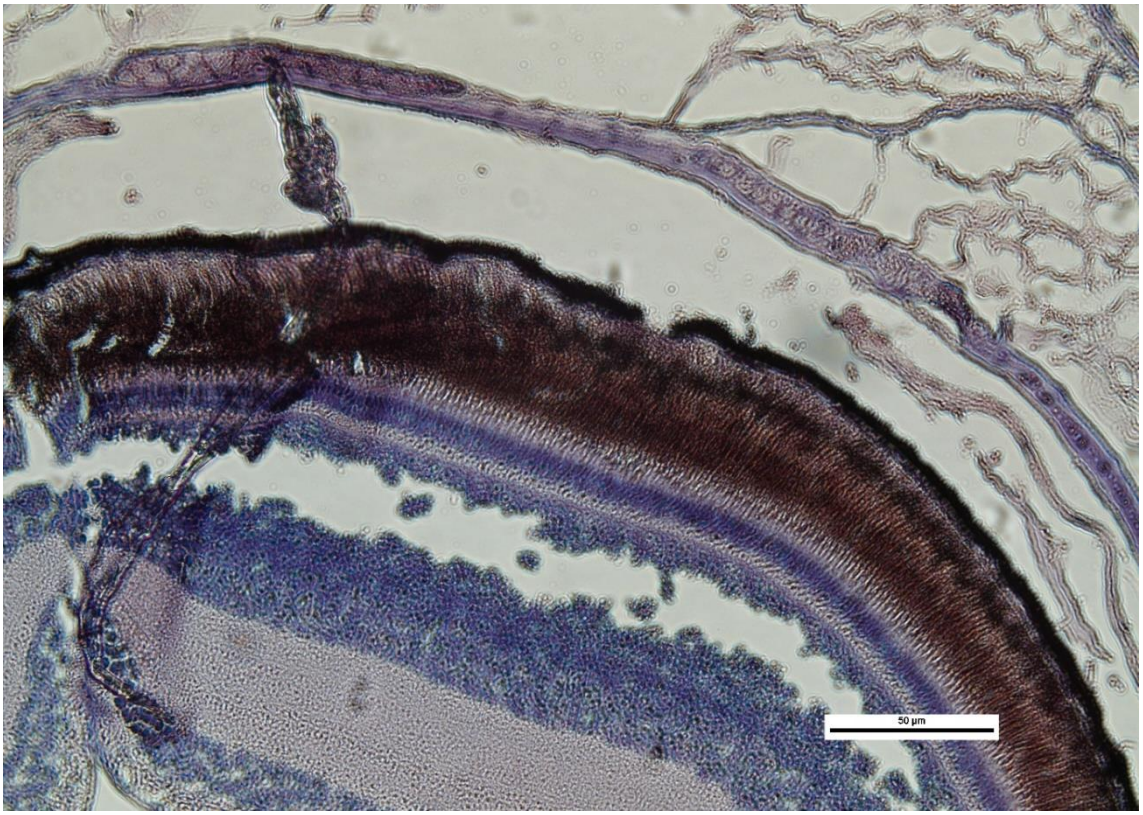

Fig. 3: A closer view of the stained area, from sample 1C.

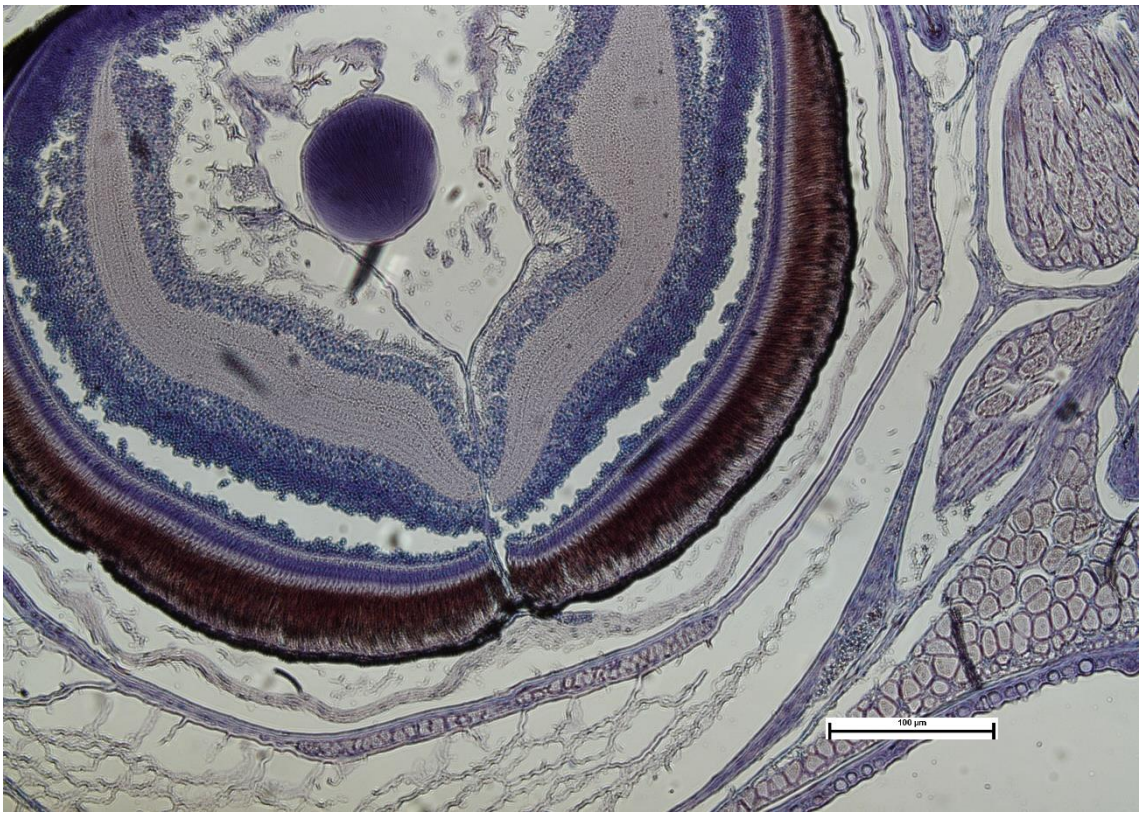

Fig. 4: Similarly to the other samples, the area around the eye is also colored in the control samples. This was the only colored area in sample 1E.

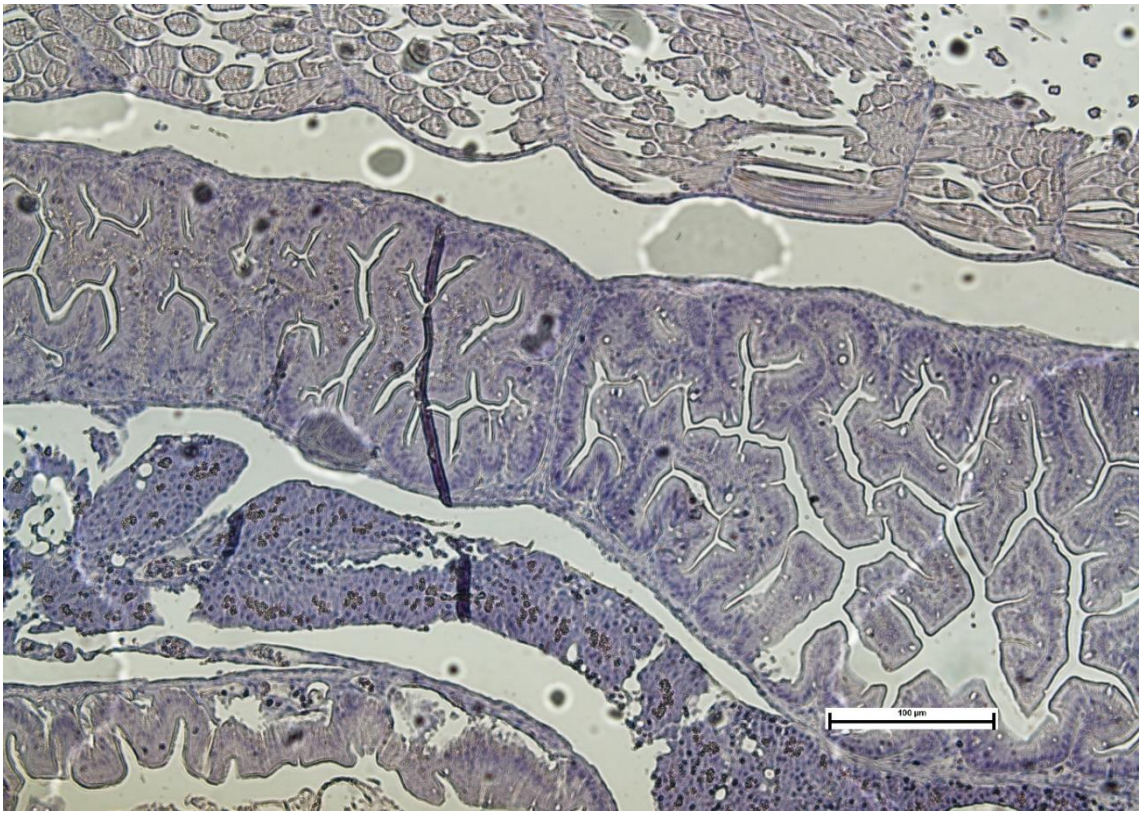

Fig. 5: Gut epithelium from sample 1A

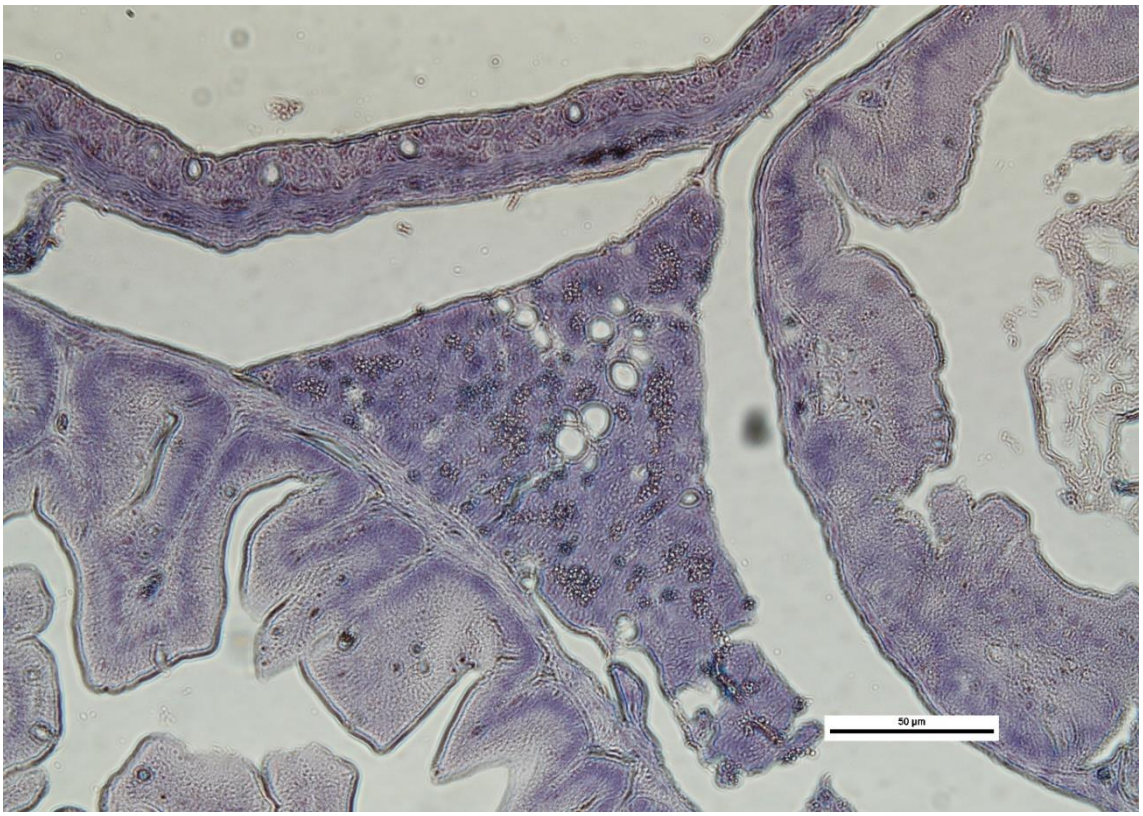

Fig. 6: Gut epithelium and bone marrow from sample 1D

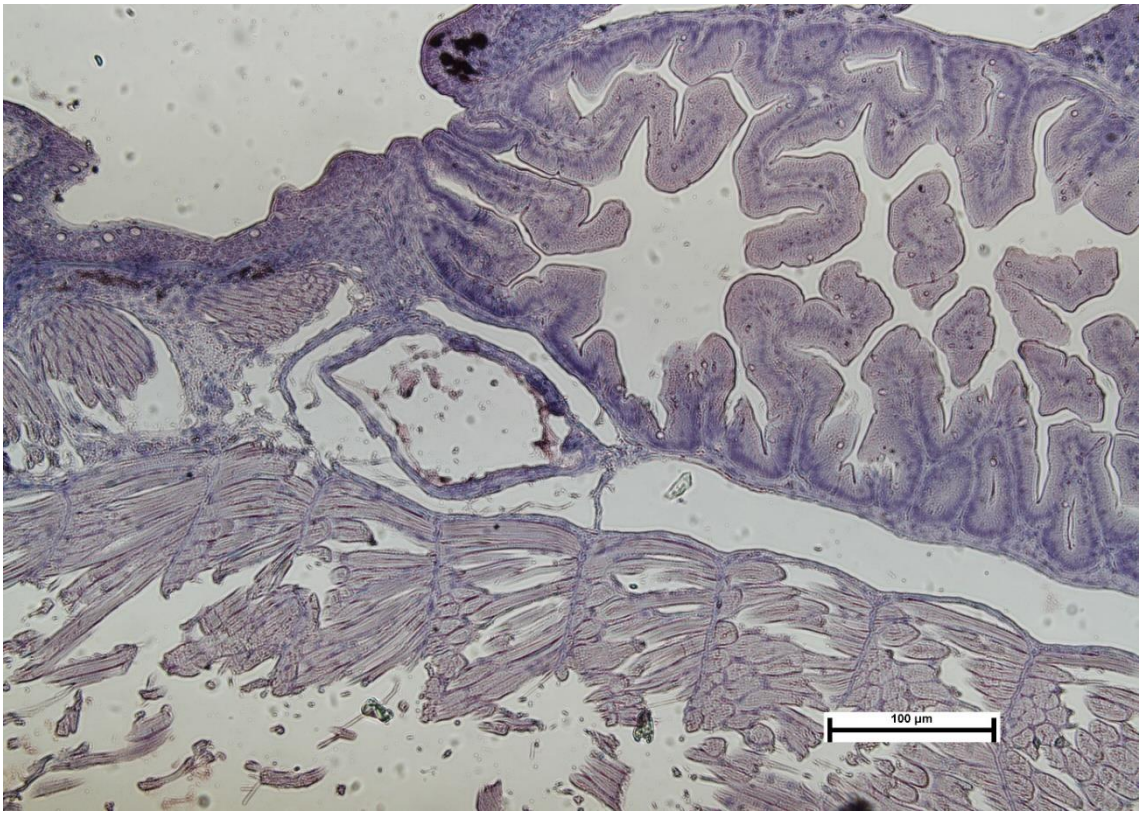

Fig. 7: gut epithelium and damaged muscle tissue in sample 1C

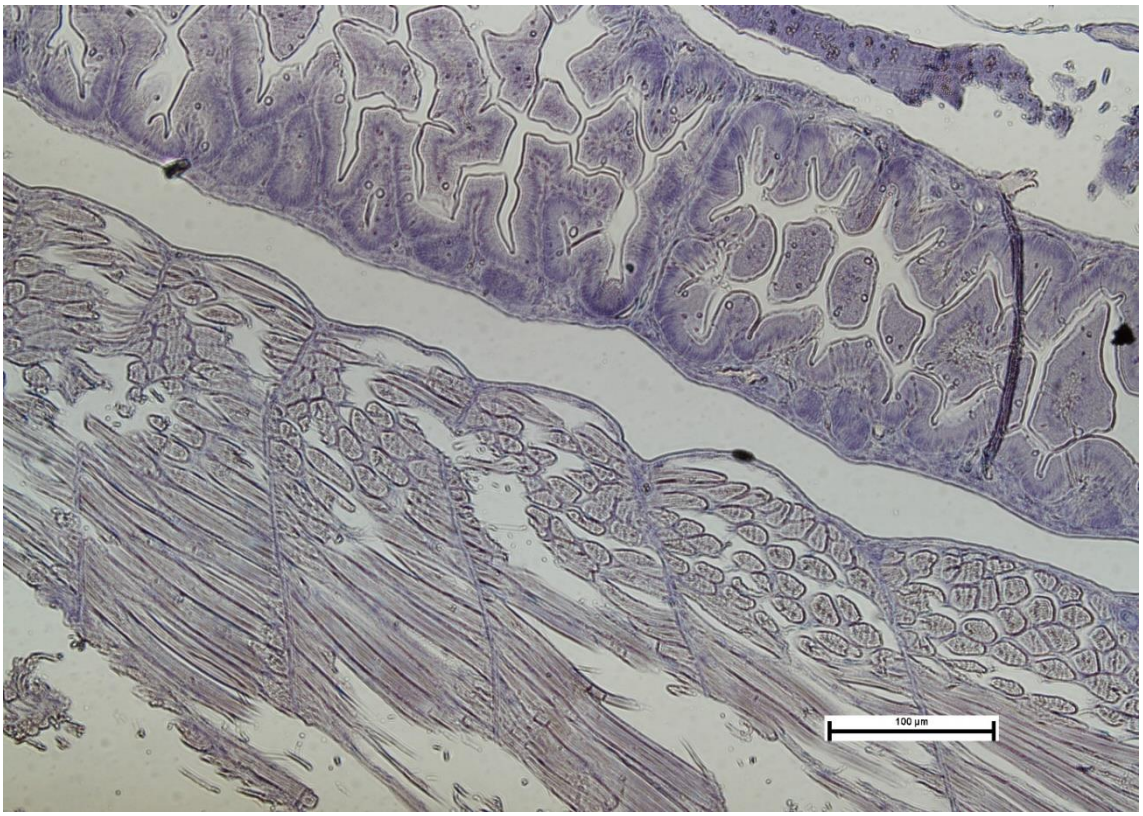

Fig. 8: gut epithelium and damaged muscle tissue in sample 1D

3.2 IHC: FIRST PRIMARY ANTIBODY CONCENTRATION TEST

The second immunohistochemistry session tested a range of different concentrations of the primary antibody. The secondary antibody concentration was 1:1000. The test had two control samples, and it used larvae of the fourth developmental stage, taken from the tank B (control diet).

Table 5: Parameters and results from the second IHC test.

	1st antibody concentration	**Result**
Sample 2A	1:200	Weak and uniform coloring
Sample 2B	1:400	Strong and uniform coloring in almost all tissue except bone marrow
Sample 2C	1:600	Fairly uniform coloring in almost all tissue except bone marrow
Sample 2D	1:800	Very weak coloring
Sample 2E	1:1000	Weak coloring. Messy and damaged.
Sample 2F	1:1200	Weak and uniform coloring
Sample 2G	1:1400	Barely any coloring
Sample 2H	1:1600	Barely any coloring
Sample 2I	1:1800	Strong coloration in a few select spots, no coloration in the rest of the tissue.
Sample 2J	1:2000	No coloration outside of a few select spots
Sample 2K	-	No coloration
Sample 2L	-	No coloration

Both the stain and the counterstain had a stronger presence in these samples. The higher concentration samples generally had higher concentration than the lower concentration samples, but this wasn't always the case. Some of the samples were damaged, and sample 2E was particularly messy.

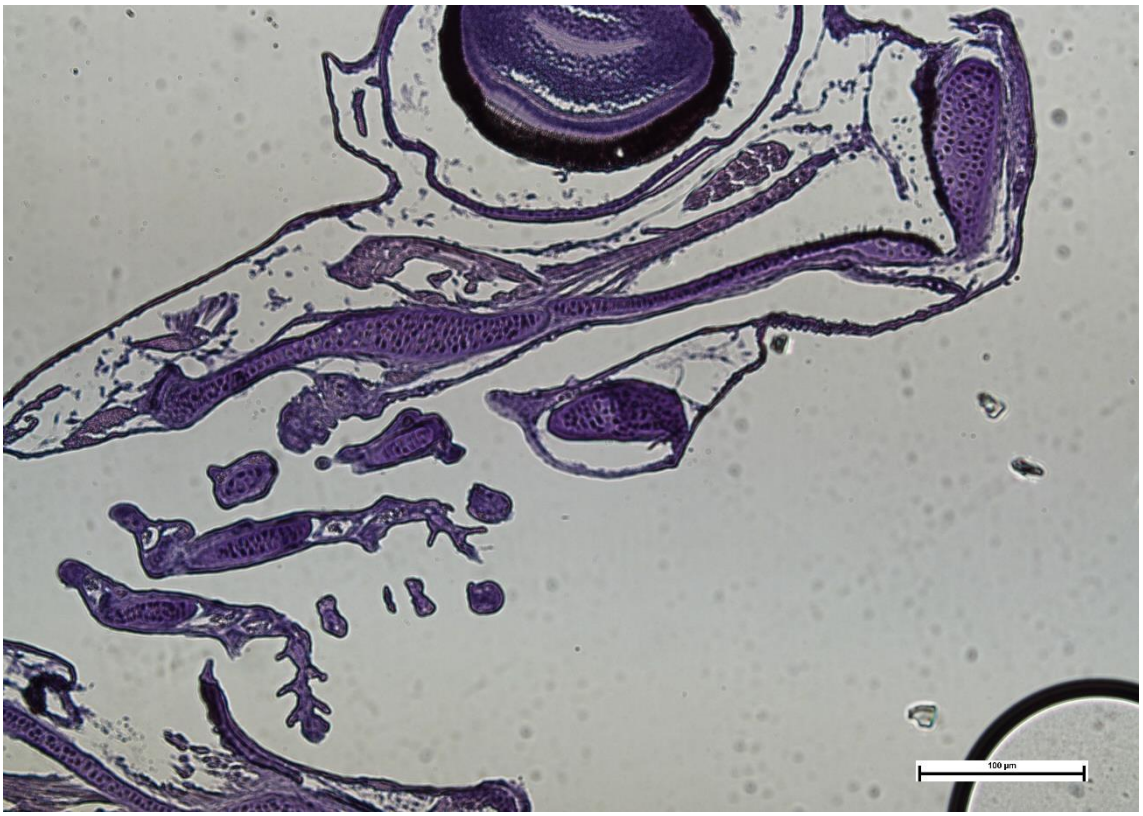

Fig. 9: There was some color present in most tissue in sample 2A, but it is difficult to discern.

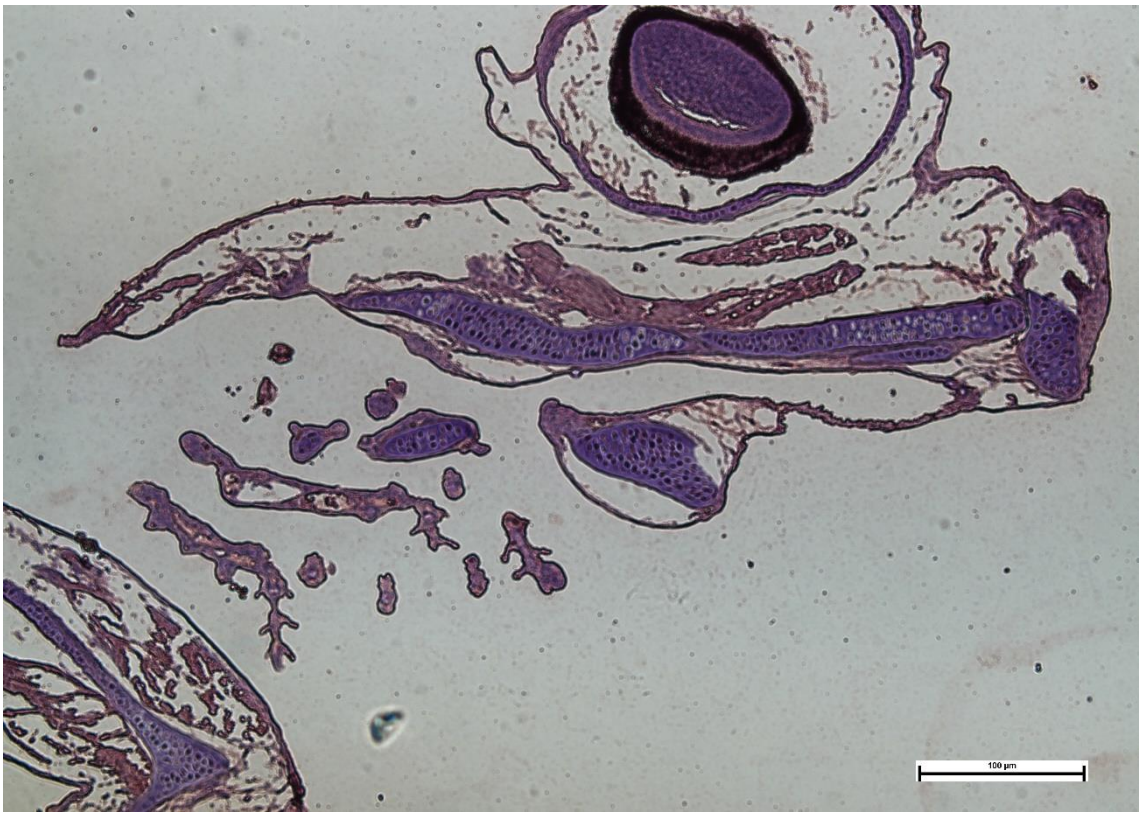

Fig. 10: Most of sample 2B was also uniformly stained in purple, and it is much more distinct here than in sample 2A.

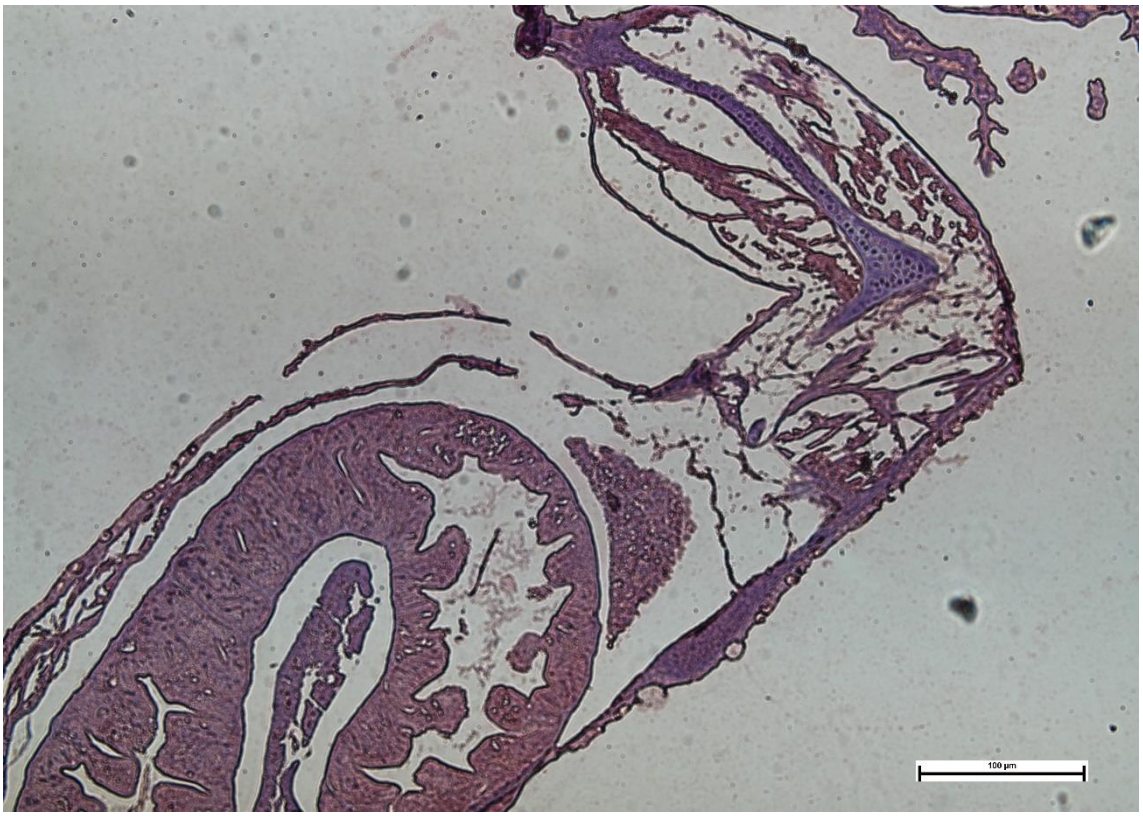

Fig. 11: As with the rest of sample 2B, its gut was uniformly purple.

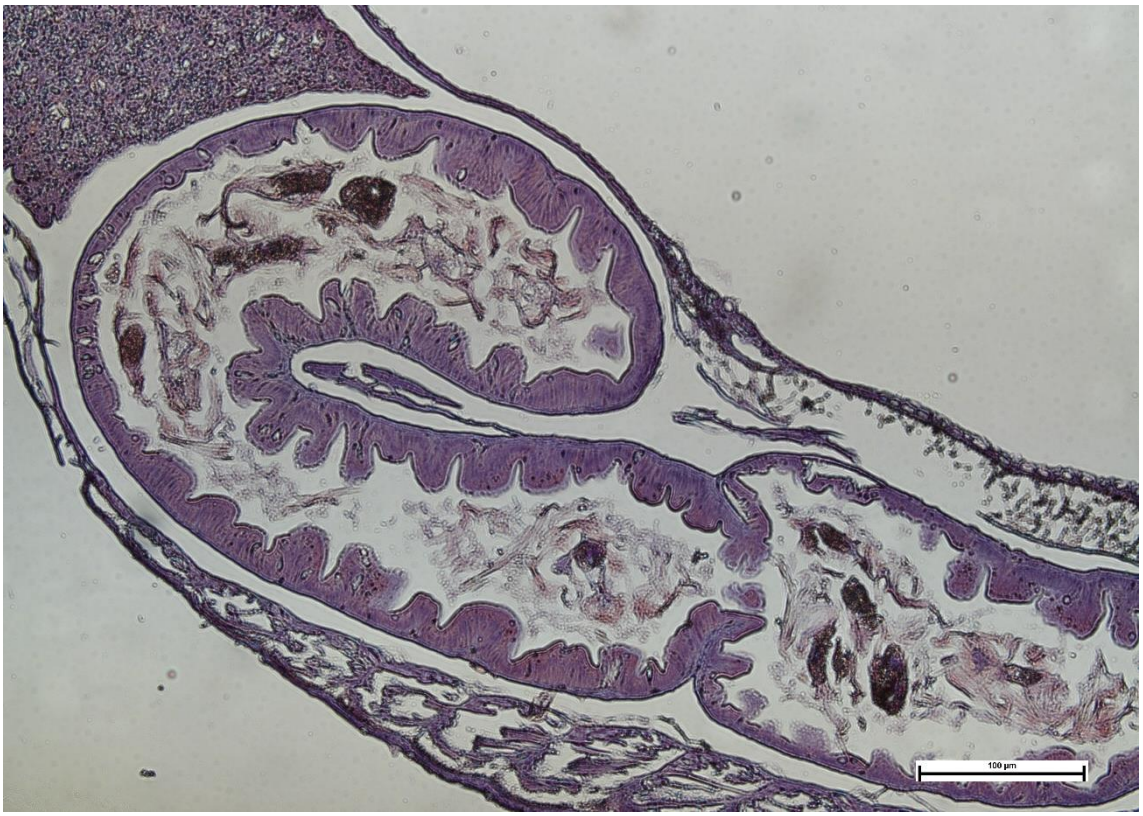

Fig. 12: The staining in sample 2C was stronger than 2A, but weaker than 2B.

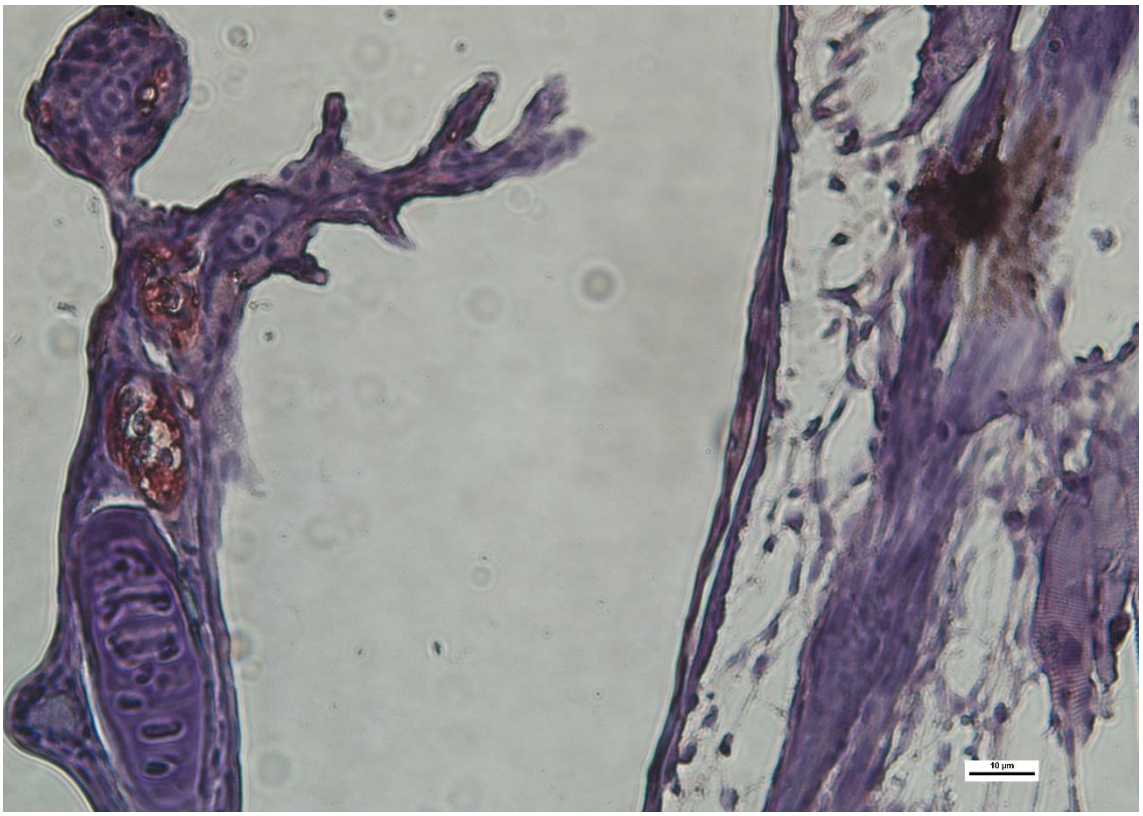

Fig. 13: Although most of sample 2C was uniformly colored, there are some spots of brighter red/purple, such as here in the gills.

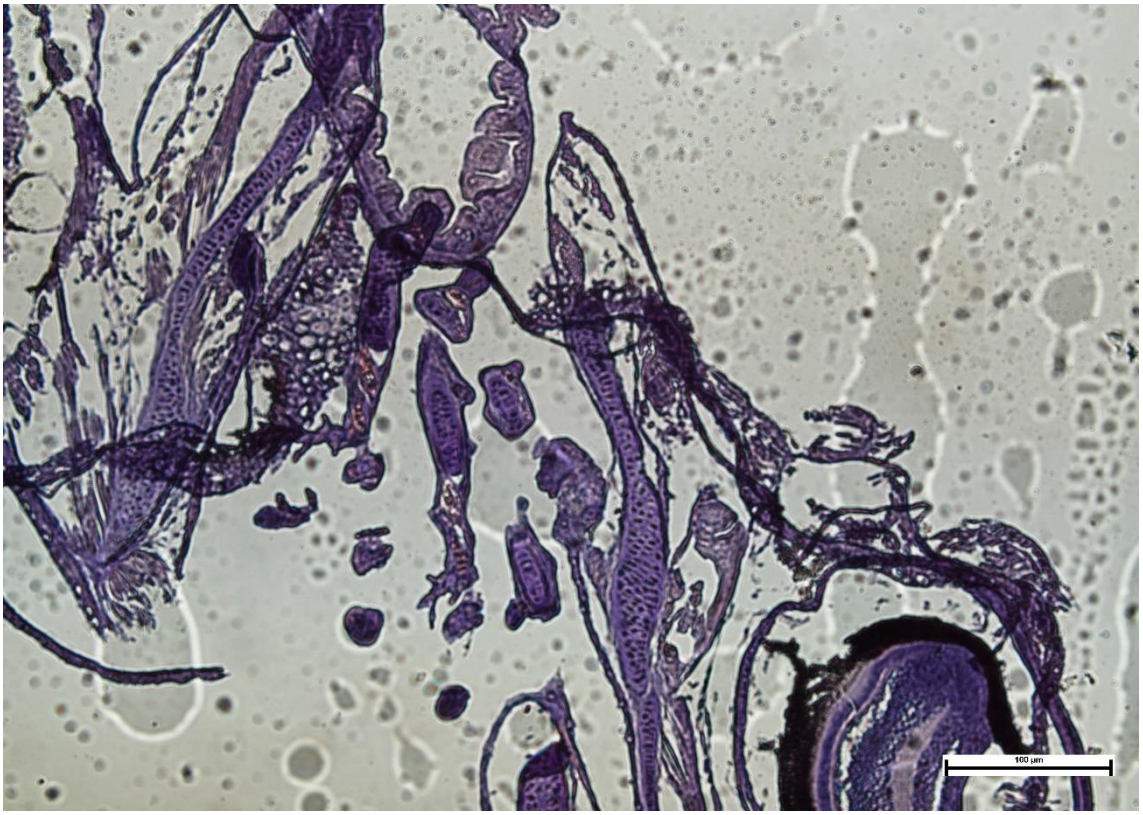

Fig. 14: Sample 2E was very messy and had little color.

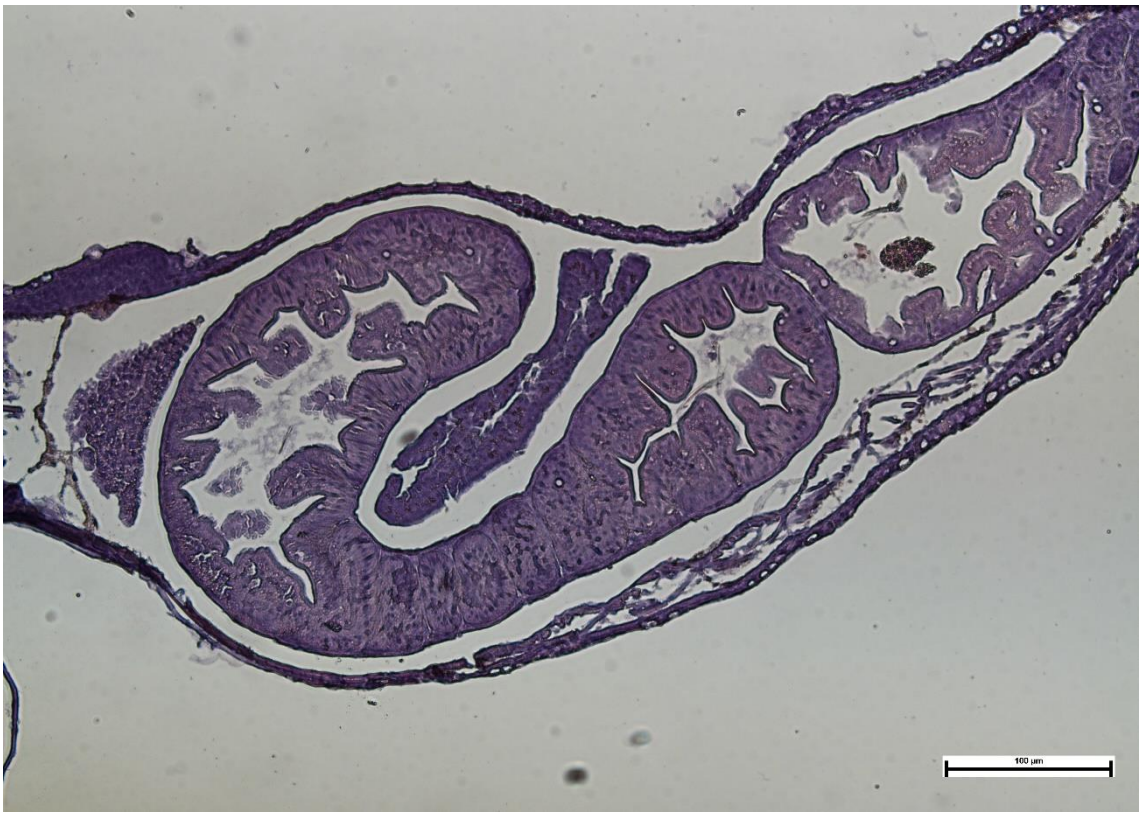

Fig. 15: The staining in sample 2F was uniform and weak, even in the gut.

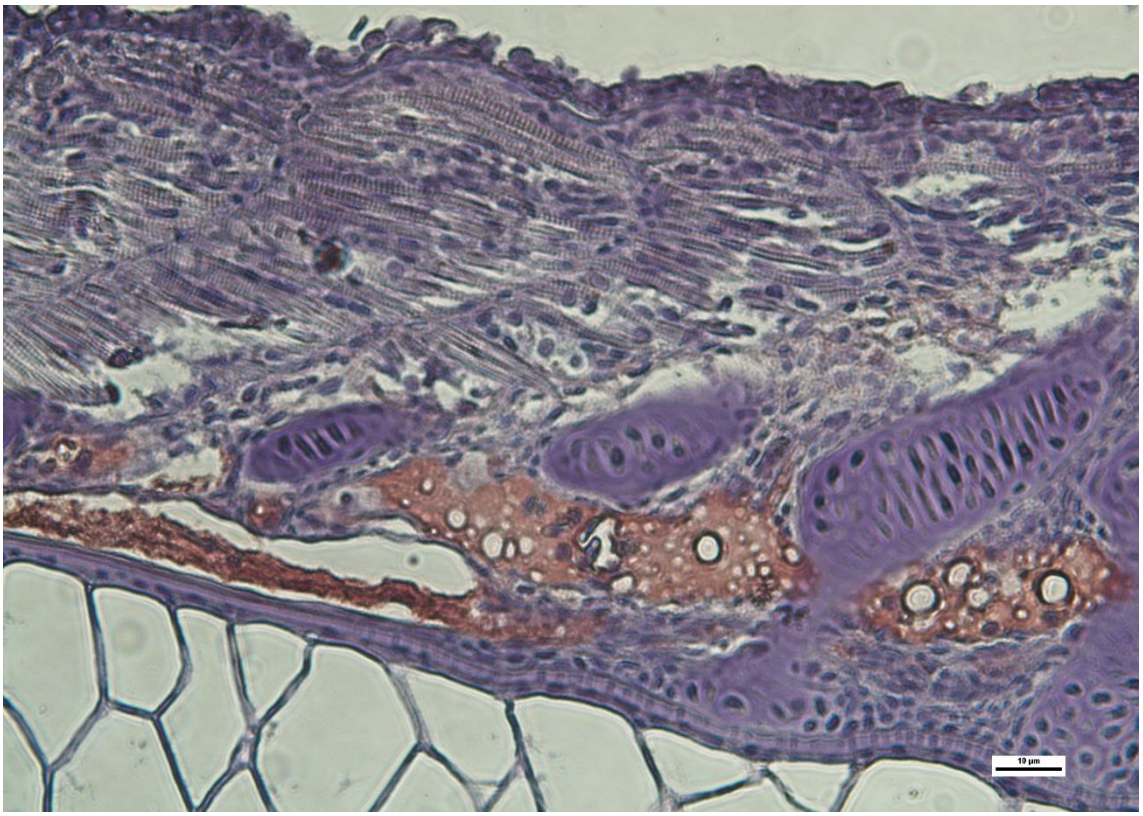

Fig. 16: Sample 2I displayed a stronger red color in some distinct spots, but no coloration in most of the tissue. The picture shows muscle tissue, bone marrow, and epithelium.

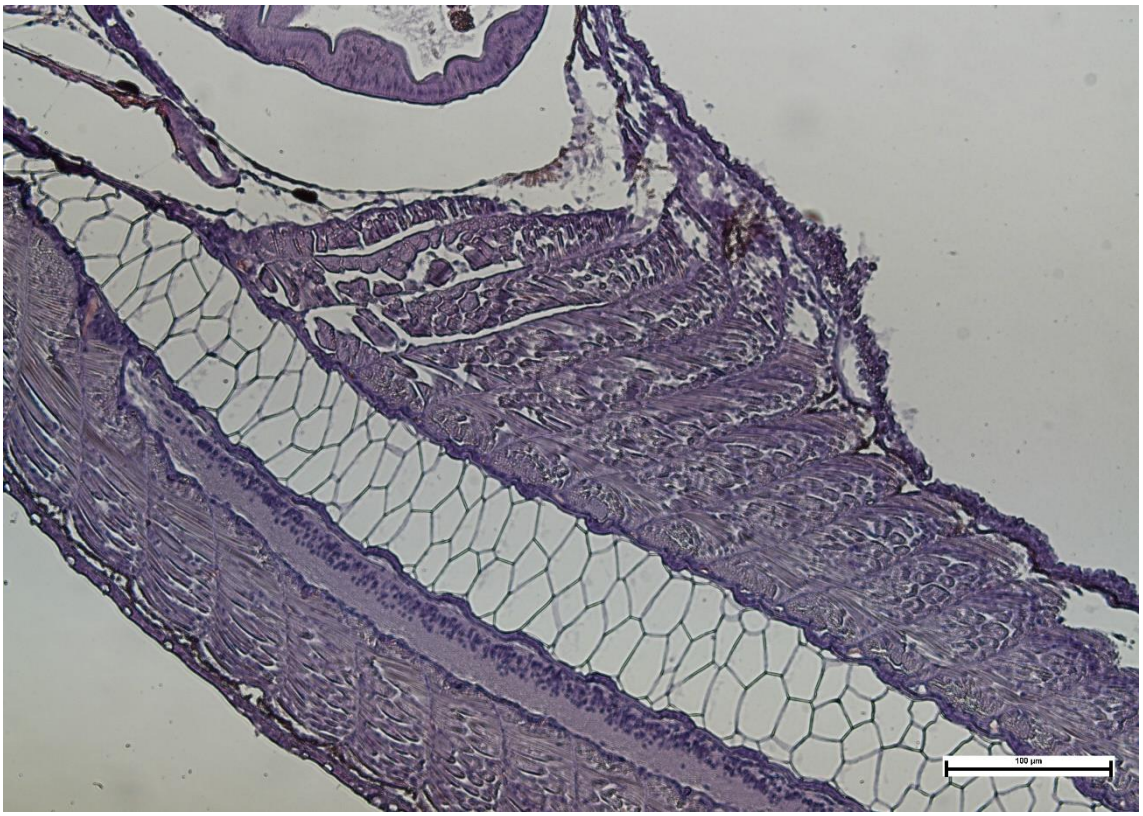

Fig. 17: Most of the tissue in sample 2J displays no red/purple color.

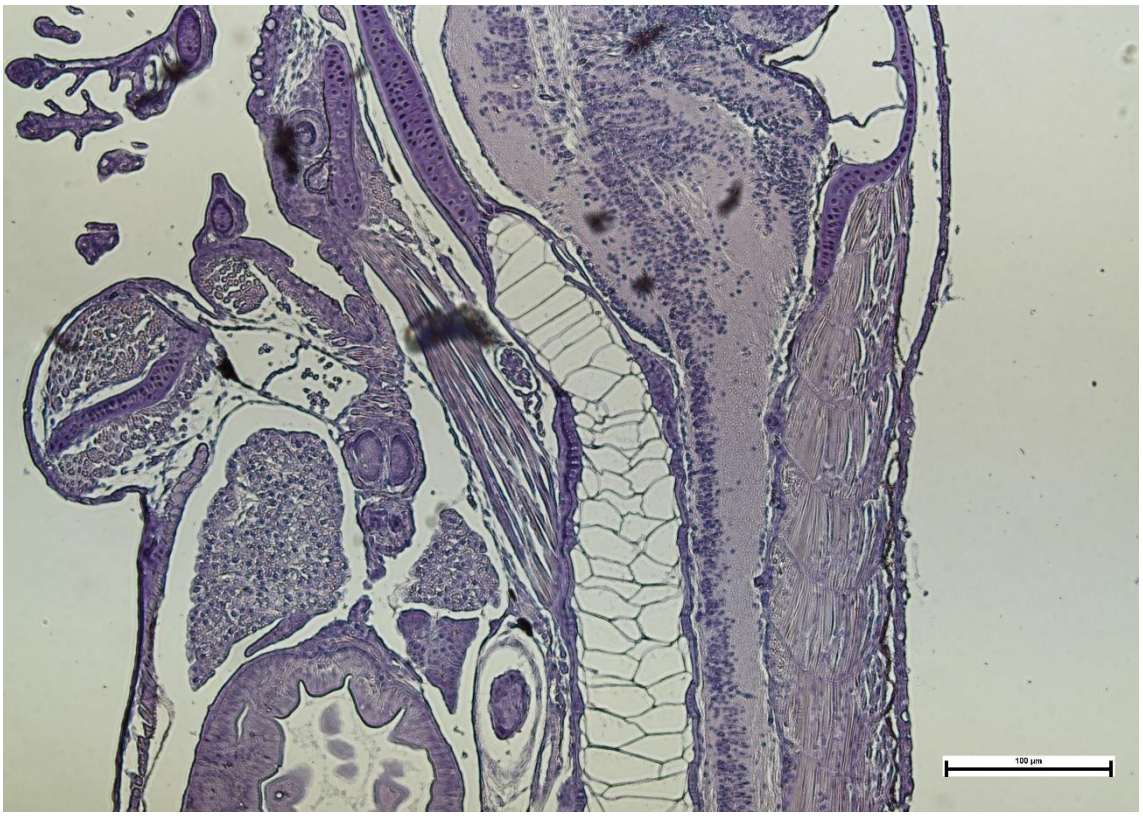

Fig. 18: The control samples, such as sample 2K, showed no red coloration.

3.3 IHC: SECOND PRIMARY ANTIBODY CONCENTRATION TEST

The third immunohistochemistry session tested various lower primary antibody concentrations than the second session. The secondary antibody concentration was 1:1000, and the blocking buffer was applied to the samples for two hours. The test had two control samples, and it used larvae of the seventh developmental stage, taken from the tank F (control diet).

Table 6: Parameters and results from the third IHC test.

	1st antibody concentration	**Result**
Sample 3A	1:1000	Very strong and uniform coloring in the entire sample
Sample 3B	1:1200	Very strong coloring in small distinct areas throughout the sample.
Sample 3C	1:1400	Extremely strong and uniform coloring in the entire sample
Sample 3D	1:1600	Extremely strong and uniform coloring in the entire sample
Sample 3E	1:1800	Very strong coloring in the entire sample
Sample 3F	1:4000	Distinct coloring in small distinct spots throughout the sample
Sample 3G	1:5000	Very distinct coloring in distinct areas
Sample 3H	1:6000	Distinct coloring in small areas
Sample 3I	1:7000	Distinct coloring in small areas
Sample 3J	1:8000	Distinct coloring in small areas
Sample 3K	1:9000	Weak coloring
Sample 3L	1:10.000	Weak coloring
Sample 3M	1:15.000	Very weak coloring
Sample 3N	1:20.000	Very weak coloring
Sample 3O	Control	No coloring
Sample 3P	Control	No coloring

All the samples below 1:2000 primary antibody concentration had very strong and uniform coloration, apart from sample 3B. This is a very different result from the previous IHC test. Some of the samples were very badly damaged, and the damaged areas often had particularly strong coloration.

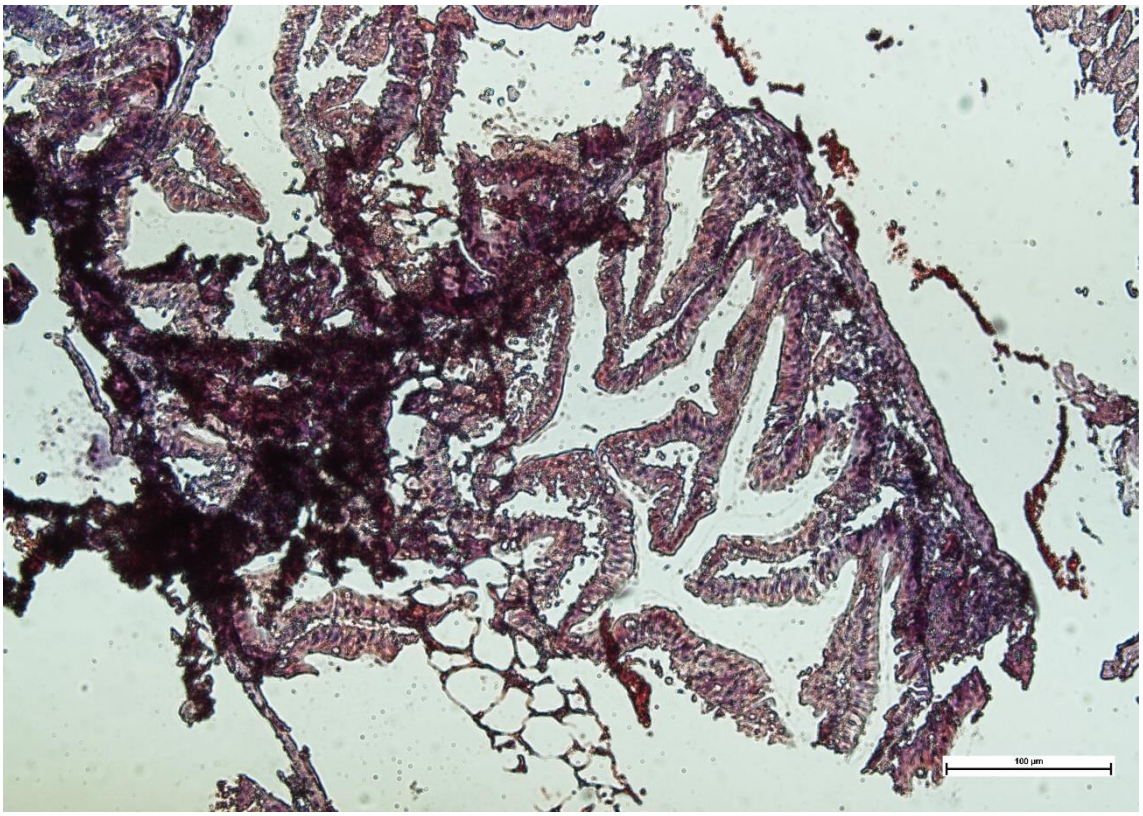

Fig. 19: Sample 3A was severely damaged and covered in strong color.

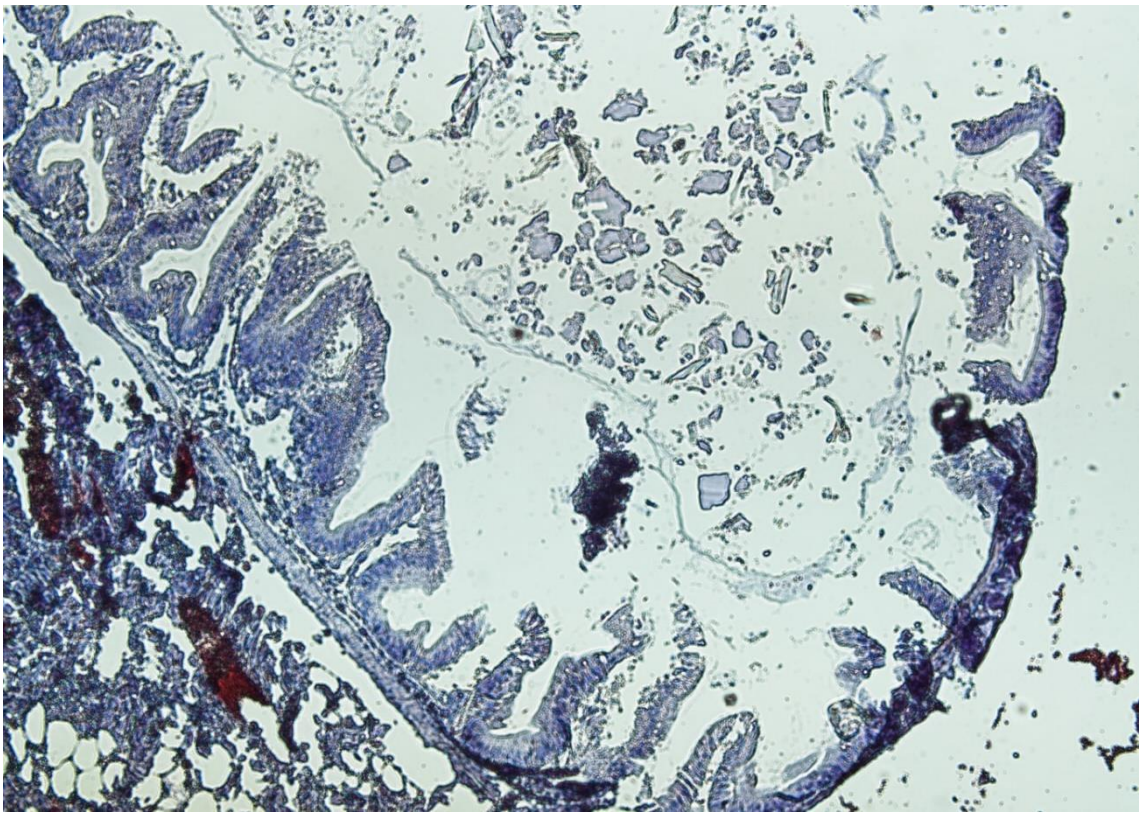

Fig. 20: Sample 3B had barely any color compared to other samples of similar concentration, apart from some distinct spots. It was also very badly damaged.

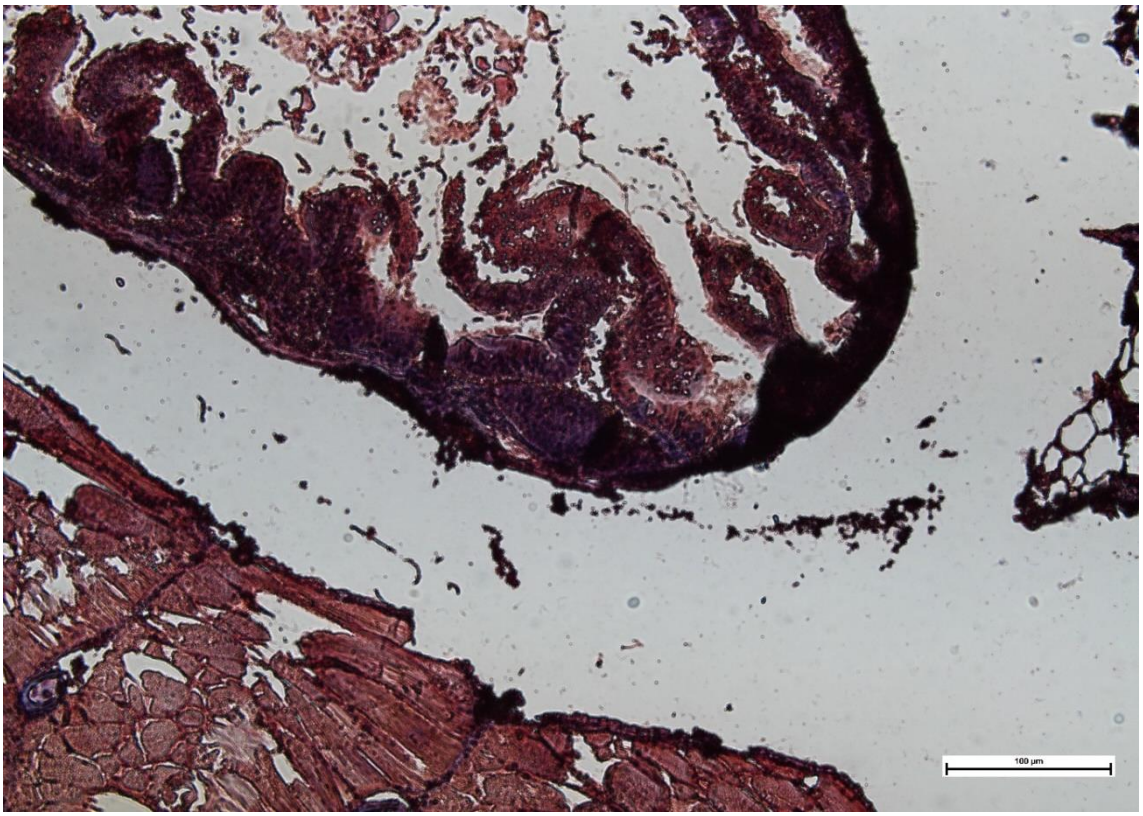

Fig. 21: Sample 3C was completely covered in extremely strong coloration.

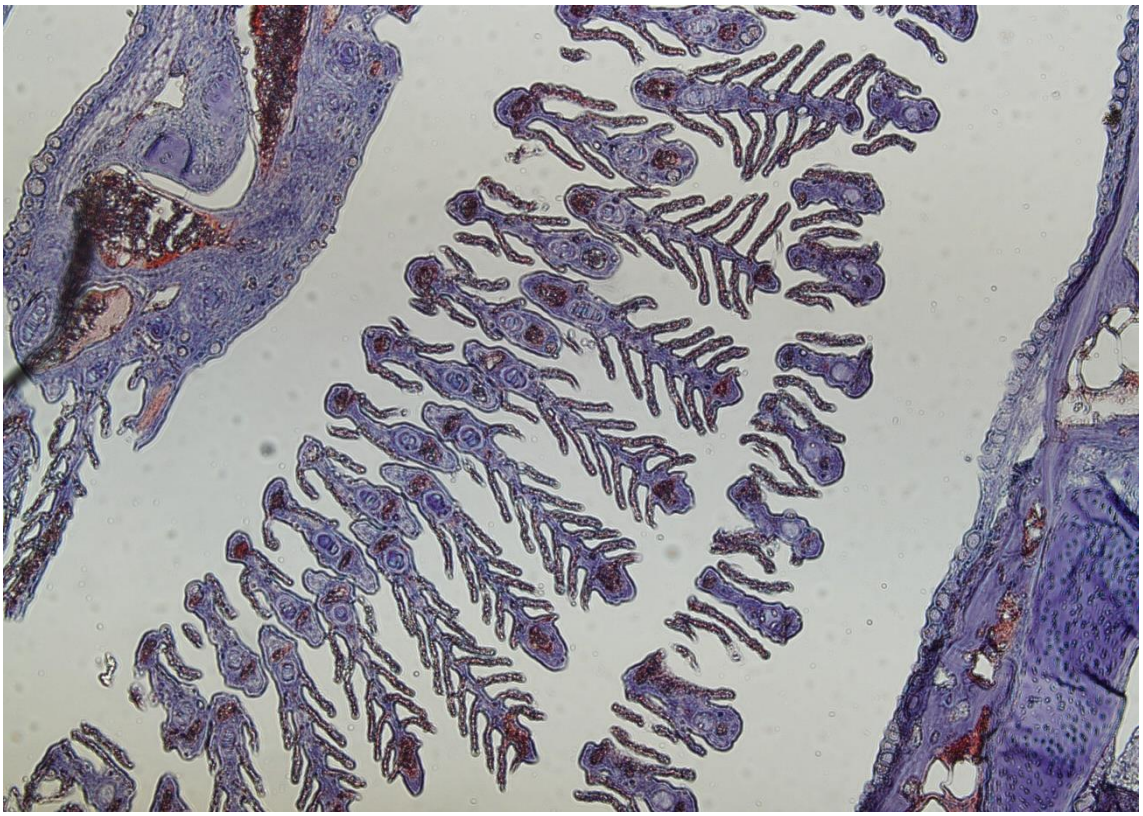

Fig. 22: In contrast to the earlier samples, the red signal in sample 3F isn't uniform. The gill shows distinct spots of coloring.

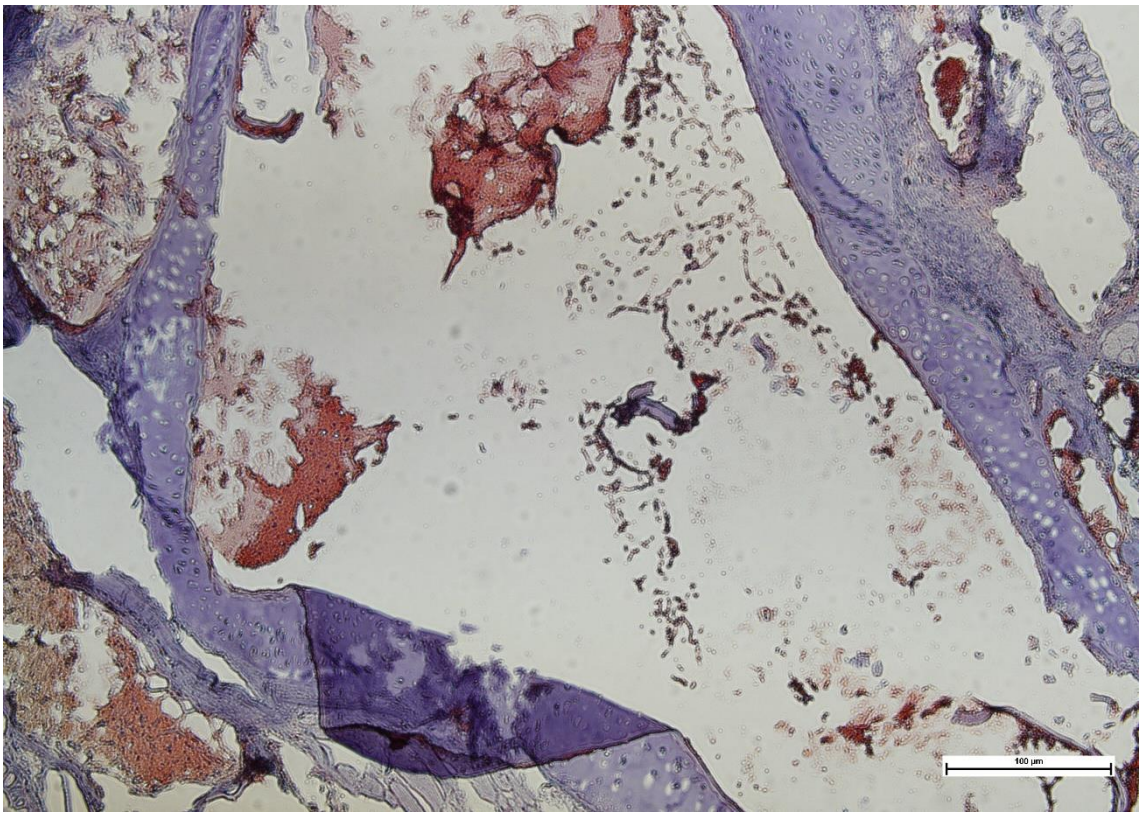

Fig. 23: Some of the most damaged areas in sample 3F show strong coloration. This picture is from the head.

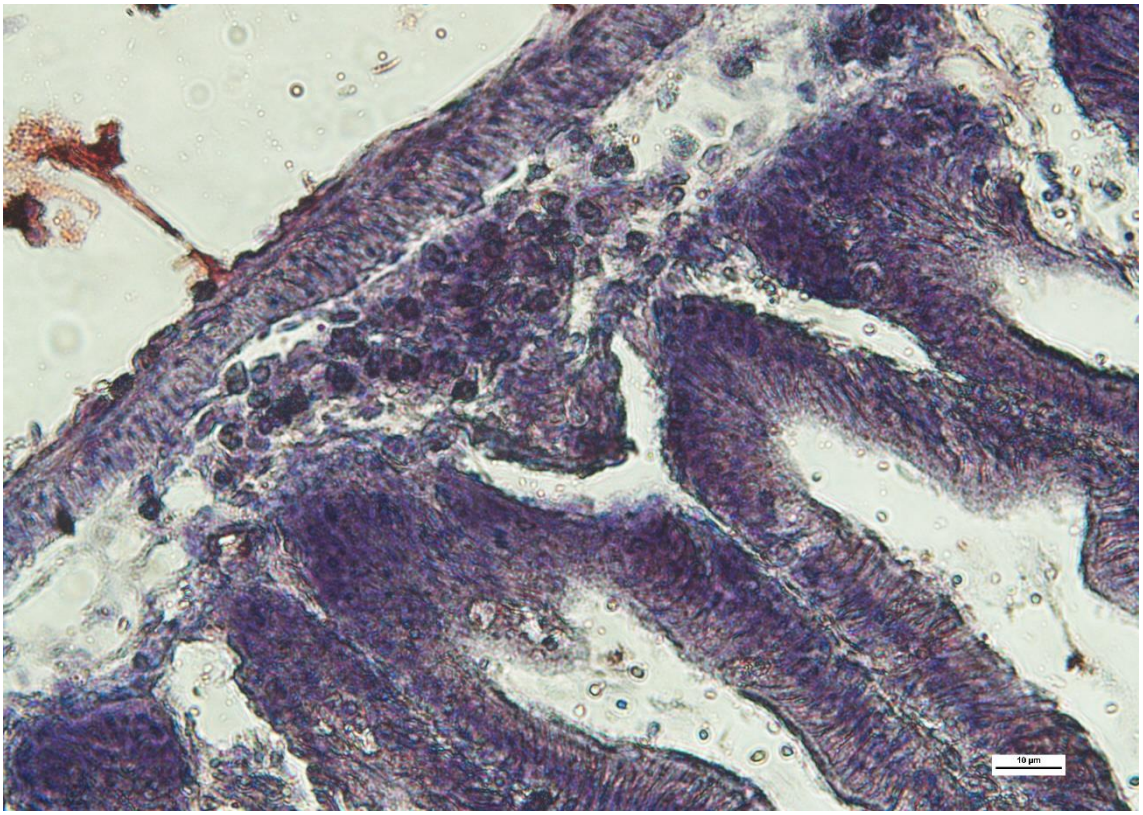

Fig. 24: None of the samples had distinct spots of coloration in the gut. This is sample 3I.

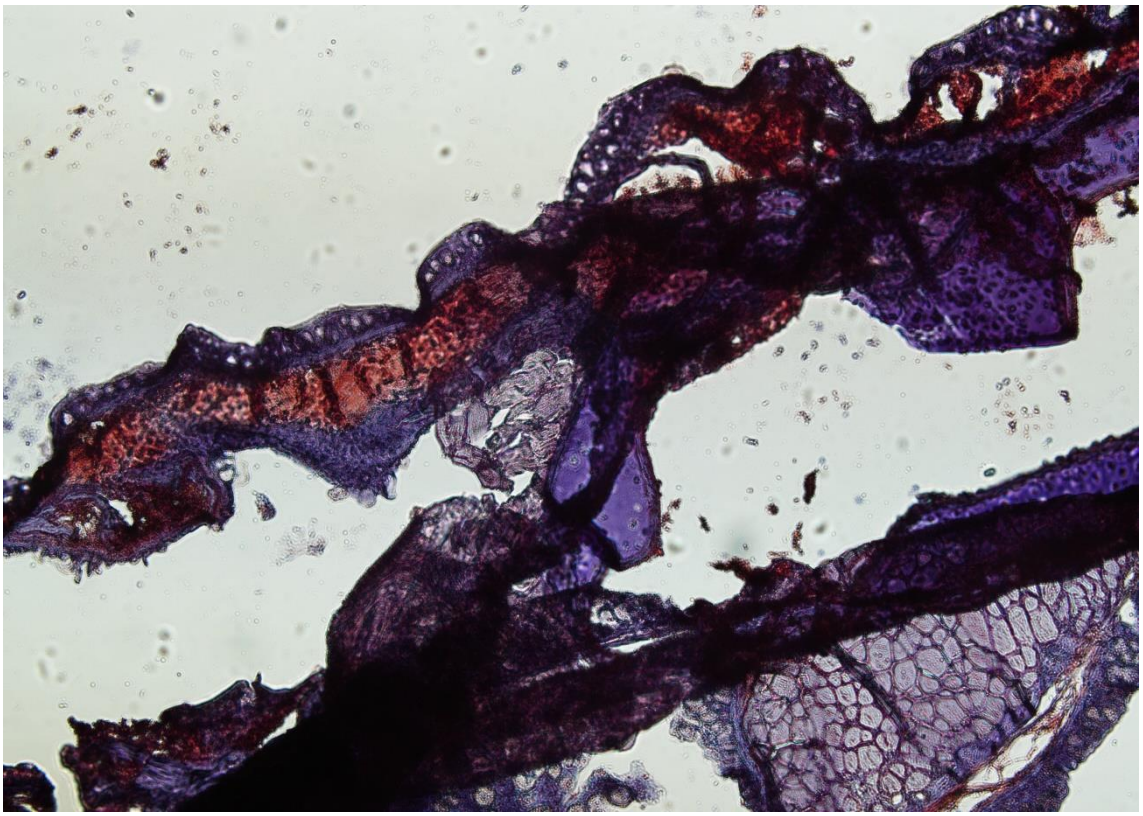

Fig. 25: A bunched up area of sample 3I displayed strong signal.

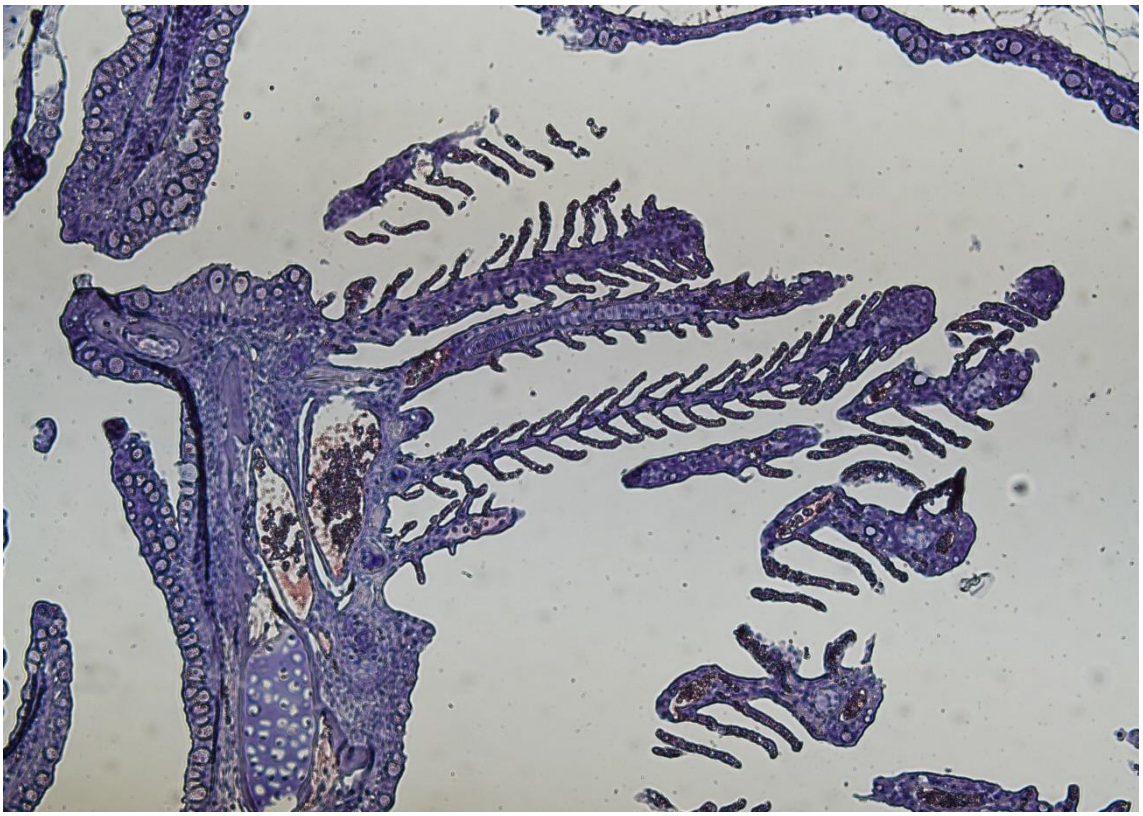

Fig. 26: Sample 3J displayed weak coloration.

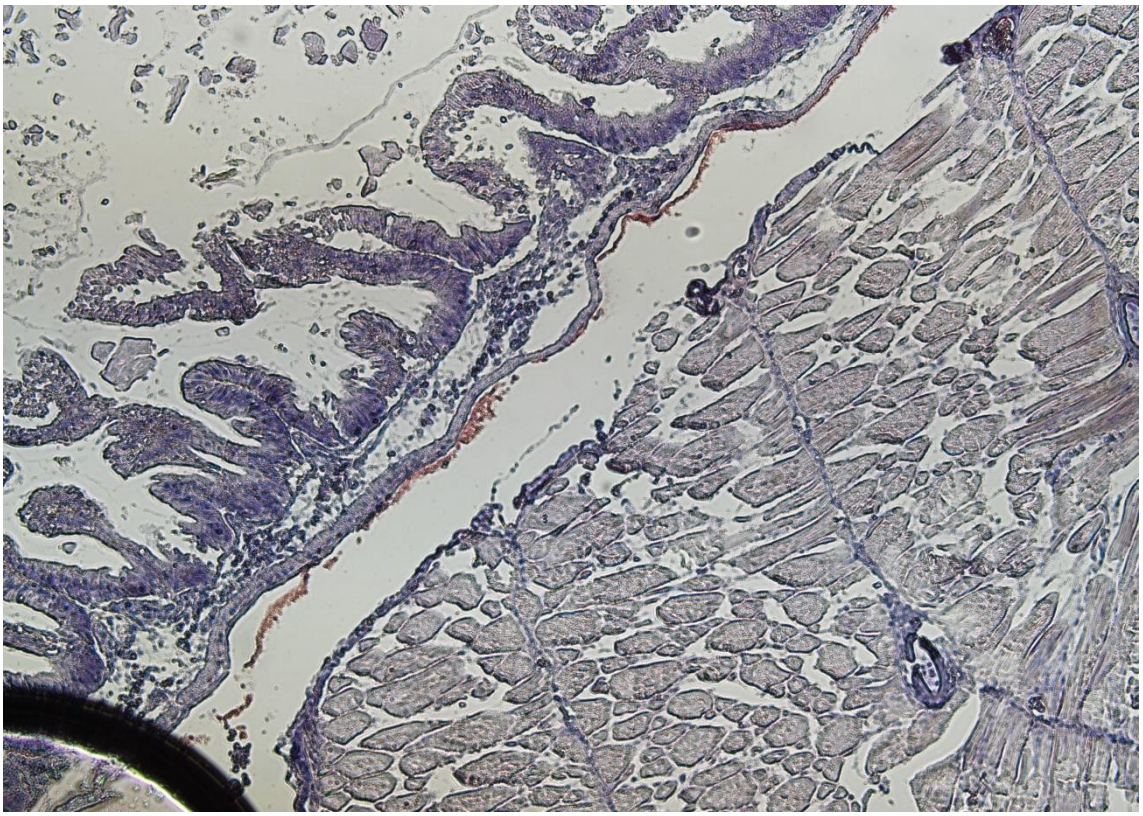

Fig. 27: Any sample with a primary antibody concentration below 1:10.000 had very weak coloration. This is gut and muscle tissue from sample 3L.

3.4 IHC: BLOCKING BUFFER TEST

In the fourth ICH test, the samples were kept in the blocking buffer for 3 hours. The test encompassed four samples with primary antibody concentrations ranging from 1:5000 to 1:8000, as well as a negative control. It used larvae of the seventh developmental stage, taken from tank B (control diet).

Table 7: Parameters and results from the fourth IHC test.

	1st antibody concentration	**Result**
Sample 4A	1:5000	Very strong and distinct coloring in some select areas
Sample 4B	1:6000	Very strong and distinct coloring in some select areas
Sample 4C	1:7000	Very strong and distinct coloring in some select areas
Sample 4D	1:8000	Very strong and distinct coloring in some select areas
Sample 4E	-	No color

Samples 4A to 4D were very similar. Although the signal was somewhat stronger in the samples with higher primary antibody concentration, the difference was hardly noticeable. The coloration was very strong, and completely contained within certain distinct areas of the larvae. Across all samples except the control, these include several large continuous fields of tissue within the head, many small spots within the gills, and one specific spot in the tail. Additionally, coloration lines a lot of surfaces where tissue seems to have torn apart. In all these five samples, the gut was lost due to damage.

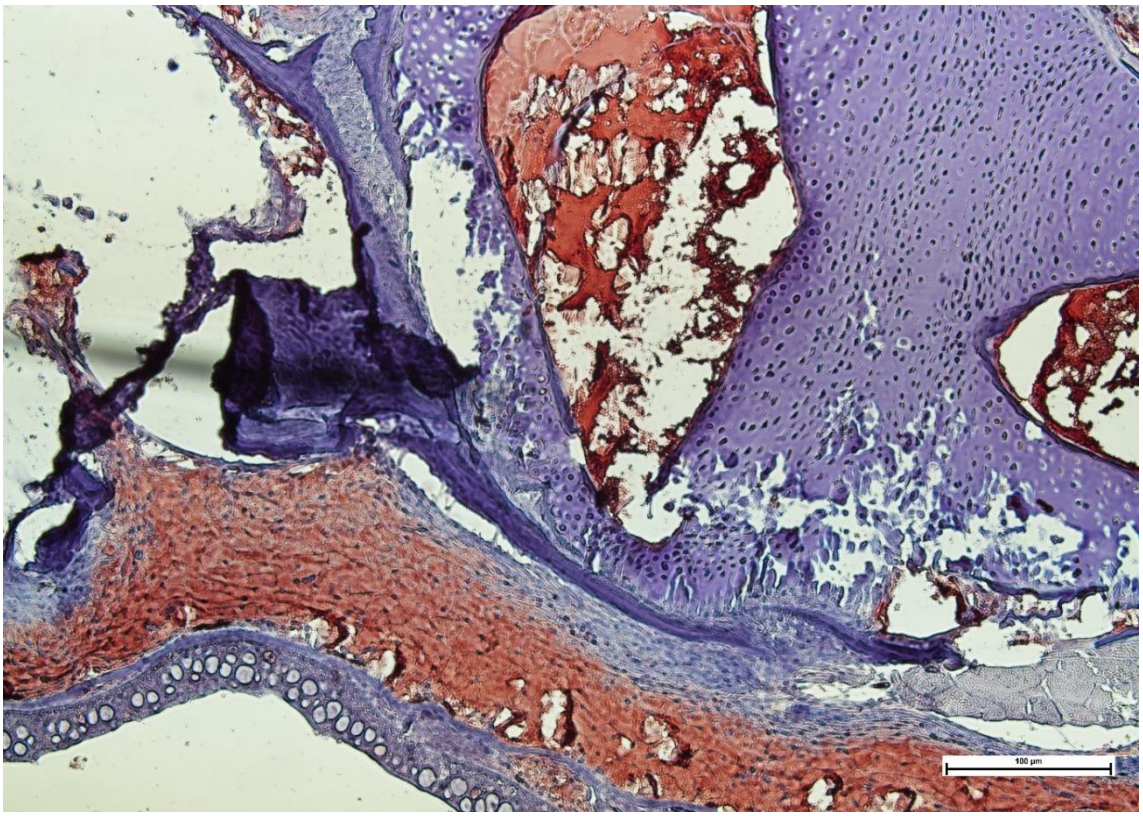

Fig. 28: There were large fields of continuous coloration in the larva's head in all the samples. Badly damaged tissue also tended to be brightly colored. This is sample 4A.

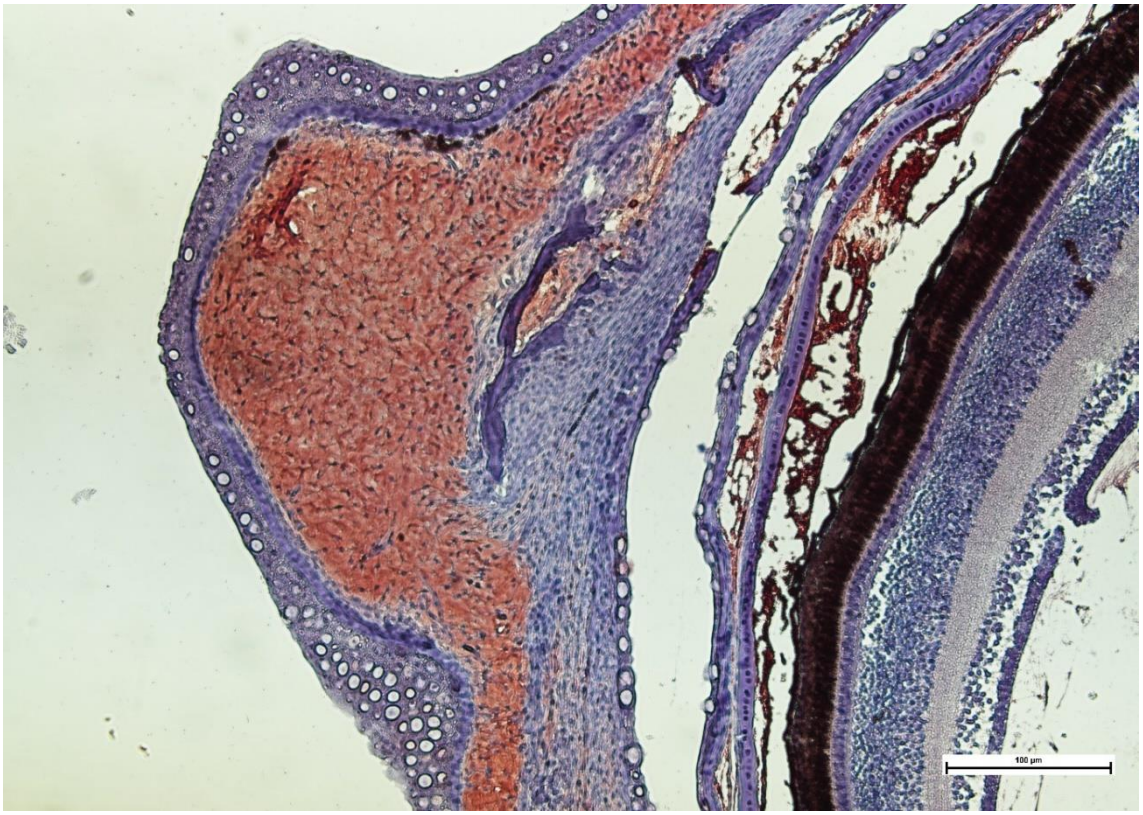

Fig. 29: A large field of continuous coloration in the larva's nose tip in sample 4A

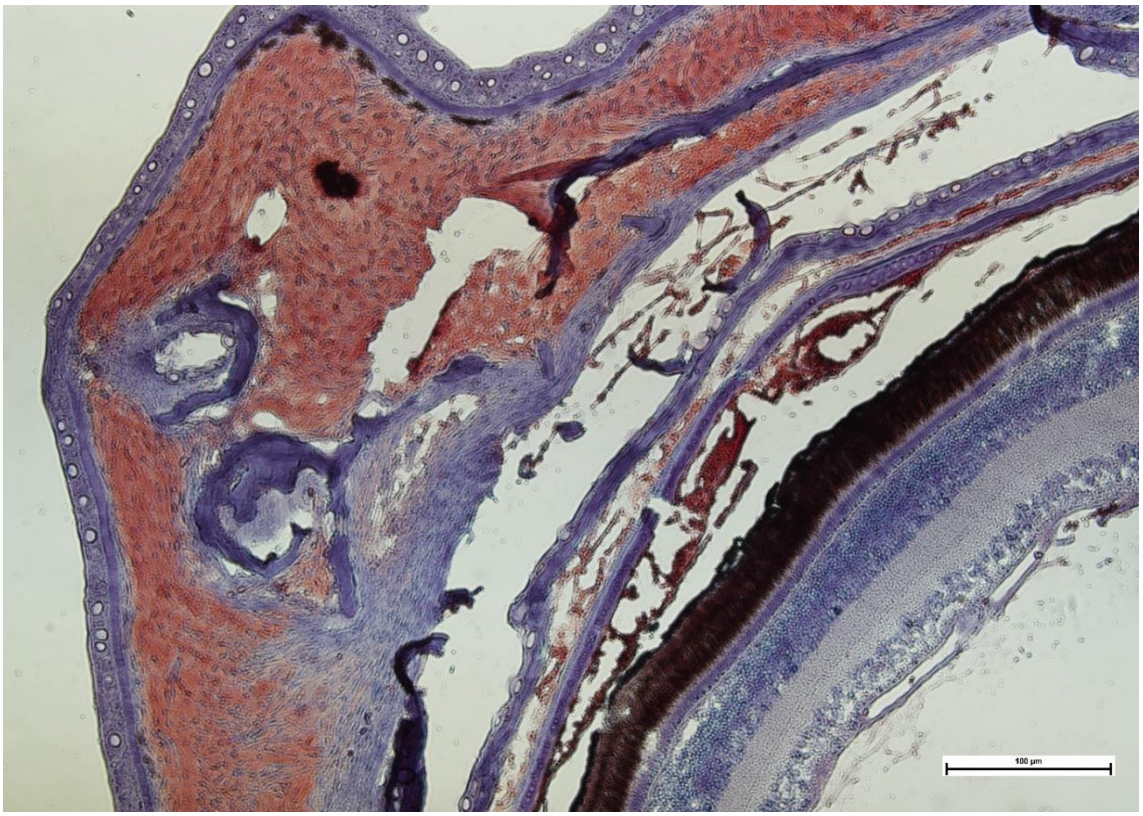

Fig. 30: All the samples displayed similar patterns of coloration. This is the nose tip of sample 4D.

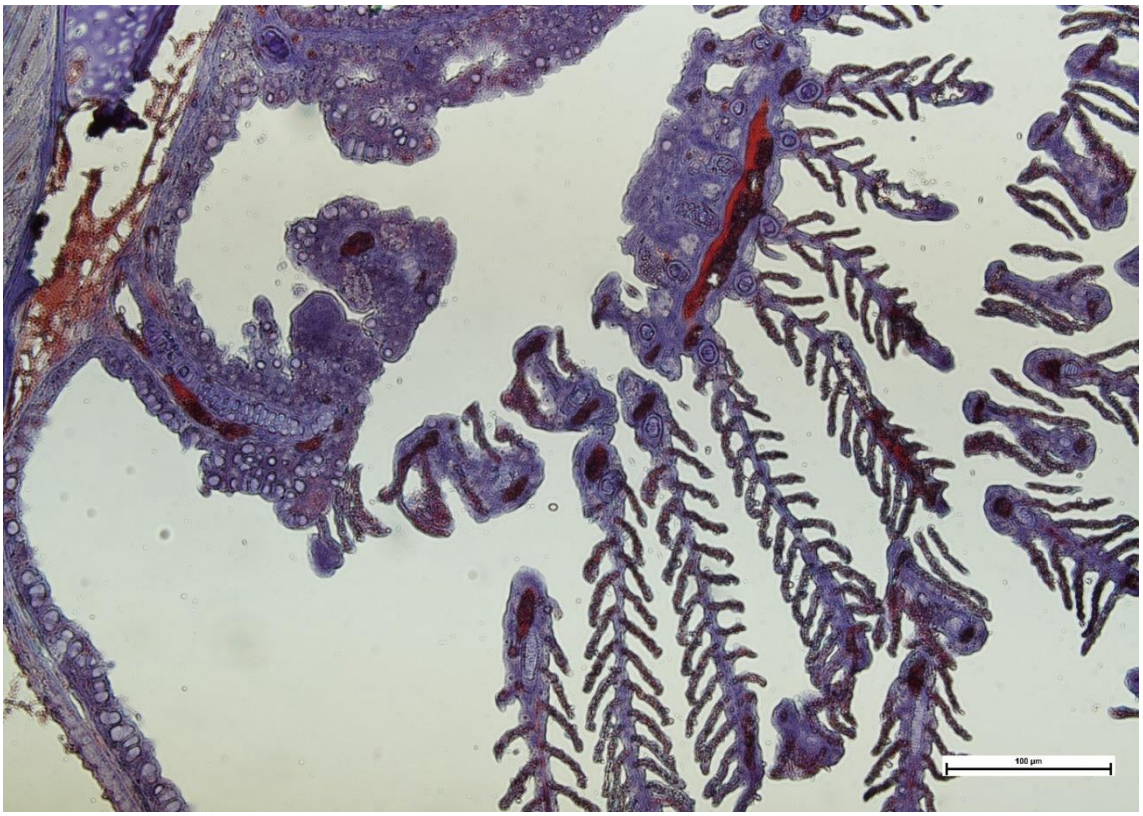

Fig. 31: All samples had distinct spots of coloration dotting the gills. These spots were in the same locations across all samples. This is sample 4C.

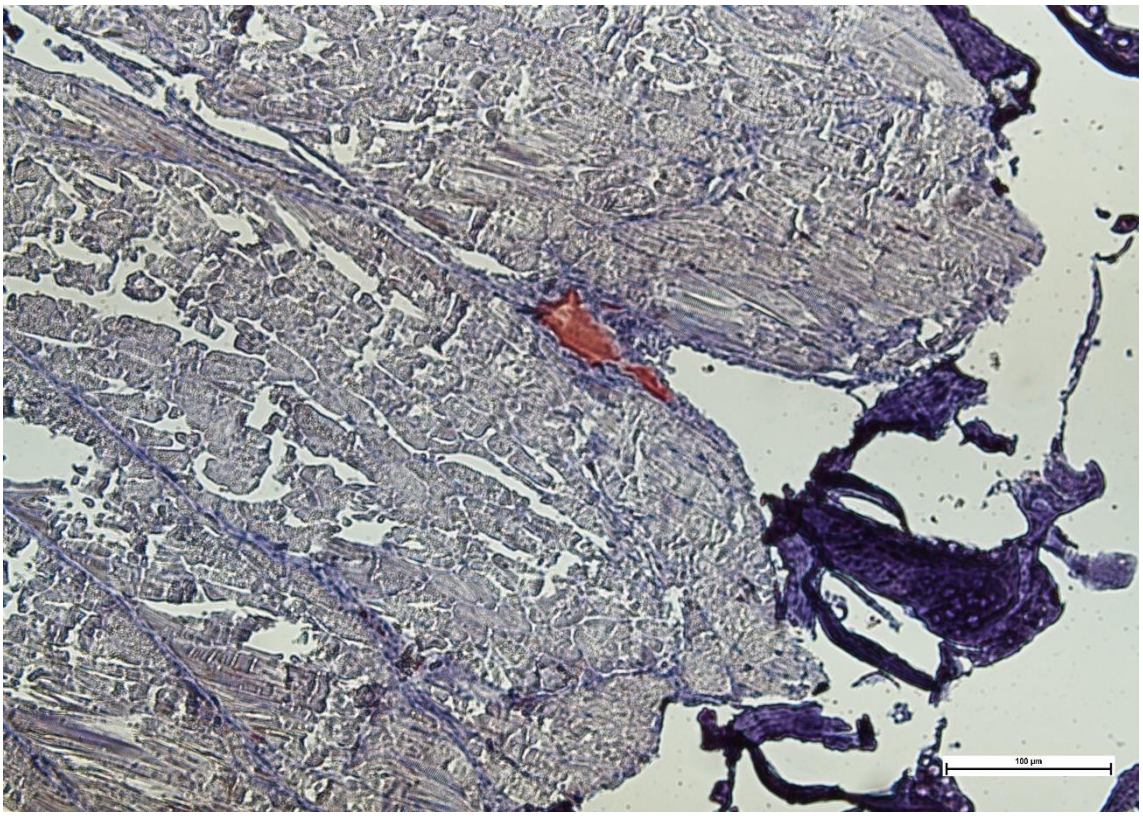

Fig. 32: All samples had a distinct spot of coloration in the same position on the tail. This is sample 4D.

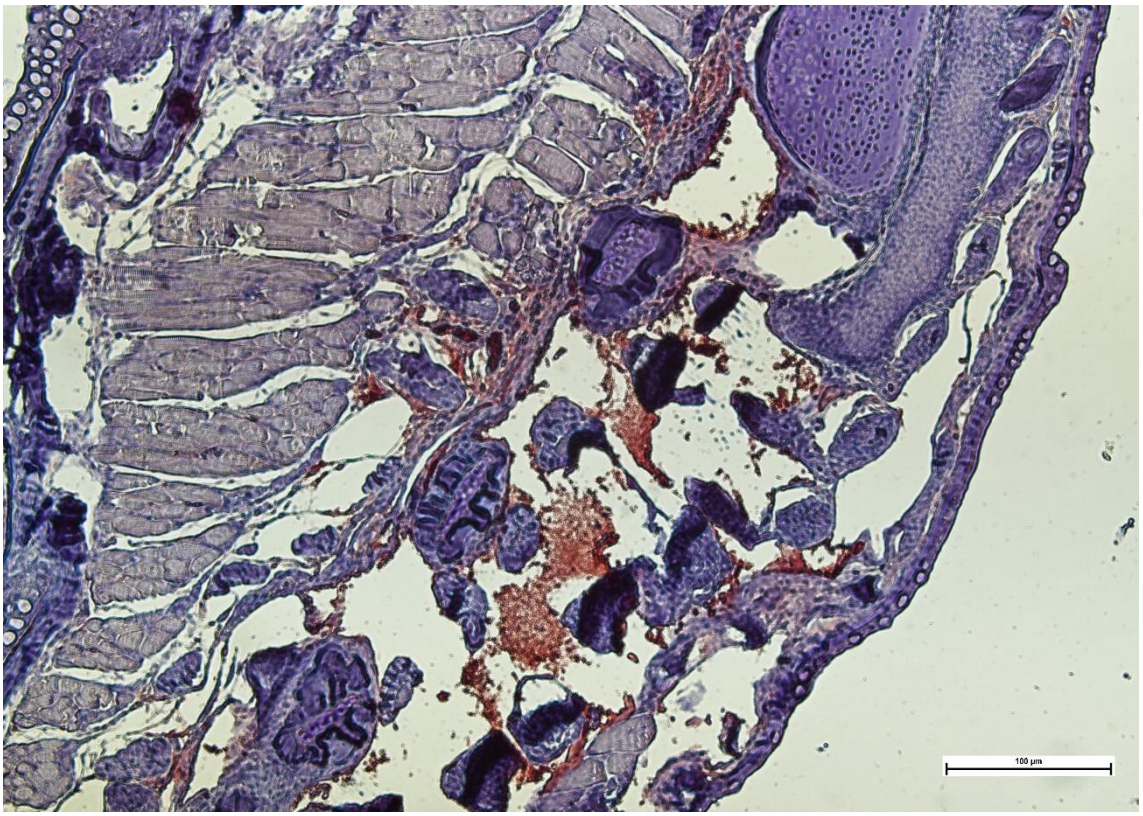

Fig. 33: Across all the samples, mangled tissue would often have strong color. This is sample 4C.

3.5 IHC: A DIFFERENT CHROMOGEN

In the fourth ICH test, the brown DAB chromogen was used instead of the usual red AEC. The test encompassed six samples. Most of the samples in this test had a primary antibody concentration of 1:7500, but two of them instead had concentrations of 1:5000 and 1:10.000. Additionally, some samples had altered timings for some of the steps in the IHC process: incubation, staining, and counterstaining. Lastly, there was a negative control. Blocking buffer was applied to all the samples for two hours. This test used larvae of the seventh developmental stage, taken from tank F (control).

Table 8: Parameters and results from the fifth IHC test.

	1st antibody concentration	**Timing**	**Result**
Sample 5A	1:7500	2x staining time	Completely ruined by structural damage
Sample 5B	1:7500	2x counterstaining time	Very strong blue stain. Little distinct coloration.
Sample 5C	1:7500	1 hour incubation	Weak coloration
Sample 5D	1:5000	Normal	Weak coloration
Sample 5E	1:7500	Normal	Weak coloration
Sample 5F	1:10000	Normal	Weak coloration
Sample 5G	Control	Normal	No coloration

None of the samples had clearly distinct coloration, and many of them were very severely damaged. Whatever coloration patterns there was appeared similar to the patterns in the previous test, although they were much less distinct in this one. None of the samples had any clear signal in the gut.

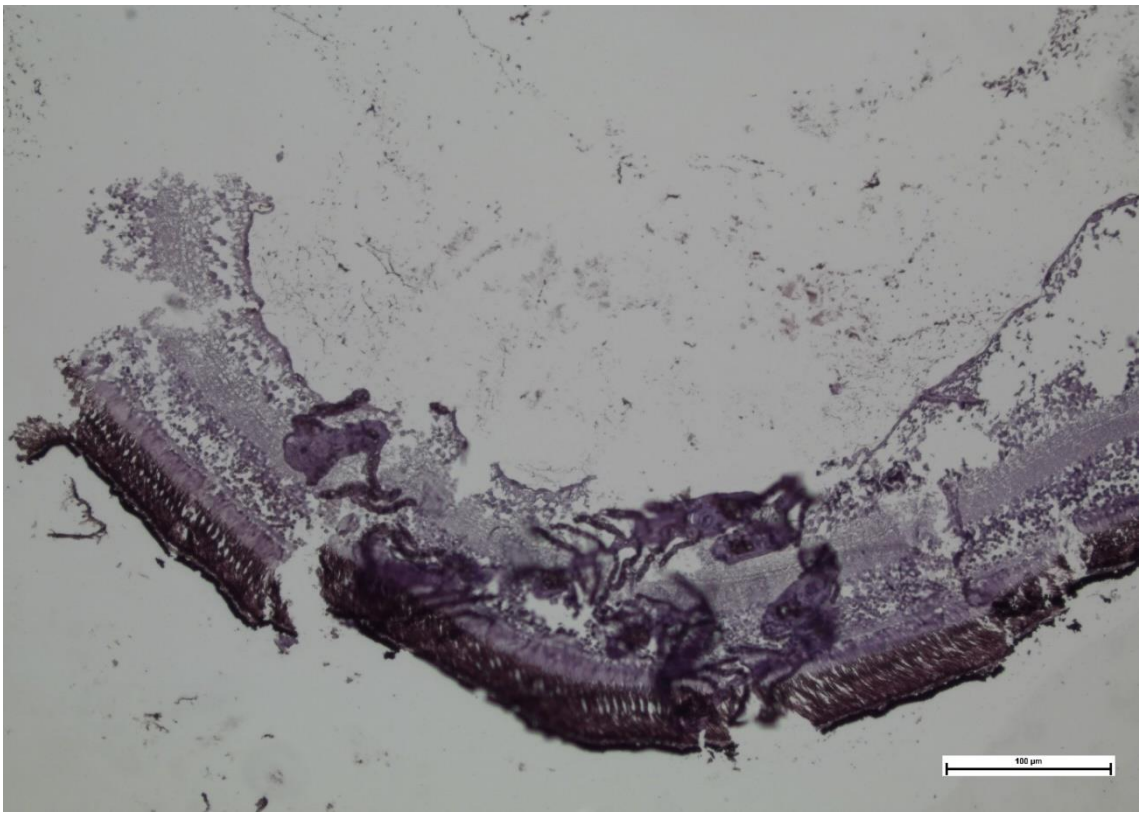

Fig. 34: Sample 5A was completely mangled. This picture shows parts of the eye and the gills.

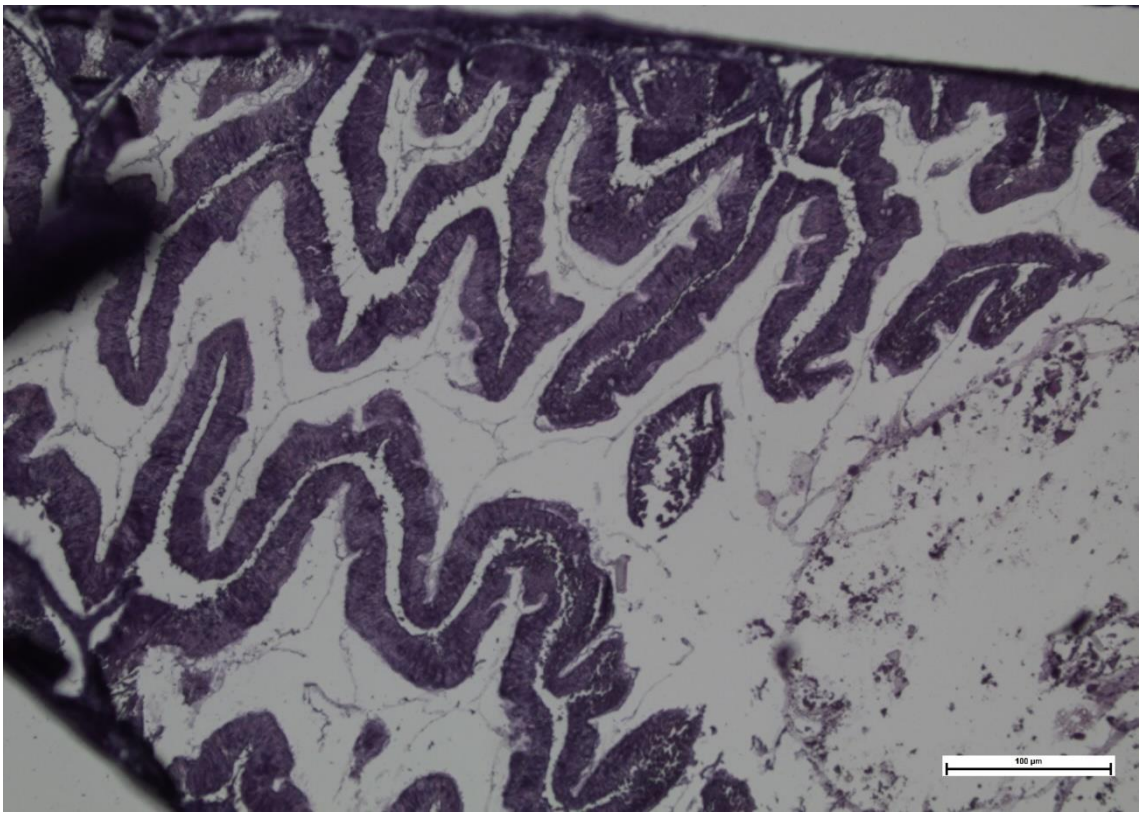

Fig. 35: Sample 5B had a strong blue color from the counterstain, making it difficult to discern any areas of signal.

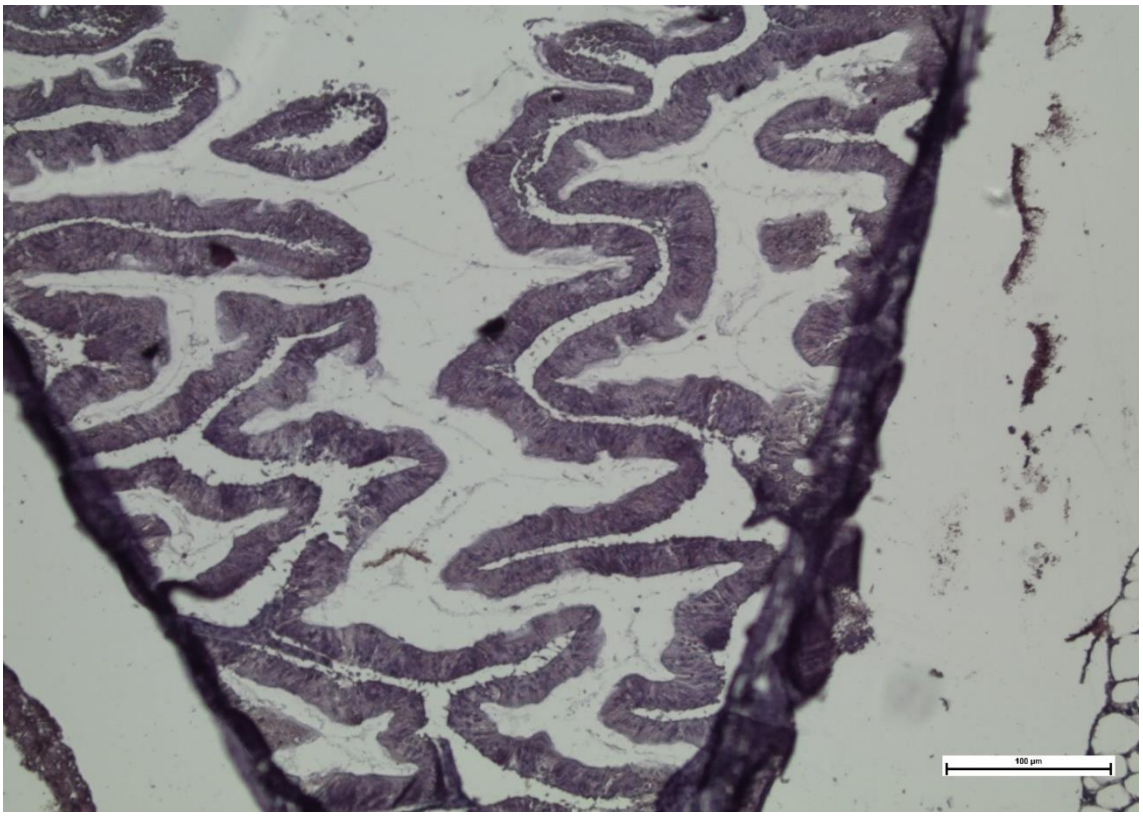

Fig. 36: Even sample 5D, which had the highest primary antibody concentration, shows no strong signal in the gut.

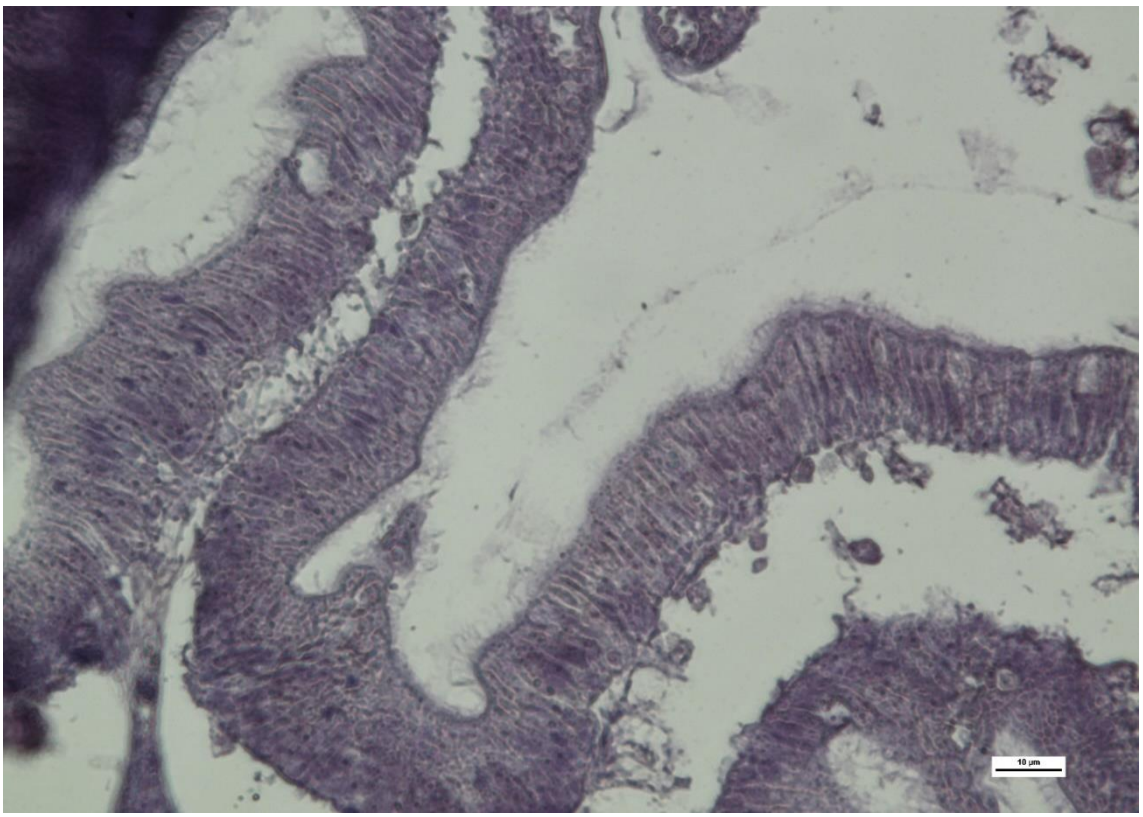

Fig. 37: There was no distinct signal in the gut in sample 5E.

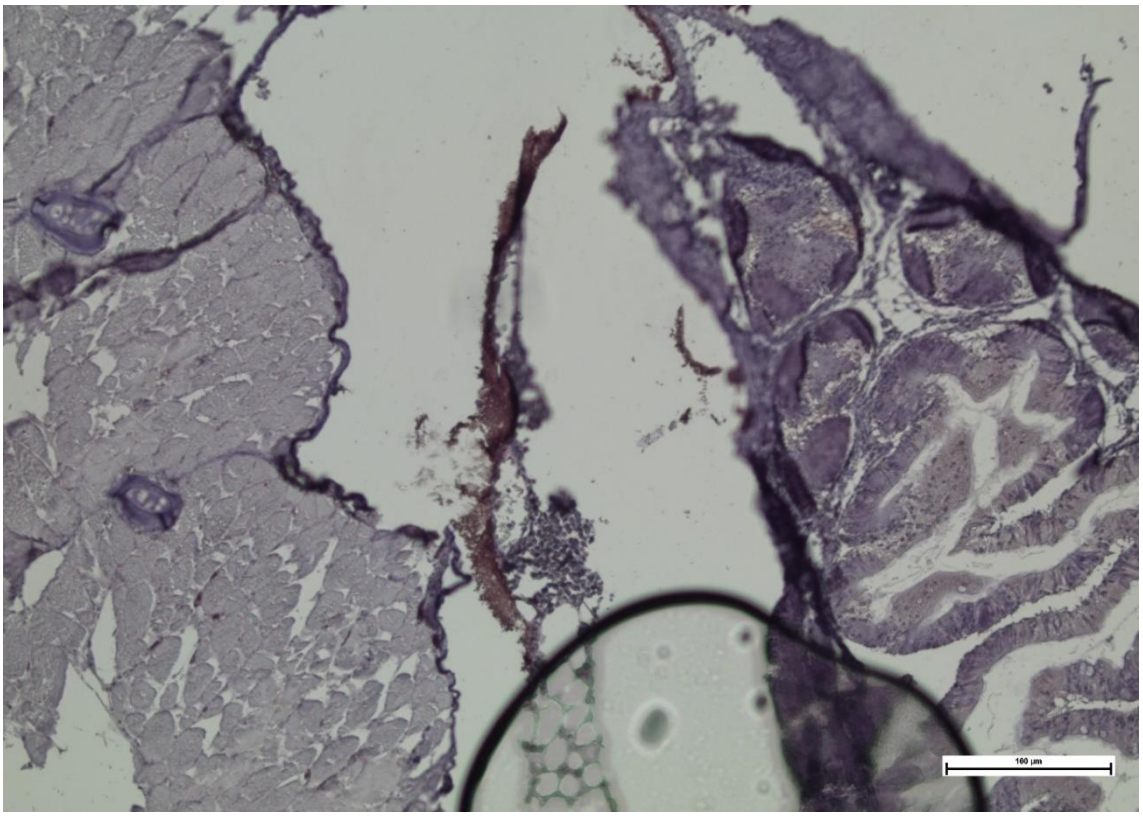

Fig. 38: Brown coloration lines mangled tissue in sample 5F.

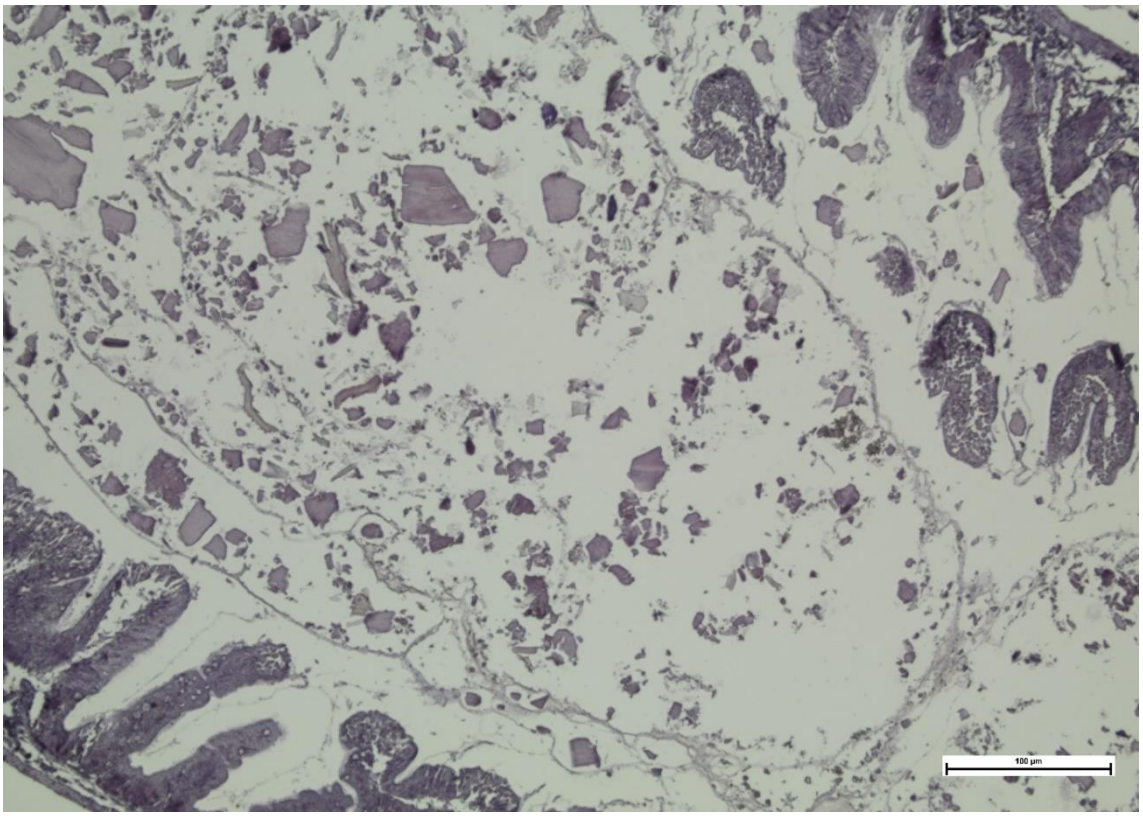

Fig. 39: Parts of the slides were completely ruined. This is sample 5D.

3.6 TRANSCRIPTOME DATA

The transcriptome data is presented on a logarithmic scale and measured in reads per million. It was analyzed using a two-way anova test with 95% confidence interval.

Most results were consistent across all the gene data. There were found no significant interaction between diet and developmental stages in any of the data. The lowest P value for this was 0,19, found in the Tcrd gene data. Across all gene data except secreted IgT, the gene expression increased in higher developmental stages. Excluding secreted IgT, the highest P value was 0,0000016, meaning they are all clearly significant. The secreted IgT data had a P value of 0,46, making the results inconclusive. The last factor examined by the two-way anova test was the effect of diet on gene expression. Only two sets of gene data gave significant results here. These were from secreted IgT, with a P value of 0,012, and 492 pIgR, with a P value of 0,0024.

All data except the IgT data shows that gene expression positively correlates with developmental stage. The 492 pIgR data showed a positive correlation between diet and gene expression (F value = 9,90). Here, the plankton diet led to decreased gene expression. The secreted IgT data showed a similarly positive correlation between diet and gene expression (F value = 6,68). Here however, the plankton diet led to increased gene expression.

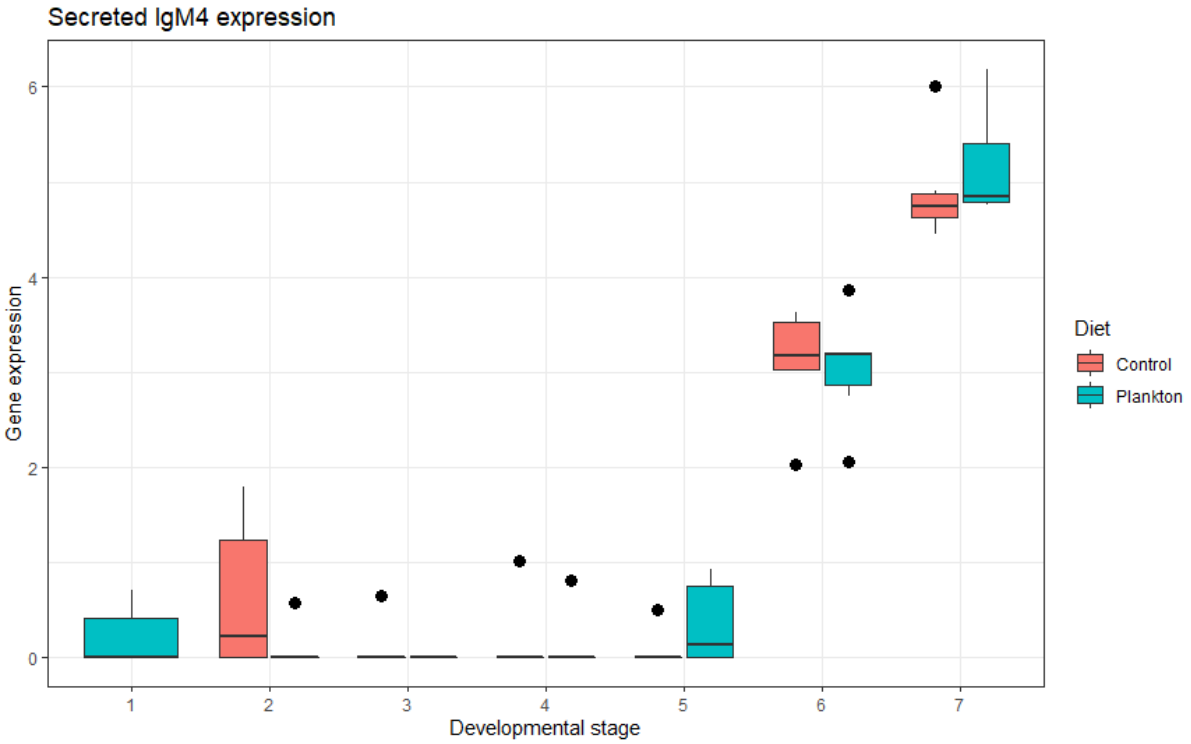

Fig. 40: Expression of secreted IgM4 in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

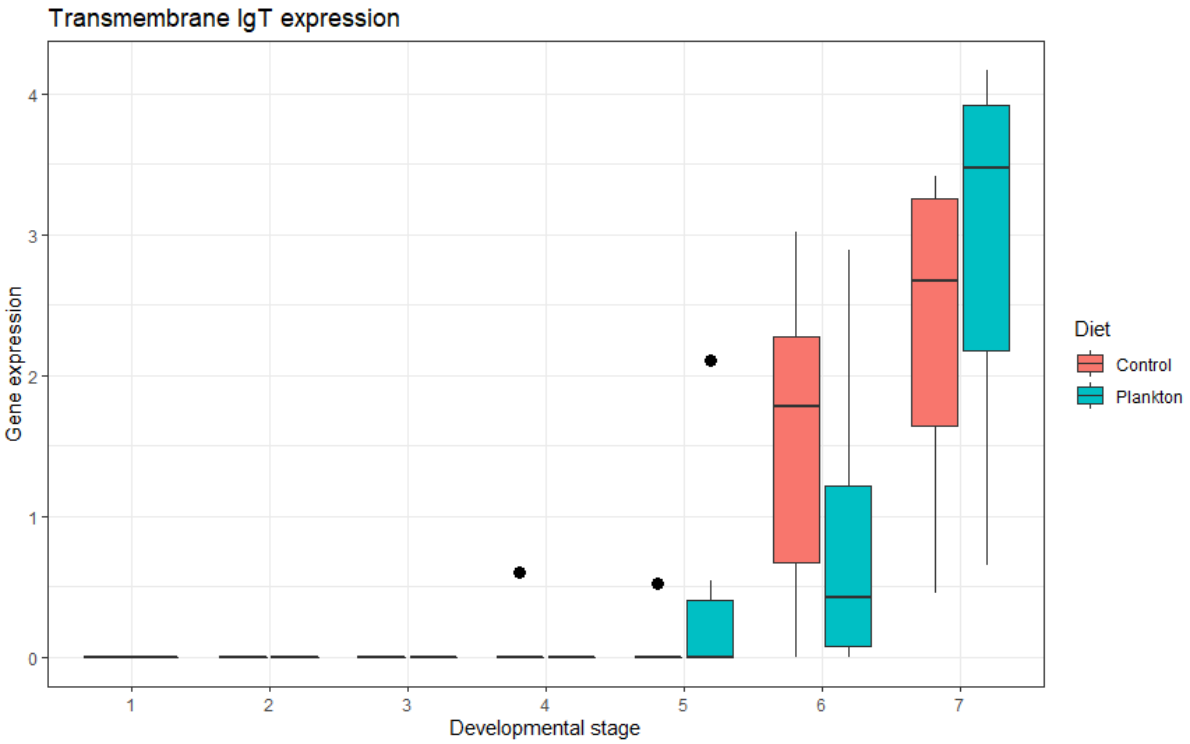

Fig. 41: Expression of transmembrane IgT in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

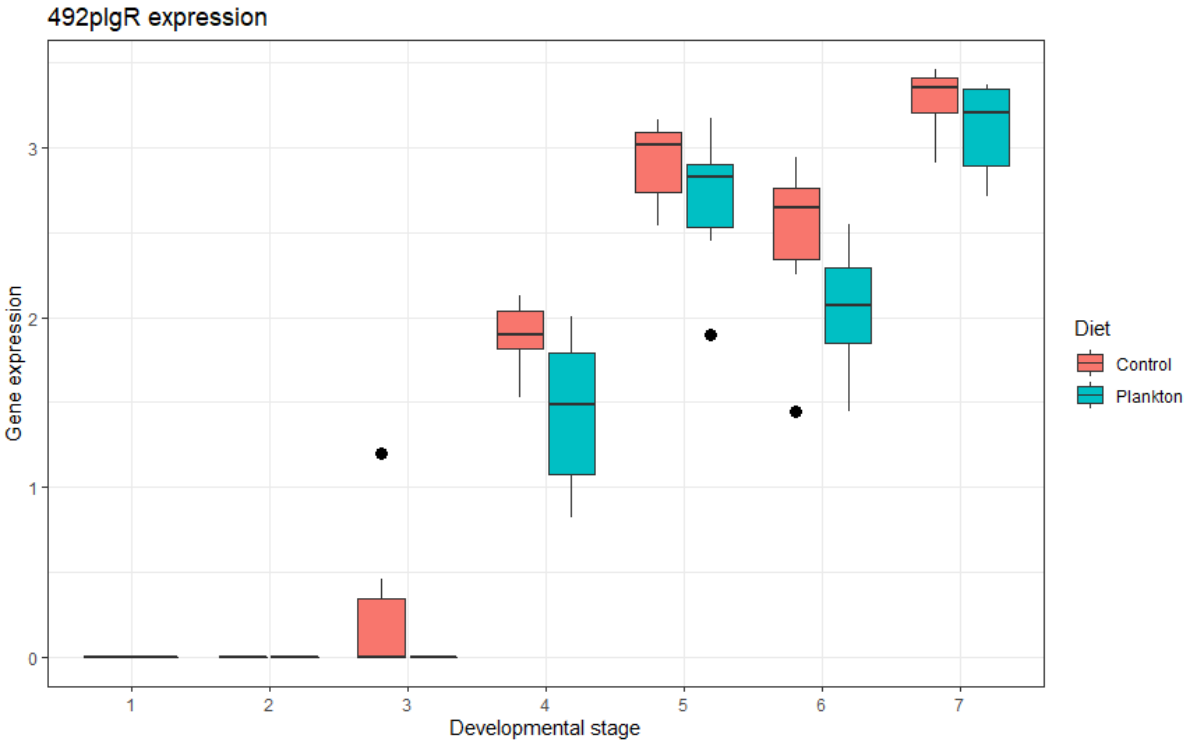

Fig. 42: Expression of 492 pIgR in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

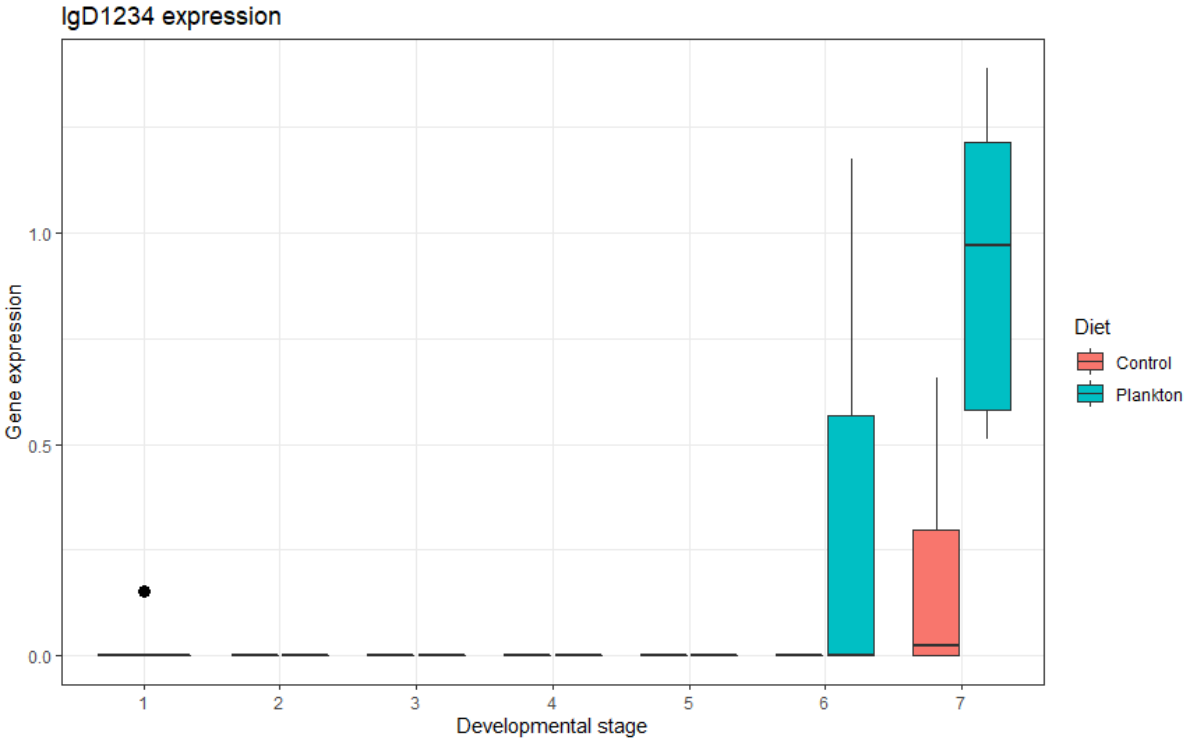

Fig. 43: Expression of secreted IgD1234 in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

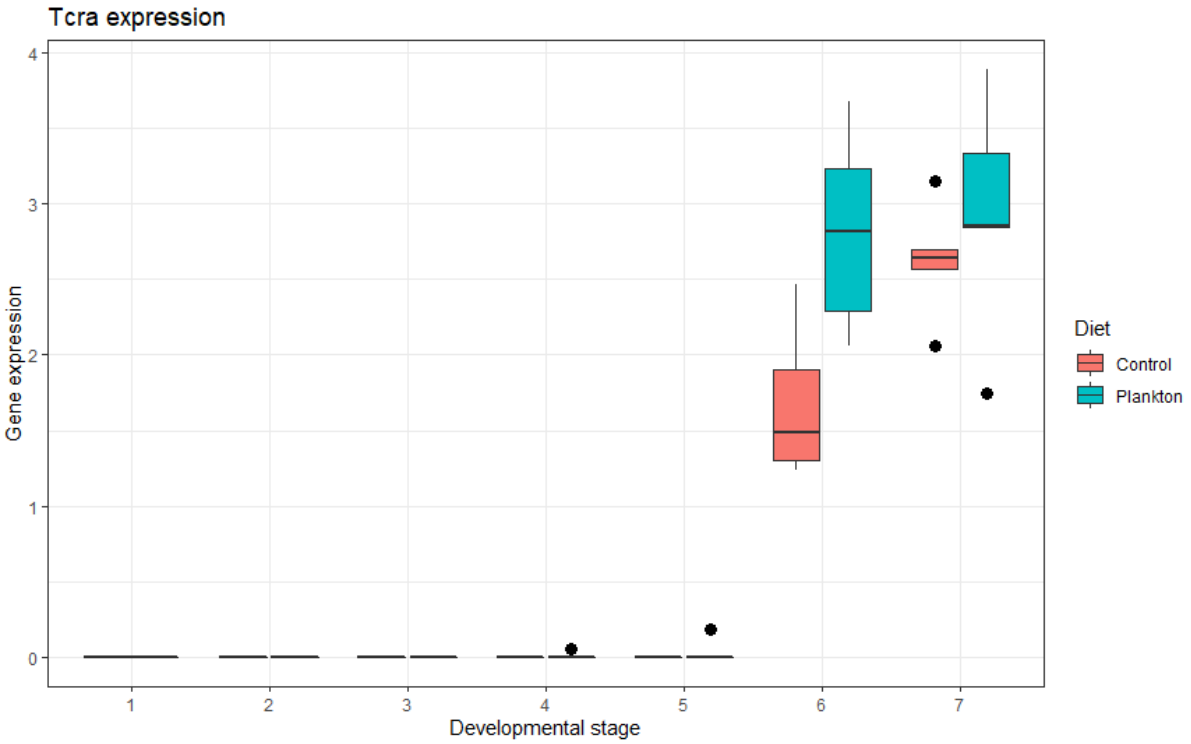

Fig. 44: Expression of Tcra in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

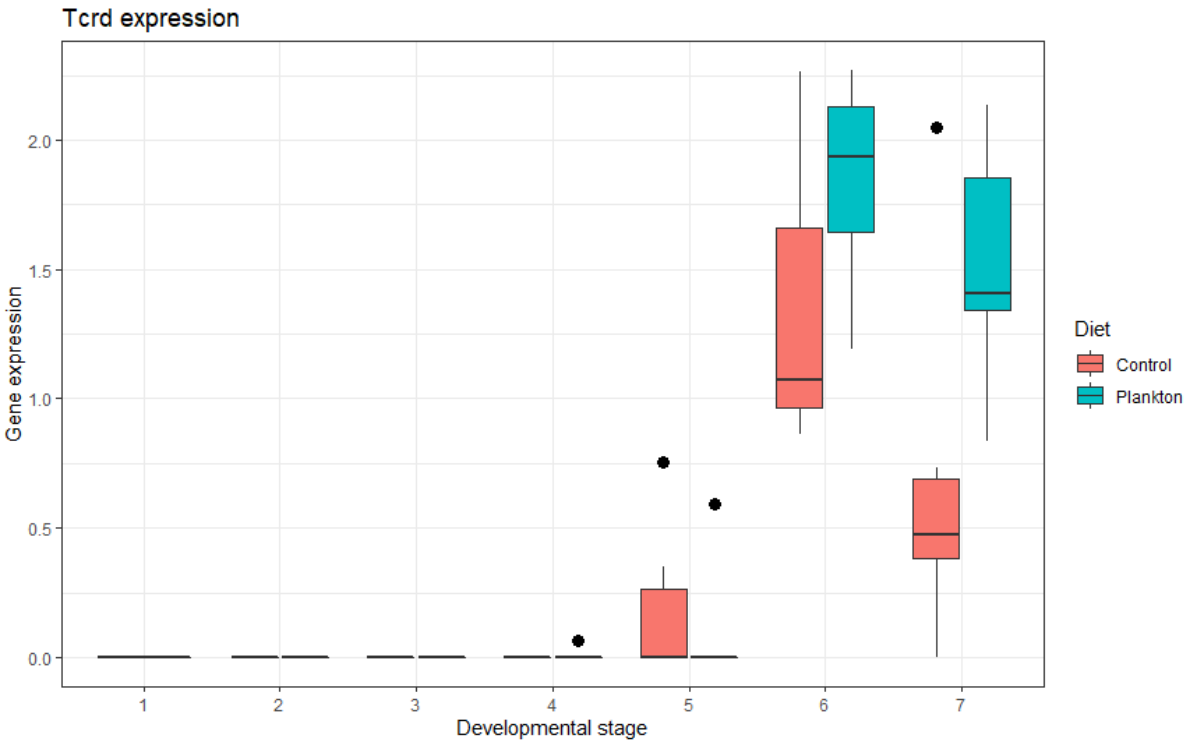

Fig. 45: Expression of Tcrd in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

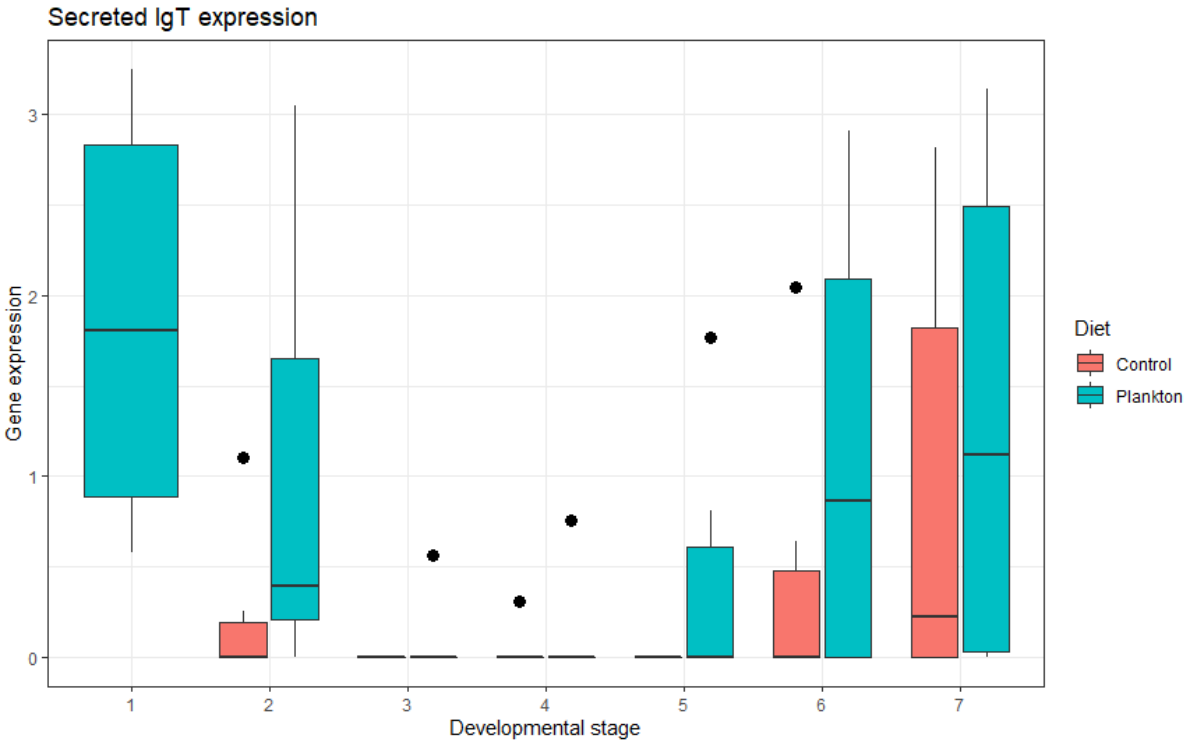

Fig. 46: Expression of secreted IgT in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

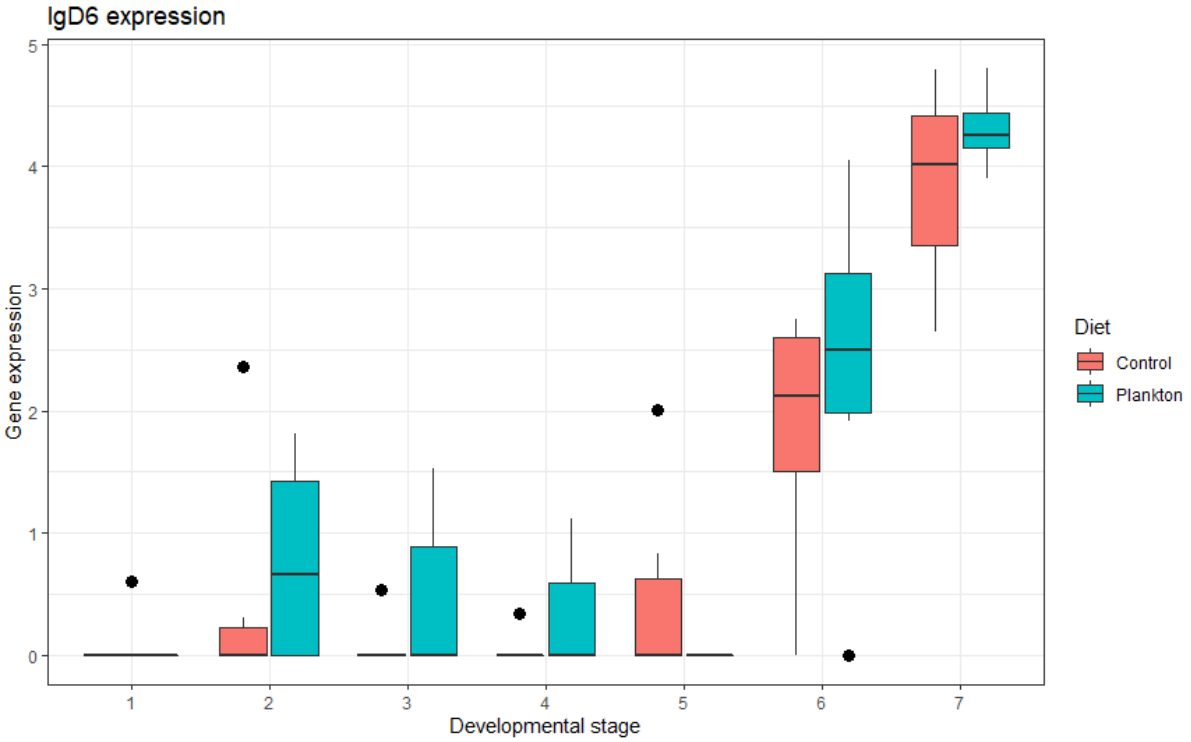

Fig. 47: Expression of IgD6 in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

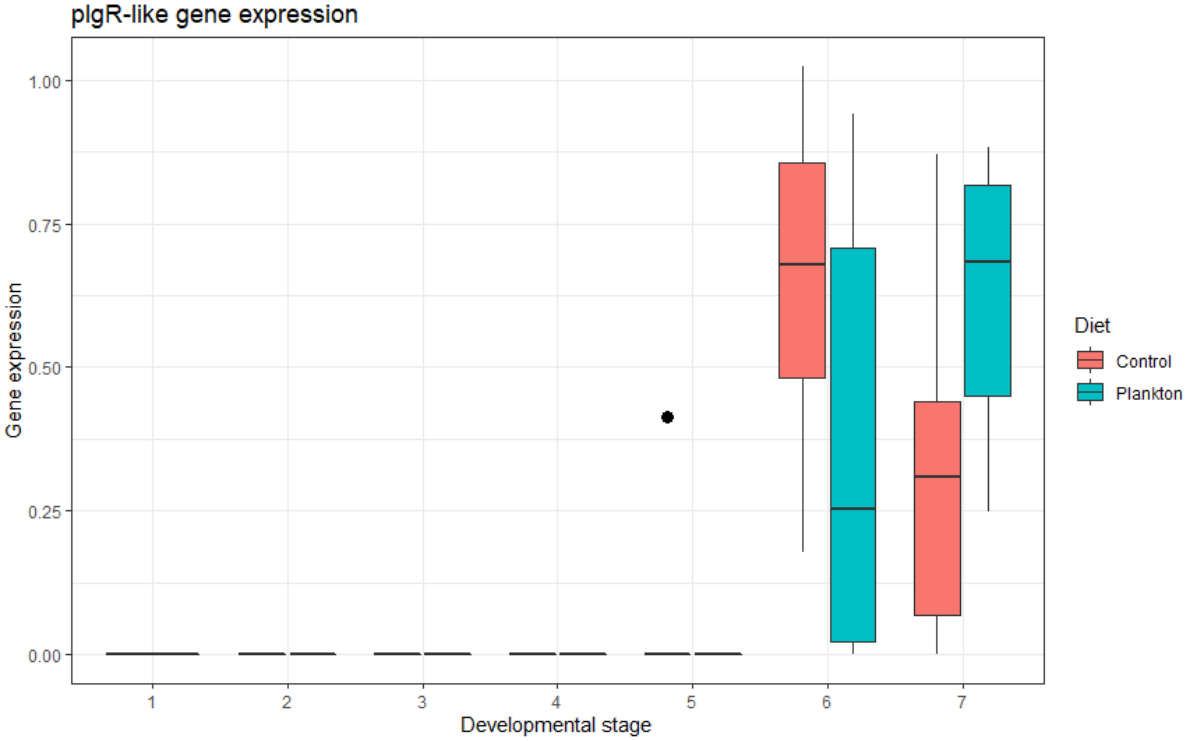

Fig. 48: pIgR-like gene expression in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

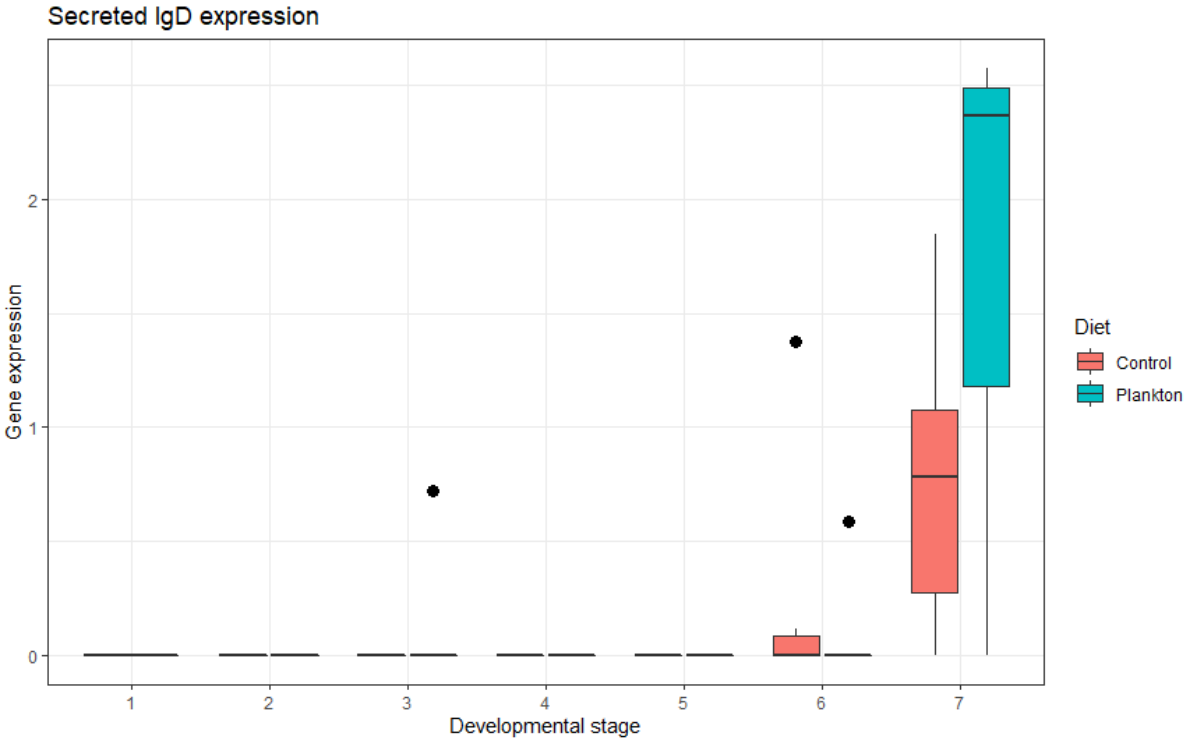

Fig. 49: Expression of secreted IgD in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

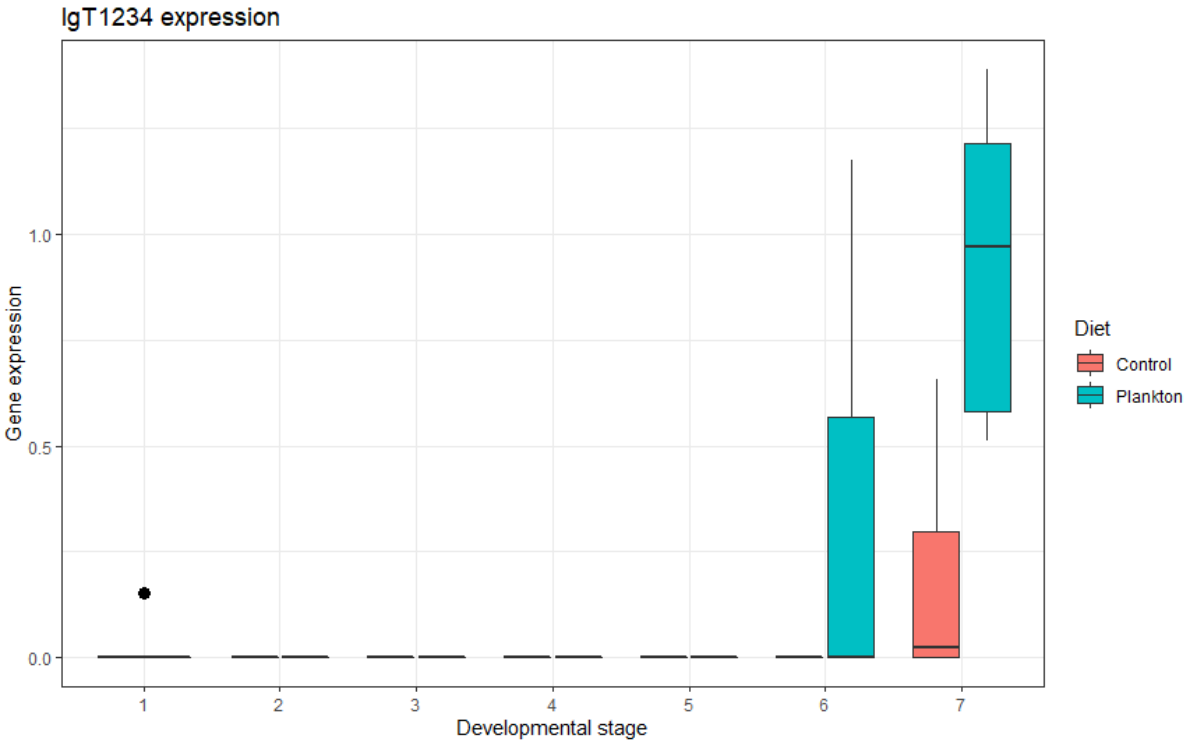

Fig. 50: Expression of IgT1234 in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

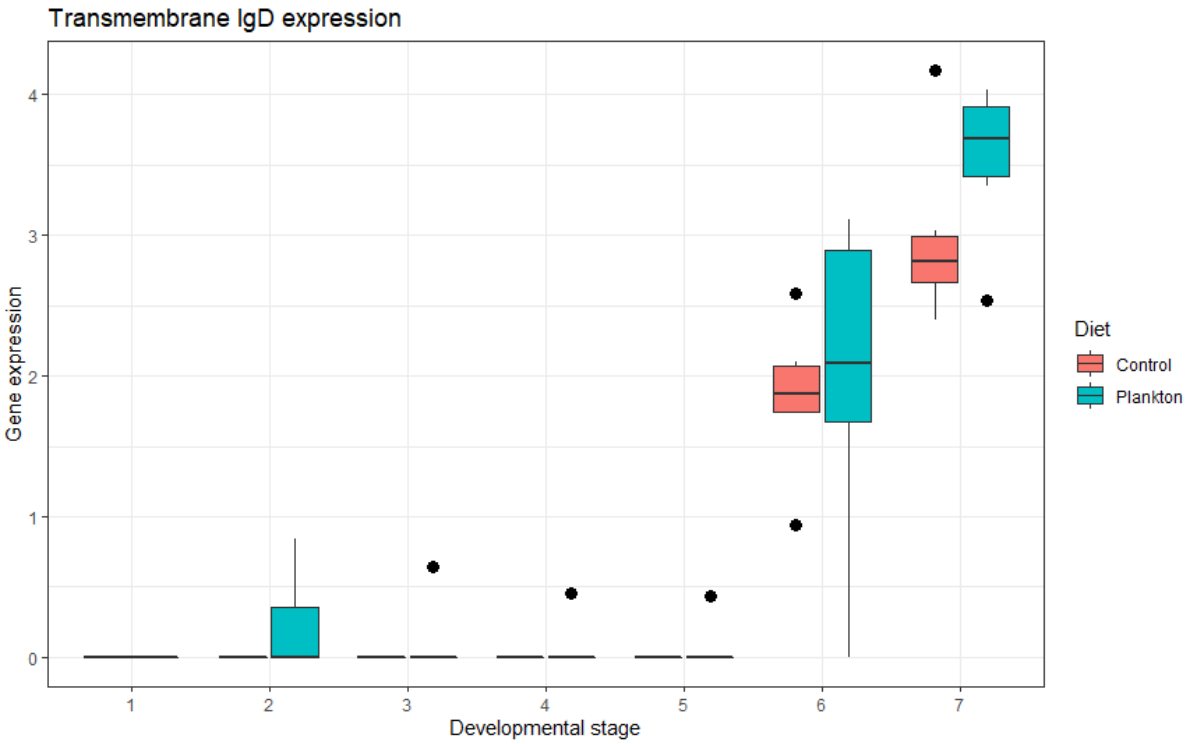

Fig. 51: Expression of transmembrane IgD in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

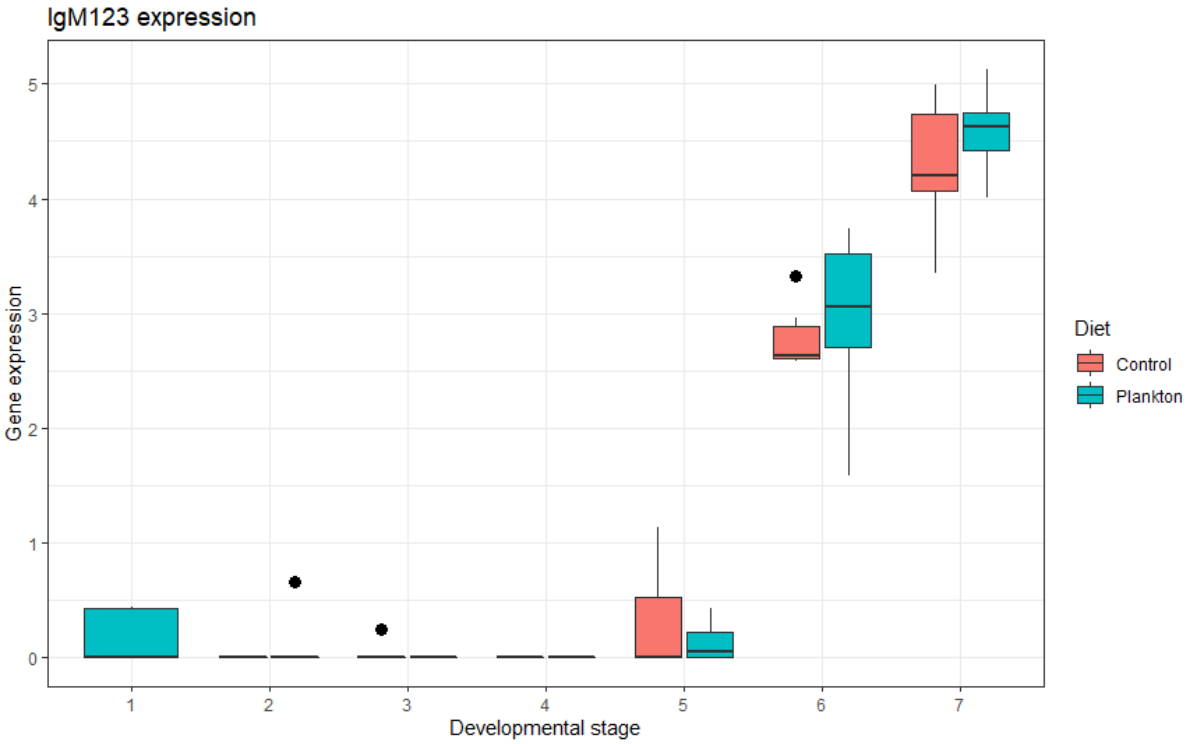

Fig. 52: Expression of IgM123 in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

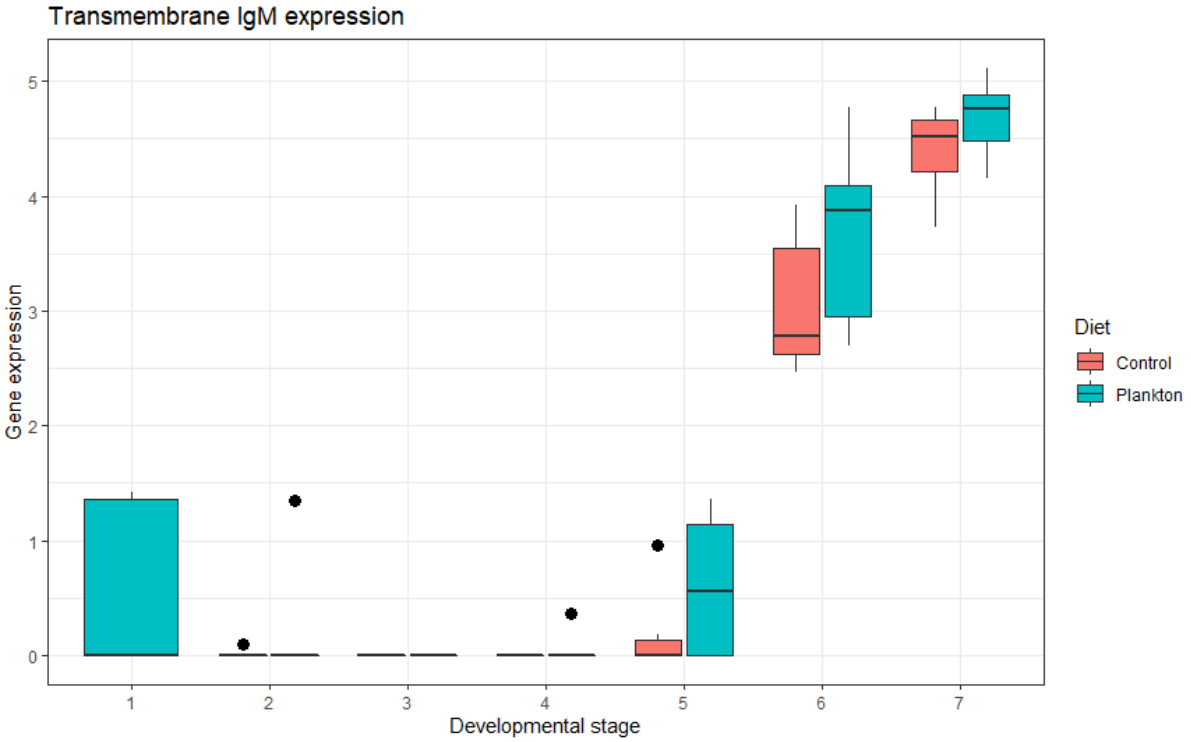

Fig. 53: Expression of transmembrane IgM in the transcriptome data. Gene expression is measured in reads per million and presented on a logarithmic scale. The black dots represent outliers.

4 DISCUSSION

4.1 IMMUNOHISTOCHEMISTRY

The five ICH sessions had mixed results. The first IHC test netted clear pictures, and there was little tissue damage. Whatever tissue damage there was may have been caused by rough sectioning, or by rough handling when putting on the cover slip. The coloration was very weak, with seemingly no color outside of one specific section of the eye. This section was also colored in the control samples, meaning it wasn't caused by the primary antibody binding to anything. Although significant IgM concentrations have been found in teleost eyes before (Gendron et al., 2020), ballan wrasse have been known to have much higher concentrations in other parts of their body (Bilal et al., 2019). This means the red stain in the eye was very likely false signal, where the secondary antibody was binding to something unrelated to IgM.

Since only the secondary antibody was binding to anything in the first IHC test, the second test used various increased concentrations of the primary antibody. Some of the highest concentrations in the second IHC test displayed very strong coloration throughout the entire sample. This was expected, and is clearly false signal. One would then expect the lower concentration samples to have gradually weaker coloration, with a few distinctly red/purple spots remaining, such as in the gut. This was not the case. Although the lower concentration samples had generally weaker coloration, it was not a continuous spectrum. Instead, there were some clear outliers, such as sample 2D and sample 2I. Even when the coloration was concentrated in distinct spots, only some of them made any sense for IgM. It isn't surprising that the gills had a high IgM concentration, since they make up one of the first lines of defense against invading pathogens (Uribe et al., 2011). However, it's surprising to see higher signal in the gill than in the gut. Since this test deployed very high concentrations of the first antibody, it's possible that this was false signal. It's also surprising to see strong signal in the tail of the fish larvae, such as in sample 2I. This was likely false signal, as the antibodies attached to thin, stretched out bits of tissue. The damage on sample 2E was likely due to rough handling when attaching the cover slip, since it seems to have been folded over itself.

Compared to the second IHC test, the third IHC test had a longer blocking period and lower concentrations of the primary antibody. One would expect a longer blocking period to lead to less non-specific binding, and this seems to have worked here. This test's results had less uniform color than the previous one. Samples 3H, 3I, and 3J had no uniform coloring, while they still retained the distinct color in spots where there was signal. These spots of color were significantly less distinct on any samples with a primary antibody concentration above 1:10.000. Samples 3H, 3I, and 3J had primary antibody concentrations of 1:6000, 1:7000, and 1:8000, meaning these concentrations seem ideal for the procedure. Some of the color spots are un unusual locations for IgM, which means they likely indicate false signal. As with the previous test, the negative control samples had no coloration. Unfortunately, many samples in this test were severely damaged. Since it seemed to have affected all the samples to some degree, it's unlikely that this was caused by rough handling. It's possible that the hot water bath loosened the samples somewhat from the glass, thereby making them more prone to structural damage. Some samples were worse off than others. Sample 3G produced less focused pictures with more signal than the other samples with similar concentrations. This means

the sample was probably cut too thick, and the likely reason for this is that the knife blade in the epitome was swapped out immediately before cutting it.

In the fourth IHC test, the blocking time was further increased to three hours. This produced the most distinct coloration patterns yet. All the samples netted very clear images with obvious distinctions between the stain and the counterstain. They all had quite similar levels of coloring, regardless of their primary antibody concentrations. However, these clear areas of coloration were mostly in unexpected places. Previous ICH tests on adult ballan wrasse have produced vastly different results, where most of the IgM was concentrated in small spots within the gut (Bilal et al., 2019). Although this isn't the first time IgM has been detected in the head of a fish larva (Shrøder et al., 1998), the size of the enormous bright red fields that this test produced are unprecedented. The spot in the tail seems similarly bizarre. It's likely both of these colored areas were caused by false signal. The coloration lining the torn-up tissue was also likely false signal, as seen in the previous test. It's possible that the signal in the gills is genuine, since adult ballan wrasse have IgM in their gills (Bilal et al., 2019). However, the concentration of IgM in adult gills is low, whereas the signal in the IHC test was strong. Overall, the signal patterns from this test were consistent but unexpected.

The fifth and final IHC test applied a different chromogen to the samples, and tested various parameters. It had little success, as the test produced very little distinct coloration, and many of the samples were very badly damaged. The main reason for the damage is likely that these samples were on super frosted slides, instead of the super frosted+ slides used in previous tests. The paraffin sections likely couldn't adhere to these slides within the 84°C temperature water bath. Most of the samples produced very similar and non-conclusive results. It seems AEC is better suited for this purpose, even though other studies have successfully used DAB as a chromogen in teleosts (Thomé et al., 2012).

Out of all the IHC tests, the fourth test was the most successful in producing clear images. It appears that a secondary antibody concentration of 1:1000 and any primary antibody concentration between 1:5000 and 1:8000 net good results. Applying the blocking buffer for longer produced a stronger distinction between signal and non-specific binding. The longest blocking time tested in this study was 3 hours. Although all the different ICH tests had different results, there were some clear trends in binding pattern. The primary antibody seems to be binding to something else than IgM, creating distinct spots of false signal. The issue doesn't originate from the secondary antibody, since the control samples are free of signal. The false signal spots are found in the head, eye, skin, scattered small spots in the muscles, and a small spot in the tail. The spots in the head are by far the largest and most distinctive. Additionally, false signal often seems to line damaged tissue. Lastly, there are also spots of signal in the gills that may be false.

In this study, IHC proved an unreliable method for detecting IgM in ballan wrasse larvae. This was unexpected, since other studies have successfully used IHC to detect IgM in adult ballan wrasse (Bilal et al., 2019). It's not immediately obvious what caused the false signal in these tests. It's possible that the samples were incubated for too long, as excessive incubation can cause false signal (Kim et al., 2016). The only sample in this test that was incubated for a shorter period was 5C, and the results

from that test was inconclusive. Other possible causes include antigen diffusion before fixation, chromogen or undissolved counterstain deposits, and inadequate rinsing (Kim et al., 2016). If this work was to continue, all of these issues would have to be examined. Additionally, it could be useful to test the effect of blocking times longer than 3 hours.

Another step of the process that presented difficulties was the epitome sectioning. Here, the most consistent results were achieved by cutting 4 µm sections after cooling the paraffin block over ice and dipping it in water. These sections were then floated on water heated to 36℃, and attached to the slides. Some of the later larval stages contained hard bones which would damage the knife, making sectioning difficult. This issue was solved by re-embedding these samples after decalcifying them to soften the bones.

4.2 DATA ANALYSIS

Unlike the IHC tests, some of the transcriptome data gave results that it is possible to draw conclusions from. It's clear from the boxplots that most of the examined genes displayed higher expression in the plankton group than in the control group. Unfortunately, a lot of the results had high P values. This means there was a high possibility the outcomes were by random chance, and they were not significant when using a 95% confidence interval. The only thing the analysis shows completely clearly across all gene data is that gene expression increased in later developmental stages, which was expected.

The three IgM coding genes, secreted IgM4, IgM123, and transmembrane IgM, were detected as early as stage 1. However, there were very few reads in early stages, and the number of reads didn't increase significantly for any of the three genes until stage 6 (58 days post hatch). Similar results are seen in most of the genes examined. Expression for most immune components is low or non-existent in early stages, but increases drastically in stages 6 (58 dph) and 7 (78 dph). The clearest exception to this was the 492 pIgR gene, which increased significantly in terms of reads as early as stage 4 (25 dph). This early expression of a pIgR gene isn't unprecedented. In turbot (*Scophthalmus maximus*), pIgR genes are expressed before the larvae have even hatched (Qin et. al., 2019).

It takes time for a fish larva to develop its immune system, and in a typical fish species, the larvae are around 20-30 mm at the first appearance of IgM (Magnadóttir et. al., 2005). In this study, IgM was present at hatching, and the larvae were typically between 15 mm and 19 mm at stage 6, when IgM expression increased significantly. Although some fish species display early expression of IgM (table 9), it's unusual for fish to express it from the day they hatch. Ballan wrasse stands out even more because they are among the marine fish, which typically express IgM significantly later than freshwater fish (Magnadóttir et. al., 2005). This is exemplified by the rainbow trout (*Oncorhynchus mykiss*), which is the only freshwater fish in table 9.

Table 9: The time when IgM coding mRNA has first been found in various fish species, measured in days post hatch (Grøntvedt and Espelid, 2003, Corripio-Miyar et al., 2007, Schrøder et. al., 1998, Patel et. al., 2009, Heinecke et. al., 2014, Lee et. al., 2014, and Saha et. al., 2005)

Species	Time of first IgM expression
Atlantic halibut (*Hippoglossus hippoglossus*)	73 dph
Atlantic cod (*Gadus morhua*)	58 dph
Gadoid haddock (*Melanogrammus aeglefinus*)	29 dph
Spotted wolffish (*Anarhichas minor Olafsen*)	7 dph
Olive flounder (*Paralichthys olivaceus*)	5 dph
Rainbow trout (*Oncorhynchus mykiss*)	4 dph
Pufferfish (*Takifugu rubripes*)	Before hatching

The early IgM expression in ballan wrasse is not entirely unprecedented. The pufferfish larvae express IgM coding genes at 4 days post fertilization, before they have hatched from their eggs (Saha et. al., 2005). Similarly, wrasse isn't the only species to express low amounts of IgM before eventually increasing expression drastically in later developmental stages. A comparable development is found in olive flounder larvae, which express small amounts of IgM as early as 5 dph, but don't significantly increase this expression until about 35 dph (Lee et. al., 2014). These two species stand in stark contrast to the Atlantic halibut, where IgM is first expressed at 73 dph, and then increases slowly but steadily (Patel et. al., 2009).

When it comes to the ANOVA test, there were no conclusive results about IgM. The analysis of the transmembrane IgM data shows that the plankton diet led to higher IgM expression, but the P value was 0,58, meaning there's a 58% chance these results came from random chance. The other IgM coding genes show similar results. The P value for secreted IgM4 was 0,51, and the P value for IgM123 was 0,74.

Even though the ANOVA offered nothing conclusive about IgM, it gave some insight into two other immunoglobulin related genes. These were secreted IgT and 492 pIgR. Secreted IgT is another one of ballan wrasse's antibodies, along with secreted IgM. It is specialized in mucosal immunity (Salinas, 2015). The larvae exposed to the plankton diet had significantly more expressed IgT than those exposed to the control diet. However, this difference was already clear before the feeding trial started (fig. 46). It's therefore hard to tell whether their diet led to higher expression of secreted IgT, or if it was caused by a different factor. The other gene that was significantly impacted by the feeding trial was 492 pIgR. This gene codes for the polymeric immunoglobulin receptor, which is a protein that supports immunoglobulins such as IgM, mainly with transport (Turula and Wobus, 2018). The pIgR expression was lower in larvae subjected to the plankton diet, but although it can be speculated that less pIgR indicates less IgM, this doesn't reveal anything about IgM directly.

4.3 CONCLUSIONS

Overall, few clear conclusions about diet and IgM can be drawn from this thesis. Even though the transcriptome data seems to show slightly higher IgM expression in the plankton-fed larvae than in the control group, analysis shows that this is nowhere near statistically significant. The immunohistochemistry trials, on the other hand, had too many issues with false signal to be used reliably.

Although it's impossible to draw any clear conclusions about diet from this thesis, the transcriptome data does give some information about the ballan wrasse immune system. It shows that most ballan wrasse immune components, including IgM, are expressed very early in development, but their expression doesn't increase significantly until about 58 dph. This is unusual for a marine fish species, but not unprecedented.

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