# Development of the adaptive immune system in ballan wrasse (*Labrus bergylta*) and the impact of different start-feed diets

Angela Etayo

Thesis for the degree of Philosophiae Doctor (PhD)
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Name: Angela Etayo

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# Scientific Environment

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Angela

# Table of Contents

# Abstract in English

Ballan wrasse (*Labrus bergylta*) primarily feeds on molluscs, echinoderms, and crustaceans, while also targeting ectoparasites on other fish. The latter trait has made ballan wrasse the preferred cleaner fish for managing salmon lice infestations within salmonid aquaculture. Cultivating this species is relatively new and faces challenges common to other marine species, including slow growth and high early-life mortality. During early larval stages, the adaptive immune system remains underdeveloped, making larvae more susceptible to infections. The present study investigates the development of the main primary lymphoid organs, the thymus and head kidney, the emergence of T- and B-cells, and transport of IgM into the gut lumen of ballan wrasse. The effect of early nutrition on these parameters has also been investigated.

The sequence of lymphoid organ appearance started with the kidney present at hatching, followed by thymus and spleen at stage 3 (20-30 days post-hatching, dph). Transcriptomic data revealed very low baseline expression levels of genes related to adaptive immunity until larval stage 5 (50 to 70 dph). At this stage, a significant increase of *RAG1* and *RAG2* transcripts, as well as transcripts that are T-cell markers (including *CD3ε, ZAP70, LCK, CD4-1*, and *CD8β*), markers of antigen presentation (*MHC-IIα*, *CD74α*), and B-cell markers (*IgM, IgT,* and *IgD*) appeared, indicating lymphoid activity at this stage. The localization of *RAG1* mRNA in wrasse larvae revealed a clear corticomedullary structure in the thymus with a $RAG1^{+}$ cortex and $RAG1^{-}$ $CD3\varepsilon^{+}$ medulla. Interestingly, *RAG1* was abundantly expressed in small cells within pancreatic tissue. This detected expression together with the identification of a high number of $IgM^{+}$ cells in the exocrine pancreas strongly suggest that B-lymphopoiesis also occurs in pancreatic tissue of ballan wrasse.

When analysing nutritional differences between two start-feed diets, one being a commonly used combination of rotifers and *Artemia*, and the other an experimental diet comprising barnacle nauplii, variations primarily existed in the levels of omega-3 poly-unsaturated fatty acids (PUFAs) and specific minerals. These differences were reflected in specific parameters of the adaptive immune system, including an increase

of the level of specific T-cell marker transcripts and an enlarged thymus size in barnacle fed larvae.

In fish, mucosal immunity plays a vital role with mucosal barriers, particularly mucosa-associated lymphoid tissues (MALTs), serving as primary sites for encountering pathogens. Through the examination of ballan wrasse lymphocyte migration to developing MALTs, it was found that $IgM^+$ B-cells reached the gut before helper T-cells. This observation indicates that B-cells play an early role in protecting young larvae, possibly by generating natural antibodies (i.e., B-cells producing IgM prior to exposure of antigens). Natural antibodies are presumably important for protecting the gut of larvae in the absence of a functional stomach, as proposed for adult ballan wrasse.

The presence of secreted immunoglobulins in the mucus of gut, gills, and skin, is essential for combating pathogens and maintaining homeostasis. A molecule named pIgR mediates active transport of immunoglobulins across the epithelium in higher vertebrates, and a teleost counterpart has been reported in many species. Although wrasse pIgR was found to be structurally similar to that in other teleosts, the present results indicate that IgM is primarily transported to the gut lumen through the hepato-biliary route involving the liver, or through the pancreatic route involving the exocrine pancreas.

IgM was present in eggs of ballan wrasse and transcripts of *sIgT* were detected in larvae prior to first feeding (4 dph), indicating that maternal transfer also occurs in ballan wrasse. The innate and adaptive arms of the immune system are closely integrated, and an extremely complex interaction of players. Fish larvae rely on innate defensive mechanisms before adaptive immunity matures, and this is also the case for ballan wrasse. Results from this work indicate when immunocompetence might be achieved during ballan wrasse development (> 90-100 dph), which is important for developing efficient vaccination strategies. Furthermore, cultivation of ballan wrasse larvae might benefit from using a barnacle diet as start-diet.

# Abstract in Norwegian

Berggylt (*Labrus bergylta*) spiser hovedsakelig bløtdyr, pigghuder og krepsdyr, der også ektoparasitter som plukkes fra andre fisker kan nyttes. Denne egenskapen har ført til at berggylt nå er brukt som rensefisk innen lakseoppdrett for å begrense lakselus infestasjoner. Oppdrett av berggylt har imidlertid møtt på de samme utfordringene som ved oppdrett av andre marine arter, inkludert sen vekst og høy dødelighet i de tidlige livsstadiene. I disse larvestadiene er det adaptive immunsystemet underutviklet, og larvene er derfor mer mottakelige for infeksjoner. Denne studien har undersøkt utviklingen av de viktigste primære lymfoide organene, tymus og hodenyre, utviklingen av T- og B-celler, samt transporten av IgM til tarmlumen hos berggylt. Effekten av alternative næringskilder på disse parameterne ble også evaluert.

Nyren viste seg å være det første lymfoide organet som ble utviklet, da den var til stede ved klekking, etterfulgt av tymus og milt som begge ble synlig ved stadium 3 (20-30 dager post klekking, dpk). Svært lave mRNA nivåer av gener relatert til adaptiv immunitet ble observert frem til larvestadium 5 (50 til 70 dpk). På dette stadiet ble en betydelig økning av *RAG1* og *RAG2*, så vel som T-cellemarkørtranskripter (*CD3ε, ZAP70, LCK, CD4-1* og *CD8β*), markører for antigenpresentasjon (*MHC-IIα*, *CD74α*) og B-cellemarkører (*IgM, IgT* og *IgD*) funnet, og lymfopoiesen starter derfor trolig på dette larvestadiet. Lokaliseringen av *RAG1* i berggyltlarver viste dessuten en tydelig sonering av tymus, med en $RAG1^{+}$ korteks og $RAG1^{-}CD3\varepsilon^{+}$ medulla. Det ble også observert $RAG1^{+}$ immunceller samt et stort antall $IgM^{+}$ celler i berggyltens eksokrine pankreas, noe som antyder at berggyltens B-celler også kan utvikles her.

Effekten av to startfôrdietter på berggyltens lymfopoiese ble også evaluert, der larver av rur som er ansett som en mer optimal diett ble sammenlignet med en tradisjonell diett av rotatorier og *Artemia*. Høyere nivåer av omega-3 flerumettede fettsyrer (PUFA) og spesifikke mineraler ble observert når næringsinnholdet ble sammenlignet mellom disse to diettene. Disse forskjellene ble gjenspeilet i en moderat økning av visse T-cellemarkører samt en økning i tymus-størrelsen i berggylt som ble foret med rur.

I fisk spiller mukosal immunitet en vital rolle, der slimhinnebarrierer med mukosa-assosierte lymfoide vev (MALT) fungerer som et primært sted for å fange opp eventuelle patogener. I berggyltlarver ble det funnet at $IgM^+$ B-celler migrerte til tarmen tidligere enn hjelper T-celler, og disse tidlige mukosale B-cellene produserer derfor sannsynligvis naturlige antistoffer (det vil si B-celler som produserer IgM før eksponering for antigener). Disse naturlige antistoffene er trolig viktig for å beskytte larvens tarm i fravær av en funksjonell mage, som foreslått for voksen berggylt. Tilstedeværelsen av spesifikke immunglobuliner i tarmens, gjellenes og hudens slim er også avgjørende for en effektiv bekjempelse av patogener. Et molekyl kalt pIgR medierer aktiv transport av immunglobuliner over epitel hos høyerestående vertebrater, og pIgR er også identifisert i mange fiskearter. Men selv om berggyltens pIgR ble funnet å være strukturelt lik den hos andre teleoster, indikerte resultatene i denne studien på at IgM primært transporteres til tarmlumen via leverens galleganger eller gjennom bukspyttkjertelens utførselskanaler.

Påvisning av IgM i berggyltegg, samt identifikasjon av sekretoriske IgT transkripter i berggyltlarver før første fôring (4 dpk), indikerer at maternal overføring av antistoffer er viktig for overlevelse av tidlige berggyltstadier. Den medfødte og adaptive delen av immunsystemet er imidlertid tett integrert. Fiskelarver er derfor avhengige av medfødte forsvarsstrategier før adaptiv immunitet modnes, og dette gjelder også for berggyltlarvene. Resultatene fra dette arbeidet indikerer når immunkompetanse tidligst kan være oppnådd under berggyltens utvikling (> 90-100 dph), noe som er viktig for å utvikle effektive vaksinasjonsstrategier. Resultatene indikerer dessuten at en startfôrdiett basert på rur kan være fordelaktig for en optimal T-celle modning i tidlige livsstadier.

# List of Publications

**Paper I**

Etayo, A., Lie, K. K., Bjelland, R., Hordvik, I., Øvergård, A.-C., & Sæle, Ø. (2023). The thymus and T-cell ontogeny in ballan wrasse (*Labrus bergylta*) is nutritionally modelled. Frontiers in immunology, 14, 2240.

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**Paper II**

Etayo, A., Bjørgen, H., Koppang, E. O., Lie, K. K., Bjelland, R., Hordvik, I., Øvergård, A.-C., & Sæle, Ø. (2023). The ontogeny of lymphoid organs and $IgM^+$ B-cells in ballan wrasse (*Labrus bergylta*) reveals a potential site for extrarenal B-cell lymphopoiesis: The pancreas.

Submitted manuscript.

**Paper III**

Etayo, A., Bjørgen, H., Koppang, E. O., & Hordvik, I. (2022). The teleost polymeric Ig receptor counterpart in ballan wrasse (*Labrus bergylta*) differs from pIgR in higher vertebrates. Veterinary immunology and immunopathology, 249, 110440.

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# Abbreviations

Immunoglobulin- **Ig**

Days post hatching- **dph**

Days post fertilization- **dpf**

Standard length- **SL**

Myotome height- **MH**

Free amino acid- **FAA**

Triacylglycerol- **TAG**

Phospholipids- **PL**

Poly-unsaturated fatty acids- **PUFAs**

Docosahexaenoic acid- **DHA**

Eicosapentaenoic acid- **EPA**

Arachidonic acid-**ARA**

Lymph node- **LNs**

Germinal center- **GC**

Mucosa associated lymphoid tissues-**MALT**

Plasma cell- **PC**

Head kidney- **HK**

Melanomacrophage center- **MMC**

Gut associated lymphoid tissue- **GALT**

Gill associated lymphoid tissue- **GIALT**

Skin-associated lymphoid tissue- **SALT**

Nasopharynx-associated lymphoid tissue- **NALT**

Inter-branchial lymphoid tissue- **ILT**

Amphibranchial lymphoid tissue- **ALT**

Pattern recognition receptors- **PRR**

Pathogen-associated molecular patterns- **PAMPs**

Toll-like receptors- **TLRs**

Antimicrobial peptides- **AMPs**

Interleukin- **IL**

Interferon- **IFN**

Tumor necrosis factor- **TNF**

Natural killer cells- **NK**

T-cell receptor- **TCR**

Recombination Activating Gene- **RAG**

B-cell receptor- **BCR**

Immunoglobulin heavy chain- **IgH**

Immunoglobulin light chain- **IgL**

Transmembrane- **TM**

Common lymphoid progenitor- **CLP**

Vitellogenin- **Vg**

Somatic hypermutation- **SHM**

Poly-immunoglobulin receptor- **pIgR**

Secretory component- **SC**

Recombination signal sequence- **RSS**

Major histocompatibility complex- **MHC**

Double positive thymocytes**- DP**

Double negative thymocytes**- DN**

Helper T cell- **Th**

Cytotoxic T cell- **Tc**

Regulatory T cell- **Treg**

Trimmed Mean of M values- **TMM**

Liquid chromatography-mass spectrometry- **LC-MS/MS**

Joining chain- **J-chain**

Biliary Epithelial Cell- **BEC**

Antigen Presenting Cell- **APC**

Dendritic Cell- **DC**

# 1. Introduction

## 1.1 Fish immunology- Preface

The aquaculture industry has experienced significant growth over the past few decades, successfully cultivating various marine species around the world. Adoption of novel practices and feeding regimes, however, introduce new risks of diseases with potential impact on fish health. Research on fish immunology has consequently grown during the last 30 years from the first description of the IgM gene and other key immune genes in a few species in the early 90's, to the relatively high number of species-specific immune studies. For example, it is currently acknowledged that there are huge differences in the expression patterns of different immunoglobulins (Igs) among teleost fish (Bilal et al., 2021). It is not surprising that knowledge on mammals has been used as a template for fish immunology. Although this has been useful in many ways, there are significant differences between fish and higher vertebrates as well as within teleost fish regarding anatomical organization, cell characterization, and gene functions (Bilal et al., 2021; Rauta et al., 2012). The high genetic diversity among teleost species together with lack of tools such as specific antibodies against leukocyte populations, have hampered fish immunology research. Despite this, important discoveries of teleost immune structures and their organization have been made during the last decade, especially in cyprinids and salmonids (Bilal et al., 2021; Bjørgen and Koppang, 2022; Salinas et al., 2021). In a broader perspective, research on teleosts representing the different branches of teleost phylogeny will certainly benefit the field of comparative immunology.

## 1.2 Cleaner fish in salmon aquaculture

The salmon louse (*Lepeophtheirus salmonis*) is a major problem for the Atlantic salmon (*Salmo salar*) farming industry as it causes important health issues. Losses due to this blood-feeding ectoparasite costed USD 525 million in Norway during 2019, becoming an economic burden in Norwegian aquaculture (Abolofia et al., 2017; Myhre Jensen et al., 2020). Pharmaceuticals were effective at preventing occurrence of sea

lice the first years of use, but it rapidly led to resistant strains of lice (Aaen et al., 2015), as well as a growing awareness of the negative environmental impacts of chemical discharge. This motivated the use of cleaner fish as a biological control for ectoparasite removal as they graze on salmon louse exhibiting a "cleaning" behaviour. Cleaner fish is used in salmon farms in Norway, Scotland, and Ireland, and it is one of the few current non-medical strategies to cope with salmon lice that is not harmful nor causes stress to salmon (Bolton-Warberg, 2018; Skiftesvik et al., 2014). The species used for this purpose are lumpfish (*Cyclopterus lumpus*) that perform well in cold waters (as low as 4 ˚C), and several species from the wrasse family (labrids) that thrive in somewhat warmer waters (Powell et al., 2018; Skiftesvik et al., 2013). Lumpfish is the most used cleaner fish compared to wrasses as exemplified for 2022 (Fig. 1A) despite increasing challenges with diseases (Fiskeridirektoratet, 2022).

There are 6 species of wrasses in Norwegian waters, of which mainly 3 have been used as cleaner fish in salmon aquaculture over the last 30 years; ballan wrasse (*Labrus bergylta,* Ascanius 1767), goldsinny wrasse (*Ctenolabrus rupestris*) and corkwing wrasse (*Symphodus melops*) (Bjordal, 1990; Skiftesvik et al., 2014). First, all wrasse species were caught from their natural habitat by fisheries and were provided to farms (Skiftesvik et al., 2014). Ballan wrasse is considered the most efficient at removing lice, and thus, it has the highest value for the industry. The growing demand of wrasse, especially ballan wrasse, increased fishing pressure on wild stocks provoking the emergence of ballan wrasse farming. Another crucial motivation to start wrasse farming was the opportunity to control size of individuals and implement vaccination strategies (Skiftesvik et al., 2013). Up to now, ballan wrasse (and lumpfish) are being farmed in North Atlantic countries; Norway, UK, Ireland, Iceland, and Faroe Islands (Brooker et al., 2018). It is important to highlight that while the majority of lumpfish used as cleaner fish is farmed, due to their rapid growth and relatively straightforward breeding process, this differs from wrasses (Powell et al., 2018). When ballan wrasse farming started about 20 years ago, the industry was small and very new, and the high demands from the salmon aquaculture sector could only be met by relying on fisheries (Skiftesvik et al., 2013). Over the past decade, there has been a gradual transformation

of this scenario, marked by an increase in number of cultivated ballan wrasse due to improvements in wrasse farming (Fig. 1B) (Fiskeridirektoratet, 2022).

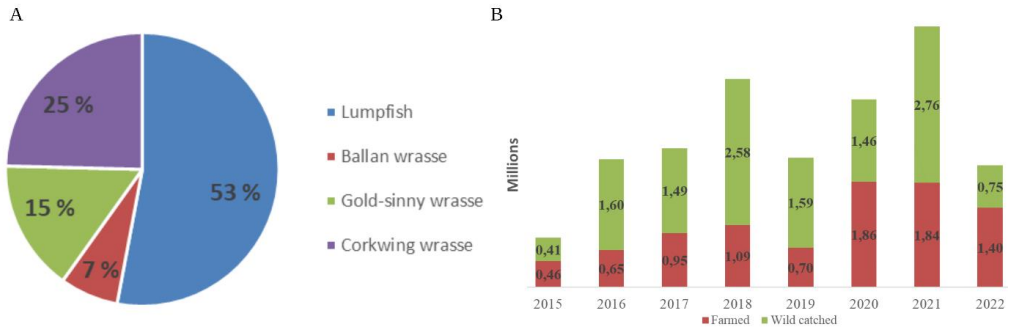


**Figure 1. A)** The use of cleaner fish in 2022. **B)** The use of wild and farmed ballan wrasse from 2015-2022. Data retrieved from (Fiskeridirektoratet, 2022).

## 1.3 Ballan wrasse

The ballan wrasse falls under the family Labridae, which is the third most diverse family within the Perciformes order. Perciformes itself is the largest order of vertebrates, accounting for approximately 41% of all bony fishes (Parenti and Randall, 2018). Ballan wrasse is distributed around the eastern North Atlantic, ranging from Norway to southern Spain, including various islands such as Azores, Madeira, Selvagens, and the Atlantic coast of Morocco (Brooker et al., 2018). Ballan wrasse is a protogynous hermaphrodite marine fish species that occupy shallow waters during summer and move to deeper water during winter. It does not cope well with cold waters and it stops feeding when water temperature is below 6 °C.

### 1.3.1 Intestinal physiology and feeding behaviour

Ballan wrasse is an agastric species meaning that they lack stomach and pyloric caeca, and they have a peculiar short intestine accounting for roughly 2/3 of their body length (Le et al., 2019). The stomach is the main site for food storage, protein digestion, osmoregulation, and an important barrier against pathogens among others in gastric fish (Koelz, 1992; Rønnestad et al., 2013).

The formation of cranial bones and development of the jaw, oral teeth (for catching prey), and pharyngeal teeth (for crushing prey) in ballan wrasse is associated with their digestive system and their feeding behaviour (in the wild) (Norland et al., 2022). There is a transition from wrasse larvae consuming zooplankton to juveniles and adults feeding on crustaceans and invertebrates such as gastropods, bivalves, and echinoderms (Figueiredo et al., 2005; St. John et al., 2020). As a consequence of being agastric, ballan wrasse should be offered small meals regularly rather than few and large. Ballan wrasse have different feeding habits compared to other commercial marine species making farming of this species quite challenging (Hamre et al., 2013a). Atlantic cod (*Gadus morhua* L.) larvae and wrasse larvae are of similar size when they start feeding. Therefore, the same feeding strategy as for Atlantic cod larvae was used during the first attempts of rearing wrasse. However, it was soon discovered that formulated feeds that had been designed for other marine species were not optimal. Hamre et al. (2013a) advised that suitable diets for juveniles and broodstock ballan wrasse should contain a low lipid content of 12% (similar to what they eat in the wild), 16 % of carbohydrates, and 65 % of dietary protein. A highly protein rich diet is successfully eaten by ballan wrasse, but it is rather expensive. It is a challenge to formulate adequate diets for wrasse as they are “picky” eaters and can voluntarily starve to death if food is not appetizing enough.

The intestine is a key organ where both digestion and absorption take place in the lack of a functional stomach. Up to date, studies on the intestinal physiology of ballan wrasse have revealed important evolutionary traits of this stomach-less fish. For instance, ballan wrasse does not retain food in the anterior intestine as seems to happen in other agastric fish species (Le et al., 2019). Instead, they have the capacity to modulate intestinal motility (peristaltic contractions) in order to increase the time that the feed is in the intestine for optimal digestion and absorption of nutrients (Le et al., 2021). Not only the proximal intestine but also the middle and distal midgut seem to have a similar absorptive function in wrasse (Lie et al., 2018). While these characteristics may be common among agastric fishes, there is a lack of studies in other species. Cholecystokinin (CCK) is a key hormone at regulating gut motility and food

passage ratios in ballan wrasse (Le et al., 2019), and serotonin levels increased in the gut after a lipid rich meal which might also regulate gut motility (Etayo et al., 2021). Moreover, wrasse has lost important genes for protein digestion and appetitive regulation in the stomach (Lie et al., 2018). Interestingly, the liver and exocrine pancreas in ballan wrasse show a larger relative volume to the digestive system compared to other gastric teleosts (Norland et al., 2022). Authors suggested this trait might enhance digestion and nutrient absorption.

Physiological and molecular studies are important to understand the evolution of the functional adaptations of agastric fish. This knowledge will also add valuable information for optimizing feed formulation to achieve more robust and healthy fish in the aquaculture sector.

### 1.3.2 Ballan wrasse development

Early life stages of marine teleosts are critical bottlenecks with a significant "unexplained" high mortality (Rojo-Cebreros et al., 2018; Vadstein et al., 2013), especially when farming new species, as knowledge in their physiology and nutritional requirements is limited. Like other marine pelagic fish, wrasse larvae show an immature digestive tract and low production of digestive enzymes when they start feeding (Dunaevskaya, 2012; Hansen et al., 2013). A recent study on the ontogeny of wrasse larvae described 6 larvae stages based on cranial ossification (Norland et al., 2022) (Fig. 2).

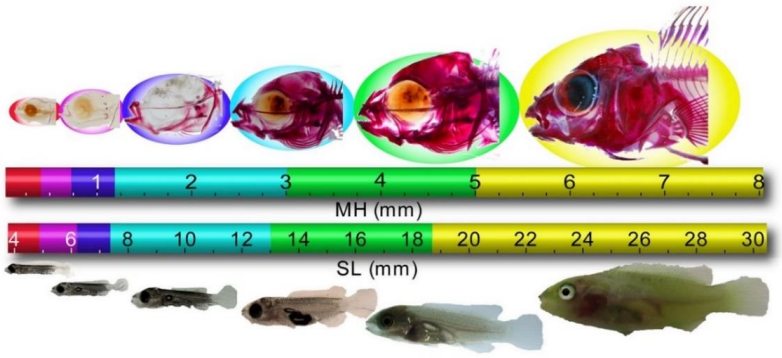


**Figure 2**. Developmental stages of ballan wrasse larvae based on cranial ossification. Each colour indicates a new developmental stage (stage 1 to 6). SL; standard length, MH; myotome height. Adapted from (Norland et al., 2022).

### 1.3.3 Knowledge gaps and challenges in farming ballan wrasse

When farming fish, it is crucial that we can ensure the best possible health and welfare. This is especially difficult when new species are introduced in captivity. The enormous demand of cleaner fish from the ambitious salmon industry over the past two decades led to a significant expansion of ballan wrasse farming. Despite the efforts, this rapid growth has not always been accompanied by optimal husbandry practices as it has been driven, generally speaking by the try-fail kind of farming. There are several challenges in wrasse farming that are hereby addressed. Up to date, a common procedure in cleaner fish hatcheries, ballan wrasse and lumpfish, is to use wild-caught adult fish as broodstock (Powell et al., 2018). Ballan wrasses have a complex reproductive behaviour as they have a strong sexual hierarchy making management of the broodstock difficult and sometimes unpredictable (Brooker et al., 2018). Therefore, developing the optimal conditions for successful and stressless breeding in captivity can be challenging. Recently, some hatcheries have been using farmed F1 populations as potential broodstock as reviewed in Brooker et al. (2018). Breeding techniques including controlling water temperatures, light cycles, feeding regimes, and providing appropriate spawning substrates, need to be optimized to facilitate natural reproduction. There are still many unknowns in the reproductive success of wrasse broodstock fish.

Secondly, similar to other farmed marine teleost species, ballan wrasse exhibits poor growth and high mortality, particularly during early life stages (Rojo-Cebreros et al., 2018; Vadstein et al., 2013). In recent years, considerable progress has been done in addressing slow growth and deformities of wrasse larvae through feed optimization (Hamre et al., 2013a; Hansen et al., 2013). However, the underlying reasons for the prevalent high larvae mortality remain largely unknown, making prevention rather difficult.

Thirdly, providing suitable and sustainable feed is also a challenge. Research on the organogenesis of ballan wrasse (Dunaevskaya, 2012; Norland et al., 2022) as well as intestinal functionality (Hansen et al., 2013; Le et al., 2019; Lie et al., 2018) has provided valuable insights into establishing appropriate feeding practices for ballan

wrasse. Developing nutritionally balanced and cost-effective (cheaper) feed that triggers appetite and at the same time meets the dietary requirements of ballan wrasse is not easy. Furthermore, larval nutrition can be challenging as the available start-feeds (live preys) have limitations. Establishing optimal feeding protocols for the first feeding of ballan wrasse larvae may significantly impact wrasse survival and welfare. Also, our knowledge of digestive physiology and nutritional requirements must improve. Disease management and maintaining fish health is also a challenge. The current production of wrasse juveniles during the hatchery phase mainly faces problems related to fin erosion and suboptimal care leading to poor welfare, high mortality, and weak fish that is more susceptible to diseases (Sommerset et al., 2023). In the sea pen phase, crowded conditions in salmon cages are stressful and favour bacterial and viral outbreaks affecting ballan wrasse. Apart from mechanical injury mainly related to handling, non-medical delousing methods, and fin erosion, the bacterial disease caused by *Atypical Aeromonas*, and the Amoebic gill disease (AGD) caused by the amoebae *Neoparamoeba perurans,* are potentially fatal for fish. For example, AGD outbreaks in sea pens are usually controlled by freshwater baths which work efficiently in salmon and lumpfish but ballan wrasse is very little tolerant to fresh water and very susceptible to handling, stressing the fish. In this context, authorized antimicrobial treatments can be used to control certain diseases, but sometimes the treatments are long, and diseases are recurrent. All of these situations can reduce the welfare of wrasses leading to high mortality rates in farms; some farmers report the near total loss of cleaner fish (100% mortality) after sea deployment (Sommerset et al., 2023). There is a dire need of acting by regulating the use of ballan wrasse as cleaner fish as well as the need for effective vaccines that can prevent fish from getting infected. Detailed knowledge of the immune system of this species is vital for vaccine development and disease control.

## 1.4 The importance of start-feed diets in larvae rearing

Altricial marine fish larvae are undeveloped when they hatch. For example, ballan wrasse and Atlantic cod have a primitive digestive system and a straight short tube after

their yolk-sac is reabsorbed (Dunaevskaya, 2012; Govoni et al., 1986). At this stage, the ability of larvae to eat food and therefore survive and develop depends on anatomical and physiological characteristics that should be in place (Rønnestad et al., 2013). For instance, a somehow developed visual and/or olfactory system to detect prey, a basic developed locomotory system that allows swimming after the prey, anatomical features such as mouth opening for prey capture and a functional digestive system for digestion and absorption of nutrients. All of these are needed for a successful first feeding in larvae as extensively reviewed in (Rønnestad et al., 2013). The moment of first feeding or start-feed is crucial and often a bottleneck in larvae rearing. The ideal larval nutrition for marine larvae such as Atlantic cod is the nauplii stages and later copepodite stages of various zooplankton. However, most reared marine larvae are provided with live feed such as rotifers and *Artemia* as these prey organisms are easy to produce (Rønnestad et al., 2013; Samat et al., 2020). As we know that rotifers and *Artemia* offer suboptimal nutrition for early larval development (Karlsen et al., 2015), it is important to explore if alternative live feed regimes may offer a more favourable development.

It is evident that studies on larvae development (physiology and functionality) are needed to improve feeding regimes, and importantly, that this is done specifically for each cultured species as teleost physiology and adaptations have evolved greatly.

### 1.4.1 Dietary requirements for larvae

One of the biggest efforts in aquaculture research is to meet the dietary requirements for healthy and robust fish, especially in larvae where requirements are poorly understood and difficult to investigate (Hamre et al., 2013b; Kvåle et al., 2007; Waagbø, 2010). Studies addressing this matter are scarce (Hamre et al., 2008). The growth of marine fish larvae is very fast and requires abundant dietary proteins and amino acids as they are the building blocks for growth and tissue development (Olsen et al., 2004). In addition to the amino acids making up proteins, taurine is an amino sulphonic acid classified as a free amino acid (FAA) that has gained attention in larvae rearing in the last decade (Hamre et al., 2013b). Taurine is involved in many biological functions. It improves larvae growth by increasing protein retention and has a general

positive effect on marine larvae development (El-Sayed, 2014; Hawkyard et al., 2015; Mæhre et al., 2013). Taurine possesses antioxidative protective effects and its deficiency can cause oxidative stress and lipid accumulation among others (Espe et al., 2012; Militante and Lombardini, 2004).

Larvae have high metabolic rates and require energy for various physiological processes. Lipids serve as the primary energy source for marine fish larvae (Sargent et al., 1999). It is well known that the lipid composition of diets needs to be balanced between the lipid classes triacylglycerol (TAG), and phospholipids (PL). Dietary PL is essential for the larvae's ability to metabolize TAG and should make up 40 to 50 % of dietary fat in the larvae diet (Sæle et al., 2018). PL play a vital role in growth as structural components in cell membranes (Watanabe and Kiron, 1994), and are involved in many other mechanisms such as skeleton development, and stress resistance (Hamre et al., 2013b). However, the exact requirements for PL and the type of PL are still uncertain. Poly-unsaturated fatty acids (PUFAs), specifically n-3 PUFAs, are essential for marine fish larvae and must be supplied through diet as fish cannot synthesize them (Bell and Sargent, 2003). Among the n-3 PUFAs, docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are considered essential fatty acids. The total amounts of PUFAs (n-3 and n-6) as well as the dietary DHA/EPA (n-3) ratios are important for larvae and its deficiency may affect fish growth, reproduction, and survival (Luo et al., 2019; Samat et al., 2020). As one of many examples, the low level of dietary n-3-PUFA, especially DHA, could be the direct cause of several developmental errors in Atlantic halibut larvae *(Hippoglossus hippoglossus*, L.) (Hamre et al., 2002). The amounts of other fatty acids such as arachidonic acid (ARA, 20:4 n-6) also deserves attention as a diet with unbalanced n-3/n-6 ratio can affect larvae performances in many different levels (Hamre et al., 2013b).

Extensive work on minerals and vitamin requirements for marine larvae has been done and yet, requirements and the effect of deficiencies remain unclear. Mæhre et al. (2013) recommended that the composition of wild zooplankton, which is a natural prey of most marine larvae should be used as reference. Vitamins such as vitamin C are strong

antioxidants and immunity modulators, and deficiencies can cause large losses in marine larvae production as reviewed in (Hamre et al., 2008; Waagbø, 2010). Minerals such as Ca, P, Se, Cu, Zn, I, and Mn are essential for normal development of larvae and deficiencies are the main cause of skeletal abnormalities (Lall and Kaushik, 2021; Moren, 2011). Minerals are also involved in many different biological processes. For example, I supplementation in larvae has been reported to improve the thyroid hormone status in Atlantic halibut and Senegalese sole (*Solea senegalensis*) larvae (Moren et al., 2006; Ribeiro et al., 2012), but can be toxic when given in too high doses as shown for Atlantic cod larvae (Penglase et al., 2013). Mn, Cu and Se are essential for enzymes involved in red-ox regulation (Hamre et al., 2008).

It is important to note that the nutritional requirements of larvae can vary greatly depending on the species. Therefore, it is crucial to understand the specific dietary needs of the larvae species and provide them with appropriate and well-formulated diets to ensure their health, growth, and development.

### 1.4.2 First feeding (start-feeds) in fish larvae rearing

Live feed such as rotifers (wheel animals) and *Artemia* (brine shrimp), are commonly used to nourish most marine fish larvae. Studies have demonstrated that incorporating a combination of live feed and formulated feed promotes growth and survival rates in marine fish larvae compared to those fed solely on live feed (Rosenlund et al., 1998). Rotifers are chosen as live food for many marine fish larvae as they are easy to cultivate, small and swim slowly, which make them a suitable prey for small larvae. However, rotifers lack essential fatty acids, including EPA and DHA which are crucial for the early-stage growth of marine fish larvae (Mæhre et al., 2013; Piccinetti et al., 2017; Sargent et al., 1999). Additionally, rotifers are usually low in amino acids, and deficient in vitamins and minerals necessary for the early developmental stages of fish (Hamre et al., 2008). *Artemia*, another commonly used live feed which is usually given to older larvae that have reached a certain size, has naturally low content of EPA and DHA (Hamre et al., 2002; Rønnestad et al., 1998). To address these nutritional deficiencies, live feed is enriched to improve the nutrient content in an attempt to meet the nutritional requirements of fish larvae (Mæhre et al., 2013).

Several studies support the notion that extensively harvested copepods are optimal for promoting larvae growth due to their superior nutritional composition compared to rotifers and *Artemia* (Busch et al., 2010; Hamre et al., 2008; Mæhre et al., 2013), but the use of copepods as live feed is still complicated and expensive. The harvesting of wild populations of copepods is not accessible for all farmers, it is seasonal and relies upon the weather conditions being difficult to predict. Furthermore, they might be carriers of pathogens (Karlsbakk et al., 2001). Intensive rearing of copepods is difficult and very costly (Øie et al., 2017). However, new technology and better production procedures are being developed with the potential of making production of copepods cheaper (Øie et al., 2017).

As of now, the regular feeding practice for most marine species with small eggs/embryos in hatcheries consists of enriched rotifers followed by *Artemia* and posterior weaning to dry feed (Olsen et al., 2004). Ballan wrasses seem to be quite picky when it comes to what they like to eat in captivity compared to other cultivated fish species, presenting challenges in start-feed formulation. Some hatcheries have managed to produce high quality enriched rotifers and *Artemia* that seem suitable for young wrasse larvae, but this is not always the case. Alternative start-feeds including or completely replacing rotifers with copepods (*Acartia tonsa*) have been tested in ballan wrasse. Øie et al. (2017) reported that wrasse larvae fed a diet with a complete replacement of rotifers with copepods as well as diets with different levels of copepod inclusion achieved more favourable growth and improved survival rates compared to the classic rotifer and *Artemia* diet. This together with previous experiments in other marine fish larvae, for example in Atlantic cod (Sæle et al., 2017) and Atlantic halibut (Sæle et al., 2003), where a copepod diet during early larvae stages greatly affected skeletal development, show the importance of the start-feed diets.

## 1.5 Diseases and vaccines in ballan wrasse

Several pathogens have been identified in both wild and farmed ballan wrasse (Brooker et al., 2018). Atypical strains of *Aeromonas salmonicida* (aAs) are currently the most significant cause of mortalities in hatchery and post cage deployment of farmed wrasse

(Hjeltnes et al., 2017). In addition to aAs, bacterial pathogens from the *Vibrionaceae* family are recurrent in wrasse adults (Brooker et al., 2018; Papadopoulou et al., 2020). Other pathogens such as the intracellular bacteria *Candidatus Similichlamydia* labri sp. nov. that affect gills, the parasite causing Amoebic Gill Disease (AGD), and the viral cardiomyopathy syndrome (CMS) have been found in farmed wrasse (Brooker et al., 2018; Steigen et al., 2015). Vaccine is the most effective measure to prevent illness and has the highest potential for a sustainable and competent aquaculture (Midtlyng, 2022). Commercial vaccines for this species are not yet available and the only current prophylactic treatment is the use of autogenous vaccines. Autogenous vaccines consist of antigens derived from pathogens recovered from sick fish. These vaccines can be rapidly produced but they are limited as they are meant to be used on the site (or near sites) where the pathogens have been isolated from, helping to control secondary breakouts. An autogenous multivalent injection vaccine containing *Aeromonas salmonicida* and one strain of *Vibrio splendidus* previously isolated from ballan wrasse in several farms in Scotland, was intra-peritoneally injected to wrasse (25-50g) and proved to control diseases caused by aAs *vapA* type V and likely other aAs *vapA* types in hatcheries and cage sites (Papadopoulou et al., 2020; Ramirez-Paredes et al., 2020). Wrasse has been observed to encounter pathogens at earlier life stages before it is possible to vaccinate fish by injection. For this reason, the efficiency of an immersion (bath) vaccine was tested by Papadopoulou et al. (2021). Authors designed a polyvalent autogenous vaccine against *Aeromonas salmonicida* and tested it in ballan wrasse juveniles at 80 dph (0,5 g) and 170 dph (1,5 g). Authors did no report protection of the vaccine at any of the tested stages. This reflects the urgent need for comprehensive studies on the immune system of ballan wrasse. Thus, understanding when the adaptive immunity develops is crucial to optimize vaccination strategies. This together with the characterization of species-specific pathogens (Brooker et al., 2018; Haugland et al., 2018) affecting wrasse, and the general on-going improvement in vaccine formulation in fish, will set the basis for efficient vaccine development with the potential of improving health of ballan wrasse.

## 1.6 The immune system in fish

The teleost immune system is formed by cell-mediated and humoral components working cooperatively to fight pathogens and re-establishing the healthy status of the body. The immune system is classically divided into innate and adaptive parts. The innate system is evolutionary primitive, and it aims at preventing infection by quickly responding to pathogens. Innate means that the response is non-specific, “quick and dirty” which is the first attempt to eliminate antigen. It corresponds to the main mechanism of defence against pathogens in plants, fungi, insects, and very simple multicellular organisms. The concept of innate immunity as it was classically understood has been challenged in the last years. New research shows that the innate system can also mount resistance to reinfection meaning that certain “innate” immunological memory might also exist to some extent in teleosts (Netea et al., 2011). The adaptive immune system is slower-acting but has a longer-lasting and more specific response. It generates memory, meaning it will recognize the antigen in future infections and will specifically eliminate it more efficiently. The innate and adaptive immune systems work together through direct cell contact and humoral components such as chemical mediators, cytokines, and chemokines. Moreover, the adaptive system is partly dependent on earlier innate pathways (Clark and Kupper, 2005; Rauta et al., 2012; Uribe et al., 2011).

### 1.6.1 Lymphoid organs in teleosts

Primary lymphoid organs are anatomical sites where lymphoblasts proliferate and become mature lymphocytes which involve the acquisition of antigen specific receptors (by gene rearrangement). T-cells are developed in the thymus both in mammals and in fish and therefore, it is a primary lymphoid organ (Trede and Zon, 1998). The development of B-cells occurs in the bone marrow of mammals, which is lacking in fish (Uribe et al., 2011). Instead, B-lymphopoiesis classically occurs in the head kidney of teleosts. On the other hand, secondary lymphoid organs are sites where circulating mature lymphocytes encounter antigens and become activated, initiating immune responses. This takes place in the spleen, lymph nodes (LNs), and mucosa-associated lymphoid tissue (MALT) of mammals. Germinal centres (GCs) and LNs

play a pivotal role in facilitating B-cell activation and further differentiation into plasma cells (PCs) and memory cells but as of now, none of these structures have been identified in teleost fish. It is worth noting that cartilaginous fish (the oldest vertebrate group with Ig-based adaptive immunity) undergo B-cell selection upon antigen encounter in the center of follicles in spleen, resembling GC-like structures (Matz and Dooley, 2023). These findings suggest that teleost might also rely on structures similar to GCs, although there is currently no supporting evidence. Classically secondary lymphoid organs in fish are spleen, posterior kidney, and MALTs (Zapata and Amemiya, 2000; Zwollo et al., 2005; Zwollo et al., 2010). Although the focus henceforth is on classical lymphoid organs, it is important to emphasize the recent discovery of two novel secondary lymphoid organs in several teleost species as described later (in section 1.6.4) (Garcia et al., 2022; Løken et al., 2020; Resseguier et al., 2023), as well as newly lymphoid structures in salmonids (Bjørgen and Koppang, 2021, 2022).

### *Kidney*

The fish kidney is a paired organ located ventrally to the spinal cord that is fused in most species giving the appearance of a single structure. It is divided into anterior or head kidney which lacks nephrons and posterior or trunk kidney where nephrons accumulate and have “renal” functions that filter blood and produce urine (Bjørgen and Koppang, 2022).

A maturation gradient of B-cells from anterior to posterior kidney has been reported in rainbow trout (*Oncorhynchus mykiss*) (Zwollo et al., 2005), with the anterior or head kidney (HK) mostly hosting developing B-cell precursors and the posterior or trunk kidney consisting mostly of activated B-cells, plasmablasts, and plasma cells (PCs) (Zwollo, 2005). The existence of a B-cell maturation gradient might seem arbitrary and should be studied in more fish species. Head kidney host both B-lymphopoiesis, which occurs within hematopoietic tissue, and endocrine cells. The posterior or trunk kidney functions as both excretory (with nephrons) and as a secondary lymphoid structure with T- and B-cells (Bjørgen and Koppang, 2022). Notice that hematopoietic tissue is also found in trunk kidney making the organ fascinating and complex.

Interestingly, PCs have been observed in the HK of some teleosts where they are stored for long periods and serve as long-term humoral protection, as it happens in mammals (Abós et al., 2022; Zwollo et al., 2005). It has been proposed that the population of PCs in HK migrate back from secondary lymphoid organs after antigen-dependent activation (Bromage et al., 2004; Zwollo et al., 2005). A peculiarity of teleost kidney compared to mammalian bone marrow is the presence of melanomacrophage centers (MMCs). MMCs are aggregates of highly pigmented macrophages (dark stained) that have been suggested as primitive GCs (Steinel and Bolnick, 2017). On-going research is being done in several groups to elucidate this matter.

### *Thymus*

The teleost thymus is a primary lymphoid organ where T-cell precursors undergo maturation. Thymus was first observed in rainbow trout (Grace and Manning, 1980), and later in other teleosts as a paired organ located dorsally near the gill cavity (Bowden et al., 2005; Zapata, 1981). Like in higher vertebrates, the teleost thymus appears to be zoned into cortex- and medulla-like regions (Flajnik, 2018), although available research on salmonids have revealed an atypical organization of the thymus characterized by three distinct layers (Koppang et al., 2010). The thymus is enveloped by a capsule that is composed of both epithelial cells and connective tissue. Epithelial cells are specifically located in the area where the organ interfaces with the gill cavity, while connective tissue surrounds the other parts of the thymus. The connective tissue forms invaginations within the thymus known as trabeculae which contain capillaries. These trabeculae provide structural support to the thymus and facilitate its vascularization.

The thymus in freshwater fish seems to be the first lymphoid organ during larvae ontogeny although lymphohematopoietic precursor cells appear in kidney before the thymus differentiates into a lymphoid organ (Chantanachookhin et al., 1991; Zapata et al., 2006). Interestingly, in marine teleosts, the thymus develops later than the kidney (Bjørgen and Koppang, 2021; Patel et al., 2009; Zapata et al., 2006).

The involution process of thymus is well known in mammals and implies dysfunction of the organ in adulthood, mainly related to reduction in organ size, decrease in thickness, and reduced number of lymphocytes. Thymus involution in fish has been reported in a few species (Lam et al., 2002; Trede and Zon, 1998). A recent review pointed out that water temperature, photoperiod, age and sexual maturity seem to be key factors potentially affecting thymus function in fish (Barraza et al., 2020). For example, the size of thymus was reduced in carp adult fish (*Cyprinus carpio* L.) whereas they kept the capacity to generate T-cells (Huttenhuis et al., 2005). Interestingly, a reduction in the number of lymphocytes but not in thymus size was observed in 3 species of tilapia (Fishelson, 1995; Sailendri and Muthukkaruppan, 1975). A correlation between the size of thymus and its capacity to produce mature T-cells is lacking in teleosts and needs further investigations.

### *Spleen*

The spleen in teleost fish is regarded as the primordial secondary lymphoid organ and main site for blood filtration (Bjørgen and Koppang, 2022; Flajnik, 2018). Although the structure and distribution of cells in spleen is not well characterized in teleosts, white and red pulp together with certain immune cells such as macrophages, T-cells, B-cells, and MMCs have been reported in several species (Koppang et al., 2003; Steinel and Bolnick, 2017).

### *Mucosa-associated lymphoid tissues -MALTs*

MALTs are formed by B- and T-cells that are phenotypically different from their systemic counterparts, appear diffusely distributed within tissue and can respond to mucosal infection (Salinas, 2015). MALTs in mammals can be diffuse or appear organized in more advanced structures such as Peyer's patches and tonsils. In teleosts, MALTs have received different names depending on the tissue, as following; gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT) and nasopharynx-associated lymphoid tissue (O-NALT). It was long believed that such a thing as organized MALT did not exist in teleost fish, until 2008 when the interbranchial lymphoid tissue (ILT) was first discovered in Atlantic salmon, and later in other species (Dalum, 2017; Haugarvoll et

al., 2008). Intriguingly, ILT in salmonids share common characteristics with secondary lymphoid organs but its structure and its response to infection does not reflect functional similarity to secondary lymphoid organs (Bjørgen and Koppang, 2022). Different opinions exist on whether ILT is regarded as a part of GIALT or should be considered as an independent organ. Nevertheless, follow-up studies strongly suggest the presence of organized lymphoid structures in teleosts (Garcia et al., 2022; Løken et al., 2020; Resseguier et al., 2023).

### 1.6.2 Innate immunity in brief

The ectotherm nature of teleosts, together with their relatively limited adaptive immune system compared to higher vertebrates, make the innate system a fundamental mechanism of defence in fish that deserves attention (Uribe et al., 2011). The innate immune system in teleosts is composed of physical barriers (skin, flakes etc), immune cells, and humoral molecules. One of the key "innate" receptors are the germline-encoded pattern recognition receptors (PRRs) which recognize small structures of microbial agents called pathogen-associated molecular patterns (PAMPs). There are several types of PRRs that are specialized in recognizing different PAMPs. For instance, Toll-Like Receptors (TLRs) are probably the most important signalling PRRs. TLRs is a very diverse family of proteins that can recognize a large diversity of PAMPs (Lydyard et al., 2004). In mice and humans 12 and 11 TLRs have been identified correspondingly. However, it seems to be more complex in fish probably due to the early genome duplication of teleost linage (Lieschke and Trede, 2009). The reported TLRs in different teleosts are extensively summarized in Dalmo and Bøgwald (2022). Although not all of them are properly characterized in fish, they are assumed to be similar to mammalian counterparts regarding localization in the cell and ligand specificity (whether they recognize virus, bacteria, parasites) (Kanwal et al., 2014). Once PRRs have bound to the non-self/microbial agent, a cascade of intracellular signals results in transcription of genes coding for different proteins such as lectins, cytokines, antimicrobial peptides (AMPs), acute-phase proteins (APPs) and others, that are released and act simultaneously in different pathways to fight pathogens. Cytokines are an important and broad family of small proteins produced by different immune cells and are involved in cell signalling. Several cytokines have also been reported in

different fish species, interleukin 1β (IL-1β) in Atlantic salmon is analogous to that in mammals, and interferon α1 (IFNα-1) also from Atlantic salmon, have been reported to have similar properties to IFNα/β and IFNγ in mammals (Uribe et al., 2011). Up to date, cytokines have not been investigated in cleaner fish (Haugland et al., 2018). Among cytokines, the interferon (IFN) system is one of the most important mechanisms of defence in both innate and adaptive immunity and has been investigated in several fish species over the last decades (Pereiro et al., 2019; Robertsen, 2006). IFN are a large group of cytokines that act against viral infections. There are two families; type I IFN which is merely involved in innate responses and type II IFN (IFN γ) which can be produced by natural killer cells (NK) taking part of the innate response, or by lymphocytes playing a role in adaptive immunity (Pereiro et al., 2019). Other cytokines involved in immune responses are the tumour necrosis factor α (TNFα), proteins of the complement system, chemokines, and interleukins that are produced by both innate and adaptive cells connecting both systems. There are several cells that are classically considered as innate cells such as macrophages/monocytes, dendritic cells, NK, and granulocytes. Furthermore, more recent studies have suggested that red blood cells, thrombocytes, B-cells, and some type of T-cells also have innate-like immune functions (Chan et al., 2023; Shen et al., 2018).

### 1.6.3 Adaptive immunity

The appearance of jawless fish, the most ancient living vertebrate species, involved the first form of a rather rudimentary adaptive immune system with no immunoglobulins nor T-cell antigen receptors. Genes that are usually used as markers for adaptive immunity firstly appeared in gnathostomes (jawed vertebrates) and remained in higher vertebrates (Abós et al., 2022; Cooper and Alder, 2006).

The adaptive immune system is characterized by the presence of antigen receptors on lymphocytes, T-cell receptor (TCR) in T-cells and B-cell receptor (BCR) in B-cells. The success of the adaptive system at recognizing millions of different antigens lies in the organization of genes encoding for BCR and TCR receptors. These genes, referred to as VDJ genes, undergo a random recombination process that results in a vast array of specific receptor repertoires in lymphocytes, contributing to significant diversity

(Abós et al., 2022; Bilal et al., 2021). The recombination activation genes (*RAG1* and *RAG2*) are crucial at activating recombination processes in both B- and T-lymphocytes as further described (Owen et al., 2013). Although it is accepted that teleosts can specifically recognize antigens and develop immunological memory, there are several important differences with the mammalian counterparts and among fish species, making research on fish immunology complex but exciting.

### *Immune cells for adaptive responses*

Adaptive immune responses rely on specialized immune cells collaborating to recognize and eliminate specific pathogens or antigens. T-cells and B-cells constitute the primary components of adaptive immunity.

For an effective immune response, antigen-presenting cells (APCs) are indispensable. Dendritic cells (DCs) play a critical role in presenting antigens in mucosal locations in mammals. While DC-like cells have been identified in the intestine of some teleost fish, their function is still not clear (Salinas and Magadán, 2017). Parra et al. (2015) proposed that macrophages and B-cells could serve as the primary antigen-presenting cells at mucosal sites in fish.

### *Teleost T-cells*

The main distinctive feature of T-cells is their ability to recognize antigens when they are presented by the major histocompatibility complex molecule (MHC), described as MHC restriction. MHC class I and II present antigen and specifically bind to T-cells through the T-cell receptor complex (TCR/CD3) and other T-cell co-receptors (CD4 and CD8) present on their surface. Teleost T-cells resemble mammalian T-cells. Some of the pioneer studies demonstrating structural homology was the discovery of *MHC* in carp (Hashimoto et al., 1990), *TCRαβ* in salmonids (Hordvik et al., 1996; Partula et al., 1995), *TCRγ* gene in Japanese flounder (*Paralichthys olivaceus)* (Nam et al., 2003), and the recombination activating genes (*RAG1* and *RAG2*) in thymus of rainbow trout and zebrafish (*Danio rerio*) (Hansen and Kaattari, 1995; Willett et al., 1997). The latter discovery was ground-breaking because it demonstrated that the molecular mechanisms involved in generating antigen receptor diversity through *V(D)J*

recombination also existed in fish. Like in mammals, teleost T-cells are divided into helper T-cells that are $CD4^+$, cytotoxic T-cells that are $CD8^+$, and regulatory T-cells (Treg) (Kasheta et al., 2017; Nakanishi et al., 2002; Takizawa et al., 2016). Upon antigen encounter, naïve T-cells are activated, proliferate, and undergo various effector responses. Helper T-cells secrete cytokines to coordinate immune response by assisting other immune cells, cytotoxic T-cells directly fight the pathogen (virus, bacteria etc) by releasing cytotoxic molecules, and Treg suppress or regulate immune responses (Ashfaq et al., 2019; Nakanishi et al., 2002; Nakanishi et al., 2015; Xiong and Bosselut, 2012). It is important to notice that while helper and cytotoxic T-cells have been partly characterized in fish, the presence of Treg is so far based on the expression of specific markers of mammalian Treg cells, such as *FOXP3* and *GATA3* (Ortiz et al., 2014). Further characterization of teleost T-cells is needed (Laing et al., 2006; Nonaka et al., 2008). A comprehensive review in the topic described the current T-cell markers that have been identified in different teleost species, including those previously mentioned, such as TCRαβ, TCRγδ, RAG, MHC, CD3 (ε, γ, and δ), CD4 (-1 and -2), and CD8 (α and β) (Barraza et al., 2020). Moreover, Lymphocyte-specific protein tyrosine Kinase (LCK) and the 70 kDa Zeta-Associated Protein (ZAP70) are considered T-cell markers and crucial proteins implicated in T-cell signalling and activation, and have also been successfully cloned in teleost fish (Bajoghli et al., 2019; Barraza et al., 2020).

### *Teleost B-cells and immunoglobulins*

B-cells, a type of lymphocyte, are specialized to produce immunoglobulins. They consistently express membrane-bound receptors known as B-cell receptors (BCR) that specifically recognize antigens. B-cell activation occurs only when antigens are recognized by BCR. Upon activation, B-cells produce glycoproteins called immunoglobulins, which are subsequently secreted and commonly known as antibodies. The basic structural unit of immunoglobulins/antibodies consists of four polypeptide chains; 2 identical heavy chains (encoded by IgH genes) and 2 shorter identical light chains (encoded by IgL), bound together by covalent disulfide bridges and non-covalent interactions. Heavy chains comprise a variable domain (VH) as well as constant domains. These constant regions composing the heavy chain of Ig determine the Ig isotype. Light chains comprise a variable domain (VL) followed by a

constant domain (CL) (kappa or gamma) (Bilal et al., 2021). Antibodies have several functions such as inactivation and opsonization of pathogens, activation of complement, and activation of cytotoxic cells acting as a mediator of cellular cytotoxicity (Lydyard et al., 2004). In mammals, there are five major immunoglobulins: IgE, IgA IgM, IgD and IgG. However, the number of Ig isotypes has increased gradually during vertebrate evolution. Thus, it is not surprising that fish which are evolutionarily more primitive than mammals, do not have the same Igs as higher vertebrates. Teleost presents three types of immunoglobulins: IgM, IgD, and IgT. IgM is mostly found as a tetramer in fish, and it is the predominant systemic immunoglobulin. IgM plays a crucial role as a natural antibody. The teleost isotype IgT was first discovered in 2005 in zebrafish, rainbow trout, and common carp, and it was proposed as an ancient isotype analogous of IgA in mammals, which is predominant in mammalian gut mucosa (Danilova et al., 2005; Hansen et al., 2005; Savan et al., 2005; Woof and Kerr, 2006). Trout IgT was found as a monomer in serum and a tetramer in gut (Zhang et al., 2011). IgD is as ancient as IgM and it is found in all jawed vertebrates, both as membrane bound and secreted (Ohta and Flajnik, 2006).

Teleost and mammalian B-cells are antigen presenters (APC) to T-cells via MHCII (Rodríguez-Pinto, 2005; Wu et al., 2020). Unlike in mammals, teleost B-cells are characterized by having a potent phagocytic and microbicidal activity (Li et al., 2006; Scapigliati et al., 2018). Nevertheless, one of the most, if not the most distinctive feature of B-cells is their ability to specifically recognize antigens in their native form through BCRs. Teleost B-cells can be divided into IgM/IgD$^+$ cells and IgT$^+$ B-cells. Upon antigen encounter, double positive naïve IgM$^+$/IgD$^+$/IgT$^-$ B-cells get activated and only express IgM (IgM$^+$/IgD$^-$/IgT$^-$) or IgD (IgM$^-$/IgD$^+$/IgT$^-$). This is the result of alternative splicing between the recombined V(D)J region and the C region of the heavy chain (IgH) of IgM (Cμ) or IgD (Cγ) (Bilal et al., 2021). On the other hand, IgT$^+$ B-cells are the result of recombinatorial exclusion. IgM$^+$ B-cells are predominant in systemic lymphoid organs, serum, and the peritoneal cavity, while IgT$^+$ B-cells are proposed to be the principal B-cell subset in MALTs (Salinas et al., 2021; Scapigliati et al., 2022; Zhang et al., 2010).

## *Development of T- and B-cells*

**T-cell development:**

T-cell lymphopoiesis occurs in the thymus, and it is a complex process that is strictly regulated and requires constant contact of T-cell precursors with both stromal and thymic epithelial cells (Owen et al., 2013; Pearse, 2006). Like in higher vertebrates, the thymus of most studied teleosts is organized into distinct zones, comprising a cortex and a medulla (Barraza et al., 2020). T-cell development begins in the cortex with a common lymphoid progenitor (CLP), where recombination-activating genes (*RAG1* and *RAG2*) are activated. As these cells progress towards the medulla, they undergo phenotypical changes (Bommhardt et al., 2004; Nagaoka et al., 2000). The ultimate goal of this progression is for medullar T-cells to effectively express a functional TCR capable of recognizing peptides bound to MHC molecules. This achievement relies on two pivotal steps: positive and negative selection that require interactions with MHC molecules (Takaba and Takayanagi, 2017). Key processes and various stages of T-cell development within the teleost thymus have been illustrated in Figure 3. There is much evidence supporting the fact that T-cell lymphopoiesis in teleosts is conserved and similar to that in higher vertebrates. This has mostly been demonstrated by the localization of specific T-cell markers within the teleost thymus and the characterization of thymocytes in different developmental stages (Abelli et al., 1998; Bajoghli et al., 2019; Barraza et al., 2020; Dalum et al., 2016; Fischer et al., 2005; Huttenhuis et al., 2005; Koppang et al., 2003; Picchietti et al., 2015; Picchietti et al., 2009; Romano et al., 2013; Takaba and Takayanagi, 2017; Takizawa et al., 2011; Takizawa et al., 2016; Toda et al., 2011; Øvergård et al., 2011; Aas et al., 2014).

Altogether, literature supports the idea that T-cell development is to some degree evolutionary conserved between fish and mammals. However, teleosts are very diverse, and studies comprising thymic structure, zonation and T-cell development are restricted to few species and yet, with certain contradictions (Barraza et al., 2020; Bowden et al., 2005).

**B-cell development:**

The letter "B" in B-cells is derived from the Bursa of Fabricius in chickens, which is a unique site for B-cell development in birds. As reviewed in detail in Melchers and Kincade (2004), mammalian B-cells develop in the bone marrow. B-cell lymphopoiesis is strictly regulated through a series of phenotypic alterations involving distinct protein expression patterns. It all starts with a CLP cell in the bone marrow that matures to pro B-cells, pre B-cells, and immature B-cells in an antigen-independent manner. Immature B-cells possess membrane-bound IgM on their surface and leave the bone marrow towards the spleen where they become mature naïve B cells ($IgM^+$ $IgD^+$) (Honjo et al., 2004; Melchers and Kincade, 2004; Owen et al., 2013).

In teleosts, the process of $IgM^+$ B-cell lymphopoiesis is believed to be similar to the mammalian pathway described above (Zwollo, 2011), and illustrated in Figure 3. As mentioned previously, teleosts lack bone marrow and B-cells seem to develop within the HK (Zapata, 1979; Zapata and Amemiya, 2000). Zwollo et al. (2005) characterized the different stages of developing B-cells in teleosts. For this, authors used five transcription factors that are highly conserved with the higher vertebrates' counterparts. This work was important as it established markers to distinguish between developing B-cells (CLP, pro B-cells, pre B-cells, naïve mature B-cells), and activated B-cells (plasmablasts and plasma cells) (Zwollo, 2011). Multiple recent studies on salmonids employing different methodological approaches, have proposed new markers for identifying B-cells at different stages (Herranz-Jusdado et al., 2023; Morel et al., 2023; Peñaranda et al., 2019). As highlighted in Scapigliati et al. (2022), there has been a predominant focus on understanding the development of $IgM^+$ B-cells, leaving the ontogeny of $IgD^+$ and $IgT^+$ B-cells relatively unexplored. A single basic description of the ontogeny of $IgT^+$ B-cells in rainbow trout is currently available (Heinecke et al., 2014). Nonetheless, further research is needed to accurately characterize B-lymphopoiesis process in teleosts and better define the different subtypes of B-cell populations in fish, as well as species-specific differences (if any) (Zwollo, 2011).

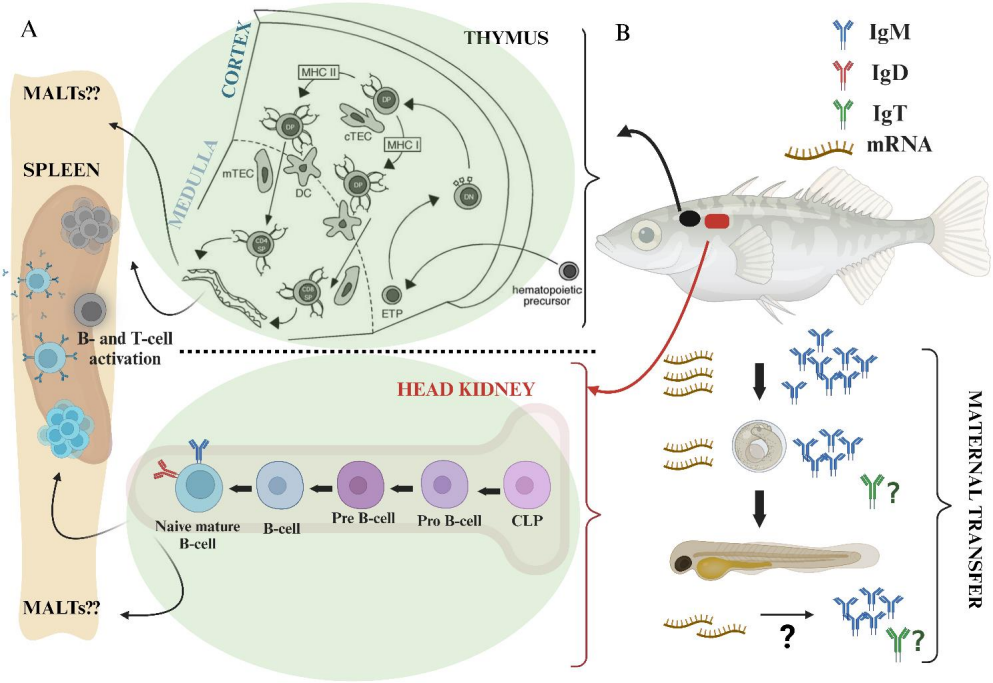


**Figure 3**. **A)** Developmental stages of T-cells in thymus (above) and B-cells in head kidney (below). Initially, the thymus cortex host thymocytes that are double-negative (DN), lacking CD4 and CD8 molecules. DN thymocytes with rearranged TCRβ chains express it on their surface, along with CD3 chains, forming a pre-TCR complex, leading to proliferation. They also increase CD4 and CD8 expression, becoming double-positive (DP) $CD4^{+}CD8^{+}$ thymocytes, undergoing positive selection based on affinity for self-MHC molecules, while those not reacting with self-MHC undergo apoptosis. Surviving DP thymocytes mature into single-positive (SP) T-lymphocytes ($CD4^{+}$ or $CD8^{+}$) and migrate to the medulla, where negative selection eliminates strongly self-reactive T-cells (with self-MHC). Mature SP T-cells lose *RAG* expression and enter circulation. T-cell activation and proliferation is triggered upon antigen recognition in secondary lymphoid organs (i.e., spleen). The original figure shows thymocyte development in a mammalian thymus and has been adapted from (Bommhardt et al., 2004). Below, teleost $IgM^{+}$ B-cell lymphopoiesis is illustrated. From a common lymphoid cell (CLP), and through several phenotypical changes, naïve mature B-cells express both IgM and IgD on their surface ($IgM^{+}$ $IgD^{+}$) and are now prepared to encounter antigen. B-cell activation and proliferation is triggered upon antigen recognition in secondary lymphoid organs. Green indicates primary lymphoid organs (thymus and HK), and yellow indicates secondary lymphoid organs, represented by the spleen. It remains elusive whether teleost naïve B- and T-cells can be directly activated in MALTs. **B)** Schematic illustration depicting transfer of passive maternal immunoglobulins and mRNA to the offspring before larva can initiate its autonomous lymphopoiesis process.

### *Maternal transfer of protective factors to the offspring*

Eggs and newly hatched larvae (yolk-embryonic larvae) are most vulnerable in a hostile environment exposed to pathogens. The size of eggs and yolk is species-specific and it is related to the reproductive strategy of a given species (Duarte and Alcaraz, 1989). For instance, larger and fewer eggs in demersal species and smaller and many eggs in pelagic fish. This might also be accompanied by a higher or lower rate of maternal transfer of several immune factors to the eggs that contribute to early defence of the embryo. Although the focus henceforth is maternally transferred molecules that belong to the immune system, there is a large variety of other factors that are maternally transferred and seem to have important implications for embryonic development and other physiological processes in the offspring. For instance, hormones can regulate embryonic development and influence timing of hatching, and antioxidants such as vitamin C and E might protect developing embryos from oxidative stress (Fan et al., 2019; McCormick, 1999; Waagbø, 2010). Similarly, transferred microbiota (via mother- chorion or during spawning) is important for gut development, nutrition and immunity (Liu et al., 2014; Wang et al., 2018).

Studies on several teleost species have shown the transfer of maternal innate protective molecules such as complement proteins, lysozymes, lectins, AMPs, cytokines, and serine proteases, that likely protect larvae before they develop their own functional defence mechanisms (Huttenhuis et al., 2006a; Løvoll et al., 2006; Mulero et al., 2007; Swain and Nayak, 2009; Valero et al., 2023). Maternal immunoglobulins (Igs) have also been reported in fish larvae. IgM protect developing embryos from pathogens and microbial infections by directly binding to their surface (opsonization) and facilitating phagocytosis as shown in zebrafish (Wang et al., 2012b). Similarly, maternal IgT in zebrafish was efficient against microbes (Ji et al., 2021).

Not only Ig-protein but also *Ig* mRNAs are maternally transported to the oocyte in several teleosts. For instance, maternal *IgM* mRNA was present in released eggs of sea bass (*Dicentrarchus labrax*) (Picchietti et al., 2004) and sea bream (*Sparus aurata*) (Picchietti et al., 2006), and *IgT* mRNA in zebrafish zygotes (Ji et al., 2021). On the other hand, unfertilized eggs of Atlantic cod were completely deprived of maternal *IgM*

and *IgD* transcripts (Seppola et al., 2009). Currently available data does not allow to conclude whether maternally transferred mRNA is a common mechanism in teleosts or if it varies among different species. It is important to remark that maternal Igs and mRNA are transferred to the offspring in a passive manner before the larvae's own B-cells mature and begin producing their own antibodies (Fig. 3).

Moreover, survival and development of fish offspring heavily rely on the nutrients they inherit from the mother through the breakdown of vitellogenin (Vg). This process yields various components such as yolk proteins, phosvitin, and lipovitellin, which serve as crucial nutrients for the growing embryos. Additionally, a review on this subject highlighted the role of Vg, as well as its derived proteins, in protecting the embryos and larvae (Zhang et al., 2013).

### 1.6.4 Mucosal Immunity and the poly-Ig Receptor (pIgR)

In fish, mucosal surfaces are the first defensive lines against invading organisms (Ángeles Esteban, 2012). Fish thrive in a changing environment, and it is crucial that they can maintain mucosal homeostasis. Anatomically, the mucosal immune system in mammals is well divided into two regions; the inductive mucosal sites where naïve B- and T-lymphocytes are exposed to antigens for the first time (ie; mesenteric lymphoid nodes), and effector mucosal sites that are areas where already differentiated B- and T-cells execute an immune response. This differentiation seems to be lacking in teleosts where antigen presentation and effector actions might happen in the same anatomical place along the MALT (Salinas, 2015).

Mucus contains many humoral innate-like active molecules as well as secreted immunoglobulins (sIg). Teleost MALTs are populated by immune and non-immune cells such as epithelial cells, mucus-producing cells and neurons that play an active role in immune responses, reviewed in (Salinas et al., 2022). Both adaptive and innate immune cells such as lymphocytes, macrophages, natural killer cells, granulocytes, and dendritic-like cells among others have been found in teleost MALTs. Furthermore, in more recent years, it has become clear that microbial communities established in mucosal tissues are crucial for a healthy mucosa (Salinas et al., 2022).

Mucosal organs such as gut, skin, gills, and olfactory mucosa are very rich in T-cells that are diffusely distributed or accumulated in clusters as reported in the ILT (Haugarvoll et al., 2008) and the trout olfactory lamellae (Sepahi et al., 2016). While the main subsets of T-cells ($CD4^+$ and $CD8^+$) exist in mucosal sites of several teleosts, it remains unknown whether fish T-cells play a role in the induction of mucosal immune responses (Salinas et al., 2022). Furthermore, a proper phenotypical characterization of these mucosal lymphocytes is still unclear (Sepahi et al., 2016). Regarding mucosal B-cells, four subsets of B-cells have been identified at different mucosal sites ($IgM^+$, $IgT^+$, $IgD^+$, and $IgM^+$ $IgD^+$) as recently reviewed elsewhere (Salinas et al., 2022; Salinas et al., 2021).

The first step for mounting a mucosal immune response is the uptake of antigens and subsequent presentation to initiate adaptive immune responses. In teleosts, there are limited studies that specifically investigate antigen uptake. However, some studies suggest that antigens may be taken up by enterocytes and/or by cells resembling dendritic cells or macrophages within the fish gut (Løkka and Koppang, 2016). In the gills of rainbow trout, two distinct cell types demonstrated the ability to present antigens. One of these cell types exhibited a profile resembling monocytes/macrophages, or dendritic cells, while the other displayed characteristics of mammalian M cells (Kato et al., 2018). In line with this, another interesting question is whether T- and B-cell activation, proliferation and differentiation takes place "*in situ*" in mucosal infected sites (after antigen presentation) or somewhere else (secondary lymphoid organs). Few investigations have addressed this matter. In trout, challenged with bacteria, Xu et al. (2016b) targeted DNA during cell division with EdU (an analogue of thymidine incorporating in the DNA during cell division). Authors observed that $IgT^+$ B-cells locally proliferated in the gills whereas the number of proliferative $IgM^+$ B-cells did not increase correspondingly. The opposite pattern was observed in lymphoid organs upon the same challenge where a significant number of proliferative $IgM^+$ B-cells was found but the number of $IgT^+$ B-cells remained unchanged (Xu et al., 2016b). A later study in Japanese flounder demonstrated local proliferation of $IgM^+$ $EdU^+$ B-cells in the lamina propria of hindgut upon infection with *Vibrio anguillarum* (Sheng et al., 2022). These two studies demonstrated the local

proliferation of B-cells in teleosts MALT in response to antigens. However, it is still unknown whether antigen presentation and subsequent activation of B-cells occurs at the infected mucosal site or if activated B-cells migrate from lymphoid organs to the infected MALT for further proliferation (Salinas and Magadán, 2017). It is interesting to note that the populations of B-cells (type and amount) in different MALTs might also be species-specific.

### *Mucosal immunoglobulins*

Secretory immunoglobulins (sIgs) or antibodies are crucial humoral components of mucosal defence in mammals and teleosts (Braathen et al., 2007; Salinas et al., 2021). Generally speaking, polymers of Igs (poly-Igs or pIgs) are produced by plasma cells residing in the mucosa underneath the mucosal epithelium that are transported towards the lumen of mucosal surfaces. In mammals, IgA and IgM form polymers (dimeric IgA and pentameric IgM) when secreted to mucosal sites being considered mucosal Igs (Woof and Mestecky, 2005). Appearance of polymeric IgA and IgM in mucosal tissue such as gut and/or skin is a well-accepted criterion for the existence of a mucosal immune system (Rombout et al., 2014). In teleost, although IgM is the predominant systemic immunoglobulin, it is also abundantly present in mucosal sites, where it plays a pivotal role as an effector molecule in immune responses (Bilal et al., 2021; Flajnik, 2002). Moreover, recent research has highlighted the role of mucosal IgM as an ancient regulator of microbiota homeostasis (Ding et al., 2023).

Based on studies on rainbow trout, IgT has been reported as a specialized mucosal Ig (Salinas et al., 2011; Zhang et al., 2010). In this regard, the ratio of $IgT^+$ to $IgM^+$ B-cells was higher in MALTs compared to spleen, head kidney, and serum (Zhang et al., 2011). For example, around 70-80 % of all B-cells in systemic lymphoid organs are $IgM^+$ B-cells whereas the proportion of $IgT^+$ and $IgM^+$ B-cells are 54% and 46% respectively in the gut of rainbow trout (Zhang et al., 2011). Similarly, the ratio IgT/IgM is much higher in mucus when compared to serum (Zhang et al., 2010). However, the overall IgM concentration exceed IgT in both serum and mucosal sites (Bilal et al., 2021; Salinas et al., 2021). While IgT is reported as a specialized Ig in mucosal surfaces in rainbow trout, further research is required to elucidate its specific

role in immune responses (Abós et al., 2022). Furthermore. similar studies on other species are crucial to clarify whether the characteristics observed in trout IgT are universally shared among different fish or if they are species-specific. IgD has been suggested to be involved in microbiota homeostasis in rainbow trout, though its specific role in mucosal responses has not been clarified yet (Abós et al., 2022; Perdiguero et al., 2019; Rombout et al., 2014; Xu et al., 2016b).

It is worth emphasizing that our understanding of B-cell subsets as well as the proportion of Ig subtypes in fish lymphoid tissues (systemic and MALTs), has focused on a limited number of species primarily salmonids and zebrafish, and remains somewhat under-studied in most teleosts (Abós et al., 2022).

### *Transport of mucosal immunoglobulin- the poly-Ig Receptor (pIgR)*

Receptors interacting with immunoglobulins (via the C-terminal constant region of the Ig heavy chains (Fc)) have important effector functions in vertebrates. For example, neutralized microbes can be phagocytosed (opsonization), killed via interactions with soluble factors such as complement proteins, and can activate antibody-dependent cellular cytotoxicity (Kaetzel, 2014; Rombout et al., 2014; Salinas and Parra, 2015) among others. The poly-immunoglobulin receptor (pIgR) is the most evolutionary ancient Fc receptor, transporting polymeric Igs (IgA/IgM/IgT/IgX) (Kaetzel, 2014). Mammalian pIgR is a transmembrane protein consisting of an extracellular, intracellular and a transmembrane region (Kaetzel et al., 1997) as shown in Figure 4A. The extracellular domain contains either five (D1-5) or three (D1, D4, and D5) Ig-like domains as it happens in rabbits and bovine due to alternative splicing (Norderhaug et al., 1999). The pIgR of lower vertebrates such as chicken (Wieland et al., 2004) and African clawed frog (*Xenopus laevis*) (Braathen et al., 2007) shows 4 Ig-like domains. Mammalian pIgR is expressed in mucosal epithelia and in hepatocytes or biliary epithelial cells where it is crucial for the transport of polymeric Igs (dimeric IgA and pentameric IgM) from the lamina propria to the luminal sites and gall bladder, respectively, in a process called transcytosis (Rojas and Apodaca, 2002). Monomer-Igs are not transported by transcytosis and they are exclusively present in serum and not in mucosal secretions (Ghumra et al., 2009). After the pIgR-pIg complexes reach the

apical surface, the complex is proteolytically cleaved and the extracellular domains of the pIgR (D1-5) are released bound to the pIgs. The excised region is called the secretory component (SC). The SC is present in mucosal sites of higher vertebrates both in association with pIgs and as a free element (Eiffert et al., 1984; Musil and Baenziger, 1987). The formation of the SC-pIg complex is crucial to facilitate excretion to the mucus, enhance secreted Ig stability and protect it from proteolytic activity. Disruption of the pIgR-pIg system can evoke serious consequences for mucosal homeostasis as it happens during inflammatory bowel disease (Johansen and Kaetzel, 2011). Although the pIgR is well characterized and known to be a key component in mucosal defence of mammals and other higher vertebrates, research on pIgR in teleosts is somewhat new and faces several challenges. The first investigated pIgR gene among teleosts was in fugu (*Takifugu rubripes)* (Hamuro et al., 2007) followed by a considerable number of species in the past decade including orange-spotted grouper (*Epinephelus coioides*) (Feng et al., 2009), rainbow trout (Zhang et al., 2010), Atlantic salmon (Tadiso et al., 2011), carp (Pei et al., 2019; Rombout et al., 2008), Japanese flounder (Sheng et al., 2018; Xu et al., 2013a), zebrafish (Kortum et al., 2014), and others. Teleost pIgR has been found to exhibit only 2 domains (Fig. 4A) which seem to correspond to mammalian D1 and D5 pIgR (Feng et al., 2009; Kong et al., 2018; Rombout et al., 2008). Moreover, Hamuro et al. (2007) suggested that the second domain described in teleosts (often referred as D5) might be an ancestral domain of mammalian D5, and that tetrameric IgM might bind in a different manner than D5 bind dimeric IgA in mammals.

The hypothesis that fish primarily depend on pIgRs to initiate mucosal antibody responses is widely recognized, although some have raised doubts about its functionality due to the current lack of supporting evidence. Important structural differences between teleost pIgR and its counterpart in higher vertebrates as well as the lack of gene synteny (Fig. 4B), challenge the generally accepted functional homology between pIgR in teleosts and that in higher vertebrates (Flowers et al., 2021; Kaetzel, 2014; Kortum et al., 2014). Furthermore, the little knowledge on the mechanisms of

polymerization of mucosal IgM and IgT and how polymeric Igs are transported to mucosal sites in the lack of J-chain adds uncertainty to the matter.

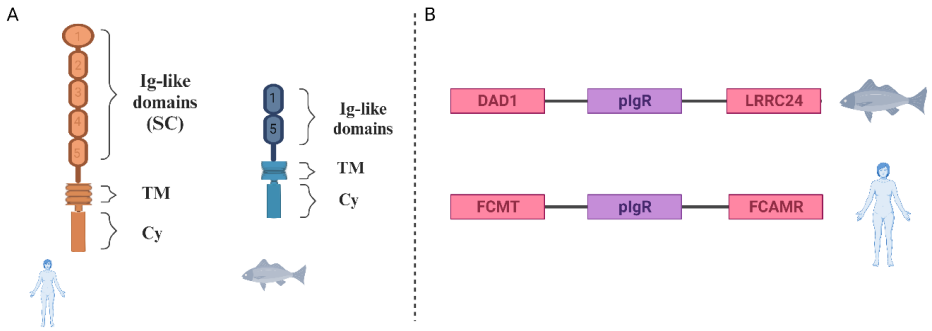


**Figure 4**. Comparison of the pIgR between human and teleost fish. **A)** The human pIgR protein structure exhibits 5 extracellular Ig-like domains compared to only 2 in teleosts. **B)** Lack of pIgR gene synteny between humans and teleosts. SC (secretory component), Cy (cytoplasmatic region), TM (transmembrane region), *DAD1* (Defender Against Cell Death 1), *LRRC24* (Leucine Rich Repeat Containing 24), *FCMT* (Farnesyl cysteine carboxyl methyltransferase), *FCMR* (Fc Alpha and Mu Receptor).

### *Implications of pIgR in maternally transferred IgM and IgT*

Maternal transfer of IgM and IgT to the offspring has been presented previously (section 1.6.3). In oviparous fish, maternal IgM is transferred to oocytes through the yolk to the mature ovaries, eggs, and yolk sac larvae (Swain and Nayak, 2009). In mammals, the transport of IgG through the placenta to the foetal site involves an Fc receptor- mediated transcytosis. Like higher vertebrates, teleost transport of IgM (and recently suggested for IgZ) to the offspring is also believed to occur by transcytosis across the follicle cells, although the mechanism behind the transport remains elusive. In this regard, the expression of the pIgR in ovaries and gonads during embryogenesis have been reported in zebrafish (Ji et al., 2021; Kortum et al., 2014), fugu (Hamuro et al., 2007), dojo loach (*Misgurnus anguillicaudatus*) (Yu et al., 2018), and orange-spotted grouper (Feng et al., 2009), suggesting its likely implication in maternal Ig transfer. Qin et al. (2019) investigated the expression of pIgR in developing embryos of both immunized and non-immunized turbot broodstock. Results showed an increasing expression of pIgR in the embryo over time, but parental immunization did

not influence the levels of pIgR expression. This did not elucidate the potential role of pIgR in the transport of maternal Ig, and direct evidence remains elusive.

### 1.6.5 Start-feeds and larval immunity

There is a noticeable lack of research on how various start-feeds (live prey) affect the immune system of larvae. This gap in knowledge is likely attributed to the prevalent underdevelopment of marine larvae upon hatching and the widespread adoption of using rotifers and *Artemia* in hatcheries. This is likely due to the prevalent underdevelopment of marine larvae upon hatching and the well-established use of start-feed diets, such as rotifers and *Artemia* in hatcheries. Consequently, attention has shifted towards investigating the use of immunostimulants, particularly β-glucans, to facilitate early activation of innate immunity in larvae (Martin and Król, 2017; Rojo-Cebreros et al., 2018). Concerning the overall development of larvae, it is reasonable to assume that the quality and nutritional composition of live feeds may significantly influence the evolving immune system, particularly the innate system, thereby contributing to the overall health of the fish. Therefore, it is important to explore the potential of start-feed diets in stimulating the onset of immune parameters to ensure health of farmed fish larvae.

### 1.6.6 Immunological studies in ballan wrasse

A basic characterization of the immune system in ballan wrasse has been made over the last years where some central components of the adaptive immune system were identified and characterized, and tools have been developed to analyse immune responses (Bilal et al., 2019; Bilal et al., 2016; Bilal et al., 2018). Several subsets of leukocytes were identified in the head kidney, spleen, and peripheral blood of ballan wrasse, resembling other teleosts (Haugland et al., 2014). Authors highlighted the "striking" abundant number of eosinophil granulocytes and suggested a likely important role in innate immune defence against microbes. Bilal et al (2016) developed IgM purification protocols for Norwegian wrasses. Authors identified the heavy chain of IgM with a molecular weight of 75 kDa and the light chain of 25-27 kDa which corresponds well with the molecular weight of IgM from other teleosts. A remarkable higher IgM concentration in wrasse serum compared to other bony fish species was

reported (IgM in ballan wrasse was about 36% of total serum protein compared to only about 3% of total serum protein in lumpfish) (Bilal et al., 2016). Later, central immune genes such as IgM, IgT, IgD, TCRs, and CD3ε were characterized. The TCRα of ballan wrasse presents somatic hypermutation (SHM) suggesting that this mechanism is involved in the diversification of TCR repertoire as well as in affinity maturation of antibodies (Bilal et al., 2018). Both TCRα and TCRδ are single copy genes in this species and correspond to the two main populations of T-cells, TCRα/β and TCRγ/δ respectively. The relative quantification of IgM, IgD, IgT, TCRα, TCRδ, and CD3ε transcripts was measured in various organs of ballan wrasse with special focus on the gut, as this site is an important site for immunological barriers against pathogens in the absence of stomach (Bilal et al., 2019). TCRδ and CD3ε were consistently present along the gut whereas TCRα was more abundant in the hindgut. Above all, the main finding was extraordinarily high levels of IgM mRNA within the gut, especially in the hindgut as well as abundant mucosal and intraepithelial $IgM^+$ cells in the same organ (Bilal et al., 2019). A working hypothesis is that this elevated levels of IgM within the gut might compensate for the lack of stomach in ballan wrasse as the stomach is the first line of defence in most gastric species.

Although certain immune parameters have been described in this species, the ontogeny of the immune system during larvae stages, where they are most vulnerable, has not yet been addressed.

# 2. Research Aims

The primary objective of the present study was to describe the development of the adaptive immune system in ballan wrasse and explore the potential impact of nutrition on its development. To this end, histological examinations were conducted on a series of larvae to describe the ontogeny of thymus, kidney and spleen, relevant T- and B-cell markers were analysed to investigate the timeline for the emergence of lymphocytes and antibodies (utilizing transcriptomic analyses, *in situ* hybridization, immunohistochemistry and proteomics), and the potential influence of an alternative start-feed diet on the ontogeny of adaptive immunity was evaluated.

**Secondary aims:**

- Analysis of the thymus development and appearance of zonation
- Analyses of kidney development
- Analyses of lymphocyte appearance and subsequent migration
- Analyses of maternal transfer of IgM to the offspring
- Characterization of pIgR as a potential marker for mucosal immunity
- Analyses of IgM in mucosal secretions and bile
- Assessment of the nutritional content of a barnacle nauplii start-diet.

In the course of the present study some exciting findings indicating extrarenal B-cell lymphopoiesis during larvae development and alternative transport routes of IgM to the gut lumen warranted further investigation.

# 3. Methodological Approach and Considerations

## 3.1 Studies on larvae (Paper I and II)

The ontogeny of thymus (**paper I**), kidney, and spleen (**paper II**) was histologically described in detail. The appearance of T- and B-cells during ontogeny was firstly indicated by analysing RNA transcriptomic data. Based on the up-regulation of selected transcripts (B- and T-cell markers), *in situ* hybridization was used to identify the sites of lymphocyte development and further migration to other organs during larvae development. Furthermore, a wrasse anti-IgM antibody was parallelly used to describe the presence of $IgM^+$ cells, IgM-secreting cells, and systemic IgM during development (**paper II**). Finally, the effect of an alternative start-feed diet was assessed based on transcriptomic data (**paper I and II**) and histological comparisons of the thymus (**paper I).**

### 3.1.1 Experimental design- start feed diets

Start-feed diets consisting on copepods and wild zooplankton had a superior effect on development of Atlantic cod and Atlantic halibut larvae when compared to rotifers and *Artemia* regimes (Sæle et al., 2017; Sæle et al., 2003). To see whether ballan wrasse larvae benefited from an alternative zooplankton start-feed diet, an experimental diet composed solely of barnacle nauplii was tested. The experimental set up is summarized in Figure 5A and thoroughly explained in **paper I**. Briefly, newly hatched larvae (4 days post hatching (dph)), approximately 30,000 to 34,000 individuals were transferred to six separate tanks, and two start-feed diets were provided in triplicates (n=3). The control diet consisted of rotifers and *Artemia* cultivated and enriched at the in-house facility at IMR in Austevoll, Bergen, Norway. The experimental diet composed of barnacle nauplii of two different sizes (small and large barnacle) was obtained from the Planktonic AS company (see **paper I** for details regarding the culturing conditions of live preys and the enrichment of rotifers and *Artemia*). Dry commercial feed was introduced approximately at the end of stage 4 in a co-feeding regime with live feed until the midpoint of stage 5. Afterward, only commercial feed was provided to all tanks (Fig. 5A). A total of six sampling points were established corresponding to the

six developmental stages of wrasse larvae, which were based on cranial ossification. At each sampling point, multiple larvae (3 to 15 larvae per pool) were collected from each tank for the different analyses. The nutritional content of both start-diets is available in **paper I**.

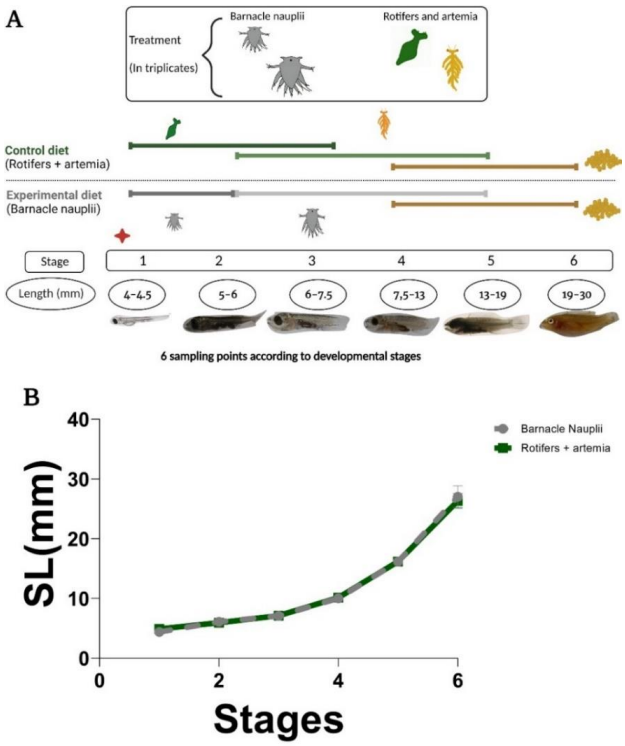


**Figure 5. A)** Experimental sampling based on wrasse larvae stages. The feeding regime is also illustrated. Asterisks indicates the initiation of feeding (4 dph). **B)** The dietary effect on growth performance. Adapted from **paper I.** SL; larvae standard length (mm).

### 3.1.2 Larvae sampling

In the present study, sampling was conducted by stages that were based on cranial ossification published elsewhere (Norland et al., 2022). When describing the development of organs and/or a specific system in fish larvae, it is crucial that a previous description of the larvae ontogeny is available for the species in question, but not any kind of ontogenetic description is valid. Many developmental studies in larvae are set up in a way that samples are taken exclusively based on larvae age (dpf or dph) or standard length (SL) which are considered rather poor parameters of development

(Norland et al., 2022; Sæle et al., 2010). Fish larvae might develop at different speed even when an identical environment is kept, for example, large size variation among larvae can be seen in the same tank. This accounts for huge differences among the same batch. Therefore, an (almost) invariable and robust parameter to characterize different developmental stages such as the sequence of cranial ossification of larvae should be used as successfully demonstrated for Atlantic halibut (Sæle and Pittman, 2010; Sæle et al., 2004), Atlantic cod (Sæle et al., 2017) and recently for ballan wrasse (Norland et al., 2022). Only when the correct description of stages (i.e. based on cranial ossification) exists, a correlation of the stages with fish size can be done to simplify practical applications (sampling) (Sæle and Pittman, 2010).

The growth data of larvae collected in this work (Fig. 5B) corroborates the correct execution of sampling (per stages). Furthermore, the transcription (mRNA) data of selected genes with a key role in larvae bone ossification (Olsvik et al., 2021) is shown in Figure 6. The general gradual up-regulation of bone formation genes in early life stages of ballan wrasse verifies the expected ossification process and validates our experimental sampling.

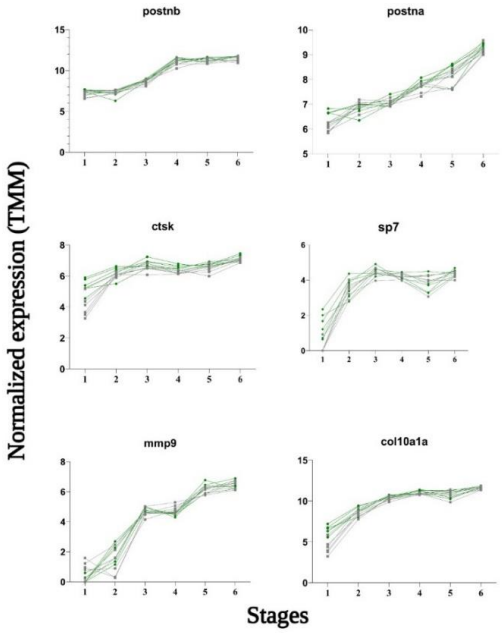


**Figure 6**. Transcriptomic data of genes related to bone ossification in ballan wrasse larvae (stages 1 to 6). Data correspond to transcription levels that were logarithm converted, normalized for differences in library size applying weighted trimmed mean expression ratios (trimmed mean of M values (TMM)), and presented as individual data points. Green corresponds to control diet (rotifers and *Artemia*), and grey represent the barnacle nauplii diet.

### 3.1.3 Transcriptomic analyses

Transcriptomic analyses, more specifically Illumina RNA-sequencing, was used in this thesis with two purposes. Firstly, investigate the appearance of genes related to the adaptive immune system during larvae ontogeny and secondly, investigate the effect of the experimental start-feed diet in the expression of the selected transcripts. For this purpose, genes that were markers of T-cells (**Paper I**) and B-cells (**Paper II**) were selected. Pools of whole larvae were taken at each developmental stage as thoroughly described in **Paper I** and used for RNA extraction and following RNA-seq analyses. Developing larvae undergo constant metabolic and physiological changes accompanied by significant changes in gene expression. A single gene can be part of various pathways being alternatively expressed among different tissues in the different developmental stages, regulating a wide variety of biological mechanisms. All of this

means that one must be very careful when drawing conclusions with whole-larvae transcriptomic data. For this reason and to describe adaptive immune parameters, it was crucial to include key genes that were specifically expressed in lymphocytes (**paper I and paper II).**

Real- time qPCR (RT-qPCR) analysis was not included in the presented developmental studies. Validation using qPCR was the gold standard for transcriptomic studies applying microarray technology, mainly due to the inherent bias following hybridization and use of probes which, however, is not a problem with Illumina RNA-sequencing. RNA-seq and qPCR analysis from the same samples have previously demonstrated a high degree of correlation (Coenye, 2021). Authors concluded that RNA-seq data is quite robust and not always precise qPCR data for validation. Lastly, although only selected genes related to adaptive immunity were thoroughly analysed in this study, a large amount of transcriptomic data is now available (accession number to the dataset: https://www.ncbi.nlm.nih.gov/,GSE200208). This data can be relevant for example, for future pathway analyses studies describing innate immune parameters or investigating the potential widespread impact of the alternative start-diet.

### 3.1.4 *In situ* hybridization

Based on transcriptomic data, *in situ* hybridization was used to localize lymphocytes in the last two developmental stages (stage 5 and 6) of larvae ontogeny (**paper I and II**). The novel RNAscope® technology was applied to whole larvae. RNAscope® *in situ* hybridization is a novel, highly specific and sensitive method that successfully detect small amounts of mRNA compared to conventional *in situ* methods (Wang et al., 2012a). It has been used in several studies in Atlantic salmon targeting low-expressed mRNAs in paraffin-embedded tissues with satisfactory results as exemplified in Løken et al. (2020). The RNAscope® workflow for a duplex chromogenic assay (Fig. 7) allows for detection of two different targets that are independently amplified and differentially detected by two chromogenic enzymes (HRP- and AP- labelled).

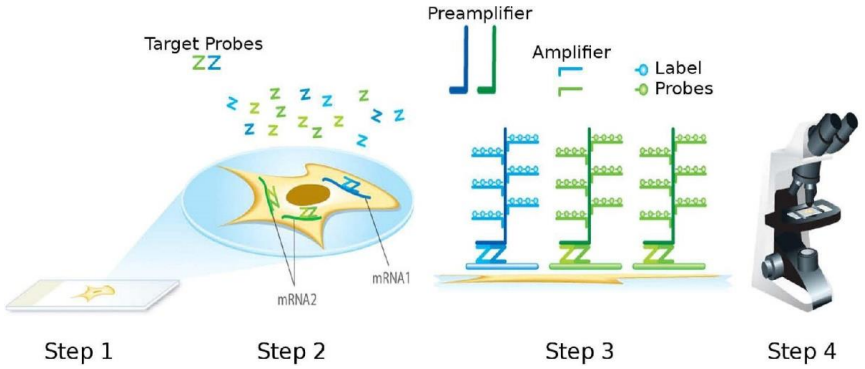


**Figure 7**. Overview of the RNAscope® procedure illustrating a duplex chromogenic assay (two targeted mRNAs). From (Wang et al., 2012a).

RNAscope® probes have a novel design called a **double-Z design** (ZZ) (Wang et al., 2012a). A single RNAscope® probe consists of a series of target probes that hybridize with the target RNA molecule. A RNAscope® probe consists of 20 ZZ pairs, each ZZ spans roughly 50 base pairs (bp) which makes up for a total probe length of roughly 1000 base pairs. Each of the target probes "ZZ" (50 bp) is designed to hybridize contiguously to a target region (part of the RNA molecule), and only when both "ZZ" have specifically bound to the target for hybridization, an optimal site for binding the pre-amplifier is created. The pre-amplifier contains 20 binding sites for the amplifier and the amplifier contains 20 binding sites for the label molecule achieving a great exponential amplification of the signal as schematically shown in Figure 7. An off-target RNA hybridization where both "Z" binds un-specifically to a putative RNA molecule is very unlikely. Simultaneously, if there is only one "Z" (18-25 bp) binding non-specifically to a random molecule of RNA, there will not be amplification or "the preamplifier will not bind with sufficient strength to result in successful signal amplification" (Wang et al., 2012a). Therefore, this technique is quite specific compared to traditional methods. In this way, anti-sense controls are not necessary or irrelevant and the negative control is substituted for a negative probe detecting a bacteria gene (DapB).

## 3.2 Studies on eggs- Maternal transfer of IgM (Paper II)

To investigate maternal transfer of IgM to the offspring, newly spawned eggs were placed in 3 incubators as thoroughly explained in **paper II**. Eggs were taken at 0.5, 3, 6, and 9 days post fertilization (dpf) (Fig. 8), and snap-frozen for further protein extraction and Western blot analyses using our anti-IgM antibody. Unfortunately, eggs from one of the incubators were unfertilized decreasing the biological replicates to only 2 (n=2).

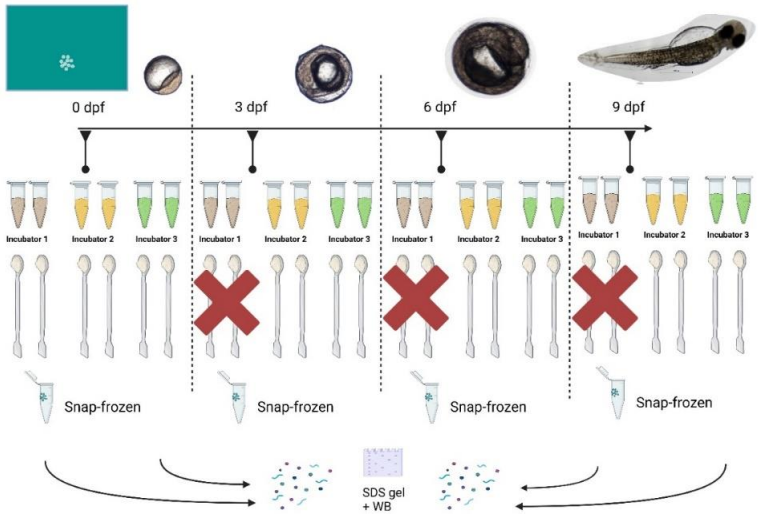


**Figure 8.** Experimental set-up of egg collection. Samples were taken at 0.5, 3, 6, and 9 dpf. Unfertilized eggs from a specific incubator were identified at 3dpf (illustrated with an "x"), and as a result, duplicate samples (n=2) were taken consequently.

## 3.3 Investigation of poly-IgR in ballan wrasse (paper III)

The poly-Ig receptor was characterized in ballan wrasse adults using several approaches. Firstly, molecular cloning, sequencing, and sequence analyses (gene synteny, exon/intron organization, protein domains and glycosylation predictions) of pIgR cDNA together with alignments with other teleosts and higher vertebrates was done. RT-qPCR and RNAscope® *in situ* hybridization were performed to measure tissue-specific expression and localization of pIgR in several organs (gut, gills, hindgut, liver, and head kidney). In an attempt to identify the secretory compound (SC) of the

pIgR (small peptide of approx. 30 KDa), untargeted Liquid chromatography-mass spectrometry (LC-MS/MS) of mucus, bile and serum of ballan wrasse was performed. To concentrate the amount of IgM and try to remove as many other proteins as possible from mucus, bile, and plasma, a "gentle" (one-step) purification of IgM by immunomagnetic precipitation was conducted as followed explained.

### 3.3.1 One-step purification of IgM by immunomagnetic separation

Staphylococcal protein A (prot-A) coated Dynabeads (Invitrogen™10002D) referred hereby as prot-A beads were used to purify IgM. The "one-step purification" refers to the fact that Prot-A beads do not need to be covered by IgG (anti-wrasse IgM in this case) to get a successful purification of wrasse IgM. Instead, wrasse IgM can directly bind to protein-A that is coating the beads, allowing for a faster extraction method of IgM. This one-step purification method (graphically shown in Fig. 9) has proven highly effective in extracting relatively pure IgM from Norwegian wrasses whereas it is not applicable to salmonid species or Atlantic cod (Bilal et al., 2016). By using dynabeads coated with streptavidin instead of prot-A as a control, IgM did not bind to the beads, strongly indicating that IgM binds to prot-A with certain affinity. In the present work, prot-A beads were used for one-step IgM purification from serum, bile, and mucus from gut, gills, and skin. This method was also used to extract putative $IgM^+$ leukocytes from blood (PBL), presumably eliminating RBC contamination from the leukocyte fraction (see section 2.6, 2.10, and 2.13 in **paper III** for detailed description of the methods).

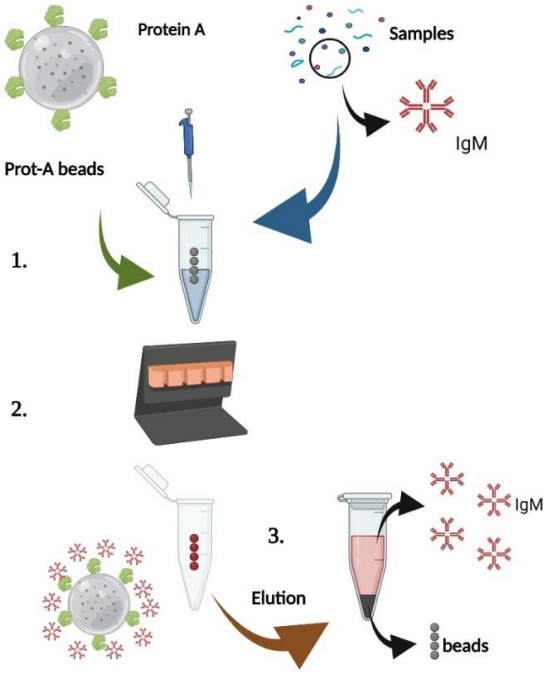


**Figure 9**. One-step purification of IgM workflow performed in this thesis work. Different samples (serum, mucus, bile, or leukocytes) were incubated with prot-A coated Dynabeads at 4 °C for 1 h **(1)**. After binding, the supernatant was removed with the help of a magnet **(2)**, beads were washed several times and prot-A beads "rich" in IgM were eluted with 1 x SDS sample buffer **(3)**. The supernatant contained purified IgM.

The secretory component (SC) of pIgR theoretically binds covalently to secreted IgM and remains bound to IgM after transport to mucosal sites. Consequently, when IgM transport is mediated by the pIgR, the SC peptide is expected to be found in mucosal secretions. A high-throughput proteomics approach, liquid chromatography-mass spectrometry (LC-MS/MS), was used to identify the SC. For this, a fraction of bile, serum, and mucus extracted from gills, skin, and intestine underwent a "gentle" one-step IgM purification. Subsequently, Western blot analyses were performed to verify the presence of IgM, followed by LC-MS/MS. The remaining fraction of mucus samples was directly subjected to LC-MS/MS without prior purification of IgM. Protein lysates extracted from organs of wrasse (liver, gills, and spleen) were subjected to LC-MS/MS without prior IgM purification, serving as a semi-control since the

detectability of pIgR protein was expected. A summary of all the samples, pre-treatments, and subsequent proteomic analyses are illustrated in (supplementary material 1in **paper III).**

### 3.3.2 Liquid chromatography-mass spectrometry (LC-MS/MS)

Untargeted LC-MS/MS was used in this work to identify the SC of pIgR, the pIgR-Like, and immunoglobulins (IgM, IgT, and IgD) in different wrasse samples. Untargeted mass-spectrometry does not target any specific protein but rather provides a complete protein profile of a given sample. The untargeted MS approach, for instance, is effectively employed to identify and quantify fraudulent ingredients in the animal feed industry and in many other situations where targeted assays are not available (Belghit et al., 2021; Varunjikar et al., 2022). Comparisons of samples that were “rich” in IgM (prot-A purified samples) with samples without IgM purification were useful for relative comparisons.

# 4. Results and Discussion

There is a limited number of studies on the immune system of ballan wrasse. Up to date, key genes that are markers for T- and B-cells (*TCRα, TCRδ, CD3ε, IgM, IgD,* and *IgT*) have been characterized showing that both α/β and γ/δ T-cells, and all teleost Ig isotypes are present (Bilal et al., 2019; Bilal et al., 2016; Bilal et al., 2018). However, the ontogeny of the adaptive immune system in this species has been largely unexplored. The present thesis reveals the timing and localization of B- and T-cell lymphopoiesis as well as the early migration of lymphocytes in wrasse larvae (**Paper I** and **Paper II**). The effect of an alternative start-feed diet on the ontogeny of the adaptive system was also assessed (**Paper I** and **Paper II**). During molecular characterization of a potential marker for measuring mucosal immune responses, the pIgR, unexpected results pointed towards alternative transport routes of IgM to the gut lumen (**Paper III**). A summary of the main developmental findings of adaptive immunity in ballan wrasse performed during this PhD work is illustrated in Figure 10.

In the present work, the developmental sequence of the lymphoid organs (thymus, kidney, and spleen) was histologically analysed during wrasse larvae ontogeny as graphically summarized in (Fig. 10).

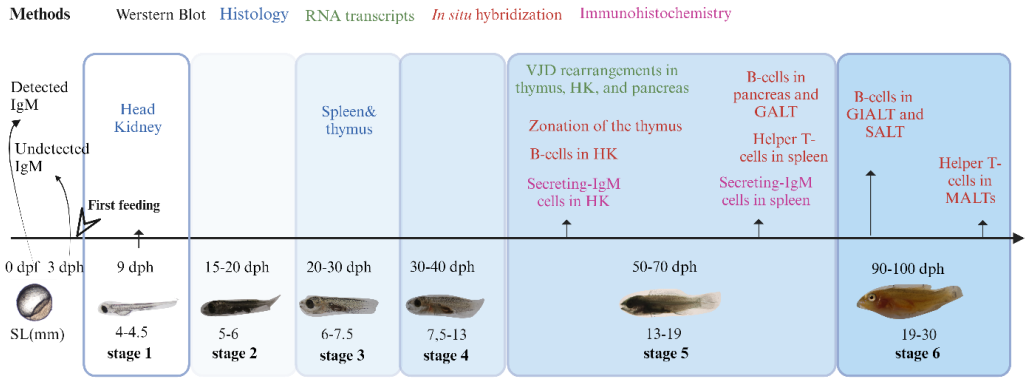


**Figure 10.** Schematic timeline illustrates key developmental milestones in the adaptive immunity of ballan wrasse larvae. The methods employed are distinguished by different colours. Initial feeding started at 4 dph. Weaning started at the end of stage 4 through a co-feeding regimen involving either *Artemia* or barnacle. By stage 5, all larvae were exclusively provided with dry commercial food.

The kidney was already present at hatching. Later, the thymus was first observed during larval stage 3 (20-30 dph), exhibiting similarities to thymus development in other marine fish species. Notably, there was a noticeable delay compared to freshwater species. (Fig. 11) (Barraza et al., 2020; Bowden et al., 2005).

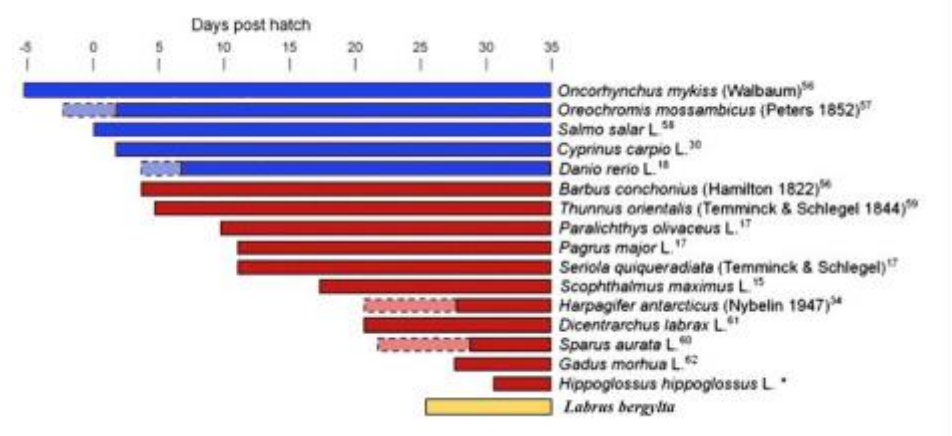


**Figure 11.** Diagrammatic representation illustrating the timeline for thymus development in fresh water (blue) and sea water (red) species, and ballan wrasse (yellow). Dashed regions denote variable time intervals. Adapted from (Bowden et al., 2005).

The order of appearance of spleen and thymus in fish seems to vary among species (Bjørgen and Koppang, 2021; Patel et al., 2009). In the present work, the primordial spleen was observed at the same stage as the thymus anlage (stage 3) coinciding with the time when the gut starts rotating and acquire the characteristic intestinal loop of this agastric species (Norland et al., 2022). Although important anatomical processes seem to happen in wrasse larvae during stage 3, it is not until stage 5 (50-60 dph) when the primary lymphoid organs (thymus and kidney) became lymphoid (**Paper I and Paper II**). The transcription factor *ikaros* (*IKZF1*) and the recombination-activating genes (*RAG1* and *RAG2*) are essential for early B- and T-cell development in mammals and fish (Kirstetter et al., 2002; Willett et al., 2001). However, *Ikaros* is not exclusively expressed in lymphoid organs as it was found to be expressed in zebrafish brain (Trede et al., 2001). Therefore, *RAG* genes serve as key markers for investigating lymphocyte development in the thymus and kidney of teleost fish (Danilova and Steiner, 2002; Huttenhuis et al., 2006b; Lam et al., 2002; Rombout et al., 2005; Wang et al., 2014).

The V (D) J region of both Ig and TCR have specific regions for binding RAG enzymes that are called recombination signal sequences (RSS). RAG1 is capable of independently recognizing and binding to RSS, whereas RAG2 only binds to DNA when RAG1 is present. RAG1 and RAG2 are typically co-regulated and co-expressed in the same cells and tissues and rarely appear independently of each other (Carlson et al., 1991; Greenhalgh et al., 1993). In fish, few studies have measured the expression of both *RAG1* and *RAG2* genes. For example, in grass carp, *RAG1* appeared earlier in thymus compared to *RAG2* (4 and 7 dpf respectively) whereas in zebrafish larvae, *RAG1* was expressed in both thymus and kidney while *RAG2* was mainly expressed in the thymus (Huttenhuis et al., 2005; Willett et al., 1997). Furthermore, RAG1 shows a higher degree of conservation across species from elasmobranchs to mammals (Carlson et al., 1991; Hansen and Kaattari, 1996; Willett et al., 1997). As *RAG1* showed a higher number of transcripts compared to *RAG2* in developing wrasse larvae (**Paper I**), *RAG1* was regarded as a better candidate for further localization of maturing lymphocytes. In the present work, the transcriptomic upregulation of *RAG* genes from larval stage 5 strongly indicated the start-point of recombination processes leading to a wide repertoire of TCR in T-cells and Ig-receptors in B-cells. Transcripts of other T-cell (*CD3, ZAP70,* and *TCR*) (**Paper I**), and B-cell markers (*IgM, IgT, IgD, CD79*, *CD40*) (**Paper II**) supported these findings.

T-cell maturation occurs in the thymus of higher vertebrates through a maturation gradient from the cortex (outer region) to the medulla (inner region) (Owen et al., 2013). Numerous studies support that T-cell lymphopoiesis within the thymus of teleost fish is to some degree evolutionary conserved with that in higher vertebrates (Fischer et al., 2005; Grace and Manning, 1980; Koppang et al., 2003; Picchietti et al., 2015; Picchietti et al., 2009; Willett et al., 1997). However, teleosts are diverse and certain contradictions and uncertainties, for example regarding the thymic structure and zonation, are still present (Barraza et al., 2020; Bowden et al., 2005). In the current study, histological analyses revealed a notably dense cortex and a relatively less dense medulla in the thymus of wrasse larvae from stage 5 and onwards (**Paper I**). This density difference was attributed to a lower thymocyte presence in the medulla compared to the cortex, mirroring observations in seabass, rainbow trout, and Atlantic

halibut (Picchietti et al., 2009; Picchietti et al., 2008; Toda et al., 2011; Øvergård et al., 2011). Moreover, a distinct regionalization of cortex and medulla regions determined through *in situ* hybridization for *RAG1* was evident (**Paper I**). Targeting RAG1 for investigating thymus zonation has been a commonly employed tool in other fish species, showing similar results to those presented in this study (Barraza et al., 2020; Huttenhuis et al., 2005). Furthermore, this cortico-medullary demarcation of the wrasse thymus was also evident when investigating the abundance of putative helper (CD4-1$^+$) T- and cytotoxic (CD8β$^+$) T-cells (**Paper I**).

Interestingly, simultaneous to the strong *RAG* expression in the thymus at stage 5, positive cells appeared in the head kidney (**Paper I**). This coincided with the presence of numerous *Igμ*$^+$ (IgM heavy chain (μ)) and IgM$^+$ cells within the organ demonstrating recombination of B-cell receptors in kidney at stage 5 (**Paper II**). Altogether, histological examinations corroborate the previously mentioned transcriptomic findings, indicating that B- and T-cell lymphopoiesis in ballan wrasse start at the same stage during larvae ontogeny. This contrasts with earlier observations in carp, where *RAG1* was noted in thymus before its detection in kidney (Huttenhuis et al., 2005). Similarly, lymphocytes were observed in the thymus of rainbow trout, carp and zebrafish before kidney (Zapata et al., 2006), but kidney was the first immune organ becoming lymphoid in Rice-Field Eel (*Monopterus albus*) (Liu et al., 2022), and in rock bream (*Oplegnathus fasciatus*) (Xiao et al., 2013). These different observations might indicate species-specificity, or it might be attributed to the use of different techniques making comparisons among fish species challenging. It is important to highlight that the upregulation of *RAG1* mRNA occurred significantly earlier when larvae were fed a barnacle diet (**Paper I**). Considering the pivotal role of *RAG1* in initiating lymphocyte development, the barnacle diet presents an intriguing aspect, as discussed later.

***RAG* expression in the pancreas**

During this work, unexpected *RAG1*$^+$ *CD3ε*$^-$ cells were observed in pancreatic tissue of the earliest investigated wrasse larvae from stage 5 and onwards. Additionally, there was a notable presence of *Igμ*$^+$ cells in pancreatic tissue, strongly indicating BCR

recombination (**Paper II**). Furthermore, mRNA of the examined T-cell markers was not detected in pancreatic tissue at stage 5, while scattered helper T-cells were evident in the spleen during the same stage (**Paper II** and **Fig. 12**). These findings support the conclusion that our results depict pancreatic B-cell and not T-cell lymphopoiesis. Unfortunately, the presence of T-cells in pancreatic tissue was not examined in later developmental stages.

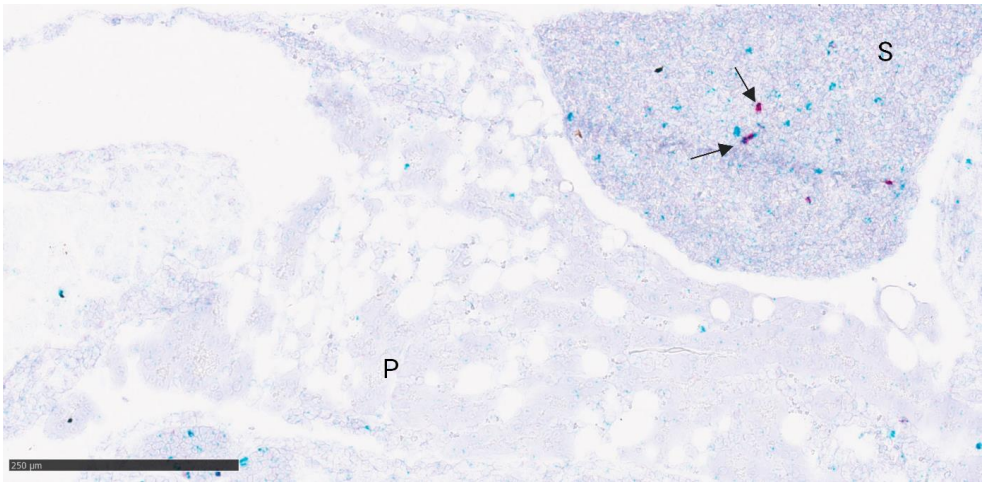


**Figure 12.** *In situ* hybridization targeting helper T-cells in larvae at stage 5. *In situ* hybridization targeting *CD3ε* (red) and *CD4-1* (blue) positive cells. Co-staining of *CD3ε* and *CD4-1* shows few putative helper T-cells in the spleen (arrows) but were not identified in pancreatic tissue. P (pancreas), and S (spleen).

The expression of *RAG1* in pancreas is not exclusive of wrasse as it was reported in zebrafish larvae over 20 years ago where it was concluded that B-cells also develop in the pancreas (Danilova and Steiner, 2002). Several other studies in zebrafish have explored the expression of *RAG* during larvae ontogeny but these studies did not include pancreatic tissue in their analysis (Jessen et al., 1999; Lam et al., 2004; Trede et al., 2001; Willett et al., 1997). For instance, Willett et al. (1997) exclusively examined *RAG* expression in cross sections of heads, omitting the body of larvae where the pancreas is located. Consequently, it is incorrect to interpret these papers as evidence for the lack of *RAG* expression in zebrafish pancreas. Besides zebrafish, *RAG* expression was reported to be absent in pancreas of adult Japanese pufferfish (*Fugu rubripes*) by RT-PCR (Peixoto et al., 2000), and low *RAG2* but no *RAG1* expression

was observed (also by RT-qPCR) in the hepatopancreas of carp larvae (Huttenhuis et al., 2005). In mice, B-cell progenitors are found in the foetal liver in the early stages and restricted to the bone marrow in adult mice (Hardy and Hayakawa, 2001). Similarly, B-cell development in zebrafish was detected in pancreas by 4 dpf, although it seems to be confined to the kidney in adult fish (Danilova and Steiner, 2002; Langenau and Zon, 2005). Based on this, B-cell lymphopoiesis in pancreatic tissue of ballan wrasse larvae may be different to that in adult individuals. Nevertheless, the presented results together with a recent report of Rice-Field Eel where *RAG1* and *RAG2* were expressed in liver (Liu et al., 2022), implies that B-cell development might occur at different sites among teleost fishes. Whether pancreatic B-lymphopoiesis is restricted to larvae stages or also occurs in adult wrasse remains unexplored.

B-cells are commonly found in lymphoid tissues associated with the gut and other immune organs in fish. In the present work, the head kidney showed the greatest concentration of $Ig\mu^+$ cells throughout larval development, followed by spleen and pancreatic tissue (Fig. 13 and **Paper II**), demonstrating its pivotal function as a primary lymphoid organ. Even though a quantification of $Ig\mu^+$ cells across these organs was not performed, it was striking that a substantial population of $Ig\mu^+$ cells was present within pancreatic tissue. Scientific literature reporting the presence of B-cells in pancreas of teleost fish is scarce. To the best of our knowledge, there is only one study reporting the presence of $IgT^+$ and $IgM^+$ B-cells in the pancreas of a healthy adult gastric fish, and their occurrence was noted to be at a very low level (Bakke et al., 2020).

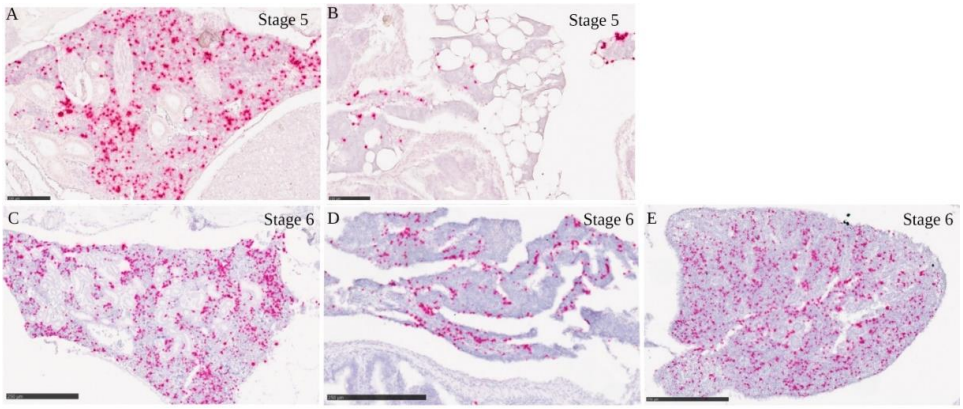


**Figure 13.** *In situ* hybridization showing *Igμ* (red) expression in larvae at stage 5 (A-B) and stage 6 (C-E). **A)** Head kidney. **B)** Pancreatic tissue. **C)** Head kidney. **D)** Pancreatic tissue. **E)** Spleen. Scale bars: A;100 µm, B; 100 µm, C; 250 µm, D;250 µm, E; 250 µm.

The exocrine wrasse pancreas is initially visible as a compact organ in newly hatched larvae, and then it diffuses along the intestine within the abdominal cavity and liver (Norland et al., 2022). This transition reveals a distinct morphology commonly found in fish without stomach (Al-Hussaini, 1949; Gagnat, 2012). Overall, this work demonstrates the recruitment of abundant $Ig\mu^+$ and $IgM^+$ B-cells to the exocrine pancreas soon after the organ starts to expand along the gut (**Paper II**), suggesting that the population of pancreatic B-cells is relevant at maintaining immune homeostasis in the peritoneal cavity. Moreover, the fraction of $IgM^+$ B-cells presumed to be locally developed within pancreatic tissue might enhance protection within the peritoneal cavity. Lastly, abundant IgM staining was found in pancreatic tissue within the liver around what seems to be blood capillaries and pancreatic ducts (**Paper II**), as discussed below.

All in all, there is indeed a clear need to explore the immune function of the pancreas in different teleost species at different developmental stages as previously stated in Huttenhuis et al. (2005).

**Unspecific *RAG* signal**

During the *in situ* hybridization work, weak and scattered *RAG1* and *Igμ* expression was found in the gut epithelium of wrasse larvae to a much lesser extent than in

lymphoid organs (Fig. 14 and Fig. 15). Positive signal did not correspond to leukocytes nor to another cell type. Importantly, similar positive signal was found in non-lymphoid tissue such as brain and eye in the same sections (Fig. 14 and Fig. 15).

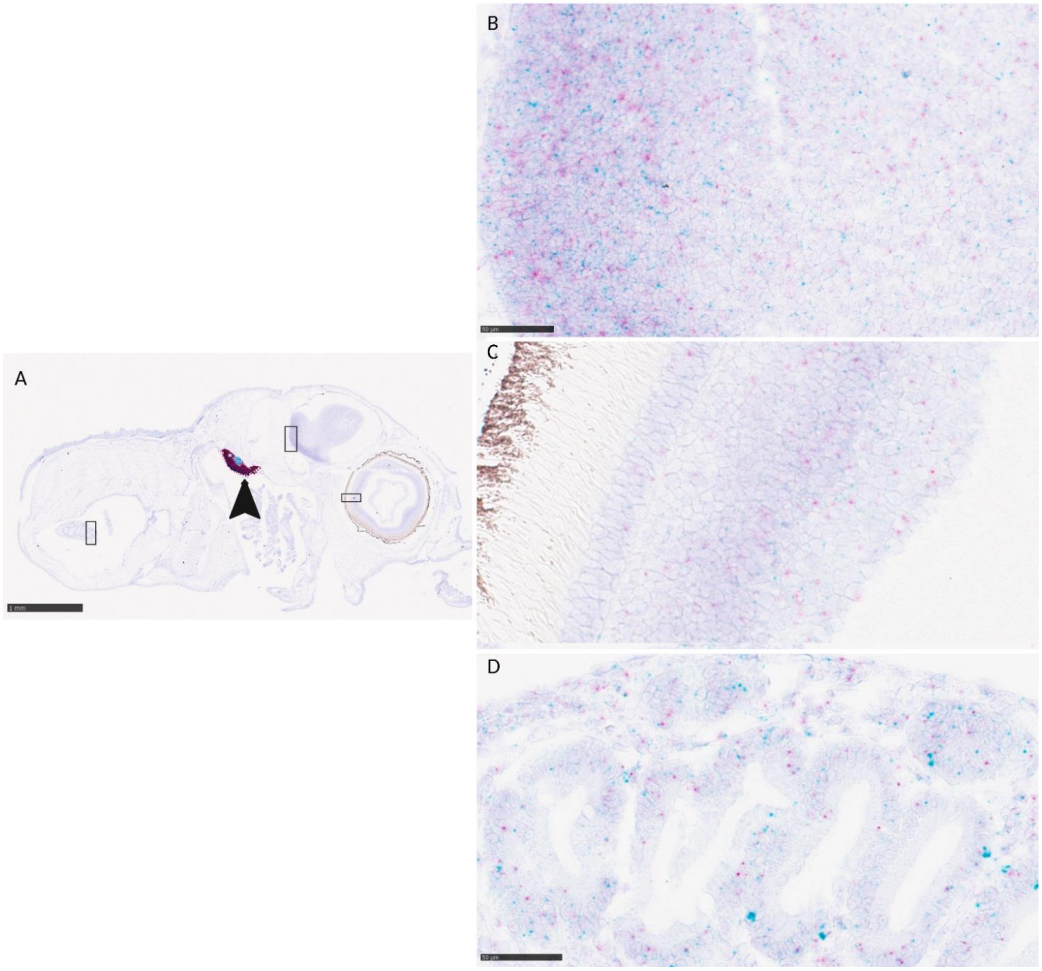


**Figure 14**. RNAscope *in situ* hybridization on ballan wrasse larvae at early substage 5 (SL: 1.6 cm) depicting *RAG1* (red) and *cd3ε* (blue) signal. **A)** Overview of whole larvae. Black arrow indicates the thymus and areas investigated at higher magnification are squared. **B)** Weak signal can be observed in the brain and in the eye (C**). D)** *RAG1*$^{+}$ (red) and *cd3ε*$^{+}$ (blue) cells in the gut. Scale bars: A;1mm, B; 100 µm, C; 100 µm, D;100 µm.

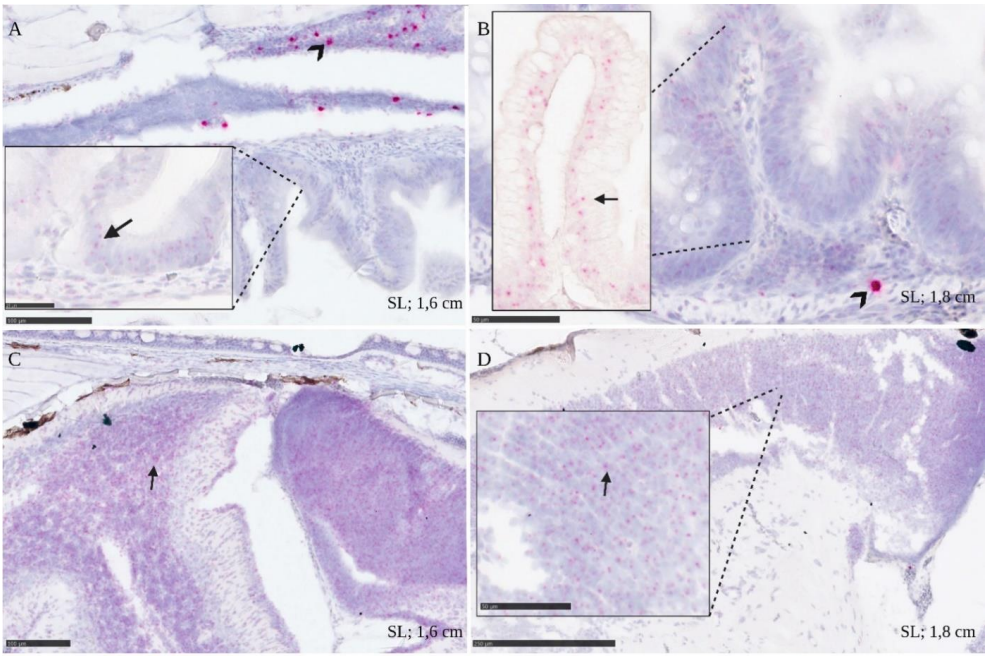


**Figure 15**. RNAscope *in situ* hybridization showing *Igμ* positive signals in ballan wrasse larvae at stage 5; divided in early substage 5 (larvae with standard length (SL) of 1,6 cm) and late substage 5 (larvae with SL of 1,8 cm). Sections in **A** and **B** correspond to the hind gut whereas sections in **C**, and **D**, correspond to brain tissue. **A)** Head kidney and pancreatic tissue showing $Ig\mu^+$ cells (head arrow) and weaker positive signal within the epithelium (black arrow). **B)** Hindgut epithelium with positive *Igμ* signal (black arrow) and a clear $Ig\mu^+$ cell in the lamina propria (head arrow). **C)** and **D)** correspond to brain tissue where black arrows indicate *Igμ* positive signal. Scale bars: A;100 µm, B; 50 µm, C; 100 µm, D;250 µm.

Research in mice found **immature** B-cells that were $RAG^+$ $Ig\mu^+$ in the spleen and intestinal lamina propria (Notarangelo et al., 2001; Schlissel, 2013). Based on this, the low level of both *RAG1* and *Igμ* expression detected within the gut epithelium in wrasse larvae could possibly be attributed to immature B-cells. However, a thorough examination of *Igμ* expression in both the gut epithelium and brain has not been conducted. Although it seems unlikely, the possibility that this less pronounced positive signal might correspond to immature B-cells cannot be entirely ruled out.

Literature support the extrathymic T-cell maturation in the gut epithelium of humans and mice (Guy-Grand et al., 2003; Lundqvist et al., 1995; Rocha et al., 1994). In accordance with this, putative $RAG1^+$ T-cells were found in the gut of adult European sea bass (Picchietti et al., 2011) and in the gut of carp at 1week post fertilization (wpf)

(by *in situ* hybridization and RT-qPCR) (Huttenhuis et al., 2006b). Scapigliati et al. (2018) suggested the gut of adult fish as a lymphoid tissue that likely retained primordial lymphopoietic function throughout evolution. In the present work, the weak *RAG1* signal identified in the gut epithelium of wrasse larvae at stage 5 decreased considerably in stage 6, and it was almost undetectable in the gut of juvenile fish. Furthermore, explorative analyses of the intestinal transcriptome from adult ballan wrasse available in Lie et al. (2018), indicated an absence of *RAG* transcripts within the gut of adult individuals. All in all, these findings suggest lack of *RAG1* expression in mucosal organs of ballan wrasse, further substantiating the lack of evidence for extrathymic development of T-cells in this species. An overview of the *in situ* hybridization findings, depicting the presence or absence of positive signal in different organs of ballan wrasse larvae with all probes employed in this work, is provided in (Table 1). Note that positive signal has been divided into putative lymphocytes ($IgM^+$ B-cells and T-cells) and signal resembling acellular characteristics.

**Table 1**. Overview of positive signals detected via *in situ* hybridization (single and double assays) in various tissues of ballan wrasse larvae (stage 6) and in the liver of adult fish. The presence of mRNA expression is denoted by (+) when identified using the provided probes. Tissues that were analysed, but no expression was observed are represented by (-). Tissues left empty indicate that they were not examined in this study.

| **PROBE** | **Igμ** | | **RAG1** | | **CD3ε and CD4-1** | **CD3ε and CD8β** | **pIgR** |
|---|---|---|---|---|---|---|---|
| **Signal type** | IgM expressing cells | Non-cellular signal | Developing lymphocytes | Non-cellular signal | Helper T-cells | Cytotoxic T-cells | |
| Thymus | + | | + | - | + | + | |
| Head kidney | + | - | + | - | + | + | |
| Trunk kidney | + | - | | | | | |
| Spleen | + | - | - | | + | - | |
| Pancreas | + | - | + | - | - | - | |
| Gut | + | + | - | + | + | - | |
| Skin | + | - | - | + | | - | |
| Gills | + | - | - | + | + | - | |
| Liver | - | - | - | - | - | - | |
| Heart | + | - | - | - | - | - | |
| Brain | - | + | - | + | - | - | |
| Eye | - | - | - | + | - | - | |
| Muscle | - | - | - | - | - | - | |
| Adult liver | + | - | | | | | - |

RNAscope is a novel sensitive technique with good results at identifying immune cells as shown in Atlantic salmon (Bakke et al., 2020; Løken et al., 2020) and in the present work, in ballan wrasse. However, the available gene map of wrasse is inferior compared to that of salmon implying that RNAscope cannot have the same level of security when designing probes for ballan wrasse and thus, the possibility of cross-hybridization is somewhat higher in wrasse. Although T- and B-cells have been successfully and trustily recognised both in lymphoid organs and mucosal tissues during this work, the presented non-cellular *RAG1* and *Igμ* positive signal within the gut and brain might reflect cross-hybridization. Further gene expression analyses, for example, RT-qPCR in the brain of perfused wrasse measuring *RAG1* and *Igμ* might elucidate this matter.

**Mucosal immunity and transport of IgM to the gut in developing larvae**

Recently, substantial advances have been made in lymphoid tissue of teleost mucosal barriers (MALTs) with the description of lymphocytic aggregates forming lymphoid structures that were previously overlooked; the ILT and the ALT within gills (Dalum, 2017; Dalum et al., 2021; Haugarvoll et al., 2008; Koppang et al., 2010), the analogue of the avian bursa in Atlantic salmon (Løken et al., 2020), the organized nasopharynx-associated lymphoid tissue (O-NALT) in rainbow trout (Garcia et al., 2022), and the newly described lymphoid organ within the sub-pharyngeal region of zebrafish, salmon, and crucian carp (*Carassius carassius*) (Resseguier et al., 2023). Furthermore, the presence of an interconnected mucosal system was reported for the first time in a teleost fish, the channel catfish (*Ictalurus punctatus*) (Lange et al., 2019). Nonetheless, our understanding of the ontogeny and sequential migration of lymphocytes to various MALTs in fish remains quite limited and has been explored in a very small number of species.

As previously stated, investigations into novel lymphoid structures or organs have primarily focused on model species, with no such studies conducted on ballan wrasse. One of the aims of the present work was to elucidate the timing and sequence of lymphocyte migration to the classical MALTs during ballan wrasse development. This is important to establish the earliest optimal timing for efficient vaccination. Furthermore, it was especially intriguing to analyse lymphocyte colonization of the wrasse gut mucosa as it presents extraordinarily high concentrations of IgM in adulthood (Bilal et al., 2019). Detailed histological or anatomical studies of the lymphoid-associated gut mucosa in ballan wrasse was not performed during this work, and thus, this structure remains as a putative GALT.

B-cell colonization of teleosts' MALTs seem to happen significantly later than B-lymphopoiesis within the kidney and subsequent migration to the spleen, indicating that the development of adaptive immunity in mucosal sites lags behind compared to systemic organs (Parra et al., 2016; Salinas et al., 2011). For example, plasma cells of spotted wolfish (*Anarhichas minor Olafsen*) and $Ig^+$ cells of common carp, seabass and mandarin fish (*Siniperca chuatsi)* appeared in the gut later than in spleen and kidney

(Grøntvedt and Espelid, 2003; Picchietti et al., 1997; Romano et al., 1997; Tian et al., 2009). In this work, the first observation of *Igμ* positive B-cells in ballan wrasse MALT was shortly after B-cell lymphopoiesis started in the early stage 5, and it was restricted to the GALT, especially to the hindgut**.** Furthermore, the identification of $Ig\mu^+$ cells within the GALT was simultaneous to their first observation in the classical secondary lymphoid organ, spleen, although less abundant **(Paper II**). Skin and gills were seeded with $IgM^+$ cells later than the gut, similar to observations in spotted wolfish (Grøntvedt and Espelid, 2003). Importantly, abundant IgM-secreting cells were present in HK of larvae already at the early stage 5, before they were identified in mucosal sites indicating the presence of systemic IgM prior to mucosal IgM (**Paper II**) as reported in other teleosts (Romano et al., 1997). B-cells populate the GALT later than T-cells in zebrafish, carp, spotted wolffish, and seabass (Dos Santos et al., 2000; Grøntvedt and Espelid, 2003; Huttenhuis et al., 2006b; Picchietti et al., 1997; Rombout et al., 2005). Distinctively, wrasse helper T-cells ($CD4\text{-}1^+$ $CD3\varepsilon^+$) were observed in the gut and other mucosal organs (gills, skin, and pharynx) later than B-cells whereas cytotoxic ($CD8\beta^+$ $CD3\varepsilon^+$) T-cells were not found at any mucosal site during larvae ontogeny (**Paper I**). The RNAscope *in situ* hybridization method does not allow for precise quantitative comparisons between T- and B-cells due to inherent limitations; working in a two-dimensional projection introduces the possibility of missing cells, and the hybridization between probes lacks calibration for accurate quantitative measurement. *Igμ* transcripts within B-cells, especially in IgM-secreting cells, are expected to be expressed in large quantities, probably much higher than T-cell receptors in T-cells, potentially accounting for certain bias in the observations. The T-cell receptor CD8 is expressed either as a CD8αα homodimer or CD8αβ heterodimer. CD8αβ is specific for T-cells whereas cd8αα is a receptor in several other innate-like cells such as mammalian dendritic cells, NK cells, monocytes and macrophages (Chang et al., 2005; Gibbings et al., 2007; Hirji et al., 1997), as well as in innate-like cells in rainbow trout (Garcia et al., 2023). Thus, only *CD8β* and not *CD8α* was targeted in the present work.

In summary, systemic IgM is detected in ballan wrasse larvae before its presence is observed in mucosal sites. Cytotoxic T-cells were not identified at any developmental

stage, and a significant number of *Igμ*$^{+}$ B-cells migrated to GALT, particularly to the hindgut, preceding the arrival of T-cells in developing wrasse larvae.

The driving migration force of B- and T-cells to mucosal sites during fish larvae ontogeny remains unexplored. In mammals, mucosal lymphoid organs develop in the presence of lymphoid tissue inducers (LTi) and stromal cells (Honda et al., 2001). Lti are innate lymphoid cells expressing chemokine receptors such as CXCR5 and CCR7 and so forth, as well as important transcription factors such as RORγ and ID2 (Randall and Mebius, 2014). Lti also express lymphotoxin (LT) which is important for enhancing expression of homeostatic chemokines and cytokines that attract populations of DCs and B-cells to developing mucosal lymphoid sites. Lymphotoxin is essential for development of Peyer's patches within the mammalian gut and it remains expressed in B-cells even after lymphoid tissue is formed, regulating cytokine communication and maintaining the structure of lymphoid organs (Randall and Mebius, 2014). *LT-β* was identified in rainbow trout but teleost fish lack *LT-α* (Kono et al., 2006; Lane et al., 2012). Although the role of LT in teleosts has not been investigated, fish possess a functional ID2 that is highly conserved to its mammalian counterpart which is involved in lymphoid tissue development (Fukuyama et al., 2002). A recent investigation in rainbow trout suggest that NALT ontogeny might be somewhat similar to the process in mice, although direct evidence is needed (Garcia et al., 2023). This could also be the case for other mucosal lymphoid organs in teleosts. In the latter study on the ontogeny of NALT, authors observed epithelial cells with low expression of *MHCII*, and reticular cells with high expression of *MHCII* that could represent populations of putative DCs, macrophages, and putative B-cells (Garcia et al., 2023). Although further direct evidence is needed, the study conducted by Garcia et al. (2023) is important as it reveals cells and mechanisms that could be involved in the ontogeny of lymphoid tissue in a teleost fish for the first time. Much work remains to be done to elucidate developing mechanisms of teleost MALTs and whether different MALTs have unique characteristics during ontogeny, as shown in mammals (Randall and Mebius, 2014).

Nevertheless, although the ontogeny of wrasse MALTs was not the focus in the present work, few indications regarding the colonization of GALT can be drawn. $IgM^+$ B-cells migrate to GALT earlier than adaptive T-cells and might therefore be activated in a helper T-independent manner (**Paper I** and **Paper II**). It is plausible that early $IgM^+$ B-cells homing to GALT respond to cytokines released by epithelial cells or other innate-like leukocytes, but this has not been addressed. Teleost B-cells have a strong phagocytic and antimicrobial activity presenting similarities to mammalian B-1 cells which produce natural antibodies without prior exposure to a specific antigen (Stosik et al., 2023). Natural antibodies are well-known to be present during embryonic and early development in mammals (Magnadóttir, 2006). In fish, they are found in serum and provide a quick initial protection against bacteria and virus in a non-specific manner as shown for Atlantic cod, rainbow trout, goldfish (*Carassius auratus* L.), and others (Magnadóttir, 2006; Uribe et al., 2011). The putative lack of helper T-cells in mucosal organs during larval stage 5 strongly suggest that the first population of $IgM^+$ cells observed within GALT have the profile of "innate-like" cells producing natural IgM or/and showing antimicrobial activity. Later in wrasse larval development, helper T-cells migrate to GALT (as observed during stage 6 and juveniles), likely triggering more antigen-specific responses (adaptive responses). Furthermore, it is expected that innate immune cells patrol lymphoid tissues in larvae prior to the migration of lymphocytes. For example, innate-like $CD8\alpha^+$ cells appeared quite early during ontogeny of NALT in rainbow trout followed by helper T-cells and $IgM^+$ B-cells (Garcia et al., 2023). This could be the case for ballan wrasse too.

In mice, B1-cells responsible for natural IgM production are predominantly located in the peritoneal cavity (Palma et al., 2018). In this study, the initial migration of "innate-like" $IgM^+$ B-cells to the peritoneal cavity, including GALT and pancreatic tissue, is shortly after the introduction of dry pellets to the feeding regime (stage 5). It is plausible that these $IgM^+$ cells producing natural IgM play a crucial role in maintaining homeostasis within the peritoneal cavity of this agastric species, serving as a defensive barrier. This mechanism may act as a compensatory response for the absence of stomach, as previously suggested in adult wrasse (Bilal et al., 2019). The current findings indicate the presence of this mechanism already from larval stages. Whether

lymphocytes within pancreatic tissue are also involved in maintaining immune homeostasis in wrasse adult fish remains elusive. Furthermore, compared to the gut, other MALTs like skin and gills might have a limited protective role in early larvae ontogeny. It is important to note that the overall role of $IgT^+$ and $IgD^+$ B-cells in MALTs remains unexplored in ballan wrasse.

Fish larvae with developed MALTs are more likely to avoid infection. Immunoglobulins can be found in the gut, gill, and skin mucus of various teleost fish, although their levels differ from those in higher vertebrates (Bilal et al., 2021). In mammals, and proposed in several teleost fish, the poly-Ig receptor (pIgR) is responsible for the active transfer of pIgs through the epithelium towards mucosal sites. As previously mentioned, analyses of the pIgR expression were planned as a suitable marker for the appearance of mucosal immunity during wrasse larvae ontogeny. This work would complement findings regarding B-cell migration to MALTs, specially GALT, and the start-point of secreted-IgM transport towards the gut lumen in this fish species. Furthermore, the relative high IgM production within the intestine of adult wrasse made this species a potential good model to better understand the working mechanisms of the pIgR among teleost fish. The pIgR gene was not annotated in the ballan wrasse genome. Consequently, we conducted an extensive characterization of the receptor, including mRNA expression analyses in various organs of adult fish and protein assessments (**Paper III**).

In mammals and other higher vertebrates, the joining chain (J-chain) together with the presence of disulfide bridges are crucial to hold together the monomers of polymeric Igs allowing for pIgR binding and transport across epithelium (Braathen et al., 2007; Rombout et al., 2014). The gene for the J-chain (*IGJ* gene) is absent in teleosts (Kaetzel, 2014) but polymers of both IgT and IgM are still formed and transported to mucosal secretions. It is worth mentioning as evidence of the great variation among vertebrates that some cartilaginous fish have both J-chain and pentameric IgM. Also, the African lungfish (*Protopterus dolloi*) which belongs to the ancient class of lobe-finned fish (Sarcopterygii) lacks IgM but present the *IGJ* gene (Tacchi et al., 2013), and amphibians have a J-chain which does not associate with IgX (Mußmann et al.,

1996). Kaetzel (2014) suggested that J-chain might not be needed for binding and transport of sIgs in teleosts. Structural studies have uncovered the precise binding of human pIgR to sIgA (Fallgreen-Gebauer et al., 1993). However, no experimental evidence is available for the binding mechanisms of sIgM and sIgT to pIgR in fish. A proposed model for IgT transport suggested that co-polymerization of tetrameric IgT with 2 molecules of pIgR might keep IgT subunits together (without disulfide bonds) facilitating the transport of IgT to mucosal sites (Kaetzel, 2014). However, this has not been experimentally proven.

Rombout et al. (2014) reviewed the characterization and expression of teleost pIgR in a total of 8 species. Since then, the pIgR of at least 8 new teleost species has been characterized (Ametrano et al., 2022; Ji et al., 2023; Pei et al., 2019; Qin et al., 2019; Wang et al., 2017; Yang et al., 2017; Yang et al., 2021; Yu et al., 2018). As studies on teleost pIgR emerged, much of the research consisted in the mere (and needed) characterization of the *pIgR* gene and mRNA expression analyses in different organs. The *pIgR* gene is mostly expressed in epithelial cells of different mammalian tissues such as gut, respiratory tract, kidney, human thymus, mammary glands, biliary epithelial cells, as well as in hepatocytes within the liver, but not in lymphoid cells (Brown and Kloppel, 1989b; Kaetzel, 2005; Nihei et al., 1996; Tomasi and Yurchak, 1972). The expression of *pIgR* mRNA in lymphoid organs and mucosal surfaces of several teleosts and higher vertebrates (determined by RT-qPCR) is summarized in (**Table 2**), and it depicts the high variation in the expression pattern of *pIgR* among fish species compared to higher vertebrates.

**Table 2.** Differential expression of *pIgR* and *pIgRL* using RT-qPCR in different tissues of teleosts. The highest level of expression described by authors is expressed by (+++), followed by (++). The lowest expression is indicated by (+). Tissues that were analysed but no expression was observed are represented by (-). Empty means tissues were not analysed in the study. Note that the level of expression is qualitative and therefore, not comparable between species. Table updated from **Paper III.**

| Teleost specie | Author | Lymphoid organs | | | Mucosal organs | | | | Other tissues | | | | Leuk | Method |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **pIgR** | | **Head kidney** | **Thymus** | **Spleen** | **Skin** | **Foregut** | **Hindgut** | **Gill** | **Liver** | **Muscle** | **Stomach** | **Gonads** | | |
| **Ballan wrasse** | | + | | ++ | + | + | + | ++ | +++ | + | | | - | RT-qPCR |
| Japanese flounder | (Xu et al., 2013a) | ++ | | +++ | ++ | +++ | | ++ | +++ | + | + | | | RT-PCR |
| Common carp | (Rombout et al., 2008) | +++ | +++ | +++ | + | + | +++ | + | +++ | - | | | - | RT-PCR |
| Orange-spotted grouper | (Feng et al., 2009) | + | + | + | ++ | + | +++ | + | + | ++ | ++ | - | | RT-PCR |
| Fugu | (Hamuro et al., 2007) | + | +++ | + | +++ | +++ | | +++ | ++ | - | | + | - | RT-PCR |
| Grass carp | (Pei et al., 2019) | + | | ++ | ++ | +++ | | + | + | + | | | | RT-qPCR |
| Zebrafish | (Kortum et al., 2014) | + | | ++ | +++ | +++ | | + | +++ | | | + | | RT-PCR |
| Atlantic salmon | (Tadiso et al., 2011) | + | | +++ | ++ | | ++ | ++ | + | | | | | RT-qPCR |
| Dojo loach | (Yu et al., 2018) | | | ++ | +++ | + | | ++ | +++ | ++ | | + | | RT-qPCR |
| Crucian carp | (Wang et al., 2017) | ++ | | ++ | + | ++ | | + | +++ | + | | | - | RT-qPCR |
| Antarctic teleost | (Ametrano et al., 2022) | + | | | | + | ++ | +++ | + | - | | | | |
| Mandarin fish | (Ji et al., 2023) | + | | +++ | | + | | + | +++ | + | | | | |
| Nile tilapia | (Liu et al., 2019a) | + | + | ++ | + | + | | + | +++ | + | | | | RT-qPCR |
| Sea bass | (Yang et al., 2017) | + | | + | + | ++ | | + | +++ | + | + | | | RT-qPCR |
| Chinese turtle | (Xu et al., 2021) | | | ++ | | +++ | ++ | | ++ | ++ | + | | | RTq-PCR |
| Human* | | | | | | +++ | +++ | | + | | ++ | | | Transcriptome |
| Bovine | (Verbeet et al., 1995) | | | | | Present | Present | | Present | | | | | Northern-blot |
| Chicken | (Wieland et al., 2004) | | Present | | | Present | | | Present | | | | | Northern-blot |
| **pIgRL** | | | | | | | | | | | | | | |
| **Ballan wrasse** | | +++ | | +++ | + | ++ | + | +++ | + | + | | | +*** | RT-qPCR |
| Zebrafish | (Kortum et al., 2014) | | | + | + | + | | + | + | | | + | +** | RT-PCR |
| Japanese flounder | (Liu et al., 2019b) | + | | ++ | +++ | + | ++ | +++ | + | + | + | | +*** | RT-qPCR |
| Atlantic salmon | (Tadiso et al., 2011) | ++ | | ++ | + | | ++ | +++ | + | | | | | RT-qPCR |

* **https:**//www.proteinatlas.org/ENSG00000162896-PIGR/tissue

*pIgRL transcripts (pIgRL1 - pIgRL4) were differentially expressed in myeloid and lymphoid cell linages.

** peripheal blood leukocytes

Regarding the existence of a secretory component (SC) in teleost pIgR (pIgR-SC), it is worth mentioning that the main approaches have been immune co-precipitation of putative SC with IgM and IgT, and immune localization of SC using antibodies raised against Igs and recombinant pIgR (rpIgR). The first reported SC in a teleost was in fugu where authors claimed that the SC had a molecular weight of 60 KDa (Hamuro et al., 2007). The later use of molecular weight calculations using online tools revealed that most teleost SC should be around 30 KDa (Rombout et al., 2014). Thus, although the molecular weight of SC can slightly vary from theory to practice, the fugu's SC (60 KDa) seem to be out of range and authors stated that their results might be the product of post-translational modifications (Rombout et al., 2014). In rainbow trout, both IgM and IgT in gill, skin, and gut mucus co-immunoprecipitated with rpIgR whereas serum Igs did not attach to the SC (Xu et al., 2013b; Xu et al., 2016b; Zhang et al., 2010). Similarly, Xu et al. (2013a) observed co-immunoprecipitation of the rpIgR with skin and mucus IgM but not with serum IgM in Japanese flounder. Later, a 800 KDa protein from bile, skin, gill, and gut mucus, co-immunoprecipitated with anti-rpIgR and anti-IgM antibodies indicating the association of pIgR with pIgs in mucosal secretions (Sheng et al., 2019). Localization (by fluorescence immunostaining) of pIgR-SC was performed in trout skin and gill (Xu et al., 2013b; Xu et al., 2016b), and in the hindgut of Japanese flounder (Sheng et al., 2018), being mostly present in epithelial cells suggesting its implications in transcytosis. Several studies on transfected cells that constitutively express pIgR have also shown co-staining of pIgR-SC with IgM and IgT. For example, this has been seen in seabass where (human) transfected cells were used (Yang et al., 2017), in Nile tilapia (*Oreochromis niloticus*) (Liu et al., 2019a), and in cell lines of mandarin fish (Ji et al., 2023).

In challenge experiments involving pathogens, an upregulated expression of *pIgR* together with an increase in IgM concentration in mucosal tissues and bile were observed in dojo loach, crucian carp, Atlantic salmon, and Japanese flounder, suggesting the involvement of pIgR in immune responses following infection (Sheng et al., 2019; Tadiso et al., 2011; Wang et al., 2017; Yu et al., 2018). In sea bass, pIgR could interact with *Escherichia coli*, *Aeromonas hydrophila*, *Staphylococcus aureus*, and *Bacillus subtilis*, in accordance with the reported binding ability of mammalian

pIgR to both Gram-negative and Gram-positive bacteria (Yang et al., 2017). In the same manner, a recombinant pIgR in largemouth bass (*Micropterus salmoides*) did bind both IgT and bacteria suggesting a direct role of pIgR to prevent infection (Yang et al., 2021).

One can observe that there is considerable literature pointing towards the functional homology of teleost pIgR and higher vertebrates. Nevertheless, there is still uncertainty surrounding the primary question of whether teleosts strictly require the pIgR for transepithelial secretion of sIg. A recent follow-up study in Japanese flounder using transfected (mammalian) MDCK cells that constitutively expressed pIgR, demonstrated transepithelial secretion of mucosal IgM by pIgR and the presence of a IgM-SC complex of 800 KDa that was positive stained with both anti-IgM and anti-rpIgR antibodies after transcytosis (Sheng et al., 2022). Authors also found that their recombinant antibody against pIgR recognized a 37 kDa protein, the putative SC, in both skin mucus and in the supernatant of MDCK-pIgR cells implying secretion of free SC as in higher vertebrates. In the same study, authors monitored IgM and pIgR in the hindgut of Japanese flounder (using fluorescence staining) following immunization with inactivated vibrio (Sheng et al., 2022). This experimental set-up allowed for semi-quantitative analyses where the level of expression and co-localization of IgM and pIgR (within the epithelium) increased post-vaccination supporting transepithelial transport of IgM-pIgR complexes. Altogether, these studies based on binding of Ig to recombinant pIgR proteins suggest functional homology of fish pIgR with that in higher vertebrates and indicate that teleost pIgR is involved in immune responses during bacterial challenges. However, key aspects regarding transport mechanisms of teleost pIgs remain elusive.

Studies in teleosts (common carp, zebrafish, Japanese flounder, and Atlantic salmon) identified regions in the genome that are similar to pIgR and have been designated as “Poly Immunoglobulin Receptor-Like” (pIgRL) containing 2 ILDs (Kortum et al., 2014; Liu et al., 2019b; Tadiso et al., 2011; Zhang et al., 2015). In zebrafish, researchers found a single gene that encodes *pIgR* on chromosome 2, and a substantial group of 29 *pIgRL* genes, most of which were located in close proximity to the *pIgR*

gene (Kortum et al., 2014). Knowledge on the functional role(s) of pIgRL is limited, but authors observed that some pIgRL proteins bound to phospholipids and not immunoglobulins, indicating that pIgRL is functionally different from pIgR and might be a result of gene differentiation. The expression pattern of *pIgRL* in zebrafish was found to be different from that of *pIgR*. *pIgRL* transcripts were detected in blood leukocytes, whereas *pIgR* expression was confined to mucosal organs. This is consistent with the pIgR role in Ig transport across mucosal epithelium. In Atlantic salmon, a *pIgRL* gene (SalsalpIgRL) was highly expressed in lymphoid organs such as spleen and head kidney (Tadiso et al., 2011). Challenge experiments with bacteria in zebrafish and Japanese flounder showed an increase of *pIgRL* expression (Kortum et al., 2014; Liu et al., 2019b). Accordingly, the abundance of *pIgRL* mRNA increased in Atlantic salmon after exposure to salmon lice (Øvergård et al., 2023). Interestingly, zebrafish pIgRL seem to play a role during bacterial challenge but not in viral infections (Kortum et al., 2014). Nevertheless, these studies as well as the *pIgRL* mRNA expression patterns in ballan wrasse (**Paper III**), suggest that pIgRL is somehow involved in mucosal immunity. However, studies on *pIgRL* genes in teleosts are very limited and further identification and characterization of these genes in other fish species will help to clarify their functionality and implications in immune responses.

Unexpectedly, the present PhD work indicated that transport of IgM was not mediated by pIgR in ballan wrasse, and a potential pIgR-like gene was neither an alternative functional pIgR homolog, as thoroughly discussed in (**Paper III).** The expression patterns of the *pIgR* gene among fish species seem to vary more than in higher vertebrates (Table 2). For example, a subpopulation of leukocytes in gut mucosa of carp was suggested to express *pIgR* (Rombout et al., 2008) whereas it is strictly restricted to epithelial cells or hepatocytes in higher vertebrates. Similarly, the gut of ballan wrasse showed a relatively low expression of *pIgR* mRNA and a rather weak signal was observed in the gut mucosa but not within the epithelium (**Paper III).** For comparisons, *in situ* hybridization was also performed in mucosal tissues of Atlantic salmon showing a similar pattern in the location of *pIgR* mRNA (**Paper III**). In addition, lymphoid organs of salmon, such as head kidney and spleen were investigated and showed *pIgR*-positive cells. It is worth noting that while a positive *pIgR* signal was

detected in salmon gut mucosa, there was also some degree of detection in intestinal epithelial cells, highlighting once again the significant variations among fish species. Although suggested by some authors, the discussion regarding an additional alternative role of pIgR in teleosts has been toned down. It is reasonable to assume that teleost pIgR can bind to pIgs but this does not imply that pIgR is a constitutive effector for Igs transport to mucosal sites in all teleost fish. This leaves room for potential alternative routes of Ig transport.

In short, results from **Paper III** did not enlighten whether pIgR is involved in the transport of sIgM to the gut lumen in wrasse. Instead, results pointed towards an alternative transport of mucosal IgM to the gut lumen together with bile, the hepato-biliary route. Equally interesting, results from **Paper II** suggested the existence of a second alternative route for IgM towards the gut lumen of ballan wrasse larvae, hereby referred to as the pancreatic route.

Bile is produced in the liver and transported to gallbladder before it is released in the anterior gut. Bile does not only aid digestion of fats, but it is highly involved in transport of Igs to the intestine (Reynoso-Paz et al., 1999). Some vertebrates, such as **rat, rabbit, and chicken**, have an active hepatic or hepato-biliary transport of pIgA (Brown and Kloppel, 1989a; Kühn and Kraehenbuhl, 1981). The mechanism of this transport is believed to be as follows; Polymeric IgA is produced by plasma cells in the intestinal mucosa and respiratory tract, migrates to blood and is transported to the liver. Once in the liver, transcytosis of pIgA from blood to bile through hepatocytes is mediated by pIgR. This results in pIgA release into bile in complex with the SC of pIgR (Brown and Kloppel, 1989a). In contrast, other species including **sheep, guinea pig, dog and humans** present a much less active hepato-biliary transport of circulatory IgA (Scicchitano et al., 1984). For example, in humans, most of pIgA found in the gut lumen is actively transported through epithelial cells by pIgR. The different transport routes among species is likely driven by the location of pIgR as well as the location and number of plasma cells producing IgA. In those species with high hepato-biliary pIgA transport (**rat**), few local plasma cells are observed in the liver and the pIgR-SC is associated with hepatocytes. Therefore, the main source of pIgA in bile derives from

blood (systemic transport). On the other hand, those species with a less-effective hepato-biliary transport of Igs (**humans**), present abundant plasma cells within the liver mostly in close contact with biliary ducts. In this case, the pIgR-SC is expressed in biliary epithelial cells (BECs) but not in hepatocytes (Brown and Kloppel, 1989a). Thus, in humans, pIgA locally produced by plasma cells within the liver dominates the bile. However, the majority of pIgA present in the gut lumen is transported from the gut mucosa across enterocytes.

The biliary system has its evolutionary origin in fish (Akiyoshi and Inoue, 2004). Interestingly, authors suggested that variations in the biliary tract structure among vertebrates is due to dietary habits and might have adapted the hepatic function differently across vertebrates. The presence of Igs in bile has only been reported in few teleost species. The Antarctic teleost *(Trenlatomus bernacchii)* contains tetrameric IgM in bile together with a low number of IgM positive cells throughout the anterior intestine, strongly suggesting the hepato-biliary transport of IgM in this fish (Abelli et al., 2005; Coscia and Oreste, 2000). A later study revealed the presence of *pIgR* transcripts (by ISH) mainly in hepatocytes suggesting its implication in transcytosis of IgM across hepatocytes (Ametrano et al., 2022). Similarly, the presumed lack of plasma cells within the liver of carp together with relatively abundant IgM in bile, indicated that the hepato-biliary route is the main source of mucosal IgM in carp intestine (Rombout et al., 2008; Rombout et al., 1993). This hepato-biliary transport suggested in carp and Antarctic teleost might be like in higher vertebrates where IgM binds to pIgR in hepatocytes and/or BEC, travels across by transcytosis and is discharged into bile ducts in complex with the pIgR-SC (Brown and Kloppel, 1989b; Reynoso-Paz et al., 1999). However, direct evidence of this hepatic transport as well as the formation of SC-pIgs complexes in teleosts liver is still missing.

In this work, the liver showed the highest mRNA expression of *pIgR* in ballan wrasse adults, but *in situ* hybridization failed at localizing transcripts in hepatocytes or BECs, making results difficult to interpretate (**Paper III**). Liver has repeatedly been reported to have a higher expression of *pIgR* mRNA compared to intestine and other MALTs in several teleost fish (Table 2), which suggests the hepato-biliary transport route as a

common mechanism for Igs transport to the gut in at least some teleost species (Abelli et al., 2005; Rombout et al., 2008). Yet, most literature do not address or suggest this matter.

The presence of IgM in bile and the putative lack (or very low presence) of *pIgR* in mucosal organs of ballan wrasse (**Paper III)** pointed towards an alternative transport of IgM similar to the hepato-biliary route. Equally interesting, abundant leukocyte infiltration together with $Ig\mu^+$ and $IgM^+$ cells was observed within the exocrine pancreatic tissue of ballan wrasse. IgM positive cells were abundant in pancreatic tissue embedded in the liver of adult ballan wrasse (Fig. 16A-C, unpublished). Pancreatic tissue within the liver is closely associated with blood vessels. Fig 16A shows the presence of clear clusters of acini characteristic of exocrine pancreas, strongly indicating the presence of pancreatic ducts (Fig.16A). In liver, abundant $Ig\mu^+$ and $IgM^+$ cells were notably present in embedded exocrine pancreas, likely located in close proximity to pancreatic ducts (Fig. 16B and C). Additionally, developing larvae showed abundant $Ig\mu^+$ cells within pancreatic tissue in the peritoneal cavity (**Paper II**) and embedded in the liver, but none in the liver parenchyma (Fig. 16D). Altogether, these results suggest a feasible direct release of IgM from pancreatic-associated B-cells towards the lumen of pancreatic ducts that might be ultimately released into the gut lumen.

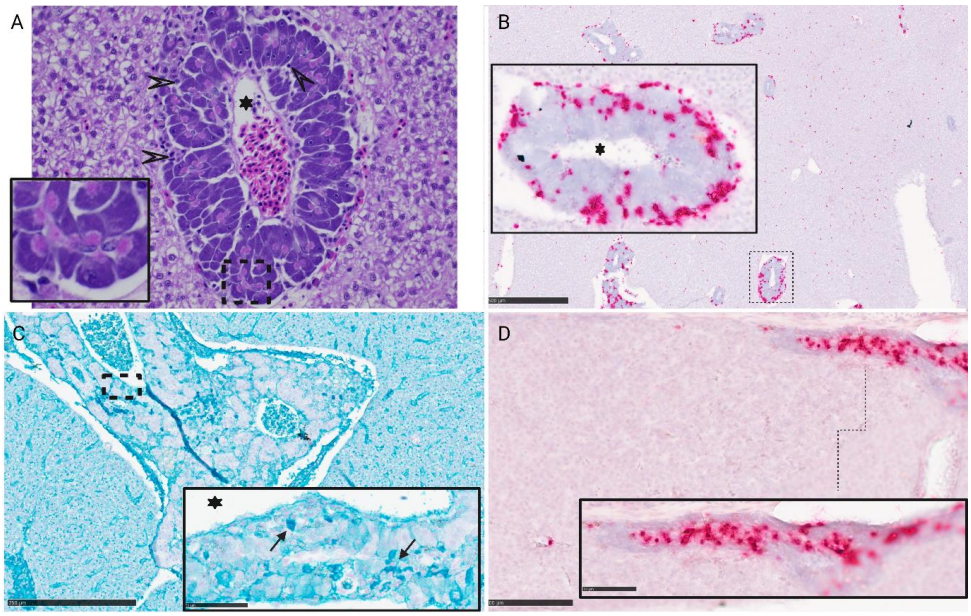


**Figure 16.** Pancreatic tissue embedded in the liver of ballan wrasse and in close association with blood vessels. **A**) Histological section of liver of an adult ballan wrasse showing pancreatic tissue with infiltrated leukocytes (indicated with head arrows). The insert is a higher magnification showing structures resembling acinus of exocrine pancreas. **B)** *In situ* hybridization performed in the same liver as in A, showing scattered *Igμ* positive cells in the liver and notably, abundant *Igμ*$^{+}$ cells within pancreatic tissue. **C)** Immunohistochemistry using anti-wrasse IgM performed in the same liver as in A, showing IgM positive cells within pancreatic tissues. The insert is a higher magnification. Arrows point to putative plasma cells infiltrated in pancreatic tissue. **D)** *In situ* hybridization in the liver of larvae (stage 5) showing *Igμ* positive cells restricted to pancreatic tissue within the liver. Asterisk indicates a blood vessel. Scale bars: B;500 µm, C; 250 µm, D;100 µm.

Enzymes that are crucial for digestion in fish lacking stomach primarily originate from pancreatic secretions, with the pancreas assuming a more significant role than in gastric fish (Lie et al., 2018). Furthermore, Norland et al. (2022) suggested that the proximity of pancreatic tissue and intestine in this species might facilitate the transport of signalling molecules such as hormones that regulate release of digestive enzymes from the exocrine pancreas to the gut lumen. A similar communication between the gut mucosa and pancreatic tissue in the peritoneal cavity likely mediated by cytokines, could also regulate immune activity. Ballan wrasse might have adopted this pancreatic route for IgM secretion as a compensatory mechanism for the lack of stomach. The two proposed alternative transport routes of IgM to the gut in ballan wrasse have been

illustrated in Figure 17 where it is plausible that this transport happens in the lack of pIgR.

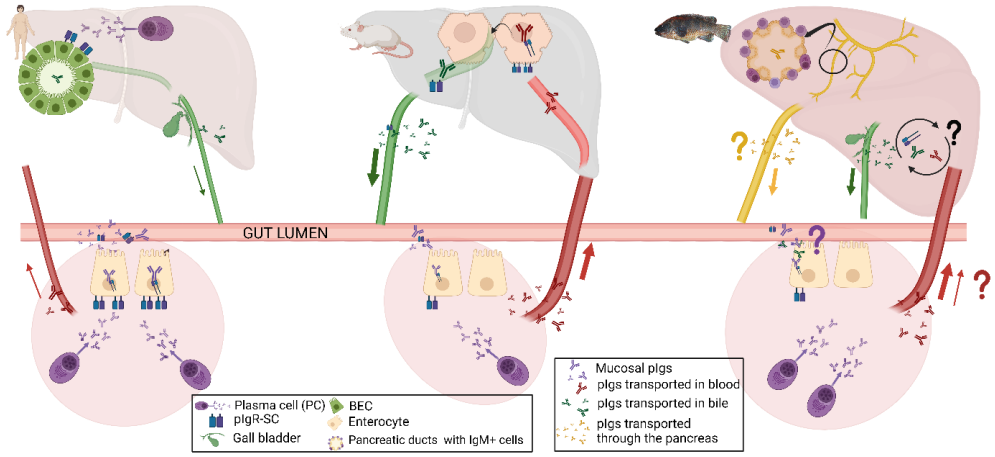


**Figure 17.** Illustration comparing IgM transport routes to the gut lumen in rats, humans, and proposed routes in ballan wrasse. In humans, a higher proportion of pIgA is transported through enterocytes to the gut lumen compared to mice. Simultaneously, plasma cells (PCs) in the human liver serve as the primary source of IgA, that is transported through biliary epithelial cells (BECs) via the pIgR-SC, eventually reaching the bile and gut (depicted by green arrows). While this transport pathway is significantly more prominent in mice, mice lack substantial PCs in the liver. Instead, mucosal IgM, secreted by PCs in the gut mucosa, travels through the bloodstream (illustrated by red arrows) to the liver and then to the bile before being secreted into the gut lumen, a process known as **hepato-biliary route**. This transport is suggested to be present in ballan wrasse. In mice, pIgR facilitates transport of pIgA from the bloodstream through hepatocytes to be discharged into bile. However, this mechanism may be different in ballan wrasse. Additionally, a second alternative route is proposed for this species, the **pancreatic route** (depicted in yellow). Here, $IgM^+$ cells in the exocrine pancreas may release IgM towards the lumen of pancreatic ducts, which might subsequently be secreted into the anterior gut. The thickness of the arrows and the abundance of pIgs symbolize the significance of each respective route. Additional investigation is required to further characterize the suggested routes, as well as to determine whether IgM is transported through the gut in ballan wrasse. This need for clarification is represented by the inclusion of question marks in the illustration.

While the pancreatic transport of IgM is here suggested for the first time in a vertebrate species, Ametrano et al. (2022) suggested that the hepato-biliary route is the oldest phylogenetically route for mucosal Ig transport toward the intestinal lumen. Thus, it is not surprising that mechanisms behind the hepato-biliary route in teleosts are somehow different to that in higher vertebrates. In the same manner, differences might also be

present among teleost species. For example, in the Antarctic teleost, where a similar hepato-biliary transport to that in rats have been suggested, plasma B-cells were observed in the liver (Abelli et al., 2005) whereas no plasma cells have been reported in rats' liver (Brown and Kloppel, 1989a). On the other hand, studies on Japanese flounder reported positive pIgR expression in BEC within the liver but not in hepatocytes, suggesting a similar hepato-biliary transport to that reported in humans (Sheng et al., 2019). All in all, investigation in different teleost species might elucidate the mechanisms and evolution of the hepato-biliary transport as well as alternative transport routes of Igs in teleost fish.

**Are larvae protected before adaptive immunity develops?**

As earlier stated, the start-point of adaptive immune parameters in ballan wrasse was at larval stage 5 (50-70 dph) (**Paper I** and **Paper II**) which is relatively late but similar to other marine fish larvae (Magnadóttir, 2006). The intriguing question is what mechanisms wrasse larvae use to protect themselves from pathogens prior to the development of adaptive immunity. It is well accepted that fish rely on maternally transferred molecules during the first stages of embryogenesis. IgG is maternally transferred in mammals (Swain and Nayak, 2009), IgY in chicken (Hamal et al., 2006), and IgM in teleosts (Hanif et al., 2005; Olsen and Press, 1997; Picchietti et al., 2006; Picchietti et al., 2004). A recent study also showed for the first time that IgT was maternally transferred to eggs in zebrafish (Ji et al., 2021). However, the amount of maternal Igs seem to be species-specific and their prevalence in the embryo is rather short (Swain and Nayak, 2009). Apart from Igs, complement proteins, lysozymes, lectins, AMPs (i.e. cathelicidin), cytokines, and serine proteases, are part of the onset of maternally active molecules that can be transferred to the offspring (Løvoll et al., 2006; Mulero et al., 2007; Swain and Nayak, 2009; Valero et al., 2023). For example, lysozyme activity was detected in eggs and embryos of Atlantic cod and in unfertilized eggs of coho salmon (*Oncorhynchus kisutch*) (Seppola et al., 2009; Yousif et al., 1991). Similarly, complement proteins from zebrafish eggs showed bacteriolytic activity suggesting a protective role of larvae (Wang et al., 2008). In the present work, IgM was detected in ballan wrasse eggs but decreased to extremely low levels after hatching (2

dph) (**Paper II**). This indicates that although maternal IgM might protect wrasse embryos, newly hatched larvae might rely on their own innate immune system shortly after birth, as proposed for other species (Uribe et al., 2011). The presence of maternal proteins other than IgM in eggs of ballan wrasse have not been investigated in the present work. Interestingly, *sIgT* transcripts were significantly more abundant compared to transcripts of *sIgM* in wrasse larvae at 4 dph, before the start of exogenous feeding (**Paper II**). The detection of *IgT* mRNA may indicate a greater prevalence of these transcripts among the mRNA pool transferred to ballan wrasse eggs. Recent findings in zebrafish suggest that IgT could play a role in protecting embryos (Ji et al., 2021). Exploring maternal transfer of IgT to the offspring of teleost fish could reveal a tissue with specific *IgT* expression, offering valuable insights into the ongoing research on the functions and distribution of IgT.

Irrespective of the potential importance of other maternally transferred molecules in ballan wrasse, exploring the emergence of self-innate immunity in developing larvae becomes intriguing and aligns as a natural continuation of this PhD work. Driven by my curiosity and with the aim of partially addressing this topic, a brief examination of transcript levels for innate immune genes was conducted using transcriptomic data employed in the analyses of B- and T-cell development in **Paper I** and **Paper II** (Fig. 18, unpublished). For this, several genes coding for innate protective proteins such as cytokines, the complement protein C3, antimicrobial peptides, as well as potential markers for macrophages and other cytotoxic innate-like cells in fish were selected (Barreda et al., 2004; Hu et al., 2018; Løvoll et al., 2006; Mulero et al., 2007; Seppola et al., 2009; Swain and Nayak, 2009; Valero et al., 2023; Wentzel et al., 2020; Wiegertjes et al., 2016).

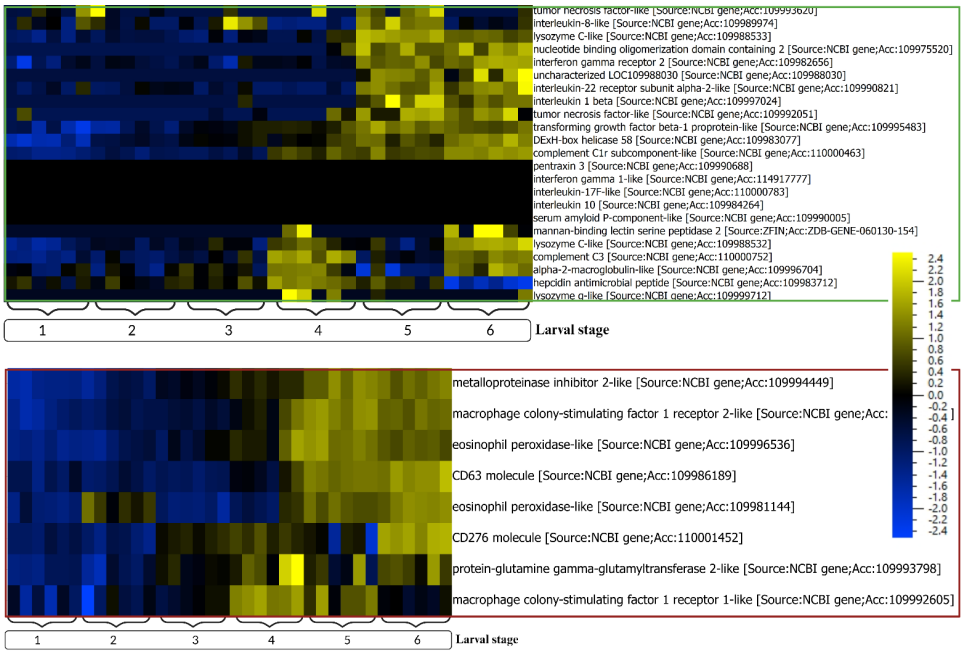


**Figure 18.** Heat map showing expression of selected innate-related genes during ballan wrasse ontogeny. Log2-transformed normalized counts per million (cpm) of RNA-seq data from wrasse larvae (stage 1 to 6). Transcripts are divided into general innate-like genes (green box), and genes that are potential markers for cytotoxic innate-like cells in teleost (red box). Gene sequences are predicted in the wrasse genome. Commercial feed was introduced at the end of stage 4 and adaptive-related genes are upregulated from stage 5 and onwards. Unpublished.

It is worth noting that reference genes used in previous studies on ballan wrasse (Bilal et al., 2019; Etayo et al., 2021) were detected as early as stage 1 (data not shown). Certain innate-related genes seem to be present well before adaptive immunity (Fig. 18, unpublished). Some genes showed an initial upregulation peak at stage 4, corresponding to significant anatomical changes in the wrasse body (such as the gut rotating into a Z-shape). The majority of examined genes experienced a second upregulation peak around stage 5, aligning with both the introduction of dried pellets to the feed, and an increase in transcripts of adaptive immune genes. The process of weaning is an intricate physiological transition that could induce stress and introduce potential contaminants or pathogens, possibly triggering a reaction from the innate immune system, which is active during this stage. This could explain the upregulation of certain innate immune genes. However, the downside effects of weaning are usually

attributed to suboptimal husbandry practices. Here, weaning was done in a co-feeding regime with live feed (barnacles or *Artemia*) for 16 days and therefore, the upregulation of innate genes could rather be explained by the intimate interaction between innate an adaptive system. The complement C3 (C3), lysozyme, and alpha-2-Macroglobulin (A2M) proteins have been proposed to actively defend young larvae of Atlantic cod, rainbow trout, and common carp (Huttenhuis et al., 2006a; Løvoll et al., 2006; Seppola et al., 2009). Interestingly, genes coding for the latter proteins showed the relatively highest number of transcripts from larval stage 1 and onwards (Fig. 18, unpublished), indicating a likely important role in protecting young ballan wrasse. Genes shown in Figure 18 are predicted sequences and need further characterization. All in all, although innate parameters have only been semi-analysed from a transcriptomic approach, ballan wrasse count on certain innate mechanisms that presumably protect larvae during early stages before the adaptive arm is active as demonstrated in other teleosts (Magnadóttir et al., 2005; Swain and Nayak, 2009; Uribe et al., 2011).

**Effects of a barnacle start-diet on adaptive immune parameters of ballan wrasse**

Feed and feed additives can modulate immunity in vertebrates and deficiency of one single nutrient might be enough to alter the function of the immune system as demonstrated for mice (Blewett and Taylor, 2012; Noor et al., 2021; Rodrigues et al., 2020). Also in humans, nutrition modulates innate parameters such as phagocyte function and cytokine production, as well as adaptive parameters by regulating B- and T-cell development (Noor et al., 2021; Tourkochristou et al., 2021). In teleost fish, modulation of the dietary fatty acid composition, especially DHA/EPA ratio and level of ARA, has an effect on certain immune responses mostly related to the innate system (Dantagnan et al., 2017; Montero et al., 2008; Xu et al., 2016a) but also suggested to affect T-cell markers and IgM expression during Atlantic halibut ontogeny (Patel et al., 2009; Øvergård et al., 2011).

In the present work, there were minimal differences in larvae growth between the two start-feed diets indicating no apparent benefit (based on growth) of using a barnacle diet compared to control, composed of rotifers and *Artemia*. However, when investigating parameters of the adaptive immune system, some key markers of T-cells,

i.e., *RAG1* transcripts, were earlier upregulated by the barnacle diet. In line with this, the thymus of larvae fed barnacle was larger in size compared to larvae fed the control start-diet (**Paper I**). Diets did not affect mRNA expression of the analysed genes identified as B-cell markers (**Paper II**). Although this work was not intended to set nutritional requirements for ballan wrasse larvae, it was intriguing to explore potential nutrients that might contribute to a more robust adaptive immune system, thereby enhancing health during early stages. The barnacle start-feed diet had a higher EPA and lower ARA profile (higher n-3/n-6 ratio), but levels of other macronutrients were similar between diets. Regarding micronutrients, greater variations in mineral content was present when examining the start-feed diets in relation to the timing of administration to the tanks. First, small barnacles and rotifers, and later, large barnacles and *Artemia*.

Extensive studies have explored the anti-inflammatory properties of n-3 polyunsaturated fatty acids (n-3PUFAs), specifically DHA and EPA, in various animal models. PUFAs exhibit diverse mechanisms of action affecting gene expression, membrane organization, cellular signalling, and eicosanoid metabolism (Shaikh and Edidin, 2006). It is well established that an increase in the dietary ratio of n-3/n-6 favour anti-inflammatory responses and autoimmunity, and that derivates from n-3PUFAs are generally considered anti-inflammatory compared to those derived from ARA, that are pro-inflammatory (Ramakers et al., 2007). Furthermore, PUFAs can modulate membrane fluidity and membrane organization at a cellular level (Shaikh and Edidin, 2006). Thus, it is not strange that immune cells are also affected by dietary fatty acids leading to modulation of immune functions. More specifically, n-3PUFA inhibits antigen presentation, activation, and proliferation, while reducing the expression of cytokines produced by T-cells (Kew et al., 2004; Petursdottir and Hardardottir, 2008). For example, EPA and DHA modulate the expression of specific receptors and induce changes in the membrane composition, affecting signalling proteins, and ultimately decreasing $CD4^+$ T-cell activation (Hou et al., 2012; Kim et al., 2010). Similarly, research on mice demonstrated that a diet rich in DHA and EPA modified membrane composition and signalling events that consequently altered the migration patterns of $CD4^+$ T-cells in such a manner that favoured the mitigation of a pro-inflammatory

immune response (Cucchi et al., 2020). Besides promoting direct modifications on immune cells, n-3PUFA can modulate the production of ceramides which are strong mediators of cell differentiation. These mediators interact closely with T-cells through membrane receptors affecting its activation and functionality (Cucchi et al., 2020; Nicolaou et al., 2014). Furthermore, n-3PUFAs similarly influence other immune cells including DCs. For example, Kong et al. (2010) found that exposing mice DCs to DHA and EPA prevented maturation, significantly reducing the release of proinflammatory cytokines. Similarly, various in vitro and in vivo experiments revealed that elevated n-3PUFA levels led to a decrease in MHCI and MHCII expression in immune cells, evidencing once more the anti-inflammatory effects of n-3PUFAs (Shaikh and Edidin, 2006). Curiously, n-3PUFA also impact B-cells by enhancing B-cell activation and antibody production as shown in mice and humans (Whelan et al., 2016). The effect of PUFAs in B-cells has received much less attention compared to that in T-cells.
In the present study, the ratio n-3/n-6 was higher in the barnacle diet suggesting the presence of anti-inflammatory mechanisms that might be beneficial for the overall body health as larvae might encounter stressors during development in captivity (i.e., physical stress such as water flow, turbulence, light regime etc).

The growth of thymus, spleen and bursa was prompted in chicks (< 4weeks) fed higher level of n-3PUFAs (Wang et al., 2000). However, changes in thymus and bursa size did not affect lymphocyte functionality (Wang et al., 2000). Interestingly, a Zn deficient diet caused atrophy of human thymus as well as apoptosis of DP thymocytes in the thymus cortex (Savino and Dardenne, 2010; Tourkochristou et al., 2021). In the present study, larvae were fed with small barnacles/rotifers for 15 days (from 4 dph to 19 dph) before a swift to larger preys (large barnacles and *Artemia*). Small barnacle showed higher Zn levels compared to rotifers, whereas Zn level was similar in large barnacles and *Artemia* (**paper I**). Given that the thymus anlage was firstly observed during larval stage 3 (20-30 dph), it is plausible that higher zinc levels administered during the initial feeding phase through the barnacle diet could persist and impact thymus development later on, even as the dietary zinc amounts became comparable in both diets when fed with large barnacles and *Artemia*. Measuring the size of wrasse

thymus was only feasible from stage 4 and beyond, and the most notable differences in size between the two diets was observed during stage 6 of larval development. Whether a high zinc level maintained throughout all developmental stages of wrasse would result in more substantial differences in thymus size remains elusive, but it is intriguing to explore further. An alternative theory, which is not mutually exclusive, suggests that the n-3/n-6 ratio could directly stimulate a larger thymus, as observed in young chicks (Wang et al., 2000). However, it is unknown whether a bigger thymus might offer any advantage for T-cell development, as in number of T-cells being produced. In summary, the current results suggest that T-cell lymphopoiesis, as opposed to B-cell lymphopoiesis, may be more susceptible to dietary influences during larvae development.

Additionally, small barnacle, representing the first food item offered to larvae was high in other minerals such as Mn, Co, and Se compared to rotifers. Mn, Co, and Se protect against lipid oxidation and Se has the potential to enhance innate response (Hamre et al., 2008; Tourkochristou et al., 2021). Furthermore, a deficiency in Se was shown to reduce the number of B-cells in humans (Tourkochristou et al., 2021). Taurin is essential for a successful development of larvae and copepod nauplii are naturally high in this free amino acid (El-Sayed, 2014; Hawkyard et al., 2015; Mæhre et al., 2013). However, contrary to our expectations, the barnacle diet presented a rather low level of taurine. One might speculate that the low detected levels of taurine supress more significant advantages in the overall larval development of those fed barnacle compared to the control group.

Altogether, the present work demonstrated that start-feed diets with different nutritional profiles can be reflected in the development of adaptive immunity in ballan wrasse larvae. Second, different levels of specific nutrients might lead to a superior quality diet compared to the control start-feed diet (rotifers and *Artemia*). However, it is important to remark that based on the lack of significant differences in larvae growth nor in mortality between diets, the control start-diet used in the present study, should also be considered as adequate, meaning that its nutritional content most likely meets the requirements for larval development. Third, the fact that nutrients can modulate

both innate and adaptive immune systems (as exemplified above by Se) and keeping in mind that innate system and not adaptive immunity is prevalent in eggs, yolk-embryonic larvae, and fry, it is likely that innate parameters are even more exposed to dietary modulation. Nevertheless, it is important to explore alternative start-feed diets (live preys). These diets should meet the nutritional requirements of larvae, promote larval health, and ensure the best possible larval development. Furthermore, they should be straightforward to produce and maintain under hatchery settings. Copepods are optimal for larvae growth as they have a superior nutritional composition compared to other live preys, but as of now, they are rather expensive and complicated to use in hatcheries (Busch et al., 2010; Hamre et al., 2008; Mæhre et al., 2013; Øie et al., 2017). On the other hand, the barnacle diet employed in this study is more affordable and ready to be pumped in tanks after an effortless thawing process. This feature is particularly attractive for hatcheries. Although copepods have been proposed to be the most successful start-feed in ballan wrasse larvae (Malzahn et al., 2022), the use of barnacle nauplii as start-feed diet ensures a robust development of ballan wrasse larvae and could be further explored in other marine species. As earlier mentioned, the barnacle diet appears to be deficient in taurine, and methods for its supplementation should be developed.

# 5. Concluding Remarks

The present work described parameters of the adaptive immune system of ballan wrasse with a special focus on developing larvae. The main findings are here highlighted:

1. The thymus and HK became lymphoid at larval stage 5 (50-70 dph)
2. Thymus zonation into cortex and medulla became clear at stage 5
3. Th T-cells appear more abundant than Tc T-cells in early larval stages
4. B-cell lymphopoiesis occurs in HK and pancreatic tissue of wrasse larvae
5. $IgM^+$ B-cells migrate to secondary lymphoid organs and MALTs before T-cells
6. $IgM^+$ B-cells are abundant in the peritoneum in early larval stages (stage 5 and 6)
7. Maternal IgM was detected in eggs but undetectable in newly hatched larvae
8. IgM is most probably transported to the gut lumen of wrasse through the liver (hepato-biliary route) and pancreas (pancreatic route).
9. A barnacle nauplii diet rich in n-3/n-6 PUFAs, Zn and other microminerals is optimal for wrasse development, with potential applications for cultivating other marine species.

Collectively, the present results indicate that immunocompetence is likely reached in juveniles of ballan wrasse (> 100 dph). It can be inferred that $IgM^+$ B-cells in the peritoneal cavity might play a vital role in protecting larvae, perhaps by producing natural IgM before T-cells migrate to this site. The subsequent presence of T-cells could initiate adaptive responses, thereby contributing to the acquisition of immunocompetence. The presented insights improve the groundwork for optimizing prophylactic strategies, but additional challenge studies are needed to establish the precise timing for effective vaccination of ballan wrasse.

# 6. Future Perspectives

This PhD work has continuously brought intriguing questions to the table with opportunities for future research. A challenge trial would be interesting to perform in order to investigate when immunocompetence is achieved. The lymphoid nature of the pancreatic tissue should be studied in more detail. To elucidate the transport function of pIgR in teleosts, several key tasks need to be accomplished. First, the identification of the secretory component (SC) in mucosal secretions should be studied by methods other than recombinant proteins or human cell models, employing for example modern proteomics tools. Second, the specific binding between IgM (IgT) and pIgR needs further explanation, and there is a need for additional exploration into the interaction between pIgs and pIgR in scenarios where the J-chain is absent, and very few, if any, N-glycosylation sites are present (which are crucial in higher vertebrates). These efforts are essential to gain a comprehensive understanding of the transport role of pIgR in teleosts. However, based on the results in this thesis, emphasis on alternative routes of IgM transport to the gut mucus seem more relevant in ballan wrasse. Could IgM produced by B-cells near pancreatic ducts be released directly into the pancreatic duct lumen?  particularly in the absence of SC? These questions are intriguing and should be further explored.

Specific micronutrients (vitamins and minerals) and fatty acids in the diet can modulate DNA methylations inducing epigenetic changes (Adam et al., 2019; Beaver et al., 2017; Panserat et al., 2017; Saito et al., 2021). Although in its infancy, trained immunity and epigenetics are relevant issues within aquaculture, with clear links to nutrition. To further elaborate on the potential benefits of a barnacle diet, it would be intriguing to conduct enrichment analyses using the previously generated transcriptomic data. This analysis would aim to investigate whether the barnacle diet has any discernible impact on innate parameters and compare this to the control start-feed diet. Another interesting topic which has attracted attention is the immunization of brood stock fish to enhance maternally transferred antibodies and other maternal immune factors. Since ballan wrasse show maternal transfer of IgM (this study) and an

extraordinarily high concentration of IgM compared to other teleosts, this approach might be interesting to look at in the future.

From a broader perspective, the presented findings have the potential to enhance the management of ballan wrasse in captivity, from the early larval stages to reaching the appropriate size for transfer to the sea. Nevertheless, challenges arise when wrasses are introduced to sea cages for delousing, making it difficult to ensure fish welfare. This challenge raises questions about the suitability of employing cleaner fish in salmonid aquaculture.

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# Paper I

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EDITED BY
Pietro Ghezzi,
University of Urbino Carlo Bo, Italy

REVIEWED BY
Xu-Jie Zhang,
Huazhong Agricultural University, China
Uwe Fischer,
Friedrich-Loeffler-Institute, Germany
Zuobing Zhang,
Shanxi University, China

*CORRESPONDENCE
Angela Etayo
angela.etayo@uib.no

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# The thymus and T-cell ontogeny in ballan wrasse (*Labrus bergylta*) is nutritionally modelled

Angela Etayo[1,2]*, Kai K. Lie[1], Reidun M. Bjelland[3], Ivar Hordvik[2], Aina-Cathrine Øvergård[2] and Øystein Sæle[1]

[1]Feed and Nutrition group, Institute of Marine Research, Bergen, Norway, [2]Fish Health Group, Department of Biological Sciences, University of Bergen, Bergen, Norway, [3]Institute of Marine Research, Austevoll Research Station, Storebø, Norway

Marine fish larvae often experience high mortality unrelated to predation during early life stages, and farmed ballan wrasse (*Labrus bergylta*) is no exception. Knowing when the adaptive immune system is developed and fully functional, and how nutrition may modulate these processes is therefore of importance to establish effective prophylactic measures and will also extend the relatively limited knowledge on the immune system in lower vertebrates. The thymus anlage of ballan wrasse was found to be histologically visible for the first time at larval stage 3 (20–30 days post hatch, dph) and becomes lymphoid at stage 5 (50–60 dph) correlating with an increase of T-cell marker transcripts. At this stage, a clear zonation into a *RAG1*$^{+}$ cortex and a *RAG1*$^{-}$ *CD3ε*$^{+}$ medulla was distinguished, indicating that T-cell maturation processes in ballan wrasse are similar to other teleosts. The higher abundance of *CD4-1*$^{+}$ compared to *CD8β*$^{+}$ cells in the thymus together with the apparent lack of *CD8β*$^{+}$ cells in gill, gut, and pharynx, where *CD4-1*$^{+}$ cells were identified, indicates that helper T-cells have a more prominent role during larval development compared to cytotoxic T-cells. As ballan wrasse lacks a stomach but has an exceptionally high IgM expression in the hindgut, we hypothesize that helper T-cells are crucial for activation and recruitment of IgM$^{+}$ B-cells and possibly other leukocytes to the gut during early development. Nutritional factors such as DHA/EPA, Zn and Se may lead to an earlier expression of certain T-cell markers as well as a larger size of the thymus, indicating an earlier onset of adaptive immunity. Including live feeds that supplies the larva with higher amounts of these nutrients can therefore be beneficial for ballan wrasse farming.

KEYWORDS

**adaptive immunity, lymphoid, thymocytes, larval ontogeny, early nutrition**

# 1 Introduction

Infections with the marine ectoparasitic copepod, the salmon louse (*Lepeophtheirus salmonis*), is a major problem for the salmon farming industry. In order to avoid heavy infestations of salmon, ballan wrasse (*Labrus bergylta*) is used as a cleaner fish for ectoparasite countermeasure. Ballan wrasse farming was initiated to decrease the fishing pressure on wild wrasse stocks, but as of now it is a relatively new industry with room for improvement. Efforts have been made to optimize feeding practices during early life stages (1), and the development of wrasse larvae has been described with focus on the ontogeny of the digestive system (1, 2). In more recent years, research on wrasse intestinal physiology and functionality has described some of the evolutionary traits of this stomach-less fish (3–5). However, wrasse farming still faces many challenges such as poor growth and a high mortality, especially during early life stages as in many other farmed marine teleost species (6, 7). The bacterial diseases Atypical *Aeromonas salmonicida* (aAs) and *vibrio anguillarum* are the primary challenge in farmed wrasse in sea pens and sporadic outbreaks have also occurred in hatcheries (8). It is believed that maternal transfer of defense molecules such as lectins and IgM to the oocytes can improve robustness at embryonic and larval stages until adaptive immunity (B-cells and T-cells) becomes functional (9, 10). After the appearance of B- and T-cells, long lasting memory is believed to be established, and the larva becomes better protected against pathogens and can be vaccinated. Understanding the ontogeny of the adaptive immune system, and specifically the appearance of functional lymphocytes, is therefore crucial for the development of efficient vaccination protocols.

T-cells are together with B-cells, the key cellular fraction of the adaptive immune system in vertebrates. Mammalian T-cells are characterized by having a T-cell receptor complex (TCR/CD3) that recognizes antigenic peptides on the surface of the major histocompatibility complex (MHC) molecules, known as MHC restriction. The majority of mammalian T-cells contain a TCR formed by the αβ heterodimer, whereas the TCR-γδ T-cell populations is relatively small (11). Moreover, there are two main subsets of T-cells distinguished by the expression of two coreceptors, CD4 and CD8. $CD4^+$ T-cells can be regulatory cells (Treg) that are key in mucosal homeostasis and immune regulation, and helper cells (Th) that secrete cytokines stimulating other immune cells. $CD8^+$ T-cells, the so-called cytotoxic cells (Tc), directly kill cells infected by pathogens such as viruses and intracellular bacteria (12). Teleost T-cells seem to resemble those in mammals. Several genes expressed in T-cells, such as *TCRαβ*, *TCRγδ*, *CD3* (ε, γ, and δ), *CD4* (*-1* and *-2*), and *CD8* (*α* and *β*) have been described in several teleosts as recently reviewed in Barraza et al. (13). Ballan wrasse *TCRα*, *TCRδ*, and *CD3ε* have also been characterized (14, 15).

The thymus is the major site for T-cell development, and thus a key organ in the immune system. The mammalian thymus is a bi-lobed organ divided into two zones: the cortex (outer zone) and the medulla (inner zone), each of them with well-defined microenvironments. Only around 5% of the cells entering the thymus will exit the thymus as mature T-cells expressing a functional TCR able to recognize peptides bound to MHC molecules while being tolerant to self-MHC/self-peptides (11). T-cell maturation is a complex process that is strictly regulated and requires constant contact of T-cell precursors with both stromal and thymic epithelial cells. The development of mammalian T-cells starts with the activation of the recombination-activating genes (*RAG1* and *RAG2*) involved in *TCR* locus rearrangement (11). Early lymphocytes or thymocytes are double negative (DN), not expressing CD4 nor CD8 ($CD4^-CD8^-$). DN thymocytes that are able to rearrange their TCR-β chain and express it on their surface together with CD3 chains, form a pre-TCR complex and will proliferate becoming double positive (DP) $CD4^+CD8^+$ thymocytes. Cortical DP thymocytes that survive to positive selection, mature to single positive (SP; $CD4^+$ or $CD8^+$) T-lymphocytes and migrate to the medulla where further negative selection occurs. As a result, mature SP T-cells are $RAG^-$, and can now enter circulation and migrate to secondary lymphoid organs where maturation of T cells is finalized upon antigen recognition (16, 17).

The teleost thymus is a paired organ located dorsally near the gill cavity (18, 19), enclosed by a capsule which consist on both epithelial cells and connective tissue. Epithelial cells are exclusively found in the region where the organ faces the gill cavity, whereas connective tissue appears surrounding the remaining portions of the thymus. Invagination of connective tissue in the thymus are called trabeculae and contain vascularized capillaries. Similar to higher vertebrates, the teleost thymus seems to be zoned into a cortex and medulla. Although the first description of a teleost thymus was in the eighties with the characterization of the rainbow trout (*Oncorhynchus mykiss*) thymus by Grace and Manning (20), it took some years before researchers started to address the molecular mechanisms regulating T-cell maturation in fish. *RAG* genes have been shown to be expressed in the early thymus of zebrafish (*Danio rerio*) (21), medaka (*Oryzias latipes*) (22), common carp (*Cyprinus carpio*) (23), Atlantic halibut (*Hippoglossus hippoglossus*) (24), and Atlantic salmon (*Salmo salar*) (25) among others, with a cortex restricted expression shown in species with a zoned thymus. DP thymocytes and both subsets of SP T-cells ($CD4^+$ and $CD8^+$ T-cells) were found in sea bass (*Dicentrarchus labrax*) (26), ginbuna crucian carp (Carassius *auratus* langsdorfii) (27), and rainbow trout thymus (28, 29). The expression of MHC class I and II in cortical thymic epithelial cells (cTECs) is crucial for positive selection of thymocytes during T-cell maturation (30). In accordance with this, MHC $II^+$ cells have been observed in the outer zone of the thymus in sea bass (31), Atlantic salmon (32) and rainbow trout (33). Furthermore, a series of genes coding for molecules that are either T-cell markers such as *LCK* and the 70 kDa zeta-associated protein (*ZAP-70*), but also play relevant roles in T-cell maturation as do the c-c chemokine ligand 25a (*CCL25α*) and the c-c chemokine receptors 9 (*CCR9*), have been cloned in teleost fish (13, 22). Altogether, findings support the idea that T-cell maturation is to some degree evolutionary conserved between fish and mammals. However, teleosts are very diverse, and studies comprising the thymic structure, zonation and T-cell development are restricted to few species and yet, with certain contradictions (13, 18).

Malnutrition is known to cause atrophy of the developing thymus and massive death of $CD4^+CD8^+$ thymocytes (34, 35) in mammals. Interestingly, nutrition has an important role at initiating and regulating B- and T-cell lymphocyte development in humans (36, 37). It is then assumable that nutrition affects development of adaptive immunity in teleosts, but corresponding studies are lacking. A well-known inherent challenge in larvae requiring live feed is to provide good nutrition (38). The most common dietary practice for small marine larvae is to give enriched rotifers followed by enriched Artemia. Rotifers and artemia are prey animals that are easy to provide for the larvae, but offer suboptimal nutrition (39) and therefore, they are enriched with essential nutrients before being administered to larvae.

To understand the timing when functional lymphocytes appear in ballan wrasse larvae, we here present the histological and molecular ontogeny of the thymus and developing T-cells, as well as the potential of a barnacle nauplii start-feed diet to boost an earlier ontogeny of the adaptive immune system compared to larvae fed with enriched rotifers and artemia.

# 2 Materials and methods

## 2.1 Experimental design

Brood stock ballan wrasse (30 females and 6 males) were kept in tanks at 8°C from October to the end of February of 2020 (2 weeks before spawning start). The temperature was then raised to 12°C during the spawning season and until September. Brood stock fish were fed to saturation every day. After spawning, eggs were placed in incubators with a capacity of 250 L at 12°C, water flow of 5 L/min and natural light until hatching. At 4 days post hatching (dph), 30 000 to 34 000 individuals were transferred into six different tanks and two different start-feed diets were given in triplicates. At this stage, most of the larvae had completely depleted their yolk-sac. The control diet consisted of rotifers enriched with algae (*Nannochloropsis* and *Tetraselmis*) from Microalgae AS, Vigra, Norway, followed by artemia enriched with LARVIVA Multigrain (BioMar), cultivated, and enriched at the in-house facility at IMR, Austevoll, Bergen (Norway). The experimental diet was barnacle nauplii of two different sizes (small and large barnacle) from Planktonic company that were frozen in liquid nitrogen and revived before being added to the tanks. Detailed information regarding cultured conditions of rotifers, artemia and barnacles can be found in Supplementary Data 1. The larvae were kept in tanks with a capacity of 500 L at 15°C, with a starting water flow of 50 L/h that increased as the larvae grew, and a light regime of 24 hours (h). Commercial formulated feed (dry feed) was introduced at 40 dph in a co-feeding regime until 56 dph. After this time point, only commercial feed was supplied to all tanks. A total of six sampling points were set according to the six developmental stages of wrasse larvae that are based on the ontogeny of cranial ossification (2). The larval stages and the experimental feeding regime are summarized in Figure 1. At each sampling point a series of pooled larvae (3 to 15 larvae per pool) in replicates were collected from each tank, rinsed with distilled water, and immersed in RNA later at 4°C overnight and kept at -20°C until further use.

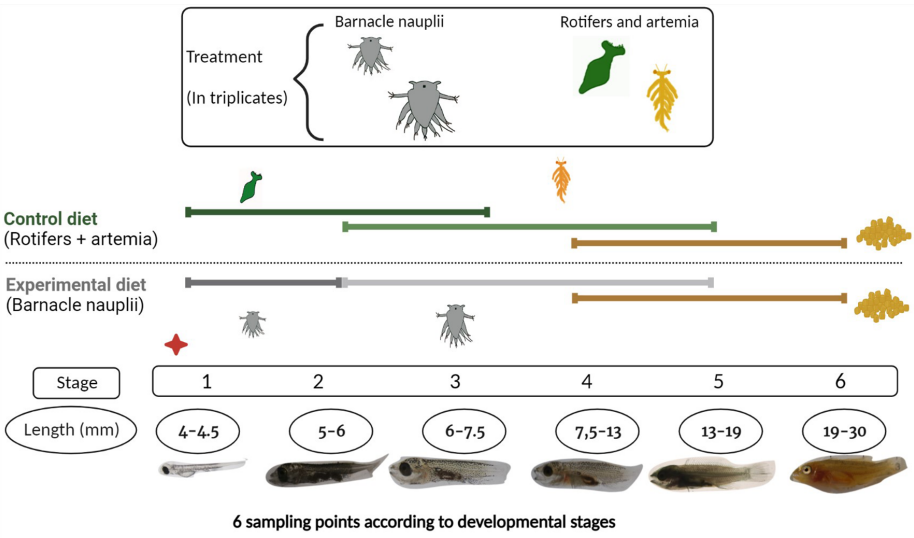


FIGURE 1
Experimental design. Two start-feed diets were given in triplicates: a control diet (rotifers and artemia), and an experimental diet, so-called barnacle diet (barnacle nauplii). The control and barnacle diet are represented in green and grey color respectively and colors are used henceforth for easier interpretation of results. Six sampling points were done according to developmental stages of wrasse larvae. After stage 6 they were considered juveniles. The red dot indicates the start of the feeding trial at 4 dph with rotifers and small barnacle correspondingly. Artemia and large barnacles were introduced at stage 2 and commercial feed (pellets) were introduced in a co-feeding regime at stage 4. Created with BioRender.com.

## 2.2 Nutrient analyses

Rotifers, artemia and both small and large barnacles were sampled in triplicates (n=3). Within each replicate, rotifers, artemia and barnacles were taken from their corresponding hatcheries the same day in the morning after feeding the larvae (approx. 10:30 am). Samples were passed through a sieve to concentrate the live prey and rinsed with distilled water to get rid of seawater. Samples were then aliquoted in different tubes for the different nutrient analyses and rapidly placed in dry ice and further stored at -80°C. One of the replicates was taken in the spring of 2020 and the other two replicates were taken in the spring of the following year. Analyses of proteins (aa composition), vitamins, pigments, and fatty acid composition was done on wet material. Analyses of minerals (ICP), total lipid and ash were done on dry material. References of the methods for nutrient analysis are in Supplementary Table 1.

## 2.3 RNA isolation and RNA-seq analyses

Two replicates of pooled larvae were collected from each tank (technical replicates) at each sampling point. The number of pooled larvae (per replicate and tank) varied from 15 individuals at stage 1, to 3 individuals at stage 6, sampling three biological replicates (tanks) for each time point (n=3). Pools of larvae were individually crushed in a mortar kept at –80°C. Fine powder was collected and used to isolate total RNA with QIAzol reagent® (Invitrogen, Waltham, MA, United States) including DNase treatment (TURBO DNase, Ambion) according to the manufacturer's protocol. RNA quality and integrity were assessed using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE,United States) and the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Total RNA samples were sent to Novogene Europe, Cambridge, UK, for sequencing using the Illumina NovaSeq 6000 platform for 150 bp paired end reads. CDNA libraries were prepared from individual samples and sequenced following manufacturer's instructions and according to the Novogen pipeline (Novogene Europe, Cambridge, UK). Raw sequence reads were mapped against the ensemble wrasse gene build (Labrus_bergylta.BallGen_V1.104) using the Hisat2 mapper (40). Gene counting was conducted using feature counts v1.6.0 (41) as previously described (42). The count data was further normalized for differences in library size applying weighted trimmed mean expression ratios [trimmed mean of M values (TMM)] featured in the EdgeR package v 3.34 (43). Due TCR genes not being predicted in the wrasse ensemble gene build and the fragmented nature of the current wrasse assembly, especially for the immune genes with variable domains, a modified version of the original transcriptome (3) was made by extracting sequences related to *IgD, IgM, IgT, pIgR, TCRα* and *TCRδ* and replacing them with recently curated sequences (14, 44). To analyze the presence of *TCRα* and *TCRδ* in the different stages of wrasse, we conducted a re-mapping of all samples against the modified transcriptome using Salmon version 0.11.3 for mapping and quantification according to (45).

The raw data are available from the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) (Accession ID: SRX14748182). In this study, 18 genes of interest related to T cell development (*RAG1, RAG2, IKZF1, LCK, ZAP70, CD3δ, CD3ζ, CD3ε, CD4-1, CD4-2, CD8β, MHCII-α, MHCIIβ, CD74α, TCRα, TCRδ, CCR9β*, and *CCL25α*) were extracted from the RNA-seq data set and studied through larvae development using the Qlucore Omics Explorer v3.2.

## 2.4 Histology

For histological analyses of the thymus a total of 36 larvae across the six sampling points (n=3) were fixed and stored in Karnovsky fixative until further processing. Larvae in stage 3 to 6 were beheaded and the head was decalcified in EDTA 0.4 M, pH 7.2 for 2 to 7 days at 4°C. The solution was changed every other day. Larvae in stage 1 and 2 were used as whole and did not require decalcification. All larvae were then dehydrated through an ethanol gradient series up to 96% ethanol. Technovit 7100 kit (Kulzer GmbH) was used for resin embedding according to the manufacturer instructions. In short, dehydrated specimens were placed in the pre-infiltration solution (ethanol 96% and 50% Technovit 7100 basic solution 1:1) for 1.5 h followed by infiltration solution (hardener 1) overnight. The larvae and heads were orientated vertically with the mouth facing down in the mold and polymerization of the resin with hardener 2 solution was done in a desiccator for 24 h at room temperature (rt).

To localize and visualize the whole of the organ thymus, serial cross-sections of 2 µm were done using a Leica RM2165 microtome. Sections were collected from the cranial end, right at the back of the fish larvae's eyes, until the thymus was not present in the sections. They were further stained with borax buffered toluidine blue.

## 2.5 Volumetric analyses

The volume of the thymus was investigated in a total of 18 larvae belonging to stage 4, 5, and 6 (3 larvae per diet and per stage) using the previous histological sections. Every fourth (for stage 4), every seventh (for stage 5), and every eleventh (for stage 6) sections were scanned using a semi-automatic scanner Nano Zoomer S60 (Hamamatsu, Japan) and visualized using NDP.view2 (Hamamatsu, Japan). Volumetric analyses were done by manually drawing a line around the thymus surface on selected slides, and the program further calculated the size of the marked area. The volume of the whole organ was then estimated as the thickness of each section times the total number of serial sections including the thymus.

## 2.6 Production and validation of antisera raised against wrasse CD3ε peptide

A polyclonal anti-wrasse CD3ε antibody was made as described in (46, 47). Wrasse CD3ε contains a cytoplasmatic peptide which is

phylogenetically conserved among humans, higher vertebrates, and to a large extent, teleost fish (Figure 2). The corresponding peptide in ballan wrasse (GRAPPLPSPDYEP) was synthetically produced and used to immunize two rabbits according to the standard protocol of the producer. The resulting sera (anti-wrasse CD3ϵ) was subsequently affinity purified using the corresponding peptide (Davids Biotechnologie GmbH, Regensburg, Germany).

The anti-wrasse CD3ϵ was further validated by western blot analysis of different wrasse tissues. Wild ballan wrasse (700 - 900 g) were caught from fjords close to Bergen, Norway. They were anaesthetized with MS-222 (30 mg/ml) and sacrificed by a blow to the head. Thymus, head kidney, liver, muscle, spleen, gills and hindgut were excised and washed in cold PBS mixed with protease and phosphatase inhibitors (Pierce™). Tissues were homogenized in lysis buffer (4% SDS, 0.1M Tris-HCl pH 7.6) using a tissue disruptor and further sonicated using an ultrasonication rod (Q55 Sonicator, Qsonica, CT, USA) at 30% amplitude for 30 sec. Tissue lysates were centrifuged at 400 x *g* for 10 min at rt, the upper fraction incubated at 95°C for 5 min and further centrifuged at 15 000 x *g* for 10 min. The supernatant containing proteins was collected and quantified using the Bradford assay according to the manufacturer's guidelines. Approximately 30 µg protein from each tissue were run on reducing, denaturing, 4–15% gradient gels. Western blotting was performed at 22 V and 1.3 A for 7 min at 22°C using a Trans-Blot Turbo System (Bio-Rad). To avoid unspecific binding of antibodies, the PVDF membrane was blocked for 30 min and incubated with rabbit anti-wrasse CD3ϵ (1:5000) for 2 h. The membrane was washed and incubated with HRP-conjugated anti-rabbit IgG (1:2000) for 1 h. The PVDF membrane was developed using ECL reagents (Pierce™ ECL Western Blotting Substrate).

## 2.7 Immunohistochemistry

Larva from stage 6 fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.2) were paraffin-embedded and sectioned at 3 µm thickness using standard procedures. The slides were incubated on a heating plate at 37°C for 24 h, followed by 58°C for 1 h, before deparaffinization in xylene and hydration in graded ethanol dilutions to distilled water. Heat-induced epitope retrieval was performed at 80°C for 40 min in 0.01 M citrate buffer (pH 6) using a water bath. The slides were cooled down and subsequently washed in 0.01 M PBS (pH 7.3). Unspecific binding was prevented by incubating the tissues in 0.05 M tris-buffered saline (TBS, pH 7.6) with 2% BSA and 2% goat serum at rt for 1 h. Polyclonal anti-wrasse CD3ϵ primary antibody was diluted 1:100 in TBS with 1% BSA before application, and the slides were incubated for 1 h at rt. After rinsing with TBS with 0.05% tween (TBS-T), endogenous peroxidase was blocked by incubation in 1,5% Hydrogen peroxide solution (Merck KGaA, Darmstadt, Germany) at rt for 10 min, and several washes with TBS-T. The slides were then incubated with goat polymer-HRP anti-rabbit (abcam, Cat. No.: ab97051) 1:1000 for 45 min and developed with DAB substrate (Cell signaling, Cat. No.: 8059). Between each step, the slides were washed in TBS-T. Slides were further dipped in 0.01 M citrate buffer, pH 4.8, and then counterstained in Methyl green solution (Vector Laboratories, Cat. No.: H-3402) at 60°C for 20 min. Slides were quickly immersed in 0.01 M citrate buffer, pH 4.8, and blot dried before dehydration through 95% and 100% ethanol before mounting in non-aqueous VectaMount® Mounting Medium (Vector Laboratories, Cat. No.: H-5000). As negative control, primary antibody was omitted from the procedure. The sections were imaged using a Leica DM 2500 LED with associated camera Leica DMC 6200. The software Leica Application Suite X was used.

## 2.8 *In situ* hybridization

*In situ* hybridization was performed on larvae in stage 5 and 6. To investigate development in more detail, the stages were divided into 2 substages according to larvae standard length (SL) and referred as early and late substage throughout the text. A total of 8 individual larvae were run in duplicates as followed; 2 larvae from early substage 5 (SL: 1,6), 2 larvae from late substage 5 (SL:1,8 cm), 2 larvae from early substage 6 (SL: 2,6), and 2 larvae from late substage 6 (SL: 3,5 cm) which are considered juveniles. The larvae were fixed in 4% PFA (pH 7.4) at rt for 24 to 32 h. Samples were then dehydrated, embedded in paraffin wax and 3 µm thick sections were made using standard procedures.

For *in situ* hybridization, RNA Scope 2.5 HD (Advanced Cell Diagnostics, Newark, CA, USA) probes for *RAG1, CD3ϵ, CD4-1* and

```
humanCD3e          NKERPPPVPNPDYEPIRKGQR--DLYSGLNQRRI--          207
ChickenCD3e        KMQRPPPVPNPDYEPIRKGQR--DVYAGLEHRGF--          175
salmonCD3e         AGRGPPVVPSPDYEPLSVATRSSDIYATTQTSTQRTG         181
FuguCD3            GGRA-PPLPSPDYEPLNPHTRSQGTYSEVHPKRMG--         172
FlounderCD3ε       VGRA-PPVPSPDYEPLNPHTRAQDPYSIVNRTG----         164
wrasseCD3ε         GGRA-PPLPSPDYEPLNPHTRNQDPYSTVSRTG----         175
                     .  ** :*.*****:    *  . *:
```

FIGURE 2
Alignment of the cytoplasmatic tail of wrasse CD3ϵ and corresponding sequences from human, chicken, salmon, fugu, and flounder. Residues identical in all sequences, highly conserved sequences, and conserved sequences are indicated by stars (*), colons (:), and periods (.) respectively. A commercial human antibody was raised against the peptide indicated in bold. In salmon, a successful antibody was raised against the peptide indicated in red. The corresponding sequence in wrasse is underlined and was used to raise an antibody (anti-wrasse CD3ϵ).

*CD8β* were designed and produced by the manufacturer based on the provided sequences of ballan wrasse (Table 1). The *in situ* hybridization procedure was slightly modified from Løken et al. (48). In short, the paraffin-embedded tissue sections were mounted on positively charged glass slides (Superfrost, Mentzel), dried at 37°C for 48 h and further incubated at 60°C for 1 h. Subsequently, samples were de-paraffinized in 2 x 5 min xylene and 2 x 1 min 100% ethanol. Samples were treated for endogenous peroxidase blocking (10 min at rt), followed by target retrieval (15 min at 100°C), and protease digestion (30 min at 40°C) to allow permeabilization of cells. For probe hybridization, samples were incubated with the RNA scope probe for 2 h at 40°C, either as Duplex assays for simultaneous detection of two probes with the following combinations (*RAG1/CD3ϵ*, *CD3ϵ/CD4-1*, and *CD3ϵ/CD8β*), or as single assays targeting either *CD8β*$^{+}$ T- cells or *CD4-1*$^{+}$ T-cells. A series of hybridizations were performed using different incubation times according to the manufacturer´s instructions (49) to allow amplification of the signal. For signal detection, samples were then treated with chromogenic substrates bound to HRP (green color) and AP enzymes (red color) for 10 min each and subsequently stained with a 25% Gill´s hematoxylin solution for 30 sec. Samples were then dehydrated and mounted with non-aqueous VectaMount® Mounting Medium (Vector Laboratories, Cat. No.: H-5000). The sections were scanned using a semi-automatic scanner Nano Zoomer S60 (Hamamatsu, Japan) and visualized using NDP.view2 (Hamamatsu, Japan).

## 2.9 Statistical analyses

Statistical analyses were performed using Prism 9 (GraphPad Software Inc., CA, USA). The dry weight data violated the Shapiro-Wilk normality test and therefore, data were log-transformed before significance testing. Log-transformed data were normal distributed but presented unequal variances (F > F Critical one-tail using F-test for homogeneity of variances). As the main objective was to test the effect of the two start-feed diets on larvae growth at each developmental stage, the non-parametric Mann-Whitney test was selected as appropriate. A significant level of 0.05 was used.

For RNA-transcriptomic data, the aim was to see whether there was an effect of the start-feed diets on those genes related to T-cell development. For most of the selected genes, there were no transcript counts before stage 4. Therefore, statistics were only applied to the last three larvae stages (stages 4, 5, and 6). Data were log-transformed and are presented as means ± standard deviation (SD). F tests were performed to check for homogeneity of variances while normality was checked by the D'Agostino-Pearson test. Out of all the genes of interest, only *RAG1* and *ZAP70* were normally distributed and presented equal variances, and the parametric multiple t-test (Holm-Šídák t-test) was used for analyses of significances between the two start-feed diets within each stage. For *RAG2, CD3ϵ, TCRα*, and *IKZF1* normality or homogeneity in variance was not achieved, and the non-parametric Mann-Whitney test was performed. A significant level of 0.05 was used for all the tests.

Generalized Linear Model (glm) was applied to measure the effect of the start-feed diets on the volume of the thymus, considering the diets as categorical factor and larval myotome height (MH) (mm) as the continuous predictor. An ANOVA followed by Tukey's multiple comparisons *post-hoc* test was used to compare the nutrient content among the life-preys (rotifers, artemia, small barnacle, and large barnacle) of the start-feed diets.

# 3 Results

## 3.1 Nutrient analyses

The complete nutrient analyses of the experimental start-feed diets (rotifers, artemia, small barnacle and large barnacle) are shown in Supplementary Table 2. An overview of the nutrients that are further discussed are shown in Table 2. Start-feed diets did not differ in the total amount of lipids nor in the amount of saturated fatty acids (SFA). Rotifers showed the lowest percent of monounsaturated fatty acids (MUFA) and the highest percent of n-3 docosahexaenoic acid (DHA). Both small and large barnacles showed significantly higher levels of n-3 eicosapentaenoic acid (EPA) and lower levels of the n-6 arachidonic acid (ARA) compared to rotifers and artemia (Table 2). As a result, the n-3/n-6 ratio was significantly higher in the barnacle diet.

Iodine was the only mineral that was significantly higher in both small and large barnacle diets compared to rotifers and artemia. Ash content was similar in all start-feed diets. The rest of the investigated minerals varied more between the four start-feed diets. Small barnacle was significantly higher in V, Mn, Co, Zn, As, Se, and Ca compared to rotifers (Table 2). As was found to be higher in large barnacle compared to artemia whereas artemia was higher in Na and Mg (Supplementary Table 2). Variation in nutrient content from batch to batch was especially high in

TABLE 1 Probes used in *in situ* hybridization (*Labrus bergylta*).

| | Probe | Accession no. | Target region (bp) | Catalogue no. |
|---|---|---|---|---|
| **Target** | RAG1 | XM_020642835.2 | 1526-2425 | 1194681-C2 |
| | CD4-1 | XM_020649070.2 | 369-1437 | 1194661-C2 |
| | CD8β | XM_020647965.2 | 131-1194 | 1194671-C2 |
| | CD3ϵ | XM_020644379.2 | 103-1140 | 1194651-C1 |
| **Control** | DapB (negative) | EF191515 | 414 - 862 | 310043 |
| | EF-1a (positive) | XM_029279947.1 | 600-1592 | 1185171-C1 |

10.3389/fimmu.2023.1166785

TABLE 2 Overview of the selected nutritional analyses in rotifers, artemia, small barnacle (*Balanus crenatu*), and large barnacle (*Semibalanus balanoides)*.

| | Rotifers | Artemia | Small barnacle | Large barnacle |
|---|---|---|---|---|
| Proximate composition (g/100g DW) | | | | |
| Protein | 51 ± 0$^{a}$ | 36 ± 9$^{b}$ | 42 ± 17$^{abc}$ | 52 ± 22$^{ac}$ |
| Lipid | 16 ± 4 | 13 ± 6 | 8 ± 4 | 9 ± 2 |
| Ash | 36 ± 11 | 23 ± 1 | 30 ± 18 | 23 ± 6 |
| Dry weight (g/100g WW) | 6 ± 4 | 9 | 22 ± 13 | 16 ± 5 |
| Fatty acids (% of TFA) | | | | |
| ΣSFA | 22 ± 5 | 22 | 22 | 19 |
| 20:5n-3 EPA | 7 ± 1$^{a}$ | 9$^{a}$ | 28$^{b}$ | 32 ± 4$^{b}$ |
| 22:6n-3 DHA | 34 ± 6$^{a}$ | 11 ± 2$^{b}$ | 15$^{b}$ | 19 ± 2$^{b}$ |
| 20:4n-6 ARA | 2$^{a}$ | 3$^{b}$ | 1$^{c}$ | 1$^{c}$ |
| n-3/n-6 | 2$^{a}$ | 2$^{a}$ | 26 ± 2$^{b}$ | 21 ± 7$^{b}$ |
| Micro-mineral composition (µg kg$^{-1}$ DW) | | | | |
| V | 5 ± 1$^{a}$ | 34 ± 47$^{ab}$ | 88 ± 5$^{b}$ | 26 ± 12$^{ab}$ |
| Mn | 98 ± 29$^{a}$ | 313 ± 429$^{a}$ | 1170 ± 142$^{b}$ | 159 ± 84$^{ac}$ |
| Co | 2 ± 1$^{a}$ | 7 ± 9$^{ab}$ | 16 ± 1$^{b}$ | 6 ± 4$^{ab}$ |
| Zn | 203 ± 85$^{a}$ | 2220 ± 2536$^{ab}$ | 4343 ± 667$^{b}$ | 5825 ± 843$^{b}$ |
| Se | 3 ± 1$^{a}$ | 10 ± 7$^{ab}$ | 19 ± 3$^{b}$ | 15 ± 2$^{b}$ |
| Jod | 58 ± 26$^{a}$ | 244 ± 300$^{a}$ | 2819 ± 523$^{b}$ | 736 ± 154$^{c}$ |
| Pigments (µg kg$^{-1}$ DW) | | | | |
| Astaxanthin | 1374 ± 912$^{a}$ | 41 ± 2 $^{ab}$ | 28 ± 20$^{b}$ | 60 ± 10 $^{ab}$ |
| Canthaxanthin | tr | tr | 4* | 7* |
| Non-essential amino acids (µg/Kg dw) | | | | |
| Protein-bound amino acids (PAA) | | | | |
| Proline* | 676 ± 92 | 195 ± 1 | 188 ± 86 | 226 ± 10 |
| Free amino acids (FAA) | | | | |
| Proline* | 52 ± 7 | 42 ± 2 | 35 ± 15 | 67 ± 4 |
| Taurine* | 3 ± 1 | 35 | 17 ± 7 | 25 ± 2 |

Values are relative to dry weight (DW) and are given as mean ± SD when value is >1. The number of replicates is 3 (N=3) unless otherwise specified by *(N=2). ANOVA test was applied only when N=3 and significances are indicated by letters.

artemia as batches were unfortunately taken in different years (same season). Rotifers and artemia were richer in all investigated vitamins compared to barnacles. Barnacles were devoid of vitamin $D_3$, and Vitamin A was only found in trace levels in all diets. Enriched rotifers presented high levels of astaxanthin compared to artemia and barnacles whereas canthaxanthin was only detected in small and large barnacles at low levels (Table 2).

The concentration of both protein-bound amino acids (PAA) and free amino acids (FAA) was highest in rotifers and lowest in barnacles except for proline in its free form (FAA) that was highest in large barnacle, and the free amino acid taurine that was lowest in rotifers and highest in artemia (Table 2). The amino acid profile was similar among the start-feed diets where lysine, aspartic acid, and glutamic acid was the most abundant in the PAA fraction followed by leucine in rotifers and artemia, and glycine and proline in small and large barnacles respectively (Supplementary Table 2). The profile for FAA was similar between rotifers and artemia being arginine, lysine, and glutamic acid the most abundant amino acids. Differently, the FAA fraction of barnacle diets were rich in proline and alanine followed by glycine in small barnacles and taurine in large barnacles (Supplementary Table 2).

## 3.2 Growth and effect of start-feed diets during larvae ontogeny

The two start-feed diets did trigger a significanlty higher dry weight (DW) of those larvae fed barnacle nauplii at stage 6 (Mann-

Whitney; p value= 0.0287) (Figure 3B). Mortality peaked in larvae fed barnacle nauplii shortly after first feeding but decreased fastly to a mortality rate lower than the control diet group (rotifers and artemia) (Figure 3C). Mortality during weaning was similar between the two groups. Data corresponding to the standard length (SL) of sampled larvae (Figure 3A) is a confirmation of the successful execution of larvae collection at each given stage.

## 3.3 Transcription of T-cell markers and thymus-associated genes

RNAseq data of wrasse larvae fed the control diet was analysed to study the starting point of T-cell development during ontogeny. In the present work, 18 genes corresponding to T-cell markers (*RAG1, RAG2, IKZF1, LCK, ZAP70, CD3δ, CD3ε, CD4-1, CD4-2, CD8β, TCRα, TCRδ*) and markers for thymic epithelial cells (*MHCII-α, MHCIIβ, CD74α, CCL25α*, and *CCR9β*) were examined (Figure 4A). Allthogether, the transcriptomic data indicated that T-cell maturation processes started at or just prior to stage 5 of wrasse development. The recombination-activating genes *RAG1* and *RAG2* were found to be upregulated from stage 5, though *RAG2* displayed a lower transcript level (Figure 4B) (the ratio *RAG1/RAG2* is 2.4 in stage 5, and 1.8 in stage 6). Expression of the T-cell markers *ZAP70*, the three CD3 chains (*CD3δ, CD3ζ, CD3ε*), *CD4-1, CD4-2, CD8β*, and *TCRα* also appeared at stage 5 but with fewer reads compared to *RAG1*, while *LCK* was the only gene displaying a similar level of expression as *RAG1* from stage 5 and onwards. Interestingly, only a small increase in the transcript level of the *TCRδ* chain was observed at stage 5, declining again at stage 6. On the opposite, transcripts of genes expressed in cortical and medullar epithelial cells important for thymus homing (*CCL25α*) and selection processes (*MHCIIα, MHCIIβ, CD74α* and *CCR9β*) were observed before stage 5, prior to the induction of *RAG1* and T-cell specific markers (Figure 4A). Similarly, the ikaros (*IKZF1*) transcription factor which is critial for early T-cell development appeared before stage 5.

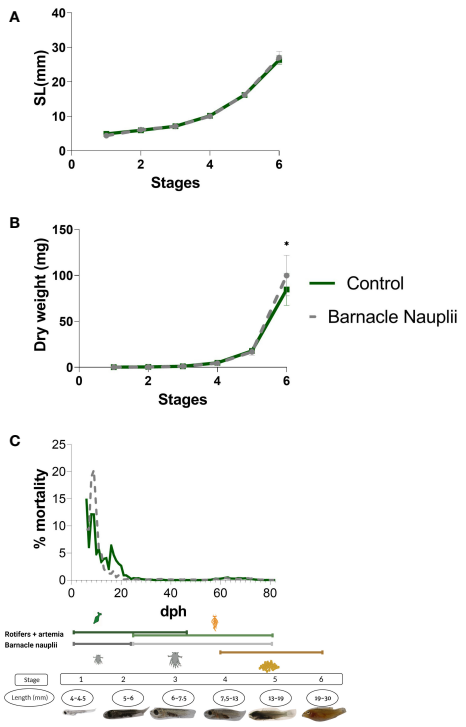


FIGURE 3
Figure 3. The dietary effect on growth performance and larvae mortality through ontogeny. **(A)** Larvae standard length (SL). **(B)** Larvae dry weight (DW) which was significantly higher in the barnacle nauplii diet in the last developmental stage (Mann-Whitney test, p value= 0.0287, indicated by *). **(C)** Mortality expressed in percentage. Stages and the feeding regime is included in the figure for easier interpretation.

## 3.4 Histology

Based on the mRNA expression data, morphological studies of the thymus ontogeny were only done from stage 3 and onwards, which correspond to the stage prior to where the first transcripts were found to be slightly elevated in whole larvae. The first sign of the thymus anlage in ballan wrasse larvae was observed dorsally in the opercular cavity at stage 3 (Figure 5A), where distinct large undifferentiated cells and few thymocyte-like cells with high nucleus to cytoplasm ratio were detected. At stage 4, the thymus as an organ became morphologically distinguished and small cells with the characteristic morphology of thymocytes were more abundant. A high density of mucous-like cells was observed in the epithelium delimiting the thymus at stage 4 (arrow in Figure 5B) which corresponds to the start point of formulated feed, whereas none were observed at older stages. The thymus of larvae fed barnacle nauplii also showed abundant mucous-like cells in the epithelium exclusively at stage 4 (data not shown). The thymus became more prominent and a weak demarcation into cortex- and medulla- like zones was observed at stage 6 (Figure 5D), with a darkly stained cortex due to the higher density of thymocytes, and a paler stained medulla less densely packed with thymocytes. At stage 5 and 6, blood vessels were found to be more visible in the thymus parenchyma (Figures 5C, D). No apparent differences were observed in the morphology of the thymus between the two start-diet groups.

## 3.5 Validation of anti-wrasse cCD3e antibodies and immunohistochemistry of the thymus

The anti-wrasse CD3ε was found to react with a protein of the expected molecular mass of CD3ε in thymus (theoretical peptide weight 19,62 KDa), whereas no reactivity was observed in muscle used as control, gills, nor spleen (Supplementary Figure 1). A weak

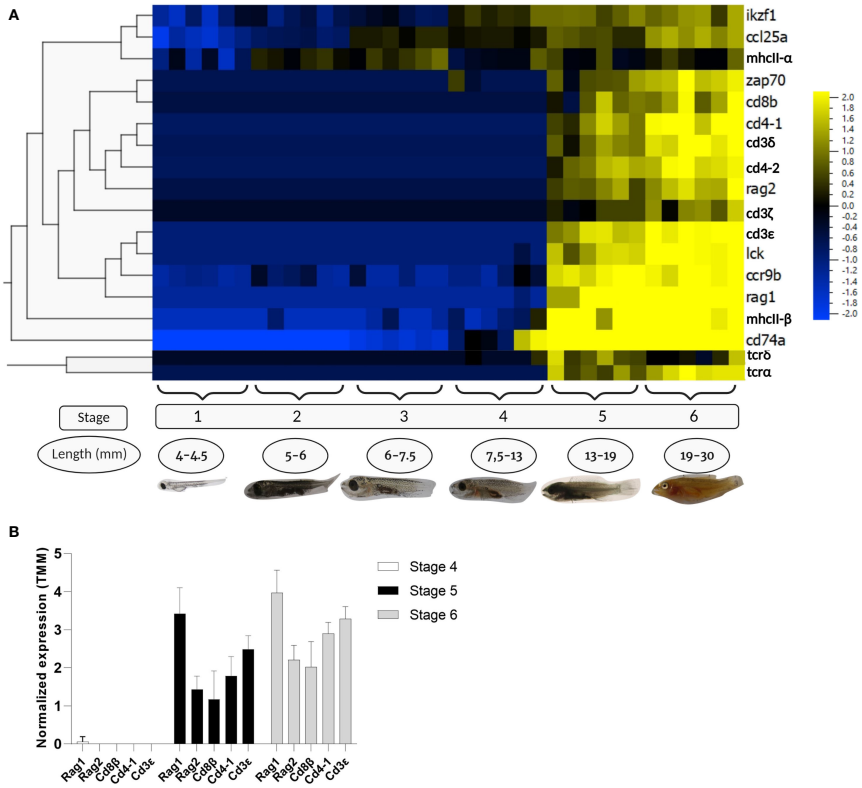


FIGURE 4
RNA transcriptomic data from different stages of wrasse larvae. Data were logarithm converted, normalized for differences in library size applying weighted trimmed mean expression ratios [trimmed mean of M values (TMM)], and expressed as mean = 0. **(A)** Heat map of RNA transcripts showing hierarchical clustering of the selected T-cell markers and non-lymphocytic thymus cells markers in the developmental stages of ballan wrasse. The figure was generated using Qlucore omics explorer software. **(B)** RNA transcripts of T-cell markers corresponding to *RAG1, RAG2, CD8β, CD4-1*, and *CD3ε* from larvae at stage 4, 5, and 6. Created with BioRender.com.

cross-reactivity was, however, detected in gills at approximately 75 kDa. The polyclonal anti-wrasse CD3ε antibody was used to identify CD3ε$^+$ cells in the thymus of ballan wrasse larvae (Supplementary Figure 1). Cross reaction of the antibody was observed in neural tissue (especially in the ganglion) and epithelial cells, and therefore a monoclonal anti-wrasse CD3ε antibody is needed to be developed to avoid background staining.

## 3.6 *In situ* hybridization

Based on transcriptomic data, *in situ* hybridization on lateral and cross-sections of ballan wrasse larvae was done on stage 5, 6, and juvenile fish, coinciding with the upregulation of transcripts corresponding to T-cell specific markers. *In situ* hybridization shows a clear demarcation between cortex and medulla in the thymus of ballan wrasse larvae (Figure 6). *RAG1* signal was prominent in the cortex at stage 5 but very scattered in the medulla whereas *CD3ε* was detected in the whole organ being clearly visible in the medulla (Figures 6A, B). The same pattern was observed in juveniles (SL > 3,5 cm) (Figures 6C, D). In accordance with this, *CD4-1*$^+$ and *CD8β*$^+$ cells were more abundant in the cortex than in the medulla (Figures 6E–H), probably staining DP and SP T-cell populations in the cortex and medulla, respectively. CD4-1$^+$ cells were found to be more abundant compared to *CD8β*$^+$ in the thymus of investigated larvae (Figures 6E–H). Scattered *RAG1*$^+$ cells and *CD3ε*$^+$ cells were also observed in the head kidney of investigated larvae from stage 5, and onwards but to a much lesser extent compared to the thymus (Figure 7). *In situ* hybridization using the negative probe (*DapB*) did not give any signal.

Localization of *CD4-1*$^+$, *CD8β*$^+$, *CD3ε*$^+$, and *RAG1*$^+$ cells were also analyzed in serial sections of gill and gut in a total of 8 larvae corresponding to stage 5, 6, and juveniles. *CD4-1*$^+$ cells were not observed in gill nor in gut at the early substage 5 (SL: 1,6cm) and very few were seen at late substage 5 (SL: 1,8 cm) (data not shown). The number of *CD4-1*$^+$ cells and putative helper T-cells (*CD4-1*$^+$ *CD3ε*$^+$) increased in gill and gut throughout stage 6 and juveniles

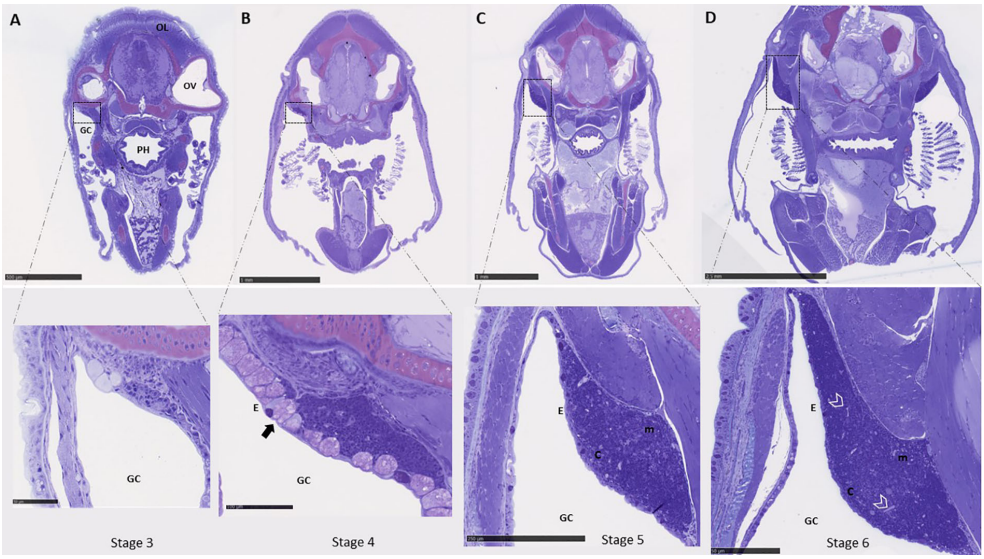


FIGURE 5
Histological examination of the thymus ontogeny in ballan wrasse. **(A-D)** show cross-sections of the whole head where the paired thymus can be observed dorsally in the opercular cavity. The left-side thymus is shown in higher magnification. **(A)** Correspond to larvae in stage 3 (SL: 6-7,5 mm). **(B)** larvae in stage 4 (SL: 7,5-13 mm). Mucous-like cells within the epithelium are indicated by an arrow. **(C)** larvae in stage 5 (13-19 mm), and **(D)** larvae in stage 6 (19 to 30 mm). Blood vessels are delimited by arrow heads. OL, Optic lobes; OV, Optic vesicle; PH, Pharynx; GC, Gill cavity; E, Epithelium; c, cortex; and m, medulla. Scales bars are as follows: **(A)** above 500 µm and below 50 µm, **(B)** above 1mm and below 100 µm, **(C)** above 1 mm and below 250 µm, and **(D)** above 2.5 mm and below 50 µm.

(Figures 8D–I). *CD8β*$^+$ cells were not found in gill, gut, and pharynx of juveniles (Figures 8A–C). *CD8β*$^+$ and *CD4-1*$^+$ cells were observed in the head kidney of larvae at stage 6 (Figure 7).

## 3.7 Differential expression of RNA-transcripts

The two start-feed diets triggered large differences in the transcriptome within stages 1-4 whereas few genes were significanlty affected by diets (q< 0.05) in stage 5 and none in stage 6 (Figure 9A).

Among the selected T-cell markers in this study, the barnacle nauplii diet triggered earlier expression of the recombination-activating genes *RAG1* and *RAG2* (Figure 9B). Significances were as follow; *RAG1* (Holm-Šídák; p values of 0.01, 0.01, and 0.03 in stage 3, stage 4 and stage 5 correspondingly. *RAG2* (Mann-Whitney; p values of 0.01 in stage 5). Accordingly, *TCRα, CD3ϵ, ZAP70*, and ikaros (*IKZF1*) transcripts were significantly more abundant in the larvae fed barnacle nauplii (Figure 9B). Significances were as follow; TCRα (Mann-Whitney; p values of 0.02 in stage 5). CD3ϵ (Mann-Whitney; p values of 0.03 and 0.03 in stage 5 and stage 6 correspondingly). *ZAP70* (Holm-Šídák; p values of 0.04 in stage 6). *IKZF1* (Mann-Whitney; p value of 0.01 in stage 6). Only p values < 0.05 are hereby presented.

## 3.8 Thymus volume

The volume of the thymus was measured in 3 larvae at each of the developmental stages 4, 5 and 6. The barnacle nauplii diet resulted in a significantly larger volume of both right and left side thymus (glm; p value = 0.0008) (Figure 10).

# 4 Discussion

The study describes the timing of thymus and T-cell development in ballan wrasse, a process that seems to be similar to that in other teleosts (50–53). The first histological observation of the wrasse thymus anlage was at stage 3 with few thymocyte-like cells which increased considerably in numbers at stage 4. During these stages (stage 3 and 4), mRNA transcripts of T-cell specific markers were absent indicating that T-cell maturation had not yet started. Although the development of other lymphoid organs has not been addressed in ballan wrasse, the thymus appeared at the same time as the gut starts rotating and acquire the characteristic intestinal loop of this a-gastric species (2) which is an important morphological trait of the digestive system. Stages 3 and 4 were however, characterized by the expression of genes with important implications in early T-cell development such as ikaros (*IKZF1*), *CCL25α, MHCIIβ*, and *CD74α. IKZF1* is required for lymphocyte

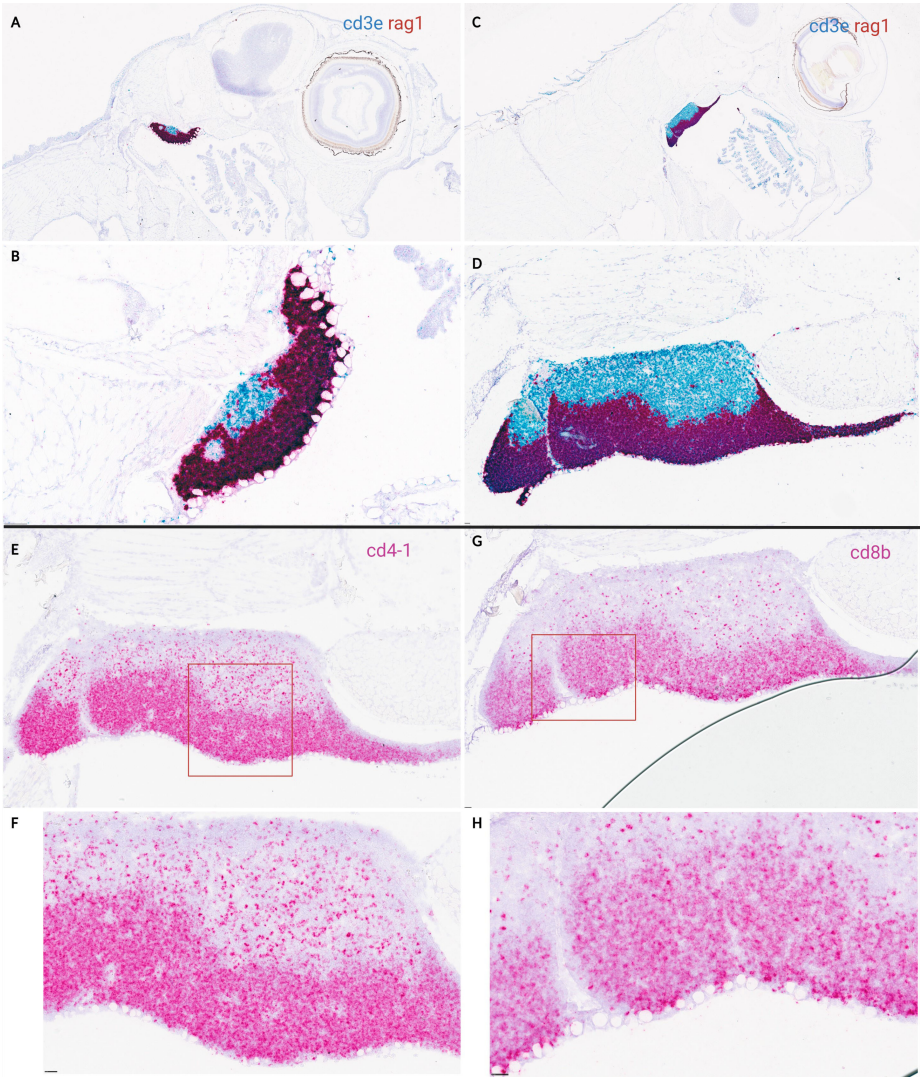


FIGURE 6
*In situ* hybridization on lateral sections of ballan wrasse thymus in larvae at stage 5 and juveniles. **(A-D)** shows duplex assays using RAG1(red) and CD3ε (blue) RNA scope probes. **(E-H)** shows *in situ* hybridization (single assay) on serial sections of juveniles' thymus using either CD4-1probes **(E-F)** or CD8β **(G-H)** probes. **(A)** Larvae corresponding to early substage 5 (SL: 1,6 cm). **(B)** Higher magnification of **(A)**, **(C)** Juvenile (SL: 3,5 cm). **(D)** Higher magnification of **(C)**, **(E, G)** are juveniles (SL: 3,5 cm), and the area delimited by a red box is shown in higher magnification in **(F, H)** correspondingly. Scale bars: **(B)** 50 µm, and **(D-H)** 25 µm.

development in mice (54) and zebrafish (55, 56). CCL25α is a chemokine expressed in the thymic epithelium that attracts dendritic cells, thymocytes, and macrophages in mammals (22, 57), and seems responsible for thymus homing in zebrafish (58). The expression of both *CCL25α* and *IKZF1* in ballan wrasse coincides with the colonization of the thymus by precursors of lymphoid cells and precede the expression of the recombination activating gene-1 (*RAG1*) as it was described in zebrafish (56). The characterization of the *MHCII-β* chain in several teleosts allowed researchers to study the distribution of the main TCR-αβ T-cell populations within the thymus. For example, Atlantic salmon (32) and rainbow trout (33) presented abundant $MHCII^+$ cells in the medulla compared to the outer region of the thymus suggesting that $MHCII^+$ cells are involved in positive selection of developing T-cells in a similar fashion as in higher vertebrates (30, 32, 59). In line with this, Picchietti et al. (31) also demonstrated the higher abundance of

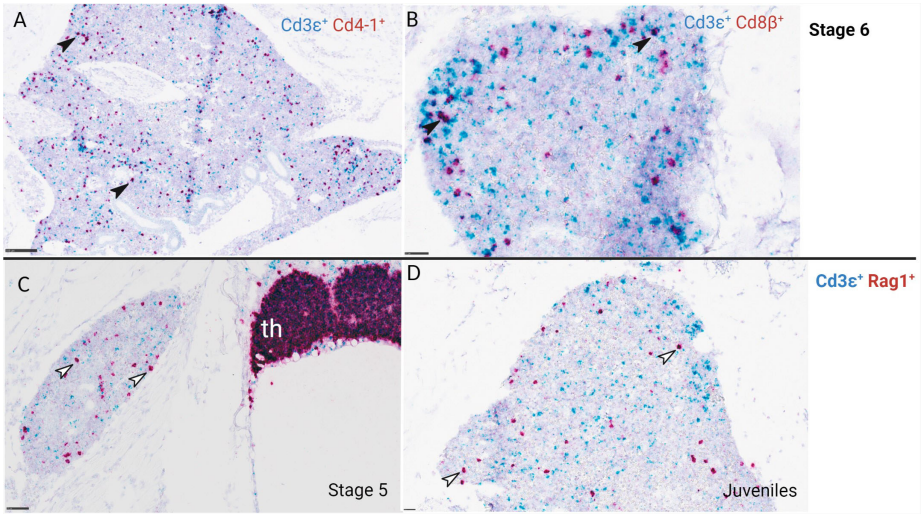


FIGURE 7
RNA scope *in situ* hybridization of ballan wrasse head kidney from larvae at stage 5, 6, and juveniles. **(A)** *CD3ε*$^+$ (blue) and *CD4-1*$^+$ (red) cells, larva at stage 6. **(B)** *CD3ε*$^+$ (blue) and *CD8β*$^+$ (red) cells, larva at stage 6. Black arrows indicate double stained lymphocytes that are either *CD3ε*$^+$ *CD4-1*$^+$ T-cells or *CD3ε*$^+$ *CD8β*$^+$ T-cells. **(C, D)** show *CD3ε*$^+$ (blue) and *RAG1*$^+$ (red) cells of larvae at stage 5 and juveniles correspondingly. White arrows indicate *RAG1*$^+$ cells in the head kidney. Th, thymus. Scale bars: **(A)** 50 µm, **(B)** 50 µm, **(C)** 100 µm, **(D)** 25 µm.

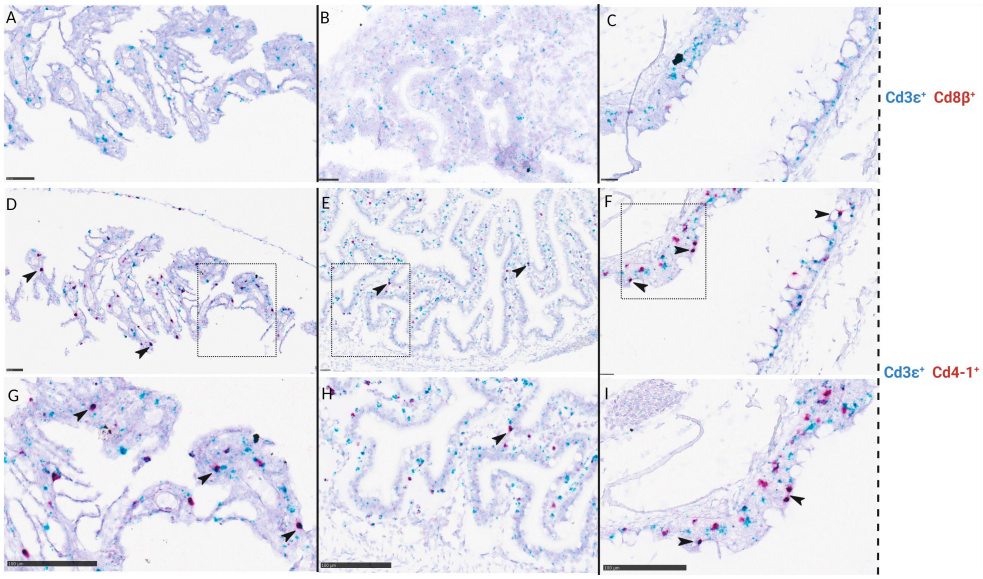


FIGURE 8
*In situ* hybridization of ballan wrasse juveniles (SL> 3,5 cm) shows differences in the amount of *CD4-1*$^+$ cells compared to *CD8β*$^+$ cells on serial sections of gills **(A, D,** and **G)**, gut **(B, E,** and **H)**, and pharynx **(C, F,** and **I)**. **(A-C)** are duplex assays using CD3ε (blue) and CD8β (red) RNA scope probes. **(D-F)** are duplex assays using CD3ε (blue) and CD4-1 (red) RNA scope probes. Areas delimited by a box in **(D-F)** are shown in higher magnification in **(G-I)** respectively. Arrows indicate putative *CD3ε*$^+$ *CD4-1*$^+$ lymphocytes. Scale bars: **(A)** 50 µm, **(B)** 25 µm, **(C)** 25 µm, **(D)** 50 µm, **(E)** 25 µm, **(F)** 25 µm, **(G-I)** 100 µm.

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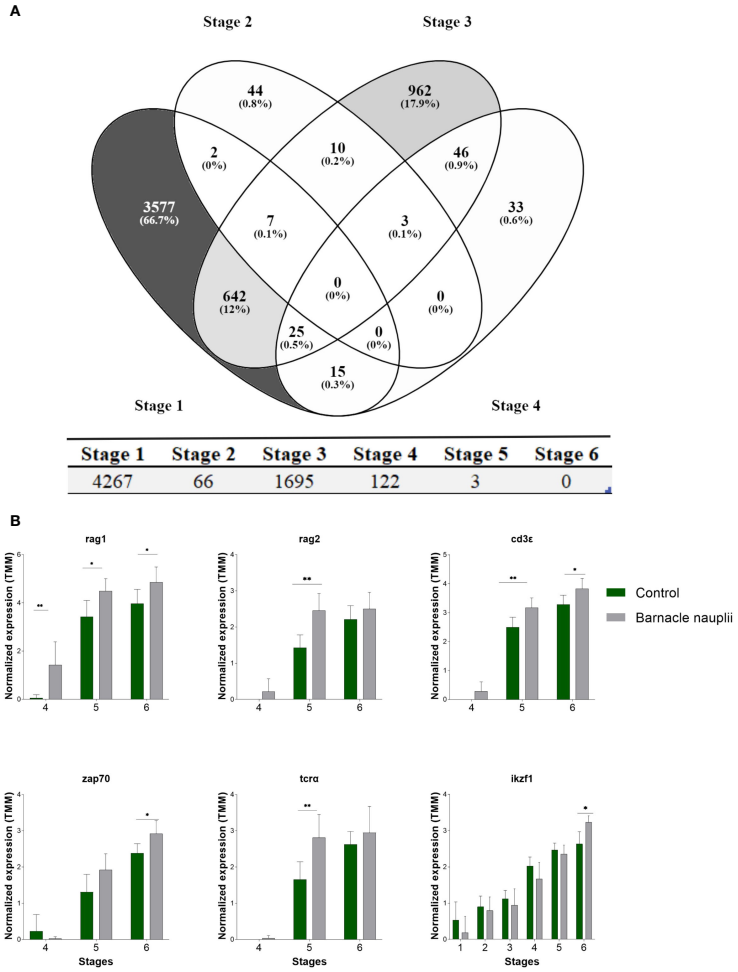


FIGURE 9
Effect of the start-feed diets on the transcriptome of ballan wrasse larvae at different developmental stages. **(A)** Venn diagram showing differentially expressed genes (q <0.05) in the first four larval stages triggered by diets (above panel). The total number of differentially expressed genes within each developmental stage (stage 1 to 6) is displayed below. **(B)** T-cell markers that were significantly affected by diets. Data correspond to transcription levels that were logarithm converted, normalized for differences in library size applying weighted trimmed mean expression ratios (trimmed mean of M values (TMM)), and presented as mean = 0, ± SD (n=3). When P ≤ 0.05 significances are represented by * and when P ≤ 0.01 significances are represented by **.

MHCII$^+$ cells in the medulla compared to the cortex in sea bass larvae. Interestingly, the authors observed an increase of *MHCII-β* transcripts at the same time as lymphoid precursors colonized the primordial thymus that was still devoid of cortex and medulla regions. Similarly, transcripts of wrasse *MHCII-β* and the *CD74α* gene, which codes for proteins involved in the formation of MHCII peptide complexes, increased extensively at the end of stage 4, shortly after thymocytes were observed in the thymus and before the organ became more prominent. Taking all together, the results indicate that the migration of lymphoid cell precursors and the creation of the optimal thymic environment for T-cell development starts at stage 3 and continues during stage 4, prior to the initiation of T-cell maturation. *MHCII-α* transcripts were present at developmental stage 2 and onwards, probably corresponding to populations of innate-like leukocytes.

A morphological change of the thymus occurs at the same time (stage 5) as transcripts of T-cell specific markers (*RAG1, RAG2, LCK, ZAP70, CD3δ, CD3ζ, CD3ε, CD4-1, CD4-2, CD8β, TCRα, and TCRδ*) increased significantly evidencing the start-point of T-cell maturation. Therefore, it seems likely that the thymus contributes to the overall expression of these genes. Wrasse developmental stage 5 include larvae that vary in size with a standard lentgh between 13

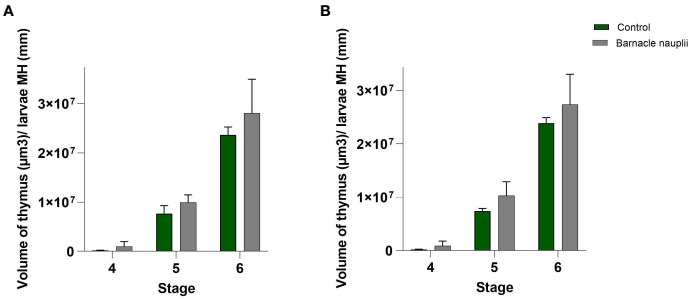


FIGURE 10
Volumetric analysis of the wrasse larvae thymus (n=3). **(A)** Left-side thymus and **(B)** Right-side thymus. MH: Myotome height. The barnacle nauplii diet triggered larger volume of the thymus (glm; p value=0.0008).

and 19 mm accounting for slightly different developmental sub-stages. This is the reason why transcriptomic data at this stage do not provide the exact order of each T-cell marker during T-cell maturation. However, it is plausible to assume that *RAG1* initiates the rearrangment of the *TCR* genes followed by the upregulation of other T-cell markers such as *LCK, ZAP70, CD3, TCRα* and *TCRδ* as it happens in other teleosts (13). Interestingly, *TCRδ* transcripts decreased whereas *TCRα* increased during stage 6, leading to a higher level of *TCR-αβ* transcripts compared to *TCR-γδ* transcripts in juveniles of ballan wrasse as reported in mammals (11). The larvae period from stages 4 to 5 is also when growth increases and the volume of the digestive organs such as gut, liver and pancreas increase dramatically (2).

The classical zonation of the thymus into medulla (inner zone) and cortex (outer zone) seem to vary within species. Several species such as carp (23), zebrafish, rainbow trout, sea bass, halibut, and turbot (*Scophthalmus maximus*), show distinction between zones as reviewed in Barraza et al. (13), and in larvae of rice-field eel (*Monopterus albus*) (60), whereas the cortico-medullary boundary in Atlantic salmon thymus still remains unclear (46) and contradictory results were published in flounder (*Paralichthys olivaceus*) (52). The present work shows a clear zonation into cortex and medulla in the developing thymus of ballan wrasse larvae from stage 5 and onwards, both regarding thymic morphology and gene expression patterns. The cortical region was densely packed with thymocytes, while the emergent medullary region had an increasing number of cells with a smaller nuclear to cytoplasm ratio. Moreover, RAG*1*$^+$ cells were restricted to the outer cortex area, whereas the medulla appeared almost *RAG1*$^-$ in all investigated larvae and juveniles. *CD4-1*$^+$ and *CD8β*$^+$ cell zones within the thymus also evidence a clear demarcation between cortex and medulla as seen with *RAG1*, alike previously reports in seabass, ginbuna carp, rainbow trout and Atlantic halibut (24, 26–29). The detection of *RAG1*$^+$ thymocytes in the cortex is likely to correspond to both DN (CD4$^-$ CD8$^-$) and DP (CD4$^+$ CD8$^+$) thymocytes as reported in mammals and other teleosts (16, 17, 27). Not surprisingly, there were fewer cells in the medulla compared to the cortex that were *CD4-1*$^+$ or *CD8β*$^+$, as only a limited number of thymocytes survive both positive and negative selection processes, and can leave the thymus migrating towards secondary lymphoid organs. The C-C chemokine receptor 9β (CCR9β) has been suggested as a potential marker for thymocyte selection within the thymus of fish (61). Accordingly, ballan wrasse *CCR9β* transcripts were upregulated at stage 5 coinciding with the start-point of T-cell maturation where these two selection processes are needed.

The increased expression and localization pattern of the different T-cell marker genes within the thymus of wrasse larvae implies that the thymus becomes lymphoid at larval stage 5. Moreover, the detection of *CD3*$^+$ cells in the head kidney at stage 5, indicates that mature T-cells have migrated out from the thymus at this timepoint, supported by the identification of *CD4-1* and *CD8* positive cells in the head kidney at stage 6. Interestingly, more *CD4-1*$^+$ cells were found in the thymus of juveniles compared to *CD8β*$^+$ cells, and transcripts of *CD4-1* were more abundant compared to *CD8β* during larvae ontogeny. This higher abundance of *CD4-1*$^+$ cells were also seen in head kidney, suggesting a higher production of mature helper T-cells compared to cytotoxic T-cells in developing ballan wrasse larvae and juveniles. In the present study we also investigated the distribution of helper T-cells (*CD4-1*$^+$ *CD3ε*$^+$) and cytotoxic T-cells (*CD8β*$^+$ *CD3ε*$^+$) in mucosal organs of developing larvae. Helper T-cells (*CD4-1*$^+$ *CD3ε*$^+$) were observed in the gut, gill, and pharynx of wrasse juveniles whereas cytotoxic T-cells were not found in any mucosal tissue at any investigated stage. However, we cannot exclude the possibility that cytotoxic T-cells are present in gut and gill of wrasse larvae. Although the method allowed for identification of *CD8β*$^+$ cells within the thymus, this organ contains an extraordinarily high number of *CD8β*$^+$ cells which is different to mucosal organs where much fewer T-cells are expected. Therefore, the sensitivity of the method might not have been high enough to allow detection of *CD8β*$^+$ T-cells in mucosal organs. Nevertheless, in accordance with these observations, our transcriptome data showed a higher number of *CD4-1* transcripts compared to *CD8β*. CD4 is also expressed by few sub-populations of dendritic cells and macrophages in teleosts (29, 62) and therefore, it is itself not an exclusively marker of helper T-cells. However, the fact that transcripts of both *CD4-1* and *CD8β* were upregulated at the same time during larvae development, and that *CD4-1* was not

detected at an earlier stage as part of possible innate-like leukocytes, suggest that T-cells contribute to the overall expression of these genes at least during the investigated larvae stages. Therefore, the higher abundance of *CD4-1* transcripts supports the higher presence of helper T-cells in the thymus and mucosal organs of wrasse juveniles compared to cytotoxic T-cells indicating an important role of helper T-cells during early larval stages. This is in agreement with the fact that adaptive immunity needs to be stimulated by helper-T cells (62). Furthermore, ballan wrasse is a stomach-less species with a remarkably elevated immune activity in the hindgut (14, 15) that has been proposed to strategically compensate for the lack of stomach (14). One possibility is that the plausible high concentration of secreted immunoglobulins in the gut efficiently act as first line of defense against pathogens that are not inactivated by the acidic environment in the stomach. Abundant intraepithelial $IgM^+$ cells were observed within the gut of adult wrasse together with an extraordinary high amount of IgM in plasma compared to other teleosts (15). We hypothesize that helper T-cells are especially important to boost B-cell activation and antibody production in gut and other mucosal organs in early stages of wrasse larvae when they are most susceptible to diseases and pathogens.

RAG1 and RAG2 are crucial for T- and B-cell receptor rearrangment in developing lymphocytes, processes that are described in primary lymphoid organs, the thymus and head kidney in teleosts (18, 19, 48, 63). However, intraepithelial lymphocytes in the gut of humans (64) and mice (65, 66) are $RAG^+$ suggesting the presence of maturing B- and T-cells within the intestine. Similarly, T-cells isolated from the gut of adult European sea bass express *RAG1* (67). In zebrafish, $RAG1^+$ cells were reported in the gut of adult individuals (68) and a few putative T-cells expressing *RAG1* were reported in the gut of carp at 1 week post fertilization (69). Furthermore, the same authors used a monoclonal antibody for putative intraepithelial T-cells and positive cells were found within the gut prior to the identification of thymocytes in the thymus. Even though the gut is not considered a lymphoyd tissue but rather a tissue containing abundant lymphoid cells in higher vertebrates, Scapigliati et al. (70) suggested the gut of adult fish to be a lymphoid tissue that has retained a primordial lymphopoietic function throughout evolution. The expression of *RAG1* in mucosal organs was also investigated in ballan wrasse larvae (data not shown). There was a weak expression of *RAG1* in gill and gut that did not seem to correspond to leukocytes. Noticeable, positive signals were found in non-lymphoid tissue such as brain and eye which are typically used as internal negative controls for the expression of these immune genes. Furthermore, *RAG1* transcripts in the gut of adult wrasse appear to be absent (3). Althogether, the results indicate lack of *RAG1* expression in mucosal organs and no evidence for extrathymic development of T cells in ballan wrasse.

It is well established that the innate and adaptive immune systems are extensively related with no clear border between them. For instance, T-cells that are classically described as adaptive immune cells may have adaptive (TCR based) functions as well as innate (cytokine based) functions making interactions between innate and adaptive systems crucial for a successful immune response. When we keep an animal in captivity we have the obligation to meet the dietary requirements for the animal at all developmental stages to ensure healthy and robust fish. This is enherentley difficult, especially in larvae where requirements are poorly understood (71). Feed and feed additives can modulate the immune system as indicated in many studies (37, 72, 73).

The total amounts of PUFAs (n-3 and n-6) as well as the dietary DHA/EPA (n-3) ratios are important for growth, reproduction, and survival (74, 75). Modulation of the dietary DHA/EPA ratio and the level of ARA has been reported to affect certain immune responses, however, mostly related to the innate immune system (76–78). In Atlantic halibut larvae, it was suggested that the low level of dietary n-3-HUFA, especially DHA, could be the direct cause of several developmental errors (79). Later, Øvergård et al. (24) analysed T-cell development of halibut larvae and suggested that dietary fatty acid composition of the live feed seemed to modulate the expression of several T-cell marker genes during larvae ontogeny. In the present work, total levels of PUFAs and DHA were highest in rotifers and similar in artemia and barnacles, due to good enrichment practices. However, barnacles showed higher EPA and lower ARA levels compared to rotifers and artemia. EPA and ARA are precursors of eicosanoids and can be anti- and pro-inflammatory, respectively. Thus, the ratio of dietary EPA/ARA determines eicosanoid production and health status accordingly (80). As a general rule, derivates from EPA have been considered anti-inflammatory, while ARA derivates are considered pro-inflammatory (81). Although there may be evidence that this is different or varies in teleosts (82), the abundant levels of EPA found in barnacle nauplii used in the present work could trigger the production of anti-inflammatory eicosanoids as well as enhancing the total amount of PUFA boosting earlier development of adaptive immunity.

Rotifers and artemia were higher in all protein bound amino acids, but not the FAAs proline and taurine. Proline was the most abundant FAA in the barnacle diet which is similar to that reported in wild copepods and zooplankton (83). Taurine is involved in many biological functions and it is important for a successful development of marine larvae (84–86). Its deficiency can cause oxidative stress and lipid accumulation among others (85, 87, 88). Northern rock sole (*Lepidopsetta polyxystra*) larvae performed better when fed with rotifers enriched with taurine (84). Authors observed that taurine supplementation yielded larvae with higher dry weight but not higher standard length, as observed in the present work in ballan wrasse. Taurine was higher in small barnacle compared to rotifers but higher in artemia compared to large barnacle, in contrast to copepod nauplia that are well characterized for having high levels of taurine compared to rotifers and artemia (86). Taking into account the beneficial role of taurine for larvae development (84, 86), we speculate that the relatively low levels of taurine present in barnacles might cause the lack of a clear positive effect of the barnacle diet on the performance of wrasse larvae.

Extensive work on the requirements for minerals and vitamins for marine larvae has been reported (71, 86). Mæhre et al. (86) recommended that the composition of zooplankton, which is the natural prey of most marine larvae should be used as reference. Vitamins have broad implications such as antioxidants and immune modulators, and deficiencies can cause large losses in marine larvae production as reviewed in (89). Enriched rotifers and artemia showed higher amount of all investigated vitamins compared to barnacles in the present work. Vitamin A was under the detection limit which is comon in zooplankton although ballan wrasse can probably convert both astaxanthin and canthaxanthin to vitamin A as it happens in other fish species (90, 91). The astaxanthin levels in the barnacle diets were similar to enriched rotifers and artemia whereas canthaxanthin was only present in barancles at low levels. This indicates that a diet composed solely of barnacle nauplii might contain lower levels of natural carotenoids or at least, a different profile than wild zooplankton (83, 92). All the vitamins in barnacles were present at a much lower levels than in wild harvested copepods (71, 83).

Overall, iodine was the only mineral that was higher expressed in both small and large barnacle compared to rotifers and artemia. Iodine supplementation in larvae has been reported to improve the thyroid hormone status in halibut and Senegalese sole larvae (*Solea senegalensis*) (93, 94). Furthermore, thyroid hormones are modulators of the innate immune system and have implications on cells that are important for mounting an adaptive response (95). More differences in the mineral content were observed when comparing the start-feed diets based on the timeline when they were administrated to the tanks; small barnacle were significantly higher in V, Mn, Co, Zn, As, Se, and Ca compared to rotifers. Rotifers and small barnacle nauplii were the first foods given to larvae. Ma, Co and Se are important co-factors in antioxidant enzymes and therefore, important to protect against lipid oxidation (71). Optimal levels of selenium enhance the innate response and deficiency decreases the number of B-cells in humans (36). Zinc and selenium are naturally more abundant in copepods compared to enriched rotifers (71, 86) which is in accordance with our results. Interestengly, zinc deficiency seems to affect primarily T-cells, leading to apoptosis of double positives thymocytes in the cortex and thymic atrophy (34, 36). Artemia and large barnacle nauplii were introduced at 19 dph and 15 dph correspondingly and had similar mineral profiles.

Nutritional requirements for larvae are difficult to investigate and studies adressing this matter are rather scarce (83, 89, 96). In this study, barnacle-fed larvae showed that important genes related to T-cell development were upregulated earlier and the size of the thymus was larger. A clear correlation between the size of the thymus and its capacity to produce mature T-cells is lacking in teleosts (13, 23, 97). Although we did not address the abundance of lymphocytes in the thymus of investigated larvae, it is likely that the experimental barnacle feed was favourable regarding T-cell development and possibly reflecting a healthier larvae production. The experimental diet composed solely of barnacle nauplii seems to be somewhat low in important nutrients characteristic of a zooplankton diet such as taurin, vitamins and carotens. On the other hand, the barnacle nauplii diet was higher in EPA with a high n-3/n-6 ratio, it contained higher levels of iodine as well as microminerals such as Mn, Co, Se, and zinc. These nutrtional traits might be directly related to the earlier onset of adaptive immunity in ballan wrasse larvae.

## 5 Conclusion

Similar to other teleosts, the thymus of ballan wrasse becomes lymphoid at stage 5 of larvae development. At this stage, there is a clear distinction between the cortex, where TCR rearrangement takes place and thymocytes are *RAG1*$^{+}$, and the medulla, which is involved in negative selection processes where most T-cells are *RAG1*$^{-}$. Although it seems that a cortico-medullary division is present in most teleosts being a potential common feature of bony fish, there is not yet consensus on its organization. The localization of *RAG* is a key tool for thymus zonation (98) and should be used for elucidating existing disagreements. Wrasse larvae at stage 6 and juveniles possess helper T-cells in mucosal organs which might be crucial to activate antibody-secreting B-cells and recruit other leukocytes to the gut of this a-gastric species. Although results indicate that immunological competence is present at least, to some extent in juveniles of ballan wrasse, the study of B-cell development and the timing of appearance of IgM$^{+}$cells able to secrete IgM is needed for establishment of effective prophylactic measures. Interestingly, a start-feed diet composed of barancle nauplii seems to trigger an earlier onset of adaptive immunity in ballan wrasse larvae.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, GSE200208.

## Ethics statement

The animal study was reviewed and approved by The Directorate of Fisheries of Norway (permission nr. VL-AV-0011 given to the Institute of Marine Research station at Austevoll (location nr. 16195)). The experiment and sampling followed the Norwegian animal welfare act guidelines, in accordance with the Animal Welfare Act of 20th December 1974, amended 19th June 2009. The facility has a general permission to conduct experiments involving all developmental stages of fish (code 93) provided by the Norwegian Animal Research Authority (FDU, www.fdu.no).

## Author contributions

ØS, IH, and AE designed the experiments. AE and RB participated in carrying out the experiment. AE, ØS, KL, and IH: analyses. AE wrote the manuscript. AE, IH, A-CØ, KL, and ØS: editing. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1166785/full#supplementary-material

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*Supplementary Material*

# The thymus and T-cell ontogeny in ballan wrasse (*Labrus bergylta*) is nutritionally modelled

**Angela Etayo[1,2]*†, Kai K. Lie[1], Reidun M. Bjelland[3], Ivar Hordvik[2], Aina-Cathrine Øvergård[2] and Øystein Sæle[1]**

[1] Institute of Marine Research, Bergen, Norway

[2] Fish Health group, Department of Biological sciences, University of Bergen, Norway

[3] Institute of Marine Research, Austevoll Research Station, 5392 Storebø, Norway

*** Correspondence:**

Angela Etayo

Email: angela.etayo@uib.no

## 1 Supplementary Data

**Supplementary Data 1.** Cultured conditions of rotifers, artemia and barnacles used in the experiment.

A deep inlet of full seawater from the proximity of the facility in Austevoll was used for rearing of rotifers, artemia and barnacles. The same water was used for larvae production. The culture conditions of live prey feed were as following:

- Small and large barnacle nauplii (planktonic diet) were provided by @planktonic as cryoPlankton shipped in cytogenetic dewars (liquid nitrogen at -196 ºC). Preparing the live feed was done in a few simple steps that included thawing, rinsing, and revitalizing the nauplii until their normal swimming activity was resumed. Thawing and rinsing was done for few minutes with continuous stirring followed by revitalization of the nauplii with > 50 % oxygen saturation overnight at <5 ºC (Revitalized nauplii have a maximum of 36 hours storage). At this stage the nauplii were ready to be fed to the fish larvae and were brought to the tank facility where they were bumped into the tanks.

- The rotifer strain at the Austevoll Research Station is a uniform culture of *Branchonus plicatilis* "cayman". Rotifers are fed on algae *Nannochloropsis* and *Tetraselmis* that is shipped as a frozen product from the Norwegian supplier Microalgae AS, which is based at Vigra. Dry yeast is also used as growth feed (egg development) in rotary culture. Multigrain is used as enrichment (https://www.biomar.com/en/larviva/hatchery-for-fish/emea/). The amount of Multigrain was 0.15 g per million rotifers. The production tanks were kept at 22.5 ± 1ºC with an oxygen saturation of 80%. Thoroughly washed rotifers were transferred to the tank and fed every hour by automatic feeding. The rotifers in the production tank were transferred to the enrichment tank through a washing station where rotifers were washed before enrichment. The

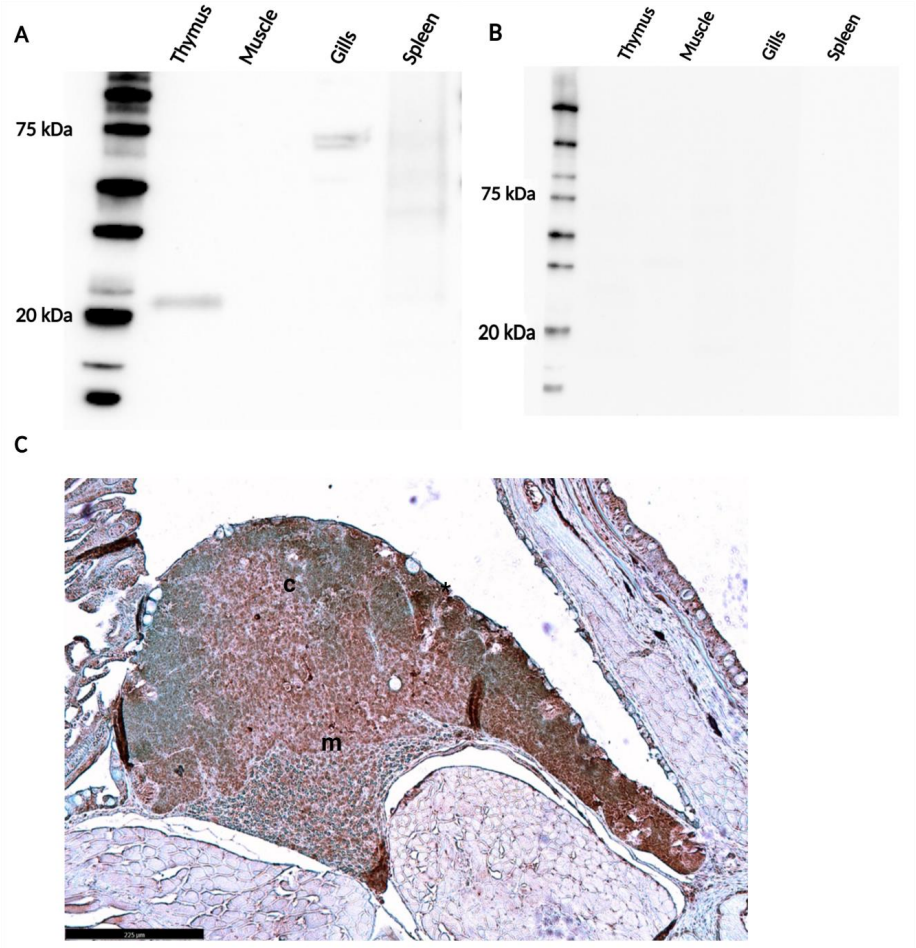
enrichment tanks have a density of 1500 ± 500 rotifers/ml = 1.5 ± 05 million rotifers/liter, and a temperature of 22.5 ± 0.5 ºC with an oxygen saturation of 100% was kept constant. A total of 0.15 g Multigrain per million rotifers is added to the tanks. Quality controls of enriched rotifers were made to check density, vitality and contamination before being administrated to the larvae. The vitality of the rotaries is assessed based on swimming activity.

- Artemia cyst (EG Artemia Cysts) are bought from INVE Aquaculture (https://www.inveaquaculture.com/product/artemia-eg-gsl-inve-blue/ ). The cyst density in the hatching tank did not exceed 2.5g cysts/ liter of seawater. The hatching tank was kept at 28 ºC with an oxygen saturation of 100-200 %, continuous light and salinity level of 33-35%. The cysts hatched after 24-26 hours, then the artemia was washed at least for 10 min before being transferred to the enrichment tanks. Enrichment tanks are kept at 26 ºC, with an oxygen saturation of 100-200 % and a salinity level of 33-35 %. The artemia nauplii were transferred to the enrichment tank in the afternoon where 1L of the multigrain was delivered automatically 4 times per day, the last feeding the following day at 6 am. The artemia was washed first in temperate seawater for 3 min, posteriorly with freshwater for 10 min or until the salinity was <0,3 and a last wash in cold seawater until the salinity was >33%. The artemia was then quality checked and concentrated into the feeding tank. Vitality, and the degree of gut filling with multigrain was checked at this point and administered to the larvae.

Live prey (rotifers, artemia, and barnacle) was administrated to the corresponding tanks by slowly pumping 3 times per day (morning, afternoon, and evening). The evening meal was given at a slower speed so that the larvae had food available overnight until the next morning feeding occurred.

## 2 Supplementary Figures and Tables

### 2.1 Supplementary Figures




**Supplementary Figure 1. (A)** Western blot of protein extracts from ballan wrasse tissues incubated with an affinity purified rabbit polyclonal anti-CD3ε antibody. The expected molecular mass of wrasse CD3e is 19,6 kDa. A positive band at the expected molecular weight was observed in thymus while being absent in the rest of the investigated tissues. **(B)** Immunoblot omitting primary antibody. **(C)** Cross-sections of head from ballan wrasse larva at stage 6 showed CD3ε$^{+}$ cells expressed in the thymus visualized with brown staining. Epithelial staining is due to a cross reaction with a 75 kDa protein. Background stain: methyl green. Antibody dilution 1:100. m: medulla. c: cortex. Asterisks indicate gill cavity. Scale bar = 225 µm.

## 2.2 Supplementary Tables

**Supplementary Table 2.** References of the methodology used for nutrient analysis.

| Analyses | Principle | Reference |
|---|---|---|
| Vitamin C | HPLC | (Mæland and Waagbø, 1998) |
| Biotin | Microbiological fertilization | (Mæland et al., 2000) |
| Folate | Microbiological fertilization | (Mæland et al., 2000) |
| Niacin | Microbiological fertilization | (Mæland et al., 2000) |
| Pantoten | Microbiological fertilization | (Mæland et al., 2000) |
| Vitamin B6 | HPLC | (CEN, 2005) |
| Thiamine | HPLC | (CEN, 2003a) |
| Riboflavin | HPLC | (CEN, 2003b) |
| Cobalamin | Microbiological fertilization | (Mæland et al., 2000) |
| Vitamin A | HPLC | (Moren et al., 2004) |
| Vitamin D | HPLC | (CEN, 1999) |
| Vitamin E | HPLC | (Hamre et al., 2010) |
| Vitamin K | HPLC | (CEN, 2003c) |
| Astaxanthin | HPLC | (CEN,2011) |
| Canthaxanthin | HPLC | (CEN,2011) |
| Minerals | ICP-MS | (Long and Martin 1990) |
| Iodine | ICP-MS | (Julshamn et al., 2004) |
| HAA (hydrolysed amino acids) | HPLC-UV | Waters, AccQ-TagTM Method. 715001320 |
| Fatty Acid | GC-FID | (Torstensen, Espe et al. 2011) |
| Crude Protein | Combustion | (Simonne et al., 1997) |
| Crude Lipid-ethyl acetate | Isopropanol extraction | NS 9402:1994 |
| Ash | Combustion | (AOAC, 1942) |
| Dry weight | Gravimetric after freeze drying | (Hamre et al., 2006) |

**Supplementary Table 2.** Nutrient analyses of the start-feed diets

Analyzed dietary proximate in rotifers, Artemia, small barnacle (*Balanus crenatu*), and large barnacle (*Semibalanus balanoides).*

| | Rotifers | Artemia | Small barnacle | Large barnacle |
|---|---|---|---|---|
| **Proximate composition (g/ 100g DW)** | | | | |
| Protein | 51±0[a] | 36±9[b] | 42±17[abc] | 52±22[ac] |
| Lipid | 16±4 | 13±6 | 8±4 | 9±2 |
| Ash | 36±11 | 23±1 | 30±18 | 23±6 |

Values are relative to dry weight (DW) and are given as mean ± SD when value is >1. The number of replicates is 3 (N=3). Significances are indicated by letters.

Analyzed fatty acid composition and total fatty acids (TFA) in rotifers, Artemia, small barnacle (*Balanus crenatu*), and large barnacle (*Semibalanus balanoides).*

| | | Rotifers | Artemia | Small barnacle | Large barnacle |
|---|---|---|---|---|---|
| **Fatty acids (% of TFA)** | | | | | |
| **ΣSFA** | | **22±5** | **22** | **22** | **19** |
| 14:0 | myristic acid | 2±1[a] | 1[a] | 6[b] | 3±1[a] |
| 16:0 | palmitic acid | 18±4 | 14 | 13 | 13±1 |
| 18:0 | Stearic acid | 2 | 5 | 2 | 2 |
| **ΣMUFA** | | **9±1[a]** | **28±1[b]** | **25[b]** | **20±2[c]** |
| 16:1n-7 | Palmitoleic acid | 3[a] | 5[a] | 11[b] | 5±2[a] |
| 18:1n-9 | Oleic acid | 3[a] | 13±1[b] | 4[ac] | 5±1[c] |
| 18:1n-7 | Vaccenic acid | 1[a] | 8[b] | 7[b] | 6[b] |
| **ΣPUFA** | | **65±6[a]** | **45±1[b]** | **50[bc]** | **58±3[ac]** |
| **Σn-3** | | **46±5[a]** | **31±1[b]** | **47±1[a]** | **55±3[a]** |
| 18:3n-3 | α-Linolenic acid | 0[a] | 9±2[b] | 0[a] | 1[a] |
| 18:4n-3 | Stearidonic acid | 0 | 1 | 2 | 2 |
| 20:5n-3 | EPA | 7±1[a] | 9[a] | 28[b] | 32±4[b] |
| 22:6n-3 | DHA | 34±6[a] | 11±2[b] | 15[b] | 19±2[b] |
| **DHA/EPA** | | 5 | 1 | 1 | 1 |
| **Σn-6** | | **19±1[a]** | **14[b]** | **2[c]** | **3±1[c]** |
| 18:2n-6 | LA | 4±1[a] | 6[b] | 1[c] | 1[c] |
| 20:4n-6 | ARA | 2[a] | 3[b] | 1[c] | 1[c] |
| **n-3/n-6** | | **2[a]** | **2[a]** | **26±2[b]** | **21±7[b]** |
| **TFA (µg/Kg DW)** | | **4593±934** | **1323±144** | **631±289** | **777±252** |

Values are relative to dry weight (DW) and are given as mean ± SD when value is >1. The number of replicates is 3 (N=3). Values indicated as trace amounts (tr) correspond to values that were detected but are below quantification limits of the analytical method. Significances are indicated by letters. TFA - total fatty acids; SFA – saturated fatty acids; MUFA - monounsaturated fatty acids; LA – linoleic acid, ARA-arachidonic acid, EPA - eicosapentaenoic acid; DHA - docosahexaenoic acid; PUFA - polyunsaturated fatty acids.

Analyzed minerals in rotifers, Artemia, small barnacle (*Balanus crenatu*), and large barnacle (*Semibalanus balanoides).*

| | Rotifers | Artemia | Small barnacle | Large barnacle |
|---|---|---|---|---|
| **Micro-mineral composition (µg kg$^{-1}$ DW)** | | | | |
| V | 5±1$^{a}$ | 34±47$^{ab}$ | 88±5$^{b}$ | 26±12$^{ab}$ |
| Cr | 4±2 | 32±30 $^{*}$ | 49±6 | 87±102 |
| Mn | 98±29$^{a}$ | 313±429$^{a}$ | 1170±142$^{b}$ | 159±84$^{ac}$ |
| Fe | 2020±586 | 11496±15911 | 26590±1421 | 15313±9672 |
| Co | 2±1$^{a}$ | 7±9$^{ab}$ | 16±1$^{b}$ | 6±4$^{ab}$ |
| Ni | 3* | 90** | 65±8 | 45±13 |
| Cu | 122±39 | 158±76 | 136±4 | 181±96 |
| Zn | 203±85$^{a}$ | 2220±2536$^{ab}$ | 4343±667$^{b}$ | 5825±843$^{b}$ |
| As | 1$^{a}$ | 84±61$^{ab}$ | 165±52$^{bc}$ | 295±53$^{c}$ |
| Se | 3±1$^{a}$ | 10±7$^{ab}$ | 19±3$^{b}$ | 15±2$^{b}$ |
| Mo | 5±1* | 15** | 10 | 8±1 |
| Ag | tr | 1* | 1* | 0.5* |
| Cd | 1 | 8** | 6±1 | 4±2 |
| Hg | tr | tr | 0.3 | 0.2 |
| Pb | 6±2 | 14±14 | 19±4 | 5±4 |
| Jod | 58±26$^{a}$ | 244±300$^{a}$ | 2819±523$^{b}$ | 736±154$^{c}$ |
| **Macro-mineral composition (mg kg$^{-1}$ DW)** | | | | |
| Ca | 39$^{a}$ | 83±36$^{ab}$ | 460±225$^{b}$ | 284±139$^{ab}$ |
| Na | 584±105$^{a}$ | 1721±451$^{b}$ | 753±322$^{a}$ | 673±234$^{a}$ |
| K | 109±6 | 111±17 | 73±7 | 80±26 |
| Mg | 66±10$^{a}$ | 207±35$^{b}$ | 117±32$^{a}$ | 99±32$^{a}$ |
| P | 115±16 | 66±16 | 94±23 | 115±13 |

Values are relative to dry weight (DW) and are given as mean ± SD when value is >1. The number of replicates is 3 (N=3) unless otherwise specified by *. Values indicated as trace amounts (tr) correspond to values that were detected but are below quantification limits of the analytical method. ANOVA test was applied only when N=3 and values are numeric (nutrients with trace elements in at least one of the diets, were not legit for statistics). Significances are indicated by letters.

$^{*}$ N=2. One out of the three samples analyzed were found in trace amounts and the expressed value corresponds to two replicates.

$^{**}$ N=1, no SD. Two out of the three samples analyzed were found in trace amounts and the expressed value corresponds to only one replicate.

Analyzed vitamins and pigments in rotifers, artemia, small barnacle (*Balanus crenatu*), and large barnacle (*Semibalanus balanoides).*

| | | Rotifers | Artemia | Small barnacle | Large barnacle |
|---|---|---|---|---|---|
| **Water soluble vitamins (µg kg$^{-1}$ DW)** | | | | | |
| Vitamin C | Ascorbic acid | 23363±3575$^{a}$ | 7275±1138$^{b}$ | 512±373$^{b}$ | 5209±6130$^{bc}$ |
| Vitamin B1 | Thiamine | 1374±934 | 76±12 | 36±16 | 35±4 |
| Vitamin B2 | Riboflavin | 479±228 | 312±23 | 322±123 | 471±113 |
| Vitamin B3 | Niacin | 4111±1820$^{a}$ | 1413±161$^{ab}$ | 310±129$^{b}$ | 660±145$^{b}$ |
| Vitamin B6 | Pyridoxine | 412±224 | 230±90 | 79±45 | 76±6 |
| Vitamin B7 | Biotin | 53±28$^{a}$ | 36±2$^{ab}$ | 4±1$^{b}$ | 7±1$^{b}$ |
| Vitamin B9 | Folate | 141±15$^{a}$ | 93±1$^{b}$ | 22±24$^{c}$ | 9±8$^{c}$ |
| Pantoten | | 2622±1041$^{a}$ | 904±24$^{b}$ | 343±190$^{b}$ | 658±213$^{bc}$ |
| Vitamin B12 | Cobalamin | 59±33$^{a}$ | 53±2$^{a}$ | 4±1$^{b}$ | 6±3$^{b}$ |
| **Lipid soluble vitamins (µg kg$^{-1}$ DW)** | | | | | |
| Vitamin A1 | | tr | tr | tr | tr |
| Vitamin A2 | | tr | tr | tr | tr |
| Vitamin D3 Cholecalcipherol | | 4±3 | 2±1 | tr | tr |
| Vitamin E [1] | | 24536±16785$^{a}$ | 5360±1855$^{ab}$ | 889±393$^{b}$ | 884±173$^{ab}$ |
| Vitamin K [2] | | 105±7$^{a}$ | 13±6$^{b}$ | 8±2$^{b}$ | 3±1$^{b}$ |
| **Pigments (µg kg$^{-1}$ DW)** | | | | | |
| Astaxanthin | | 1374±912 $^{a}$ | 41±2 $^{ab}$ | 28±20$^{b}$ | 60± 10 $^{ab}$ |
| Canthaxanthin | | tr | tr | 4** | 7** |

Values are relative to dry weight (DW) and are given as mean ± SD when value is >1. The number of replicates is 3 (N=3) unless otherwise specified by *. Values indicated as trace amounts (tr) correspond to values that were detected but are below quantification limits of the analytical method. ANOVA test was applied only when N=3 and values are numeric (nutrients with trace elements in at least one of the diets, were not legit for statistics). Significances are indicated by letters.

[1] The value is the sum of tokoferol-α/β/γ/δ, and tocotrienol- α/β/γ/δ

[2] The value is the sum of vitamin k1, ß,Y-Dihydro vitamin K1, and the different Vitamin K2 (MK4, MK5, MK6, MK7,MK8, MK9, and MK10).

** N=1, no SD. Two out of the three samples analyzed were found in trace amounts and the expressed value corresponds to only one replicate.

Analyzed protein-bound amino acids (PAA) and free amino acids (FAA) in rotifers, artemia, small barnacle (*Balanus crenatu*), and large barnacle (*Semibalanus balanoides).*

| | Rotifers | Artemia | Small barnacle | Large barnacle |
|---|---|---|---|---|
| **Protein-bound amino acids (PAA)** | | | | |
| **Essential amino acids (µg/Kg dw)** | | | | |
| Histidine | 207** | 76** | 63±28 | 62±2 |
| Arginine | 695±92 | 255±1 | 200±88 | 203±7 |
| Threonine | 570±76 | 197±1 | 150±67 | 146±5 |
| Lysine | 1007±183 | 378±2 | 214±102 | 218±18 |
| Methionine | 266±34 | 92 | 65±29 | 69±4 |
| Valine | 683±105 | 225 | 152±69 | 155±8 |
| Isoleucine | 653±103 | 206±1 | 110±50 | 108±6 |
| Leucine | 1018±152 | 315±1 | 199±89 | 203±12 |
| Phenylalanine | 612±73 | 175±1 | 118±52 | 112±4 |
| **Non-essential amino acids (µg/Kg dw)** | | | | |
| Serine | 681±91 | 205±1 | 164±72 | 157±1 |
| Glycine | 549±77 | 203±1 | 226±99 | 198±2 |
| Aspartic acid | 1461±232 | 440±5 | 288±133 | 272±16 |
| Glutamic acid | 1845±281 | 598±10 | 370±170 | 329±17 |
| Alanine | 611±86 | 270±3 | 186±88 | 190±9 |
| Hydroxyproline | tr | tr | tr | tr |
| Proline | 676±92 | 195±1 | 188±86 | 226±10 |
| Tyrosine | 490±54 | 155 | 121±55 | 121±5 |
| **Free amino acids (FAA)** | | | | |
| **Essential amino acids (µg/Kg dw)** | | | | |
| Histidine | 28±3 | 20±1 | 4±2 | 4 |
| Arginine | 93±21 | 88±1 | 23±10 | 27±2 |
| Threonine | 50±9 | 34±2 | 4±2 | 5 |
| Lysine | 108±18 | 74 | 10±4 | 13 |
| Methionine | 34±6 | 28±1 | 4±2 | 5±1 |
| Valine | 55±8 | 46±2 | 5±2 | 6 |
| Isoleucine | 57±10 | 44±2 | 3±1 | 3 |
| Leucine | 96±18 | 64±1 | 7±3 | 6 |
| Phenylalanine | 66±13 | 43±1 | 4±2 | 5 |
| Tryptophan | 10±2 | 11±1 | 3±1 | 3 |
| **Non-essential amino acids (µg/Kg dw)** | | | | |
| Serine | 76±15 | 41±2 | 6±3 | 6 |
| Glycine | 37±6 | 25±1 | 19±8 | 19±1 |
| Aspartic acid | 54±9 | 25±1 | 3±1 | 2 |
| Glutamic acid | 149±24 | 74±4 | 15±7 | 16±1 |
| Alanine | 80±10 | 64 | 21±9 | 24±1 |
| Proline | 52±7 | 42±2 | 35±15 | 67±4 |
| Tyrosine | 61±10 | 47±1 | 10±4 | 9±1 |

| | | | | |
|---|---|---|---|---|
| **Glutamine** | 66±13 | 47±2 | 5±2 | 3 |
| **Gamma-amino butyric acid** | 0 | 0 | 1 | 1 |
| **Asparagine** | 61±10 | 38±2 | 3±1 | 3 |
| **Taurine** | 3±1 | 35 | 17±7 | 25±2 |

Values are relative to dry weight (DW) and are given as mean ± SD when value is >1. The number of replicates is 2 (N=2) unless otherwise specified by *. Values indicated as trace amounts (tr) correspond to values that were detected but are below quantification limits of the analytical method. Significances were not applied as N was considered too low (N=2).

** N=1, no SD. Two out of the three samples analyzed were found in trace amounts and the expressed value corresponds to only one replicate.

# Paper II

**Etayo, A.,** Bjørgen, H., Koppang, E. O., Lie, K. K., Bjelland, R., Hordvik, I., Øvergård, A.-C., & Sæle, Ø. (2023).

The ontogeny of lymphoid organs and $IgM^+$ B-cells in ballan wrasse (*Labrus bergylta*) reveals a potential site for extrarenal B-cell lymphopoiesis: The pancreas.

**Submitted manuscript.**

II

# The ontogeny of lymphoid organs and IgM$^+$ B-cells in ballan wrasse (*Labrus bergylta*) reveals a potential site for extrarenal B-cell lymphopoiesis: The pancreas

**Angela Etayo[1,2]*, Håvard Bjørgen[3], Erling O. Koppang[3], Kai K. Lie[1], Reidun M. Bjelland[4], Ivar Hordvik[2], Aina-Cathrine Øvergård[2] and Øystein Sæle[1]**

[1] Institute of Marine Research, Bergen, Norway

[2] Fish Health group, Department of Biological sciences, University of Bergen, Norway

[3] Section of Anatomy, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

[4]Institute of Marine Research, Austevoll Research Station, 5392 Storebø, Norway

*** Correspondence:**

Angela Etayo

Email: angela.etayo@uib.no

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## Abstract

Vaccination of farmed fish is the most effective prophylactic measure against contagious diseases but requires specific knowledge on when the adaptive immune system is fully developed. The present work describes kidney and spleen morphogenesis as well as B-cell development in the ballan wrasse (*Labrus bergylta*). The kidney was present at hatching (0 days pot hatching, dph) but was not lymphoid before larvae was 50-60 dph (stage 5), containing abundant *Igμ*$^+$ cells. The spleen anlage was first observed in larvae at 20-30 dph and was later populated with B-cells. Unexpectedly, we found strong *RAG1* signal together with abundant *Igμ*$^+$ and IgM$^+$ cells in the exocrine pancreas of larvae from when the kidney was lymphoid and onwards, suggesting that B-cell lymphopoiesis occurs not only in the head kidney (HK) but also in pancreatic tissue. In this agastric fish, the pancreas is diffused along the intestine and the early presence of IgM$^+$ B-cells in pancreatic tissue might have a role in maintain immune homeostasis in the peritoneal cavity, making a substantial contribution to early protection. IgM-secreting cells in HK indicate the presence of systemic IgM at stage 5, before the first IgM$^+$ cells were identified in mucosal sites. This work together with our previous study on T-cell development in this species indicates that although T- and B-cells start to develop around the same time, B-cells migrate to mucosal tissues ahead of T-cells. This early migration likely involves the

production of natural antibodies, contributing significantly to early protection. Moreover, a diet composed of barnacle nauplii did not result in an earlier onset of B-cell lymphopoiesis, as seen in the previous study analysing T-cell development. Nevertheless, components for adaptive immunity indicating putative immunocompetence is likely achieved in early juveniles (> 100 dph). Additionally, maternal transfer of IgM to the offspring is also described. These findings provide important insights into the development of the immune system in ballan wrasse and lay the foundation for optimizing prophylactic strategies in the future. Furthermore, this work adds valuable information to broaden the knowledge on the immune system in lower vertebrates.

## 1. Introduction

The ballan wrasse is the largest of the European labrids, a family of more than 600 species. It spawns small eggs, and the larvae is only 4 mm long when they start feeding on small planktonic prey. As the cyprinids the labrids are agastric, however the labrid intestine remains short also in the adults. Another singular trait in adult labrids is that they feed on prey living on the surface. This ability is taken advantage of to clean Atlantic salmon (*Salmo salar*) for the ectoparasite salmon louse (*Lepeophtheirus salmonis*) that causes severe health problems and major economic losses to the salmon farming industry. Farming of ballan wrasse started to decrease the pressure on wild wrasse stocks, however, fish farming also comes with disease problems. As of now, atypical strains of *Aeromonas salmonicida* (aAs) causing atypical furunculosis, and bacterial pathogens from the *Vibrionaceae* family are currently the most significant cause of mortalities in farmed ballan wrasse in Scotland [1] and Norway [2]. To avoid overuse of antibiotics, ballan wrasse are vaccinated. However, since there are no existing commercial vaccines for this species, preventive treatments for ballan wrasse rely on autogenous vaccines. Although autogenous multivalent vaccines have been somewhat successful to control diseases in adult individuals [3], wrasse have been observed to encounter pathogens at earlier life stages before it is possible to vaccinate fish by injection. Thus, it is important to understand the timing when the adaptive immune system (T- and B-cells) is functional to optimize vaccine formulation and vaccination strategies. The development of T-cells in ballan wrasse larvae has recently been addressed [4]. To gain further knowledge on the adaptive immune system in this species, the development of B-cells is here investigated.

The “B” in B-cells originates from the avian bursa of Fabricius, but this site of B-cell development seems unique to birds, although a teleost structural analogue has been identified in Atlantic salmon (*Salmo salar*) [5]. In mammals, B-cells develop from the bone marrow, but as teleost fish lack this structure, the hematopoietic tissue in the head kidney (HK) is believed to be the main site for B-cell development [6, 7]. The fish kidney is a complex organ located ventrally to the spinal cord, divided into the anterior or head kidney (HK) which is rich of hematopoietic tissue but lacks nephrons, and the posterior or trunk kidney with abundant nephrons as well as hematopoietic tissue, functioning both as an excretory and lymphoid organ [8]. The B lymphopoiesis pathway is strictly regulated in mammals and consists of a series of phenotypical changes and variations in the expression of characteristic proteins. In the bone marrow, common lymphoid progenitor (CLP) cells mature to pro-B cells, pre-B cells, and immature B-cells in an antigen-independent manner [9]. Immature B-cells are characterized by the presence of membrane IgM and by MHC class II expression [10, 11]. They leave the bone marrow and enter circulation where they migrate between the spleen and lymphoid follicles to encounter antigen and further mature to plasmablasts [12] and eventually

plasma cells and memory cells [13]. In teleosts, although the B-cell differentiation process is not as well characterized, Zwollo, Cole, Bromage and Kaattari [14] described a series of markers that are highly conserved within higher vertebrates and can be used to describe the sub-stages of developing B-cells. Developing B-cell precursors are abundant in the hematopoietic tissue of the HK, whereas the posterior kidney contained mainly B-cells and plasma cells [14]. This is in accordance with the dogma that the trunk kidney (or permanent mesonephros) together with the spleen are considered classical secondary lymphoid organs with T- and B-cells, as recently reviewed in [8]. Plasma cells (PCs) have been observed in the head kidney of some teleosts where they are stored for long periods and contribute to long-term humoral protection, similar to PC in the mammalian bone marrow [14-16]. It seems that there are certain similarities between teleost B-cells and those in higher vertebrates, especially regarding B-cell differentiation processes. However, much research is still needed to properly characterize the phenotype of teleost B-cells that may help to define different subtypes of fish B-cells, e.g. knowledge on memory B-cells in fish is very limited [17]. Genes such as *CD22, CD79A, CD40* among others have, however, been characterized in different populations of teleost B-lymphocytes and might be used as potential markers of B-cells in fish [18, 19].

Immunoglobulins are glycoproteins produced in B-cells; secreted as antibodies, or produced as membrane-bound receptors, forming the B-cell receptor (BCR) complex. Three immunoglobulins are present in teleosts; IgM, IgD, and IgT. Most mature B-cells express either IgM/IgD or IgT [20]. While $IgM^+$ B-cells are most abundant in lymphoid organs (kidney and spleen), blood and peritoneal cavity, $IgT^+$ B-cells are predominantly found in mucosa associated lymphoid tissue (MALT). Accordingly, IgT is referred to as a specialized mucosal Ig [21]. It is important to remark that $IgM^+$ B-cells and secreted IgM are also found in mucosal sites and are important to maintain mucosal homeostasis. However, the ratio of $IgT^+$ B-cells to $IgM^+$ B-cells was found to be superior in the gut of rainbow trout compared to systemic compartments [21]. IgD has also been found at mucosal sites and proposed to interact with microbiota [22-24] but its function remains unclear [15]. IgM is by far, the main effector and most abundant systemic Ig that acts as a natural antibody and as part of adaptive immunity. IgM is also important at the early stages of fish larvae. It is the first Ig isotype expressed in developing (immature) $IgM^+/IgD^+$ B-cells [15, 20], meaning that it can be used as a marker for the appearance of adaptive immunity during larvae ontogeny. Notice that $IgT^+$ B-cells emerge independently from $IgM^+/IgD^+$ lymphocytes and using IgM as a marker exclude the $IgT^+$ B-cell sub-population [17]. Furthermore, IgM is the main antibody reported to be maternally transferred in teleosts. In mammals, IgG is transferred from mother to fetus during pregnancy [25]. Likewise, it has been reported that IgM protects developing zebrafish embryos from pathogens by directly binding to their surface (opsonization) and facilitating phagocytosis [26]. They do, however, have a short duration completely

disappearing during larval stages [25]. Immunization of brood-stock fish to enhance maternal transfer of innate compounds and IgM has successfully been demonstrated in several teleosts, as reviewed in Swain and Nayak [25], and later demonstrated in Tiger grouper (*Epinephelus fuscoguttatus*) [27], zebrafish [26], and turbot [28]. Although maternal transfer of immune protection was not observed in Atlantic salmon [29], the immunization of females appears to generally enhance the immune defense of offspring, potentially reducing mortality in marine fish larvae. Farmed ballan wrasse is not an exception and experiences elevated mortality during the larvae stages.

Interestingly, adult ballan wrasse possess an extraordinarily high amount of plasma IgM compared to other teleosts and a high IgM mRNA expression in the gut [30], possibly compensating for the lack of a functional stomach that is an important protective barrier in gastric fish [31, 32]. It is therefore intriguing to understand the migration patterns of the first $IgM^+$ B-cells to the gut of larvae during ontogeny, as well as the role of IgM in offeringprotection to early larvae when they are most vulnerable. In the present study, we attempt to fill in the knowledge gap on when the adaptive immune system is developed and becomes fully functional in ballan wrasse. More specifically, we address the development of lymphoid organs such as kidney and spleen, together with the appearance of IgM-secreting B-cells and their localization during larvae ontogeny. Furthermore, we investigate maternal transfer of IgM in this species.

## 2. Material and Methods

### 2.1. Experimental design

#### 2.1.1 Egg collection

Brood stock ballan wrasse (70 females and 12 males) were kept in tanks for a year under optimal conditions before the spawning season (mid-March 2022). A standard in-house procedure to collect eggs from wrasse during spawning season is to place several mats in the brood stock tanks where females lay eggs that are thereafter fertilized by males. In this experiment, several synthetic mats (50x50 cm) were placed on the bottom of 3 different brood stock tanks in the early evening and collected the following morning. Mats from the same brood stock tank were placed in the same incubator with a capacity of 250 L at 12 °C, water flow of 5 L/min and natural light. Fertilization of the eggs was checked after 2 days. Unfortunately, eggs from one of the brood stock tanks were unfertilized and therefore excluded from the analyses (n=2). The wealthiest mat in each of the incubators was chosen and eggs were collected at 4 different time points (sampling points) in a nested experimental design by spooning the mat. An average of 1.65 ± 0,17 grams of eggs were collected in each sample. Eggs were collected in duplicates at 0.5 dpf, 3 dpf, 6 dpf, and 9 dpf. At 9dpf, eggs had already hatched and became yolk-embryonic larvae. At each sampling point, eggs were placed in an empty

Eppendorf tube that was snap-frozen in nitrogen liquid and kept at -80 °C for protein extraction.

### 2.1.2 Larvae collection

The experimental design and larvae collection in the present work has been published elsewhere [4]. Briefly, newly spawned eggs were placed in incubators with a capacity of 250 L at 12 °C, water flow of 5 L/min and natural light until hatching. At 4 days post hatching (dph), 30 000 to 34 000 individuals were transferred into six different tanks and two different start-feed diets were given in triplicates. The control diet consisted of enriched rotifers and enriched *Artemia*, and the experimental diet was barnacle nauplii of two different sizes (small and large barnacle) from Planktonic company. For more details on the diet composition see [4]. The larvae were kept in tanks with a capacity of 500 L at 15 °C, with a water flow of 50 L/h and a light regime of 24 hours. Commercial formulated feed (dry feed) was introduced at 40 dph in a co-feeding regime until 56 dph. After this time point, only commercial feed was supplied to all tanks. A total of six sampling points were set according to the six developmental stages of wrasse larvae based on cranial ossification [33]. At each sampling point a series of pooled larvae (3 to 15 larvae per pool) in replicates were collected from each tank, rinsed with distilled water, and immersed in RNA-later at 4 °C overnight and kept at -20 °C until further use. For a summary of the experimental sampling points and the feeding regime see (Fig. 1 in [4]).

## 2.2. Nutrient analyses of the start-feeds

The complete nutrient profile of the two (life-prey) start-feed diets consisting of enriched rotifers/enriched *Artemia* and small/large barnacles have been published elsewhere [4]. A selection of the main nutritional components that varied among the four life-preys are shown in Table 1 previously published in [4]. Briefly, the total amount of lipids and monounsaturated fatty acids (MUFA) was similar in all the life-preys. However, both small and large barnacles had higher levels of EPA and lower levels of ARA compared to rotifers and *Artemia* while rotifers contained the highest percent of DHA. Minerals varied extensively but small barnacles were significantly higher in V, Mn, Co, Zn, As, Se, and Ca compared to rotifers. Rotifers and *Artemia* were richer in all investigated vitamins compared to barnacles, and the amino acid profile was somewhat similar among the life-preys [4].

## 2.3. Protein extraction, SDS-PAGE and Western blot analyses of eggs

The eggs were collected and rapidly snap-frozen as described above. Proteins were extracted as described elsewhere [4]. In short, eggs were homogenized in lysis buffer (4% SDS, 0.1M Tris-HCl pH 7.6) and sonicated at 30 % amplitude for 30 sec. The resulting egg lysates were centrifuged at 400 x g for 10 min at rt, the upper fraction

incubated at 95 °C for 5 min and further centrifuged at 15 000 x g for 10 min. The supernatant was then collected and quantified using the Bradford assay. A protein lysate of larvae at 48 dpf (corresponding to developmental stage 4) from the same batch as the collected eggs, together with a protein lysate of head kidney from adult ballan wrasse previously processed was included in the analyses. Approximately 20 µg protein from each egg-lysate and head kidney were run on reducing, denaturing, 4–15 % gradient TGX Stain-Free precast gels (Bio-Rad). Western blotting was performed at 22 V and 1.3 A for 7 min at 22 °C using a Trans-Blot Turbo System (Bio-Rad). To avoid unspecific binding of antibodies, the PVDF membrane was blocked for 30 min and incubated with rabbit anti-ballan wrasse IgM [34] diluted to 1:5000 in blocking buffer for 90 min. The membrane was washed and incubated with HRP-conjugated anti-rabbit IgG (ab97051, Abcam) diluted to 1:5000 for 1 hour. The PVDF membrane was developed using ECL reagents (Pierce™ ECL Western Blotting Substrate) and visualized using the ChemiDoc MP Imaging system (Bio-Rad). Image data were analysed using image Lab 4.1 Software (Bio-Rad).

Protein normalization was done against total band protein with stain-free technology (Bio-Rad) using one of the lanes as an internal calibrator as described in Hammond, Kohn, Oh, Piatti and Liu [35]. Levels of reactive IgM are hereby shown as relative intensity of protein bands. Total protein normalization using stain-free technology has been shown to be more accurate than protein normalization using housekeeping genes [36].

## 2.4. RNA-seq analyses of larvae

RNA-seq analyses from developing wrasse larvae was previously done and described in [4]. In short, total RNA from larvae at 4 dph (prior to the start of exogenous feeding) and larvae collected at each given developmental stage (stage 1 to 6) were sent to Novogene Europe, Cambridge, UK, for sequencing using the Illumina NovaSeq 6000 platform for 150 bp paired end reads. Complementary DNA (cDNA) libraries were prepared from individual samples and sequenced following manufacturer's instructions and according to the Novogen pipeline (Novogene Europe, Cambridge, UK). Raw sequence reads were mapped against the ensemble wrasse gene build (Labrus_bergylta.BallGen_V1.104) using the Hisat2 mapper [37]. Gene counting and subsequent normalization was conducted using feature counts v1.6.0 [38] as previously described [39]. The count data was further normalized for differences in library size applying weighted trimmed mean expression ratios (trimmed mean of M values (TMM)) featured in the EdgeR package v 3.34 [40]. Due to Ig-genes not being precisely predicted in the wrasse ensemble gene build and the fragmented nature of the current wrasse assembly (probably affected by the high number of variable Ig domains) a modified version of the original transcriptome [41] was made by extracting sequences related to *IgD, IgM, IgT*, and replacing them with recently curated sequences [30, 42].

To analyse the presence of *IgD, IgM*, and *IgT* in the different stages of wrasse development, we conducted a re-mapping of all samples against the modified transcriptome, using Salmon version 0.11.3 for mapping and quantification according to [43].

The raw data is available from the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) (Accession ID: SRX14748182). In this study, 18 genes of interest related to B-cell development were extracted from the RNA-seq data set and analysed through larvae development (stages 1 to 6) using the Qlucore Omics Explorer v3.2.

## 2.5. Histogenesis of Head Kidney and Spleen

Histological serial sections of ballan wrasse larvae corresponding to stages 1 to 6 [33] were kindly donated to us for this purpose. In short, individuals were fixed and stored in 70 % ethanol until dehydration in 96 % ethanol, followed by embedding in Technovit 7100. Longitudinal serial sections (2µm) were stained with toluidine blue [33]. A total of 6 to 9 sections were carefully selected from each stage (1 to 6) making sure that the selection was a good representation of the organ development state. Selected sections were scanned using a semi-automatic scanner Nano Zoomer S60 (Hamamatsu, Japan) and visualized using NDP.view2 (Hamamatsu, Japan).

## 2.6. Immunohistochemistry and *in situ* hybridization

The main purpose was to identify $IgM^+$ B-cells in tissues of developing larvae. Based on the expression of relevant genes from transcriptomic data analysis, immunohistochemistry and *in situ* hybridization was only performed on larvae in the last 2 developmental stages (stage 5 and 6). Furthermore, to study development in more detailed, larval stage 5 and 6 were divided into two sub-stages as followed; early substage 5 (standard length (SL): 1,6 cm/54 dph), late substage 5 (SL:1,8 cm/67dph), early substage 6 (SL: 2,6 cm/96 dph), and late substage 6 (SL: 3,5 cm/109 dph) which were considered juveniles. The larvae were fixed in 4 % PFA (pH 7.4) at rt for 24 to 32 hours. Samples were then dehydrated, embedded in paraffin wax and 3 µm thick sections were made using standard procedures. The paraffin-embedded tissue sections were mounted on positively charged glass slides (Superfrost, Mentzel), dried at 37 °C for 48 hours and further incubated at 60 °C for 1 hour. Subsequently, samples were de-paraffinized in 2 x 5 min xylene and 2 x 1 min 100 % ethanol. Samples were treated for endogenous peroxidase blocking (10 min at rt), followed by target retrieval (15 min at 100 °C). For immunohistochemistry, unspecific binding was prevented by incubating the slides in 0.05M tris-buffered saline (TBS, pH 7.6) with 5 % BSA at rt for 2 hours. Anti-ballan wrasse IgM primary antibody [34] was diluted 1:15 000 in TBS with 1 % BSA before application, and the slides were incubated overnight at 4 °C. As negative control, primary antibody was omitted from the procedure. After several washes in TBS

with 0.05 % tween (TBS-T), slides were incubated with a goat-HRP detection reagent (SignalStain® Boost IHC 8114, cell signalling) for 30 min at rt, and thoroughly washed in TBS-T. For signal detection, sections were then treated with chromogenic substrates bound to HRP enzymes (green colour) for 5 min and subsequently stained with a 25 % Gill´s haematoxylin solution for 30 sec. Slides were shortly washed in tap water, dried at 60 °C for 30 min and mounted with non-aqueous VectaMount® Mounting Medium (Vector Laboratories, Cat. No.: H-5000).

For *in situ* hybridization, an RNA probe using RNAscope™ technology (Advanced Cell Diagnostics, Newark, CA, USA) targeting both the secreted and the membrane-bound forms of *Igμ* (heavy chain of IgM) was designed and produced by the manufacturer based on the provided sequences of ballan wrasse (Table 2). The *in situ* hybridization procedure was slightly modified from Løken, Bjørgen, Hordvik and Koppang [5]. In short, slides were protease-digested (30 min at 40 °C) after target retrieval to allow permeabilization of cells. For probe hybridization, slides were incubated with the RNAscope probe for 2 h at 40 °C. A series of hybridizations were performed using different incubation times according to the manufacturer´s instructions [44] to allow amplification of the signal. For signal detection, sections were then treated with chromogenic substrates bound to AP (red colour) for 10 min and subsequently stained with 25 % Gill´s haematoxylin solution for 30 sec. Slides were then dehydrated and mounted with non-aqueous VectaMount® Mounting Medium (Vector Laboratories, Cat. No.: H-5000). Slides were scanned using a semi-automatic scanner Nano Zoomer S60 (Hamamatsu, Japan) and visualized using NDP.view2 (Hamamatsu, Japan).

### 2.7. Statistics

Two-way ANOVA was used to test whether there was an effect of the start-feed diets on genes related to B-cell development during larvae ontogeny. RNA-transcriptomic data from larvae corresponding to stage 1 to 6 was used for this purpose with a previous F-test to check for homogeneity of variances and a D'Agostino-Pearson test to check normality of the dataset. One-way ANOVA was used to test significances in the expression of immunoglobulin transcripts (*IgM, IgT,* and *IgD*) in newly hatched larvae before the start of exogenous feeding (pre-stage 1). A significant level of 0.05 was used.

## 3. Results

### 3.1 Growth and effect of start-feed diets during larvae ontogeny

The growth results and effect of the two different start-feed diets have been published elsewhere [4]. In short, data corresponding to the standard length (SL) of sampled larvae is a confirmation of the successful execution of larvae collection at each given stage (Fig. 1A). The two start-feed diets did trigger a significantly higher dry weight (DW)

of those larvae fed barnacle nauplii at stage 6 (Fig. 1B). Mortality varied slightly between the two start-feed diets, but it was similar during weaning.

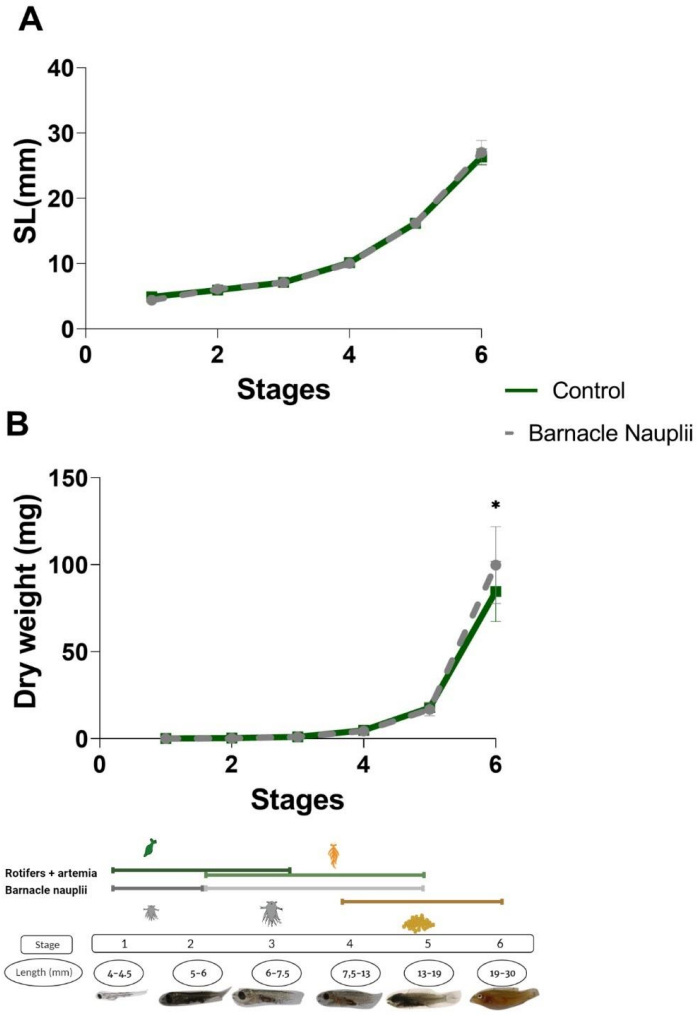


**Figure 1**. The dietary effect on growth performance during larvae ontogeny. **A)** Larvae standard length (SL). **B)** Larvae dry weight (DW) which was significantly higher in the barnacle nauplii diet in the last developmental stage (Mann-Whitney test, p value= 0.0287, indicated by *). Stages and the feeding regime are included in the figure for easier interpretation. Previously published in [4]

### 3.2 Morphogenesis of the head kidney and spleen

During the first stage of development, the excretory portion of the kidney was visible, characterized by the presence of few tubular structures (Fig. 2A, B). Notably, there were

no indications of hematopoietic cells at this early stage. Tubular structures in the kidney became more abundant at stage 2 (Fig. 2C, D) and here incipient hematopoietic tissue was observed. Furthermore, tubular structures were identified along the length of the body cavity indicating that the kidney at this stage could be divided in anterior, middle, and posterior kidney. The anterior kidney developed throughout stage 3 and 4 presenting abundant lymphoid cells (Fig. 2E, F). During the last 2 larvae stages, the anterior kidney became more prominent with high abundance of blood sinusoids and tubules (Fig. 3A, B). Lymphocytes, granulocytes, and other leukocytes could be observed in the anterior kidney (head kidney) at stages 4 and 5 (data not shown) and became more abundant in stage 6 (insert in Fig. 3C).

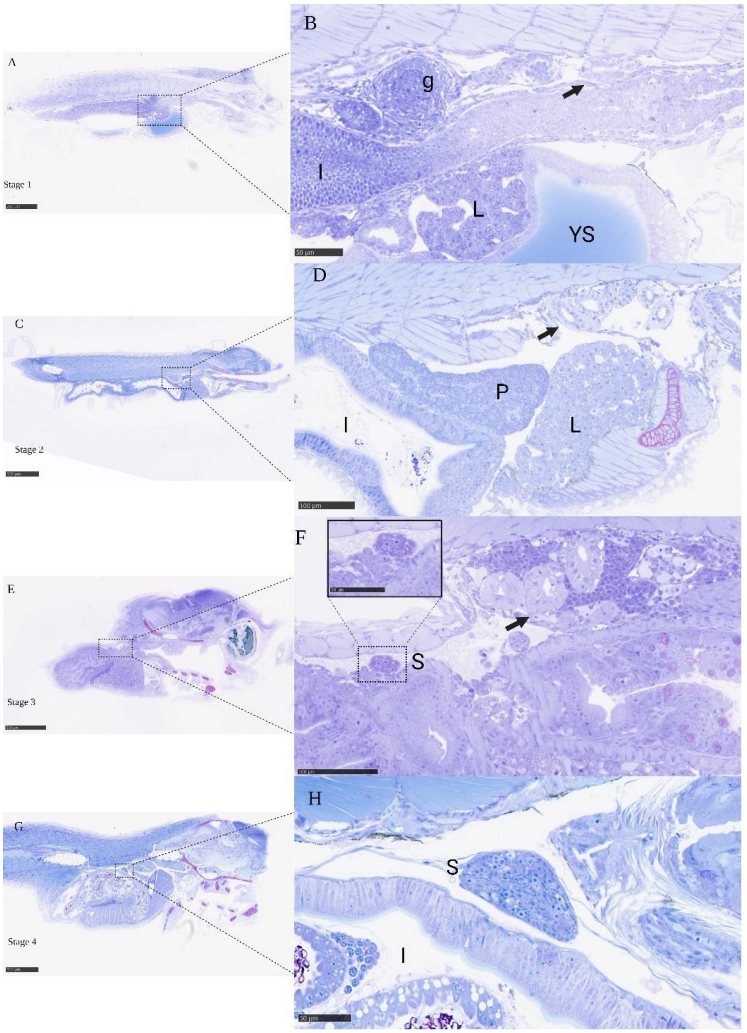


**Figure 2.** Morphological development of kidney and spleen in ballan wrasse larvae (stage 1 to 4). Black arrows indicate tubules in the kidney; **A)** Larvae at stage 1 where

the head kidney is squared. **B)** Higher magnification of A where tubules and a structure with morphology of a glomerulus surrounded by Bowman's capsule can be observed. **C)** Larvae at stage 2. **D)** Higher magnification of C with incipient hematopoietic tissue. **E)** Larvae at stage 3, where the spleen anlage could be seen adjacent to the intestine. **F)** Higher magnification of E. The inset shows the spleen anlage (scale bar is 50 µm). **G)** Larvae at stage 4. The spleen is marked with a square. **H)** Higher magnification of G showing a more developed spleen adjacent to the intestine and gas bladder. Intestine (l), liver (L), yolk sac (ys), glomerulus (g), pancreas (p), spleen (s). Scale bars are as follows; A; 250 µm, B; 50 µm, C; 500 µm, D; 100 µm, E; 500 µm, F; 100 µm, G; 500 µm, H; 50 µm.

The first observation of the spleen anlage was at stage 3 (Fig. 2E, F) adjacent to the intestine and gas bladder. At stage 4, the spleen developed into a small spherical organ (Fig. 2G, H) where erythrocytes and thrombocytes were identified. Later, at stage 5, the organ was more abrupt and both white pulp (WP) and red pulp (RP) areas could be observed (Fig. 3B). The spleen developed fully at stage 6 where it appeared closely associated with pancreatic tissue. Multiple blood sinusoids, visible ellipsoids in RP as well as leukocytes such as granulocytes, macrophages and lymphocyte-like cells were identified at this stage (Fig. 3D).

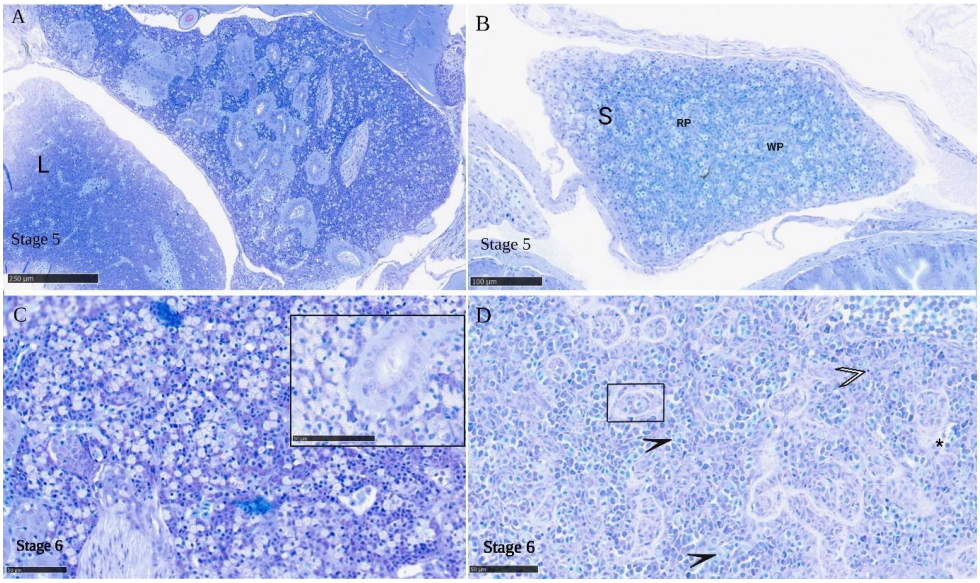


**Figure 3.** Morphological development of kidney and spleen in ballan wrasse larvae at stage 5 and 6. **A)** Head (anterior) kidney of larvae at stage 5 with abundant blood sinusoids and tubules. **B)** Spleen of larvae at stage 5 with red (RP) and white pulp (WP) areas. **C)** Head kidney of larvae at stage 6. The inset shows the high presence of granulocytes (scale bar is 50 µm). **D)** Spleen of larvae at stage 6 with several ellipsoids (marked in a box). Black arrows indicate granulocytes, white arrows point to

lymphocyte-like cells, and the asterisk shows a macrophage. liver (L), spleen (S). Scale bars are as follows; A; 250 µm, B; 100 µm, C and D; 50 µm.

Although the main goal of this work was to describe the kidney and spleen ontogeny in ballan wrasse, it is worth noticing that the pancreatic tissue was already visible at stage 1 being abundant in the body cavity at all larval stages as shown for stage 2 (Fig. 2D), stage 5 (Fig. 5B, E), and stage 6 (Fig. 6D, E).

### 3.3 Expression of B-cell markers during ontogeny

The two start-feed diets did not trigger differences in the expression of the chosen B-cell markers (including the Ig-genes) in this study (Two-way ANOVA >0,05). Therefore, RNA-seq data of wrasse larvae fed the control diet was selected to study the starting point of B-cell development during ontogeny. RNA-seq revealed that *IgM, IgT*, and *IgD* transcripts appeared at stage 5 of larvae development (Fig. 4), indicating the appearance of B-cells at this stage. Among the Ig genes, both membrane (*TM-IgM*) and secreted (*sIgM*) IgM were highest expressed followed by membrane IgD (*TM-IgD*) and membrane IgT (*TM-IgT*) (Fig. 4C). The secreted forms of IgD (*sIgD*) and IgT (*sIgT*) showed the lowest expression at stages 5 and 6 (Fig. 4C). Expression of the recombination activating genes *RAG1* and *RAG2* was upregulated from stage 5 (Fig. 4A). *CD40* and *CD79A* have been suggested as markers for teleost $IgM^+$ B-cells and they were upregulated at stage 5 and 6, respectively (Fig. 4A). In a similar way, the upregulation of *CD40LG* was only observed at the latest developmental stage, stage 6.

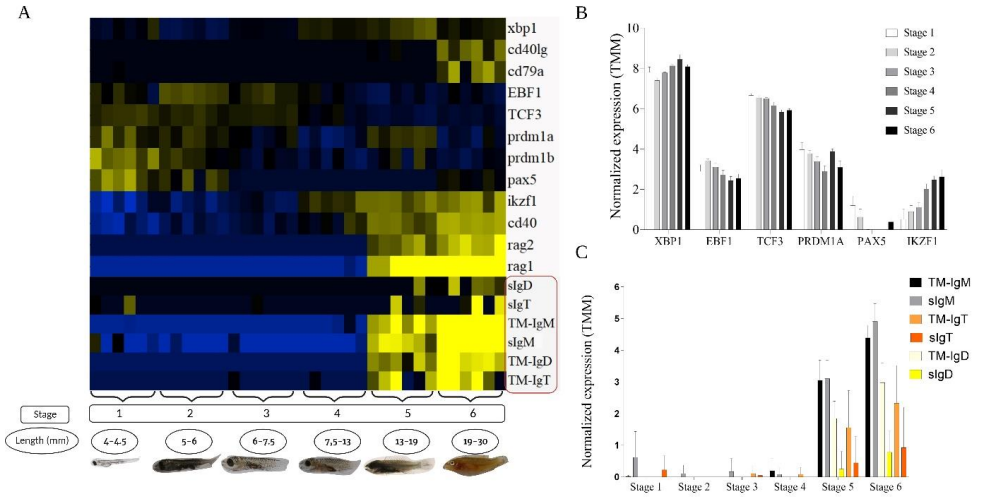


**Figure 4.** RNA-seq data from developmental stages of ballan wrasse. Data were log-transformed, normalized for differences in library size applying weighted trimmed mean expression ratios (trimmed mean of M values (TMM)), and expressed as mean = 0. **A)** Heat map of RNA transcripts showing the selected B-cell markers. Note that transcripts corresponding to immunoglobulins (IgM, IgT, and IgD) are delimited in a

red box. Data were logarithm normalized and expressed as mean = 0. The Figure was generated using Qlucore omics explorer software. **B)** RNA transcripts corresponding to the transcription factors *XBPL, EBF1, TCF3, PRDM1A, PAX5*, and *IKZF1* of different stages of developing B-cells at larvae stages 1-6. **C)** RNA transcripts of immunoglobulins; *sIgM* (secreted IgM), *TM-IgM* (membrane IgM), *sIgD* (secreted IgD), *TM-IgD* (membrane IgD), *sIgT* (secreted IgT), and *TM-IgT* (membrane IgT).

In an attempt to describe the different stages of developing B-cells in ballan wrasse ontogeny, a total of 6 genes (*XBPL, EBF1, TCF3, PRDM1, PAX5, and IKZF1*) corresponding to transcription factors that are differentially expressed during B-cell development were selected (Fig. 4A, B). From stage 1 and onwards, larvae consistently exhibited expression of all these genes. *XBPL, EBF1, TCF3*, and *PRDM1* had a consistent number of reads across all developmental stages, while the expression patterns of *PAX5* and IKAROS (*IKZF1*) varied distinctly (Fig. 4B). The transcript level of PAX5 was highest expressed at stage 1 and drastically dropped during stage 3, 4, and 5 where transcripts were not detected until stage 6 (where a slight upregulation in transcription could be detected). The *IKZF1* transcript level increased gradually from stage 1 to 6. Altogether, transcriptomic data indicates that B-cell development started at stage 5 and that the early expression of these 6 transcription factors is presumably important in other processes unrelated to B-cell development as discussed further below. No differences were found in the level of expression of B-cell markers between diets (Two-way ANOVA > 0.05).

### 3.4 Localization of *Igμ*+ cells in wrasse larvae

Based on the expression of relevant genes detected by analysis of transcriptomic data, *in situ* hybridization on lateral sections of ballan wrasse larvae were done on stages 5, 6, and juvenile fish, coinciding with the upregulation of immunoglobulin transcripts. Most of the *IgM (Igμ)* mRNA expression seemed to correspond to IgM-secreting B-cells and was restricted to the head kidney in the earliest investigated larvae (early substage 5) (Fig. 5A). Positive cells were also found in pancreatic tissue (Fig. 5B), especially in pancreatic tissue embedded in the liver (Fig. S1), trunk kidney (Fig. S1), the lamina propria of the hindgut (Fig. 5F), and to a lesser extent, in the lamina propria of the middle gut (Fig. 5C) at the late substage 5. Scattered *Igμ* mRNA was observed in the hearth at this stage (data not shown). The number of $Ig\mu^+$ cells increased along the kidney (Fig. 6A), pancreatic tissue (Fig. 6E), and in the lamina propria of the hindgut at stage 6 and juveniles (SL > 3,5 cm), respectively shown in (Fig. 6C, F). Spleen was only investigated at stage 6 by *in situ* hybridization, showing abundant $Ig\mu^+$ cells (Fig. 6B). Interestingly, *RAG1* mRNA expression was found in pancreatic tissue in larvae in the early stage 5 (Fig. 5G) and more abundantly in stage 6 (Fig. 6D). From stage 6 and onwards, scattered $Ig\mu^+$ cells were also identified in other parts of the larvae such as

skin, pharynx, mouth, gill arch and in more abundance in the heart (Fig. S1). *In situ* hybridization using the negative probe (DapB) did not give any signal (Fig. 6G).

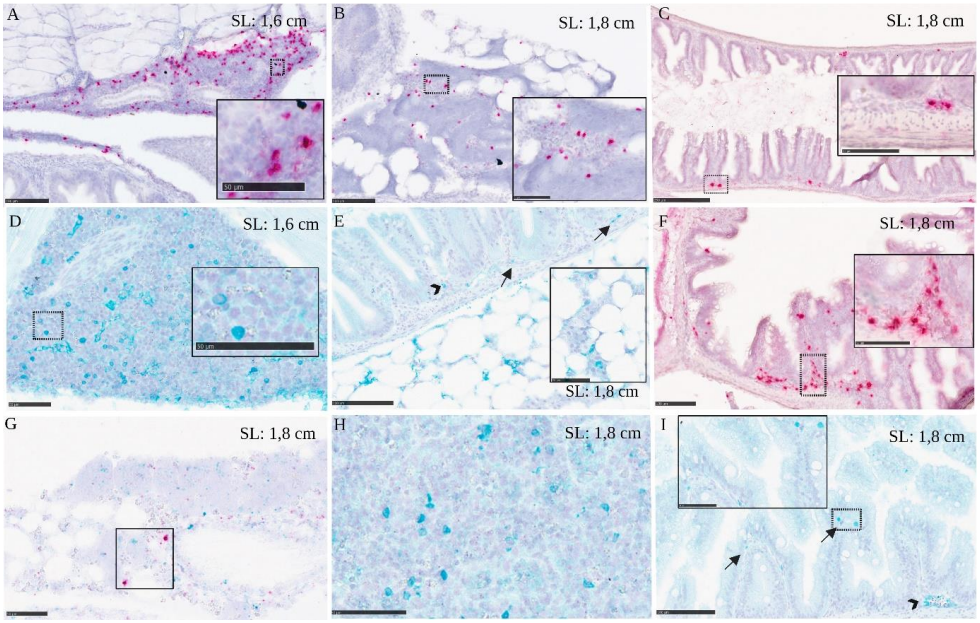


**Figure 5.** *In situ* hybridization and immunohistochemistry showing IgM positive cells of wrasse larvae at stage 5 that is divided in early substage 5 (larvae with a standard length (SL) of 1,6 cm) and late substage 5 (larvae with SL of 1,8 cm). Sections in A, B, C, and F correspond to RNAscope *in situ* hybridization targeting ballan wrasse Igμ, and sections in D, E, H, and I correspond to immunohistochemistry using an anti-wrasse IgM antibody (1:15 000). RAG1 probe was used in G. The image depicted in the insert is a higher magnification of the area delimited by a dash square. **A)** Head kidney. **B)** Pancreatic tissue. **C)** Middle gut. **D)** Head kidney. The inset shows an IgM+ cell with prominent staining localized in the cytoplasm, presumably an IgM-secreting cell as well as an IgM+ cell that is strongly stained around the surface (membrane bound IgM-B-cell). **E)** Middle gut and adjacent pancreatic tissue. **F)** Hindgut. **G)** Positive cells for *RAG1* mRNA (red signal) in pancreatic tissue of larvae. **H)** Spleen. **I)** Hindgut. Black arrows in E and I indicate IgM+ cells and head arrows indicate positive signals surrounding blood capillaries. Scale bars as follows (scales bars of the inserts are indicated by ()); A; 100 μm (50μm), B; 100 μm (50 μm), C; 250 μm (50μm), D; 50μm (50μm), E; 100 μm (50 μm), F; 100 μm (50μm), G; 50 μm, H; 50 μm, I; 100 μm (50 μm).

### 3.5 Immunolocalization of IgM$^+$ cells in wrasse larvae

Based on transcriptomic data and *in situ* hybridization, stages 5, 6, and juveniles were selected for immunohistochemistry analysis (anti-wrasse IgM). The smallest investigated larvae by immunohistochemistry corresponded to early substage 5 (SL: 1,6

cm) where abundant IgM$^+$ cells were observed in the anterior kidney (Fig. 5D) with prominent staining on the surface (**membrane bound-IgM B-cells**) or prominent staining in the cytoplasm **(IgM-secreting cells**). Few IgM$^+$ cells were identified in pancreatic tissue adjacent to the gut (data not shown) and positive signal was not identified in the spleen nor in the trunk kidney at this stage. At the late substage 5 (SL: 1,8 cm), IgM$^+$ cells were also observed in the middle and posterior kidney (data not shown) and was now identified in the spleen (Fig. 5H). Few scattered IgM$^+$ cells were observed in the lamina propria of the gut and hindgut (Fig. 5E, I). Staining not associated with cells was also seen in blood capillaries (Fig. 5E, I).

Later in larvae development, at stage 6 (SL: 2,6 cm) and in juveniles (SL > 3,5 cm), abundant IgM$^+$ cells were observed in the kidney (Fig. 6H) and more IgM$^+$ cells appeared in the lamina propria of the hindgut (Fig. 6I). Positive IgM cells were also observed in the thymus of juveniles mainly limited to the medulla although scattered positive signals could also be observed in the cortex (Fig. S1). Compared to earlier stages, abundant IgM positive cells were found in the primary and secondary lamellae of gills and heart (Fig. S1). Positive signal in the liver appeared delimiting blood sinusoids and what seems to be pancreatic and/or biliary ducts embedded in the liver as shown for juveniles (Fig. S1). Negative control omitting the IgM antibody was negative (Fig. S2).

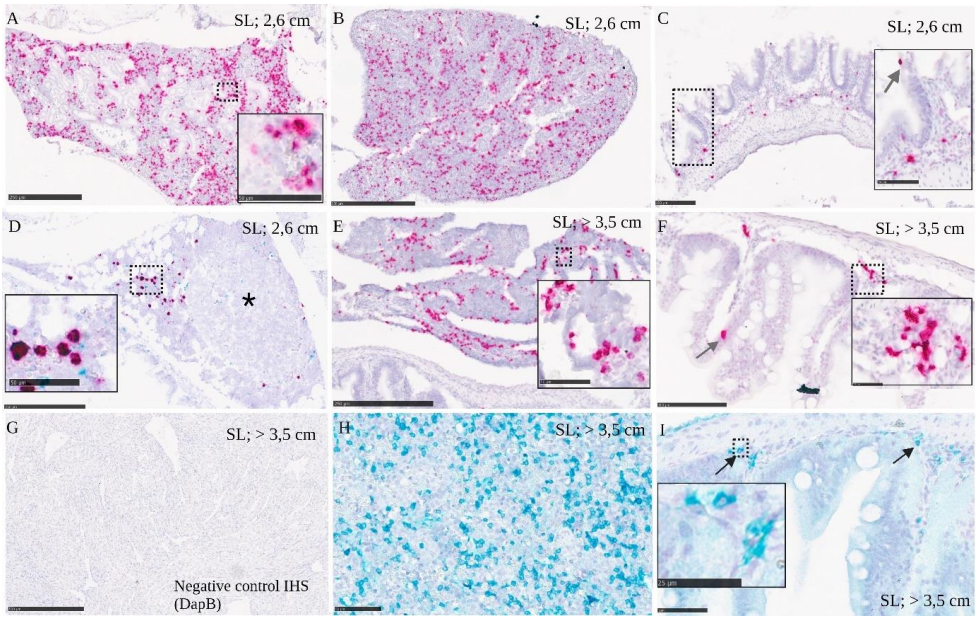


**Figure 6.** *In situ* hybridization and immunohistochemistry showing IgM positive cells of wrasse larvae at stage 6 (larvae with a standard length (SL) of 2,6 cm) and juveniles (SL > 3,5 cm). Sections in A-F correspond to RNAscope *in situ* hybridization targeting ballan wrasse Igμ except for D where RAG1 probe was used. Sections in H-I correspond

to immunohistochemistry using an anti-wrasse IgM antibody (1:15 000). The images depicted in the insets are a higher magnification of the area delimited by a dash square. **A)** Head Kidney. **B)** Spleen. **C)** Hindgut. **D)** Positive cells for *RAG1* mRNA (red signal) in pancreatic tissue surrounding a structure similar to an islet of Langerhans (indicated by *). **E)** Pancreatic tissue adjacent to the middle gut. **F)** Hind gut. **G)** Larvae used as negative control using DapB probe. **H)** Head kidney **I)** Hind gut. Black arrows in I indicate IgM+ cells, and grey arrows in C and F indicate intraepithelial IgM+ cells (IEL). Scale bars as follows (scales bars of the inserts are indicated by ()); A; 250 µm (50µm), B; 250 µm, C; 100 µm (50µm), D; 250µm (50µm), E; 250 µm (50 µm), F; 100 µm (25µm), G; 500 µm, H; 50 µm, I; 50 µm (25 µm).

### 3.6 Maternal transfer of IgM to the offspring

Western blot analysis revealed that eggs at 0.5 days post-fertilization (dpf) contained the highest amount of IgM (Fig. 7). The abundance of IgM in eggs at 3 and 6 dpf decreased 40% and 55% respectively compared to eggs at 0.5 dpf. Ballan wrasse larvae hatched around 7 dpf. Yolk-embryonic larvae (9 dpf) and larvae at 48 dpf (stage 4 of larvae development) presented extremely low levels of IgM (Fig. 7) with 99% less IgM than eggs at 0.5 dpf. A protein lysate of head kidney from an adult wrasse contained 10 times more IgM than eggs at 0.5 dpf (Fig. 7). Original SDS-PAGE and Western blot are shown in Fig. S3.

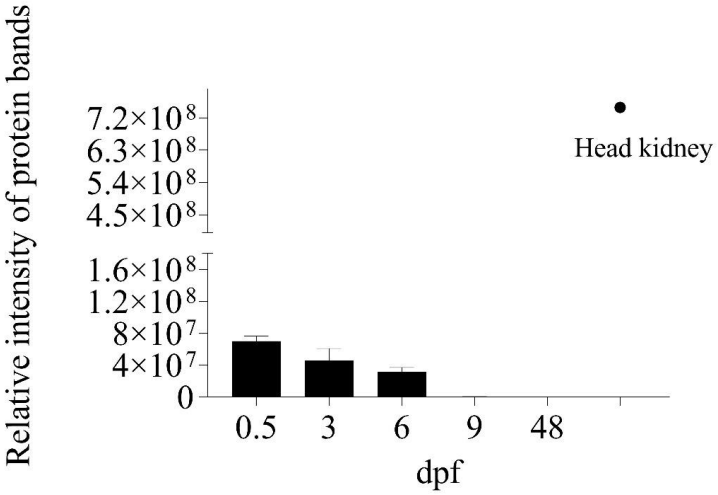


**Figure 7.** IgM protein levels in eggs of ballan wrasse at 0.5, 3, 6, 9, and larvae at 48 dpf (stage 4) measured by Western blot analysis (n=2). A head kidney lysate from adult wrasse was included for comparisons. The relative intensity of protein bands was

obtained by normalizing IgM signal against total protein band using stain-free technology (Bio-Rad). A decrease of 40% and 55% in the amount of IgM was observed in eggs at 3 and 6 dpf respectively compared to eggs at 0.5 dpf. Eggs at 9 dpf (pre-stage 1) and 48 dpf (Stage 4) showed 99% less IgM than newly hatched eggs at 0.5 dpf.

A meta-analysis of RNA-seq data from ballan wrasse larvae at 4 dph collected before the start of exogenous feeding was performed. Interestingly, transcripts of s*IgT* were significantly more abundant than *IgM* transcripts (One-way ANOVA; p value= 0.0181) indicating that IgT might potentially be maternally transferred (Fig. 8).

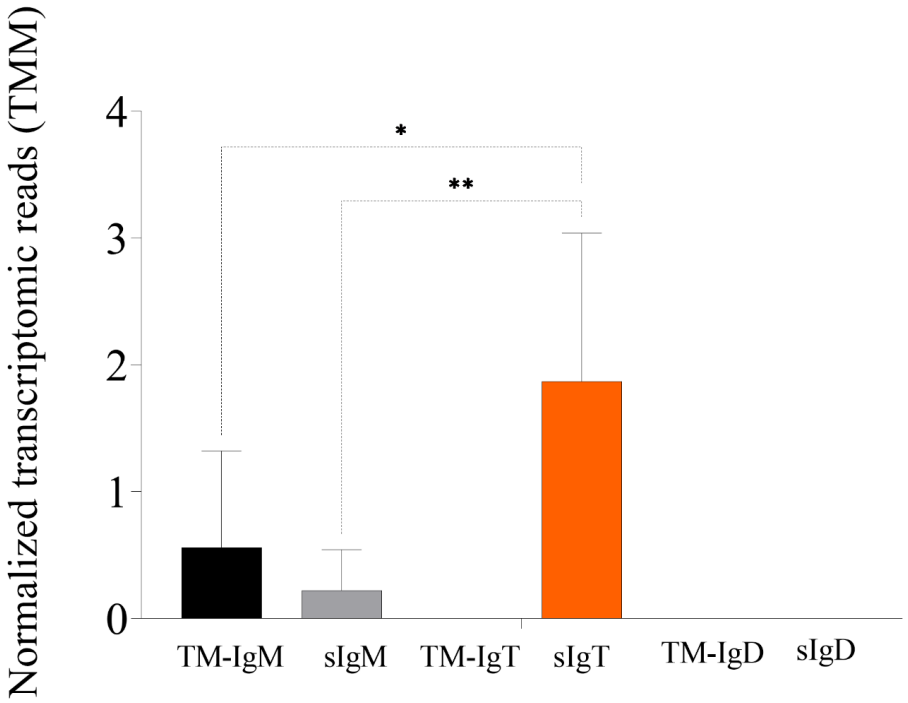


**Figure 8.** RNA transcripts of immunoglobulins from newly hatched larvae at 4 dph (pre-stage 1) (RNA-seq data; Accession ID: SRX14748182) corresponding to larvae collected before the start of exogenous feeding; *sIgM* (secreted IgM), *TM-IgM* (membrane IgM), *sIgD* (secreted IgD), *TM-IgD* (membrane IgD), *sIgT* (secreted IgT), and *TM-IgT* (membrane IgT). Significances tested by One-way ANOVA are indicated by asterisks (* = p value of 0.0181, and ** = p value of 0.0020, n=5).

## 4. Discussion

The aim of the present work was to characterize key components related to the development of B-cells and antibody-producing cells in ballan wrasse larvae, with focus on the immune organs, namely kidney and spleen. The development of thymus, the primary lymphoid organ where T-cells mature, was recently described [4]. Surprisingly,

513 we found abundant IgM-positive cells and *RAG-1* expression in pancreatic tissue.
514 Another unexpected finding was a significantly higher amount of *IgT* mRNA transfer
515 to eggs compared to IgM.

516 In marine teleost fish, the kidney develops prior to the spleen and thymus [45], though,
517 the order of spleen and thymus development seems to vary among fish species [8, 46].
518 In the present study, the ballan wrasse kidney was first visible whereas the first
519 histological observation of the spleen anlage coincides with the appearance of the
520 primordial thymus [4]. Although the kidney and spleen were observed in early stages
521 of larvae, it was not until stage 5 when immunoglobulin transcripts were distinctly
522 upregulated, indicating the start of successful Ig recombination and first B-cell
523 appearance. Our findings thus, confirm that the development and presence of lymphoid
524 organs does not imply a functional immune system as previously reported for Atlantic
525 halibut (*Hippoglossus hippoglossus*) [46].

526 The head kidney is the primary lymphoid organ for B-cell maturation in fish. The
527 transcription factor ikaros (*IKZF1*) is essential for early B-cell development in both
528 mammals [47] and fish [48], and is needed to initiate *RAG* expression [49].
529 Simultaneously, the activation of RAG is necessary for V(D)J rearrangement of Ig
530 genes [50]. However, *IKZF1* and *RAG* genes are not exclusively expressed in B-cells
531 as they are also involved in TCR rearrangements in developing T-cells and therefore,
532 cannot be considered as exclusive B-cell markers. B-cells are characterized by the
533 ability to fully rearrange heavy and light chain genes which are needed to produce
534 immunoglobulins (Igs). Hence, Ig-genes can be used as markers of B-cells.
535 Accordingly, we demonstrate that *IKZF1* upregulation precedes the upregulation of
536 *RAG1, RAG2*, and Ig-genes (*IgM, IgD*, and *IgT*). *CD79A* is present from early stages of
537 B-cell development to plasma cells in mammals [51], also suggested to be a potential
538 marker of pan-B-cells in teleosts [18]. CD40 is expressed in mature naïve B-cells while
539 its ligand CD40L is expressed in follicular T-cells (Tfh) of mammals. Upon binding,
540 this interaction facilitates plasma cell differentiation [52]. Also in fish, co-stimulation
541 of $IgM^+$ B-cells with CD40L and specific cytokines have shown to promote $IgM^+$ B-
542 cell survival, proliferation, and IgM secretion [19, 53]. In the present work, *CD40* was
543 upregulated from stage 5 coinciding with the upregulation of *Igs* and *RAG* genes,
544 whereas *CD40L* and *CD79A* showed a later upregulation, specifically at stage 6.
545 Altogether, mRNA expression of Ig-genes together with the expression pattern of the
546 mentioned transcripts confirm the emergence of developing B-cells starting from stage
547 5 and continuing thereafter. From stage 6 and onwards, IgM-staining showed cells
548 within the HK with strongly stained cytoplasm, indicating a higher number of IgM-
549 secreting cells at this stage.

550 Analysing the expression level of several stage-specific transcription factors is useful
551 to identify B-cells at different developing stages [54]. In an attempt to identify different

B-cell developmental stages in wrasse larvae, the level of *PRDM1, XBPL, EBF1, TCF3*, and *PAX*5 transcripts were analysed in the present study. PRDM1 also known as BLIMP, together with XBPL are necessary for B-cell terminal differentiation in mammals. These proteins present conserved homology of functional domains across species which might indicate a comparable function to that in teleosts [54, 55]. The Early B-cell factor (*EBF1*), the transcription factor *TCF3* (also known as E2A), and *PAX5* are present in common lymphoid progenitors (CLP), pro-B-cells and immature B-cells but absent in plasma cells of higher vertebrates. They have been suggested to play a crucial role in the commitment of the B-cell lineage in piscine species [54, 56]. All these transcription factors were notably expressed in wrasse larval stages 1 and 2 where neither *RAG* nor *Ig*-genes were present, indicating the absence of B-cells. It is noteworthy that these genes also play roles in the development of brain and muscle cells in humans, mice, and in zebrafish [57-61]. Additionally, XBPL functions as a regulator of innate immune responses in both humans and common carp [62, 63]. Altogether, our whole-larvae transcriptome does not allow for identification of the different stages of developing B-cells across the larval phases of ballan wrasse. Nevertheless, data indicate that the presented transcription factors have similar functions in both higher and lower vertebrates.

Taking into account that B-cells are predominantly $IgM^+$ in teleosts [20, 64], and that our transcriptome analysis indicated that IgM is the most abundant Ig isotype during wrasse development, *in situ* hybridization targeting *Igμ*, the heavy chain of IgM, was considered to be optimal to follow B-cell development, as previously reported in zebrafish [65], Atlantic cod (*Gadus morhua*) [66], Atlantic halibut [46], and other teleosts recently reviewed in [67]. It is important to notice that targeting IgM exclude $IgT^+$ B-cells and $IgM^-/IgD^+$ B-cells [17]. Immunocompetence refers to the ability to mount a fully functional immune response. The detection of *IgM* mRNA and secreted IgM, which is present at mucosal sites in all investigated teleosts, are important markers for immunocompetence [46]. The present results suggest that humoral immunity in wrasse larvae is present from the late substage 5, as there were abundant *Igμ* positive cells, membrane bound-IgM positive cells, and IgM-secreting cells in kidney and spleen at this stage. Moreover, the gut of wrasse, especially the hindgut showed many Ig*μ* positive cells in the lamina propria at late stage 5 although membrane bound-IgM positive cells were scarce and appeared mostly around blood vessels of the lamina propria, presumably accounting for secreted IgM. It was not until stage 6 and juveniles when higher numbers of $IgM^+$ cells were observed in the hindgut together with $IgM^+$ cells in other mucosal organs such as gill, oesophagus, and skin. Overall, our results indicate that systemic IgM as well as $IgM^+$ B-cells within the hindgut mucosa are present from stage 5 whereas migration of $IgM^+$ B-cells to other mucosal tissues occurs later in development, during stage 6 and juveniles.

In the present work, strong expression of *Igμ* as well as *RAG1*$^{+}$ CD3ε$^{-}$ cells were unexpectedly found within the exocrine pancreas from the early stage 5 and onwards. Accordingly, scattered positive membrane bound-IgM cells were also observed in pancreatic tissue at this stage when IgM protein appeared otherwise restricted to the kidney and blood capillaries. Simultaneously, in stage 5, T-cells were not found in pancreatic tissue, and only a minimal number of putative helper T-cells were detected in the spleen [4]. Altogether, the fact that *RAG*1 and *Igμ* were expressed in pancreatic tissue from stage 5 and onwards, but T-cells appear limited to the thymus until stage 6, strongly suggests BCR recombination in pancreatic tissue. This implies that, to some extent, B-cell lymphopoiesis, but not T-cell development, occurs in pancreatic tissue of ballan wrasse larvae. Pancreatic tissue is considered a non-lymphoid organ in fish and the occurrence of natural immune cells in this tissue has not been given much attention [8]. Danilova and Steiner [65] reported for the first time in a fish species, the occurrence of B lymphopoiesis in the pancreas of zebrafish. However, *RAG* expression was reported as absent in pancreatic tissue of Japanese pufferfish (*Fugu rubripes*) [68], and only minimal expression of *RAG2* but no *RAG1*, was observed in the pancreas of common carp (*Cyprinus carpio*) [69]. In an agastric fish species where food is driven directly from the oesophagus to the intestine, enzymes released by the exocrine pancreas are even more crucial for digestion demonstrating the importance of this organ [70] and this is also true for ballan wrasse [41, 71]. The exocrine pancreas in this species is firstly observed in newly hatched larvae (stage 1) as a compact organ posterior to the liver. It develops into a scattered organ located along the intestine in the abdominal cavity and also within the liver [33]. These features closely resemble the morphology observed in other agastric teleosts [72, 73]. Interestingly, Norland, Sæle and Rønnestad [33] reported a high abundance of immune cells, especially eosinophilic granular cells within the pancreatic tissue in wrasse larvae. The present study also demonstrated that there is a large population of *Igμ*$^{+}$ cells within the pancreatic tissue revealing a previously overlooked immune activity in the pancreas of this species.

A high immune activity in the intestine of ballan wrasse has been previously reported, suggesting a compensatory mechanism for the loss of stomach [30]. In accordance with this, the present study showed that *Igμ* was expressed in the lamina propria of the hindgut and to a lesser extent, in the gut of all the investigated larvae (stage 5 and 6) indicating that the gut mucosa (gut-associated lymphoid tissue (GALT)) is populated with *IgM*$^{+}$ B-cells rapidly after B-cell lymphopoiesis starts. The identification of B-cell lymphopoiesis within pancreatic tissue might be of crucial importance for early stages of larvae as they are likely exposed to pathogens entering the gut. The fact that fish lack lymph nodes suggests that B-cell terminal differentiation can occur at different sites [74]. Therefore, B-cells developed within the pancreatic tissue could be differentiated to mature B-cells upon antigen encounter at the infection site in the proximity of the body cavity acting as a fast route of defence or at least, helping the population of B-

cells "classically developed in the head kidney" for an enhanced protection. Pancreatic B-cells might also strategically help to maintain immune homeostasis in the peritoneal cavity of ballan wrasse as the first observation of positive *Igμ* cells and $IgM^+$ cells in the connective tissue of the pancreas was soon after the organ starts to expand and appears scattered within the abdominal cavity. Equally interesting is the high abundance of $Ig\mu^+$ cells and $IgM^+$ cells in the exocrine pancreatic tissue within the liver (hepatopancreas) that is closely associated with blood vessels. These results indicate the possible transport of IgM that is released from $IgM^+$ B-cells localized in pancreatic tissue within the liver, in the proximity of ducts, towards either blood or the lumen pancreatic ducts. Pancreatic ducts form a complex net of vessels that are connected to the intestinal bulbous through one main duct [33]. Transport of IgM through pancreatic ducts towards the intestine might be similar to the hepatobiliary route of IgM previously suggested in ballan wrasse [75] and in the Antarctic teleost fish, *Trematomus bernacchii* [76].

Despite the high sensitivity of RNAscope *in situ* hybridization [44], the method does not allow for a precise quantitative comparison between T- and B-cell ontogeny. Nevertheless, during the stage when both the thymus and head kidney became lymphoid (stage 5), *RAG1* expression was notably more abundant in the thymus [4]. In the present study, numerous *Igμ* positive cells were observed in the intestinal mucosa during late stage 5, whereas extremely few putative helper T-cells ($CD3\varepsilon^+$ $CD4\text{-}1^+$) were detected at the same stage [4]. Similarly, at larval stage 6, many *Igμ* positive cells but very few putative helper T-cells were observed in other mucosal organs such as skin, gill, and oesophagus. Altogether, this indicates that B-cells migrate to mucosal organs slightly earlier than T-cells in ballan wrasse. This is opposite to migration patterns in common carp and sea bass where B-cells were observed in the gut mucosa later than T-cells by immunohistochemistry [69, 77]. This difference might reflect both species-specific features and the use of different methods as mRNA levels may not necessarily reflect protein levels in the cell. Nevertheless, we suggest that the initial population of $IgM^+$ B-cells migrating to the gut might possess characteristics typical of "innate-like" B-cells, producing natural antibodies that are important for early protection. Subsequently, as T-cells migrate to the intestine, more specific (adaptive) responses are more likely to happen.

Overall, the presented results together with earlier work [4] indicates that although systemic IgM (humoral immunity) might be present from stage 5, immunocompetence in wrasse is likely achieved later in juveniles. Papadopoulou et al. [78] tested a polyvalent autogenous vaccine against *Aeromonas salmonicida* in ballan wrasse individuals at 80 dph (0,5 g) and 170 dph (1,5 g) by immersion vaccination, and authors reported no protection of the vaccine at any of the tested stages. We have demonstrated that components for adaptive immunity seems to appear during larval stage 6 and

juveniles (90-100 dph) and might partly explain the insufficient protection after vaccination. Nevertheless, other factors related to vaccine design could influence the lack of action. Importantly, further studies involving pathogen challenges would prove when wrasse juveniles are able to mount an efficient adaptive immune response.

Prior to development of adaptive immunocompetence, it is known that both innate and adaptive immune molecules are transferred to eggs and protect fish larvae against pathogens. Studies on oviparous species have demonstrated the transfer of complement proteins, lysozymes, lectins, and immunoglobulins (Igs) to the offspring as reviewed in detail in [25, 79]. Previously, IgM was thought to be the exclusive antibody isotype maternally transferred in teleosts, similar to maternally transferred IgG in mammals and IgY in chicken [25, 80]. However, a recent study revealed that IgT rather than IgM appeared to be the primary maternal Ig involved in early defence in zebrafish [81]. Maternally transferred Igs have been reported in newly fertilized eggs from non-stimulated brood stock in several teleosts [81-85]. In the present study, we demonstrated the presence of maternal IgM protein in newly released eggs of ballan wrasse indicating the potential role of IgM at protecting wrasse embryo. However, this presumed protection is short-term as IgM was not traceable in newly hatched larvae in the yolk-embryonic stage where they are still uncapable of producing IgM themselves. Maternal transport of mRNA transcripts to the offspring has also been observed in several teleost species. For instance, maternal *IgM* mRNA was present in eggs of sea bass and sea bream at very low levels and disappeared much faster than IgM protein [82, 83]. The same pattern was observed with maternally derived IgT in zebrafish: *IgT* mRNA was not detected after 6 hpf whereas IgT protein was still detected at 12 hpf [81]. Maternally transferred Ig mRNA has been considered as a likely common mechanism in early defence of fish larvae. However, evidence of the protective role of Igs mRNA are difficult to address and remains elusive. The first RNA-seq study on maternal immune parameters in a teleost species showed that unfertilized eggs of Atlantic cod were completely deprived of *IgM* and *IgD* transcripts indicating the lack of maternally transferred Ig mRNA molecules [86]. We unexpectedly discovered a significantly higher abundance of *sIgT* compared to *IgM* transcripts in newly hatched larvae at 4 dph (pre-stage 1), similar to findings in zebrafish [81]. Further studies investigating the abundance of maternally transferred IgT is needed to understand the implications (if any) in ballan wrasse. The fact that ballan wrasse larvae are able to produce IgM from stage 5 and onwards is in agreement with the assumption that humoral IgM appear relatively late in seawater teleosts [87], indicating that other immune molecules might have a major protective role during early stages. It is likely that maternally transferred innate parameters such as lysozyme, cathelicidin, and complement proteins protect fish eggs and larvae [26, 86, 88-90]. Thus, it is plausible to assume that innate molecules are also maternally transferred in ballan wrasse although this has not been addressed.

It is well documented that nutrients can modulate immune responses in fish [91-93]. Our previous work on the development of the thymus in ballan wrasse revealed that the start-feed diet composed of barnacle nauplii, rich in e.g. EPA, Se, and Zn, triggered an earlier expression of certain T-cell markers as well as a larger size of the thymus [4]. In the present work, the morphological comparison of lymphoid organs such as kidney and spleen in larvae fed the two start-diets was not performed, as there was a lack of dietary effect in the expression of Ig-genes and other selected B-cell markers. This indicates that the kidney together with B-cell lymphopoiesis was not as affected as the thymus by the barnacle nauplii diet. Nevertheless, it is reasonable to think that malnutrition may negatively impact the kidney functionality.

## Conclusion

In the present work, we have described the development of lymphoid organs and B-cell lymphopoiesis in ballan wrasse, processes that do not seem to be dietary modulated by a barnacle nauplii diet. The kidney was observed at hatching, and it was populated with abundant *Igμ* positive cells in the earliest investigated larvae at stage 5 (50-60 dph), which coincides with the notably upregulation of *Ig*-transcripts during wrasse larvae development. The spleen developed later (20-30 dph) and $IgM^+$ cells (mRNA and protein) were observed later than in the head kidney (HK) as reported in other teleosts. We found abundant *Igμ* cells and unexpected *RAG1* mRNA in the connective tissue within the pancreatic tissue of larvae from stage 5 and onwards indicating that B-cells are developed both in the HK and pancreatic tissue in wrasse larvae. The characteristic localization of the pancreatic tissue in this agastric species (around the gut along the body cavity) together with the high immune activity earlier reported in the gut of this species, might strategically help to maintain immune homeostasis in ballan wrasse. Protein IgM was abundantly observed in lymphoid organs (kidney and spleen), and in pancreatic tissue from late stage 5 indicating humoral immunity is present at this stage. However, the migration of $IgM^+$ cells to mucosal organs became more prominent later during stage 6 (90-100 dph) and juveniles (> 100 dph). The lymphoid transition in wrasse larvae occurs simultaneously in the thymus, head kidney, and pancreatic tissue, suggesting that the initiation of TCR and Ig rearrangements takes place around the same developmental stage. Nonetheless, $IgM^+$ B-cells migrate to MALTs slightly earlier than T-cells, presumably accounting for B-cells producing natural IgM that are important at defending early larvae. In summary, the present study indicates that components for adaptive immune responses in ballan wrasse emerge during the final larval developmental stage and in juveniles, aligning with our earlier research on T-cell development. This information is relevant for optimizing prophylactic strategies. Furthermore, IgM was maternally transferred to the offspring but decreased to insignificant levels after hatching, and results pointed towards IgT having a potential role as maternally transferred immunoglobulin.

## 5. Conflict of Interest

*The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest*.

## 6. Author Contributions

**ØS, IH** and **AE** designed the experiments. **AE** and **RB** participated in carrying out the experiment. **EOK** and **HB** performed the *in situ* hybridization. **AE, ØS, KL**, and **IH**: analyses. **AE** wrote the manuscript. **AE, IH, AØ, KL** and **ØS**: editing. All authors read, commented, and approved the final manuscript.

## 7. Ethics Statement

Ethical review and approval for the experiment was covered by permission nr. VL-AV-0011 given by the Directorate of Fisheries of Norway to the Institute of Marine Research station at Austevoll (location nr. 16195). The experiment and sampling followed the Norwegian animal welfare act guidelines, in accordance with the Animal Welfare Act of 20th December 1974, amended 19th June 2009. The facility has a general permission to conduct experiments involving all developmental stages of fish (code 93) provided by the Norwegian Animal Research Authority (FDU, www.fdu.no).

## 8. Funding

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## 9. Acknowledgments

We thank Sissel Norland for kindly providing the histological sections for morphogenesis studies.

## 10. Supplementary Material

1.1. Fig. S1
1.2. Fig. S2
1.3. Fig. S3

## 11. Data Availability Statement

The transcriptomic data for this study (accession number GSE200208) can be found in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE200208)

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**Table 1**. Overview of the selected nutritional analyses in rotifers (*Branchonus plicatilis)*, *Artemia* (*Artemia salina*), small barnacle (*Balanus crenatu*), and large barnacle (*Semibalanus balanoides).* Previously published in [4].

| | Rotifers | *Artemia* | Small barnacle | Large barnacle |
|---|---|---|---|---|
| **Proximate composition (g/ 100g DW)** | | | | |
| Protein | 51±0[a] | 36±9[b] | 42±17[abc] | 52±22[ac] |
| Lipid | 16±4 | 13±6 | 8±4 | 9±2 |
| Ash | 36±11 | 23±1 | 30±18 | 23±6 |
| **Fatty acids (% of TFA)** | | | | |
| ΣSFA | 22±5 | 22 | 22 | 19 |
| 20:5n-3 EPA | 7±1[a] | 9[a] | 28[b] | 32±4[b] |
| 22:6n-3 DHA | 34±6[a] | 11±2[b] | 15[b] | 19±2[b] |
| 20:4n-6 ARA | 2[a] | 3[b] | 1[c] | 1[c] |
| n-3/n-6 | 2[a] | 2[a] | 26±2[b] | 21±7[b] |
| **Micro-mineral composition (µg kg$^{-1}$ DW)** | | | | |
| V | 5±1[a] | 34±47[ab] | 88±5[b] | 26±12[ab] |
| Mn | 98±29[a] | 313±429[a] | 1170±142[b] | 159±84[ac] |
| Co | 2±1[a] | 7±9[ab] | 16±1[b] | 6±4[ab] |
| Zn | 203±85[a] | 2220±2536[ab] | 4343±667[b] | 5825±843[b] |
| Se | 3±1[a] | 10±7[ab] | 19±3[b] | 15±2[b] |
| I | 58±26[a] | 244±300[a] | 2819±523[b] | 736±154[c] |
| **Pigments (µg kg$^{-1}$ DW)** | | | | |
| Astaxanthin | 1374±912 [a] | 41±2 [ab] | 28±20[b] | 60± 10 [ab] |
| Canthaxanthin | tr | tr | 4* | 7* |
| **Non-essential amino acids (µg/Kg dw)** | | | | |
| **Protein-bound amino acids (PAA)** | | | | |
| Proline* | 676±92 | 195±1 | 188±86 | 226±10 |
| **Free amino acids (FAA)** | | | | |
| Proline* | 52±7 | 42±2 | 35±15 | 67±4 |
| Taurine* | 3±1 | 35 | 17±7 | 25±2 |

Values are relative to dry weight (DW) and are given as mean ± SD when value is >1. The number of replicates is 3 (N=3) unless otherwise specified by * (N=2). One-way ANOVA test was applied only when N=3 and significances are indicated by letters.

**Table 2**. Probes designed for RNAscope® in *situ* hybridization.

| | Probe | Accession no. | Target region (bp) | Catalogue no. |
|---|---|---|---|---|
| Control Target | IgM | KX688616.1 | 301-1321 | 1185161-C1 |
| | DapB (negative) | EF191515 | 414 - 862 | 310043 |
| | EF-1a (positive) | XM_029279947.1 | 600-1592 | 1185171-C1 |

*Supplementary Material*

# The ontogeny of lymphoid organs and IgM$^+$ B-cells in ballan wrasse (*Labrus bergylta*) reveals a potential site for extrarenal B-cell lymphopoiesis: The pancreas

**Angela Etayo[1,2]*, Håvard Bjørgen[3], Erling O. Koppang[3], Kai K. Lie[1], Reidun M. Bjelland[4], Ivar Hordvik[2], Aina-Cathrine Øvergård[2] and Øystein Sæle[1]**

[1] Institute of Marine Research, Bergen, Norway

[2] Fish Health group, Department of Biological sciences, University of Bergen, Norway

[3] Section of Anatomy, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

[4]Institute of Marine Research, Austevoll Research Station, 5392 Storebø, Norway

*** Correspondence:**

Angela Etayo

Email: angela.etayo@uib.no

**Supplementary Material**

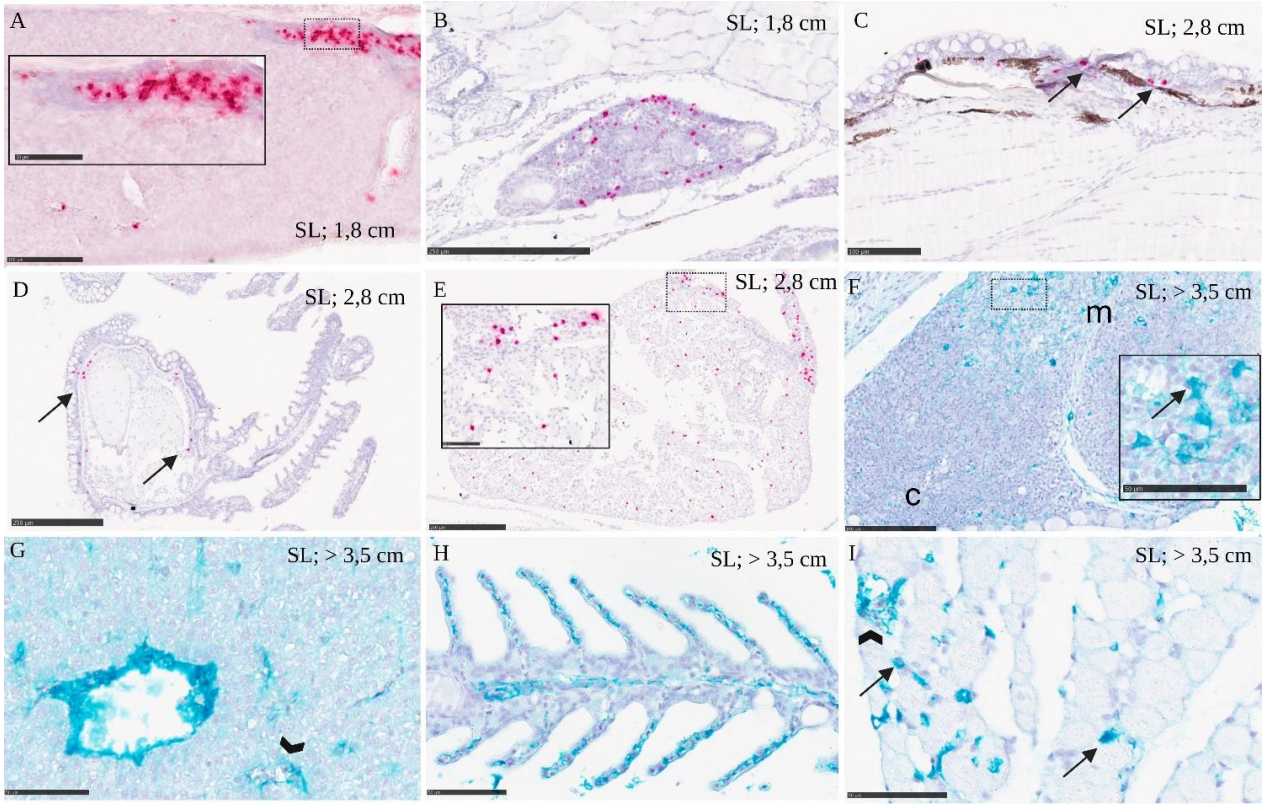


**Fig. S1.** *In situ* hybridization and immunohistochemistry showing IgM positive cells of wrasse larvae at late substage 5 (larvae with a standard length (SL) of 1,8 cm), stage 6 (SL: 2,6 cm) and juveniles (SL > 3,5 cm). Sections in **A-E** correspond to RNAscope *in situ* hybridization targeting ballan wrasse *Igμ*. Sections in **F-I** correspond to immunohistochemistry using an anti-wrasse IgM antibody (1:15 000). The images depicted in the insets are a higher magnification of the area delimited by a dash square. **A)** Pancreatic tissue embedded in liver. **B)** Trunk kidney. **C)** Skin. **D)** Gill arches where black arrows indicate $IgM^+$ cells. **E)** Hearth. **F)** Thymus showing higher IgM positive signal and $IgM^+$ cells in the medulla (m) compared to the cortex (c). **G)** Liver showing IgM positive signal within pancreatic tissue that appear delimiting a prominent blood vessel. **H)** Gills **I)** Heart. Black arrows indicate $IgM^+$ cells and head arrows indicate positive IgM signal surrounding blood capillaries. Scale bars as follows (scales bars of the inserts are indicated by ()); A; 100 µm (50µm), B; 250 µm, C; 100 µm, D; 250µm, E; 250 µm (50 µm), F; 100 µm (50 µm), G; 50 µm, H; 50 µm, I; 50 µm.

**Supplementary Material**

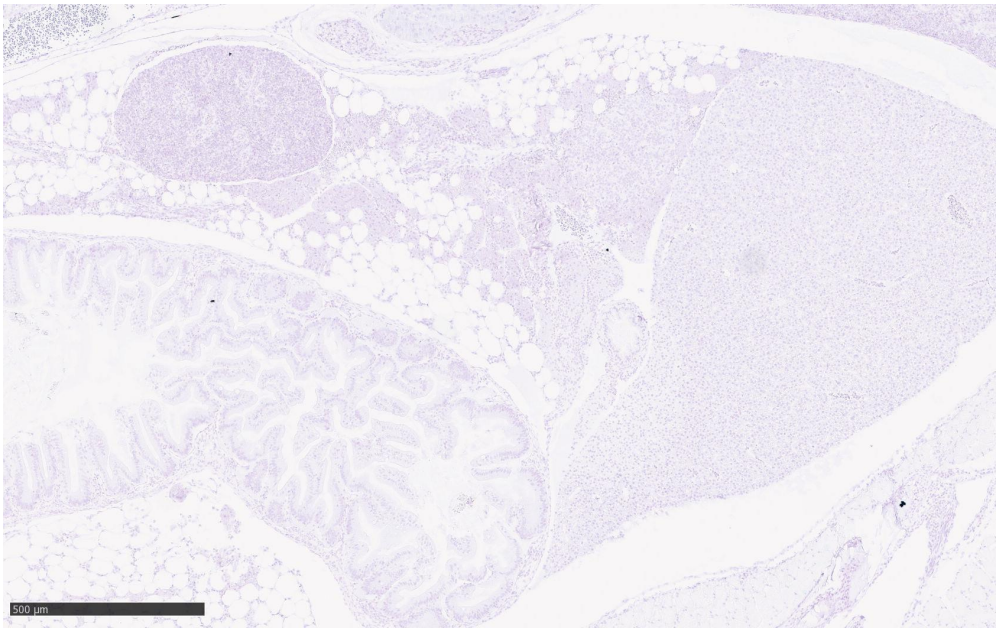


**Fig. S2.** Ballan wrasse larvae at late substage 5 (SL; 1.8 cm) used as negative control for immunohistochemistry analyses where IgM antibody was omitted. Scale bar is 500 µm.

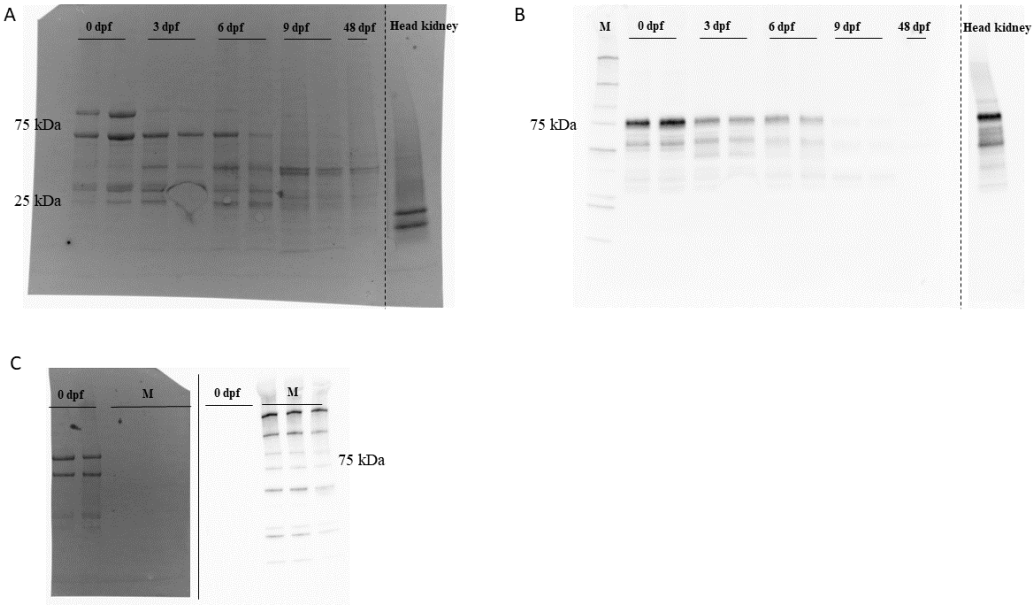

**Fig. S3.** SDS-PAGE analyses and Western blot analyses of protein extracts from ballan wrasse eggs. **(A)** SDS-PAGE analysis of egg lysates at 0.5, 3, 6, 9, 48 dpf (larvae stage 4) and head kidney (n=2). **(B)** Western blot of corresponding samples incubated with an affinity purified rabbit anti wrasse IgM antiserum and developed using ECL reagents. The expected molecular mass of the wrasse heavy and light chain IgM is 75 kDa and 25 kDa, respectively. Antibody dilution 1:5 000. **(C)** SDS-PAGE (left) and immunoblot (right) of egg lysates at 0.5 dpf omitting primary antibody. Three different dilutions of unstained marker (M) were applied on the gel.

# Paper III

**Etayo, A.**, Bjørgen, H., Koppang, E. O., & Hordvik, I. (2022).

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III

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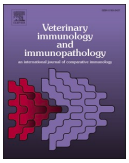

# The teleost polymeric Ig receptor counterpart in ballan wrasse (*Labrus bergylta*) differs from pIgR in higher vertebrates

Angela Etayo [a,1,*], Håvard Bjørgen [b,2], Erling O. Koppang [b,3], Ivar Hordvik [a,4]

[a] Department of Biological Sciences (BIO), University of Bergen, Bergen, Norway
[b] Section of Anatomy, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

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ABSTRACT

As mucosal barriers in fish are the main sites where pathogens are encountered, mucosal immunity is crucial to avoid infection in the aquatic environment. In teleost fish, immunoglobulins are present in gut, gill and skin mucus, although not in the same amounts as in higher vertebrates. In mammals, the poly-Ig receptor (pIgR) is synthesized in epithelial cells and mediates the active transport of poly-immunoglobulins (pIgs) across the epithelium. During transport, a component of the pIgR, the secretory component (SC), is covalently bound to pIgs secreted into the mucus providing protection against proteases and avoiding degradation. The teleost pIgR gene does not show synteny to higher vertebrates, the overall structure of the protein is different (comprising two Ig domains) and its functional mechanisms remain unclear. The J-chain which is essential for pIgR-mediated transport of IgA and IgM in higher vertebrates is absent in teleost fish. The aim of the present study was to characterize the ballan wrasse (*Labrus bergylta*) pIgR and use it as a marker for further studies of mucosal immunity in this species. The pIgR gene was unambiguously identified. Unexpectedly, reverse transcription real time PCR (RT-qPCR) revealed highest abundance of pIgR mRNA in liver and significantly lower expression in mucosal organs such as foregut, hindgut, and skin. *In situ* hybridization showed pIgR-positive cells dispersed in the lamina propria while it was undetectable in epithelial cells of foregut and hindgut of ballan wrasse. A similar pattern was observed in Atlantic salmon. Liquid Chromatography-Mass Spectrometry (LC-MS/MS) analysis of IgM enriched mucus samples from gut, gill, skin, and bile gave relatively few matches to wrasse pIgR. Notably, the matching peptides were from the transmembrane (TM) and cytoplasmatic (Cy) region as well as the putative SC, indicating leakage from lysed cells rather than covalent bonds between IgM and SC. Altogether, the results indicate that pIgR has another (or at least an additional) function in wrasse. Another pIgR-like molecule (pIgRL) in ballan wrasse (comprising three Ig domains) was analyzed to see if this could be an alternative functional pIgR homolog. However, the presence of pIgRL mRNA in blood leukocytes and a relatively high expression in immune organs like spleen and head kidney pointed to a receptor function on a circulating leukocyte population. As significant amounts of IgM were found in bile of ballan wrasse further studies should consider the hepato-biliary route regarding IgM delivery to the gut lumen.

## 1. Introduction

The poly-immunoglobulin receptor (pIgR) is a key player during mucosal immune responses mediating transport and secretion of mammalian dimeric IgA (SIgA) and pentameric IgM across the epithelia to mucosal surfaces (Johansen and Kaetzel, 2011; Kaetzel, 2001). The pIgR binding sites, CDR-like (complementary determining region-like) loops, for IgA and IgM are highly conserved throughout mammalian species, and these binding sites together with the presence of J-chains (joining chains) are crucial for binding to pIgs and subsequent transport (Braathen et al., 2007; Mostov et al., 1984; Rombout et al., 2014). In fish, mucosal surfaces are essential barriers against pathogen entry

* Corresponding author.
*E-mail address:* angela.etayo@uib.no (A. Etayo).
[1] 0000-0001-6988-3057.
[2] 0000-0003-4683-0088.
[3] 0000-0003-4859-1455.
[4] 0000-0001-9361-7656.

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**Table 1**
Primers used for cloning and SYBR Green RT-qPCR.

| | Gene | Accession no. | Primer sequence 5′ to 3′ | Amp size |
|---|---|---|---|---|
| RT-qPCR | pIgR | XM_020653429.2 | F: GACCCAAAGATACGCTGCCT<br>R: GGAGTCTTGGCTGATGTGCT | 144 |
| | pIgRL | XM_020654174 | F: TGTTCATCCTGTGTATTGCTCT<br>R: AACATCATTGCTACCGCAGTC | 164 |
| Cloning | pIgR | XM_020653429.2 | F: GACCCAAAGATACGCTGCCT<br>R: ATGTAAGCGACCAGCAGGAC | 288 |
| | pIgRL | XM_020654174 | F: AGAGCATGAAGATGTTGAGCCG<br>R: GGATTTTGGTTCCTTGTGCTC | 1112 |

through gut, skin, and gills. Genes designated as pIgR have been characterized from different teleost species in the last two decades, as reviewed by (Rombout et al., 2014), later by (Kong et al., 2018) and more recently by (Xia et al., 2020).

The extracellular part of the pIgR, also called the secretory component (SC), is covalently bound by a disulphide bridge to pIgs during transport across the epithelium being further cleaved and secreted together with the pIgs, providing protection against proteases and avoiding rapid degradation of pIgs in mucosal sites (Johansen and Kaetzel, 2011; Musil and Baenziger, 1987). The pIgR mediated transport of pIgs is J-chain dependent in mammals. In teleosts, injection experiments suggested that IgM antibodies are not able to reach the surface by passive transport, implicating that fish have some type of secretory system (Lin et al., 1996; Lobb and Clem, 1981). However, polymerization of Igs occur in the absence of J-chains, and pIgR-mediated transport to mucosal sites in teleosts is not well understood (Rombout et al., 2014). Notably, there is a lack of conserved synteny between human and zebrafish pIgR, as flanking genes to the teleost pIgR (DAD1 and LRRC24) are located at different chromosomes in humans (Kortum et al., 2014). The lack of synteny with higher vertebrates is also recently supported by Flowers et al. (2021). Yet, it is supposed to resemble pIgR in higher vertebrates where the SC is secreted together with pIgs. Recombinant proteins for the pIgR-SC have been produced from different teleosts to investigate the reactivity of the SC to mucus pIgs, as reported in trout (Xu et al., 2013b; Xu et al., 2016; Zhang et al., 2010) and flounder (Xu et al., 2013a). Recombinant pIgR protein in Grass carp (Xu et al., 2021a) and Nile tilapia (Liu et al., 2019a) interacted with recombinant IgM and IgT in a concentration dependent manner.

Higher expression of the pIgR gene in mucosal tissues and increased IgM production was found in skin, gut and/or gill mucus after challenge experiments with pathogens indicating that the teleost pIgR is involved in mucosal immune responses (Leya et al., 2021; Liu et al., 2019a; Sheng et al., 2018; Wang et al., 2017; Yang et al., 2017; Yu et al., 2018). This together with the association of pIgR from some teleosts with mucosal IgT and/or IgM have supported the idea that teleost pIgR is functionally homologous to mammalian pIgR. Nevertheless, evidence describing the binding mechanism of IgM and IgT to the pIgR in the absence of the J-chain, as well as the cleavage site of the pIgR and the role of the SC are needed to elucidate the function of pIgR in teleosts.

Poly Immunoglobulin Receptor-Like (pIgRL) molecules with similar structures to teleost pIgR have been reported from common carp, zebrafish, flounder and Atlantic salmon (Kortum et al., 2014; Liu et al., 2019b; Tadiso et al., 2011; Zhang et al., 2015). In zebrafish a single gene encoding pIgR was identified on chromosome 2 along with a large multigene family consisting of 29 pIgRL genes of which the majority were identified adjacent to pIgR (Kortum et al., 2014). Knowledge on the functional roles of pIgRL is limited, but the authors observed that recombinant pIgRL proteins bound phospholipids and not immunoglobulins, and that the pIgRL gene was expressed in blood leukocytes in contrast to pIgR. These observations indicate that pIgRL is functionally different from pIgR.

Large amounts of SIgA are produced at mucosal sites in higher vertebrates (Woof and Kerr, 2006). A human adult secretes up to 3 g of SIgA per day due to the extremely high turnover of this antibody (Johansen and Kaetzel, 2011). The gut of the stomach-less fish, ballan wrasse (*Labrus bergylta)* shows a large number of IgM-positive intraepithelial cells and an extraordinarily high abundance of IgM mRNA (Bilal et al., 2019). The aim of the present study was to characterize the pIgR gene and use this sequence information for further studies of mucosal immunity in this species. We expected to find a high expression of pIgR in the gut of wrasse, and a clear-cut distribution of the mRNA in epithelial cells since we had observed an extraordinarily high IgM expression in the gut, and a high serum concentration of IgM in this species (approximately 10 times higher than in Atlantic salmon, in wild catch fish). However, in the course of the present work we found surprising results different from previous reports of other teleosts.

## 2. Materials and methods

### 2.1. Fish samples

For the experiments, both wild ballan wrasse (700–900 g) caught from fjords close to Bergen, Norway (during the months of September and October 2019 and 2020) and farmed ballan wrasse (32–100 g) reared in tanks and fed commercial feed from the Institute of Marine Research (IMR), Austevoll Research Station, Norway were used. Fish were anaesthetized with MS-222 (30 mg/ml) and killed by a blow to the head. For RNA extraction and further molecular characterization of the pIgR gene, tissues were quickly collected and placed in RNA later (Ambion) at 4 °C overnight and stored at −20 °C until further use. Collected tissues were gill, foregut, hindgut, skin, liver, kidney, spleen, and muscle.

### 2.2. Molecular cloning and sequencing of pIgR cDNA

Based on a predicted mRNA sequence (GenBank accession number: XM_020653429.2), a pair of primers for cloning pIgR were designed near the start/stop codon (Table 1) using NetPrimer software (http://www.premierbiosoft.com/NetPrimer/AnalyzePrimer.jsp). Total RNA was extracted using a tissue homogenizer (Tissue lyser II) and TRIzol reagent® (Invitrogen) including DNase treatment (TURBO DNase, Ambion) according to the manufacturer's protocol. RNA quality was checked using an agarose gel. 500 ng of total RNA were used to generate first-strand cDNA, utilizing SuperScript™ II reverse transcriptase (Invitrogen) and an oligo $dT_{16}$ primer. Negative controls were performed in parallel by omitting RNA or enzyme. Obtained cDNA was diluted 1:10 and stored at −20 °C for further use. The amplified cDNA was obtained using AccuPrime™ *Taq* DNA Polymerase. Amplification was performed over 30 cycles of 94 °C for 14 s, 55 °C for 15 s and 68 °C for 1 min, followed by a final elongation step at 68 °C for 7 min. Amplified cDNA from kidney and gut were cloned using pCR™ 4-TOPO® vector (Invitrogen) according to the manufacturer's guidelines. Positive clones were identified using a 3% agarose gel stained with ethidium bromide. Plasmid DNA isolation and purification from chosen colonies was performed using the NucleoSpin® EasyPure protocol (Macherey-Nagel). Sequencing was performed at an in-house sequencing facility using Big Dye termination chemistry (Applied Biosystems).

### 2.3. Sequence analysis

The location of the genomic sequence (synteny) encoding ballan wrasse pIgR was identified using the Integrative Genomics viewer (**IGV**) (Robinson et al., 2011). DNA/amino acid sequences were compared to sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Madden, 2002). The exon/intron organization of wrasse pIgR was investigated using the Splign tool from NCBI (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi) (Kapustin et al., 2008). DNA was translated into amino acids using the computer program ExPASy-Translate tool (https://www.expasy.org/) (Gasteiger et al., 2003). Multiple sequence alignments were performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Madeira et al., 2019). Ig domains were predicted using SMART tool (http://smart.embl-heidelberg.de/smart/show_motifs.pl) (Schultz et al., 1998). N-glycosylation and O-glycosylation sites were predicted using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) (Gupta and Brunak, 2001) and NetOGlyc 4.0 (http://www.cbs.dtu.dk/services/NetOGlyc/) (Steentoft et al., 2013) servers respectively. Transmembrane (TM) regions were predicted by use of the TMHMM Server v. 2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) (Sonnhammer et al., 1998). The phylogenetic tree was constructed using the "Molecular Evolutionary Genetics Analysis" (MEGA X) software program (Kumar et al., 1994), utilizing 1000 "bootstrap" replicates, available at (https://www.megasoftware.net).

### 2.4. Analysis of mRNA expression by RT-qPCR

Gene expression was determined by means of RT-qPCR using a QuantStudio ™ 3-RealTime PCR instrument (Thermo Fisher Scientific) with the following protocol: UDG (Uracil-DNA glycosylase) activation at 50 °C for 2 min, AmpliTaq ® Fast DNA Polymerase UP activation at 50 °C for 2 min followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing/extension). Melting curves from 65 °C to 95 °C were run to evaluate the results. Each 10 µl DNA amplification reaction contained 2 µl PCR-grade water, 5 µl of SYBR® Select Master Mix (2X), 2 µl of 1:10 diluted cDNA template and 0.5 µl (final concentration of 500 nM) of forward and reverse primers. Samples were run in triplicates with NTC, NAC and genomic DNA as controls. Primers used for RT-qPCR are shown in Table 1. The relative mRNA expression was calculated following normalization to the Ribosomal Protein L37 (rpl37) and Ubiquitin (ubi) previously used in Etayo et al. (2021) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Gut was used as a calibrator. One-way ANOVA and the post hoc Tukey's test were used to show statistically significant differences ($p < 0.05$) in the target gene expression between tissues.

### 2.5. Isolation of leukocytes

Leukocytes were isolated from peripheral blood from 6 wrasse individuals. Immediately after fish were killed, blood was collected using a syringe containing heparin and kept on ice. 0.5 ml of blood was diluted in 2 ml of L15 medium (L-15 media without L-Glutamine adjusted to 370 mOsm by adding 5% (v/v) of a solution consisting of 0.41 M NaCl, 0.33 M NaHCO3 and 0.66% (w/v) D-glucose). The medium was supplemented with 100 mg/ml of penicillin/streptomycin, 2 mM L-glutamine (Lonza Biowhittaker), 10 U/ ml heparin (Sigma Aldrich) and 15 mM HEPES (Sigma Aldrich) (final concentrations of the total L-15 medium volume). L-15 medium is a widely used, and commercially available cell growth medium buffered by phosphates and free of base amino acids. Leukocytes from blood were isolated using two percoll solutions with different densities (1.05 and 1.07 g/ml) as previously reported in (Haugland et al., 2014). The diluted blood in L-15 medium was carefully layered on top of the percoll gradient in 10 ml centrifuge tubes. Centrifugation of the gradients was performed at 400 x g, 4 °C for 55 min. After centrifugation, the leukocyte fraction was collected from the percoll gradient and washed in PBS-380 (380 mOsm) by centrifugation at 200g, 4 °C for 10 min. Leukocytes were then resuspended in 0.5 ml of L-15 medium and a small aliquot (80 µl) was taken for quality control using a cytospin to verify the success of the isolation showing a minority of red blood cells (RBC) in the leukocyte fraction. The rest of the leukocyte suspension was mixed with PBS-380 and washed a second time. The cells were finally resuspended in 1 ml of PBS 0.1% BSA and kept on ice.

**Table 2**
Probes used in *in situ* hybridization.

| | Probe | Accession no. | Target region (bp) | Catalogue no. |
|---|---|---|---|---|
| **Target** | pIgR (Ballan wrasse) | XM_020653429.2 | 81–1086 | 845441 |
| | pIgR (Atlantic salmon) | XM_014189417.1 | 119–1145 | 845451 |
| **Control** | DapB (negative) | EF191515 | 414–862 | 310043 |
| | PPIB (positive) | NM_001140870 | 20–934 | 494421 |

### 2.6. Immunomagnetic separation of leukocytes

Staphylococcal protein A (prot-A) coated Dynabeads (Invitrogen™ 10002D) (30 mg/ml) were used for one-step extraction of IgM+ leukocytes. A fraction of freshly isolated leukocytes (500 µl) suspended in PBS 0.1% BSA was mixed with 100 µl of prot-A beads and incubated on a rotor at 4 °C for 20 min. After incubation of the prot-A beads/leukocyte mix, prot-A beads were washed 5 times with PBS 0.1% BSA. The presence of RBC in the leukocyte fraction was likely eliminated after prot-A beads incubation and washing steps. These leukocyte fractions were finally kept in PBS 0.1% BSA until further treatment.

### 2.7. RNA isolation from immune cells and RT-qPCR

RNA was extracted from 6 aliquots of purified leukocytes and 6 aliquots of IgM+ leukocytes, using HiBind® RNA Mini Columns (Omega Bio-Tek). The leukocytes were pelleted and mixed with 700 µl of TRK lysis Buffer (Omega Bio-Tek), and RNA was extracted following the manufacturer's guidelines. The quantity of total RNA was measured using a Nanodrop spectrophotometer. For cDNA synthesis, 160 ng of total RNA was used in a total reaction volume of 20 µl. First strand cDNA was synthesized using SuperScript™ II reverse transcriptase (Invitrogen) and an oligo $dT_{16}$ primer. cDNA was diluted 1:5 and RT-qPCR was performed as previously described for 45 cycles of amplification. Rpl37 and ubi were used as reference genes and the data were analysed using the $2^{-\Delta\Delta Ct}$ method.

### 2.8. In situ hybridization

Farmed ballan wrasse samples from liver, gut, and gills were fixed in 4% paraformaldehyde PBS, pH 7.4 at room temperature (rt) for 24–48 h. Samples were then dehydrated and embedded in paraffin wax. Sections (4 µm) were mounted on glass slides and de-waxed. All samples were histologically examined by staining with haematoxylin and eosin (HE) as a regular procedure for tissue quality. For comparison, tissues (gut, gill, head kidney and spleen) from Atlantic salmon (*Salmo salar*) were similarly included.

For *in situ* hybridization, RNA Scope 2.5 HD Assay-Red (Advanced Cell Diagnostics, Newark, CA, USA) probes were designed and produced by the manufacturer based on the provided pIgR sequences of ballan wrasse and Atlantic salmon (Table 2). *In situ* procedures were followed as described by (Løken et al., 2020). In short, paraffin-embedded tissue sections (4 µm) were mounted on positively charged glass slides (Superfrost, Mentzel), dried at 37 °C for 48 h and further incubated at 60 °C for 90 min. Subsequently, samples were de-paraffinized by incubation in 2 × 5 min xylene and 2 × 1 min 100% ethanol. Samples were

treated for endogenous peroxidase blocking (10 min at rt), followed by target retrieval (15 min at 100 °C), and protease digestion (30 min at 40 °C) to allow permeabilization of cells. For probe hybridization, samples were incubated with the RNA scope probe for 2 h at 40 °C. A series of hybridizations were performed using different incubation times according to the manufacturer's instructions (Wang et al., 2012) to allow amplification of the signal. For signal detection, samples were then treated with Fast Red chromogenic substrate for 10 min and subsequently stained with a 50% Gill's hematoxylin solution for 2 min. Samples were then dehydrated and mounted with EcoMount (BioCare Medical, Pacheco, CA, USA).

### 2.9. Collection of mucus and serum samples

An illustration gathering the methodology described below in section 9–15 is shown in Supplementary material 1.

Mucus was collected from external surfaces (skin and gills) and gut (foregut and hindgut) from wild ballan wrasse. Mucus from skin was collected by placing fish in a heavy-duty plastic bag with 5 ml of PBS mixed with protease inhibitors (Pierce TM, Thermo Fisher Scientific) for 5–10 min with gentle rubbing of the fish. The skin mucus was scraped off the fish with a spatula, collected in the bag and further centrifuged at 400 x g, 4 °C for 10 min to pellet scales and fish cells.

Gills and gut were excised and washed 3 times in cold PBS (the gut was reversed inside-out). Subsequently, gill and gut were incubated in PBS with protease inhibitors (at a ratio of 1 g of gill/gut tissue per ml of PBS) at 4 °C for 12 h with gently shaking (Xu et al., 2016). The suspension was then collected and centrifuged at 400 x *g*, 4 °C for 10 min. For serum isolation blood was collected by venepuncture, allowed to clot at 4 °C overnight. The serum was then obtained by centrifugation at 16,000 x *g* for 5 min and diluted 1:1 v/v with PBS. All mucus samples from skin, gill, and gut were split into three aliquots and kept at −20 °C until further use. One aliquot referred to as mucus extract was analysed by liquid chromatography-mass spectrometry (LC-MS/MS) and the remaining two were separately subjected to either one-step IgM purification or SDS-PAGE electrophoresis as explained below.

### 2.10. One-step purification of IgM from mucus and serum

Serum and mucus samples (500 µl) were incubated with prot-A coated Dynabeads (30 mg/ml) on a rotating mixer at 4 °C for 1 h. After binding, the supernatant was removed and beads were washed 3 x in PBS 0.02% Tween®-20, pH 7.4. After the last wash, beads were mixed with PBS and stored at −20 °C. A fraction of the prot-A purified samples were eluted with 1 x SDS sample buffer and boiled for 5 min before SDS-PAGE. The remaining fractions of the prot-A purified serum and mucus samples were analysed by LC-MS/MS.

### 2.11. SDS–Polyacrylamide Gel Electrophoresis (PAGE) and Western blot analysis

Prot-A purified samples (serum and mucus) and mucus extracts were run on reducing, denaturing, 4–15% gradient gels. Western blotting was performed at 25 V for 30 min at 22 °C using a Trans-Blot Turbo System (Bio-Rad). The PVDF membrane was blocked in 5% dry milk in PBS Tween®20 for 30 min and incubated with rabbit anti-ballan wrasse IgM diluted 1:5000 in blocking buffer for 2 h as described in (Bilal et al., 2016). The membrane was washed 3 times in PBS 0.02% Tween®20 at 22 °C for 5 min each time and incubated with HRP-conjugated anti--rabbit IgG secondary antibody diluted 1:2000 in wash buffer. The PVDF membrane was developed using ECL reagents (Pierce™ ECL Western Blotting Substrate). Prot-A purified IgM from wrasse serum was used as a positive control.

### 2.12. Tissue lysate preparation

Liver, spleen, and gills from ballan wrasse were excised and washed in cold PBS mixed with protease and phosphatase inhibitors (Pierce™). One hundred milligrams of each tissue were lysed in 1 ml lysis buffer (4% SDS, 0.1 M Tris-HCl pH 7.6) using a tissue disruptor and further sonicated using an ultrasonication rod (Q55 Sonicator, Qsonica, CT, USA) at 30% amplitude for 30 s. The lysed tissue was centrifuged at 400 x g for 10 min at rt, the supernatant incubated at 95°C for 5 min and further centrifuged at 15 000 x *g* for 10 min. The supernatant was collected and stored at −20 °C until further use.

### 2.13. Bile extraction and purification of IgM

Initial experiments showed that IgM was present in bile of every individual of wrasse (>5 individuals; results not shown). For the present study, bile was sampled from three individuals by puncturing the gall bladder. Bile was pooled and centrifuged at 400 x g for 10 min at 4 °C. The supernatant (250 µl) was divided into 2 fractions; one of them (non-treated bile; NT) was directly applied on a SDS gel and the other one was subjected to one-step IgM purification using prot-A coated Dynabeads (30 mg/ml) as previously described. Non-treated (NT) bile and prot-A purified bile were analysed by SDS-PAGE and three pieces of the preparative gel; containing proteins between 20 and 50 kDa, 20–30 kDa, and 30–70 kDa, were excised from the gel and analysed by LC-MS/MS. To corroborate the presence of IgM in bile, a fraction of prot-A purified bile was subjected to Western blot analysis as previously described. In this case, the PVDF membrane was developed using TMB solution and purified IgM from wrasse serum was used as a positive control.

### 2.14. Liquid chromatography-mass spectrometry (LC-MS/MS)

For the identification of proteins, samples purified by prot-A beads (serum, mucus, and bile (preparative gel bands)) as well as non-purified samples (mucus extracts, tissue lysates, and NT bile (preparative gel)) were sent to the Proteomics Unit at the University of Bergen, Norway (PROBE) for LC-MS/MS analyses. A recapitulation of all the samples subjected to LC-MS/MS, preparation techniques and quantitative information is shown in Supplementary material 1 to facilitate the interpretation of the results. Methodology on the LC-MS/MS analyses workflow can be found in supplementary material 2.

### 2.15. Molecular analyses of wrasse pIgR-Like (pIgRL)

Based on the predicted sequence of the pIgRL in GenBank (XM_020654174), cDNA was copied and cloned from liver, gut (foregut and hindgut) and gills as previously described in Section 2. The pIgRL gene was studied using bioinformatic tools as described in Section 3. After cDNA synthesis, SYBR® Select Master Mix was used for RT-qPCR analysis in different tissues of ballan wrasse and the relative abundance of pIgRL mRNA was calculated as described in Section 4. Forward and reverse primers for pIgRL are shown in Table 1.

## 3. Results

### 3.1. Cloning and molecular characterization of the putative ballan wrasse pIgR

BLAST searches in GenBank utilizing teleost pIgR polypeptides as queries against ballan wrasse *(Labrus bergylta)* resulted in hits with predicted mRNAs automatically annotated as "CMR35-like transcripts". The corresponding gene in scaffold 492 (Acc. nos. **FKLU01000493.1/NW_018114907.1**) was annotated as "pIgR" in the first version of the ballan wrasse genome and revealed conserved synteny to other teleosts as it was flanked by the *DAD1* and *LRRC24* genes. The wrasse pIgR mRNA was transcribed from a single-copy gene. The structure was

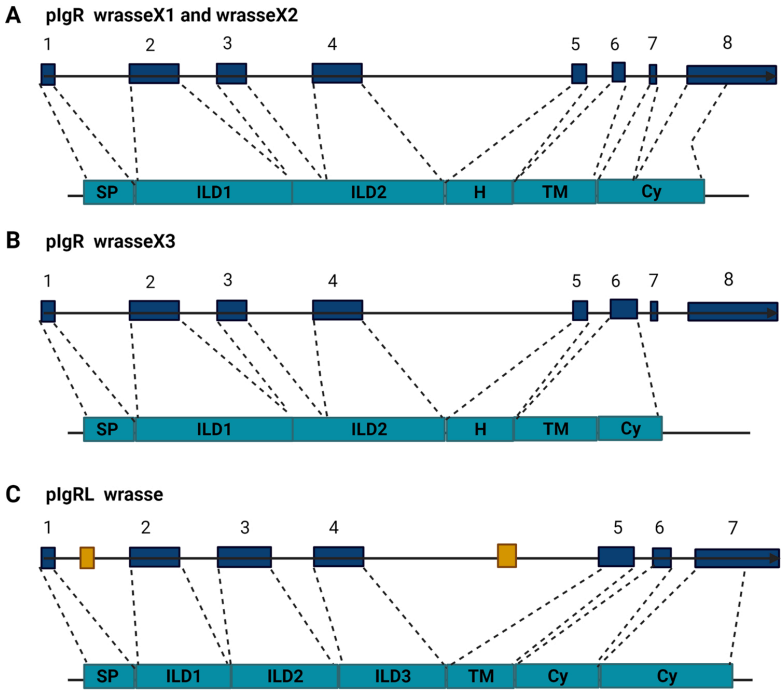



**Fig. 1.** Schematic illustration of the pIgR and pIgRL genes in ballan wrasse. The pIgR gene (Scaffold 492) with its mRNA splice variants and correspondence between exons and domains in ballan wrasse is shown in A and B. The three splice variants of the gene were designated as wrasseX1, wrasseX2 and wrasseX3; A) WrasseX1 and wrasseX2 differed with respect to an alternative use of splice acceptor sites in exon 7, resulting in a 7 amino acid shorter cytoplasmatic tail in wX2. B) WrasseX3 used an alternative splice donor site in exon 6, resulting in a stop codon and a short cytoplasmatic tail. SP (Signal peptide), ILD1 and ILD2 (Ig- like domain 1 and 2), H (Hinge region), TM (Transmembrane region), Cy (Cytoplasmatic region). C) pIgRL gene in ballan wrasse and the correspondence between exons and protein domains. Exons marked in dark blue show exons that are in the predicted pIgRL mRNA sequence (XM_020654174). Each ILD is encoded by a separate exon. Exons in yellow (between exons 1–2 and between exons 4–5) show two additional exons that were present in some of the cDNAs that were cloned in the present study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

equivalent to other teleost pIgR genes, with 8 exons and 7 introns (Fig. 1). Three slightly different predicted transcripts (annotated as wrasseX1, wrasseX2 and wrasseX3) were confirmed by cloning. An alignment of the pIgR gDNA and the three mRNA splice variants with the location of the exons/introns and designed primers is shown in Supplementary material 3.

The predicted full-length mRNA of WrasseX1 is 1484 nucleotides, wrasseX2 is 1463 nucleotides while wrasseX3 is slightly shorter and contains 1233 nucleotides, encoding 345, 338 and 302 amino acid long polypeptides, respectively. The translated pIgR variants of ballan wrasse are aligned with corresponding polypeptides from other teleosts in Fig. 2. The three splice variants were cloned from foregut and kidney and appeared to be expressed in all the investigated tissues of adult healthy fish (Fig. 3).

### 3.2. Differential expression of pIgR in ballan wrasse

RT-qPCR analyses showed differential expression of pIgR in a series of tissues (Fig. 4a). The highest expression was observed in liver, followed by gills and spleen. The expression of pIgR in liver was significantly higher than in muscle, head kidney, and other mucosal organs such as skin, foregut, and hindgut. The mRNA expression of pIgR was below the cut off value selected as a reliable detection limit (Fig. 4b).

### 3.3. In situ hybridization

RNAscope *in situ* hybridization was performed in a series of tissues from ballan wrasse (Fig. 5). Positive signal (pink) was found in the lamina propria of gut (Figs. 5a,5d) and hindgut (Figs. 5c,5f), and scattered positive cells were detected in the gill lamellae (Fig. 5b). Positive signal was also found in liver (Fig. 5e). Control probes are shown in supplementary material 4. *In situ* hybridization was applied to mucosal tissues of salmon showing a similar pattern in the location of the pIgR mRNA (supplementary material 4). In addition, lymphoid organs of salmon, such as head kidney and spleen were investigated and showed relatively high levels of pIgR-positive cells, also shown in supplementary material 4.

### 3.4. Purification of IgM from mucus

Staphylococcal protein A has been successfully used for isolation of Igs from several teleosts (Bilal et al., 2016; Bromage et al., 2004). Prot-A coated magnetic beads were used to obtain IgM-enriched samples from mucus of skin, gills and gut of adult fish. After partial purification of mucus IgM, both prot-A purified samples (mucus and serum) and mucus extracts (not treated with prot-A beads) were analysed with specific antibodies against wrasse IgM. Enriched IgM was found in all prot-A purified samples (Fig. 6). The presence of IgM was also confirmed in mucus extracts (data not shown) before LC-MS/MS analyses.

### 3.5. Purification of IgM from bile

Bile IgM was purified with prot-A beads revealing 2 clear bands at 75 kDa and 25 kDa with reactivity to rabbit antiserum against wrasse IgM (Fig. 6d). Two preparative gel segments (20–30 kDa and 30–70 kDa) from the prot-A purified bile, and one larger gel segment (25–50 kDa) from the non-treated bile were analysed by LC-MS/MS (Fig. 6c).

### 3.6. Liquid chromatography-mass spectrometry (LC-MS/MS)

Non-targeted mass spectrometry (MS) is a highly sensitive method for the detection of specific peptides (Belghit et al., 2021). Peptide Spectra Matches (PSMs) identified IgM as the most frequent hit in mucus and serum, whereas IgT and IgD had relatively few numbers of PSMs (Table 3).

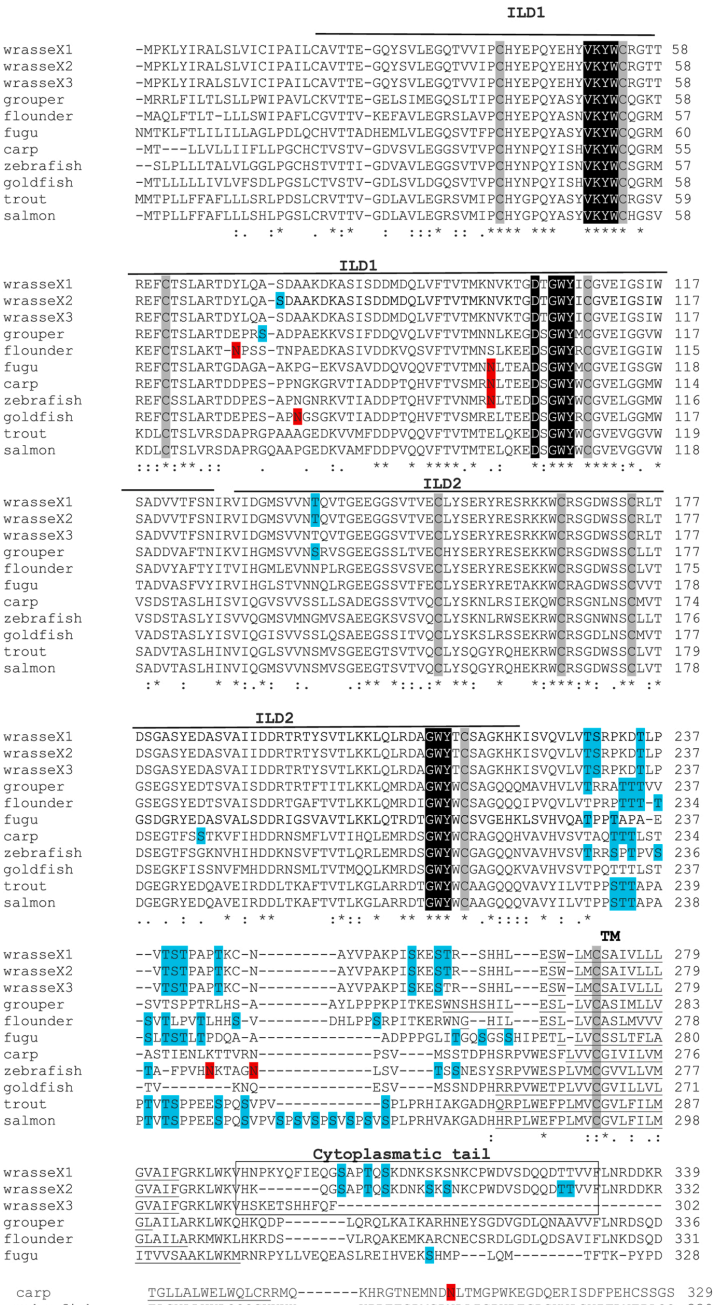



**Fig. 2.** Alignment of three ballan wrasse pIgR variants (wrasseX1, wrasseX2 and wrasseX3) and corresponding polypeptides from other teleost fish. The alignment shows the two Ig-like domains (ILDs) present in teleost pIgR (ILD1 and ILD2), the transmembrane region (TM) and the cytoplasmatic tail (Cy). Residues identical in all sequences, highly conserved sequences, and conserved sequences are indicated by stars (*), colons (:), and periods (.) respectively. Conserved motifs in ILDs are shaded in black with white font. The positions of fully conserved cysteine (C) residues are shaded in dark gray. The O-glycosylation sites are shaded in light blue and asparagine residues (N) predicted to be N-glycosylated are highlighted in red (only predicted glycosylated sites with scores higher than 0.5 are shown). The amino acid sequence corresponding to the TM region is underlined. Differences in the sequences of the three splice variants of wrasse are marked with a box. GenBank accession numbers of the pIgRs are: wrasseX1, wrasseX2, and wrasseX3, *Labrus bergylta* (XM_020653428.2, XM_020653429.2, XR_002278599.2 respectively); grouper, *Epinephelus coioides* (FJ803367.1); flounder, *Paralichthys olivaceus* (HM536144.1); fugu, *Takifugu rubripes* (AB176853.1); carp, *Cyprinus carpio* (GU338410.1); zebrafish, *Danio rerio* (NM_001302250.1); goldfish, *Carassius auratus* (KY652915.1); trout, *Oncorhynchus mykiss* (FJ940682.1); salmon, *Salmo salar* (GQ892056.1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

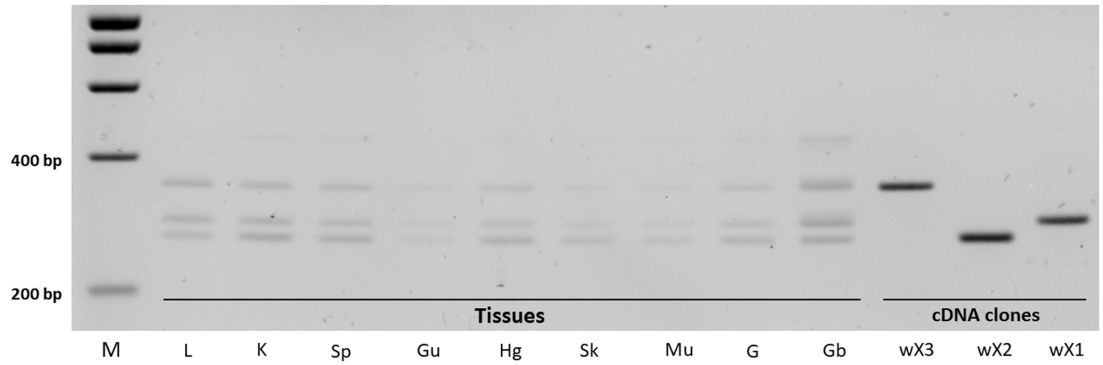



**Fig. 3.** Expression of three pIgR splice variants in different tissues of ballan wrasse (wX1, wX2 and wX3). The tree splice variants of the pIgR gene were present in all investigated tissues. From left to right; M, marker; L, liver; K, kidney; Sp, spleen; Gu, foregut; Hg, hindgut; Sk, skin; Mu, muscle; G, gills; Gb, gall bladder. Different amounts of the RT-PCR products were applied on the gel to optimize the presentation of the band patterns. On the right of the gel, the cDNA clones corresponding to the three splice variants are shown (equal amounts of PCR amplified inserts were applied). wX3, splice variant wrasseX3 (access. XR_002278599); wX2, splice variant wrasseX2 (access. XM_020653429); wX1, splice variant wrasseX1 (access. XM_020653428).

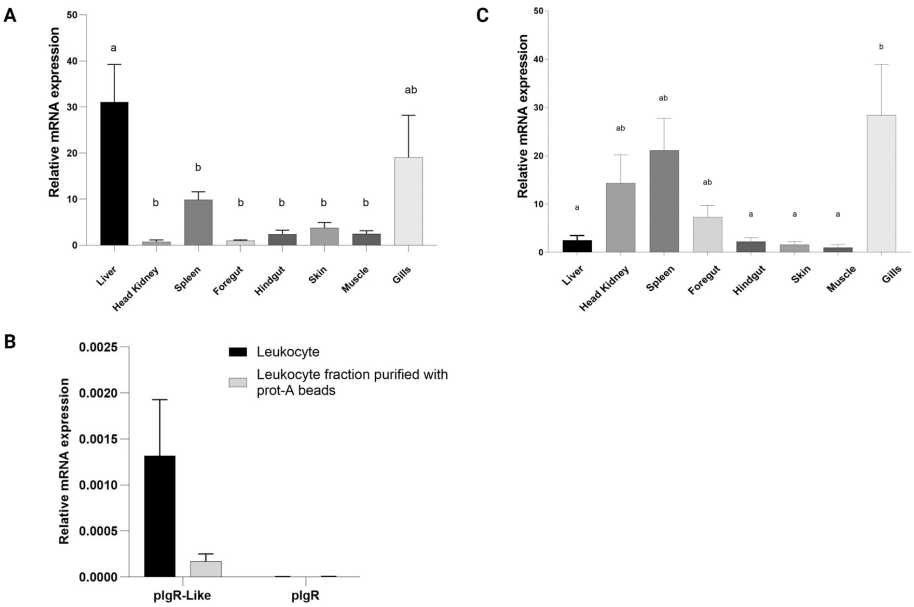



**Fig. 4.** Relative mRNA expression of pIgR and pIgR-L (pIgRL) in different tissues and blood leukocytes from ballan wrasse measured by RT-qPCR. A) Relative abundance of pIgR mRNA in different tissues. Data are expressed as mean values (±SEM) of n = 6 individuals and gut was used as calibrator. B) Relative abundance of pIgR and pIgRL mRNA in blood leukocytes. Total leukocytes and leukocytes captured with prot-A coated magnetic beads are shown. Data are expressed as mean values (±SEM) of n = 6 individuals. C) Relative abundance of pIgRL mRNA in different tissues. Data are expressed as mean values (±SEM) of n = 5 individuals, and muscle was used as calibrator. Significances were tested by One-way ANOVA and Tukey's test in A and C and indicated by letters as a ‡ b where columns with different letters are significantly differently expressed. Ubi and rpl37 were used as reference genes for normalization in all cases.

When comparing mucus extracts (not purified with prot-A beads), the IgT/IgM ratios were 30, 40, and 50-fold higher in gill, foregut, and skin mucus respectively, compared to serum. Following a similar trend, the IgD/IgM ratios were 50, 70, and 80-fold higher in mucus than in serum. At the same time, the number of PSMs for IgM varied between 15 and 70 in the different mucus samples (prot-A purified) and mucus extracts (not incubated with protein A beads), and only 2–9 PSMs corresponded to the pIgR except for skin which showed 22 PSMs (Table 3). In order to identify the location of the peptide matches in wrasse pIgR, all peptides (PSMs) recognized by LC-MS/MS were manually curated (Supplementary material 2 and Supplementary material 5). Results showed that not only ILD1/ILD2, but also TM and Cy were present in all mucus samples indicating the presence of un-cleaved pIgR.

Bile IgM was efficiently purified using prot-A coated beads (Figs. 6c and 6d). The putative SC of ballan wrasse pIgR is estimated to be 30 kDa. Those protein bands obtained from bile that were in the range of the

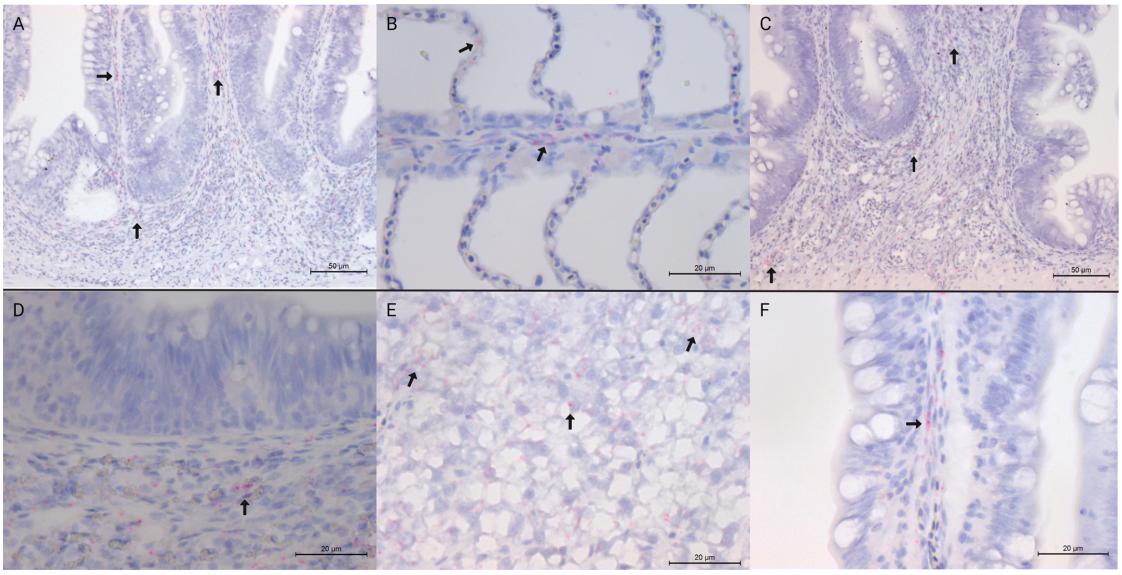
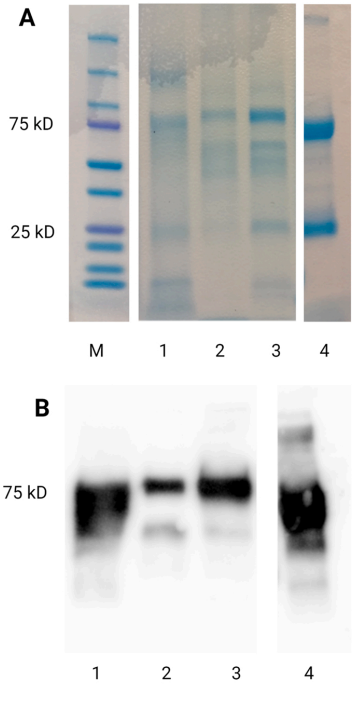



**Fig. 5.** RNAscope *In situ* hybridization demonstrating pIgR mRNA distribution in ballan wrasse; A) Gut, B) Gills, C) Hindgut, D) Gut epithelium (negative for pIgR) and the lamina propria with pIgR positive cells E) Liver F) Hindgut epithelium with pIgR positive cells in the lamina propria. Arrows indicate pIgR-positive cells. Scale bars are as followed; A) 50 µm, B) 20 µm, C) 50 µm, D) 20 µm, E) 20 µm, and F) 20 µm.



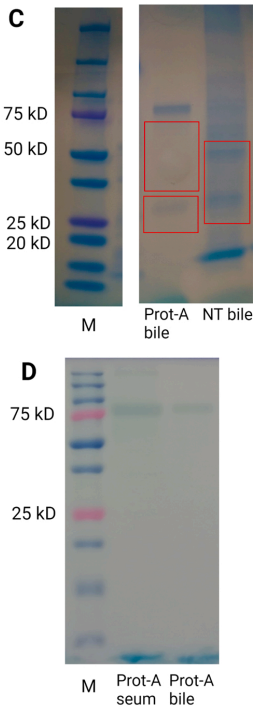




**Fig. 6.** SDS-PAGE analysis of protein-A purified mucus, protein-A purified bile (Prot-A bile) and non-treated bile (NT bile) from ballan wrasse. Serum was purified with prot-A and used as a positive control in A, B, and D. A) Prot-A purified mucus from hindgut, skin and gills were separated by SDS-PAGE and stained with Coomassie blue. B) Western blot of corresponding samples (hindgut, skin and gills) incubated with rabbit anti wrasse IgM antiserum and developed using ECL reagents. All samples were subjected to the same SDS-PAGE and WB-blot analysis, but the original image has been modified for easier interpretation. C) Prot-A purified bile and NT bile were separated by SDS-PAGE and stained with Coomassie blue. Red boxes indicate gel segments that were excised from the polyacrylamide gel for LC-MS/MS analysis. Marker and samples were applied on the same gel. D) Western blot of protein-A purified serum (Prot-A serum) which was used as a positive control, and protein-A purified bile (Prot-A bile) incubated with rabbit anti wrasse IgM antiserum and developed with TMB solution. M; Marker, lane 1; prot-A hindgut mucus, lane 2; prot-A skin mucus, lane 3; prot-A gill mucus, lane 4; prot-A serum (control).

**Table 3**
Peptide Spectra Matches (PSMs) for immunoglobulins, pIgR and pIgRL in foregut, hindgut, gill and skin mucus, serum, organ lysates (liver, spleen, and gill) and bile of ballan wrasse by LC-MS/MS.

| Protein | Accession no. | Prot-A Serum[a] | Prot-A Hindgut mucus[a] | Prot-A Gill mucus[a] | Prot-A Skin mucus[a] | Gut mucus extract[b] | Gill mucus extract[b] | Skin mucus extract[b] | Liver lysate[c] | Spleen lysate[c] | Gill lysate[c] | NT Bile (25–50 kDa)[d] | Prot-A purified bile (20–30 kDa)[d] | Prot-A purified bile (30–70 kDa)[d] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IgM | AOW44093 | 295 | 69 | 53 | 15 | 25 | 56 | 52 | 140 | 140 | 124 | 38 | 2 | 128 |
| IgT | XM_029282586.1 | 3 | 8 | 6 | 9 | 10 | 17 | 27 | 81 | 84 | 85 | 21 | 10 | 12 |
| IgD | XM_020658986.2 | 2 | 7 | 8 | 7 | 13 | 19 | 29 | 95 | 93 | 76 | 18 | 10 | 7 |
| pIgR | XM_020653429.2 | 2+(2) | 2+(2) | 2 | 2+(4) | 9 | 4 | 12+(10) | 31+(9) | 21+(15) | 26+ (10) | 5+(2) | 3+(4) | 2+(2) |
| pIgRL | XM_020654174 | 1 | 8 | 8 | 9 | 6+(1) | 12+(1) | 18+(3) | 50+(6) | 31+(4) | 35+(5) | 11+(3) | 4+(1) | 7 |

Note: Only PSMs with probabilities higher than 0.7 were considered. The total number of PSMs corresponding to the transmembrane region (TM) and the cytoplasmatic region (Cy) of the pIgR and pIgRL are included in (), while the total number of PSMs corresponding to ILD1 and ILD2 are not.

[a] Serum and mucus from hindgut, gill, and skin that were incubated with protein A for IgM purification.

[b] Mucus extracts (not incubated with protein A beads) from gut, gill, and skin.

[c] Protein lysates from liver, spleen, and gill.

[d] Preparative gel segments (from SDS-PAGE) from non-treated (NT) bile and prot-A purified bile.

putative wrasse SC were subjected to LC-MS/MS and the PSMs manually curated (Supplementary material 5). PSMs indicated the presence of pIgR (Table 3), however TM and Cy were also present following the same pattern as described in mucus.

Overall, the unexpected gene expression pattern, the relatively low number of matches with the pIgR-SC in mucus and bile, together with the fact that not only peptides corresponding to the putative SC of pIgR were found, led us to look for other possible pIgR candidates.

### 3.7. Analysis of a pIgR-like gene in ballan wrasse

BLAST searches using the pIgR polypeptides from mouse, human, chicken and frog as queries against the translated ballan wrasse genome shotgun sequence database produced higher scores with scaffold 539 containing a pIgRL gene than scaffold 492 containing the pIgR gene (Lie et al., 2018). To investigate whether wrasse pIgRL could be a possible homologue to higher vertebrate pIgR, the gene and corresponding mRNAs were further analysed.

Structural analyses of the predicted pIgRL mRNA showed that the transcript consisted of 1679 bp, of which 45 bp was a 5′ untranslated region, 1323 bp an open reading frame and 311 bp an untranslated 3′ tail. The predicted pIgRL gene included 7 exons and 6 introns where no Ig domain exons were split, like in other teleost pIgRL genes (i.e. in contrast to the teleost pIgR gene) (Kortum et al., 2014; Liu et al., 2019b; Tadiso et al., 2011). In addition to the predicted pIgRL transcript (XM_020654174), two other splice variants were identified in the present study, and two different pre-transcripts were also cloned from liver, gut, and gills. The mRNA splice variants revealed two additional exons in the pIgRL gene, as schematically shown in Fig. 1c. Among the cDNA clones from liver, gut, and gills, there was a dominant variant with an extra exon encoding an elongated (and possibly O-glycosylated) hinge region (Fig. 1c).

The pIgRL mRNA of *Labrus bergylta* (XM_020654174) encoded 441 amino acids comprising three extracellular Ig domains (ILD1, ILD2, and ILD3) as opposed to two ILDs reported in other teleost pIgRL. Thus, the wrasse pIgRL sequence showed relatively low similarity to published pIgRL sequences from other teleosts: 28% with Atlantic salmon (*Salmo salar*, HM452379), 21.8% with zebrafish (*Danio rerio,* XM_021466400) and slightly higher similarity (38%) with Japanese flounder (*Paralichthys olivaceus,* HM536144). To identify sequences that resembled the ballan wrasse pIgRL, a BLAST search was performed and similar molecules with 3 ILDs were found from lumpsucker (*Cyclopetrus lumpus*, XM_034544241), zander (*Sander lucioperca*, XM_031289048), spotty (*Notolabrus celidotus*, XM_034688120) and gilt-head bream (*Sparus aurata,* XM_030437348), all of which are automatically predicted mRNA sequences not formerly published in article form. The predicted ballan wrasse pIgRL sequence was compared with pIgRL sequences from the above-mentioned teleosts as shown in Supplementary material 6.

### 3.8. Differential expression of pIgRL in ballan wrasse tissues and leukocytes

Expression of the pIgRL gene in tissues was measured by RT-qPCR (Fig. 4c). The highest pIgRL expression was found in gills. It was also relatively high in spleen and head kidney, and much higher compared to the other tissues examined. Blood leukocytes were pIgRL positive (Fig. 4b). Furthermore, the presence of the pIgRL polypeptide was analysed by LC-MS/MS and PSMs were manually curated. The location of the peptide matches in the sequence of wrasse pIgRL are shown in Supplementary material 5.

## 4. Discussion

Teleost pIgR has been characterized in several species. In this study, the ballan wrasse counterpart was unambiguously identified based on synteny, exon/intron structure, and analysis of the translated products.

**Table 4**
Differential expression of pIgR and pIgRL genes in different tissues of teleosts, Chinese soft-shelled turtle, human, bovine, and chicken.

| Teleost specie | Author | Lymphoid organs | | | Mucosal organs | | | | Other tissues | | | | Leuk | Method |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pIgR | | Head kidney | Thymus | Spleen | Skin | Foregut | Hindgut | Gill | Liver | Muscle | Stomach | Gonads | | |
| Ballan wrasse | | + | | ++ | + | + | + | ++ | +++ | + | | | - | RT-qPCR |
| Olive flounder | (Xu et al., 2013) | ++ | | +++ | ++ | +++ | | ++ | +++ | + | + | | | RT-PCR |
| Common carp | (J. Rombout et al., 2008) | +++ | +++ | +++ | + | + | +++ | + | +++ | - | | | - | RT-PCR |
| Orange-spotted grouper | (Feng et al., 2009) | + | + | + | ++ | + | +++ | + | + | ++ | ++ | - | | RT-PCR |
| Fugu | (Hamuro et al., 2007) | + | +++ | + | +++ | +++ | | +++ | ++ | - | | + | - | RT-PCR |
| Grass carp | (Pei et al., 2019) | + | | ++ | ++ | +++ | | + | + | + | | | | RT-qPCR |
| Zebrafish | (Kortum et al., 2014) | + | | ++ | +++ | +++ | | + | +++ | | | + | - | RT-PCR |
| Atlantic salmon | (Tadiso et al., 2011) | + | | +++ | ++ | | ++ | ++ | + | | | | | RT-qPCR |
| Dojo loach | (Yu et al., 2018) | | | ++ | +++ | + | | ++ | +++ | ++ | | + | | RT-qPCR |
| Crucian carp | (Wang et al., 2017) | ++ | | ++ | + | ++ | | + | +++ | + | | | - | RT-qPCR |
| Nile tilapia | (Liu et al., 2019a) | + | + | ++ | + | + | | + | +++ | + | | | | RT-qPCR |
| Sea bass | (Yang et al., 2017) | + | | + | + | ++ | | + | +++ | + | + | | | RT-qPCR |
| Chinese turtle | (Xu et al., 2021b) | | | ++ | | +++ | ++ | | ++ | ++ | + | | | RT-qPCR |
| Human [a] | (Uhlén et al., 2015) | | | | | +++ | +++ | | + | | ++ | | | Transcriptome |
| Bovine | (Verbeet et al., 1995) | | | | | Present | Present | | Present | | | | | Northern-blot |
| Chicken | (Wieland et al., 2004) | | Present | | | Present | | | Present | | | | | Northern-blot |
| pIgRL | | | | | | | | | | | | | | |
| Ballan wrasse | | +++ | | +++ | + | ++ | + | +++ | + | + | | | +[c] | RT-qPCR |
| Zebrafish | (Kortum et al., 2014)c | | | + | + | + | | + | + | | | + | +[b] | RT-PCR |
| Olive flounder | (Liu et al., 2019) | + | | ++ | +++ | + | ++ | +++ | + | + | + | | +[c] | RT-qPCR |
| Atlantic salmon | (Tadiso et al., 2011) | ++ | | ++ | + | | ++ | +++ | + | | | | | RT-qPCR |

Note: Different methods have been used in the referred studies; gray and white shading indicate RT-qPCR and RT-PCR. The highest level of expression described by the contributing authors is expressed by (+++), followed by (++) and the lowest expression (+). Tissues that were analysed but not positive are indicated as (-). Empty spaces in the table indicate that the corresponding tissues were not analysed in the study. Higher vertebrates are shaded in blue and only tissues in common with teleosts are shown. For bovine and chicken, only those tissues reported as pIgR-positive are marked as "present".

a https://www.proteinatlas.org/ENSG00000162896-PIGR/tissue.

b pIgRL transcripts (pIgRL1 - pIgRL4) were differentially expressed in myeloid and lymphoid cell lineages.

c peripheral blood leukocytes.

(Pei et al., 2019; Uhlén et al., 2015; Verbeet et al., 1995; Wieland et al., 2004; Xu et al., 2021b)

However, the abundance of pIgR mRNA was relatively low in mucosal tissues, and significantly higher in liver and spleen. *In situ* hybridization revealed pIgR- positive cells dispersed in the lamina propria while it was undetectable in epithelial cells of foregut and hindgut. This result does not exclude that pIgR is expressed in epithelial cells, but the sensitivity of the method might not have been high enough to allow detection in these cells. Xu et al. (2016) detected positive epithelial cells in gills by immunohistochemistry, but also positive cells beneath the basement membrane seem to occur in their results. We emphasize that their results appeared in another species with another method, and mRNA levels may not necessarily reflect the protein levels in the cells. The signal obtained in our investigations in gills is difficult to interpretate as most of the pIgR-positive cells are not epithelial cells but apparently leukocytes. Interestingly, Rombout et al. (2008) observed pIgR-positive signals in both epithelium and lymphoid cells in the lamina propria of carp gut, suggesting a possible intestinal T-like cell population located in both the epithelium and lamina propria. In a search for an alternative pIgR candidate, a pIgR-like gene in ballan wrasse (having three extracellular Ig-domains) was analysed, but the expression pattern of the mRNA was not compatible with a functional role as pIgR.

The gene defined as the teleost pIgR was first characterized in fugu and carp (Hamuro et al., 2007; Rombout et al., 2008) exhibiting 2 Ig-domains suggested to correspond to ILD1 and ILD5 of the mammalian pIgR (Feng et al., 2009; Kong et al., 2018). Ballan wrasse pIgR consisted of 2 ILDs and showed 62% identity to the reported orange-spotted grouper pIgR (Blast search, results not shown). As previously described in salmon and other fish species, several predicted O-glycosylation sites were present in the hinge region (Tadiso et al., 2011).

In mammals, the pIgR gene is expressed in epithelial cells at sites where SIgA is transported to external secretions (Kaetzel, 2005; Woof and Kerr, 2006). Thus, pIgR mRNA can be localized to specific transport routes, whereas the pIgR protein shows a wider distribution. The teleost pIgR gene expression pattern appears to vary more, as summarized in Table 4. The somewhat atypical expression patterns in teleosts versus higher vertebrates have not been discussed much in previous publications.

Like in several other teleosts, ballan wrasse pIgR was highly expressed in liver and gills. Surprisingly, the abundance of pIgR mRNA was significantly lower in other mucosal tissues like skin, foregut, and hindgut. The same expression pattern was recently reported for Nile tilapia (Liu et al., 2019a). The RT-qPCR data did not fit into the typical mucosal expression pattern. On the other hand, the ballan wrasse pIgR mRNA was below the detection level in blood leukocytes as previously reported in carp (Rombout et al., 2008) and zebrafish (Kortum et al., 2014).

*In situ* hybridization of teleost pIgR has been reported in fugu (Hamuro et al., 2007), carp (Rombout et al., 2008) and turbot embryos (Qin et al., 2019) and immunostaining of the pIgR-SC has been conducted in flounder (Sheng et al., 2018), trout (Xu et al., 2013b, Xu et al., 2016; Zhang et al., 2010), sea bass (Yang et al., 2017) and Nile tilapia (Liu et al., 2019a) using antibodies against recombinant pIgR proteins. As previously mentioned, *in situ* hybridization in ballan wrasse show

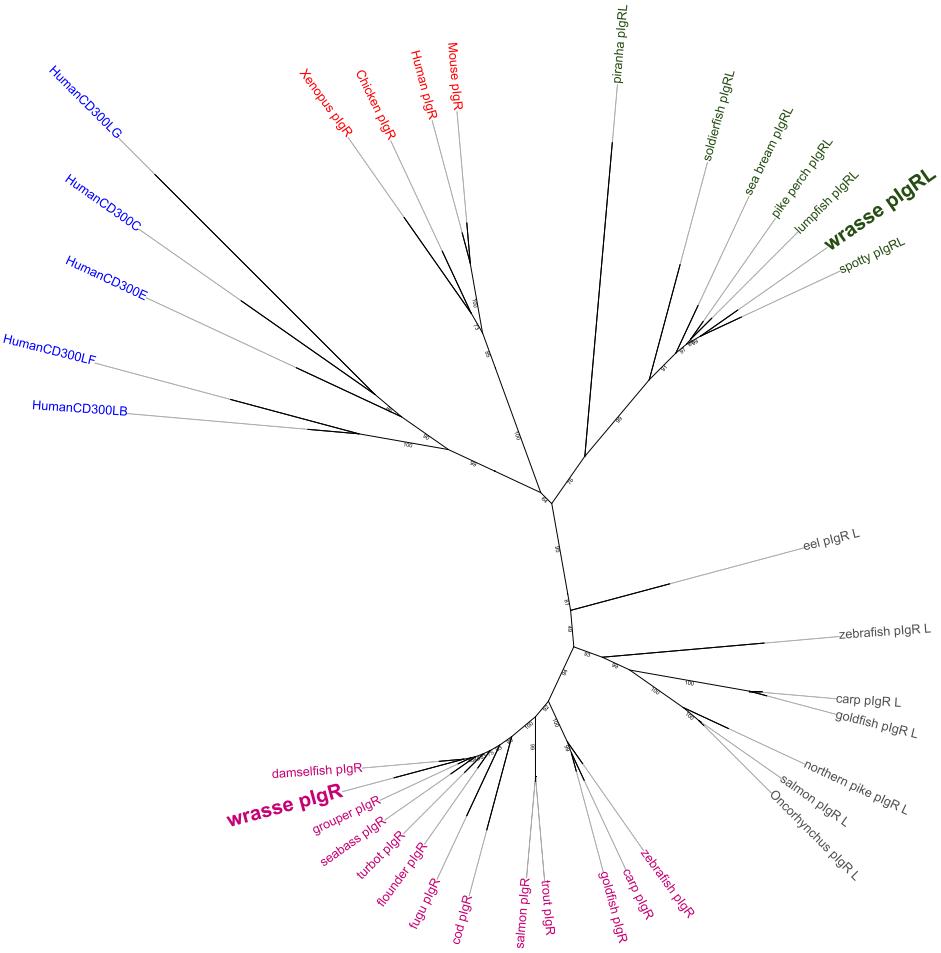



**Fig. 7.** Phylogenetic tree illustrating the relationship between pIgR and pIgRL in ballan wrasse and other related sequences, including human CMRF-35-like molecules. The pink branch shows teleost pIgR polypeptides which consists of two ILDs. The gray branch shows pIgRL in different teleosts which consists of two ILDs. The branch in green shows sequences that are most closely related to ballan wrasse pIgRL, comprising 3 ILDs. The red branch shows pIgR from human, mouse, chicken, and frog. The branch in blue shows human CMRF-35-like molecules. Ballan wrasse pIgR and pIgRL are highlighted with bigger font size. GenBank accession numbers for pIgR: Chicken, Gallus gallus (NM_001044644); Human, Homo sapiens (NM_002644); Mouse, Mus musculus (NM_011082); Clawed frog, Xenopus tropicalis (XM_031896818); Goldfish, Carassius auratus (KY652915); Common carp, Cyprinus carpio (GU338410); Zebrafish, Danio rerio (NM_001302250); Orange-spotted grouper, Epinephelus coioides (FJ803367); Atlantic cod, Gadus morhua (KJ460333); Japanese sea bass, Lateolabrax japonicus (KU516384); Rainbow trout, Oncorhynchus mykiss (FJ940682); Japanese flounder, Paralichthys olivaceus (HM536144); Atlantic salmon, Salmo salar (GQ892057); Japanese pufferfish, Fugu rubripes (NM_001280015); Turbot, Scophthalmus maximus (KC142170); Damselfish, Acanthochromis polyacanthus (XM_022205111); Ballan wrasse, Labrus bergylta (XM_020653428). GenBank accession numbers for pIgRL: Ballan wrasse, Labrus bergylta (XM_020654174); Common carp, Cyprinus carpio (XM_019071756); Atlantic salmon, Salmo salar (HM452379); Zebrafish, Danio rerio (XM_021466400); Chinook salmon, Oncorhynchus tshawytscha (XM_024417571); Northern pike, Esox lucius (XM_013134949); Goldfish, Carassius auratus (XM_026289591); European eel, Anguilla anguilla (XM_035413180); Lumpfish, Cyclopterus lumpus (XM_034544241); Pike-perch, Sander lucioperca (XM_031289048); Red-bellied piranha, Pygocentrus nattereri (XM_037543149); Gilthead seabream, Sparus aurata (XM_030437348); Pinecone soldierfish, Myripristis murdjan (XM_030056215); New Zealand spotty, Notolabrus celidotus (XM_034688120). Accession number for human CMRF-35-like molecules: Human CD300C (XM_017024033.2); Human CD300LB (NM_174892.4); Human CD300LF (NM_001289082.2); Human CD300LG (NM_001168322.2); Human CD300E (NM_181449.3).

that most of the pIgR-positive cells in gills are not epithelial cells but cells located in the gill lamellae. Similarly, pIgR-positive cells were detected in the lamina propria under the basal lamina in wrasse gut. Although we cannot exclude the possibility of pIgR being expressed in epithelial cells, results strongly indicate the presence of pIgR mRNA in a leukocyte population. Mucosal tissues of Atlantic salmon were also investigated by *in situ* hybridization following a similar distribution pattern of the pIgR mRNA as in ballan wrasse. This could indicate that our findings might have more general implications among teleost fishes.

In this study we performed a gentle, one-step purification method with prot-A coated magnetic beads, previously shown to work well to purify IgM from wrasse serum and mucus (Bilal et al., 2016). The purpose was to remove as many other proteins from the samples as possible without too harsh treatment. Due to the "sticky" nature of mucus

samples, removing background proteins is challenging, but in the present study we obtained enriched IgM from gut, skin and gill mucus, and quite pure IgM from bile. Although LC-MS/MS analysis are not strictly quantitative, comparing untreated and prot-A purified samples could offer valuable information. IgM was dominant in all samples analysed by LC-MS/MS. The ratio IgT/IgM and IgD/IgM were in the order of 20–80-fold higher in mucus compared to serum of ballan wrasse. In agreement with this observation, IgT was reported to have a relatively higher concentration in gills of rainbow trout (Xu et al., 2016) where IgT was suggested to have a potential role in gill immunity. The number of PSMs with IgT and IgD was similar in all mucosal secretions from skin, gut, and gill. The presence of secreted IgD in mucosal sites was in accordance with (Perdiguero et al., 2019) that found abundant IgD+ IgM- plasmablasts in gills and gut of rainbow trout. On the other hand, (Xu et al., 2013b) reported IgD-secreting IgD+ IgM- plasmablasts in the intestine, but not in skin.

Human pIgR can be released bound to SIgA or as free SC at mucosal sites (Kaetzel, 2005; Woof and Kerr, 2006). At least one molecule of pIgR is needed for binding SIgA (in the ratio 1:1) and further secretion to mucus (Johansen and Kaetzel, 2011). Large amounts of free SC have also been reported in trout mucus (Kelly et al., 2017). The SC corresponds to the Ig-like extracellular domains of pIgR, i.e. 5 ILDs in humans (Johansen and Kaetzel, 2011) and presumably 2 ILDs in teleosts. Thus, when teleost SC is secreted to mucosal sites, only ILD1 and ILD2 are expected to be found in mucus while the transmembrane region (TM) and the cytoplasmatic tail (Cy) remain in the epithelium. In an attempt to identify the pIgR-SC in gut, hindgut, gill and skin mucus of ballan wrasse we used the high-throughput proteomics approach, LC-MS/MS, which showed that pIgs and the pIgR-SC were present in mucus. However, results showed that not only the SC of pIgR (ILD1 and ILD2), but also TM and Cy were present in all mucus samples. Although there is a relatively low number of PSMs corresponding to the SC compared to the PSMs corresponding to Igs and the TM and Cy of pIgR, LC-MS/MS does not allow us to be strictly quantitative. We hypothesize that the presence of TM and Cy in mucosal secretions correspond to a leakage of un-cleaved pIgR from tissue during mucus extraction. Altogether, results indicate that the SC is not covalently bound to mucosal IgM.

Studies on the Antarctic teleost, *Trematomus bernacchii* (Abelli et al., 2005) indicated transport of mucosal IgM from liver into bile across the hepatocytes to be further released into the gut. This process has been characterized in higher vertebrates as well, where IgA binds to the pIgR on biliary epithelial cells (BECs), travels across by transcytosis and is discharged into the bile ducts in complex with the SC (Brown and Kloppel, 1989a; Reynoso-Paz et al., 1999). The relatively high abundance of pIgR mRNA in the liver of ballan wrasse as well as the positive staining by *in situ* hybridization could indicate a role of pIgR at mediating the transport of pIgs from liver to gut through the bile (hepato-biliary transport route). However, if the mechanism was equivalent to that described in birds, rats and rabbits it would be expected to find considerable amounts of SC in bile and gut (Brown and Kloppel, 1989b; Kühn and Kraehenbuhl, 1981). Mucosal IgM was described in the bile ducts and capillaries in carp and in the previously mentioned Antarctic teleost (Abelli et al., 2005; Rombout et al., 2008). Our results showed the presence of IgM in the bile of wrasse. Proteins from bile were separated by SDS-PAGE to narrow the number of peptides analysed by LC-MS/MS avoiding possible masking of the protein of interest, in this case, the pIgR. Liver lysate was used to confirm the sensitivity of the method (LC-MS/MS) as liver showed the highest expression of pIgR mRNA and thus, the presence of the pIgR peptide was expected to be detectable. In line with results obtained from mucus, bile samples purified with prot-A beads also showed low numbers of PSMs corresponding not only to the putative SC but also to TM and Cy. Thus, it is plausible that the few matches with the whole pIgR peptide in mucus and bile correspond to degraded molecules.

In the context of a search for alternative pIgR candidates in ballan wrasse, BLAST searches against the translated genome, using higher vertebrate pIgR polypeptides as queries, revealed high scores with scaffold 539 (corresponding to mRNA XM_020654174). Subsequently, we saw that this poly-Immunoglobulin Receptor-Like (pIgRL) molecule contained 3 predicted ILDs as opposed to 2 in other published teleost pIgRL polypeptides (salmon (Tadiso et al., 2011), carp (Zhang et al., 2015), zebrafish (Kortum et al., 2014), and Japanese flounder (Liu et al., 2019b)). The number of pIgRL genes in different teleosts seems to vary considerably (Kortum et al., 2014), indicating that there is a large variation across the phylogenetic tree, although there are distinct features of pIgRL versus pIgR. In common with other reported pIgRL genes none of the Ig-domain (ILD) coding exons were split into two in wrasse pIgRL (Kortum et al., 2014; Tadiso et al., 2011). The pIgRL mRNA was also abundant in blood leukocytes, similar to zebrafish, and in contrast to pIgR (Kortum et al., 2014). These findings indicate that ballan wrasse pIgRL shares some features with other characterized pIgRL. A phylogenetic tree was constructed to show the relationship between the pIgR and pIgRL in ballan wrasse, other teleosts, higher vertebrates, and human CMRF-35-like molecules (Fig. 7).

The ballan wrasse pIgRL showed a significant high expression in gills compared to other mucosal organs, followed by lymphoid organs such as spleen and head kidney. A similar expression pattern was reported in Atlantic salmon (Tadiso et al., 2011). Challenge experiments with bacteria in zebrafish (Kortum et al., 2014) and flounder (Liu et al., 2019b) showed an increase in the expression of pIgRL, especially in the gut of zebrafish and in the gut, gills, and skin of flounder. Accordingly, the abundance of pIgRL mRNA increased in Atlantic salmon after exposure to sea lice (Tadiso et al., 2011). All this information indicates that the pIgRL is involved in mucosal immunity. Although challenge experiments were not performed in the present study, the pIgRL of ballan wrasse shows characteristics that resemble pIgRL-genes from other teleosts. Thus, we suggest that pIgRL is likely involved in mucosal immunity, encoding a surface receptor of a leukocyte sub-population present in blood of ballan wrasse.

In summary, although the pIgR has been reported to transport pIgs through epithelial cells in humans, IgM in flounder, trout, and carp (Rombout et al., 2008; Sheng et al., 2018; Xu et al., 2013a; Zhang et al., 2015) and IgT in rainbow trout (Xu et al., 2016), the present study indicates that ballan wrasse pIgR has another (or at least an additional) function in another cell type. We suggest that both pIgR and pIgRL in ballan wrasse are receptors on different leukocyte populations involved in mucosal immunity. As significant amounts of IgM were found in bile of ballan wrasse further studies should consider the hepato-biliary route regarding IgM delivery to the gut lumen.

## Author's contributions

**IH** and **AE** designed the experiments. Material preparation, data collection and analyses were performed by **AE**. **EOK** and **HB** performed the *in situ* hybridization. **AE** and **IH** wrote the manuscript. All authors read, commented, and approved the final manuscript.

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## Conflict of interest

None.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetimm.2022.110440.

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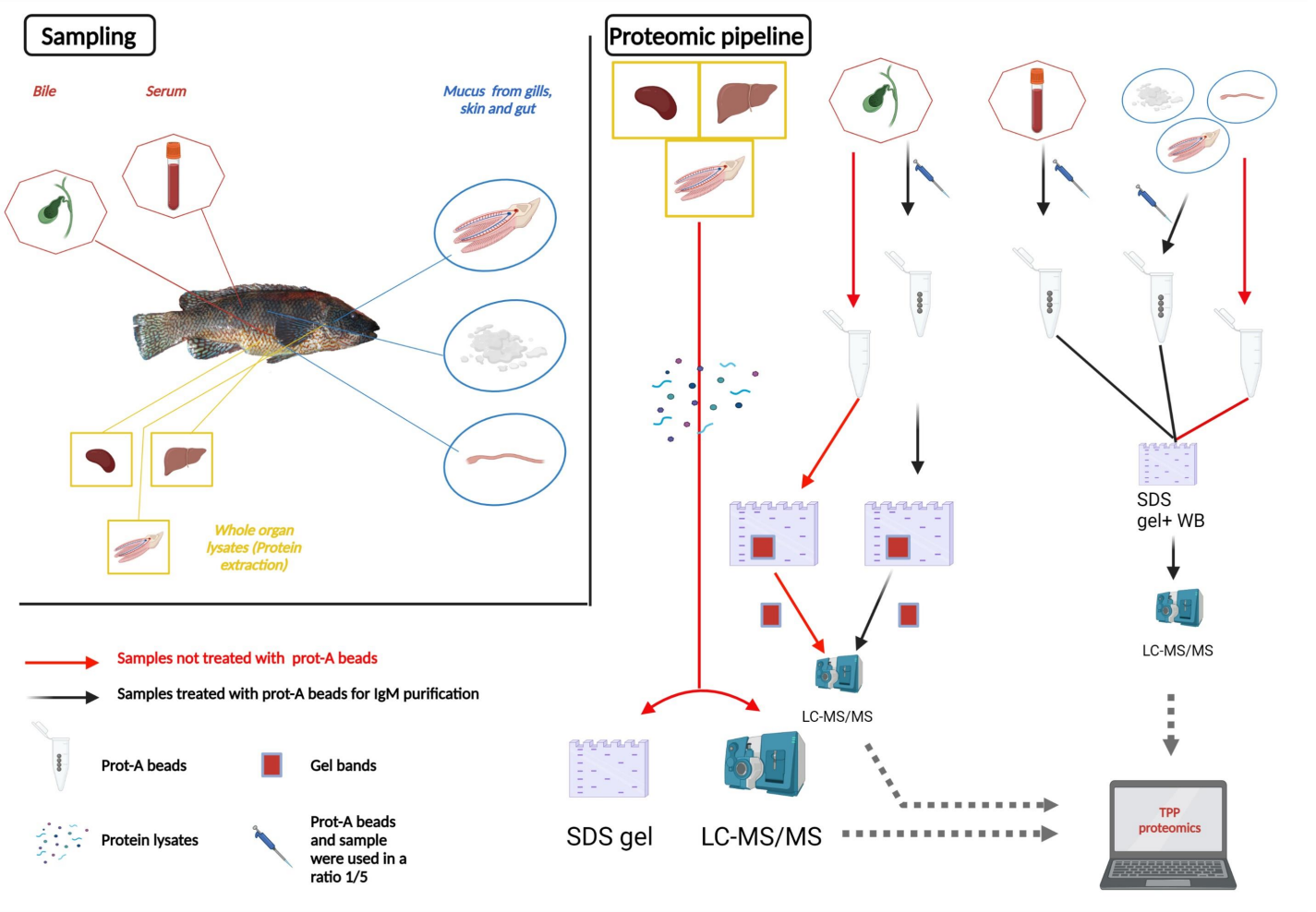
**Supplementary material 1.** Summary of the samples subjected to mass spectrometry analyses (LC-MS/MS) and its methodology. **A)** Illustration showing the origin of the samples on the left and the different pre-treatment before LC-MS/MS (proteomic pipeline) on the right. **B)** Summary of all the samples subjected to LC-MS/MS, preparation techniques, quantitative information and where to find them in the text.

**A**



**B**

| Sample name | Sample nature (Solution/gel band) | Origin | Pre-treatment | Final product | Prot-A purification (1,5 mg prot-A Dynabeads) | SDS gel | Western Blot | Figure in article | LC MS/MS | Protein concentration (µg/µl) for LC MS/MS | MS/MS instrument | Proteomic analyses |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **prot-A serum** | Solution | Serum | Diluted PBS | Serum | Yes | Yes | Yes | Fig 6a, 6d | Yes | 0,5 | Q Exactive HF Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **Prot-A Hindgut mucus** | Solution | Hindgut | Incubated in PBS with protease inhibitors + centrifugation | Mucus | Yes | Yes | Yes | Fig 6a,6b | Yes | 0,5 | Q Exactive HF Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **prot-A gill mucus** | Solution | Gill | Incubated in PBS with protease inhibitors + centrifugation | Mucus | Yes | Yes | Yes | Fig 6a,6b | Yes | 0,5 | Q Exactive HF Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **prot-A skin mucus** | Solution | Skin | Rubbing fish in PBS with protease inhibitors + scraping + centrifugation | Mucus | Yes | Yes | Yes | Fig 6a,6b | Yes | 0,5 | Q Exactive HF Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **Gut mucus extract** | Solution | Gut | Incubated in PBS with protease inhibitors + centrifugation | Mucus | No | Yes | Yes | Not shown | Yes | 0,5 | Q Exactive HF Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **Gill mucus extract** | Solution | Gill | Incubated in PBS with protease inhibitors + centrifugation | Mucus | No | Yes | Yes | Not shown | Yes | 0,5 | Q Exactive HF Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **Skin mucus extract** | Solution | Skin | Rubbing fish in PBS with protease inhibitors + scraping + centrifugation | Mucus | No | Yes | Yes | Not shown | Yes | 0,5 | Q Exactive HF Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **Liver lysate** | Solution | Liver tissue | Protein extraction in 4% SDS, 0.1M Tris-HCl pH 7.6 | Protein lysate | No | Yes | No | Not shown | Yes | 0,5 | Exploris 480 Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **Spleen lysate** | Solution | Spleen tissue | Protein extraction in 4% SDS, 0.1M Tris-HCl pH 7.6 | Protein lysate | No | Yes | No | Not shown | Yes | 0,5 | Exploris 480 Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **Gill lysate** | Solution | Gill tissue | Protein extraction in 4% SDS, 0.1M Tris-HCl pH 7.6 | Protein lysate | No | Yes | No | Not shown | Yes | 0,5 | Exploris 480 Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **Non-treated bile (NT)** | Gel band 25 to 50 kDa | Bile | Diluted PBS | Bile | No | Yes | No | Fig 6c | Yes | 0,5 | Exploris 480 Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **Prot-A purified bile (20- 30 kDa)** | Gel band 20 to 30 kDa | Bile | Diluted PBS | Bile | Yes | Yes | No | Fig 6c | Yes | 0,5 | Exploris 480 Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **Prot-A purified bile (30-70 kDa)** | Gel band 30 to 70 kDa | Bile | Diluted PBS | Bile | Yes | Yes | No | Fig 6c | Yes | 0,5 | Exploris 480 Orbitrap | Trans-Proteomic Pipeline (TPP) |
| **prot-A bile** | Solution | Bile | None | Bile | Yes | Yes | Yes | Fig 6d | No | | | |

**Article Information:**

"The teleost polymeric Ig receptor counterpart in ballan wrasse (Labrus bergylta) differs from pIgR in higher vertebrates"

Angela Etayo[1](0000-0001-6988-3057), Håvard Bjørgen[2] (0000-0003-4683-0088), Erling O. Koppang[2] (0000-0003-4859-1455), Ivar Hordvik[1](0000-0001-9361-7656)

1: Department of Biological Sciences (BIO), University of Bergen, Bergen, Norway

2: Section of Anatomy, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

Corresponding author: Angela Etayo

Email: angela.etayo@uib.no

**Supplementary material 2. A)** LC-MS/MS analyses workflow and **B)** Protein Spectra Matches (PSMs) of ballan wrasse pIgR found by LC-MS/MS. Peptides recognized by LC-MS/MS are underlined. Trypsin cleavage sites for the three splice variants of ballan wrasse pIgR are shaded in yellow. WrasseX1 wrasse X2 and wrasse X3 correspond to the three splice variants of pIgR in ballan wrasse.

**A)**

Protein concentration was first determined by Pierce BCA Protein assay kit (Thermo Scientific) and samples were adjusted to an initial concentration of 0.5 µg/µl. Trypsin solution (50mM Tris/1mM CaCl2) was added to all samples to achieve cleavage into smaller peptides before the actual MS. Samples were treated with 0.1 M DTT and subsequent incubation with 0.2 M IAA (isoamyl alcohol). Different treatments were followed for prot-A purified samples, mucus extracts and tissue lysates.

Prot-A purified samples from serum and mucus were incubated with 0.1M DTT for 10 min before adding 0.8 µg of trypsin and incubating on a shaker overnight at 37˚C. Mucus extracts and tissue lysates were digested according to the SP3 method described in (Hughes et al., 2019). After digestion, peptides were acidified with 0.5% TFA and cleaned with Oasis HLB 96-well elution plate. Dried peptides were dissolved in 2% ACN/0.5 % FA. Peptides were pre-concentrated on a 2 cm x 75 µm ID Acclaim PepMap 100 trapping column and separated on a 50 cm x 75 µm ID EASY-spray PepMap RSLC analytical column (Thermo Scientific). A Q-Exactive HF Orbitrap mass spectrometer equipped with an Easy-Spray (Thermo Scientific, San Diego, U.S.A.) and coupled to an Ultimate 3000 Rapid Separation LC system (Thermo Scientific) was used. Peptides were eluted during an 80 min binary gradient with solvent A (0.1% FA) and solvent B (0.1% FA / ACN). The gradient started at 5% B from 0-5 min and increased to 8% B from 5-5.5 min, then to 22% B from 5.5-40 min to 38% B from 40-55 min, and to 80% B from 55-57 min. Hold at 80% from 57-62 min, then ramped to 5% B from 62-65min and hold at 5% until the end of the run. The Q-Exactive HF mass spectrometer was operated in data dependent acquisition (DDA) mode. Full MS scans (scan range 375 – 1500 m/z) were acquired in profile mode with a resolution R = 120 000, a target value of 3 x 106 and maximum injection time of 100 ms. MS/MS scans were acquired in centroid mode for the top 12 precursors with intensity threshold > 5 x 104 (5.5% underfill ratio). The target ion was set to 1 x 105 with a maximum injection time of 50 ms and a resolution R = 30 000. The normalized collision energy was 28, the isolation window was 1.6 m/z with 0.3 m/z offset, and the dynamic exclusion lasted for 30 s. Peptides were eluted during a 195 min binary gradient with solvent A (0.1% FA) and solvent B (0.1% FA / ACN). The gradient started at 5% B from 0-5 min and increased to 8% B from 5-5.5 min, then to 24% B from 5.5-115 min to 35% B from 115-140 min, and to 90% B from 140-155 min. Hold at 90% from 155-170 min, then ramped to 5% B from 170-195 min.

For the mucus extracts, the same Q-Exactive HF Orbitrap mass spectrometer was used. The Q-Exactive HF mass spectrometer was operated as described before, except from the maximum injection time that was 110 ms and the dynamic exclusion period which lasted for 25 s.

For the lysates samples, Exploris 480 Orbitrap mass spectrometer equipped with an Easy-Spray Ion-source (Thermo Scientific, San Diego, U.S.A.) and coupled to an Ultimate 3000 Rapid Separation LC system (Thermo Scientific) was used instead. Peptides were eluted during a 195 min binary gradient with solvent A (0.1% FA) and solvent B (0.1% FA / ACN). The gradient started at 5% B from 0-5 min and increased to 8% B from 5-6 min, then to 32% B from 6-160 min. Elution of very hydrophobic peptides and conditioning of the column were performed during 10 min isocratic flow at 80% B (165 – 175 min) and 15 min isocratic flow at 5% B (180-195 min) respectively. The Exploris 480 mass spectrometer was operated in data dependent acquisition (DDA) mode. Full MS scans (scan range 350 – 1400 m/z) were acquired in profile mode with a resolution R = 120 000, a target value of 3 x 106 and maximum injection time of 100 ms. MS/MS scans were acquired in centroid mode for the top 15 precursors with intensity threshold > 5 x 104. The target ion was set to 1 x 105 with a maximum injection time of 75 ms and a resolution R = 15 000. The normalized collision energy was 30, the isolation window was 1.2 m/z, and the dynamic exclusion lasted for 45 s. Spray voltage were maintained at 1900 V, and the RF lens set at 40%.

For preparative gel bands (both prot-A purified bile and NT bile) the Exploris 480 Orbitrap mass spectrometer was used. The gel pieces were washed twice with 50% ACN and 25mM AMBIC solution, further treated with 0.1 M DTT and subsequent incubated with 0.2 M IAA (isoamyl alcohol) and washed. Trypsin was added to the gel bands and samples and peptides were eluted as described above.

The raw LC–MS/MS spectra files were converted to mzML using msConvert (version: 3.0., ProteoWizard (Kessner et al., 2008) in R interphase and consecutively processed with the Trans-Proteomic Pipeline (TPP) web interface (Deutsch et al., 2015). The spectra were searched against the concatenated forward and reversed decoy Fasta files containing the protein sequences of interest (pIgR, pIgRL, IgM, IgT, and IgD) were retrieved from the “proteome *Labrus bergylta*; UP000261660 (available in uniprot)” database using the Comet search engine (Eng et al., 2013). Only those Peptide Spectra Matches (PSMs) with a higher probability than 0.7 were considered as true PSM.

**B)**

```
                1        10        20        30        40        50
wrasseX3        MPKLYIRALSLVICIPAILCAVTTEGQYSVLEGQTVVIPCHYEPQYEHYVKYWCRGTTRE       60
wrasseX1        MPKLYIRALSLVICIPAILCAVTTEGQYSVLEGQTVVIPCHYEPQYEHYVKYWCRGTTRE       60
wrasseX2        MPKLYIRALSLVICIPAILCAVTTEGQYSVLEGQTVVIPCHYEPQYEHYVKYWCRGTTRE       60
                ************************************************************

wrasseX3        FCTSLARTDYLQASDAAKDKASISDDMDQLVFTVTMKNVKTGDTGWYICGVEIGSIWSAD       120
wrasseX1        FCTSLARTDYLQASDAAKDKASISDDMDQLVFTVTMKNVKTGDTGWYICGVEIGSIWSAD       120
wrasseX2        FCTSLARTDYLQASDAAKDKASISDDMDQLVFTVTMKNVKTGDTGWYICGVEIGSIWSAD       120
                ************************************************************
                121      130       140       150       160       170
wrasseX3        VVTFSNIRVIDGMSVVNTQVTGEEGGSVTVECLYSERYRESRKKWCRSGDWSSCRLTDSG       180
wrasseX1        VVTFSNIRVIDGMSVVNTQVTGEEGGSVTVECLYSERYRESRKKWCRSGDWSSCRLTDSG       180
wrasseX2        VVTFSNIRVIDGMSVVNTQVTGEEGGSVTVECLYSERYRESRKKWCRSGDWSSCRLTDSG       180
                ************************************************************

wrasseX3        ASYEDASVAIIDDRTRTYSVTLKKLQLRDAGWYTCSAGKHKISVQVLVTSRPKDTLPVTS       240
wrasseX1        ASYEDASVAIIDDRTRTYSVTLKKLQLRDAGWYTCSAGKHKISVQVLVTSRPKDTLPVTS       240
wrasseX2        ASYEDASVAIIDDRTRTYSVTLKKLQLRDAGWYTCSAGKHKISVQVLVTSRPKDTLPVTS       240
                ************************************************************
                241      250       260       270       280       290
wrasseX3        TPAPTKCNAYVPAKPISKESTRSHHLESWLMCSAIVLLLGVAIFGRKLWKVHS-KETSHH       300
wrasseX1        TPAPTKCNAYVPAKPISKESTRSHHLESWLMCSAIVLLLGVAIFGRKLWKVHNPKYQFIE       300
wrasseX2        TPAPTKCNAYVPAKPISKESTRSHHLESWLMCSAIVLLLGVAIFGRKLWKVH--K-----       293
                ************************************************.

wrasseX3        FQF-------------------------------------------      302
wrasseX1        QGSAPTQSKDNKSKSNKCPWDVSDQQDTTVVFLNRDDKRDDAFLY      345
wrasseX2        QGSAPTQSKDNKSKSNKCPWDVSDQQDTTVVFLNRDDKRDDAFLY      338
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"The teleost polymeric Ig receptor counterpart in ballan wrasse (Labrus bergylta) differs from pIgR in higher vertebrates"

Angela Etayo[1](0000-0001-6988-3057), Håvard Bjørgen[2] (0000-0003-4683-0088), Erling O. Koppang[2] (0000-0003-4859-1455), Ivar Hordvik[1](0000-0001-9361-7656)

1: Department of Biological Sciences (BIO), University of Bergen, Bergen, Norway

2: Section of Anatomy, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

Corresponding author: Angela Etayo

Email: angela.etayo@uib.no

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**Supplementary material 3.** Comparison of the nucleotide sequence of the gDNA of the pIgR in *Labrus bergylta* (NW_018114907.1) and its three splice variants which are labelled X1, X2, and X3 (XM_020653428.2, XM_020653429.2, XR_002278599.2 respectively). Exons along the gDNA sequence are indicated in yellow and the corresponding two nucleotides belonging to introns flanking each exon (splice acceptor/donor sites) are marked in blue. Primers used in this article were designed based on the predicted sequences and are marked as followed; "Forward" primers in green (F1, F2 and F3) and "Reverse" primers in colour fuschia (R1, R2 and R3). F3 and R2 were used for cloning of the three splice variants. F3 and R3 were used for SYBR Green RT-qPCR analyses

```
gDNA            GATTTGTATTTAAATCAAAAATCCAGCCAGCACCTACATGGTTAAAATCTCAAAAATCTG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

                                          EXON 1 (SP)
gDNA            AATCCAATTTATAGTCACATGACCAAGAGAAAAACTTTCATTGCGGAAACTGTAGTCCAT
X3              ------------------------------AAAACTTTCATTGCGGAAACTGTAGTCCAT
X1              -----------------------------AAACTTTCATTGCGGAAACTGTAGTCCAT
X2              -----------------------------AAACTTTCATTGCGGAAACTGTAGTCCAT
                                              ****************************

gDNA            TAACAGAAGGGAGTGGATTCACTTGGAATATCTCAAACAGATGGTGAAACATGCCGAAGC F1
X3              TAACAGAAGGGAGTGGATTCACTTGGAATATCTCAAACAGATGGTGAAACATGCCGAAGC
X1              TAACAGAAGGGAGTGGATTCACTTGGAATATCTCAAACAGATGGTGAAACATGCCGAAGC
X2              TAACAGAAGGGAGTGGATTCACTTGGAATATCTCAAACAGATGGTGAAACATGCCGAAGC
                ************************************************************

gDNA            TCTACATTCGTGCCCTGAGCCTGGTAATATGCATCCCAGGTAATAACTGAGCACATACTG
X3              TCTACATTCGTGCCCTGAGCCTGGTAATATGCATCCCAG--------------------
X1              TCTACATTCGTGCCCTGAGCCTGGTAATATGCATCCCAG--------------------
X2              TCTACATTCGTGCCCTGAGCCTGGTAATATGCATCCCAG--------------------
                ************************************

gDNA            AGGTCATAGGATTACAAAAGTTATTCAGTAAGACTTTTATTTATGAAAGAACAGATTGTT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            CAGATGACTAATATGTATTTTGCACCCTCACTTTATTGTTGTGCTTTTTGGCAAACAGTC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            ATGCTACAGTGTAAGACTTTTCTTACGTTTCACTTCTCATTATTAATTTATTGATATGTT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            TTATGTATTAATTATTTGTAATAGACAGATTTGAAAGGTTGTAAAAGCCGCCCTTTTGTC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            TGGGAGCCGCAGAGGGCTATTGTGTTAAAAATAATCTTTTAAGCCTCTGTACTCTTTGCC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            CCATTGGCAAGAGTTGGTTTGATGTTTATTTTTCTGTTCTCCAAAGGCCTGCTACATTTT
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```
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            TCTACCCCAAATCTGTTTAATAGAGGGATAATCCAATTTTGGAATGAGGGGTGCAGGCTG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            GGAAGTAGGGAGAGTGTTTTGTAAGGAAATGAAACTGAAACCATTACTGTTTTCAACATG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

                                EXON 2 (ILD1)
gDNA            AAGTCATCTTCACTTTCCCTCAGCCATCCTGTGCGCTGTTACCACAGAGGGACAGTATTC_F2
X3              ------------------------CCATCCTGTGCGCTGTTACCACAGAGGGACAGTATTC
X1              ------------------------CCATCCTGTGCGCTGTTACCACAGAGGGACAGTATTC
X2              ------------------------CCATCCTGTGCGCTGTTACCACAGAGGGACAGTATTC
                                         ************************************

gDNA            AGTCCTGGAGGGCCAAACTGTTGTCATACCGTGTCACTACGAGCCCCAGTATGAACACTA
X3              AGTCCTGGAGGGCCAAACTGTTGTCATACCGTGTCACTACGAGCCCCAGTATGAACACTA
X1              AGTCCTGGAGGGCCAAACTGTTGTCATACCGTGTCACTACGAGCCCCAGTATGAACACTA
X2              AGTCCTGGAGGGCCAAACTGTTGTCATACCGTGTCACTACGAGCCCCAGTATGAACACTA
                ************************************************************

gDNA            TGTAAAATATTGGTGTCGGGGGACGACGAGGGAGTTCTGCACCAGCTTAGCCCGAACAGA
X3              TGTAAAATATTGGTGTCGGGGGACGACGAGGGAGTTCTGCACCAGCTTAGCCCGAACAGA
X1              TGTAAAATATTGGTGTCGGGGGACGACGAGGGAGTTCTGCACCAGCTTAGCCCGAACAGA
X2              TGTAAAATATTGGTGTCGGGGGACGACGAGGGAGTTCTGCACCAGCTTAGCCCGAACAGA
                ************************************************************

gDNA            TTACCTCCAAGCATCCGATGCAGCCAAGGATAAAGCGAGCATTTCTGATGACATGGACCA
X3              TTACCTCCAAGCATCCGATGCAGCCAAGGATAAAGCGAGCATTTCTGATGACATGGACCA
X1              TTACCTCCAAGCATCCGATGCAGCCAAGGATAAAGCGAGCATTTCTGATGACATGGACCA
X2              TTACCTCCAAGCATCCGATGCAGCCAAGGATAAAGCGAGCATTTCTGATGACATGGACCA
                ************************************************************

gDNA            ACTGGTATTCACTGTGACCATGAAAAACGTGAAGACAGGGGACACTGGGTGGTACATATG
X3              ACTGGTATTCACTGTGACCATGAAAAACGTGAAGACAGGGGACACTGGGTGGTACATATG
X1              ACTGGTATTCACTGTGACCATGAAAAACGTGAAGACAGGGGACACTGGGTGGTACATATG
X2              ACTGGTATTCACTGTGACCATGAAAAACGTGAAGACAGGGGACACTGGGTGGTACATATG
                ************************************************************

gDNA            TGGTGTGGAGATAGGCAGTATATGGAGTGCTGATGTTGTCACTTTCTCAAACATCAGGGT
X3              TGGTGTGGAGATAGGCAGTATATGGAGTGCTGATGTTGTCACTTTCTCAAACATCAGGGT
X1              TGGTGTGGAGATAGGCAGTATATGGAGTGCTGATGTTGTCACTTTCTCAAACATCAGGGT
X2              TGGTGTGGAGATAGGCAGTATATGGAGTGCTGATGTTGTCACTTTCTCAAACATCAGGGT
                ************************************************************

gDNA            CATTGATGGTGAGTGATTTGAACTCTTAAACCTTTATTGACGCAATGAAGAAACCAACAA
X3              CATTGATG--------------------------------------------------------
X1              CATTGATG--------------------------------------------------------
X2              CATTGATG--------------------------------------------------------
                ********
                                                                EXON 3 (ILD2)
gDNA            ACTCTACACATTTCATATGCAAACTCTGAAAACAGGTCCAATCATTTCGTGCAGGTATGT
X3              ------------------------------------------------------GTATGT
X1              ------------------------------------------------------GTATGT
X2              ------------------------------------------------------GTATGT
                                                                      ******

gDNA            CTGTGGTGAACACCCAAGTGACTGGGGAAGAAGGGGGAAGTGTCACAGTTGAATGCCTCT
X3              CTGTGGTGAACACCCAAGTGACTGGGGAAGAAGGGGGAAGTGTCACAGTTGAATGCCTCT
X1              CTGTGGTGAACACCCAAGTGACTGGGGAAGAAGGGGGAAGTGTCACAGTTGAATGCCTCT
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X2              CTGTGGTGAACACCCAAGTGACTGGGGAAGAAGGGGGAAGTGTCACAGTTGAATGCCTCT
                ************************************************************

gDNA            ACAGTGAGAGATACAGGTACTGCATTTTCCCATTTTTAGTCTATCAATCATAACAGTTTT
X3              ACAGTGAGAGATACAG-------------------------------------------
X1              ACAGTGAGAGATACAG-------------------------------------------
X2              ACAGTGAGAGATACAG-------------------------------------------
                ************

gDNA            ATTTACCCTAAAATCCAACATTTATAAAGCATGAGTGAACCTGTAAAAAATACTTCTTTG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            TGTGAAATCTTCAATTCAAGCCAAACTCTGACGGACAGTCAGCCTCCTCTGACTCCCCTG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

                           Exon 4 (ILD2)
gDNA            TGATTGGTTGCCAACAGAGAAAGCAGAAAGAAGTGGTGTCGGAGTGGAGACTGGAGCTCC
X3              -----------------AGAAAGCAGAAAGAAGTGGTGTCGGAGTGGAGACTGGAGCTCC
X1              -----------------AGAAAGCAGAAAGAAGTGGTGTCGGAGTGGAGACTGGAGCTCC
X2              -----------------AGAAAGCAGAAAGAAGTGGTGTCGGAGTGGAGACTGGAGCTCC
                                 ********************************************

gDNA            TGTCGGCTGACAGATTCTGGAGCGAGCTACGAAGATGCTTCAGTGGCCATCATTGATGAC
X3              TGTCGGCTGACAGATTCTGGAGCGAGCTACGAAGATGCTTCAGTGGCCATCATTGATGAC
X1              TGTCGGCTGACAGATTCTGGAGCGAGCTACGAAGATGCTTCAGTGGCCATCATTGATGAC
X2              TGTCGGCTGACAGATTCTGGAGCGAGCTACGAAGATGCTTCAGTGGCCATCATTGATGAC
                ************************************************************

gDNA            AGAACTAGGACTTACTCTGTAACCCTAAAGAAGCTGCAGCTGAGAGATGCCGGCTGGTAC
X3              AGAACTAGGACTTACTCTGTAACCCTAAAGAAGCTGCAGCTGAGAGATGCCGGCTGGTAC
X1              AGAACTAGGACTTACTCTGTAACCCTAAAGAAGCTGCAGCTGAGAGATGCCGGCTGGTAC
X2              AGAACTAGGACTTACTCTGTAACCCTAAAGAAGCTGCAGCTGAGAGATGCCGGCTGGTAC
                ************************************************************

gDNA            ACTTGCTCTGCAGGAAAGCACAAGATTTCCGTGCAAGTGCTGGTGACGTCTCGACCCAAA_F3
X3              ACTTGCTCTGCAGGAAAGCACAAGATTTCCGTGCAAGTGCTGGTGACGTCTCGACCCAAA
X1              ACTTGCTCTGCAGGAAAGCACAAGATTTCCGTGCAAGTGCTGGTGACGTCTCGACCCAAA
X2              ACTTGCTCTGCAGGAAAGCACAAGATTTCCGTGCAAGTGCTGGTGACGTCTCGACCCAAA
                ************************************************************

gDNA            GATAGTAGGTTGACAAAGAACCCTTATAAATTGAAGTATGAACACATTATCCTTTCTGAT
X3              GATA--------------------------------------------------------
X1              GATA--------------------------------------------------------
X2              GATA--------------------------------------------------------
                ****

gDNA            CAATTTCTTTCACTGAATGGTAATCAAAAAAAGTTATGCATTAGCTGTTCCTTTTTATCA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            TAAGTTTTTTTCTTTTCTTGACTTTGTGGGTCTCTGTCTGCTTTTTTTCAGACTCTAGTG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            TGGTTTGTTTGTCGTAAGAACATTAACCAACCTCAATCCAACCATGACTGTAGGAAGTGT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------
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gDNA            TGCACTATTTGTGTTCATTTAAGAAAGGTATCACATAGGCGACTGTAAGGCTGCCTTTGA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            ATGTATCAACTCTACCGTCATTACACAGCATCTCTTACAGCCCCTCTTAAGTTTACTTAT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            TACAAAAGTGAAAATAATGGGAATACGAAAAACAGGTGTGAAAAAATCCTTGGATACCTC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            AGCTTCGATCAAATCTAACTCGTTTCACTGAAAGTTTATTTAGATAGTCTCTGGACACAT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            GGTGAATAGAAGTCTGTTTACCTTGTAGCTTTGTTTTGGTCTCAACCATTTCATGATACA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            ATACCAACATCATTAGCTACTGGTACTGCGATCAATCATTAGCTAACTTTGTCTATTGGT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            GGCTCTGGCAAAGAGTTTTTATCAGAACTTGAACAGTTAAGTTCCATCCATCCATCCATC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            CATCCATCCATTGTCTTTACCTTTTCACCCTGGACTGTTCACCAGTCAATCACAGGGCTG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            ACATATAGAGACAGACAACCAGCCACACTCACATTCACACTTACGGGCAATTTTAGAGTC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            ACCAATTAACCTAACGAGCATTTCTTTGACTGCGGAACAAGTTATGATTTAAAAAATTAA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            AACAAATAGCTCAAAGGCCAGTGCATTGTTCTCCAGGGTGGGCACAATTTTCACTGATAG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            ACCAGGATGTATATCTCTGTTCTTTAACTTGTTGCCTGCACATTCTGGAACAGAATTCTG
```

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X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            GCTAACAGAATTAGCATAGCTGACATTTTTACTGAGGAAACTACCTGTTTTACTTAACAT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            TCTTAACAAACAACCCCATATGCACTGTTACTTACAGTCATATGTTCCCTAAACTCTGTG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            GAGACAAATCTATAAAACTGCTGACTCATGCTAGTCTGGTCTGCTACTCTGATCTATTTC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            AATAAACAGTCTTTGCTCTTATAACATGACACTGCATGACTCACTCTTTATGTCCCCAGT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            CATGGGACGTATCTCTCTCCTGCTAACTTTTTTTCTGCAGCCATCAAAGATCCTATCGCT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            TATATTAAAGAGGAGCAATGAGTATGTGCAGCTTATAACGAGAGTCAATGGAAGCCATTC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            TTATTGCAGATAGTACATTTTACTCAATTACTGCATAATATTTACCTGGCCCCTTTAAAA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            TTGTACTTATGTATAGCCTACATTTAAAACAATTTCAAAAATATCATATTCAGTGAACAT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            CTGTCGTTTTCCGTGTGGGGAGTGGTTGGGCAGCTCCCCTAGCGCACTCTTTTGACCAGC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            CGCCACAGTCCAAGACACTAAATACTCAATAGAGTTGAAGGTAACCGCAAAACATTTTGG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------


gDNA            ATTTTCTCTGAAATTACTTACTATTTACAATATCCTTTTAAATTGAGTTTGATCTTTTGG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
```

```
X2              ------------------------------------------------------------

gDNA            TAGTGCTTAGAGGGAAAGTTTAAATTCTCTATAAAAGGAATTCCAATTTTCCCACATTTT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            CTTTTCATTGAGAAAAAGAAAAGTAGTGTGCAACAAAGGTCAAACCAGCTTTATATGCCA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            TTTTGCTTTATACTGTTGGTTGGTTGGTTGGATGGTTTGAGTGGCTACACGATGTTGTTG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            TTTTAAGTTCCAATAAGCTCTTTATTTAGTTTATTTACAGGTTGCCGTTACTGTCAAGGT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            GGGAAACTCAATAGCTGTCATACTGCTTAGTATCAATCATATCAATGGAACGACTCTGAC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            AAATGTTGTCATTTCTCCATACTTTCAAATGAATATGGATAATGATAATGGTGATAATGA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            TAATGCAAAGGATATTCATTCTTTAAATAAAGCAACATGAAATTAATTTACATCATGTAA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            CACTACTAAACAGTAATACTAGCTTTCAGCCAATTCATAGTTCATAGTTCATAGAAGTAT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            AAGGTGAATAGCTTTTTTCTACCAAAGCTAACATGCTATCAAATATATTTCTTGACCTTG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            AATTGAGGCCAGAATTCCCTTGAAGAGATGATCAAAGGACAAATGTTGAAATGTTATTGA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            TTATTCAGATACCCAGAATTTATGCAACTTACTGTCTTGAATACAGCAATATGACACTAT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------
```

```
gDNA            AGCAGCATTTTAGCCTCCCAGCCAAAGCAAGGCGTAAACCTTGCCCTGTGTGAACATGGT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            CAATATTGTCCTTAGATAAAACCTTCCAGATAGCGAATACGGAGTTTGAAATGACTGAAT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            GATTTCAGGTACAACCTTTGATGTTACAGTATCATTGTATAGTTCATTCAAAACACTGAT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            CACTGCAACTTCAAAAAAAAAAAAAAAAAAAAAATATTTTCCTTACCCATGAGCCTTGCAA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            AGCTTGCATCGATCTCAAACTCAGTGTTCAGTGGCATTTTTATTCTTGGAAACATCTTTC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            AATAGTATTTTCAGGCAAGTGTGAAAAGGAGGCCTTTGCAGACTTGATTAGATGGTGACC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            TGACATGCCAATTCAAGCTCCAGACTGCTATGCAGAACTAGTTAATCCAGGCATTGTAGG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            TGAAAAGAGGTCACATTTTGCCCTTTTGCCCACATCATTCAGCTGTATTGAGAGGTAGCC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            AAGTGCGACTTAGAGATCAATATGTGTTTGTGACAGATGGCAGGAGCTCTTTTAGCTCTC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            AAAAGTGTACAAGGTATTGATCATTTACGAGGCACTTATCAAGATTATTGACGCTATGAA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            CTCTGCGGGTGCTACTGCAAACACTACTTTGCTTGTACTTGTAACATCATTTTTCAGATG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

                                        EXON 5 (H)
gDNA            CATGTTAAATGTCTTTTTCTTCTGCAGCGCTGCCTGTGACATCCACACCTGCGCCAACTA F3
```

```
X3              ----------------------------CGCTGCCTGTGACATCCACACCTGCGCCAACTA
X1              ----------------------------CGCTGCCTGTGACATCCACACCTGCGCCAACTA
X2              ----------------------------CGCTGCCTGTGACATCCACACCTGCGCCAACTA
                                            ********************************

gDNA            AGTGTAATGCCTACGTGCCTGCCAAACCCATCAGCAAGGAGTCCACTCGTAGGTGAGAGT
X3              AGTGTAATGCCTACGTGCCTGCCAAACCCATCAGCAAGGAGTCCACTCGTAG--------
X1              AGTGTAATGCCTACGTGCCTGCCAAACCCATCAGCAAGGAGTCCACTCGTAG--------
X2              AGTGTAATGCCTACGTGCCTGCCAAACCCATCAGCAAGGAGTCCACTCGTAG--------
                **************************************************

gDNA            GAAGCAGCTGTTCAGGAGATATGGAAAGCACATTTTTTGGTTATACAATGTTAATCGGCT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            CTTCTAAAGCTTGGTAAGAATAGTGATTGTAAGTCCTTAATTAAAGGAAAAACTCCTCTT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            AAGGCCCTTAGTCCATATTTGTAACTAAATGTACACATAAGAGTTCTCAGAACCAGTCCT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            GTAACACACCCTTCATGCTTGATGGGATGCCTTGAAAGTGTCTGGAAACACTAATCCACT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            AATGTTTCTAACTACAAAAGAAAGGATCCTGTTAGTACTCAGTGTTAAATACTACTTTCT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            ATCCAAAATTGCCATTTAAGTTTATGTTTATCTTCCGGATAGCTGGCCTGATATTTACCT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            TGATGATGAATTCAGCAGTTGACCTTATAGGAAAAGCCTTATGTCTATGCGTCTGTTTGC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            TTCCAGTATTAACAGCCTGTAGGCCGTTATTGACTGTTGCAGGTGTTTAGGTTCAACTTC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            TGCTATCTGTTTTTCAGCTGTGGGAGTTTAGTGCAACTGTTGTTGATCCATTTTTCCGTG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            GCGGAGGAACATCAGCTCTAAAATAAAAATGATGTACTGCTACTATAACTGTTCATAAAC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
```

```
X2              ------------------------------------------------------------

gDNA            CCCCATGTTCTGCAGGATGTCAGCCGTGTTTAAATAATTATAGCGTTATTGTTTTACCTT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            GCTGTTGAAAACTGCGCGAGTGGCCAGAGTTTCTAGTCCCAGTTGGTTGTACAGTCTTTC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            TTGTTTTGATCTCTTGCTAGCTCTGGTTTAGACTTCAAAAGTACGTCTACTCTAAATTTG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            ACTATCTTTCAGACTGCATGAATGTCAATGTTGCAATTTTACAATCAGCACCTGCTTAAT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            GGAGACTCTGGTTTTAATCTCAAGAATAGACAAAGGGAACACAAGCATTGAAACATAGAT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            CTGCTTTGTAAAAGCCTAAAGAAGCAAATATGTACAGTGCAATTGAGAGTGCAAAGGTGC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

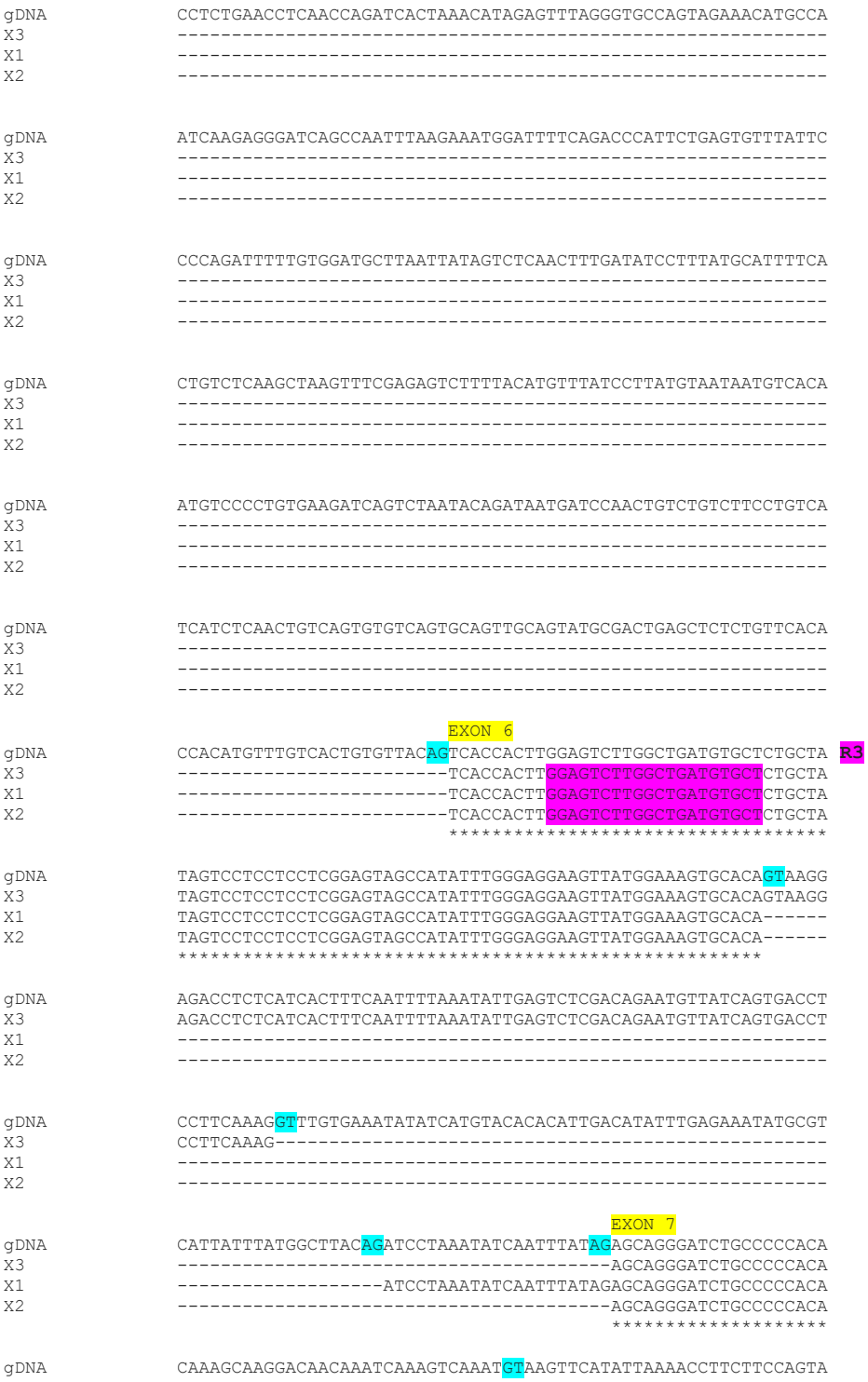
gDNA            TTACATATATATAGTTATGTATAGGTTTGCTATACAATAGTGCACTCATCAGTAAAGTAA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            AATTGAATTTAAATTTGAGTCAAGAGTAATCCCTCCTGCATGTTAACATCTGACTTTTGC
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            CCAAGATAACAACACAGCACAAATTAACACTCCATCGCCATCAGCATGCATGCATTCATA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            GCAAGGAGAAGAAAAAGGGGCATCTAAACTTACGTAGCATGTCTATGCTAACTGCTAACA
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

gDNA            ATGATATTAGGAGTAAAGCACATAGTGAGTTACTGTAATGATTGAAGGGGTTCTTTTCCT
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------
```


gDNA CCTCTGAACCTCAACCAGATCACTAAACATAGAGTTTAGGGTGCCAGTAGAAACATGCCA
X3 ----------
X1 ----------
X2 ----------
gDNA ATCAAGAGGGATCAGCCAATTTAAGAAATGGATTTTCAGACCCATTCTGAGTGTTTATTC
X3 ----------
X1 ----------
X2 ----------
gDNA CCCAGATTTTTGTGGATGCTTAATTATAGTCTCAACTTTGATATCCTTTATGCATTTTCA
X3 ----------
X1 ----------
X2 ----------
gDNA CTGTCTCAAGCTAAGTTTCGAGAGTCTTTTACATGTTTATCCTTATGTAATAATGTCACA
X3 ----------
X1 ----------
X2 ----------
gDNA ATGTCCCCTGTGAAGATCAGTCTAATACAGATAATGATCCAACTGTCTGTCTTCCTGTCA
X3 ----------
X1 ----------
X2 ----------
gDNA TCATCTCAACTGTCAGTGTGTCAGTGCAGTTGCAGTATGCGACTGAGCTCTCTGTTCACA
X3 ----------
X1 ----------
X2 ----------
EXON 6
gDNA CCACATGTTTGTCACTGTGTTACAGTCACCACTTGGAGTCTTGGCTGATGTGCTCTGCTA R3
X3 ----------TCACCACTTGGAGTCTTGGCTGATGTGCTCTGCTA
X1 ----------TCACCACTTGGAGTCTTGGCTGATGTGCTCTGCTA
X2 ----------TCACCACTTGGAGTCTTGGCTGATGTGCTCTGCTA
**********
gDNA TAGTCCTCCTCCTCGGAGTAGCCATATTTGGGAGGAAGTTATGGAAAGTGCACAGTAAGG
X3 TAGTCCTCCTCCTCGGAGTAGCCATATTTGGGAGGAAGTTATGGAAAGTGCACAGTAAGG
X1 TAGTCCTCCTCCTCGGAGTAGCCATATTTGGGAGGAAGTTATGGAAAGTGCACA------
X2 TAGTCCTCCTCCTCGGAGTAGCCATATTTGGGAGGAAGTTATGGAAAGTGCACA------
**********
gDNA AGACCTCTCATCACTTTCAATTTTAAATATTGAGTCTCGACAGAATGTTATCAGTGACCT
X3 AGACCTCTCATCACTTTCAATTTTAAATATTGAGTCTCGACAGAATGTTATCAGTGACCT
X1 ----------
X2 ----------
gDNA CCTTCAAAGGTTTGTGAAATATATCATGTACACACATTGACATATTTGAGAAATATGCGT
X3 CCTTCAAAG----------
X1 ----------
X2 ----------
EXON 7
gDNA CATTATTTATGGCTTACAGATCCTAAATATCAATTTATAGAGCAGGGATCTGCCCCCACA
X3 ----------AGCAGGGATCTGCCCCCACA
X1 ----------ATCCTAAATATCAATTTATAGAGCAGGGATCTGCCCCCACA
X2 ----------AGCAGGGATCTGCCCCCACA
**********
gDNA CAAAGCAAGGACAACAAATCAAAGTCAAATGTAAGTTCATATTAAAACCTTCTTCCAGTA

```
X3              CAAAGCAAGGACAACAAATCAAAGTCAAAT------------------------------
X1              CAAAGCAAGGACAACAAATCAAAGTCAAAT------------------------------
X2              CAAAGCAAGGACAACAAATCAAAGTCAAAT------------------------------
                ****************************

gDNA            TTCCATTAAACGTTTTATAAATAAGACTGTAATCAAGGATGTGACTTTGTGCTTATCAGG
X3              ------------------------------------------------------------
X1              ------------------------------------------------------------
X2              ------------------------------------------------------------

                                      EXON 8
gDNA            CTTTATTTTCTTTTGTCTCTCCTACAGAAATGTCCATGGGATGTAAGCGACCAGCAGGAC_R2
X3              ---------------------------AAATGTCCATGGGATGTAAGCGACCAGCAGGAC
X1              ---------------------------AAATGTCCATGGGATGTAAGCGACCAGCAGGAC
X2              ---------------------------AAATGTCCATGGGATGTAAGCGACCAGCAGGAC
                                           *********************************

gDNA            ACCACAGTCGTTTTCCTAAACAGGGATGATAAGAGGGATGACGCCTTTCTGTACTGAGCT  R1
X3              ACCACAGTCGTTTTCCTAAACAGGGATGATAAGAGGGATGACGCCTTTCTGTACTGAGCT
X1              ACCACAGTCGTTTTCCTAAACAGGGATGATAAGAGGGATGACGCCTTTCTGTACTGAGCT
X2              ACCACAGTCGTTTTCCTAAACAGGGATGATAAGAGGGATGACGCCTTTCTGTACTGAGCT
                ************************************************************

gDNA            CCGTCAGTGCAGTGTTTGATGACACTCTGCAAGTGACAATAAATCCATATATGCATGACT
X3              CCGTCAGTGCAGTGTTTGATGACACTCTGCAAGTGACAATAAATCCATATATGCATGA--
X1              CCGTCAGTGCAGTGTTTGATGACACTCTGCAAGTGACAATAAATCCATATATGCATGACT
X2              CCGTCAGTGCAGTGTTTGATGACACTCTGCAAGTGACAATAAATCCATATATGCATGACT
                ************************************************************

gDNA            ------------------------------------------------------------
X3              ------------------------------------------------------------
X1              CTATATCCAGTGCCTTTTATATCGTATACCAACACATTGAAAACAATTATAATGTTTATT
X2              CTATATCCAGTGCCTTTTATATCGTATACCAACACATTGAAAACAATTATAATGTTTATT

gDNA            ------------------------------------------------------------
X3              ------------------------------------------------------------
X1              TTCTGCTTTCATTACACAACTGAAACAGGCTGCAGATCGTAACAGAGAGCTGTTACTTTT
X2              TTCTGCTTTCATTACACAACTGAAACAGGCTGCAGATCGTAACAGAGAGCTGTTACTTTT

gDNA            ------------------------------------------------------------
X3              ------------------------------------------------------------
X1              AATTGCCAACACTGAAACCATGTAGTCTTTCATTTGTAAGGATGAGCCCCGTGAATAACT
X2              AATTGCCAACACTGAAACCATGTAGTCTTTCATTTGTAAGGATGAGCCCCGTGAATAACT

gDNA            ------------------------------------------------------------
X3              ------------------------------------------------------------
X1              CCACAGACTGTTGGATGAAGGCATTTGGGGATTTGAGTAAACGGGTGGGGTTTATAGCTA
X2              CCACAGACTGTTGGATGAAGGCATTTGGGGATTTGAGTAAACGGGTGGGGTTTATAGCTA

gDNA            ------------------------------------------------------------
X3              ------------------------------------------------------------
X1              CACGACAGGGGGAATGTTTAATTCAAAGTTTATGTTTTAAAATAAATTCAGATTTCAATT
X2              CACGACAGGGGGAATGTTTAATTCAAAGTTTATGTTTTAAAATAAATTCAGATTTCAATT

gDNA            ----
X3              ----
X1              ATGA
X2              ATGA
```

**Article Information:**

"The teleost polymeric Ig receptor counterpart in ballan wrasse (Labrus bergylta) differs from pIgR in higher vertebrates"

Angela Etayo[1](0000-0001-6988-3057), Håvard Bjørgen[2] (0000-0003-4683-0088), Erling O. Koppang[2] (0000-0003-4859-1455), Ivar Hordvik[1](0000-0001-9361-7656)

1: Department of Biological Sciences (BIO), University of Bergen, Bergen, Norway

2: Section of Anatomy, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

Corresponding author: Angela Etayo

Email: angela.etayo@uib.no

**Supplementary material 4.** RNAscope *In situ* hybridization demonstrating pIgR mRNA distribution in mucosal and lymphoid organs in Atlantic salmon (*Salmo salar*); **A)** Gut with pIgR positive cells in the lamina propria, **B)** Gills, **C)** Spleen, and **D)** Head kidney**.** Arrows indicate pIgR-positive cells. Negative and positive control (**E** and **F**) were conducted in head kidney of Atlantic salmon. Scale bars are as followed; A) 100 µm, B) 20 µm, C) 20 µm, D) 20 µm, E) 50 µm, and F) 50 µm.

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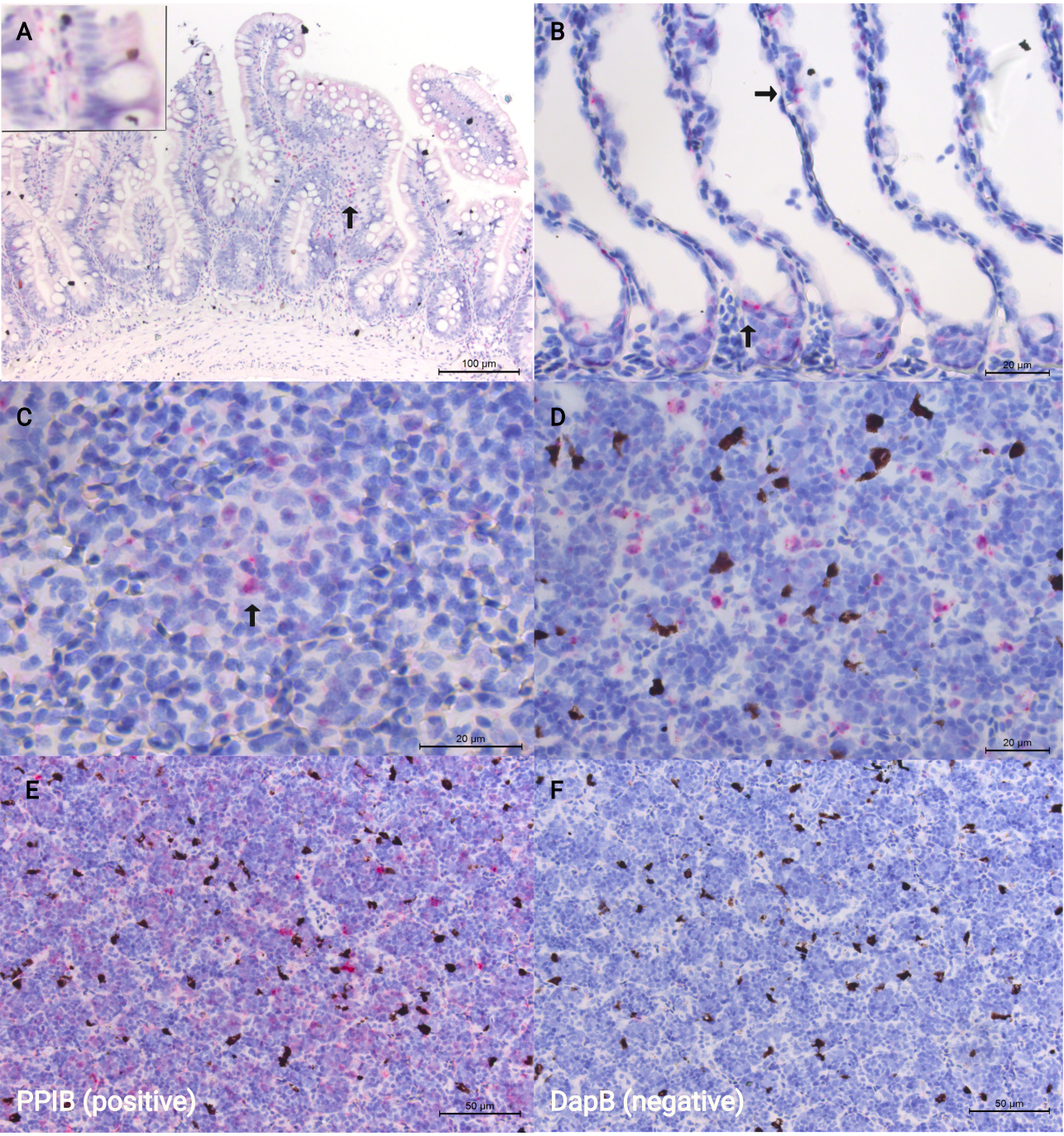
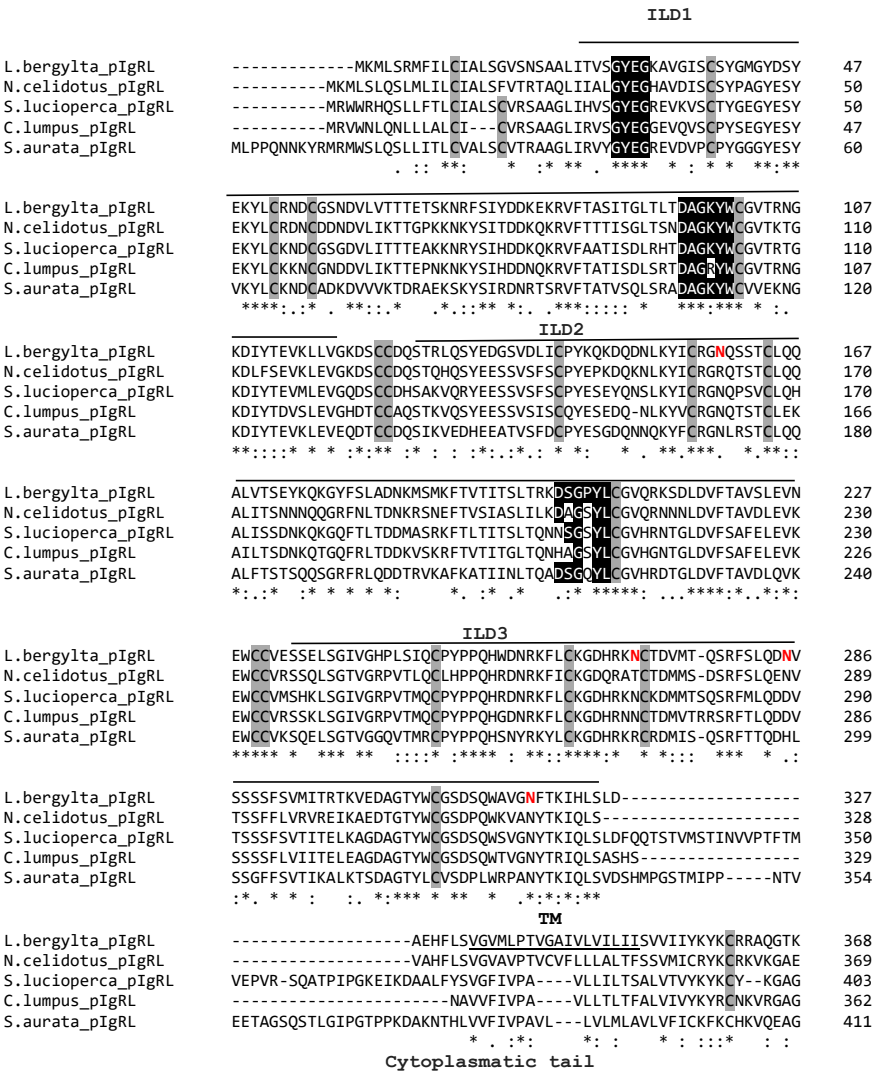
2: Section of Anatomy, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

Corresponding author: Angela Etayo

Email: angela.etayo@uib.no


A
100 µm
B
20 µm
C
20 µm
D
20 µm
E
PPIB (positive)
50 µm
F
DapB (negative)
50 µm

**Supplementary material 6.** Amino acid alignment of the predicted pIgRL sequence in ballan wrasse (*Labrus bergylta*, XM_020654174) and similar pIgRL sequences from other teleosts. The alignment shows the three Ig-like domains (ILDs) predicted in the wrasse pIgRL (ILD, ILD2, ILD3) and the transmembrane region (TM) and the cytoplasmatic tail. Residues identical in all sequences, highly conserved sequences, and conserved sequences are indicated by stars (*), colons (:), and periods (.) respectively. Conserved motifs in ILDs are shaded in black with white font. The positions of fully conserved cysteine (C) residues are shaded in dark grey. There were no predicted O-glycosylation. The asparagine residues (N) predicted to be N-glycosylated are highlighted in red (only predicted glycosylated sites with scores higher than 0.5 are shown). The amino acid sequence corresponding to the TM region is underlined. GenBank accession numbers are: spotty (*Notolabrus celidotus*, XM_034688120), zander (*Sander lucioperca*, XM_031289048), lumpsucker (*Cyclopetrus lumpus*, XM_034544241, and gilt-head bream (*Sparus aurata*, XM_030437348).



```
L.bergylta_pIgRL        IQEKRNQTKVAGVEEVIDVADIYENQGAAACSKQETSKWHSSCHQN------DNDDVYQN    422
N.celidotus_pIgRL       VSGKRNQTKKAEADEVISVADDYENSDVIVRSKQRTSRPLSPQHQN------EDDSVYQN    423
S.lucioperca_pIgRL      GNMKRNKTKTAETEEVIGVADIYQNQDV-AYSKQRTSKPQSACQHHDDAGEAEQDSVYQN    462
C.lumpus_pIgRL          ANMNRNVIKATETEEVMAGADIYENQDK-ACSQQGTSKPQSAGQHYDDVGDDQQESIYQN    421
S.aurata_pIgRL          VVDSGNKTKAVRAEKVIDVDDIYANQDV-VCKTQGTSY-----DHY---DDAGDESVYQN    462
                           . *  * . .::*:   * * *..  . . * **      .:       ::.:***

L.bergylta_pIgRL        CSTTEDMYCNQFYLKTAKR         441
N.celidotus_pIgRL       FST-DEIYCNQMYAASKR-         440
S.lucioperca_pIgRL      VTTADDIYCNQIFIKANRR         481
C.lumpus_pIgRL          FTTTDDIYCNEMYNKANRR         440
S.aurata_pIgRL          --TADDVYCNQFPMKAKR-         478
                          * :::***::   : :
```

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2: Section of Anatomy, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

Corresponding author: Angela Etayo

Email: angela.etayo@uib.no

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