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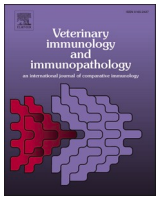

# The teleost polymeric Ig receptor counterpart in ballan wrasse (*Labrus bergylta*) differs from pIgR in higher vertebrates

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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

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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

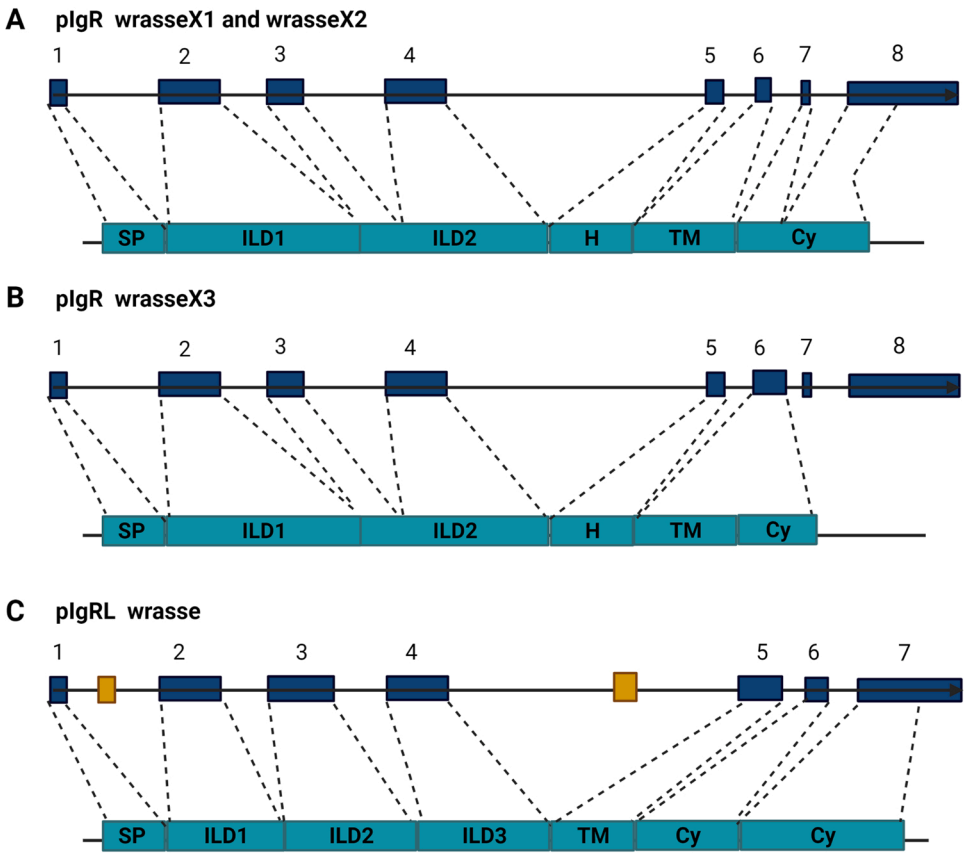



**Fig. 1.** Schematic illustration of the pIgR and pIgRL genes in ballan wrasse. The pIgR gene (Scaffold 492) with its mRNA splice variants and correspondence between exons and domains in ballan wrasse is shown in A and B. The three splice variants of the gene were designated as wrasseX1, wrasseX2 and 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).

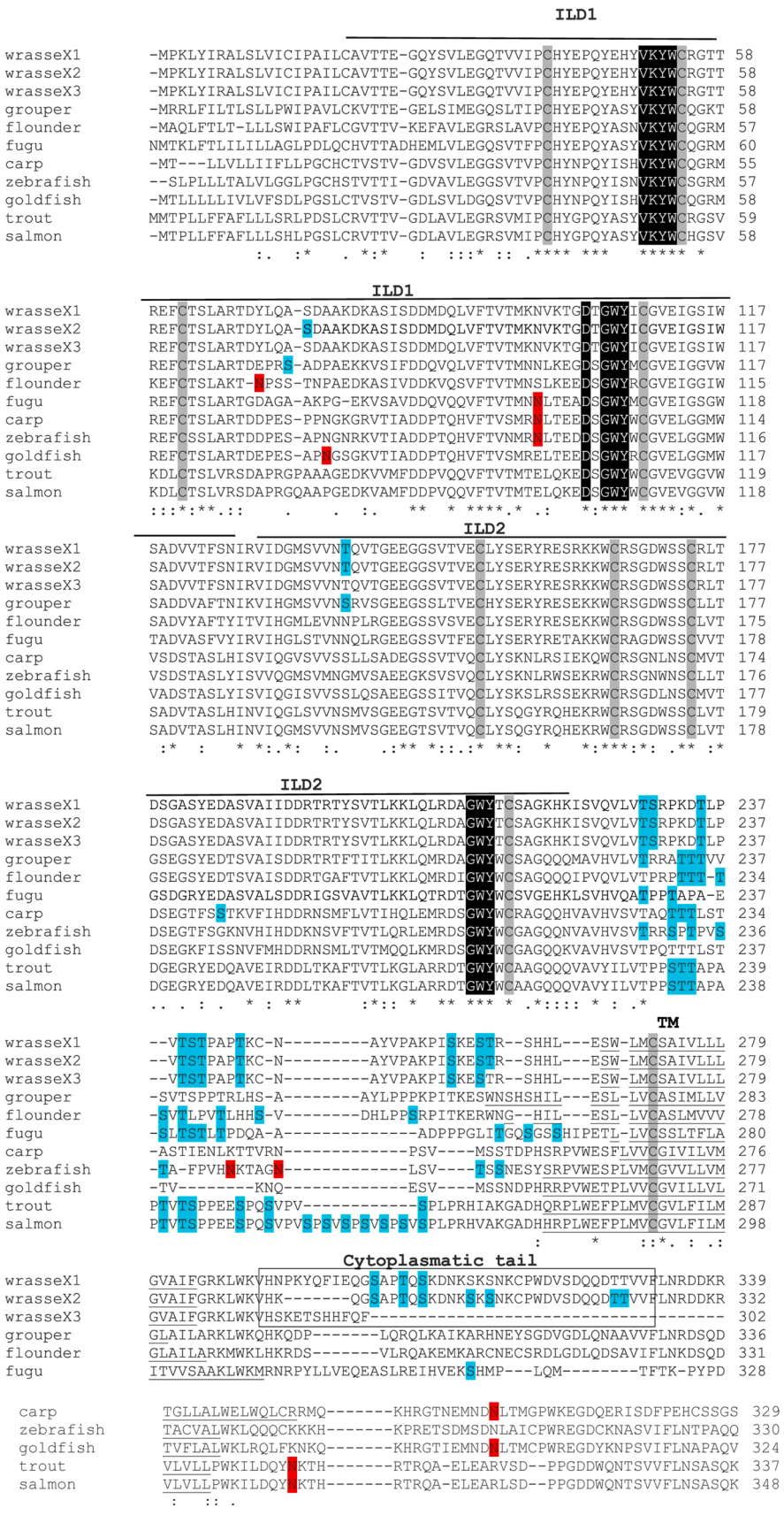



**Fig. 2.** Alignment of three ballan wrasse pIgR variants (wrasseX1, wrasseX2 and wrasseX3) and corresponding polypeptides from other teleost fish. The alignment shows the two Ig-like domains (ILDs) present in teleost pIgR (ILD1 and ILD2), the transmembrane region (TM) and the cytoplasmatic tail (Cy). 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.)

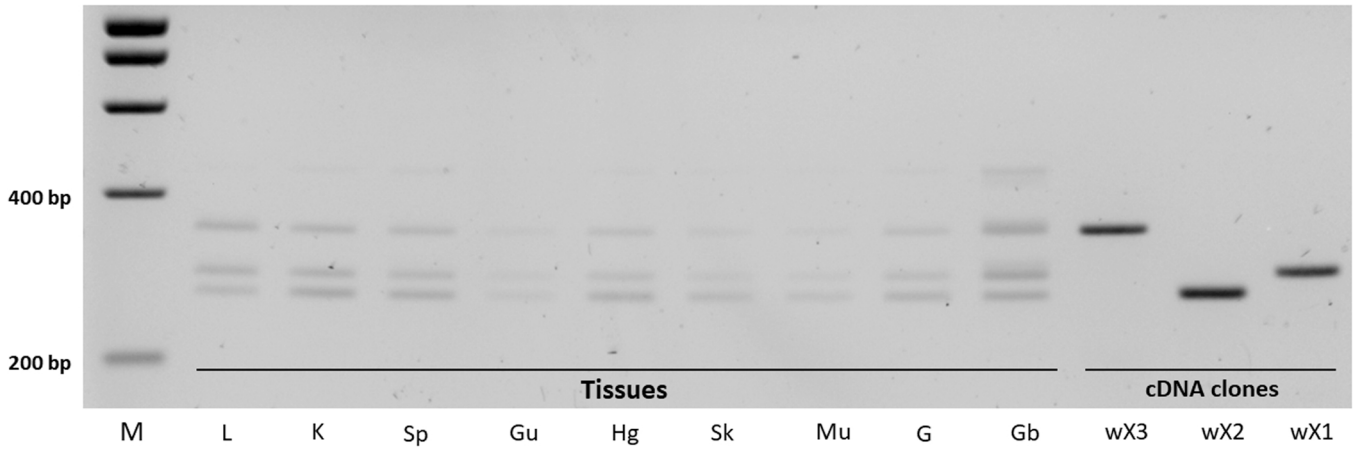



**Fig. 3.** Expression of three pIgR splice variants in different tissues of ballan wrasse (wX1, wX2 and wX3). The tree splice variants of the pIgR gene were present in all investigated tissues. From left to right; M, marker; L, liver; K, kidney; Sp, spleen; Gu, foregut; Hg, hindgut; Sk, skin; Mu, muscle; 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).

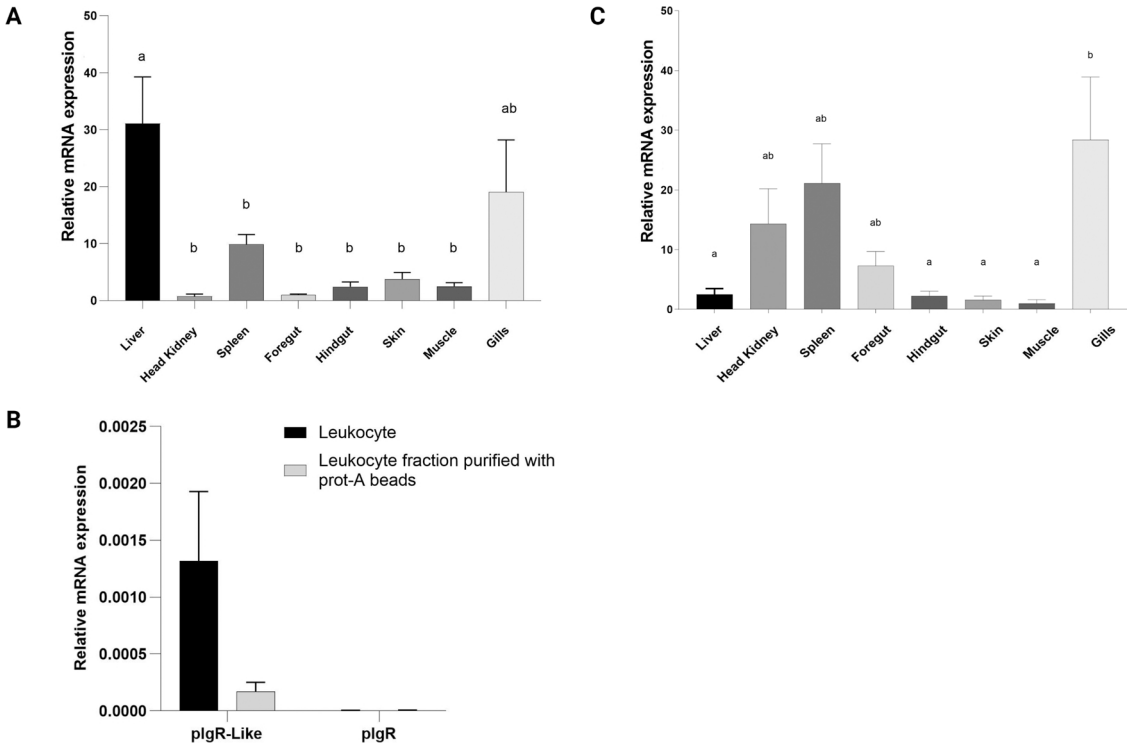



**Fig. 4.** Relative mRNA expression of pIgR and pIgR-L (pIgRL) in different tissues and blood leukocytes from ballan wrasse measured by RT-qPCR. A) Relative abundance of pIgR mRNA in different tissues. Data are expressed as mean values (±SEM) of n = 6 individuals and gut was used as calibrator. B) Relative 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

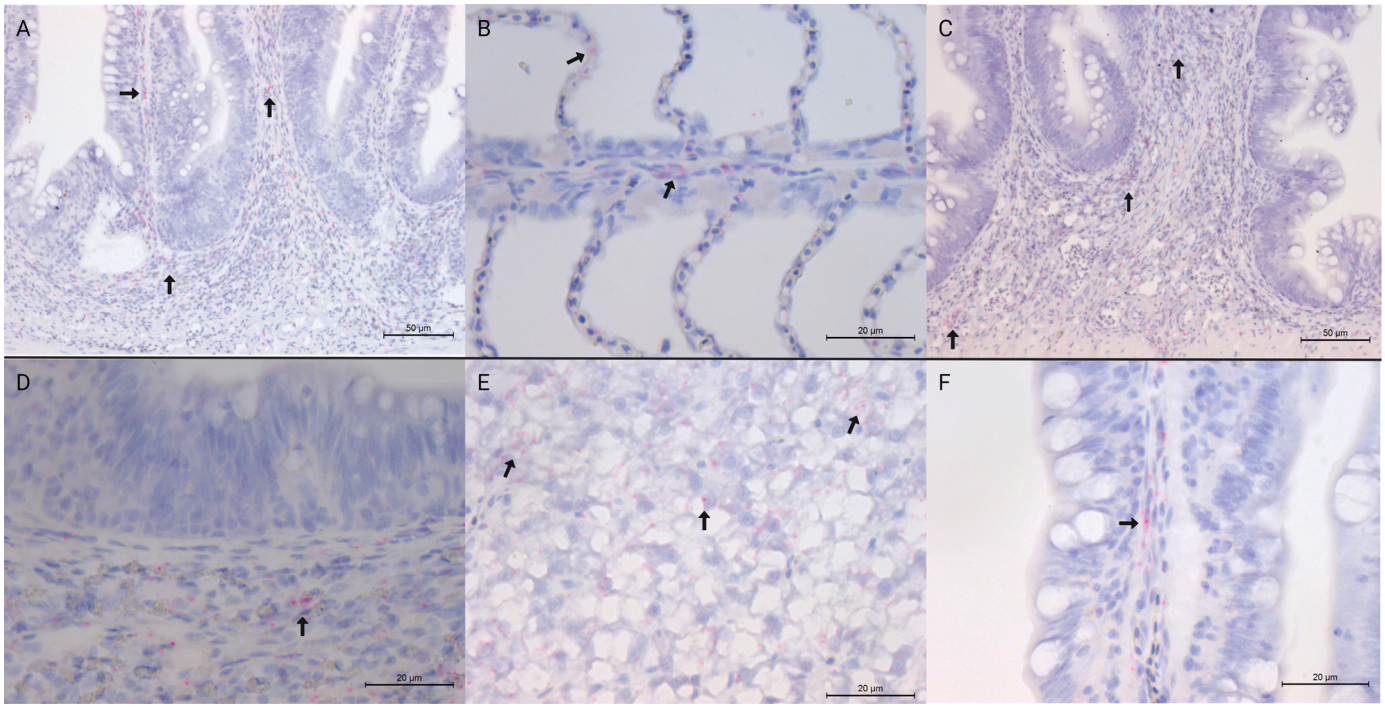
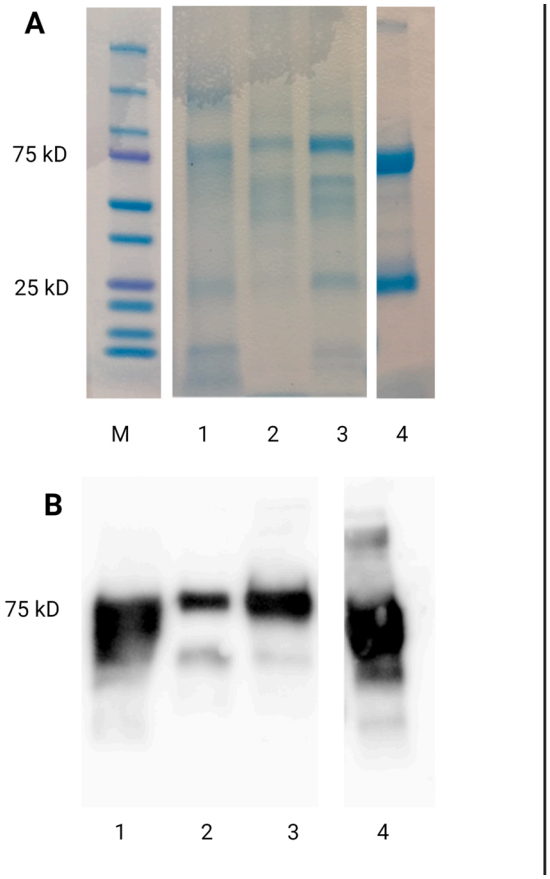



**Fig. 5.** RNAscope *In situ* hybridization demonstrating pIgR mRNA distribution in ballan wrasse; A) Gut, B) Gills, C) Hindgut, D) Gut epithelium (negative for pIgR) and the lamina propria with pIgR positive cells E) Liver F) Hindgut epithelium with pIgR positive cells in the lamina propria. Arrows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



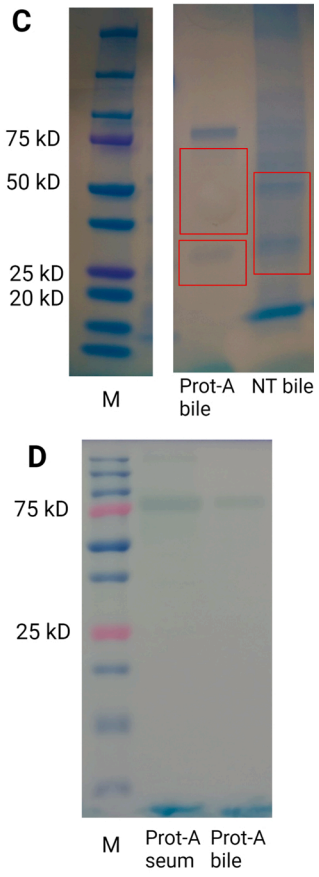




**Fig. 6.** SDS-PAGE analysis of protein-A purified mucus, protein-A purified bile (Prot-A bile) and non-treated bile (NT bile) from ballan wrasse. Serum was purified with prot-A and used as a positive control in A, B, and D. A) Prot-A purified mucus from hindgut, skin and gills were separated by SDS-PAGE 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

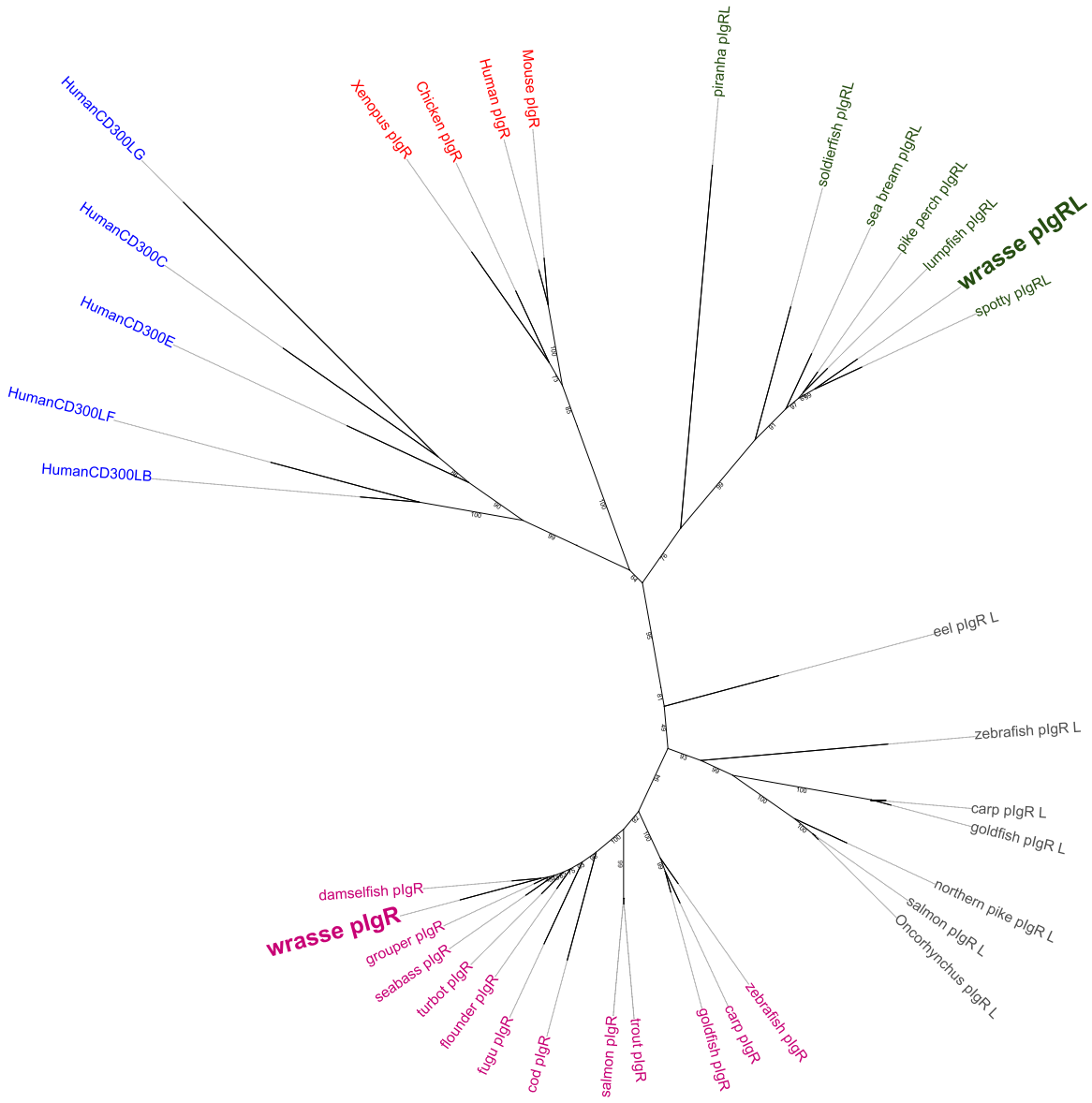



**Fig. 7.** Phylogenetic tree illustrating the relationship between pIgR and pIgRL in ballan wrasse and other related sequences, including human CMRF-35-like molecules. The pink branch shows teleost pIgR polypeptides which consists of two ILDs. The gray branch shows pIgRL in different teleosts which 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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