Ontogeny of myeloperoxidase (MPO) positive cells in flounder (*Paralichthys olivaceus*)

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Abstract

Neutrophils represent an important asset of innate immunity. Neutrophils express myeloperoxidase (MPO) which is a heme-containing peroxidase involved in microbial killing. In this study, by using real-time quantitative PCR and Western blot analysis, the flounder MPO (*Po*MPO) was observed to be highly expressed in the head kidney, followed by spleen, gill, and intestine during ontogeny – during developmental stages from larvae to adults. Furthermore, *Po*MPO positive cells were present in major immune organs of flounder at all developmental stages, and the number of neutrophils was generally higher as the fish grew to a juvenile stage. In addition, flow cytometry analysis revealed that the proportion of *Po*MPO positive cells relative to leukocytes, in the peritoneal cavity, head kidney, and peripheral blood of flounder juvenile stage was 18.3%, 34.8 %, and 6.0%, respectively, which is similar to the adult stage in flounder as previously reported. The presence and tissue distribution of *Po*MPO during ontogeny suggests that *Po*MPO positive cells are indeed a player of the innate immunity at all developmental stages of flounder.

Keywords: Ontogeny; Myeloperoxidase; Innate immunity; Fish

Introduction

One of the main problems in the worldwide production of marine fish is the high mortality rate during larviculture (Rojo-Cebreros et al., 2018; Austin, 2006; Bergh, 2000). Disease susceptibility may be due to an immature immune defense system at early stages. It has been shown the first feeding represents a milestone of organ development in gadoids, flatfishes and sparids, while a continuous differentiation during the larval stage and metamorphosis occurs (Falk-Petersen, 2005). For many fish species, the immune system is fully operational at the stage of first feeding (Martin and Król, 2017; Yúfera and Darias, 2007).

It is acknowledged that main lymphoid organs of the teleost are the head kidney, thymus, spleen, gill- and mucous-associated lymphoid tissues (GIALTs and MALTs) (Patel et al., 2009; Dalum et al., 2021) – both in larvae and developmental stages later on (Campoverde et al., 2019; Liang et al., 2022). During the early life stages, larvae have to rely solely on innate immune mechanisms and possibly maternal antibodies until they develop a functional adaptive immune system, which may take a few weeks to months (Saurabh and Sahoo, 2008). The cellular innate immunity is partly executed by phagocytic cells such as neutrophils and macrophages (Gasteiger et al., 2016; Hirayama et al., 2017; Lee et al., 2022). They are involved in antibacterial defenses, both by phagocytosis and by subsequent intracellular killing, as well as the release of extracellular traps (ETs) (Ellis, 1999; Malech et al., 2020; Sollberger et al., 2018). Myeloperoxidase (MPO), a highly cationic, heme-containing, and glycosylated enzyme is a major protein in azurophilic granules of neutrophils and is one of the key molecules

in immune response (Odobasic et al., 2016). In the phagosomes, MPO is involved in sustaining an alkaline milieu, which is optimal for the activity of serine proteases and other granule components to inactivate and kill microbes (Arnhold, 2020). In the presence of hydrogen peroxide and halides, MPO catalyzes the formation of reactive oxygen intermediates, which facilitates microbial killing by neutrophils (Aratani, 2018). MPO is also a key component in ETs capable of inhibiting the proliferation of bacteria, such as *Staphylococcus aureus* (Morales-Primo et al., 2022). In teleost, MPO possess antimicrobial activity similar to what found in mammals, where it could inhibit bacterial proliferation as constituent in ETs (Chi and Sun, 2016; Zhao et al., 2017; Gan et al., 2023).

The *MPO* gene has previously been cloned and the tissue distribution in some teleost has been described (Gan et al., 2023). However, MPO expression and content during flounder *Paralichthys olivaceus* (*Po*MPO) ontogeny have not yet been studied. Here, we studied the expression of *PoMPO* mRNA and protein at various stages during fish development and compared the fraction of MPO positive cells relative to other leucocytes from larvae to juvenile and adult stages. This study provided knowledge on early innate defense mechanisms in flounder, assessed by the occurrence of *Po*MPO - which is mainly expressed by neutrophils.

Material and Methods

Sample collection and preparation

Flounder larvae from 1 to 35 days post-hatch (dph), juveniles (~10 cm in length, ~75

63 dph) and adults (~30 cm, ~240 dph) were obtained from a farm located in Qingdao,
64 Shandong province, China, and reared in recirculating seawater at 20 °C. The larvae
65 were fed with Artemia until weaning and were switched to dry feed at approximately
66 20 dph and sampled at 1, 4, 7, 10, 14, and 35 dph. The juvenile and adult fish were fed
67 daily with a commercial dry feed pellet. By clinical examination, the fish were assessed
68 to be healthy. Before sampling, the fish were brought to euthanasia using 100 mg/L
69 ethyl 3-aminobenzoate methanesulfonic acid (MS222, Sigma, St. Louis, MO, USA).
70 Eighteen larvae were pooled at each time point and divided into three replicate groups
71 for total RNA purification. For juveniles and adults, samples of liver, spleen, head
72 kidney, gill, intestine, skin, and muscle were obtained from three fish. All samples were
73 immediately frozen in liquid nitrogen until RNA isolation. These protocols for animal
74 care and handling were approved by the Animal Care and Use Committee of Ocean
75 University of China (Permit Number: 20180101).
76 **Purification of total RNA and real-time quantitative PCR**
77 Total RNA was extracted from hatchlings or tissues (at late timepoints) from flounder
78 by the Trizol method according to the manufacturer's protocol (Vazyme, Nanjing,
79 China). The cDNA strand was synthesized by using Hiscript III RT SuperMix kit
80 (Vazyme). The expression level of *PoMPO* mRNA was measured by using specific
81 primers (forward primer: 5'- AGATCTGTCCCGATGAACGC - 3' and reverse primer:
82 5'- TTACAGCTATCACCCGAGCC - 3'), 2 × Universal SYBR Green Fast qPCR Mix
83 (Abclonal, Wuhan, China), and LightCycler® 480 II Real-Time System (Roche, Basel,
84 Switzerland). The relative transcriptional levels of *PoMPO* were normalized against the

housekeeping gene 18S (forward primer: 5'- GGTCTGTGATGCCCTTAGATGTC - 3' and reverse primer: 5'- AGTGGGGTTCAGCGGGTTAC - 3'). All reactions were carried out in triplicate. The expression levels of *PoMPO* gene were analyzed using the $2^{-\Delta Ct}$ method with 18S serving as the internal control. Finally, the lowest expression level was used as the standard to calibrate the expression of other tissues.

Western blotting

Hatchlings or tissues were lysed with RIPA Lysis Buffer Kit (Beyotime, Shanghai, China) containing a protease inhibitor. The protein concentration of the lysates was assessed by using the BCA kit (EpiZyme, Shanghai, China). The lysates (~20 μg proteins) went through SDS-PAGE and electroblotted onto polyvinylidene fluoride membrane (Merck Millipore, Darmstadt, Germany). After blocking with BSA, the mouse anti-r*Po*MPO Abs (Gan et al., 2023) or rabbit anti-GAPDH Abs (Abclonal, Wuhan, China) was incubated with the membrane overnight at 4 ºC. After washing three times, goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP (Sigma, St. Louis, Mo, USA) was added according to standard protocol. Protein bands were visualized using a chemiluminescence detection instrument (Vilber, Ile-de-France, France).

Histological preparation

Pre-metamorphic larva (14 dph, before eye migration, ~0.8 cm in length), and metamorphic climax larva (35 dph, right eye has migrated over the dorsal midline, ~1.2 cm) were fixed overnight in Bouin's fixative (Phygene, Fujian, China) at room temperature. In the case of juveniles and adults, lymphoid tissues including head kidney, spleen, intestine, and gill were also sampled and fixed. All the fixed samples were

dehydrated in ethanol solutions and embedded in paraffin (Hushi, Shanghai, China). The paraffin blocks were sectioned at thickness of 5 μm, and the sections of larvae were stained with hematoxylin-eosin (H&E) for morphological study.

Isolation of leukocytes from peritoneal cavity, peripheral blood and head kidney

The leukocytes in the peritoneal cavity (PerCs), peripheral blood (PBLs) and head kidney (HKLs) of juveniles were isolated according to the previous report (Gan et al., 2023). For peritoneal cell isolation, the cells were harvested by lavaging peritoneal cavity with L-15 medium. The peripheral blood was drawn from the caudal vein and mixed with the anticoagulant (RPMI-1640 containing 20 IU mL^{-1} heparin, 0.1% w/v NaN_3, and 1% w/v BSA) at a volume ratio of 1:2. The head kidney was cut into small pieces and grinded with adding RPMI-1640 (VivaCell, Shanghai, China). The solution was collected and filtered by a cell strainer to obtain a cell suspension. Then the leukocytes were isolated by Percoll (GE Healthcare, Uppsala, Sweden) density centrifugation, on a discontinuous density gradient (1.020/1.070 g mL^{-1}). All cells were subsequently resuspended in L-15 medium and adjusted to a concentration of 1×10^6 cells mL^{-1}. The isolated cells were processed for Wright-Giemsa staining, and then the images were taken using a microscope (Olympus, Tokyo, Japan).

Immunofluorescence staining

The cells (1×10^6 cells mL^{-1}) were seeded onto adhesion microscope slides (Citotest, Jiangsu, China) for 1 h, and then fixed with 4% paraformaldehyde (Solarbio, Beijing, China). The sections of flounder larvae and tissue were deparaffinized and rehydrated in graded series of ethanol. Antigen retrieval was performed at 95 °C in citrate antigen

retrieval solution (pH = 6.0, Beyotime, Shanghai, China) for 15 min to retrieve antigens. Endogenous peroxidase activity was quenched by incubation by a 1% H_2O_2 in methanol (Hushi, Shanghai, China) solution for 20 min. The cells were permeabilized by 0.25% Triton X-100 (Solarbio, Beijing, China) in 5% BSA. Mouse anti-r*Po*MPO Abs, or anti-rTrx Abs (Gan et al., 2023) was used as the primary antibody onto the slides for overnight at 4 ºC, followed by Alexa Fluor 649-conjugated goat anti-mouse IgG antibodies (Sigma, St. Louis, Mo, USA). Cells were counterstained with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, Thermo Scientific, Massachusetts, USA) and observed by a fluorescence microscopy (ZEISS, Oberkochen, Germany).

Flow cytometry

The PerCs, HKLs and PBLs were fixed and permeabilized using BD Cytofix/Cytoperm plus kit (BD Biosciences, San Diego, CA) to allow for intracellular examination of MPO by mouse anti-r*Po*MPO Abs. The cells were then incubated with Alexa Fluor 649-conjugated goat anti-mouse IgG secondary antibodies (Sigma, St. Louis, Mo, USA) for 45 min at 37 °C in the dark. Mouse anti-rTrx Abs were used as negative controls. Flow cytometry was performed using FACSCalibur (BD Biosciences) flow cytometer with acquisition enabled by CellQuest Pro software.

Statistical analysis

One-way analysis of variance (ANOVA) and Duncan's multiple comparisons were performed by using Statistical Product and Service Solution (SPSS) software (Version 19.0; SPSS, IBM, BY, USA). For assessment of the results, $P < 0.05$ was considered

151 statistically significant.

152 ## Results

153 **Ontogenic appearance of *Po*MPO mRNA and protein**

154 *PoMPO* mRNA levels at different time points post hatch, ranging from larvae to
155 juvenile stages, were analyzed by real-time quantitative PCR (qPCR) (Fig. 1). At the
156 larvae stage, the *PoMPO* gene was expressed in all samples. *PoMPO* gene expression
157 was observed already on 1 dph and increased continuously to 14 dph (P <0.05 between
158 each time point) (Fig. 1B). At the juvenile stage, the highest levels of *PoMPO* mRNA
159 were observed in the head kidney, followed by the gill, spleen, intestine. The expression
160 levels in the skin, muscle and liver were relatively low (Fig. 1D).

161 Western blotting revealed *Po*MPO protein presence in both larvae and juveniles.
162 Consistent with the mRNA expression pattern, *Po*MPO was detected in homogenates
163 from all sample time-points of larvae (Fig. 1A). For juveniles, the protein was found
164 mainly in the head kidney and spleen, while no to minor content were found in gill,
165 skin, muscle, and liver (Fig. 1C).

166 ***Po*MPO$^+$ cells during ontogeny**

167 To analyze the presence and distribution of *Po*MPO$^+$ cells during different ontogenic
168 stages, the immunological organs of the pre-metamorphic larvae, metamorphic climax
169 larvae, juvenile, and adult individuals were investigated by immunofluorescence. At the
170 stage just before metamorphosis, the kidney developed into an adult-like organ. The
171 kidney fused along the ventral part of peritoneal cavity, with a visible head kidney and
172 trunk kidney (Fig. 2A, B). The head kidney is an important immune organ in teleost,

thus some *Po*MPO$^+$ cells were found scattered in the kidney (Fig. 2B, C). The kidney became larger in the metamorphic climax larvae and *Po*MPO$^+$ cells were observed not only in the head kidney, but also along the renal part of the trunk kidney (Fig. 3A, B). However, *Po*MPO$^+$ cells were, at this stage, more numerous in the head kidney compared to trunk kidney (Fig. 3B, C). In juveniles and adults, head kidney seemed to be an important pool for *Po*MPO$^+$ cells (Fig. 4). The spleen of the flounder is a small spheric organ and increases in size as the fish grows (Fig. 2A, Fig. 3A). In the pre-metamorphic larvae, only a few *Po*MPO$^+$ cells were found in the spleen (Fig. 2D). During the development into metamorphic climax larva, the number of *Po*MPO$^+$ cells increased and the cells were observed scattered throughout the spleen (Fig. 3D). In juvenile and adult individuals, a relatively high number of of *Po*MPO$^+$ cells were found in the spleen (Fig. 4).

The gills of flounder are bilaterally situated on either side of the pharynx, composed of gill arches and primary lamellae (Fig. 2A, Fig. 3A). In the pre-metamorphic larva, though the differentiation of gills seemed to be inconspicuous, a few *Po*MPO$^+$ cells were detected. At the metamorphic climax larvae stage, *Po*MPO$^+$ cells were detected in the primary gill lamellae and in the gill filaments along blood vessels (Fig. 2E, Fig. 3E). As the flounder grew older, the distribution and number of *Po*MPO$^+$ cells appeared to be similar compared to juvenile and adult stages (Fig. 4). In the flounder, the intestine is composed of four layers, namely lamina epithelialis, lamina propria, lamina muscularis and serosa. In the pre-metamorphic larvae, this layered structure of the intestine was not clearly observable compared to the metamorphic climax stage. In the

metamorphic climax larvae, *Po*MPO$^+$ cells were dispersed predominantly in the lamina epithelialis (Fig. 2F, Fig. 3F). As the flounder grew older, the *Po*MPO$^+$ cells were mainly located in the lamina propria at juvenile and adult stages (Fig. 4). No fluorescence was observed in the negative controls from the selected organs (Fig. S2A-D).

Presence and distribution of *Po*MPO$^+$ cells in juvenile and adult flounder

The presence and proportion of *Po*MPO$^+$ cells from juveniles were assessed in total leucocytes extracted from the peritoneal cavity, head kidney, and peripheral blood (Fig. 5). The results of Giemsa staining showed that the PerCs and HKLs contained many granulocytes with the nuclei located on the cellular side and relatively faintly stained cytoplasm, while the PBLs mainly contained lymphoid cells possessing a large nucleus with less cytoplasm (Fig. S1). Indirect immunofluorescence assay demonstrated that *Po*MPO was dispersed throughout the entire cytoplasm. The nuclei of MPO$^+$ cells were darker compared to other leucocyte-like cells (Fig. 4, Fig. 5). No fluorescence was observed in the negative controls (Fig. S2E). Flow cytometry revealed that the proportion of *Po*MPO$^+$ cells to leukocytes in the peritoneal cavity, head kidney, and peripheral blood of juvenile flounders was 18.3%, 34.8%, and 6.0%, respectively (Fig. 5A-C).

Kommentert [RD1]: What do you mean by «cellular side»?

Discussion

Flatfish possess metamorphosis during its development, which is a gradual process of maturation of larval morphology and anatomy into a juvenile morphology. Morphological assessment is often the most used criterium to determine the early stages

of development where right eye movement and the disappearance of coronal fins are the key characteristics (Falk-Petersen, 2005; Miwa and Inui, 1987). As one of the first defensive response against invading pathogens, neutrophils are rapidly recruited to infection and inflammation foci, where they recognize, phagocytose, inactivate microorganisms, perform immunomodulatory effects, and may act as antigen-presenting accessory cells involved in the induction stage of adaptive immunity (Gan et al., 2023; Arnhold, 2020; Marcinkiewicz and Walczewska, 2020; Mantovani et al., 2011). Determining the quantity and density of MPO^+ cells in fish immune system during ontogeny is helpful to assess the maturity of immune organs and possibly to track the onset of functional innate mechanisms. The knowledge can be exploited further to find the right time for innate immunity training and vaccination.

MPO is a member of the heme peroxidase-cyclooxygenase superfamily, which could kill e.g., trapped bacteria by virtue of extracellular traps, and is involved in other functions neutrophils, such as neutrophil trafficking, activation, phagocytosis, and lifespan (Rizo-Téllez et al., 2022). In mammals, MPO is correlated well with tissue neutrophil content, can be used as a marker to assess neutrophil infiltration in the tissue (Pulli et al., 2013). In turbot, potassium iodide and oxidized pyronine Y (KI-PyY) stained MPO^+ cells exhibited characteristics of neutrophil morphology (Chi et al., 2017). In one of our previous studies *Po*MPO was found to mainly exist in granulocytes and macrophages which indicated that these two types of cells may have some function that share, such mediating microbial killing. *Po*MPO exists in ET scaffold and is involved in the antibacterial effect of ETs (Gan et al., 2023). In humans, neutrophils first appear

239 in bone marrow by 10-11 weeks of gestation, and then mature neutrophils are released
240 from the bone marrow about three weeks later (Leiding, 2017). In foals, MPO gene
241 expression is present in newborns and the expression increases with age, reaching
242 highest levels at day 30 (Ghaeli et al., 2022). Opossum are considered as a model
243 species for investigations regarding the evolution of the mammalian immune system.
244 They are born with a low number of neutrophils, but in a few weeks later the neutrophils
245 become the predominant leukocyte (Fingerhut et al., 2020). In zebrafish, the earliest
246 MPO expression has been detected in cells of the intermediate cell mass right before
247 one-day post-fertilization (18 hours post fertilization) (Shen et al., 2013). In this study,
248 *Po*MPO transcripts and protein were detected already at 1 dph and were subsequently
249 found widely expressed in major immune organs as the fish grew to a juvenile stage,
250 especially from pre-metamorphic larvae to metamorphic climax larvae. ~~It can be~~
251 ~~speculated that *Po*MPO is involved in processes related to organ e.g., development, and~~
252 ~~imply the functional enhancement in responsiveness to antigenic stimuli.~~ The similarity
253 with respect to ontogenic development in fish compared to higher vertebrate animals
254 may reflect a conserved evolutionary function of neutrophils (Fingerhut et al., 2020;
255 Buchmann, 2022; Hauser et al., 2023).
256 In teleost, the head kidney is regarded to be the bone marrow equivalent in vertebrates,
257 being hemopoietic (Rauta et al., 2012; Bjørgen and Koppang, 2022). The spleen also
258 plays a role in hematopoiesis, antigen trapping, and antibody production (Uribe et al.,
259 2011). The gills and intestine are both tissues with continuous exposure to seawater and
260 possibly pathogens. As such, a wide distribution of leukocytes, including T cells, B cells,

Kommentert [RD2]: I think you should remove this speculation.

plasma cells, macrophages and granulocytes is vital (Salinas et al., 2011) The maturation of fish immune system occurs, depending on the species under study, in a few weeks after hatching, and it has been shown that mandarin fish may require at least 3 weeks after to be able to mount a more advanced immune response involving adaptive immunity (Grøntvedt and Espelid, 2003; Tian et al., 2009). In some teleost, such as in the Atlantic halibut, the immune organs mature both morphologically and functionally in the period after the start of exogenous feeding, and at 94 dph they possess IgM positive cells and secreted IgM (Patel et al., 2009). The tissue distribution of *Po*MPO mRNA and protein in juvenile flounder at day 75 was comparable with the expression pattern in adult fish, where *Po*MPO previously has been shown to be highly expressed in head kidney and gills, followed by spleen and intestine (Gan et al., 2023). These findings suggest that 75 days after hatch, the flounder has reached a mature state – although not proven functionally. Supportively, the highest proportion of *Po*MPO$^+$ cells relative to leukocytes in the head kidney of flounder juvenile stage was similar to the adult, where *Po*MPO$^+$ cells have previously been shown to account for approximately 42% in head kidney leukocytes, which strongly indicates that the head kidney recruits neutrophils (Gan et al., 2023). However, during development, *Po*MPO$^+$ cells may likely follow the peripheral blood circulation system to patrol and settle in other organs such as heart, liver, and skin ~~may also play a role in immune or inflammatory responses~~.

A major difference between teleost and mammals relates to the relative numbers of neutrophils in circulation. In humans, neutrophils make up 40% - 70% of leukocyte in circulation, compared to fish (approx. 5%) ~~(40%-70%).~~ The kidney of teleost has been

shown to house prominent neutrophilic populations (Havixbeck etal., 2015). In our study, the head kidney of flounder was found to contain significant neutrophilic populations compared to peritoneal cavity and blood. This suggests that the neutrophil hematopoiesis occurs in head kidney which also serve as a pool of mature neutrophils in bony fish a, which can traffic to more distant site upon microbial infection or inflammation.

In conclusion, the ontogenic distribution of myeloperoxidase in flounder were analyzed by using gene expression in combination with immunolabelling methods. These methods provided evidence for the ontogenic development of MPO positive cells, thus neutrophils, in flounder. These findings deepens our understanding of the onset and development of the immune system in teleost fish and may provide means to increase the survival of flounder larvae using e.g., immunostimulants targeting receptors specific for neutrophils.

Data availability

Data will be made available on request.

Ethics Statement

The animal study was reviewed and approved by the protocols for animal care and handling were approved by the Animal Care and Use Committee of Ocean University of China (Permit Number: 20180101).

Author contribution

Q.G., H.C., and R.A.D. were associated with the conception of the study. Q.G., H.C., C.L., and L.Z. performed the experimental and statistical analyses, and wrote the original draft. R.A.D. edited the manuscript to the final version. H.C., R.A.D., J.X., X.T., X.S., and W.Z. provided the funding. All authors read and approved the final version of the manuscript.

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Declaration of competing 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.

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462

Figure legend

Fig. 1 Expression of *PoMPO* mRNA and protein during ontogeny. Lysates were harvested and subjected to western blotting analysis using by anti-r*Po*MPO and anti-GAPDH Abs was shown in larvae (A) and tissues of juvenile (C). The fold change of *PoMPO* mRNA in the larval flounder (B) and different organs of juvenile (D) by using qPCR analysis. The results were calculated by using relative expression method with 18S as the housekeeping gene. Different letters above the bar represent the statistical significance ($P < 0.05$) compared to each other, and vertical bars represented the mean ± SD, n = 3.

Fig. 2 The H&E staining shows the morphological features (A) and indirect immunofluorescence assay indicates the positive signal of *Po*MPO in the tissues of pre-metamorphic larvae (B-F). A, bar = 500 μm; B, bar = 200 μm; C-F, bar = 50 μm.

Fig. 3 The H&E staining shows the morphological features (A) and indirect immunofluorescence assay indicats the positive signal of *Po*MPO in the tissues of metamorphic climax larvae (B-F). A, bar = 500 μm; B, bar = 200 μm; C-F, bar = 50 μm. IEC: intestinal epithelial cell; LP: lamina propria.

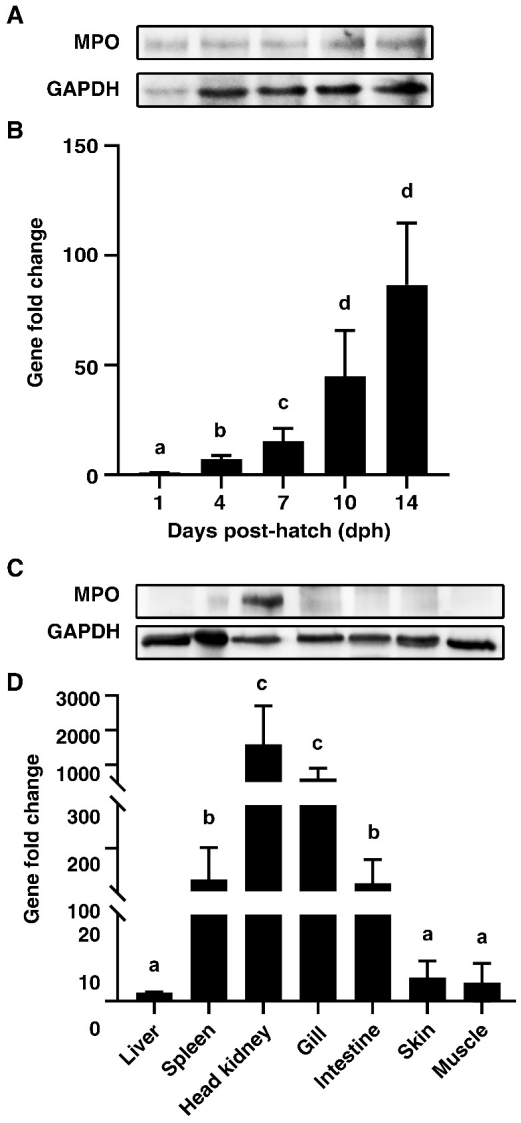
Fig. 4 *Po*MPO$^+$ cells detected by indirect immunofluorescence and flow cytometric analysis. Indirect immunofluorescence assay showed the positive signal of *Po*MPO in different tissues of juvenile and adult flounder. Bar = 50 μm. IEC: intestinal epithelial cell; LP: lamina propria.

Fig. 5 Indirect immunofluorescence assay and flow cytometric analysis of *Po*MPO$^+$ cells in PerCs (A), HKLs (B) and PBLs (C). The scatter plot showed the gate of FSC area (FSC-A)/SSC area (SSC-A) of juvenile flounder. Fluorescence histogram showed the ratio of *Po*MPO$^+$ cells in gated leukocytes. The representative results from three different individuals.

Fig. S1 The Giemsa stain analysis of PerCs, HKLs and PBLs in flounder. LY: lymphocyte; MA: macrophages; GR: granulocytes. Bar = 10 μm.

Fig. S2 The indirect immunofluorescence assay using mouse anti-rTrx Abs as the first antibody for negative controls to the positive signal of *Po*MPO in the tissues of pre-metamorphic larvae (A), metamorphic climax larvae (B), juvenile (C) and adult flounder (D), the cells in the peritoneal cavity, head kidney and peripheral blood (E). Blue indicates the nuclei stained by DAPI. Red shows the positive signal or background noise.

497 Fig. 1



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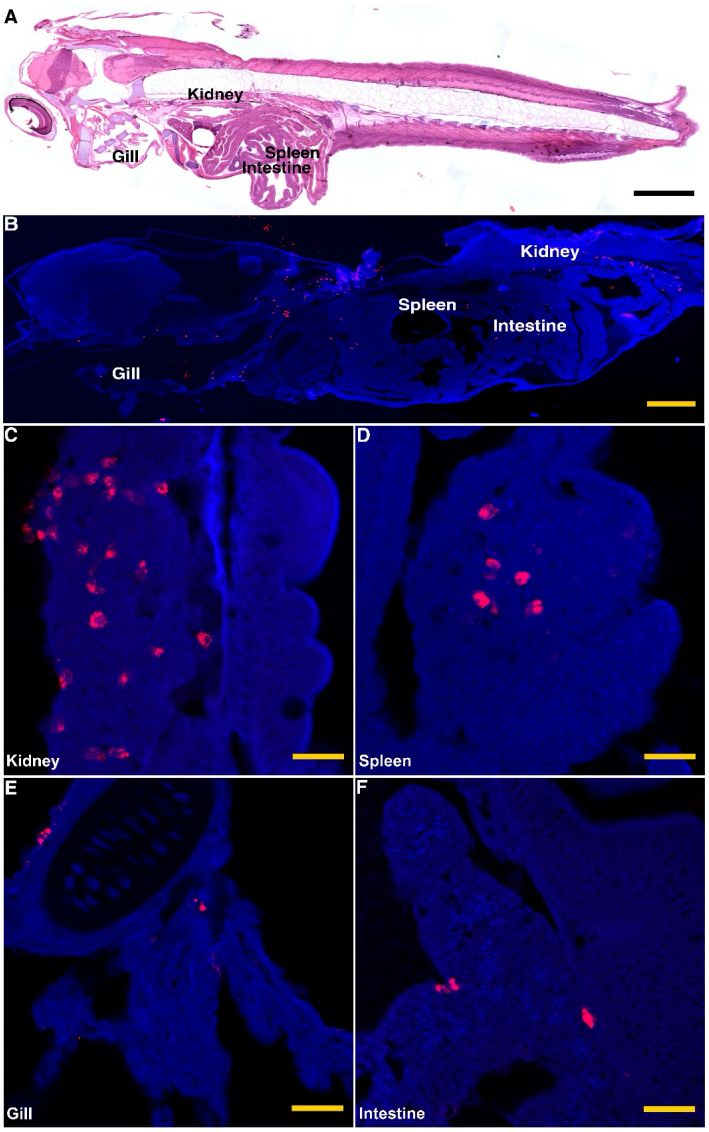
500 Fig. 2



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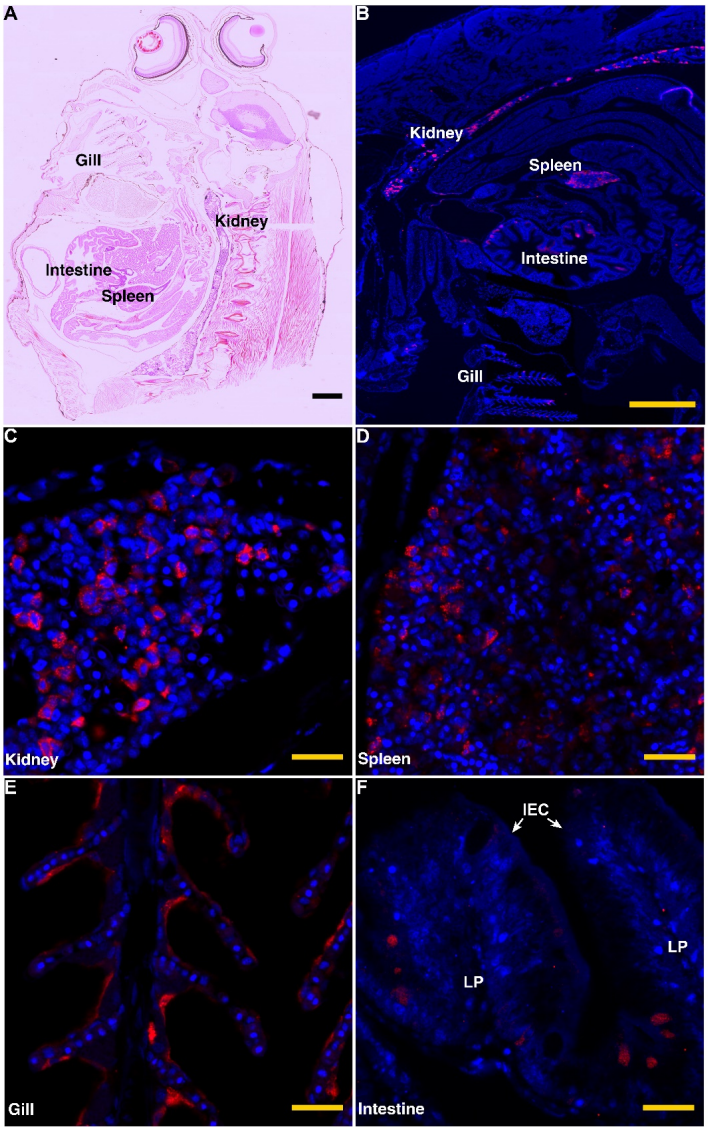
503 Fig. 3



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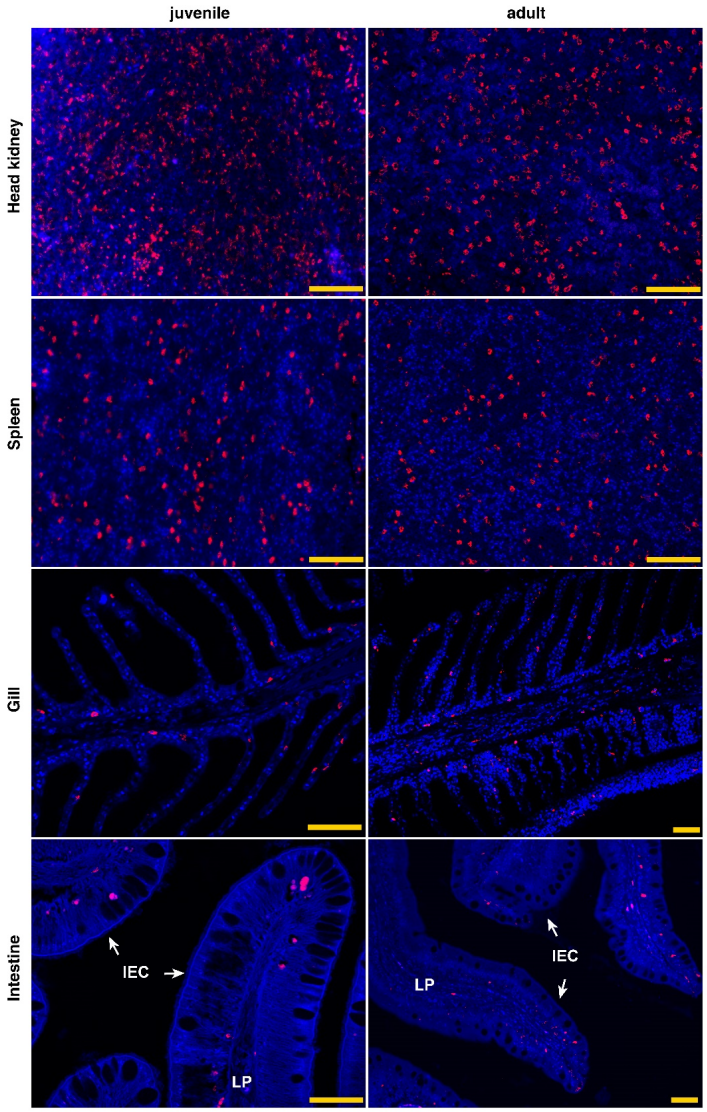

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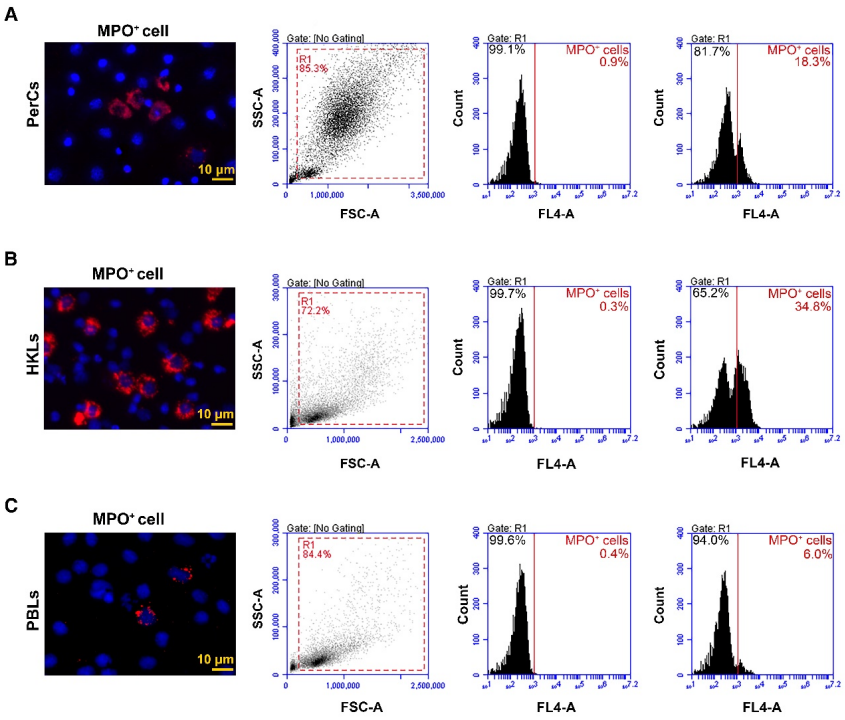
509 Fig. 5



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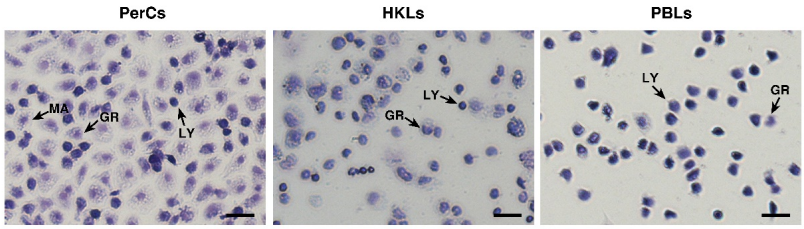
512 Fig. S1



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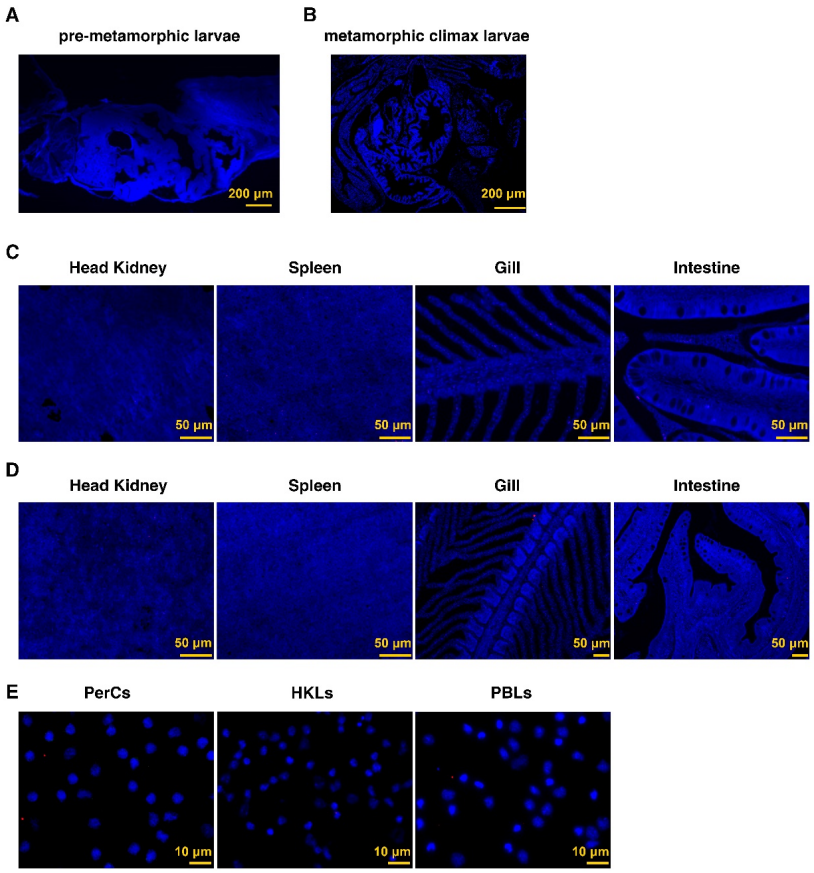
515 Fig. S2



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