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Distribution of the professional phagocytic granulocytes of the bony fish gilthead seabream (*Sparus aurata* L.) during the ontogeny of lymphomyeloid organs and pathogen entry sites

Iván Mulero[a], Elena Chaves-Pozo[a], Alicia García-Alcázar[b], José Meseguer[a], Victoriano Mulero[a,*], Alfonsa García Ayala[a]

[a]*Department of Cell Biology, Faculty of Biology, University of Murcia, Campus Universitario Espinardo, 30100 Murcia, Spain*
[b]*Spanish Oceanographic Institute (IEO), Murcia Oceanographic Centre, Mazarrón, Spain*

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Abstract

Although it is believed that fish fry depend fundamentally on their innate defence mechanisms, the ontogeny of fish innate immune cells is poorly understood. In the present study, we have used a specific monoclonal antibody against acidophilic granulocytes (AGs), the main professional phagocytic cell type of the bony fish gilthead seabream, to study their localization during the development of the main lymphomyeloid organs, namely the head kidney, spleen and thymus, and of the two major portals for pathogen entry, namely the gills and intestine. AGs were observed in the posterior intestine and in the blood earlier than in the haematopoietic kidney (21 vs. 27 days post-hatching, dph). AGs were observed scattered between other cells of the haematopoietic lineage in the head kidney of larvae, but were grouped around the blood vessels of this organ in juveniles and adults, where they were also much more numerous. In the spleen and in the thymus, AGs were observed much later (62 dph) and appeared scattered. AGs were also observed in the gill lamella and the posterior intestine near the anus throughout development.

Keywords: Innate immunity; Head kidney; Spleen; Thymus; Gills; Intestine; Ontogeny; Teleosts

1. Introduction

Fish eggs are released and embryos hatch into a pathogenically hostile environment, at a time when their immunological capacity is still severely limited. Although some studies suggest that maternal antibodies can be transferred through the yolk sac [1,2], it seems that young specimens depend fundamentally on their innate defence mechanisms.

Abbreviations: AGs, acidophilic granulocytes; BSA, bovine serum albumin; DAB, 3, 3′-diaminobenzidine tetrahydrochloride; dph, days post-hatching; H&E, haematoxylin and eosin; IHC, immunohistochemistry; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PBT, PBS + BSA + Triton X-100.

*Corresponding author. Tel.: +34 968 367581; fax: +34 968 363963.
E-mail address: vmulero@um.es (V. Mulero).

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The ontogeny of the immune cells and the lymphomyeloid organs that home them has been studied in several teleost species using mainly light and/or electron microscopy because of the lack of appropriate markers for specific cell populations of most of the species studied [3–13]. These studies have shown that, although the basic developmental mechanisms of teleosts are largely similar, there are differences with respect to the timing of developmental events. These differences are not only due to the widely varying duration of the embryonic period and larva development between teleost species, but also to the fact that larval development is strongly affected by culture conditions, such as temperature, salinity and photoperiod [14]. However, it must be emphasised that the structure of lymphomyeloid organs, as well as the presence of lymphocytes within them, does not correlate with their functional activity or degree of maturity [3,15–17].

The acidophilic granulocytes (AGs) of the adult marine teleost gilthead seabream (*Sparus aurata* L.) are the most abundant of circulating granulocytes and are rapidly recruited from the head kidney, the main haematopoietic organ in fish, to the infection site [18,19]. In addition, their main role is the phagocytosis of bacteria, which is accomplished by coordinating their attachment and internalization to the release of reactive oxygen intermediates into the phagocytic vacuole [18,19]. Therefore, these cells have been considered functionally equivalent to the neutrophils of higher vertebrates [18–20]. We report here the ontogeny of AGs in the gilthead seabream using the G7 monoclonal antibody (mAb) [18], which specifically recognizes these cells. This mAb allowed us to establish the time of appearance and the distribution of AGs in the main lymphomyeloid organs of gilthead seabream larvae, juvenile and adult specimens as well as in the major pathogen entry sites.

2. Materials and methods

2.1. Animals

Gilthead seabream (*S. aurata* L., Actinopterygii, Sparidae) fry were maintained at the Murcia Oceanographic Centre (IEO) using the "green water" technique in a 5000 l round tank with an initial density of about 60 eggs/l. Natural seawater (38‰ salinity) was heated at the beginning to $17 \pm 1\,^{\circ}C$ and filtered through mechanical and biological substrates. The temperature naturally increased and reached 26 °C at the end of the experiment. Water renewal was limited to 2% daily during the first 20 days of culture and was achieved by the addition of 70 ml/m^3 of a microalgae concentrated solution (Phytobloom, Necton) containing 80% of *Nannochloropsis oculata*. Afterwards, continuous water renewal (30%/h) and light aeration were provided in the tank. During the experiment the light intensity was 1000 lx at the water surface and the photoperiod 16:8. Larvae were successively fed with enriched (Selco, Inve Animal Health) rotifers from 6 to 24 days post-hatching (dph), *Artemia* nauplii (Inve Animal Health) from 20 to 35 dph, enriched Instar II *Artemia* from 31 to 58 dph, and commercial dry pellet diet (Skretting) from 54 dph onward. Larvae were sampled 2, 6, 8, 10, 13, 21, 27, 34, 48, 62 and 76 dph, and juveniles 92, 111, 132 and 146 dph.

Adult fish (150 g mean weight) were kept in 260 l running-seawater aquaria (flow rate 1500 l/h) at 23 °C under a 12 h light/dark cycle and fed with a commercial pellet diet (Skretting) at a feeding rate of 15 g dry diet/kg biomass of fish per day. All animal studies were carried out in accordance with the European Union regulations for animal experimentation.

2.2. Light microscopy and immunohistochemistry (IHC)

Larvae (2–76 dph) and juveniles of 92 dph (5 specimens of each age) were processed without further dissection while juveniles of 111–146 dph were sectioned into two parts before processing. Adults (3 specimens) were bled and the head kidney, spleen, thymus, gills and posterior intestine were removed and processed. Samples were fixed overnight in Bouin–Hollande fluid or 4% buffered paraformaldehyde solution, embedded in Paraplast Plus (Sherwood Medical) and sectioned at 5 μm. After being dewaxed and rehydrated, sections fixed in Bouin–Hollande fluid were stained with haematoxylin and eosin (H&E), while sections fixed in paraformaldehyde solution were subjected to an indirect immunocytochemical method [21] using the G7 mAb, which is specific to gilthead seabream AGs [19]. The samples were incubated for 40 min in peroxidase quenching solution (H_2O_2 in methanol, 1:9) to eliminate the endogenous peroxidase activity and then rinsed in phosphate-buffered saline (PBS, pH 7.2–7.4) and in PBS containing 0.01% bovine serum albumin (BSA) and 0.2% Triton X-100 (PBT). After a 30 min incubation with PBS containing 0.5% BSA to block the non-specific reaction,