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Neutrophils of diploid and triploid rainbow trouts assessed by flow cytometry

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Abstract Triploid rainbow trouts *Oncorhynchus mykiss* are reared in Brazil, but there is no data about haematological differences when compared to diploid ones. Due to the lack of information, the goal was to evaluate the neutrophils of adult diploid and triploid rainbow trouts *O. mykiss* by flow cytometry screening using a monoclonal anti-neutrophil antibody (TTL-5E9). In comparison, the population of neutrophils was proportionally larger in diploid trouts (9.94 ± 4.64% vs. 5.58 ± 1.09, p = 0.0481), but with cells showing smaller size and less complexity than the neutrophils of triploid trouts. As sorted neutrophils were not evaluated on phagocytic activity or apoptosis induction, it was not possible to establish neutrophil activity differences between ploidies.

Keywords: leucocytes, monoclonal antibody, *Oncorhynchus mykiss*, triploid fish

1. Introduction

Triploid rainbow trouts *Oncorhynchus mykiss* have been reared for more than three decades in America, Europe, and Japan. In Brazilian southern highlands, small- and medium-scale properties cultivated more than 31,000 tons of rainbow trout in 2017, representing 4.6% of total Brazilian fish production (Barbosa et al 2020; Nobile et al 2020) Triploid trouts breeding have some advantages, for instance, not using energy from stored fat in reproduction and to reach higher mean weights with lower mortality rates at the end of the production cycle (Benfey 1999; Tabata et al 1999).

The determination of hematological parameters facilitates the early detection of infectious diseases and the identification of sublethal conditions, identifying each cell value and proportion in fish bloodstream (Fazio 2019). In tissue injuries, the first immune cells are neutrophils, which perform a non-specific cytotoxic activity against pathogens (Sasaki et al 2002). Hematological data from adult trouts *O. mykiss* showed that the proportion of blood neutrophils from triploid trouts is lower than in the diploid ones, but with functional similarities (Yamamoto and Iida 1994 1995). Data related to flow cytometry neutrophil characteristics are available for trouts (Hamdani et al 1998; Sasaki et al 2002), Atlantic salmon *Salmo salar* (Pettersen et al. 2000) and cod *Gadus morhua* (Rønneseth et al 2007), however for triploid trouts are lacking.

Due to the lack of information about the hematological profile of triploid trouts reared in Brazil, the goal of our work is to sort and compare the population profile of neutrophils of healthy diploid and triploid rainbow trouts *O. mykiss* by flow cytometry.

2. Materials and Methods

2.1. Acquisition and maintenance of the rainbow trouts

Ten 24-month-old female diploid and triploid rainbow trouts *O. mykiss* (877.61 ± 166.03 g and 39.64 ± 2.19 cm total length) were acquired from the Salmoniculture Experimental Station from Campos do Jordão, Brazil, and were transported to the aquatic animals' vivarium at the Institute of Biomedical Sciences of the University of São Paulo (ICB/USP), São Paulo, Brazil. They were kept in 500 L tanks with dechlorinated water at 12 ± 3°C and used mechanical and biological filtration to maintain the water quality. This work was approved by the Animal Use Ethics Committee of ICB/USP # 40/2004.

2.2. Anesthesia and blood collection

Every rainbow trout handled experimentally was previously anesthetized by immersion in a benzocaine water solution (Benzocaine, Sigma-Aldrich, USA) at a concentration of 70 mg/L, previously dissolved in ethyl alcohol (qsp). After reaching the deep anesthesia stage, blood collection was performed through caudal vein puncture using a 5 ml syringe with 22 gauge

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heparinized needle (Liquemine, sodium heparin, Roche, Switzerland). Then, the fish was returned to the tank and was observed until recovery from anesthesia.

2.3. Flow cytometry analysis

To each 5 ml aliquot of blood, 10 ml of Ringer's solution (NaCl 0.75%, KCl 0.02%, CaCl2 0.02%, NaHCO3 0.02%, pH 6.4) was added and centrifuged at 2000 rpm for 5 minutes at 4˚C for the isolation of the buffy coat. The leukocyte layer was separated with the aid of a Pasteur pipette and resuspended with RPMI 1640 solution (GIBCO RPMI Medium 1640, Invitrogen Corporation, USA), and centrifuged three times at 2000 rpm for 5 minutes at 4˚C. The leukocyte suspension was adjusted to $2x10^6$ cells/ml, separated into two tubes and incubated with 100 µl of anti-neutrophil antibody (TTL-5E9) in 0.1% sodium azide buffer (NaN3) and 0.1% bovine serum albumin (BSA, Sigma-Aldrich, USA) for 40 minutes on ice. After washing three times in Ringer's solution, the leukocytes were incubated with 50 µl of goat anti-mouse antibody conjugated to FITC (DAKO A/S, Denmark) (1:100 dilution in phosphate buffer saline, 0.1% NaN3, 0.1% BSA) was added, and the cells were once more incubated for 20 min on ice. After washing three times in Ringer's solution, the samples were screened on a flow cytometer (FACS Excalibur, Becton-Dickinson Biosciences, USA) (Kfoury Junior et al 1999a).The threshold was visually adjusted until two populations could be identified, differentiating neutrophils from other granulocytes as reported by Kfoury Junior et al (1999b). Negative controls consisted of two tubes, one containing only cells and the other incubated only with secondary antibody (Kfoury Junior et al 1999a; Sasaki et al 2002).

Monoclonal anti-neutrophil antibody (TTL-5E9) used in this research was kindly donated by Prof. Nobuaki Okamoto, Tokyo University of Fisheries, Tokyo, Japan.

2.4. Data Analysis

The number of positive neutrophils detected by flow cytometry was assessed using the BD CellQuest Pro program (v. 5.1, Becton Dickinson, USA). The obtained data were compared using the unpaired Student t-test with Welch's correction. All values are shown as a mean ± standard deviation. The results were statistically significant at $p < 0.05$. Data analysis was performed using GraphPad Prism 6 software (GraphPad Software, San Diego, USA).

3. Results

14 blood samples (seven samples from each ploidy) were analyzed to identify size and complexity (forward scatter channel, FSC and side scatter channel, SSC) and the proportion of neutrophil populations. Triploid rainbow trouts showed larger and more complex neutrophils (higher values of forward scatter channel and side scatter channel) when compared to populations of diploid trouts (Figure 1). Neutrophils in diploid trouts were proportionally more numerous than in triploid fishes (9.94 ± 4.64% vs. 5.58 ± 1.09, $p = 0.0481$).

4. Discussion

Fish neutrophils are originated from hematopoietic progenitor cells in kidneys in highly conserved molecular pathways governing hematopoiesis. Differently from mammals, hematopoietic kidneys are a large reservoir of neutrophils in teleost fish, thus constituting less than 5% of circulating leucocytes (Havixbech and Barreda 2015). Flow cytometry is a tool to detect blood neutrophils using monoclonal antibodies in healthy (Hamdani et al 1998; Kfoury Junior et al 1999b; Sasaki et al 2002) and in bacterial challenged rainbow trouts (Moore et al 2019). Our results showed that diploid and triploid trouts have different percentages of blood neutrophils. Hematological analysis showed lower values for neutrophils in triploid trouts (Yamamoto and Iida 1994) and triploid silver catfish *Rhamdia quelen* (Fukushima et al 2012). Triploid salmonids lake trout *Salmo. trutta f. lacustris*, brook trout *Salvelinus fontinalis,* and Arctic charr *S. umbla* have identical blood granulocyte values when compared to the diploid counterparts (Lahnsteiner 2020), however, specific data about neutrophils values were not available.

Neutrophil activity can be assessed by flow cytometry as reported by Morimoto et al (2003) and Øverland et al (2010) for healthy diploid fishes, including rainbow trouts. It is likely to have a direct relationship between size and activity in fish neutrophils which may be related to the presence of a greater number of granules in the cytosol and more segmented nuclei as seen in brook trouts *S. fontinalis* (Wlasow et al 2004). Still, diploid and triploid rainbow trout neutrophils have identical phagocytic activities by visual evaluation (Yamamoto and Iida 1995). Moreover, there are conflicting data in the literature about the leucocyte activities for other triploid fishes. Triploid fish leukocytes, including neutrophils, show higher respiratory burst and phagocytic activity in ayu *Plecoglossus altivelis* (Kusuda et al 1991) and in turbot *Psetta maxima* (Budiño et al 2006). On the other hand, in diploid and triploid shi drum *Umbrina cirrosa*, there was no difference in oxygen consumption after bacterial stimulation based on assays for superoxide anions (Ballarin et al 2004).

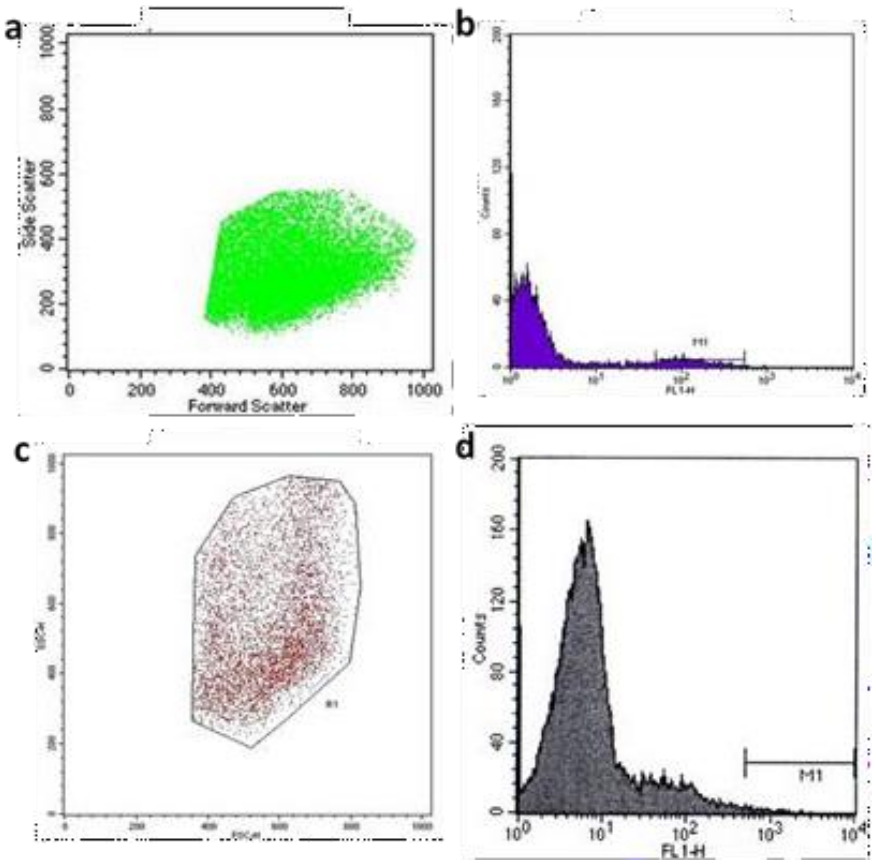

Figure 1 **a)** Population profile of the leukocyte layer of triploid trout with emphasis on the region of the predominance of granulocytes and **b)** histogram shows the specificity of the TTL-5E9 antibody (anti-neutrophil). **c)** Population profile of the leukocyte layer of diploid trout, and in **d)** histogram shows the specificity of the TTL-5E9 antibody (anti-neutrophil).

5. Conclusions

In conclusion, diploid and triploid trouts have different neutrophil proportions in the bloodstream detected by monoclonal antibodies.

Limitations: Diploid and triploid sorted neutrophils were not evaluated on phagocytic activity or apoptosis induction, limiting conclusion about superior or inferior action of triploid rainbow trout neutrophils.

Conflict of Interest

The authors declare that they have no conflict of interest.

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