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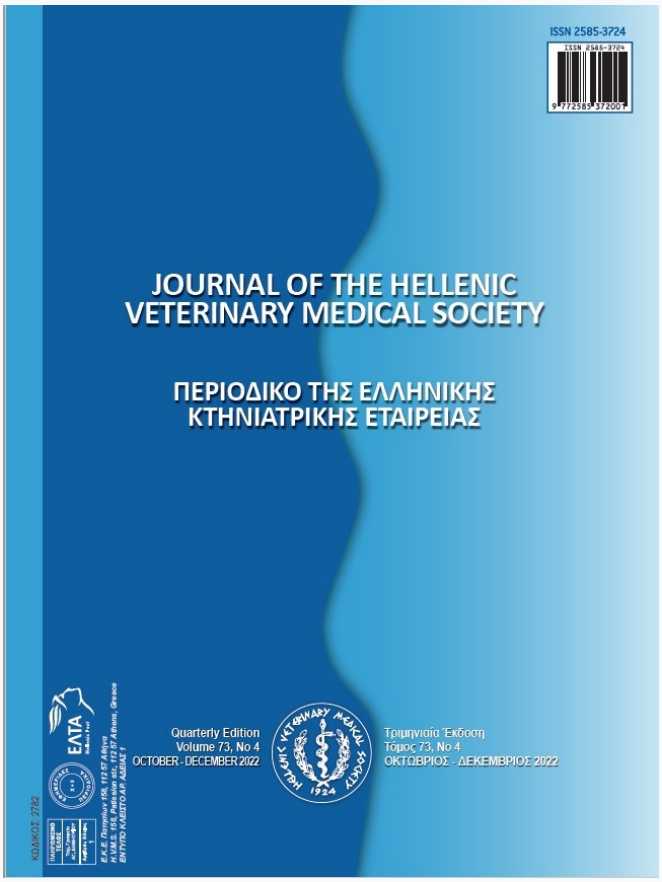


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# Density, Distribution and Staining Properties of Eosinophilic Granular Cells in Oscar Fish (*Astronotus ocellatus*) Intestine

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**ABSTRACT:** Fish eosinophilic granular cells are found in the connective tissues of several organ systems including digestive system and respiratory system associated with the external environment. These cells are similar to mammalian mast cells in terms of structural and functional characteristics. The cytoplasmic granules of these cells give different staining reactions depending on fixative type. This study aimed to determine the staining properties and densities of eosinophilic granular cells in Oscar fish (*Astronotus ocellatus* Agassiz, 1831) intestine using different fixatives and histochemical techniques. Formalin and basic lead acetate fixation-Giemsa staining indicated that eosinophilic granular cells wereabundant in anterior intestine, localizing around especially the blood vessels and submucosa. Giemsa staining of Bouin's fixed rather than other fixatives showed that eosinophilic granular cells were higher in posterior intestine. No reaction was observed in eosinophilic granular cells in Thionin and toluidine blue staining in any fixative. Eosinophilic granular cells in samples fixed with different fixatives had metachromatic alcian Blue staining. In conclusion, this study shows that fixatives may have different effects on the density, distribution and staining properties of eosinophilic granular cells in Oscar fish intestinal regions.

**Keywords:** Fixation; metachromasia; mucosal immunity; staining

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## INTRODUCTION

Mast cells are located at epithelial and mucosal tissues throughout the body, and practically could be found in all vascularized tissues with a few exceptions (Silva et al., 2014). They are involved in early immune responses, and bear granules with different staining properties (Wernersson and Pejler, 2014). Various stimuli cause them to degranulate and release their contents (DePasquale, 2017).

Mammalian mast cells can be smoothly detected in tissue sections using different histochemical staining methodssince they have distinctive staining properties. Staining with Giemsa, alcian- and toluidine blue are commonly used in the identification of mammalian mast cells (Enërback et al., 1986). Commonly used staining procedures reveal two types of cells. Eosinophilic granular cells (EGCs) in teleost fish resemble mammalian mast cells. Staining of fish mast cells (hereafter referred to as EGCs) with toluidine blue imparts them to distinctive metachromasia similar to that also seen in mammalian mast cells (Reite, 1998). Staining with alcian blue at low-pH gives the granules present in both teleost EGCs and their mammalian counterparts a unique pale blue (Reite and Evensen, 2006). Nevertheless, EGCs of fish exhibit variations in terms of staining among fish species (Reite, 1998). Granules of some fish EGCs are characterized by both eosinophilic and basophilic staining (Rombout et al., 1989). The basophilic granules are predominant in fish families such as pike, whereas the eosinophilic granules are common in fish families such as labrides. The presence of both eosinophilic and basophilic granules is regarded as intermediate cell types that give rise to either eosinophilic or basophilic cells (Temkin and McMillan, 1986).

Different fixation, embedding and staining methods utilized for histochemistry might produce a dramatic impact on staining of EGCs. Water-based fixatives lead to loss of granule contents, whereas alcohol-based ones allow metachromasia with Thionine and toluidine blue (Reite, 1998; Reite and Evensen, 2006). For example, gastrointestinal tissue samples of *Hoplias malabaricus* and *Hoplias lacerdae* were fixed in modified Karnovsky's fixative and Helly's solution. All tissue samples were embedded in glycol methacrylate. Then, tissue sections were stained with toluidine blue. EGCs of *H. malabaricus* exhibited metachromasia with toluidine blue, pH 1.5 after Helly's fixation but not Karnovsky's fixation. However, EGCs of *H. lacerdae* showed no metachromasia with toluidine blue, pH 1.5 after Helly's and Karnovsky's solution (Rocha and Chiarini-Garcia, 2007). This shows that metachromatic staining characteristics may vary depending on species. Therefore, staining results should be treated with caution as comparing staining characteristics of EGCs in different fish species. After staining of EGCs of fish species with toluidine blue, occurrence of metachromasia shows that they are truly mast cells. Fish EGCs strikingly resemble their mammalian counterparts in terms of morphology, histochemistry and granule composition, indicating that fish may use a model for investigating functions of human mast cells (DePasquale, 2017).

Oscar fish, *Astronotus ocellatus* (Cuvier, 1829), is a cichlid species with omnivorous feeding habits. It is a freshwater fish species found in habitats with warm water temperatures (Fracalossi et al., 1998; Trindade and De Queiroz, 2012).To date, EGCs have not been examined in the intestines of Oscar fish, *Astronotus ocellatus*. The present study has been conducted to understand the density of EGCs and their specific staining patterns in Oscar fish (*Astronotus ocellatus*) intestinal regions and to evaluate the effects of different fixatives and histochemical methods on their staining properties.

## MATERIALS AND METHODS

### Study protocol

The approval for this study was obtained from Animal Experiments Local Ethics Committee of Süleyman Demirel University (approval number #03.04.2012-04). Use of the animals was performed in accordance with the guidelines of the International Association for the Study of Pain.

### Tissue processing

The intestine samples harvested from six Oscar fish (*Astronotus ocellatus*),weighing 350-400g on average, were used for the histochemical study. The fish were purchased from the ornamental fisher in Isparta, Turkey. After anesthetizing with quinaldine sulphate (20 mg / L) for 1-4 minutes (Gibson, 1967), the fish were killed by decapitation. After opening the body cavity ventrally, the intestine was rapidly excised and divided into three equal-sized regions, namely anterior intestine (AI), middle intestine (MI) and posterior intestine (PI).Three intestinal regions received from 6 fishes were used for all fixatives and staining procedures. In other words, intestinal samples were taken from each region for three fixative

solutions.In brief, they were fixed in 10% of formalin (F) for 48h, Bouin's (B) solution for 12 h, and basic lead acetate (BLA) for 24 h at room temperature for light microscope study. After fixation, tissues were passed through ascending alcoholseries (70% for 24 h, 80% and 96% for 1 h each and 100% alcohol for 2 h), then cleared in xylene and embedding in paraffin. Serial sections of 5 µm in thickness from the same paraffin block belonging to each intestinal region of 6 fishes were placed onto the pre-coated slides. Serial sections of each paraffin block were utilized for four staining methods.In order to determine the density of eosinophilic granular cells, Giemsa (Giemsa, 1904), Thionin (Cooke, 1961), Toluidine Blue (TB) (pH 0.5) (Wolman, 1971) and Alcian Blue (pH 0.3) / Safranin-O (pH 1.0) (AB/SO) (Haddock, 1948) staining methods were applied to the slides. The slides were then rinsed in distilled water, passed through ascending alcohol series, followed by clearing in xylene, and permanently coverslipping with entellan. They were analysed under light microscope (Olympus, CX 41) and photographed with the help of a digital camera (DP26, Olympus, Tokyo, Japan) affixed to the microscope.

**EGC Counting**

In order to determine EGC density in the slides stained with four staining methods, we used a 40x objective and 10x eyepiece containing an ocular grid measuring 0.0625 $mm^2$ of tissue section. In each of six serial sections per intestine region regardless of the mucosal layer for three fixatives, the number of EGCs cells was counted by two blinded observers for selected random 10 microscopic fields. The data are presented as the mean number of EGCs per $mm^2$ (Pabts et al., 1989).

**Statistical Analysis**

Data was analyzed using GraphPad Prizm 8 for Windows (Version 8.0.2) software. Data on the number of EGCs were submitted into GraphPad Prizm 8 for Windows (Version 8.0.2) software in order to assess the density of EGCs in intestine region depending on fixation type. A normality test shows that data on EGCs were normally distributed. One-way ANOVA with Bonferroni's multiple comparisons test was adopted to test if there existed any significant difference in the number of EGCs between fixatives throughout the intestine. Two-way ANOVA with Bonferroni's multiple comparisons test was adopted to test if there existed any significant difference in the number of EGCs between fixatives and intestinal regions. All the values are presented as the mean±SEM. $P<0.05$ was deemed as statistically significance.

## RESULTS

Histochemical results revealed the density and distribution of EGCs and their staining characteristics in intestinal regions and mucosal layers associated with these regions. The density of EGCs in F-, BLA- and B-fixed tissue sections with Giemsa showed statistically significant differences across the intestine (for F vs BLA,F vs B, and BLA vs B:$P<0.001$) (Figure 1a). Among the intestinal regions, fixation with formalin and BLA led toa similar Giemsa staining pattern for ECGs, decreasing from anterior intestine to posterior intestine in a statistically significant manner ($P<0.05$). Bouin's solution resulted in a statistically significant Giemsa staining pattern for ECGs in posterior intestine ($P<0.001$) and anterior intestine ($P<0.004$), but did not show any difference between anterior intestine and posterior intestine in terms of Giemsa staining pattern for ECGs ($P=0.267$). (Figure 1b). As shown in Figure 1b, staining of formalin-fixed tissue sections with Giemsa showed that EGCs were highest in the anterior intestine ($P<0,001$) and Giemsa-stained EGCs were localized especially in the submucosa (Figure 2a) and around blood vessels there (Figure 2b). EGCs were observed to be absent or few in the submucosa (Figure 2c). When stained formalin-fixed tissue samples with thionine and toluidine blue, positive EGCs were absent. Metachromatic AB (+) EGCs were detected in AB/SO staining of formalin-fixed tissue sections (Figure 2d). However, SO (+) EGCs did not observe in AB/SO staining of formalin-fixed tissue sections (Figure 3a).Again, as shown in Figure 1b, staining of BLA-fixed tissue sections with Giemsa demonstrated that EGCs were highest in the anterior intestine($P<0.001$and Giemsa-stained EGCs were localized especially in the submucosa (Figure 3b) and submucosal blood vessels (Figure 3c).As a result of staining with Thionine and Toluidine blue, EGCs was lacking in BLA-fixed tissue sections. Metachromatic AB (+) EGCs were detected in AB/SO staining of BLA-fixed tissue sections (Figure 3d), while SO (+) EGCs did not detect in AB/SO staining of BLA-fixed tissue sections. However, as shown in Figure 2b, staining of Bouin's solution-fixed tissue sections with Giemsa indicated that EGCs were the highest in posterior intestine ($P<0.001$) (Figure 4a). EGCs did not stain with Thionine and toluidine blue in Bouin's solution-fixed tissue sections. Metachro-

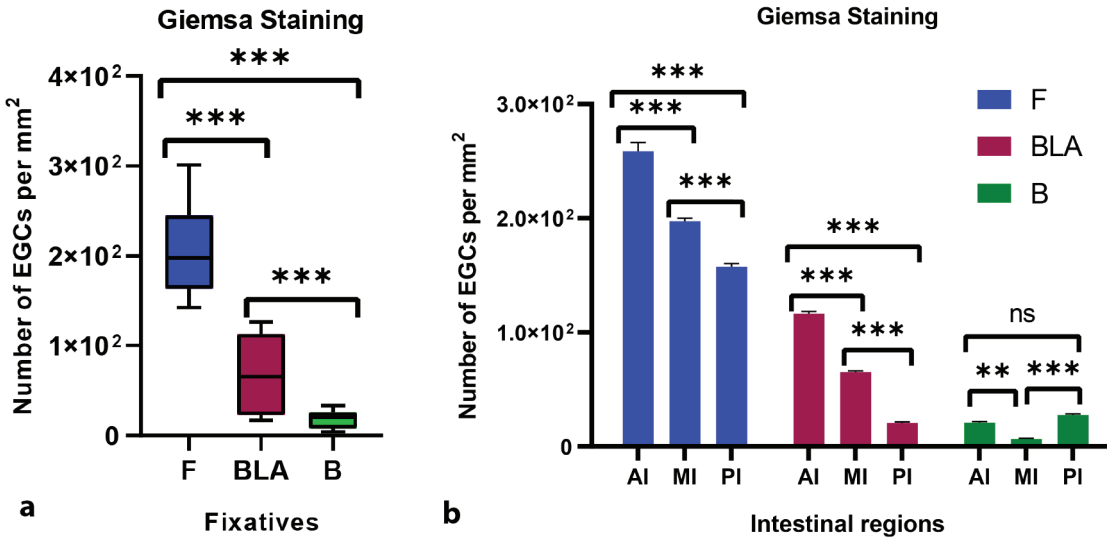


**Figure 1.** Statistical assessment of density of EGCs in intestinal regions of Oscar Fish according to different fixatives and staining methods. a) Number of EGCs per $mm^2$ in tissue sections fixed with F, BLA and B throughout the intestine. b) Comparison of number of EGCs per $mm^2$ in three intestinal regions fixed with F, BLA and B. Error bar values represent the mean±SEM. For comparisons between fixatives throughout the intestine, one-way ANOVA with Bonferroni's multiple comparisons test was used. For comparison of the number of EGCs between fixatives and intestinal regions, two-way ANOVA with Bonferroni's multiple comparisons test was used. ***$P$<0.001, **$P$<0.01, ns: non-significant.AI= Anterior Intestine; MI= Middle Intestine; PI= Posterior Intestine.

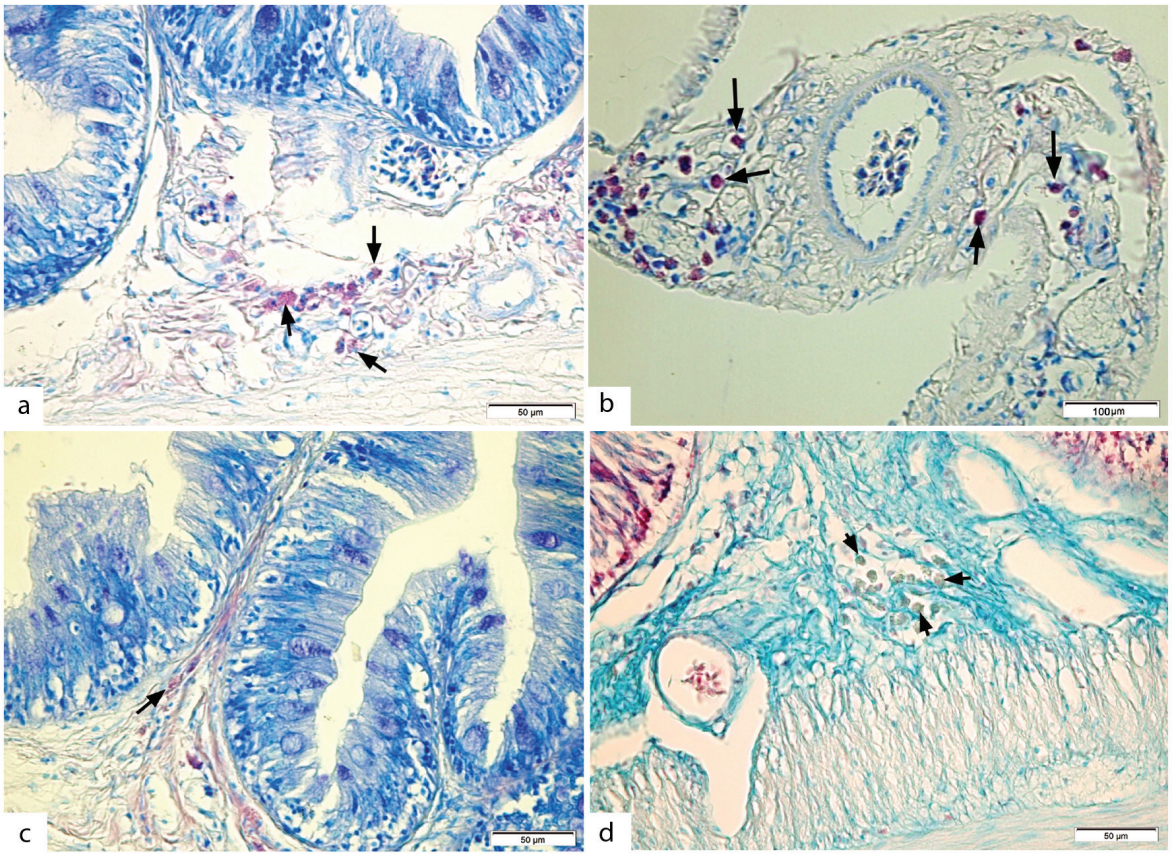


**Figure 2.** Giemsa and AB/SO staining of tissue sections fixed with 10% Formalin.a) Anterior intestine. Giemsa staining of tissue sections fixed with 10% Formalin. EGCs (arrows) present in the submucosa. Scale bar: 50 µm. b) Anterior intestine. Giemsa staining of tissue sections fixed with 10% Formalin. EGCs (arrows) around the blood vessel. Scale bar: 100 µm. c) Middleintestine. Giemsa staining of tissue sections fixed with 10% Formalin. EGCs (arrows) within the submucosa. Scale bar: 50 µm. d) Anterior intestine. AB/SO staining of tissue sections fixed with 10% Formalin. Metachromatic AB (+) EGCs (arrows) in the submucosa. Scale bar: 50 µm.

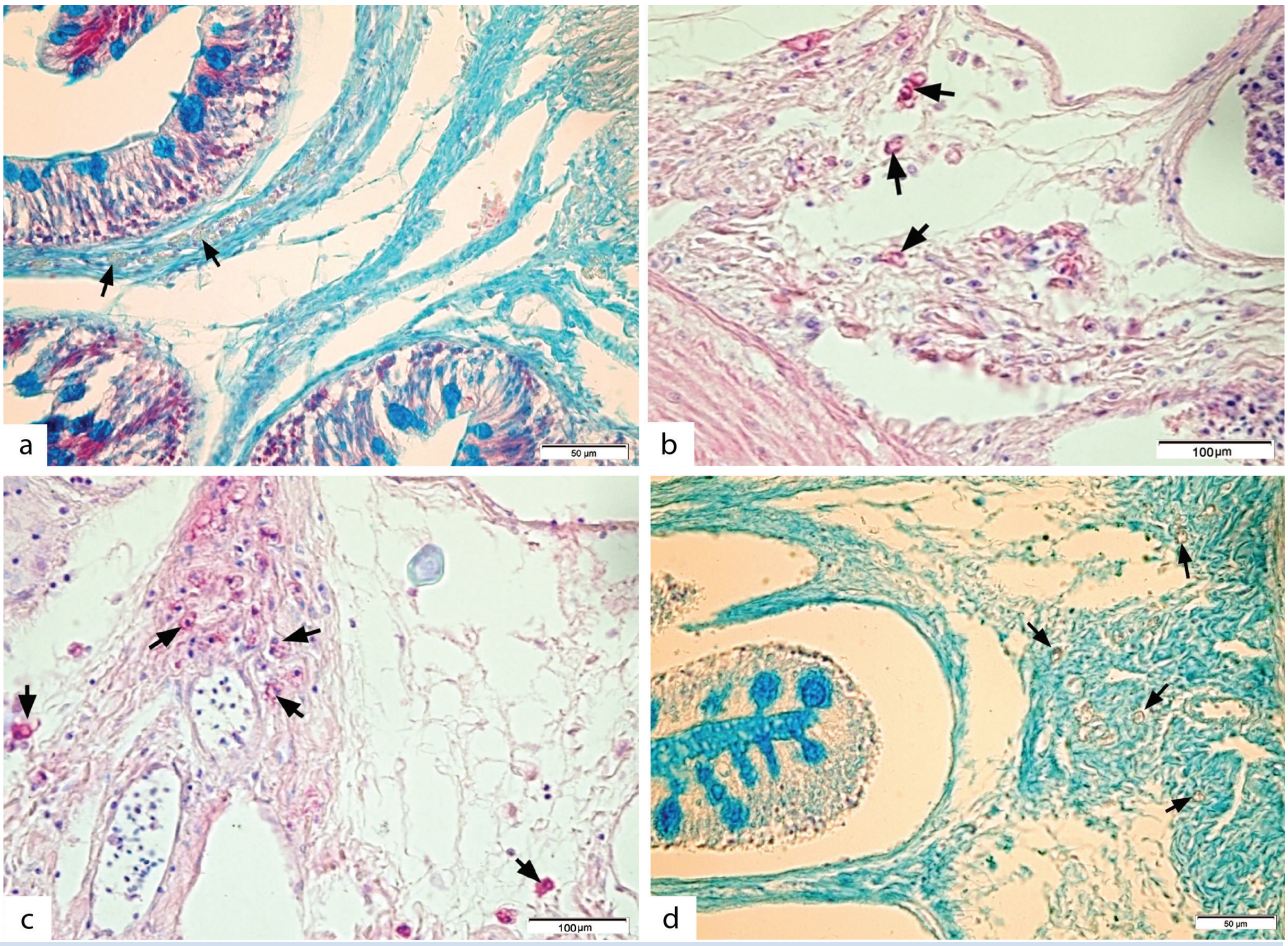


**Figure 3.** Giemsa and AB/SO staining of tissue sections fixed with 10% Formalin and BLA.a) Posterior intestine. AB/SO staining of tissue sections fixed with 10% Formalin. SO (-) EGCs (arrows) present in the submucosa. Scale bar: 50 µm. b) Anterior intestine. Giemsa staining of tissue sections fixed with BLA. EGCs (arrows) around the blood vessel. Scale bar: 100 µm. c) Middleintestine. AB/SO staining of tissue sections fixed with BLA. EGCs (arrows) in the submucosa. Scale bar: 100 µm. d) Anterior intestine. AB/SO staining of tissue sections fixed with BLA. Metachromatic AB (+) EGCs (arrows) in the submucosa. Scale bar: 50 µm.

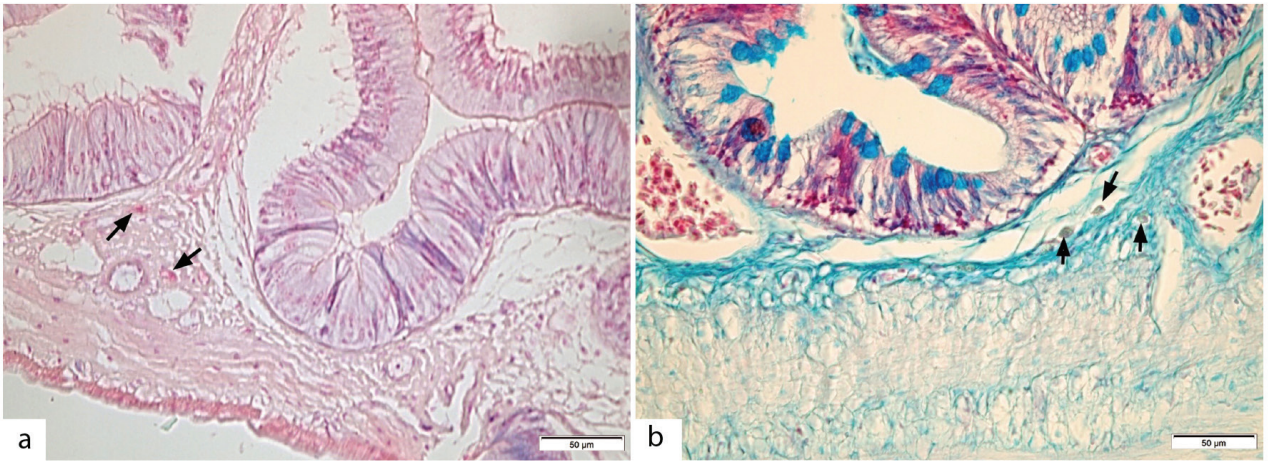


**Figure 4.** Giemsa and AB/SO staining of tissue sections fixed with Bouin's solution.a) Middleintestine. Giemsa staining of tissue sections fixed with Bouin's solution. EGCs (arrows) present in the submucosa. Scale bar: 50 µm. b) Posterior intestine. AB/SO staining of tissue sections fixed with Bouin's solution. Metachromatic AB (+) EGCs (arrows) in the submucosa. Scale bar: 50 µm

matic AB (+) EGCs were detected in AB/SO staining of Bouin's solution-fixed tissue sections, whereas SO (+) EGCs were absent in AB/SO staining of Bouin's solution-fixed tissue sections (Figure 4b).

## DISCUSSION

This study investigates the density and staining characteristics of EGCs in intestinal regions of Oscar fish. This study also reveals that formalin fixation and Giemsa staining are a better approach to the detection of EGCs in Oscar fish intestinal regions than other fixatives and staining procedures. We observed that density of EGCs significantly reduced from anterior intestine to posterior intestine ($P<0.001$) for all the tissue sections fixed with three fixatives. The abundance of EGCs at anterior intestineappears to be a species-dependent phenomenon, suggesting that it might be the first region responsible for launching immune response against food-borne pathogens and microorganisms at lower alimentary tract.

As mammalian counterparts of mast cells, fish EGCs are found in the connective tissues of body areas, especially the respiratory- and alimentary systems, associated with the external environment. They are cells of hematopoietic lineage that play a defensive role in parasitic and microbial infections (Penissi et al., 2003; Marshall, 2004). Studies on fish EGCs have revealed that these cells generate a heterogeneous group of cells (Reite, 1998; Reite and Evensen, 2006; Hopperdietzel et al.,2015; DePasquale, 2017). This heterogeneity ascribes to overt basophilic or eosinophilic staining characteristics of granules that have been identified in various species. Therefore, those cells have been named as mast cells, basophilic granular cells, or acidophilic/eosinophilic granular cells in distinct vertebrate species studied up to now (Ellis, 1977; Reite, 1998; Hopperdietzel et al., 2015; DePasquale, 2017).

Basophilic and eosinophilic (acidophilic) components present in the granules of EGCs may exhibit great differences between fish species, even fish families (Reite,1996; Reite,1997; Hopperdietzel et al., 2015; DePasquale, 2017). Some studies have reported the existence of many EGCs in some fish families (Temkin and McMillan, 1986; Vallejo and Ellis, 1989; Dorin et al., 1993; Hopperdietzel et al., 2015; DePasquale, 2017) whereas other studies have shown the lack of EGCs or few in some fish families (Sis et al., 1979; Buddington and Doroshov, 1986; Williams and Nichol, 1989; Reite, 2005). For example, eosinophilic components are predominant in EGCs of labrids (Reite, 1995), while both basophilic and eosinophilic components are found in salmonids (Reite, 1997). In this respect, our findings are consistent with those reported by Reite (1997).

Demonstration of mammalian mast cells (Rieger et al., 2013) and their fish counterparts, EGCs (Reite, 1998) relies heavily on fixative type and staining protocol. Therefore, an approach to Giemsa, thionine and toluidine blue, alcian blue / safranin O staining of sections fixed with different fixative solutions offers a practical and cheap alternative to the distinct immunohistochemical (Dezfuli et al., 2002) or cytochemical (Da'as et al., 2011) detection approaches of intestinal EGCs in fish. In this regard, the literature reports the results unique to each type of staining and fixation (Hopperdietzel et al., 2015; DePasquale, 2017). Studies comparing different fixatives and staining procedures have reported that EGCs in carp and trout tissues fixed with alcohol-based solutions were stained metachromatically with thionine (Reite and Evensen, 1994; Reite, 1997). Their findings are inconsistent with our findings because we did not use alcohol-based fixatives. On the other hand, metachromatic EGCs were observed in carp tongue sections that were fixed with paraformaldehyde vapor and stained with toluidine blue (Chiu and Laguno, 1972). Temkin and McMillan (1986) showed that goldfish (*Carassius auratus*) gut sections fixed with Helly fixative containing formaldehyde were metachromatically stained with toluidine blue. The results reported by Chiu and Laguno (1972) and Temkin and McMillan (1986) agree with our findings, suggesting that the fixative agents and staining techniques affect the staining ability of EGCs. However, EGCs in Helly-fixed intestine tissues of *Hoplias malabaricus* were metachromatically stained with toluidine blue (Chiarini-Garcia and Ferreira, 1992), which is inconsistent with our formalin-fixed and toluidine blue-stained findings. Taken together, the available literature and our results show that fixation and staining procedures could produce different effects on staining of EGCs among fish species and even families.

Our study is limited to the histochemical approach. If we could perform immunohistochemical staining for mast cell markers, it would be interesting for us to provide detailed information on similarities and differences between mammalian mast cells and fish EGCs. Because a more recent study (Romano et al., 2021) of *Oncorhynchus mykiss* tissues including

intestines reports that mast cells and EGCs are representative of well-differentiated cell populations due to differences in their staining affinities although they have some common characteristics.

## CONCLUSION

In conclusion, this study shows that distribution of eosinophilic granular cells differs largely between the intestinal regions of Oscar fish (*Astronotus ocellatus*) depending on approaches to different fixation and staining. Furthermore, the abundance of eosinophilic granular cells in the anterior midgut shows that these cells could constitute the first line of defense at lower alimentary tract and play an active role in mucosal immunity against food-borne pathogens.

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