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BRIEF COMMUNICATION

Hematological parameters and nuclear abnormalities in peripheral erythrocytes of *Achirus lineatus* (*Pleuronectiformes: Achiridae*)

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Abstract Hematological parameter of demersal fish constitutes important measures of biological effects of seasonality and contaminants in sediments. We aimed to assess the hematological parameters and nuclear abnormalities, including micronuclei levels, in peripheral erythrocytes of flatfish *Achirus lineatus* collected in São Vicente Estuary in summer and winter. The number of lymphocytes was significantly higher in summer, whereas the number of neutrophils was significantly higher in winter. For other measured parameters, no significant differences were observed in spite of the levels of erythroblasts, leukocytes, thrombocytes, micronuclei and nuclear abnormality tended to be slightly higher in winter. Integrated analysis of data suggests that genotoxicity may be related to immunosuppression, although some types of leukocytes appear to act removing anomalous cells. Considering the contamination status of the Santos–São Vicente Estuarine system, the results provide an important contribution to knowledge of the hematological aspects of *A.lineatus* and its potential use as a bioindicator for monitoring estuarine sites.

Keywords Flatfish · Hematology · Biomarkers · Seasonality · Estuary

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Introduction

Demersal fishes play an important role in food web of marine ecosystems, as they feed on benthic invertebrates and small fish, transferring energy and biomass to higher levels of the trophic web (Munroe 2007). Among them, the subtropical flatfish *Achirus lineatus* presents an intimate relationship with benthic environment as it presents a burrowing behavior (Chaves and Serenato 1998) and feeds on benthic organisms, thus increasing the exposure to hypoxic conditions and to environmental contamination, since sediments are recognized as a sink and secondary source of chemicals in aquatic systems (Burton and Johnston 2010).

Flatfishes have been used as biological models to study the effects of contamination (Moore 1992; Riba et al. 2004), as in field surveys through laboratorial sediment toxicity testing. Such studies have considered effects at biochemical, bioaccumulation, cellular, histological and also lethality of exposed

organisms (Moore 1992; Moore and Evans 1992; Belpaeme et al. 1998; Stentiford et al. 2005; Polak-Juszczak 2012).

Alterations of hematological parameters and presence of micronuclei (MN) and other nuclear abnormalities (NA) constitute relevant indicators of responses of fishes exposed to stressing agents because such changes may be associated to diseases, environmental variations (including contamination) and physiological status, and thus they may be considered an overall indicator of health of fishes (Tavares-Dias and Moraes 2004; Osman et al. 2012; Massar et al. 2012; Seriani and Ranzani-Paiva 2012; França et al. 2013). This approach has been used worldwide to assess the responses of fishes to pollutants as in laboratorial experiments with single and mixtures of contaminants in environmental assessments (Al-Sabti and Metcalfe 1995; Kirschbaum et al. 2009; Seriani and Ranzani-Paiva 2012). However, hematological changes and MN may be induced by natural causes, such as seasonality, temperature and salinity changes, reproductive cycle, feeding status, among others (Blaxhall 1972; Duthie and Tort 1985; Ogbulie and Okpokwasili 1999; Tavares-Dias and Moraes 2004; Tavares-Dias et al. 2011; Martins et al. 2010; Seriani and Ranzani-Paiva 2012; Seriani et al. 2013). Thus, the use of this approach in environmental studies requires previous knowledge on the ranges each cell type are expected to occur and how they respond to natural and anthropic factors.

The objective of this study is to evaluate the hematological parameters and micronuclei and nuclear abnormalities (Blebbed, Lobed, Vacuolated and Notched) in peripheral erythrocytes of the flatfish *A. lineatus* collected in São Vicente Estuary in two seasons, aiming to provide information to support the use of this species as a suitable biomonitor of environmental contamination.

Material and methods

The fish individuals were collected at São Vicente Estuary (SVE) (23°30′-24°S–46°05′-46°30′W). This area receives discharges of sewage and contributions of contaminants from industrial areas, marinas and percolated leachates from irregular domestic and industrial landfills (Lamparelli et al. 2001). According to Sampaio et al. (2008), the SVE presents the worst indicators of fecal pollution on the coast of São Paulo. Moreover, moderate to high concentrations of metals (Cd, Ni, Cu, Zn, Cr) and organochlorines were observed in aquatic organisms and sediments from SVE (Abessa et al. 2008; Lamparelli et al. 2001; Carmo et al. 2011).

Flatfish individuals (*A. lineatus*) with length: 12.0±1.0 cm and weight: 62.7±5.2 g were captured by 5′ trawl fishery during winter (n=15) and summer (n=15) at SVE, transferred to plastic boxes containing seawater from the estuary and immediately taken to laboratory, where they were acclimated in ambient temperature and at a density of 1 g fish/L with continuous aeration for at least 4 h to reduce stress. Then, the fishes were anesthetized with benzocaine (3 %), measured and weighed, and then, blood samples were withdrawn from the caudal vein with heparinized syringes. Blood processing was immediately initiated after collections.

Blood extensions (≅50 μL of blood) were prepared in glass slides and stained with May-Grünwald-Giemsa (Rosenfeld 1947), and the specific counts were analyzed per slide/animal under an optical microscope (1,000×). First extensions were used for the analysis of leukocytes (neutrophils, monocytes, lymphocytes, basophyls and eosinophyls) expressed in percentage. In the second set of extensions, 2,000 cells were analyzed per slide/specimen for micronuclei (MN) and nuclear abnormality (NA) rates, considering Blebbed, Lobed, Vacuolated and Notched nuclei. These extensions were also used to count erythroblasts (ERB), total leukocytes (TLC) and thrombocytes (TRB), according to the indirect method adopted by Hrubec and Smith (1998); in this case, 2,000 cells were analyzed as well.

Correlation tests were used to identify interactions between cell types under the influence of seasonality. The measured parameters were expressed as means ± standard errors (S.E.), and statistical analyses consisted in comparing the results of winter and summer by using Student's t-test with $p<0.05$.

Water sample aliquots were taken from the acclimation tanks, and salinities, pH values and temperatures (°C) were measured. These physical–chemical parameters of water samples collected in São Vicente Estuary in summer and winter are presented in Table 1. Variation was relatively low between seasons, even considering tidal cycles, and salinities were ≥25 ‰; pH values ranged between 7.3 (winter) and 7.6 (summer), and temperatures were higher than 21 °C.

Results

Regarding the differential leukocyte accounting (Fig. 1), significant differences were observed for percentages of lymphocytes and neutrophils in summer when compared to winter results (p=0.03). Lymphocytes were more numerous in summer (62.75 % ±) than in winter (16.3 ±), whereas the number of neutrophils was greater in winter (75.95±against 16.3 ±). The number of monocytes, eosinophils and basophils was generally low in both periods (summer: monocytes (6.25± 4.08), eosinophils (2.15±1.81) and basophils (0.45±0.66);

Table 1 Physical–chemical parameters of water samples collected in São Vicente Estuary in summer and winter

Parameters	Summer	Winter
Salinity	25	27
pH	7.6	7.3
Temperature (°C)	24.0	21.5

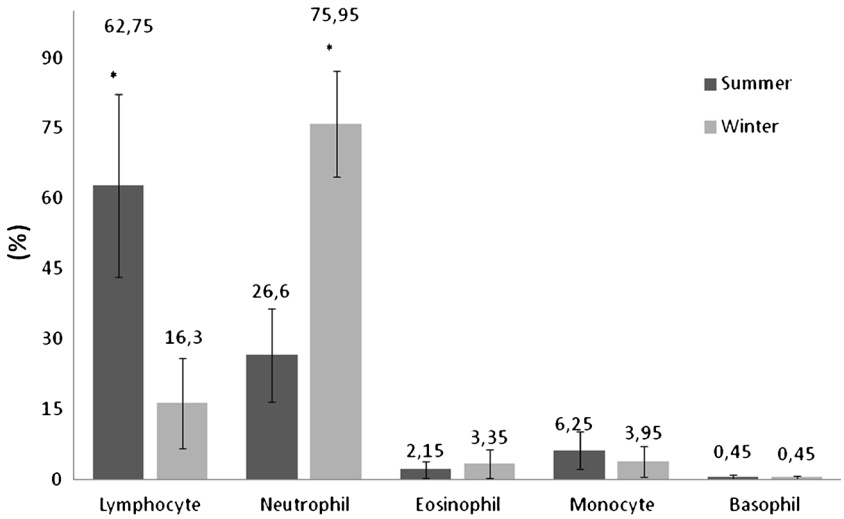

Fig. 1 Mean percentages of peripheral leukocytes of *Achirus lineatus* from São Vicente Estuary in summer and winter. *Asterisks* indicate significant differences ($p<0.05$) between summer and winter

winter: monocytes (3.95±3.28), eosinophils (1.81±3.09) and basophils (0.45±0.39)).

On the other hand, although not statistically different, the percentages of erythroblasts, leukocytes, thrombocytes, micronuclei and other nuclear abnormality of *A. lineatus* collected in winter tended to be slightly higher than those observed in summer (Fig. 2), suggesting worse conditions in winter.

Figure 3 shows cellular types measured in this study. The red blood cells of fish have similar shapes and sizes and often are oval shaped and have a central nucleus with acidophilic cytoplasm. They are nucleated and red pigmented because they carry hemoglobin (Tavares-Dias and Moraes 2004). Fish thrombocytes come in a variety of shapes, and they may be elliptic, rounded, oval shaped or fusiform (Ranzani-Paiva and Silva-Souza 2004) with acidophilic cytoplasm. Their nucleus is often large, and cytoplasm is scarce. Because of these features, they are sometimes confused with lymphocytes. These cells appear either as single cells or grouped together (Vázquez and Guerrero 2007). Leukocytes are non-pigmented, nucleated cells whose primary function is to combat infections and cellular debris, and they may assume different types: lymphocytes are spherical, with a rounded nucleus and strongly basophilic cytoplasm; monocytes are large cells, apparently with phagocytosis functions (Ranzani-Paiva and Silva-Souza 2004); neutrophils are rounded, with a segmented nucleus, and may be frequent in the blood of some fish species. Eosinophils and basophils are scarce and sometimes absent in the blood of fish, and their function is not well understood.

In the summer sampling, the only significant correlation among measured parameters occurred between micronuclei ×

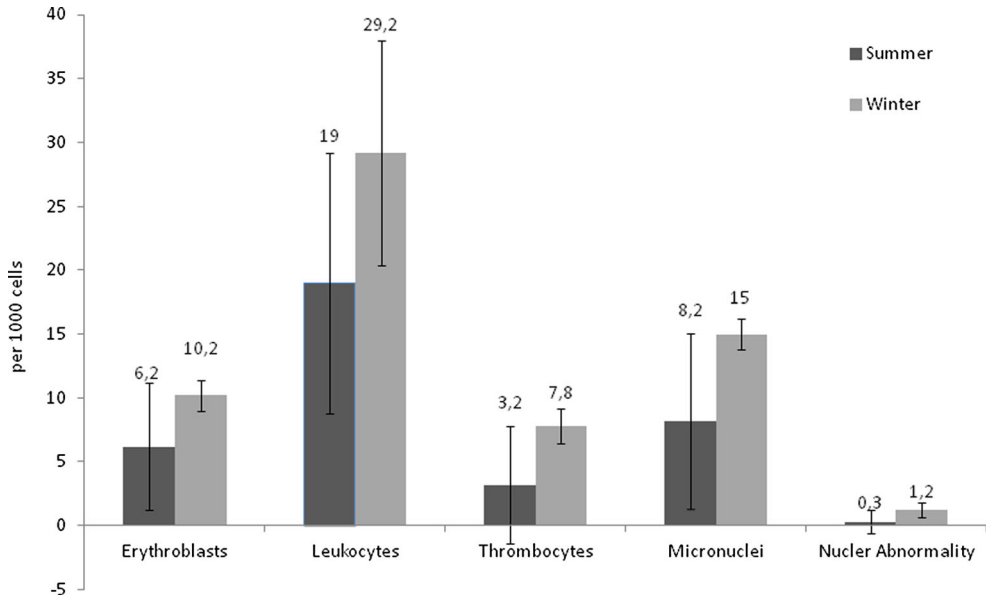

Fig. 2 Mean numbers of erythroblasts, leukocytes, thrombocytes, micronuclei and nuclear abnormality in 1,000 peripheral cells of *Achirus lineatus* from São Vicente Estuary

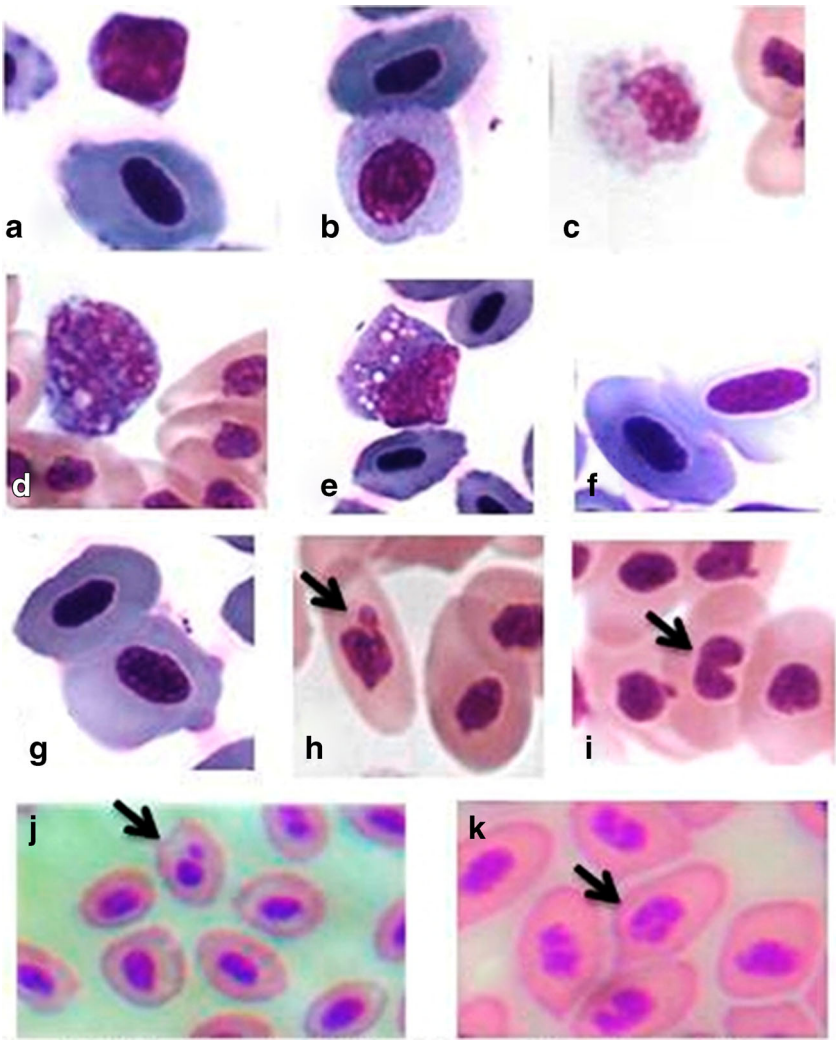

Fig. 3 Peripheral cells of *Achirus lineatus*. **a** Lymphocyte. **b** Neutrophil. **c** Eosinophil. **d** Basophil. **e** Monocyte. **f** Thrombocyte. **g** Erythroblasts. **h** Lobed nuclei. **i** Blebbed nuclei. **j** Micronuclei. **k** Notched nuclei

TLC (r=−0.70; p=0.02). In winter, positive correlations were observed for neutrophil × eosinophil (r=0.72; p=0.01); MN × NA (r=0.67; p=0.01); MN × neutrophil (r=0.76; p=0.01); NA × monocyte (r=0.83; p=0.001). Negative correlations were observed between neutrophil × lymphocyte (r=−0.88; p=0.01); TLC×NA (r=−0.84; p=0.02); erythroblasts × eosinophil (r=−0.88; p=0.001); TLC × neutrophil (r=−0.60; p=0.04); NA × eosinophil (r=−0.68; p=0.02).

Discussion

In summer, MN and WBC were negatively correlated. Such response may be a signal that leukocytes are related to the removal of anomalous or damaged cells, as observed by John et al. (2007). Another hypothesis for this correlation is that stressing agents may induce DNA damage (Normann et al. 2008; Kirschbaum et al. 2009; Osman et al. 2012; Kurteshi and Letaj 2013) and immunosuppression (Houston 1990; Wepener et al. 1992; Danion et al. 2011; Seriani et al. 2011), which is suggested by the lower TLC number.

In winter, positive correlations between NA and MN, MN and neutrophil, NA and monocyte and neutrophil and eosinophil may be evidencing that these leukocytes are removing anomalous cells with DNA damage. However, the increased number of some types of leukocytes and indicators of DNA damage may be due to the action of a stressing agent.

Differential leukocyte accounting showed significant decrease of the percentage of lymphocytes in winter and of neutrophils in summer (p<0.05). White blood cell levels (as neutrophils and lymphocytes) respond to temperature changes, and this probably may explain such results, since water temperature variation may interfere with immune response due to ectothermic metabolism (Tavares-Dias and Moraes 2004). The increase in the percentages of neutrophils in winter may be related to periods of low temperatures (Ainsworth et al. 1991). Although leukocytes have been described as more resistant to changing temperatures, our

study suggests that actually neutrophils can be related to stress due to lower temperature instead of all TLC cells. On the other hand, freshwater species such *Oreochromis niloticus* (Seriani et al. 2011), *Alburnoides bipunctatus* and *Cyprinion macrostomus* (Örün et al. 2003) showed a neutrophil increase only in summer, which is the opposite of results obtained in this investigation. Further studies are required to understand the causes of such discrepancy, which may be related to specific mechanisms of regulation in the face of external stress inductors.

Also, the decrease in the number of lymphocytes in winter probably was influenced by seasonal conditions (especially in the cooler temperatures) due to the action of adrenocorticotropic hormone (ACTH) and cortisol production, and mitogenic activity of cells (Ellis 1981). These hormones inhibit production of defense cells, thus inducing immunosuppression (Adamante et al. 2008; Diniz and Honorato 2012). Similar results were found for a catfish species (Dexiang and Ainsworth 1991).

Comparing results for summer and winter, no significant differences were observed between the numbers of erythroblasts, leukocytes, thrombocytes, micronuclei and nuclear abnormalities in *A. lineatus* from São Vicente Estuary; however, mean values tended to be higher in winter. Such higher numbers of NA and MN may suggest a slightly worse environmental conditions in winter. Seasonality may affect differently each species of fish, as well as environmental factors such as contamination, leading to changes in erythropoiesis and leucopoiesis. Under stressing conditions, the reduction of circulating lymphocytes is the most common change in hematological parameters, possibly due to its redistribution between organs or also due to lymphopoietic activity. In this study, the lower number of lymphocytes in winter corroborates to that pattern, accompanied by increased numbers of neutrophils (Tavares-Dias and Moraes 2004).

Influence of seasonality and pollution was observed in the expression of micronuclei frequencies in *Centropomus parallelus* and *Cathorops spixii* from Cananéia-Iguape Estuarine Complex; however, the regime of such estuary combines rainy season (summer), producing low salinities and higher loadings of metals via suspended particles, making it very difficult to separate the natural and anthropic effects on MN and NA (Kirschbaum et al. 2009; Kuniyoshi and Braga 2011). Anyway, MN and NA alterations were due to natural factors as it is well known that micronuclei and nuclear abnormality formation can occur spontaneously or be induced by environmental changes or pollution (Al-Sabti and Metcalfe 1995; Rybakovas et al. 2009; Kuniyoshi and Braga 2011; Osman et al. 2012; Kurteshi and Letaj 2013).

São Vicente Estuary is considered degraded (Lamparelli et al. 2001; Cesar et al. 2012; Abessa et al. 2008; Hortellani et al. 2008; Azevedo and Braga 2011; Pereira et al. 2012; Seriani et al. 2013; Buruaem et al. 2013) due to human occupation and discharges of sewage, residues of marinas and urban drainage, which produce sediment contamination (Medeiros and Bícego 2004; Bícego et al. 2006; Hortellani et al. 2008; Azevedo and Braga 2011; Buruaem et al. 2013) and incidence of biological effects (Abessa et al. 2008; Cesar et al. 2007; Seriani et al. 2011; Azevedo et al. 2012; Maranho et al. 2012; Seriani et al. 2013). However, results obtained in this investigation do not allow distinguishing if hematological changes were due to natural or anthropic factors. In this sense, it is possible that the observed effects are a product of a combination between them. Further studies are required to elucidate the causes of such variations and to allow evaluating if hematology of *A. lineatus* may be considered a good biomarker of environmental contamination.

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