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Research article

Comparative morphology of blood cells of *Ambassis nalua*, *A. vachelli*, and *Inegocia japonica*

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Abstract

Hematological investigation is an essential tool for monitoring fish health. This objective of this research was to compare morphometric traits and blood cell characteristics between two representative pelagic fish species (*Ambassis nalua* and *A. vachelli*) and one benthic species (*Inegocia japonica*) collected from seagrass meadows off Libong Island in Thailand, where they were exposed to human activity. Blood samples were collected and prepared using the smear technique. The erythrocytes of all observed specimens had elliptical or oval shapes. The largest erythrocytes were observed in *I. Japonica*. In addition, *I. japonica* and *A. nalua* had a significant degree of erythrocyte nuclear abnormalities. According to hematological profiling, lymphocytes made up the majority of leukocytes in *I. japonica*, followed by neutrophils. The morphometric erythrocyte data of *I. japonica* possibly indicated the greater oxygen requirement of fish living in demersal habitats as a result of their adaptation to the environment. The baseline parameters from the hematological data of the sampled fish will be used to monitor environmental quality.

Keywords: Environmental health, Estuarine fish, Hematological biomarker, Seagrass, Thailand

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INTRODUCTION

Coastal areas are essential ecosystems that serve marine animal activities, nursery habitats, and food sources. However, coastal environments, which are easily disturbed due to the sensitivity of their complex biochemical processes, face considerable challenges from marine pollution and intensive human activities (Li et al., 2014; Keshavarzi et al., 2015). Increasing exposure to organic and inorganic pollutants is a significant threat (Li et al., 2014). The most common pollutants found in coastal ecosystems result from mining, industrial processing, waste disposal, agricultural activities and heavy metals (Keshavarzi et al., 2015). In Thailand, pollution from industrial and urban areas flows down drainage systems directly into the sea. Because of its toxicity and accumulation in marine organisms, industrial pollution is considered the most important form of pollution in the aquatic environment (Wattanayakorn and Rungsupa, 2012).

Marine pollution can be monitored by using sentinel species as environmental bioindicators. Fish are often used as sentinel species because they are plentiful, easy to handle, tolerant of a wide range of environmental conditions (Beeby, 2001), and sensitive to specific pollutants (Carpenter, 1925). Several biomarkers can be utilized to analyze the effects of pollutants on animals. Changes in organ weight and hematological and histopathological impacts are established indicators (Auro de Ocampo and Ocampo, 1999; Kendall et al., 2001). For instance, Nussey et al. (1995) discovered that the number of white blood cells increases in a polluted environment.

Hematological profiles are commonly used to assess the health status of fish (Parrino et al., 2018). Variations in blood parameters reflect the physiology, nutrition and feeding behavior of fish, and environmental contamination (Hrubec et al., 2001; Fazio et al., 2017; Parrino et al., 2018). Hematological variations are frequently treated as hematological disorders (Witeska et al., 2011), but blood cell abnormalities can vary depending on the hematological profile, morphological characterization, and erythrocytic alteration (Singkhanan et al., 2019). Qualitative and quantitative changes in hematological parameters, including the number of red blood cells (RBCs), erythrocytic nuclear abnormalities, and the proportion of white blood cells (WBCs), also provide information about the health of the environment (Kumar et al., 2016; Ergene et al., 2007). Morphological changes in cell size, shape, and nuclear alteration have been reported in fish living in polluted areas (Ergene et al., 2007). After exposure to water pollution, impaired fish health can be reflected in nuclear abnormalities, which were found to include irregular nuclear shape, vacuolation, micronuclei, blebbed nuclei, binucleated cells, notched nuclei, and putative fragmented notched nuclei (Ergene et al., 2007). Recent data from Libong Island, Trang Province, Thailand showed that seagrass, which has a complex ecological composition, has been lost, while pollutants and hazardous waste have been dumped in the sea (Pradit et al., 2020). However, the hematological status of fish in the area is still unknown. The objective of this study was to characterize and compare the blood cell morphometries of two pelagic species and one benthic species. The results of this study provided the way for a broader understanding of hematology of fish species in Thai waters and they will be applied in further observation.

MATERIALS AND METHODS

Study area and fish collection

Our adult fish samples were collected from seagrass fields around Libong Island, Thailand. Thirty fish specimens were collected from each location between June 2020 and March 2021. The pelagic *Ambassis nalua* and *A. vachelli* and the benthic species *Inegocia japonica*, were selected based on their habitats and used for this study. Ethical approval for the work was granted by the Animal Care and Use Committee of Rajamagala University of Technology Srivijaya (ID#IAC 13-04-64).

Morphometric data and histological observation

The collected specimens were immediately subjected to a rapid cooling shock at 2–4 °C by immersion in water mixed with ice at a ratio of 1:1 (Wilson et al., 2009). The total length (TL) and total weight (TW) of each fish were recorded. Blood samples of 0.3–0.5 ml were taken from 10 fish per species/region, drawn from the heart area using a 1 ml plastic syringe with a No. 21G needle. Following the standard method of Singkhanan et al. (2019), approximately 10 µl of each sample were smeared on a glass slide and left to dry at room temperature, forming a thin film. The prepared slides were fixed with methyl alcohol for 1 min and stained with Wright's Giemsa for 15 min. The slide was then placed for 30 min in buffer solution at pH 7.4 to wash off the dye before morphological observation of the blood cells (Cossins and Gibson, 1997).

Data collection and statistical analysis

Using an Olympus BX53 light microscope (Olympus Corporation, Tokyo, Japan), erythrocyte length (EL), erythrocyte width (EW), nucleus length (NL) and nucleus width (NW), blood cell percentages, and blood cell abnormalities (100 cells per fish/ per slide) were counted in the middle area of the blood cell smear slide, recorded, and evaluated using the scientific image analysis program Image J (Version 4.0.1 for MS windows, 1998) according to the standards of Andreyeva and Mukhanov (2012). Experimental data were analyzed using SPSS statistical software version 25.0 (IBM, 2017). The data were summarized as means ± standard deviation (SD). Student's t-test was used to analyse differences in erythrocyte morphometries between species. The results were considered significantly different at $p < 0.05$.

RESULTS

Morphological characterization and morphometric analysis of erythrocytes

The morphological characteristics of mature erythrocytes were investigated under the light microscope at 100x magnification level. The erythrocyte was the most abundant cell type in all three species (Figure 1a). Mature erythrocytes were oval in shape. The nuclei were round to oval, and the cytoplasm showed a magenta to pink colour (Figure 1b). Among the three species, EL ranged from 7.05 to 9.24 µm. The longest EL was found in *I. japonica* (9.24 ± 0.96 µm) and the shortest in *A. vachelli* (7.05 ± 0.81 µm).

EW ranged from 5.50 to 6.96 µm. The longest EW was found in *I. japonica* (6.96±0.72 µm) and the shortest in *A. vachelli* (5.50 ± 0.58 µm). NL ranged from 3.06 to 3.47 µm among the three species. The longest NL was found in *A. nalua* (3.47±0.50 µm) and the shortest in *A. vachelli* (3.06 ± 0.5 µm). NW ranged from 3.10 to 2.14 µm. The longest NW was found in *I. japonica* (3.10±0.56 µm) and the shortest in *A. vachelli* (2.14±0.35 µm) (Table 1).

Table 1 Morphometric analysis of erythrocytes in three fish species from Libong Island

Station	Positions (µm)	*Ambassis vachelli* (x̄± SD.)	*Ambassis nalua* (x̄± SD.)	*Inegocia japonica* (x̄± SD.)
Libong Island	NL (N1)	3.06 ± 0.51	3.47 ± 0.50	3.38 ± 0.53
	NW (N2)	2.14 ± 0.35	2.70 ± 0.44	3.10 ± 0.56*
	EL (C1)	7.05 ± 0.81	7.69 ± 0.84	9.24 ± 0.96*
	EW C2	5.50 ± 0.58	5.50 ± 0.68	6.96 ± 0.72*

Note; EL: Erythrocyte Length, EW: Erythrocyte Width, NL: Nucleus Length, NW: Nucleus Width.
*indicates a significant difference ($p < 0.05$) when the data are compared with other groups

Characterization and morphometry of erythrocyte nuclear abnormalities

Two types of nuclear anomaly were determined by morphometric analysis of the three different fish species. Erythrocytes presented reniform and notched nuclei (Figures 1b-1f). Reniform and notched nuclei were found in all three species. Percentages of reniform and notched nuclei were higher in *A. vachelli* than in *A. nalua* and *I. japonica* (Table 2).

Table 2 Percentage proportion of abnormal erythrocyte nuclei in three fish species from Libong Island

Station	Morphometric analysis (%)	*Ambassis vachelli*	*Ambassis nalua*	*Inegocia japonica*
Libong Island	Kidney nuclei	3	1.5	2.5
	Notched nuclei	2	1	1

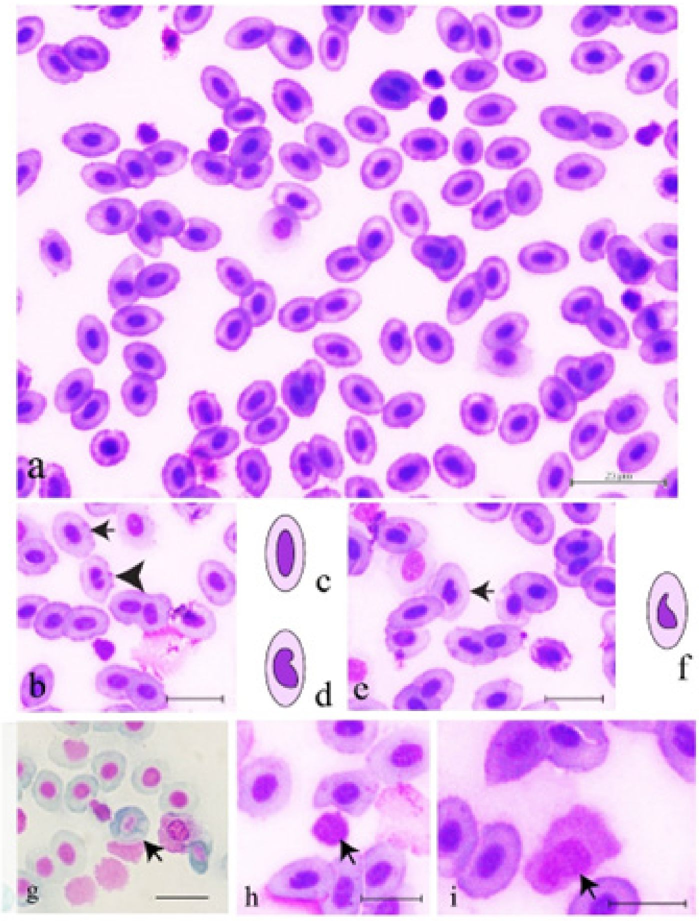

Figure 1 Erythrocytes and leucocytes from the representative specimens were morphologically compared. Micrograph a shows overall peripheral blood cells and erythrocytes of *Inegocia japonica*. Micrograph b shows representative morphology of normal and renifom erythrocyte nuclei (arrows) of *Ambassis nalua* with corresponding schematic diagrams c and d. Micrograph e shows notched erythrocyte nucleus (arrow) of *Ambassis nalua* with the corresponding schematic diagram f. Micrographs show a neutrophil (arrow, g) of *I. japonic*, lymphocyte (arrow, h) and monocyte (arrow, i) of *A. nalua.*

Comparative characteristics and morphometric analysis of leukocytes

Our observation showed that neutrophils were spherical in shape and presented multilobed nuclei (Figure 1g). Neutrophils were found in all three species. The highest percentage of neutrophils was found in *I. japonica* (3.14%). Lymphocytes were characterized by a small amount of cytoplasm with a round central nucleus, and with a large centrally located nucleus (Figure 1h). The highest percentage of lymphocytes was found in *I. japonica* (9.4%). Monocytes were also present (Figure 1i). These cells exhibited a large, generally ovoid, eccentric nucleus, as well as a mildly basophilic cytoplasm. Monocytes were found in *A. nalua* (1%) and *I. japonica* (2.15%) (Table 3).

Table 3 Percentage proportion of three major types of leucocytes in three fish species from Libong Island

Station	Morphometric analysis (%)	*Ambassis vachelli*	*Ambassis nalua*	*Inegocia japonica*
Libong Island	Neutrophil	2	2	3.14
	Lymphocyte	3	7	9.4
	Monocyte		1	2.25

DISCUSSION

Hematological parameters are widely used as biomarkers. The evaluation of blood cell morphology could provide an early indication of physiological changes in fish health status, reflecting their environmental conditions. Studies of fish adaptation to their environment have reported changes in haemoglobin properties and blood cell size (Witeska, 2013). Our work used haematological profiling and morphometric evaluation to investigate the effects of pollution on the health of two pelagic species and one benthic species.

The proportions of erythrocytes and leucocytes in the three fish species were calculated. Since alterations in the size and shape of erythrocytes are related to changes in environmental factors, specific influences could be genotoxicity, oxidative stress, and physiological adaptation (Da Silva and Fontanetti, 2006; Da Rocha et al., 2009; Fazio, 2019). Out of the three species, *I. japonica* had the biggest erythrocytes. Because it was the only benthic species in this study, the larger size of its erythrocytes could be related to its higher demand for oxygen. The observed morphometric parameters of erythrocytes from this study corresponded to those of other fish species in previous studies (Rodriguez-Cea et al., 2003; Guilherme et al., 2008; Rocha et al., 2009; Singkhanan et al., 2019). In addition, the erythrocytic nuclear abnormalities observed in this study indicated that these fish had been exposed to toxicity, contaminated water, or disease (Witeska et al., 2010). Nuclear abnormalities are known to be indicators of haematological toxicity (Parrino et al., 2018). Nuclear abnormalities such as reniform and notched nuclei are considered indicators of genotoxicity (Van Ngan et al., 2009; Witeska et al., 2010). Based on these findings, these fish have likely been directly exposed to heavy metals or other pollutants (Strunjak-Perovic et al., 2009; Witeska et al., 2010).

Three types of leucocytes were identified in this study: neutrophils, lymphocytes, and monocytes. Similar findings have been reported in another fish species (Hrubec et al., 2011). Among vertebrates, proportions of each type of leukocyte are considered to be associated with age, season, and environmental conditions (Hutchison and Szarski, 1965; Duguy, 1970). Our results confirmed a recent study (Salkova et al., 2022) showing lymphocytes were the most abundant leucocytes in *A. vachelli*, *A. nalua* and *I. japonica*. The functional explanation for this finding, however, remains unknown. Moreover, the solitary benthic species, *I. japonica*, presented had much more leucocytes than the two pelagic species; however, this is uncertain. The haematological profiles throughout population, sample size, sampling periods, habitats, and other ecological parameters should all be investigated (Hrubec et al., 2001; Gordeev et al., 2017).

CONCLUSIONS

This is the first report of morphology of blood cells of three fish species from Libong Island, Thailand, as determined by hematological profiling. It is probable that, based on their leucocyte counts, benthic species are more vulnerable to environmental stress than pelagic species. As an early sentinel system, we consider that our hematological study could be a beneficial tool for detecting the consequences of marine pollution.

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AUTHOR CONTRIBUTIONS

Supapong Imsonpang: Methodology, analysis tools and wrote in original manuscript.
Nontawat Kawjaeng: Sample collection, editing and formal analysis.
Archig Jeamah: Sample collection, editing and formal analysis.
Sinlapachai Senarat: Conceptualization, designed the analysis, formal analysis and design the experiment, supervision and finalization.
Pahol Kosiyachinda: Editing and formal analysis.
Anan Kenthao: Data analysis, Editing and formal analysis
Theerakamol Pengsakul: Data analysis, Editing and formal analysis
Wikit Phinrub: Editing and formal analysis
Sittichoke Janyong: Editing and formal analysis.

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