e-ISSN 1734-9168
http://www.isez.pan.krakow.pl/en/folia-biologica.html

Folia Biologica (Kraków), vol. 67 (2019), No 1
https://doi.org/10.3409/fb_67-1.05

Histochemical Characteristics of Macrophages of Butterfly Splitfin *Ameca splendens*

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Accepted March 18, 2019 *Published online March 29, 2019* *Issue online March 29, 2019*

Original article

LATOSZEK E., KAMASZEWSKI M., MILCZAREK K., PUPPEL K., SZUDROWICZ H., ADAMSKI A., BURY-BURZYMSKI P., OSTASZEWSKA T. 2019. Histochemical characteristics of macrophages of butterfly splitfin *Ameca splendens*. Folia Biologica (Kraków) **67**: 53-60.

The butterfly splitfin (*Ameca splendens*) is a fish species that belongs to the Goodeidae family. The biology of this species, which is today at risk of extinction in its natural habitats, has not been fully explored. The objective of the present study was to characterize melanomacrophages (MMs) and the melanomacrophage centers (MMCs) that they form, associated with the immunological system of butterfly splitfin. Butterfly splitfin is a potential new model species with a placenta for scientific research. In addition, knowledge about the location and characteristics of MMCs will allow the effective development of a conservation strategy for this species and monitoring of the natural environment. The results of histological analyses show that the immune system of fish aged 1 dph was completely developed. Single MMs were observed in hepatic sinusoids and in head kidney and their agglomerations were noticed in the exocrine pancreas and in the spleen. Hemosiderin was detected in single MMs in the head kidney of fish aged 1 dph, and in the spleen, exocrine pancreas and liver of older fish. Furthermore, histological analyses of older fish revealed the presence of lipofuscin in the head kidney, exocrine pancreas and spleen in melanomacrophages. The localization and size of MMCs may be an indicator of the health status of fish and may be a biomarker of their immune system activation. The obtained results will allow effective monitoring of the populations of this species. Furthermore our results demonstrate the contribution of liver in the iron homeostasis in fish, as evidenced by enhanced deposition of hemosiderin during butterfly splitfin ontogenesis.

Key words: butterfly splitfin, hemosiderin, immune system, lipofuscin, melanomacrophage centers, melanomacrophages.

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Melanomacrophages (MMs) and the melanomacrophage centers (MMCs) that they form are commonly observed in fish (AGIUS 1980). Their functions are reported to depend on the type of pigment deposited in them and on their localization in organs (LEKNES 2007). Their major function includes involvement in the immune response. The presence of both MMs and MMCs has been confirmed in the spleen, head kidney and liver of fish of various species (AGIUS & ROBERTS 2003). The immune system of fish may be stimulated by xenobiotics present in the environment, and may lead to MM activation and MMC formation (HANDY & PENRICE 1993), because MMs are involved in the removal of xenobiotics and metabolites from the body (PAPAGEORGIOU *et al.* 2008). The correlation between the number and area of MMCs and immunological activation is used to investigate the effect of potentially toxic substances, including herbicides, heavy metals and anthropogenic contaminants (FOURNIE *et al.* 2001; SURESH 2009; WASZAK *et al.* 2012; BANAEE *et al.* 2013; PASSANTINO *et al.*

2013; SIMONATO *et al.* 2016; SAYED & YOUNES 2017) on fish. Different pigments are deposited in the cytoplasm of MMCs-forming macrophages, such as melanin, hemosiderin, lipofuscin, and ceroid (AGIUS & ROBERTS 2003). The color intensity of melanin observed in macrophages varies depending on, e.g., the number and shape of melanophores and proliferation of xanthophore precursors (KOTTLER *et al.* 2015), whereas the presence of a given pigment is determined by processes ongoing in the body. Macrophages of blood vessels capture the degraded red blood cells and phagocytize their residues, and thus become a reservoir of iron ions linked with hemosiderin (AGIUS & ROBERTS 2003). The presence of hemosiderin in melanomacrophages was demonstrated to be strictly dependent on the content of iron which is produced during the degradation of erythrocytes. In turn, lipofuscin deposition in MMs is the indicator of fatty acid peroxidation, which results in the production of such secondary metabolites as lipofuscin (AGIUS 1985). The MMCs, composed of multiple individual macrophages, may be a deposition site for more than one pigment (AGIUS 1980).

No publication characterizing the localization and structure of MMCs in the Goodeidae, including the butterfly splitfin *Ameca splendens* is available. Therefore, it is essential to identify the localization, structure and number of its MMCs. Concerns about the conservation status of this species, raised in central Mexico at the beginning of the XXI century, were caused by decreasing populations of fish species from the Goodeidae family because of degradation of the natural environment and the narrow ecological tolerance of these species (DE LA VEGA-SALZAR 2005). As a result in 2010 the butterfly splitfin was classified as at critical risk of extinction (NOM-059-SEMARNAT-2010). However, for many years this species has been kept in captivity by aquarists and scientists. Currently, the butterfly splitfin is considered a potential model species for research, including toxicological tests. Considering the above, the objective of this study was to describe the localization, structure and histochemical characteristics of MMCs during the ontogenesis of butterfly splitfin. Due to its small size, high fertility, simple procedure of breeding during experiments and the fact the butterfly splitfin is easy to obtain from proven breeders, it is considered a potential model organism for toxicological studies. Therefore, it is necessary to describe the functioning of the butterfly splitfin's immune system.

Materials and Methods

This experiment was conducted in accordance with ethical principles, and was approved by the 3rd Local Ethics Commission regarding Experiments in Animals at the Warsaw University of Life Sciences, no 70/2015.

The fish originated from the Department of Ichthyobiology, Fisheries and Aquaculture Biotechnology. It was the fourth generation of butterfly splitfin kept at the Department. Fish of both sexes were kept in groups in 120 l aquariums (30 fish in each) in a male to female ratio of 1:2. Under such conditions they reproduce in a natural way, and the fry is transferred to new tanks, including experimental tanks. During the experiment, fish were reared in three 120 l aquariums (30 fish each), at a temperature of 24.0 ± 3.16ºC, pH 7.6 ± 0.2, dissolved oxygen concentration of 7.9 ± 0.3 mg/l, and 12h:12h day:night light regime. On the first day of the experiment, the fish density was 0.25 individual per 1 liter, while on the last day of the experiment, after taking the fish for analysis, it was 0.16 individual per 1 liter. During the experiment, fish health status was monitored (including behavior, skin surface and fins) and Fulton's Condition Index (K) was calculated according to the formula (RICKER 1975):

$$K = (W \times 100) \times L^{-3}$$

where: W – body weight (g), L – total body length (cm).

The fish were fed commercial feed mixtures TetraMin (Tetra, Germany) and Spiruline Super-Forte (Tropical, Poland), and with frozen *Artemia sp*. twice a day (*ad libitum*). For histological analyses, 10 fish of both sexes were selected on the day of hatching (1 dph), and 30 days post hatching (30 dph), as well as 5 males and 5 females aged 120 days (120 dph) and 180 days (180 dph). The periods of fish collection for analysis were selected on the basis of breeding stock observation. In fish of 30 dph, the shape of the body changes (fish become more higher), at the age of 120 dph, the first external features of sexual dimorphism appear, while at the age of 180 dph they are adult and sexually mature fish. The collected fish were euthanized in an MS 222 solution (at a concentration of 1:5000 and pH 7.5 adjusted with $NaHCO_3$). Next, their total body length and body weight were measured (Table 1). For the histology analysis, decapitated fish with open ventral shells were preserved in Bouin's solution. The preserved fish were subjected to a standard histological procedure, cut into 5 µm thick sections using a Leica RM2265 microtome (Leica Microsystems, Nussloch, Germany), and stained with hematoxylin and eosin to enable visualization of the morphology of selected organs and melanin deposition. To investigate hemosiderin deposition in macrophages, the sections were stained with Pearls' Prussian blue method (DABROWSKA *et al.* 2012), whereas to de-

Table 1

Mean total body length and body weight of fish used for histological analyses (mean ± Standard Deviation, n=10)

Parameter	Age of fish			
	1 dph	30 dph	120 dph	180 dph
Total body length (mm)	18.69±1.26	21.77±1.39	33.74±2.56	44.56±5.23
Body weight (g)	0.054±0.01	0.156±0.04	0.56±0.13	1.42±0.49
Fulton's Condition Index	0.82±0.01	1.49±0.11	1.44±0.02	1.56±0.04

dph – days post hatching

tect deposition of lipid peroxidation products – mainly lipofuscin – the specimens were stained with AB/PAS method (Alcian blue – Periodic acid-Schiff) (PEARSE 1985) and with Sudan Black B (DISBREY & RACK 1970). Observations of liver, head kidney, spleen and exocrine pancreas morphology and MMC characteristics were conducted using a Nikon Eclipse 90i microscope connected with a digital camera (Nikon Digital Sight DS-U1) and NIS-Elements AR 2.10 system for digital image analysis (Nikon Corporation, Tokyo, Japan).

Results

During the experiment, there was no disturbance of growth or behavior of the studied fish. The fish were mainly in the middle part of the aquarium. In the school, dominant behaviors were observed in males, which are typical for sexually mature fish of this species. The Fulton's Condition Index at 30 dph, 120 dph and 180 dph was over 1.20 (Table 1), indicating good or very good fish condition during the experiment.

Histological analysis of the sections of butterfly splitfin aged 1 dph demonstrated the presence of well developed: liver, spleen, head kidney, and exocrine pancreas. The liver was characterized by a tubular structure composed of polygonal hepatocytes with a central nucleus (Fig. 1A-D). In the liver parenchyma, sinusoids were located in the proximity of thin strands of connective tissue. Single melanomacrophages were observed near the hepatic peri-sinusoids area of fish aged 30 dph (Fig. 1B), 120 dph (Fig. 1C) and 180 dph (Fig. 1D), whereas MMCs were observed in fish at 120 and

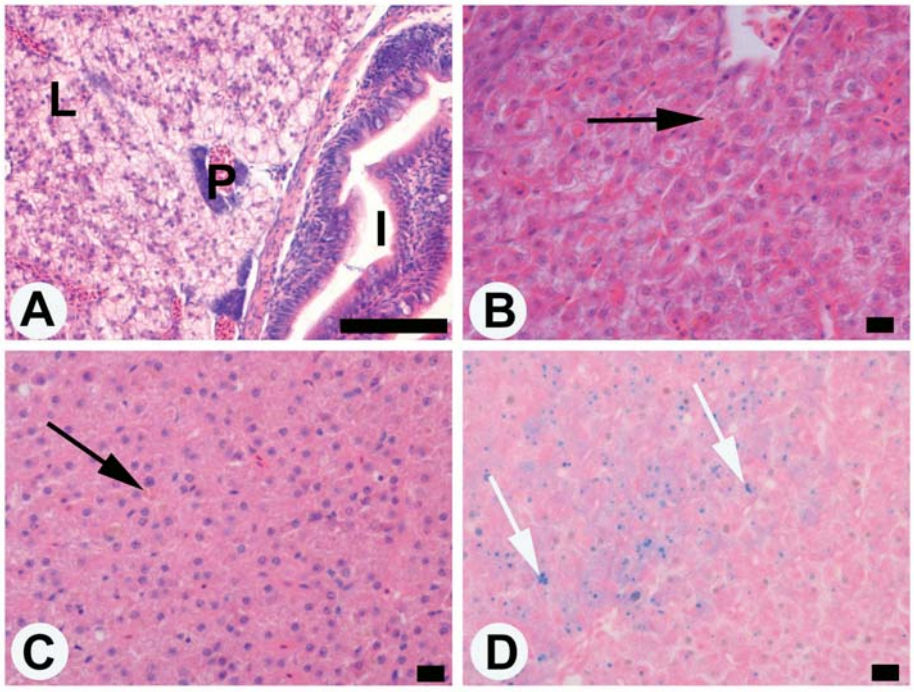

Fig. 1. The cross-section of butterfly splitfin liver. (A) 1 dph, hematoxylin and eosin staining, (B) 30 dph, hematoxylin and eosin staining, (C) 120 dph, hematoxylin and eosin staining, (D) 180 dph, Pearls' Prussian blue staining. Black arrows indicate MMs, white arrows – MMs containing hemosiderin. I – intestine, P – exocrine pancreas, L – liver. Scale bars in A – 100 μm, in B-D – 10 μm.

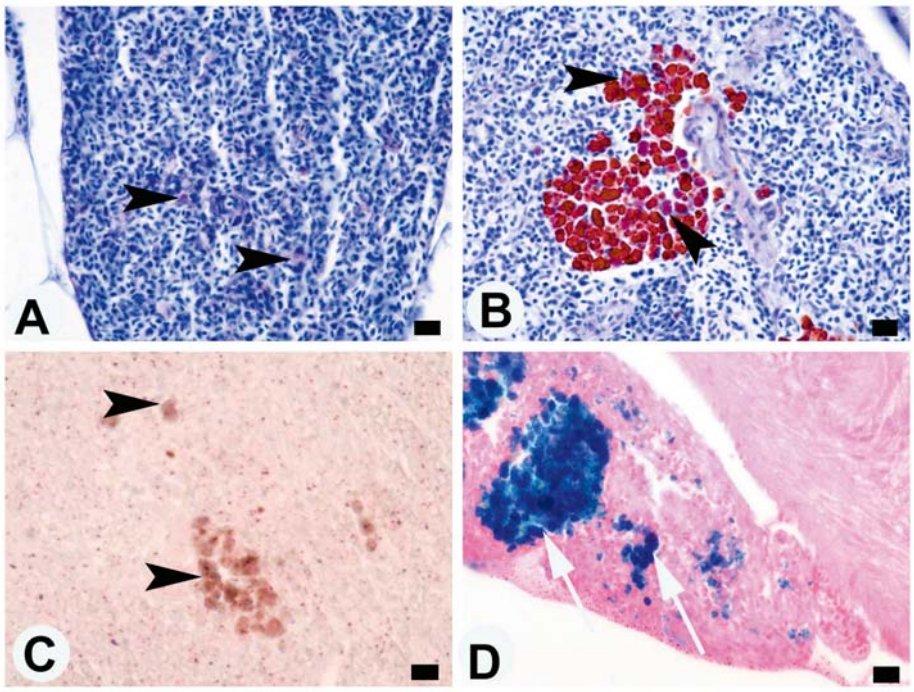

Fig. 2. The cross-section of butterfly splitfin spleen. (A) 1 dph, AB/PAS staining, (B) 30 dph, AB/PAS staining, (C) 120 dph, Sudan Black B, (D) 180 dph, Pearls' Prussian blue staining. Arrowhead – MMCs containig lipofuscin, white arrows – MMCs containing hemosiderin. Scale bars – 10 μm.

Table 2

Deposition of pigments in macrophages in the analyzed fish organs

Organ	Pigment	Age of fish			
		1 dph	30 dph	120 dph	180 dph
Liver	Melanin	–	–	+	+
	Hemosiderin	–	+	–	+
	Lipofuscin	–	–	–	–
Spleen	Melanin	–	+	+	+
	Hemosiderin	–	+	+	+
	Lipofuscin	–	+	+	+
Head kidney	Melanin	–	+	+	+
	Hemosiderin	+	+	+	+
	Lipofuscin	–	+	+	+
Exocrine pancreas	Melanin	–	+	+	+
	Hemosiderin	–	+	+	+
	Lipofuscin	–	+	+	+

dph – days post hatching

180 dph. In MMCs deposition of melanin was observed, whereas in single MMs of fish in 30 and 180 dph deposition of hemosiderin was visible (Table 2, Fig. 1D).

The spleen had a normal parenchymatic structure divided by connective tissue trabeculae. The spleen cross-section revealed clusters of white pulp near large blood vessels filled with nucleated erythrocytes (Fig. 2A, B). Large MMCs containing melanin, hemosiderin or lipofuscin were observed in the spleen of fish aged 30, 120 and 180 dph (Table 2, Fig. 2B-D).

The head kidney of butterfly splitfin was located in the anterior section of the body, behind the pericardial sac. The MMs were localized between numerous renal tubules, within blood vessels, where blood cells could be observed (Fig. 3A-D). The hemopoietic tissue of the head kidney revealed MMs with melanin and lipofuscin in fish aged 30 dph and over (Table 2, Fig. 3C, D), whereas MMs with

hemosiderin were observed in fish of all analyzed age groups (Table 2, Fig. 3A).

The exocrine pancreas was located in the region of liver horns and had a uniform vesicular structure, divided by the interstitial connective tissue (Fig. 4A). Observations of the parenchyma of the exocrine pancreas revealed the presence of MMCs with melanin and hemosiderin in fish aged 30, 120

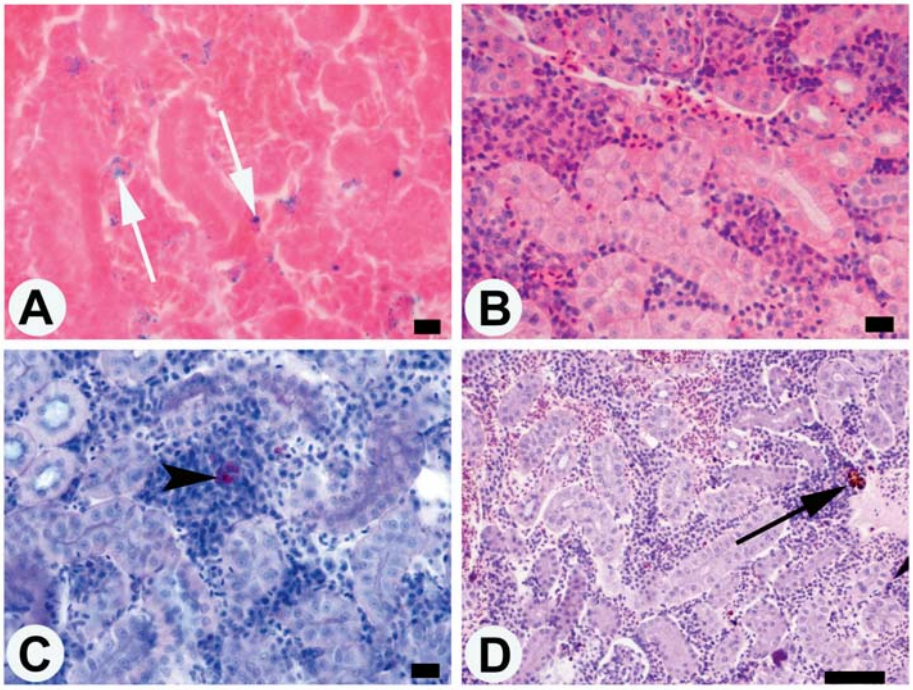

Fig. 3. The cross-section of butterfly splitfin head kidney. (A) 1 dph, Pearls' Prussian blue staining, (B) 30 dph, hematoxylin and eosin staining, (C) 120 dph, AB/PAS staining, (D) 180 dph, hematoxylin and eosin staining. Black arrows indicate MMs, white arrows – MMs containing hemosiderin, arrowhead – MMs containing lipofuscin. Scale bars in A-C – 10 μm, in D – 100 μm.

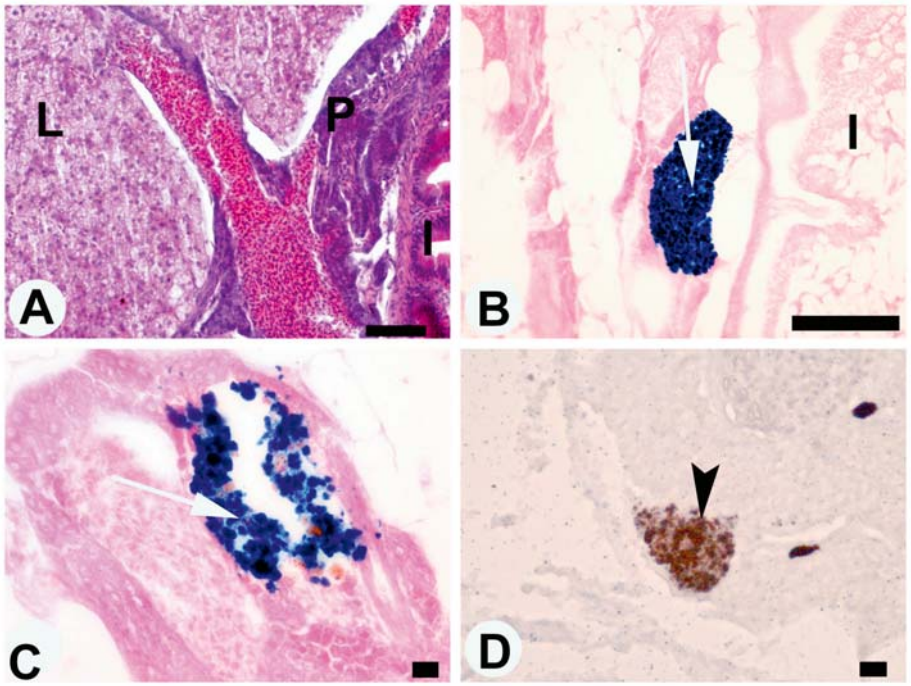

Fig. 4. The cross-section of butterfly splitfin exocrine pancreas. (A) 1 dph, hematoxylin and eosin staining, (B) 30 dph, Pearls' Prussian blue staining, (C) 120 dph, Pearls' Prussian blue staining, (D) 180 dph, Sudan Black B staining. White arrows – MMs containing hemosiderin, arrowhead – MMs containing lipofuscin. I – intestine, P – exocrine pancreas, L – liver. Scale bars in A-B – 100 μm, in C-D – 10 μm.

and 180 dph (Table 2, Fig. 4B, C), as well as MMCs with lipofuscin in fish aged 30 and 180 dph (Table 2, Fig. 4D).

Discussion

The melanomacrophage centers observed in fish are indicators of the activation of their immune system induced by a change of diet (LEDIC-NETO *et al.* 2014) and exposure to xenobiotics (LEKNES 2007; CAMARGO & MARTINEZ 2007) or pathogens (RONZA *et al.* 2013) in the natural environment. Therefore, the presence of MMCs as well as changes in their number and surface area may be used as important biomarkers and indicators of fish condition (FACEY *et al.* 2005; DABROWSKA *et al.* 2012; PASSANTINO *et al.* 2013). SCHWINDT *et al*. (2006) demonstrated changes in MMCs during ontogenesis of brook trout *Salvelinus fontinalis* and rainbow trout *Oncorhynchus mykiss*. The number of MMCs was directly proportional to the age of fish and inversely proportional to the fish condition factor. In contrast, no correlation between fish sex and sexual maturity was observed. These results were attributed to apoptosis enhancement in tissues along with age and the effects of nutritional deficiencies.

Being phagocytes, the macrophages are capable of migrating in the body and therefore may transport pigment from the skin, where melanin is released in the process of melanocyte degradation. Therefore, macrophages are needed for neutralization of melanin with a respective bound agent (AGIUS & AGBEDE 1984). The presence of MMs is used as a reliable biomarker of water quality, especially under the circumstances of chemical contamination (AGIUS & ROBERTS 2003). Moreover, melanin containing macrophages are involved in sequestration of heavy metals (HONG & SIMON 2007; LIU *et al.* 2003). Despite some morphological differences, the immunological system of fish has a physiology similar to other vertebrates (ROMBOUT 2005). In butterfly splitfins aged 1 dph, fully developed immune organs, including: head kidney, spleen and liver, with accumulated cells of the immune system were observed. The exocrine pancreas of the analyzed fish also displayed clusters of MMs, which indicates the importance of this organ in the immune response. Our observations confirm the results reported by DANILOVA and STEINER (2002). These results indicate a partial involvement of this organ in lymphopoiesis and thus, its role as a site of lymphocyte B differentiation. Microscopic observations of butterfly splitfins in 1 dph larva showed no melanomacrophages in any of the analyzed organs. Likewise, LEKNES (2004) did not observe any MMCs nor hemosiderin in the liver of prenatal larvae of platyfish *Xiphophorus maculatus*. In turn, analysis of the head kidney of 1 dph old butterfly splitfin revealed infiltration of hemosiderin-containing macrophages, most likely resulting from its function as a counterpart of bone marrow in other vertebrates (ZAPATA *et al*. 2006). According to PRESS *et al.* (1994), the MMs and MMCs formed by them are the main cells of the fish head kidney. A significant increase in hemosiderin content in MMCs was demonstrated in the spleen of roach *Rutilus rutilus* L. 1758, probably due to enhanced degradation of erythrocytes and simultaneous release of iron as a result of exposure to toxins present in water (PRONINA *et al.* 2014). AGIUS and ROBERTS (2003) reported that hemosiderin was observed in melanomacrophage centers of fish with diagnosed hemolytic anemia. In turn, KRANZ and PETERS (1984) observed single melanomacrophages with hemosiderin in the liver of Eurasian ruffe *Gymnocephalus cernua* inhabiting contaminated waters. This indicates that the liver does not play any significant role in erythrocyte degradation but, most probably, is responsible for the storage and elimination of toxins. LEKNES (2001) showed that MMCs present in the liver and spleen of *X. maculatus* naturally contained iron (III) ions, which are missing in kidney. This confirms the function of liver and spleen as organs associated with the processes of accumulation and reuse of iron in the body. A similar dependency was observed in our study in butterfly splitfin, large surface MMCs containing hemosiderin were present in both liver and spleen, and also deposition of hemosiderin was observed in head kidney and exocrine pancreas. As reported by LEKNES (2007), localization and character of MMCs varies among species. Numerous MMCs were demonstrated in the kidney and spleen of pearl gourami *Trichopodus leerii* while they were significantly smaller and less frequently observed in platyfish. The histological analysis of butterfly splitfin specimens demonstrated a similar distribution of macrophages to that described for pearl gourami. However, hemosiderin deposition was similar to pigment distribution in platyfish (LEKNES 2007). According to LEKNES (2007), such differences indicate diverse functions served by the same organs in different fish species. An accumulation of MMs, suggesting MMC formation in the head kidney of the analyzed fish, was also observed in grass carp *Ctenopharyngodon idella*. In addition, MMCs present in the hepatopancreas of grass carp were associated with blood vessels and contained lipofuscin (MOKHTAR 2015). Lipofuscin, also referred to as the wear and tear pigment (WOLKE 1992), is formed upon peroxidation of unsaturated fatty acids and upon incomplete degradation of damaged mitochondria (PEREIRA *et al.* 2014). It is

composed of oxidized proteins (30-70%) and lipids (20-50%) (HÖHN & GRUNE 2013). The oxidation process of lipid residues may be catalyzed by iron which would suggest that no lipofuscin or only very small amounts would be noticed while observing hemosiderin (TERMAN & BRUNK 2004). This is consistent with our results for butterfly splitfins, where no accumulation of lipofuscin was detected when hemosiderin was present in MMCs. According to HÖHN and GRUNE (2013) lipofuscin plays a negative role in the body by competitive binding with proteolytic enzymes (lysosomal proteases), which inhibits degradation of oxidized proteins. In addition, its deposits are not degraded, while they are transferred to new cells formed during mitotic divisions. In addition, lipofuscin was found to be an intermediate source of free radicals by providing a catalyst for Fenton's reaction owing to its capability to bind different metals, including iron, but also metal containing ferritin (BRUNK & TERMAN 2002). However, KRANZ and PETERS (1984) suggested that lipofuscin containing MMCs might play a positive role in the body. They did not notice any MMCs containing lipofuscin in the liver of Eurasian ruffe displaying *steatosis* of the surrounding hepatocytes or tumor growth. Since *steatosis* may develop in response to the presence of fat soluble xenobiotics, they may accumulate in melanomacrophages containing lipofuscin. Recently, butterfly splitfin was considered as a new species for toxicological studies. Therefore, knowing the morphology and physiology of its MMCs may allow identifying individual organs useful for MMC analysis in order to characterize the toxic effects of various types of xenobiotics on this species. Compilation of analyses of histological markers, taking into account MMC morphology and distribution, with the level of enzymes of the first stage of biotransformation – cytochromes P450 (CYP1 family) and EROD (VAN DER OOST *et al.* 2003) would provide additional information on the physiological condition of individual organs.

Author Contributions

Research concept and design: M.K.; Collection and/or assembly of data: E.L., M.K., K.M., H.S., A.A.; Data analysis and interpretation: E.L., M.K., K.P., H.S.; Writing the article: E.L., M.K. K.M., P.B.-B., T.O.; Critical revision of the article: E.L., M.K., K.P., H.S., A.A., P.B.-B., T.O.; Final approval of article: M.K.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgment

This work was financially supported by the National Science Centre (Poland) grant 2015/19/D/NZ8/03871.

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