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RESEARCH PAPER

Splenic Pigmented Macrophage Aggregates in Barbel (*Barbus peloponnesius,* Valenciennes, 1844) from River Bregalnica — Influences of Age, Sex and Season on a Pollution Biomarker

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Abstract

Macrophages are involved in innate and acquired immune responses, being key players in immunotoxicology. Changes in the quantities of pigmented macrophage aggregates (PMQ) in fish are often used as biomarkers of pollution. Most studies offer only qualitative data on PMQ changes, and effects of age, sex and season are poorly known. We present a stereological study of the relative and total volumes of PMQ in the barbel, *Barbus peloponnesius* spleen. Our focus is to disclose influences of age, sex and season on PMQ, and check if the there is any positive association with aging. The females had a significantly higher spleen and body weights and length. PMQ loaded with lipofuscin/ceroid and hemosiderin were dispersed throughout both spleen pulps. Seasons impacted on PMQ, despite no too markedly. Differences existed between sexes, with males having significantly higher relative volumes of PMQ: the total volumes had similar tendencies. The volumes of PMQ were positively associated with age. The new data reinforce the importance of studying influences of both intrinsic and extrinsic factors that model the quantities of PMQ so to better interpret their changes seen in biomonitoring works.

Keywords: Barbel, melanomacrophages, pollution biomarker.

Introduction

Human activities have impacted on the natural ecosystems for a long time and the situation tends to worsen on a global scale (Covich *et al*., 2004; Woodward *et al*., 2005; Balvanera *et al*, 2006; Brooks *et al*., 2006). Riverine ecosystems are among the most polluted ones, resulting in drastically decreased biodiversity (Dudgeon *et al*., 2006) and impaired health of the aquatic biota, naturally including fish species (Blazer *et al*., 1987). These have been used in biomonitoring studies, namely because fish can offer good and stable indicators about the pollution status of their specific ecosystems (Hinton *et al*., 1992; Hinton 1993; Meador, Carlisle and Coles 2008). Fish can have a long life (years), are located at the top of the food web, and so being prone to bioaccumulate a vast array of toxicants, and, at last, fish react to environmental changes by structural and functional adaptations, including their immune system (Chovanec, Hofer and Schemer 2003).

It is well known that negative effects of pollutants in water ecosystems have influences that cover the whole immune system, and specifically including the PMQ (Meinelt *et al*., 1997; Manera *et al*., 2000; Marth *et al*., 2001; Hung, Tu and Wang 2007). In biological investigations, PMQ in fish were often used as potential biomarkers for judging the level of pollution of the ecosystem (Blazer *et al*., 1987; Fournie *et al*., 2001; Agius and Roberts 2003; Iwanowitcz *et al*., 2012). The involved phagocytes have the ability to engulf, degrade, detoxify, and recycle and/or destruct endogenous or exogenous materials (Ferguson, 1976; Ellis, 1980; Herraez and Zapata 1986; Vogelbein, Fourine and Overstree, 1987; Wolke 1992; Haaparanta *et al*., 1996). As part of the immune system PMQ are often viewed as "metabolic dumps" (Roberts, 1975; Agius, 1980; Agius and Agbede 1984; Fulop and McMillan 1984; Zapata and Cooper 1990).

In fish, PMQ are located mainly in spleen parenchyma and in kidney, and to some extent in liver (Roberts 1975; Wolke 1992). Their number, size, shape and pigment composition vary depending on factors such as: species (Roberts 1975; Agius 1980, 1985; Agius and Roberts 2003; Iwanowitcz *et al*., 2012); organ (Agius 1979; Kranz and Peters 1984);

sex (Schwindt *et al*., 2006); age (Agius 1979; Agius and Roberts, 1981; Brown and George 1985; Blazer *et al*., 1987; Kranz and Gercken 1987; Russo, Yanong and Terrell 2007; Iwanowitcz *et al*., 2012); reproductive cycle (Jordanova, Miteva and Rocha 2008; Jordanova *et al*., 2011); nutritional status and health condition (Agius 1981; Agius and Roberts 1981; Agius 1985; Agius and Agbede 1984; Kranz 1989; Mizuno *et al*., 2002; Gregory *et al*., 2014; Omnes *et al*., 2015). Environmental deterioration, and mainly of water quality, can also influence PMQ in various ways (Agius 1985; Peters and Schwarzen 1985; Weeks and Warinner 1986; Weeks *et al*., 1986; Blazer *et al*., 1987; Kranz and Gercken 1987; Pulsford, Ryan and Nott 1992; Long *et al*., 1995; Couillard and Hodson 1996; Meinelt *et al*., 1997; Fournie *et al*., 2001; Facey *et al*., 2005, Iwanowitcz *et al*., 2012; Balamarugun *et al*., 2012; Beso *et al*., 2016). Accordingly, many authors point that not only changes in fish PMQ can be used as biomarkers, as stated above, but also that they are early indicators for stress, illness or toxicants entering in the environment (Kelly-Reay and Weeks-Perkins 1994; Anderson 1990; Wolke 1992; Fournie *et al*., 2001; Wynn, Chawla and Pollard 2013; Armero-Lituañas and Ocampo 2015).

Although histopathological studies have been examining the influences of endogenous and exogenous factors on PMQ, particularly on their frequency and size, most of the works are merely qualitative in nature; and so not able to offer unbiased objective assessments. Moreover, a relative much lower amount of quantitative studies exist, with authors investigating a variety of parameters (Krüger *et al*., 1996; Rocha, Monteiro and Pereira 1997; Jordanova, Miteva and Rocha 2008; Jordanova *et al*., 2011; Iwanowitcz *et al*., 2012; Balamarugan *et al*., 2012; Gregory *et al*., 2014; Armero-Lituañas and Ocampo 2015). All this makes it difficult to compare data and patterns between different fish species. Also, it is evident that there is yet very scarce quantitative data on amounts of PMQ in view of gender and/or year season, though qualitative analyses indicated that those factors may make a difference (Agius and Roberts 2003; Schwindt *et al*., 2006; Balamarugan *et al*., 2012). To our knowledge, there are only tree studies that, using salmonid fish and stereological techniques that provided quantitative data, supported the idea that both gender and season/reproductive status influence the amount of PMQ (Rocha, Monteiro and Pereira 1997; Jordanova, Miteva and Rocha 2008; Jordanova *et al*., 2011). Given the suitability of PMQ changes as biomarkers of exposure to contaminants, and the need to control basic confounding variables, our primary objective herein is to determine quantitatively (stereologically) if age, sex and season influence the amount of PMQ in the barbel (*Barbus peloponnesius*, Valanciennes, 1884). This is in theory an excellent bioindicator species for the western Greece and Balkans.

Materials and Methods

Fish Sampling and Dissection

Barbel samplings in river Bregalnica were performed to cover every year season (autumn, winter, spring and summer), from October 2007 to March 2008. Fish, 348 males and 310 females, were collected by electro fishing (Samus 725G Electrofisher) according to CEN EN 14011, 2003 standard. Fish capture and handling complied with the current laws of the Republic of Macedonia. After capture, fish were transported alive from field sampling sites to the laboratory, in plastic containers with well aerated river water, where necropsy and collection of biological material were made. For age determination, scales were removed from fish just below the dorsal fin. To avoid the influence of the development of gonads on some of the examined biometric parameters, body mass (BM) was measured without gonads. Total and fork length (TL and FL respectively) were also recorded. Condition factor (CF) was calculated according to the following formula: $CF = BM \times 100/FL^3$. After measurements fish were dissected by severing spinal cord just behind the operculum. Then, the spleen was carefully removed, and the organs mass (SW) were measured. Splenosomatic index (SSI) was calculated according to the following formulas: SSI= SW×100/BW.

Tissue Collection, Processing and Stereological Analysis

The spleen was removed and fixed in toto in Bouin's fluid for 48 hour. Thereafter the organ was routinely processed for embedding in paraffin. Each block (one per animal) was serially cut into 5 μm thick sections, systematically picking every 20th (and two additional ones) after a random start, so to obtain a final set of five slides per animal. These were stained with Perl's' Prussian blue for hemosiderin; extra sections were stained with haematoxylin an eosin (H&E). In each Perl's' stained section, 10 to 15 systematically sampled fields were quantified, with the first file being randomly selected. In average 50 fields per fish were studied. Stereological techniques based on differential point counting (Freere and Weibel, 1967) were used to estimate the relative volumes of PMQ, according follow formula:

$$V_V \text{ (structure, reference)} = V_V (s, r) = [P(s) \times 100] \div P(r)$$

where: $V_V (s, r)$ is the percentage of the total volume of a reference space occupied by one particular type of structure within that space; P(s) is the number of points falling over a chosen structural component; and $P(r)$ is the total number of test points lying over the reference space. Herein, the all splenic tissue was determined as reference space. Point

counting was made manually with glass grid with 180 points inserted into the microscope ocular. Total volumes of the PMQ [V (PMQ)] were estimated as:

$$V\ (PMQ) = V_V\ (PMQ, spleen) \times V\ (spleen)$$

Statistical analyses

The data are presented as a group means of individual fish values, accompanied with the respective coefficient of variation (CV = SD / Mean); the latter offering an easy assessment of the inter-individual variability. For statistical analyses, the software Statistica 7.0 for Windows was used. After checking the normality and homogeneity of variances, they were analysed by a two-way ANOVA to test the effects of the sex, season and sampling site *versus* MACs aggregates. Some variables were transformed to reduce skewness and improve homoscedasticity. Whenever the ANOVA revealed significant effects, post-hoc differences between groups were judged by the Newman-Keuls test. Spearman correlation analyses were used to find monotonic associations between two variables. Differences were considered significant whenever $P<0.05$.

Results

The two-way ANOVA showed that BW and TL depended both on season and sex, with no interaction between factors. Contrarily, the CF depended on season and sex, but there was a significant interaction between the factors. Significant sex differences were noticed for BW and TL (Table 1). The inter-animal variability was generally high, being particularly notable in weights both of body and spleen and in the SSI too (Tables 1, 2). The body length and CF were more stable. In all seasons, females were significantly heavier and longer compared with males (Table 1). As to the CF, in winter and summer females had significantly higher mean values than males. As to seasonal effects on the CF, only males showed a significantly higher value in spring. Concerning the spleen weight (Table 2), the ANOVA showed that it depended on season and sex, and that there was a significant interaction between both factors. The post-hoc testing revealed that females and males differed in autumn and winter, with the first ones displaying heavier spleens. In spring and summer the females' spleen significantly decreased compared with the previous seasons, and there were no intersex differences. For the SSI, no statistically relevant effects were revealed using the ANOVA. Anyway, there was a trend towards lower mean values in females from spring and summer, in line with the significant decrease in the organ's mass.

Histologically, PMQ seem randomly distributed in the splenic parenchyma, despite most often they appear in the close vicinity of blood vessels (Figures. 1, 2). Their shape is typically irregular, and only rarely PMQ displayed a more regular configuration. H&E staining (Fig. 1) evidenced that the cells cytoplasm in aggregates is loaded with a brownish pigmentation, highly suggestive of lipogenic pigments (ceroid/lipofuscin). Also, Perl's staining (Fig. 2) revealed profuse amounts of hemosiderin.

As to the relative volumes of PMQ (Table 3), the ANOVA showed a significant effect only for the factor sex. The post-hoc tests evidenced those volumes were significantly greater in the males, at every season. By contrast, the total volumes of PMQ (Table 3) were impacted solely by the factor season, with fish at autumn (females and males combined) having greater volumes. At last, the factors sex and season, independently, had significant effects on the mean volumes of PMQ per unit of body weight, with the males having, overall (seasons combined), greater volumes, and with autumn and summer (sexes combined) showing the greatest and smallest volumes, respectively.

The fish age was estimated to be from 2+ to 5+ years. The non-parametric correlative study demonstrated that, for both sexes, the relative and total volumes were positively associated with age. For females, the correlations were weak for the relative volume ($r=0.22$; $P<0.001$) and moderate for the total ($r=0.54$; $P<0.001$). For males, the significant correlations were just moderate for both the relative (r

Table 1. Barbel's body weight, total length, condition factor for both sexes in every season

	Body weight (g)		Total length (cm)		Condition factor (%)	
Season	Female	Male	Female	Male	Female	Male
Autumn N=106, n=133	32.30(0.55) ^{A}	15.84 (0.35) ^{B}	14.70 (0.17) ^{A}	12.09 (0.11) ^{B}	1.18 (0.11)	1.13 (0.10)^{a}
Winter N=26, n=22	29.33 (0.46) ^{A}	13.51 (0.31) ^{B}	14.48 (0.16) ^{A}	11.67 (0.11) ^{B}	1.17 (0.15)^{A}	1.08 (0.13)^{Ba}
Spring N=61, n=74	28.32 (0.66) ^{A}	14.21 (0.43) ^{B}	13.83 (0.22) ^{A}	11.50 (0.15) ^{B}	1.20 (0.10)	1.22 (0.11)^{b}
Summer N =118, n=146	27.09 (0.57) ^{A}	14.18 (0.29) ^{B}	13.79 (0.19) ^{A}	11.79 (0.11) ^{B}	1.19 (0.11)^{A}	1.11 (0.09)^{Ba}

Data given as mean (coefficient of variation). N and n are, respectively, the number of females and males. For every metric, different uppercase superscript letters represent differences between sexes at each season (read horizontally), and different lowercase superscript letters represent differences between seasons within same sex (read vertically), according to post-hoc testing after significant ANOVA.

Table 2. Barbel's spleen weight and splenosomatic index (SSI), for both sexes in every season

	Spleen weight (mg)		SSI (%)	
Season	Female	Male	Female	Male
Autumn N=106, n=133	48.5 (0.79)^{Aa}	16.6 (0.82)^{B}	0.15 (0.56)^{Aa}	0.11 (0.80)^{B}
Winter N=26, n=22	44.2 (0.94)^{Aa}	22.9 (2.15)^{B}	0.14 (0.55)^{a}	0.16 (1.89)^{a}
Spring N=61, n=74	27.9 (0.90)^{Ab}	10.2 (0.36)^{B}	0.10 (0.58)^{b}	0.09 (0.57)
Summer N =118, n=146	26.1 (1.02)^{Ab}	10.6 (1.19)^{B}	0.09 (0.62)^{b}	0.07 (0.41)^{b}

Data given as mean (coefficient of variation). N and n are, respectively, the number of females and males. For every metric, different uppercase superscript letters represent differences between sexes at each season (read horizontally), and different lowercase superscript letters represent differences between seasons within same sex (read vertically), according to post-hoc testing after significant ANOVA.

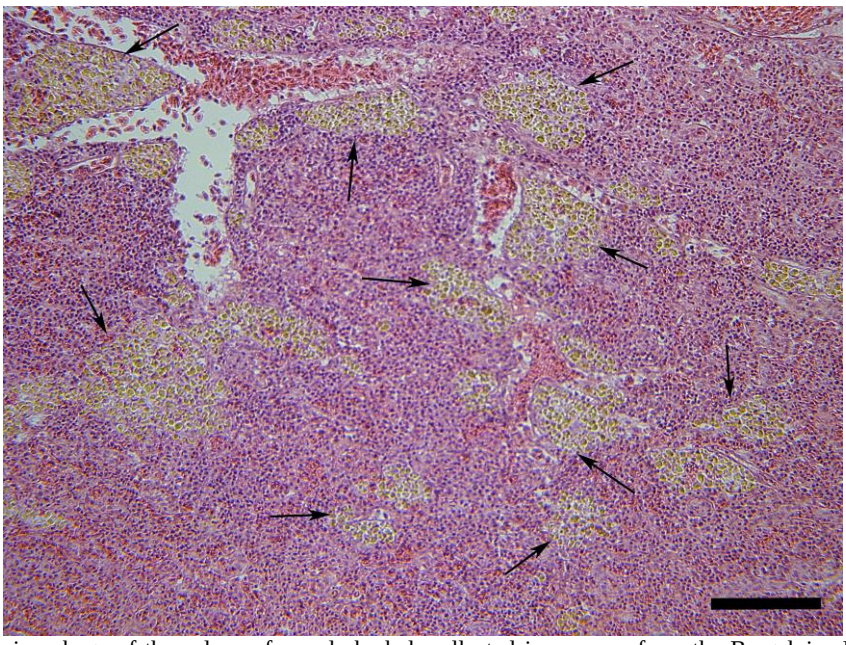

Figure 1. Light microphage of the spleen of a male barbel, collected in summer from the Bregalnica River, illustrating aggregates of pigmented macrophages with a lightly yellowish tone (arrows). Haematoxylin and eosin staining. Scale bar = 100 μm.

= 0.35; P<0.001) and total volume (r=0.38; P<0.001). Note that the animals captured at different seasons are comparable because they show both similar weights and lengths (Table 1).

Discussion

Many studies suggested that the PMQ status in fish spleen, kidney and liver are useful biomarkers of toxicant exposure (e.g., Blazer *et al*., 1987; Agius and Roberts 2003). However, PMQ may be affected by factors other than pollution. Using stereology at light microscopy level, here we evaluate the influence of age, sex and season on the relative and total volumes of PMQ in the spleen of wild barbel. These data are complemented with fish body and spleen morphometric parameters.

Females were both heavier and longer than males, in line with the pattern found in former studies focusing on barbel, e.g., on *Barbus meridionalis* (Šorić and Janković 1989), *Barbus peloponnesius petenyi* (Šorić 1992), *Barbus luteus* (Al Hazzaa 2005), *Barbus peloponnesius* and *Barbus cyclolepis* (Vasiliou and Economidis 2005). These findings indicate that our sample should be representative of the natural population of barbel from River Bregalnica. The fact that females are larger is thought to result from a faster sexual maturation in males than in females (Vasiliou and Economidis 2005).

The CF is a general and much used indicator of the fish "well-being" and also of the tolerance and response of the organism to stresses caused by environmental toxicants (Mayer *et al*., 1992; Froese 2006). Many factors can shape the CF, such as sex, season, nutrition status, or water quality and temperature (Garcia-Abiado *et al*., 2004; Swansburg *et al*., 2002; Khallaf *et al*., 2003; Nehemia *et al*., 2012). Independently of the particular influences,

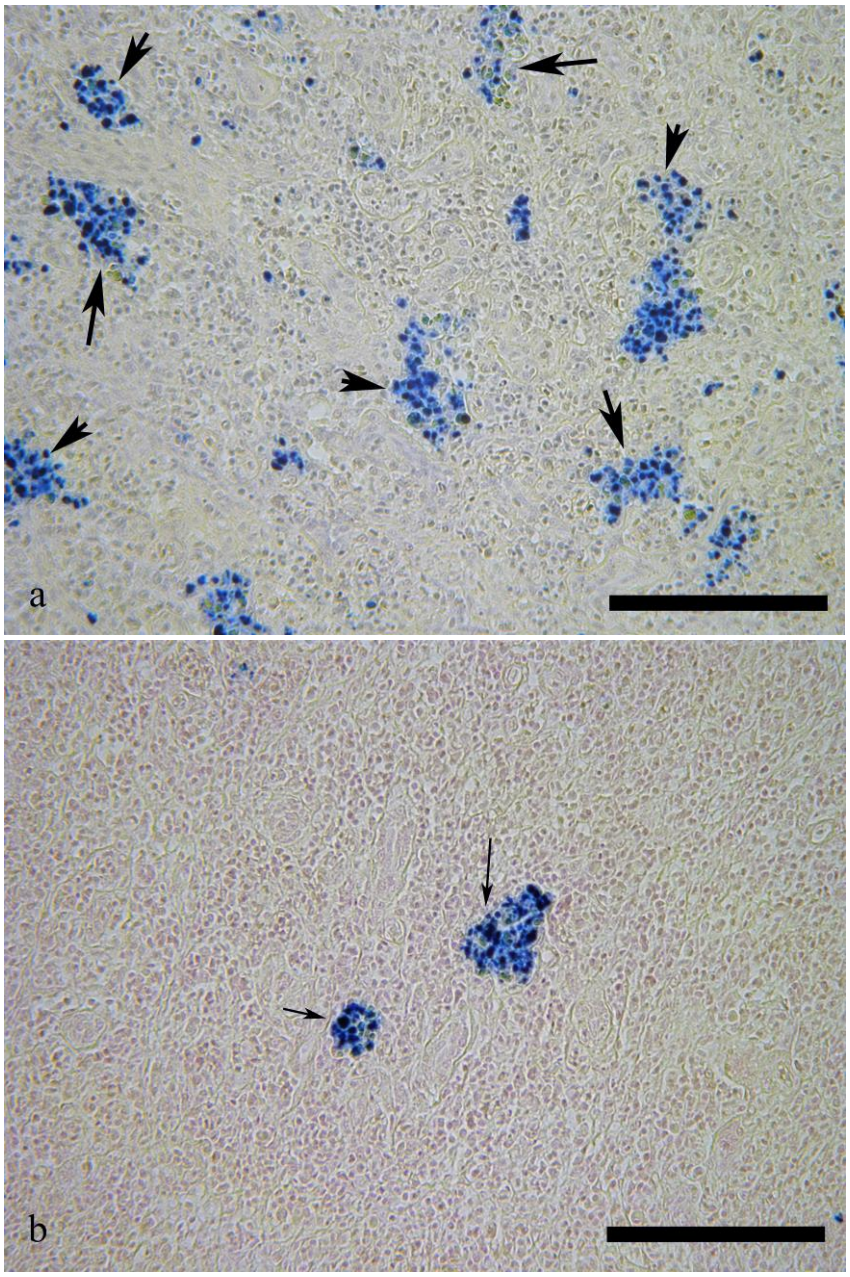

Figure 2. Light micrographs taken from the spleen of **a)** male and **b)** female barbel, collected in summer from the Bregalnica River, illustrating aggregates of pigmented macrophages (arrows) stained by the Perl's method. This selected field qualitatively illustrated the higher relative occurrence of aggregates in males. Scale bar = 100 μm.

higher values of the CF typically indicate better "condition" (health status) of the individuals (Nehemia *et al.*, 2012). Here, the CF of male barbel were generally lower than those of females, especially in winter and summer. Females had stable CF along season, but males peak at spring. Similar seasonal variations of the CF were observed in *Barbus plebejus escherichi* (Yildirim *et al*., 2006), which, once more, suggests that our fish were within ranges of normality.

Spleen weight mainly depends on the species, sex, body weight and nutrition (Tischendorf 1985). We found that the barbel spleen weight in females was roughly three times greater than in males, in all seasons. However, due to the high variability between the studied individuals, these sex differences were proved as significant only in autumn and winter. Because the SSI (%) did not vary with sex, we can suppose that differences between sexes as to total spleen weight result from intrinsic dissimilarities in immune system structure/needs/responses between females and males. With larger spleens, it was argued that females have an increased capacity for blood filtration and consequently are prone to display an a priori better immunology defence system for the organism (Hadidi *et al*., 2008). Regarding seasonal influences, females from autumn and winter had significantly heavier spleens compared with those from spring and summer. Reproductive cycle and

Table 3. Relative [V_V (PMQ, spleen)] and total splenic [V_{spleen} (PMQ)] volumes of pigmented macrophage aggregates, for both sexes in every season

	V_V (PMQ, spleen) (%)		V_{spleen} (PMQ) (mg)		$V_{mg/g\ BW}$ (PMQ)	
Season	Female	Male	Female	Male	Female	Male
Autumn N=106, n=133	0.77 (1.25)^{A}	2.18 (1.12)^{B}	42.3 (1.99)	41.5 (1.59)^{a}	1.2 (2.01)	2.3 (1.42)
Winter N=26, n=22	0.87 (1.10)^{A}	1.83 (1.36)^{B}	38.8 (1.77)	35.1 (1.72)	1.1 (1.33)	2.2 (1.55)
Spring N=61, n=74	1.12 (0.82)^{A}	2.10 (1.02)^{B}	32.8 (1.30)	22.1 (1.21)	1.1 (1.05)	1.5 (0.96)
Summer N =118, n=146	0.95 (1.55)^{A}	1.94 (1.17)^{B}	26.2 (1.30)	18.6 (1.22)^{b}	0.8 (1.54)	1.3 (1.22)

Data given as mean (coefficient of variation). N and n are, respectively, the number of females and males. For every metric, different uppercase superscript letters represent differences between sexes at each season (read horizontally), according to post-hoc testing after significant ANOVA.

seasons were both reported to cause significant fluctuations of the spleen weight (Fänge and Nilsson 1985; Tischendorf 1985; Lamkova *et al*., 2007; Rebok, Jordanova and TavcioskaVasileva 2011, Rebok 2013. According to Fänge and Nilsson (1985), based on salmonids data, before and after spawning the organ is bigger and store larger amounts of blood, but at spawning it gets smaller and with less blood (anaemic). The spawning period for our barbel was spring-summer, and so it is likely that the lowest weight of spleen is in close association with gonadal maturation. Seasonal differences in the spleen at different seasons/reproductive cycle stages were well characterized in other fish species, such as *Salmo trutta* (Álvarez *et al*., 1998), *Rutilus rutilus* (Kortet *et al*, 2003), and *Salmo letnica* (Rebok, Jordanova and TavcioskaVasileva, 2011). Anyway, we did not found in this study any seasonal differences in the SSI. One explanation could be fish age. Accordingly, Yildirim *et al*. (2006), who investigated seasonal changes in the SSI of the *Barbus plebejus escherichi*, found real differences in the oldest but not in the youngest fish.

PMQ in fish have diverse structural organizations and vary in the amount and type of pigment (Agius and Roberts, 2003). Here, barbel PMQ were largely irregular i and only rarely oval-shaped. According to Agius (1985) and Wolke (1992), PMQ are irregularly shaped in cartilaginous fish and in teleosts of the Clupeiformes and Salmoniformes orders. However, there are departures of this general rule, and it seems the three-dimensional organization of aggregates depend on the fish species (Blazer *et al*., 1987; Manera 1997; Hinck *et al*., 2007). So, our findings reflect such variability. As to the pigment type, here PMQ aggregates were filled with lipofuscin/ceroid and hemosiderin. This agrees with the expectations for spleen (Agius and Roberts 2003).

Most studies mentioning changes in number, size and frequency of PMQ is relation to influences of exogenous or endogenous factors are based on qualitative observations. Only a small amount of studies quantitatively investigated such kind of parameters (Jordanova *et al*. 2011). Because changes in PMQ may be good biomarkers of anthropogenic impacts on ichthyofaunal health (Hinton *et al*., 1992; Wolke 1992; Thilakaratne, Mclaughlin and Marcogliese 2007; Balamarugan *et al*., 2012), in depth studies of the normal ranges and influences of endogenous factors are essential for each bioindicator species. Here we discovered that the amounts (relative and total volumes) of barbel spleen PMQ were positively correlated with age, which is in line with previous reports (Blazer *et al*., 1987; Agius and Roberts 2003; Iwanowitcz *et al*., 2012). Our data also show that sex differences exist in the amount of spleen PMQ. Contrarily, in some fish species sex differences were not noted (Blazer *et al*., 1987; Haaparanta *et al*., 1996; Figueiredo-Fernandes *et al*., 2006). In agreement with our data, sex differences were observed in some other fish species too (Brown and George 1985; Hampton, Lanz and Hinton 1989; Agius and Roberts 2003, Hinck *et al*., 2007; Krüger *et al*., 1996; Rocha, Monteiro and Pereira, 1997). However, in some of the later studies the highest amount of PMQ were found in females (Hampton, Lanz and Hinton 1989; Krüger *et al*., 1996; Rocha, Monteiro and Pereira 1997), a scenario contrary to ours, as the highest mean volumes of PMQ (per g of body weight) were in males. We thus consider that universal patterns do not exist and that when using PMQ as biomarkers species specificities must be taken into account.

We did not observe seasonal differences as to the relative volumes of PMQ, despite there were significant effect of seasons (looking at males and females combined) regarding total volumes, which tended to decrease in summer. Our data partially agree with those from other species, which showed more marked changes. For instance, seasonal and/or gonad maturation-related changes in PMQ were noted by Blazer *et al*. (1987) and Balamarugan *et al*. (2012). Moreover, our own studies in PMQ of *Salmo letnica*, using similar stereological methods, showed changes along the breeding cycle in liver and kidney (Jordanova, Miteva and Rocha 2008; Jordanova *et al*., 2011). Again, facts support that major interspecies differences exist. To realize the functional meaning of

these nuances we need assays integrating various methods and fish models with contrasting reproductive strategies.

In conclusion, PMQ are useful in toxicopathology as biomarkers of contamination (Manera *et al*., 2000; Fishelson and Becker 2001; Fournie *et al*., 2001; Balamarugan *et al*., 2012; Armero-Lituañas and Ocampo 2015). However, their use is questioned (e.g., Rabitto *et al*., 2005), taking into account non-toxicant factors that can influence PMQ. This study suggest that in the studied barbel, the age and sex, but not season, directly/indirectly impact on the amount of splenic PMQ. However, season has its impact too, especially in what regards the female spleen weight. Future biomonitoring with *Barbus peloponnesius* in River Bregalnica, and other freshwater systems, should consider the potentially confounding effects of the cited factors when interpreting the data. This need is surely extendable to other fish species and biomonitoring contexts.

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