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Liver melanomacrophage centres and CYP1A expression as response biomarkers to environmental pollution in European anchovy (*Engraulis encrasicolus*) from the western Mediterranean Sea.

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Abstract

The goal of the present study was to verify the suitability of using melanomacrophage centres (MMCs) as response biomarkers of marine pollution in European anchovy, which are short-lived, migratory, small pelagic fish. This suitability was verified by analysing the MMC density and cytochrome P450 monooxygenase 1A (CYP1A) expression in livers of anchovies from four areas of southern Italy. Age 2 anchovies sampled from three areas exposed to pollutants of industrial/agricultural origin (Gulf of Gela, Mazara del Vallo and Gulf of Naples) showed liver areas occupied by MMCs and numbers of MMCs that were significantly higher than those in the anchovies from Pozzallo, which is a marine area not subjected to any source of pollution. Anti-CYP1A immunoreactivity was observed in the hepatocytes of all specimens sampled from the Gulf of Gela. These findings suggest the utility of liver MMCs as biomarkers of exposure to pollutants in this small pelagic fish.

Keywords: small pelagic fish; fish liver; melanomacrophage centres; cytochrome P450 monooxygenase 1A; marine pollution.

1. Introduction

The European anchovy *Engraulis encrasicolus* (Linnaeus, 1758) is a short-lived small pelagic fish that is distributed worldwide (Crawford, 1987; Schwartzlose et al., 1999; Palomera et al., 2007). The European anchovy represents an important fishery resource for Mediterranean countries, accounting for approximately 30% of the total fish production (Lleonart and Maynou, 2003). The annual catches of European anchovy in the Mediterranean ranged between 200,000 and 700,000 t in the 20-year period from 1980-2000 (FAO, 2005), and the annual average landings reached almost 400,000 t in the 2010-2013 period (FAO, 2016).

The Mediterranean Sea is subjected to environmental degradation that has accelerated over the last few decades of the 20th century (EEA, 2008; Cinnirella et al., 2013), and the EU member states are required to develop tools to define qualitative descriptors of "Good Environmental Status" by 2020, including the monitoring of the contamination levels in habitats and fishes (EC, 2008).

The fish liver is a key organ that controls many vital functions and plays a prominent role in fish physiology, both in anabolism and catabolism, as well as in the metabolism of xenobiotics, and it is considered a good indicator of the health status of a fish (Bruslé and Anadon, 1996; Ghosh et al., 2001; Desantis et al., 2005; Cionna et al., 2006; Kirchhoff et al., 2011; Corriero et al., 2013; Passantino et al., 2014).

Cytochrome P450 monooxygenase 1A (CYP1A) is a subfamily of the cytochrome P450-dependent monooxygenase enzymes and plays an important role in the biotransformation of many xenobiotics including dioxins, furans, polychlorinated biphenyls, polyaromatic hydrocarbons (PAHs), and dichlorodiphenyltrichloroethane (DDT) (Stegeman, 1978; Goksøyr, 1985; Parkinson, 1995; Husøy et al., 1996; Jeong and Kim, 2002). As a consequence, the expression of CYP1A in the liver may increase following exposure to a variety of organic environmental pollutants (Stegeman, 1978; Goksøyr, 1985; Parkinson, 1995; Husøy et al., 1996; Jeong and Kim, 2002).

Melanomacrophage centres (MMCs) are aggregates of macrophage-like cells, which are fragments derived from phagocytosed cells and pigments such as melanin, haemosiderin, and lipofuscin (Roberts, 1975; Fournie et al., 2001; Agius and Roberts, 2003), and MMCs are located in the

reticuloendothelial tissue of haemolymphopoietic organs of various non-mammalian vertebrates (Wolke, 1992; Christiansen et al., 1996; Rund et al., 1998; Barni et al., 2002; Loumbourdis and Vogiatzis, 2002; Fournie et al., 2001; Koppang et al., 2005; Fishelson, 2006). MMCs play a role in the destruction, detoxification, and recycling of endogenous and exogenous materials, including dead cells and cell debris (Agius and Roberts, 1981; Van der Oost et al., 2003; Mela et al., 2007). An increase in MMC density has been observed along with increases in CYP450 expression (van der Weiden et al., 1994; Passantino et al., 2014) and apoptotic cell death (Gogal et al., 1999; Corriero et al., 2013) after fishes are exposed to toxic compounds; thus, MMCs are useful response (effect) biomarkers for different kinds of stress, including environmental pollutants (Agius, 1979; Agius and Roberts, 1981, 2003; Fishelson, 2006; Passantino et al., 2014).

The present study represents the first attempt to verify the suitability of using MMCs as response biomarkers to marine pollution in a short-lived, migratory, small pelagic fish, by analysing MMCs and CYP1A expression in the livers of individuals sampled from four geographical areas of south Italy, and three of these areas are potentially affected by strong pollutant inflows.

2. Materials and methods

2.1. Sampling site selection

European anchovies were sampled in the marine waters off three villages of the southern coast of Sicily: Pozzallo (*PZ; S1*), Gulf of Gela (*GL; S2*) and Mazara del Vallo (*MZ; S3)*, and in the Gulf of Naples in the South Tyrrhenian Sea (*NA*; S4) (Fig. 1). All samples were caught during experimental surveys carried out between 50 and 100 m depth (see the next subsection for further details on fish sampling). The sampling areas were selected by considering that the area around *PZ* is devoid of industrial plants and has limited agriculture activities; hence, this sampling site was selected as a potential negative control area. The other three sampling sites were selected due to their known potential exposure to pollutants of industrial and/or agricultural origin. The Gulf of Gela, the marine area off Mazara del Vallo, and the Gulf of Naples are affected by intense inflows of chemical

pollutants of agricultural origin since they receive effluents from zones identified as “nitrate vulnerable zones” according to the Council Directive 75/440/EEC (EEC, 1975), which rules on the threshold levels of nitrate concentrations within water bodies (Fig. 1). In addition to the agricultural impacts, the Gulf of Gela receives industrial pollutants that originate from industrial and petrol extraction activities (King, 2015), and the Gulf of Naples is known as one of the most contaminated areas along the southern Tyrrhenian Sea due to anthropogenic activities (Naso et al., 2005; Tornero and Ribera d’Alcalà, 2014).

2.2. Sample collection

Sampling was conducted in 2015 within the framework of two combined small pelagic fish abundance evaluation surveys in the framework of the Mediterranean International Acoustic Surveys (MEDIAS). Catches were collected by means of an experimental mid-water pelagic trawl (vertical opening of 8 m, cod-end mesh size of 18 mm), operating at 4.0 knots. A total of 90 individuals were collected from the four selected subareas. For each fish, total length (TL, to the nearest mm) and total weight (TW, to the nearest 0.1 g) were measured. Fish ages were estimated using an age-length key available in the literature for this species (Basilone et al., 2004). Each fish was dissected immediately after death, the sex was identified by visual inspection of the gonads, and the entire liver was excised and fixed in 10% buffered formalin for subsequent histological preparation and image analysis.

2.3. *Histology, histochemistry, and immunohistochemistry*

The entire livers (small organs ranging in weight between 0.06 and 0.25 g) were embedded in paraffin wax after dehydration in increasing ethanol concentrations and clarification in xylene using a fully automated sample tissue processing system (Leica, TP1020). Five to ten sections that were 4 µm thick were cut with an automatic precision rotary microtome (Leica RM2255) and stained with haematoxylin-eosin (H&E) for the description of the liver structure. Cytochemical peroxidase (Perox) (Sigma Diagnostics, St. Louis, MO, USA) staining was used to detect lysosomal enzymes contained in macrophages. Moreover, Mallory (Merck, Darmstadt, Germany) and Perls-Van Gieson (Bio-

Optica, Milan, Italy) stainings were performed to identify lipofuscin/ceroids and ferric iron, respectively.

For the immunohistochemical detection of CYP1A, liver sections were deparaffinized in xylene, rehydrated through graded ethanol solutions, pretreated for 10 min with 3% H_2O_2 to inhibit endogenous peroxidase activity, and then rinsed with distilled water and phosphate-buffered saline (PBS, 0.01 M, pH 7.4, containing 0.15 M NaCl). Non-specific binding sites for immunoglobulins were blocked by incubation for 30 min in normal horse serum (NHS), and sections were then incubated for 60 min at 37 °C with polyclonal anti-fish CYP1A peptide (Biosense Laboratories, Bergen, Norway) diluted 1:500 in PBS containing 0.1% bovine serum albumin (BSA). After rinsing for 10 min in PBS, immuno-histochemical visualization was obtained using the Vectastain Universal Elite Kit (Vector, Burlingame, CA). This method uses the avidin-biotin-peroxidase complex (ABC) procedure. Peroxidase activity was visualized by incubating for 10 min with the Vector DAB Peroxidase Substrate Kit (Vector, Burlingame, CA), which produces a brown precipitate. Nuclear counterstaining was obtained by a quick section treatment (20 s) with a ready-to-use solution of haematoxylin (Vector, Burlingame, CA). To confirm the specificity of the immunoreaction, control-staining procedures were carried out by replacing the primary antibody with NHS and PBS. Liver visual fields of H&E-stained sections were photographed at 40X magnification with a digital camera (DFC 425, Leica.) connected to an optical microscope (DM2500, Leica). The relative surface area occupied by MMCs ($\mu m^2/mm^2$ hepatic parenchyma), their number/mm^2 hepatic parenchyma, and mean area (μm^2) were measured using the Leica Application Suite (LAS) image analysis software.

2.4. Statistical analyses

The average liver area and number of MMCs did not show normal distributions (assessed through the Shapiro-Wilk *W* test). Differences in MMC areas and numbers between sexes were assessed for each sampling area by Mann-Whitney *U* test and were not statistically significant ($P > 0.1$); hence, the MMC areas and numbers of males and females were pooled for the subsequent statistical analyses. Differences in the liver areas occupied by MMCs, as well as in MMC numbers and sizes (mean

individual MCC area), were assessed by means of a Kruskal-Wallis ANOVA test for non-parametric comparisons of multiple independent groups. The liver areas occupied by MMCs and the numbers of MMCs in the samples from *GL*, *MZ*, and *NA* were then compared with samples of the same age class from *PZ*, which was considered a control area because of the absence of any known contamination source, using the Mann-Whitney *U* test. Statistical analyses were performed by Statistica software, version 7 (StatSoft, USA), and statistical significance was identified at $P \leq 0.05$.

3. Results

The sampled European anchovies were one or two years old. Individual TL, TW and estimated age are provided in the supporting data file for each fish.

3.1. Histology, histochemistry, and immunohistochemistry

The histological appearance of anchovy liver is shown in Fig. 2. Liver parenchyma appeared to be arranged according to a heterogeneous architecture: most of the hepatocytes were arranged as cords surrounded by a network of sinusoids. Hepatocyte cords were sometimes constituted by a single cell layer and sometimes by two or more cell layers (Fig. 2a). Occasionally, several layers of hepatocytes seemed to give rise to spherical lobules surrounded by a portal vein branch and containing a vein in a more or less central position (centre-lobular vein) (Fig. 2b). Hepatic artery branches were difficult to distinguish from veins due to their very thin walls. Bile ducts were either associated with a portal vein branch (Fig. 2c) or isolated (Fig. 2d). Hepatocytes were polygonal in shape and showed one or two spherical nuclei. In most cases, the hepatocyte cytoplasm was homogenous and weakly basophilic (Fig. 2a, b); the hepatocytes of some samples showed conspicuous fat content, which was represented by one to a few lipid droplets that sometimes occupied the majority of the cell volume (Fig. 2c, d).

Aggregates of cells and pigments that constituted the MMCs were visible in the H&E sections (Fig. 2c). These aggregates were positive to Perox staining (Fig. 3a) and contained lipofuscin-ceroids

and ferric iron as demonstrated by Mallory (Fig. 3b) and Perls-Van Gieson stainings (Fig. 3c), respectively.

A strong and diffuse anti-CYP1A immunoreactivity was observed in the hepatocyte cytoplasm of all examined sections of specimens sampled in *GL* (Fig. 4a). Negative to faintly visible immunostaining was observed in hepatocytes of the anchovies sampled in *PZ*, *MZ*, and *NA* (4b).

3.2. Quantitative analyses of melanomacrophage centres

The results of the quantitative analysis of the MMCs are reported in Table 1. Significant differences in the liver area occupied by MMCs as well as in the number of MMCs, but not in the size (mean area) of MMCs, were found when the Kruskal-Wallis ANOVA test was applied using both the sampling area ($H_{3\text{-}89} = 22.5$, $P < 0.001$; $H_{3\text{-}89} = 25.8$, $P < 0.001$; and $H_{3\text{-}89} = 3.1$, $P = 0.37$, for MMCs area, number and size, respectively) and the age combined with sampling area as independent variables ($H_{7\text{-}89} = 27.7$, $P < 0.001$; $H_{7\text{-}89} = 29.6$, $P < 0.001$; and $H_{7\text{-}89} = 6.8$, $P = 0.45$, for MCC area, number, and size, respectively). No significant difference was found using the age as the independent variable ($H_{1\text{-}89} = 0.5$, $P = 0.45$; $H_{1\text{-}89} = 0.4$, $P = 0.52$; and $H_{1\text{-}89} = 0.2$, $P = 0.68$, for MMCs area, number, and size, respectively).

In *PZ*, the area occupied by MMCs showed a significant decrease from age 1 to age 2, whereas in all other examined sampling sites, the liver area occupied by MMCs increased, although not significantly (Mann-Whitney U test, $P > 0.05$) from age 1 to age 2. No difference in the area occupied by MMCs was found in age 1 fish from the different areas investigated (Mann-Whitney U test, $P > 0.05$). However, the area occupied by the MMCs in age 2 anchovies was significantly higher in *GL*, *MZ*, and *NA* compared to *PZ* (Mann-Whitney U test, $P < 0.05$).

In age 1 specimens, the number of MMCs/mm^2 liver parenchyma was higher in all the investigated areas compared to *PZ*, although the difference was statistically significant in only *NA* (Mann-Whitney U test, $P < 0.05$). The number of MMCs/mm^2 liver parenchyma of age 2 anchovies was significantly higher in all investigated areas compared to that of *PZ* (Mann-Whitney U test, $P < 0.01$).

4. Discussion

The European anchovy liver showed the classical liver architecture with parenchyma arrangement corresponding to the cord and tubular forms described by Akiyoshi and Inoue (2004), i.e., hepatocytes arranged in a single (cord-like form) or a double (tubular form) layer among sinusoids. This finding is in agreement with the liver structure described in other Clupeiformes such as *Etrumeus teres*, *Sardinella zunasi*, *Konosirus punctatus*, and *Engraulis japonicus* (Akiyoshi and Inoue, 2004). However, a third type of arrangement of the hepatic parenchyma was observed: a multilayered hepatocyte lining consisting of rounded lobule-like structures with a central (hepatic) vein surrounded by a portal vein branch (Fig. 2). To the best of our knowledge, this arrangement of liver parenchyma that was occasionally observed in the examined sections and resembles the mammalian lobular organization has never been described in teleost fish.

The histochemical analyses of European anchovy liver allowed for the identification of typical free as well as clustered macrophages, which are often located near blood vessels. The data reported in the present study confirm the information available regarding fish MMCs, which showed a variable morphological appearance and staining affinity. The histochemical analyses performed in the present study demonstrated the presence of a) lipofuscin/ceroid pigments, which are consecutive outputs of the same oxidative polymerization of polyunsaturated fatty acids and may accumulate in fish displaying a wide variety of pathological conditions, including nutritional deficiencies, bacterial and viral diseases, and disturbances caused by toxins (Agius, 1979); b) haemosiderin, which is an iron-storage complex whose accumulation can be indicative of toxic haemolysis (Pacheco and Santos, 2002); c) ferric iron that, along with haemosiderin, is considered as a residue of the catabolism of several compounds (Leknes, 2004; Ribeiro et al., 2011).

MMCs are involved in the destruction of endogenous and exogenous material (including dead cells and cell debris) and are associated with apoptosis (Gogal et al., 1999). In the rainbow trout *Oncorhynchus mykiss* (Walbaum), the association of MMCs to the exposure to different kinds of stress was proposed (Passantino et al., 2002a, 2002b, 2004). An increased density of MMCs in the

liver and CYP1A expression (Passantino et al., 2014), as well as hepatocyte apoptosis and tumour necrosis factor expression (Corriero et al., 2013), were observed in juvenile Atlantic bluefin tuna reared in captivity in the North Adriatic Sea, and these conditions were associated with the exposure to persistent organic pollutants and heavy metals. Thus, fish exposure to environmental pollutants may be responsible for an increase in both CYP1A expression and MMC density. In particular, after exposure to toxic compounds, increased CYP1A expression may occur following the increased need for detoxification activity, whereas an augmented MMC density may result from tissue damage, since macrophages, once their phagocytic activity towards damaged cells, dead cells and cell debris is accomplished, aggregate in MMCs.

In the present study, age 1 European anchovies collected in three different geographical areas off the southern Sicilian coasts and in the Gulf of Naples showed similar liver areas occupied by MMCs. Age 2 European anchovy specimens captured in *PZ*, which is a marine area that is less subjected to anthropogenic sources of contamination, showed a significantly lower MMC density compared with age 1 specimens sampled from the same area. The explanation for this finding is uncertain and can be ascribed to the migration of the examined fish to cleaner waters after the first year of life, thus testifying the ability of the anchovy to recover from the tissue damages caused by the exposure to environmental pollutants. In contrast, liver MMC density showed an increase (although not statistically significant) from age 1 to age 2 in the three other investigated areas. Furthermore, age 2 specimens sampled in the three contaminated areas had significantly higher areas occupied by MMCs compared to the individuals of the same age caught in *PZ*. This result suggests that the exposure to various sources of pollution, either of agricultural (*MZ*, *GL*, *NA*) or industrial (*GL*, *NA*) origin, resulted in an increase in MMC density. The observed increase in the liver area occupied by MMCs was due to an increase in the number of MMCs (i.e., development of new MMCs by age 2; see Table 1) rather than to an increase in the sizes of MMCs from age 1 to age 2. The highest amount of MMCs (both in terms of area and number) was shown by fish sampled in *GL* (both ages 1 and 2), whose livers also showed strong expressions of CYP1A, which is a well-established response biomarker of environmental pollutants.

Despite the economic relevance of the European anchovy and its intense fishery exploitation in the Mediterranean Sea, its population structure, dynamics, and connectivity are far from being clarified. In some marine fish species such as herring (MacQuinn, 1997) sardine (Carrera and Porteiro, 2003), and anchovy (Ribeiro et al., 2011), a new perspective of metapopulations has been proposed to explain population dynamics. The metapopulation concept was introduced by Andrewartha and Birch (1954) and further refined by Kritzer and Sale (2004), suggesting that a metapopulation is a system of discrete local populations, each showing a certain degree of mixing with other local populations.

Stock discrimination studies in the Strait of Sicily were carried out by amino-acid composition analysis, and these results suggested that the European anchovy is structured in 'local subpopulations' forming a metapopulation (Basilone et al., 2004; Ribeiro et al., 2011). A necessary condition for the persistence of a metapopulation in the face of unstable subpopulations is asynchronous local dynamics (Hanski, 1999), which means that subpopulations may experience different environmental conditions; therefore, factors that structure habitat suitability may vary among subpopulations (Ribeiro et al., 2011). Another stock discrimination study based on morphometric data showed that anchovy sampled in *PZ* were clustered apart from two other northwestern locations along the south Sicilian coast (Sciacca and Agrigento; which are located between two sampling locations of the present study, *PZ* and *GL*) (Traina et al., 2011).

The samples used for the present study were collected in July and August, during the peak of the spawning period of the species (Basilone et al., 2006) in one of the main spawning areas (Basilone et al., 2013). In fact, almost all sampled European anchovies (both age 1 and age 2 specimens) were in spawning condition, displaying ovaries with hydrated oocytes and postovulatory follicles (data not provided). European anchovies are partial spawners with a 30% daily spawning frequency (Basilone et al., 2015) from April to September (Zupa et al., 2013; Basilone et al., 2006). Although no definitive data have been reported regarding spawning site fidelity in this species, it is reasonable to assume that European anchovies spend the entire reproductive season in a restricted spawning area. If this assumption is correct, the fish sampled in the present study remained in the area for at least three

295 months before being captured; hence, the semi-quantitative analysis of MMCs, as well as the CYP1A
296 liver expression, could be affected by the exposure to environmental pollutants during their time in
297 the spawning area. This condition would explain both the higher amounts of MMCs as well as the
298 strong immunopositivity to CYP1A in the specimens sampled from *GL*, which receives effluents
299 from the main industrial site for the production of chemical and organic waste in Sicily, which is one
300 of the main industrial poles in Italy. Over the last decade, several studies have demonstrated the severe
301 state of environmental degradation off the southeastern Sicilian coast (Di Leonardo et al., 2007;
302 Sprovieri et al., 2011; Salvagio Manta et al., 2016). Specifically, the intense activity of the
303 petrochemical plant of Gela causes extremely high levels of toxic, persistent, and bio-accumulating
304 chemical pollutants in different environmental compartments (Musumeci et al., 2009), as well as an
305 excess of congenital anomalies in people the Municipality of Gela (Bianchi et al., 2014).
306 Although the effective impacts of the petrochemical plant of Gela on the fishery resources have
307 been scarcely investigated, recent studies reported high levels of mercury in fishes collected from the
308 east coast Augusta Bay (Ausili et al., 2008; Bonsignore et al., 2013) and warned of the potential risk
309 to the health of the local human populations associated with fish consumption (Bonsignore et al.,
310 2015, 2016).
311 In conclusion, the present study provides a description of the European anchovy liver and proposes
312 the use of liver melanomacrophage centres as biomarkers of exposure to environmental pollutants in
313 this small pelagic fish species. Among the investigated parameters, in addition to CYP1A liver
314 expression, liver MMC area and number (but not mean area of MMCs) appear to be reliable
315 descriptors of exposure history to environmental pollutants.
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521

Figure Captions

Fig. 1. Geographical locations of European anchovy sampling areas. Zones vulnerable to pollution caused by nitrates of agricultural origin are shown in pink (upper inset) and red (lower inset). The nitrate vulnerability maps were extracted and modified from the "Carta Regionale delle zone vulnerabili da nitrati di origine agricola – Note esplicative" document (http://www.psrsicilia.it/Allegati/CartaNitrati/Note_CartaNitrati.pdf; accessed on 21/06/2017) (upper inset) and by the institutional website of the Campania Region (http://www.agricoltura.regione.campania.it/nitrati/zone-vulnerabili.htm; accessed on 21/06/2017) (lower inset). The sampling areas are waters off Mazara del Vallo (S1), Gulf of Gela (S2), waters off Pozzallo (S3), and Gulf of Naples (S4).

Fig. 2. Micrographs of European anchovy liver sampled in Pozzallo (*PZ).* a), b) General appearance of the liver parenchyma; c) bile duct in close proximity to a portal vessel and a melanomacrophage centre: note the high lipid contents of the hepatocytes. d) Isolated bile duct (not associated with blood vessels). Haematoxylin-Eosin staining. Scale bars = 100 µm in a) and b) and 50 µm in c) and d). Arrow, hepatic vessel; arrowhead, sinusoid; asterisk, bile duct; dashed arrow, melanomacrophage centre; double asterisk, portal vessel.

Fig. 3. Melanomacrophage centres in the liver of European anchovies sampled in Pozzallo (*PZ).* a) Peroxidase (Perox) staining. b) Mallory's trichrome staining (ferric iron ions in red). c) Perls-Van Gieson staining (lipofuscin-ceroids in light blue). Scale bars = 50 µm. Arrowhead, melanomacrophage centre.

Fig. 4. Liver sections from European anchovies immunostained with anti-CYP1A antibodies. a) Strong immunopositivity in the hepatocyte cytoplasm of a specimen sampled in the Gulf of Gela (GL). b) Faintly visible immunoreaction in a specimen sampled in Pozzallo (PZ). Scale bars = 25 µm.