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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.

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 to 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;

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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 40× magnification with a digital camera (DFC 425, Leica.) connected to an optical microscope (DM2500, Leica). The relative surface area occupied by MMCs (μm^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.

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