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Digestive enzymatic patterns as possible biomarkers of endocrine disruption in the red mullet (*Mullus barbatus*): A preliminary investigation

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ABSTRACT

During two seasonal trawl surveys (April and October, 2012), red mullet specimens were caught from two sites of the northern Sicilian coast (Western Mediterranean), characterized by different degrees of pollution, to assess whether their digestive enzymes could be cost-effective diagnostic tools for endocrine disruption. Pepsin, chymotrypsin, carboxypeptidases A and B, amylase and lipase were measured in the digestive tract of each fish. During both samplings, significant differences in the digestive enzymatic patterns of fish collected from the two sites were found. In April, pepsin and lipase contents were significantly lower in fish from the most impacted site than in those from the reference site. In October, the enzymatic patterns showed trends different from spring, with controversial results for carboxypeptidases A and B and amylase. Pepsin and lipase patterns suggest a detrimental effect played by organic pollutants and the use of these enzymes as possible biomarkers of exposure to endocrine disruptors.

1. Introduction

Endocrine active substances (EAS), also known as Endocrine Disruptors (EDs), are injurious to predatory birds and fish (Bascietto et al., 1990). Therefore, there is a legitimate concern that low doses of these chemicals in daily diets may be currently impacting human health, causing endocrine disruption (Rivas et al., 2001) and detrimental effects on neurodevelopment (Colborn, 2004). In fish, the biological effects of EDs are generally evaluated through the use of a variety of molecular, biochemical and histological biomarkers, among which some liver and gonadal enzymatic activities (e.g., mixed function oxygenase system, ovarian aromatase) are most common. Several studies have reported on the effects of organic pollutants of different chemical nature on the activity of digestive enzymes in fish (see Filippov et al., 2013 for a review); a decrease has generally been observed in digestive functionality after exposure to contaminants during "in vitro" experiments simulating chronic pollution, suggesting that the activity of digestive enzymes could be used as potential biomarkers in aquatic toxicology. However, field studies supporting this hypothesis are lacking. To assess whether and at what extent digestive enzymes respond to environmental contamination in the natural environment, a preliminary study has been performed on red mullet (*Mullus barbatus*, Osteichtyes, Perciformes), in the framework of the coordinated research project "Food and environmental safety: the problem of endocrine disruptors", funded by the Italian Ministry of Health. The red mullet is a marine fish commonly found on gravel, sand and mud bottoms at a depth range of 10–270 m (Lombarte et al., 2000), which is recommended as a sentinel species in environmental monitoring programmes (UNEP/RAMOGE, 1999; Lionetto et al., 2003; Martin-Skilton et al., 2006; Zorita et al., 2008; Law et al., 2010), because of wide geographical distribution, non-migratory behaviour, and feeding habits. In fact, it feeds mainly on organisms living in close association with sediments, where most contaminants accumulate, which favours xenobiotic accumulation (Regoli et al., 2002; Esposito et al., 2014). The individuals of red mullet analysed in the present research were caught from two sites at different anthropogenic impact in western Mediterranean, one of which (Milazzo) has been recognized as a pollution hot spot by the Strategic Action Programme (SAP) of UNEP (UNEP/WHO, 2003; EEA, 2006), being subjected to contamination by polycyclic aromatic hydrocarbons (PAHs), heavy metals, and organochlorinated compounds (Caruso et al., 2004; Yakimov et al., 2005; ARPA Sicilia, 2008; Fasulo et al., 2010). This study reports preliminary data on a set of enzymatic activities measured in the gastrointestinal tract of red mullet, which suggest altered digestive capacity in fish from the most impacted site.

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2. Materials and methods

2.1. Sample collection and treatment

Specimens of red mullet were captured during two seasonal trawl surveys (April and October, 2012), from two different sites along the northern Sicilian coast (Western Mediterranean), characterized by different degrees of pollution: the first site (thereafter indicated as Impact site) was located in a dense shipping traffic area, close to city and harbour of Milazzo, in front of an oil refinery and a thermal power plant; these anthropogenic factors have been demonstrated to affect significantly the marine environment of the harbour (Yakimov et al., 2005; Fasulo et al., 2010). The second site (thereafter indicated as Control site), was located in the Gulf of Patti, a leisure area interdicted to both industrial activity and commercial fishing (Fig. 1).

The whole digestive tract — including the chyme content — of each individual (n = 10 per site) was removed and then divided into different organs (stomach, pyloric caeca and intestine), which were stored at −20 °C until enzyme analysis. After homogenization in buffer 50 mM of Tris HCl pH 7.0 and centrifugation at 3000 rpm × 20 min at +4 °C, the supernatant obtained was used as crude enzymatic extract for measurements of the pepsin, chymotrypsin, carboxypeptidases A and B, amylase and lipase, according to the analytical procedures reported below.

2.2. Enzymatic activity assays

Peptic activity was measured using the method of Anson (1938) modified according to Rick (1974a). The reaction mixture, consisting of 0.5 ml of bovine hemoglobin (2% in 0.06 N HCl; pH 2.00) as the substrate and 0.1 ml of the enzyme extract, was incubated for 10 min at 35.5 °C, then the reaction was stopped by addition of 1.0 ml of 5% trichloroacetic acid followed by centrifugation at 3500 rpm for 10 min. A control tube consisted of the same reagents, but the enzyme extract was added after incubation. Absorbance of supernatant was measured at 280 nm and L-tyrosine was used for calibration. Pepsin activity was determined as 1 Unit = micrograms of tyrosine released per minute from the substrate.

Trypsin activity was determined using the method of Hummel (1959) modified by Rick (1974b), with p-toluenesulfonyl-L-arginine methyl ester (TAME) as the substrate. The reaction started with the addition of enzyme extract (0.05 ml) to a mixture of 0.15 ml of 10.0 mM TAME solution and 1.3 ml of 46.0 mM Tris buffer at pH 8.10 with 11.5 mM $CaCl_2$ buffer. The change in absorbance was measured at 247 nm during 3 min at 25.0 °C. Trypsin activity was reported as 1 Unit = 0.001 absorbance increase per minute.

Chymotrypsin activity was determined using the method of Hummel (1959) modified by Rick (1974c), with N-benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate. The reaction started with the addition of enzyme extract (0.05 ml) to a mixture of 0.70 ml of BTEE (80.0 mM in 50% methanol) and 0.75 ml of 80.0 mM Tris buffer at pH 7.80 with 0.1 M $CaCl_2$ buffer. The change in absorbance was measured at 256 nm during 3 min at 25.0 °C. Chymotrypsin activity was reported as 1 Unit = 0.001 absorbance increase per minute.

Carboxypeptidases A and B were measured using Hyppuryl-L-arginine and Hyppuril-L-phenylalanine as the substrates, respectively (Appel, 1974). For carboxypeptidase A, 0.15 ml of enzyme extract were mixed with 1.35 ml of 1.1 mM hyppuryl-L-phenyalalanine in 27.5 mM Tris buffer 0.11 M NaCl at pH 7.60. For carboxypeptidase B, 0.15 ml of enzyme extract were mixed with 1.35 ml of 1.1 mM hyppuryl-L-arginine in 27.5 mM Tris buffer 0.11 M NaCl at pH 7.60. The change in absorbance was measured at 254 nm during 3 min at 25.0 °C. Carboxypeptidases A and B were reported as 1 Unit = 0.001 absorbance increase per minute.

Amylase activity was determined using the method of Bernfeld (1955) modified by Rick (1974d). The reaction mixture, containing 0.05 ml of enzyme extract, 1.0 ml solution of soluble starch (1% in phosphate buffer pH 6.9) as the substrate and 1.0 ml of 20 mM phosphate buffer at pH 6.9 with 10 mM NaCl, was first incubated for 10 min at 25.0 °C. After addition of Dinitrosalicylate (DNS) reagent (2.0 ml of 1% DNS, 30% sodium potassium tartrate), the mixture was placed in boiling water for 5 min and cooled at room temperature for 30 min. Absorbance of the mixture was determined at 540 nm, using maltose for calibration. Alpha-amylase activity was reported as 1 Unit = 1 μg of maltose released per minute.

Lipase activity was determined using the method of Tietz and Fiereck (1966). The reaction mixture, containing 0.5 ml of enzyme extract, 5.0 ml of olive oil emulsion as the substrate (prepared with 0.2 g of sodium benzoate, 7.0 g of arabic gum, 100 ml of demineralised water and 100 ml of olive oil) and 1.25 ml of 0.2 M Tris buffer at pH 8.0, was incubated for 2 h at 37.0 °C. After addition of 1.5 ml of

Fig. 1. Sampling areas located in Sicily (Italy) along the Tyrrhenian coast: Milazzo (Latitude 38°12′73″N, Longitude 15°17′92″E Average Depth 31 m); Mongiove-Patti (Latitude 38°09′77″N, Longitude 15°00′03″E, Average Depth 53 m).