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Multivariate approach to gill pathology in European sea bass after experimental exposure to cadmium and terbuthylazine

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ABSTRACT

The combined use of guided quantitative expert analysis and of multivariate exploratory data analysis is reported as a robust, sensitive and sufficiently specific approach to study European sea bass gill secondary lamellar pathology after exposure to incremental doses of cadmium and terbuthylazine up to 48 h. The following elementary pathological findings were considered: "epithelial lifting", "epithelial shrinkage", "epithelial swelling", "pillar cells coarctation", "pillar cells detachment", "channels fusion", "chloride cells swelling", and "chloride cells invasion". The relative spatial extension was determined according to exposure class and data were analyzed by means of canonical correspondence analysis (CCA), linear discriminant analysis (LDA) and canonical variates analysis (CVA). Histologically and ultrastructurally, cellular shrinkage/coarctation prevailed in cadmium exposed lamellae, whereas cellular swelling and epithelial lifting were predominant in terbuthylazine exposed lamellae compared to unexposed fish. Both CCA and CVA permit a good graphical data grouping according to exposure classes by means of the convex hull minimum polygons. This also reveals exposure dose and time gradients in CCA plot. Accordingly, epithelial swelling and epithelial shrinkage were comparatively associated to higher exposure time, whereas epithelial shrinkage and pillar cells coarctation were comparatively associated to higher exposure dose. LDA with only "epithelial shrinkage", "epithelial swelling" and "pillar cells coarctation" in the model classified correctly 87.5% of the cross-validated cases. A possible pathogenetic relationship between the discriminant elementary lesions and the toxic mode of action at the cellular level of both cadmium and terbuthylazine is also discussed.

1. Introduction

Fish gills are the primary site of gas exchange, osmoregulation, acid-base regulation, and excretion (Evans, 1987, 2005; Brauner and Rombough, 2012). The gills play a pivotal role in maintaining fish homeostasis and, being in direct and permanent contact with potential waterborne irritants, they account for practical biomarkers of aquatic pollution (Bernet et al., 1999). The complex structure of gills has been previously reported both in normal and in pathological conditions (Mallatt, 1985; Wilson and Laurent, 2002). The repertoire of gill responses to the multitude of pathogens (including toxicants) affecting their integrity is limited, and therefore should be treated as general biomarkers (Mallatt, 1985; Dezfuli et al., 2003, 2006; Giari et al., 2007; Gomes et al., 2012; Nascimento et al., 2012). Particular attention should be paid in avoiding both false positive (type I) and false negative (type II) errors, which can affect both the specificity and sensitivity of the adopted diagnostic technique (Mallatt, 1985; Manera et al., 2016a). With regard to histopathology, pitfalls may arise throughout the entire diagnostic process, from tissue sampling to data analysis, according to the method (human mental algorithm vs. statistical discriminant approach) used to set the discriminant level between positive (i.e. "pathologic") and negative (i.e. "normal") samples. Importantly, from a biomedical perspective, false negative errors are more serious than false positive errors and should be adequately controlled by means of proper screening test sensitivity (Mallatt, 1985; Manera, 2013; Szczypinski et al., 2014; Manera et al., 2016a). Accordingly, the need of a guided and possibly morphometrically based assessment of fish gill pathology has been stressed, in order to avoid type II errors (false negative) (Mallatt, 1985; Manera et al., 2016a).

A quantitative guided screening technique has recently been developed and proposed as a reliable method to objectively characterize fish gill pathology with regard to toxicological trials (Manera et al., 2016a). It is anticipated that guided screening will become a powerful tool in environmental biomonitoring

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programs, ensuring standardization and reproducibility.

Effectively, fish gill condition is widely used in environmental studies and ecotoxicological trials, though gill lesions are normally only qualitatively or semi-quantitatively assessed (Mallatt, 1985; Pawert et al., 1998; Pandey et al., 2008; Gomes et al., 2012; Nascimento et al., 2012). Furthermore, little or no attempt has been made to categorize discriminant elementary pathological findings according to xenobiotics (Mallatt, 1985; Manera et al., 2016a). In particular, each histopathological pattern relies on the sum of many elementary pathological findings, some of which may be artifacts and/or may be associated with many pathological patterns, whereas others may be strictly related to few or, possibly, only one pathological pattern (Manera et al., 2016a; Colin et al., 2016).

In the present study the authors describe the combined use of semithin sections, of guided quantitative expert analysis, and of multivariate exploratory data analysis as a robust, sensitive and specific approach to study sea bass [*Dicentrarchus labrax* (Linnaeus, 1758)] gill lamellar pathology. The approach discriminates among exposure classes with respect to unexposed compared to cadmium and terbuthylazine experimentally exposed fish. The pathogenetic relationship between observed discriminant elementary lesions and the toxic mode of action at the cellular level is discussed.

Multivariate exploratory data analysis relies on pattern detecting and data structure exploration. Its objectives embrace the extraction of crucial data features and the finding of latent structural relationships. Summarization, visualization and description of biological pattern in the form of mathematical constructs are also performed (Podani, 2000). Multivariate exploratory data analysis is widely used in ecological and population genetic studies, whereas it is relatively neglected or partially applied in biomedical disciplines, with particular regard to pathology, which typically utilize uni- and bivariate statistical procedures. Multivariate analysis on the other hand, is an extension of these, relying on functional relationships between a dependent variable and many independent variables and allowing significance testing of statistical hypotheses (e.g. multivariate analysis of variance – MANOVA) (Cavalli-Sforza et al., 1994; Podani, 2000; Lepš and Šmilauer 2003). ter Braaka and Šmilauerb (2014) have recently stressed the use of constrained ordination methods, based on an ANOVA/regression approach, instead of unconstrained methods relying on the least-squares (eigenvector) methods.

In the present study, multivariate exploratory data analysis and, particularly, its graphic, intuitive data ordination/classification, was shown to be a robust, sensitive and specific approach to study fish gill lamellar pathology resulting from cadmium and terbuthylazine experimental exposure, to discriminate among exposure classes, by means of the identification of the best combination of discriminant elementary pathological findings.

Cadmium is a heavy metal widely used in industry (batteries, electroplating, plastic stabilizers, pigments). Its emission into the environment has dramatically increased during the 20th century, leading to contamination of aquatic systems (Zelikoff, 1993; Jarup, 2003), and ultimately having a harmful effect on fish. Cadmium accumulates mainly in kidney, liver and gills (Cattani et al., 1996) of fish, causing pathological changes in these tissues and organs (Lemaire-Gony and Lemaire, 1992; Battaglini et al., 1993; Thophon et al., 2003; Giari et al., 2007; Manera et al., 2016a, 2016b).

Terbuthylazine (2-chloro-4-tart-butylamino-6 ethylamino-s-triazine) is a relatively widespread triazine herbicide, a common substitute of the well-studied atrazine (Steinberg et al., 1994). Though its toxicity has been addressed by several studies in terrestrial animals (Lang et al., 1996, 1997; Lalminen et al., 1996), little is known with regard to its impact on aquatic organisms (Steinberg et al., 1994; Marchini et al., 1988; Szarek et al., 2000; Dezfuli et al., 2006). The molecular basis of toxicity of both cadmium and terbuthylazine thus certainly deserve further study (Manera et al., 2016a, 2016b). Regardless of any particular toxicant, fish gills have the greatest external host-to-water interface directly exposed to waterborne agents and thus are particularly susceptible to direct toxic effects (Giari et al., 2007; Manera et al., 2016a, 2016b).

2. Methods

The present study was carried out on semi-thin sections images taken from previous experimental trials (Dezfuli et al., 2006; Giari et al., 2007). Therefore, the experimental design is only briefly summarized below.

2.1. Experimental fish and acute exposure

Specimens of intensively reared *D. labrax* (mean total length = 124.4 mm; mean mass = 18.8 g, n = 45), previously acclimated for two weeks in 200 l aquaria containing 22‰ salt water at a mean temperature of 19.9 °C under a standard photoperiod 12 h daylight, were experimentally exposed to four incremental doses of Cd (standard solution for atomic absorbance spectrophotometry, Code 497471 Carlo Erba, Milan, Italy) 4.47 mg l^{-1} (0.0398 mM), 5.63 mg l^{-1} (0.0501 mM), 7.08 mg l^{-1} (0.0630 mM), 8.91 mg l^{-1} (0.0793 mM) and three incremental doses of terbuthylazine (TERB SC, Terranalisi s. r. l., Cento FE, Italy) 3.55 mg l^{-1} (0.0155 mM), 5.01 mg l^{-1} (0.0218 mM), 7.08 mg l^{-1} (0.0308 mM), in 20 l polycarbonate exposure tanks, up to 48 h. Fish were fed daily with commercial feed (crude protein 62%, crude fat 11%, fiber 0.8%, ash 10%, phosphorus 1.1%) and starved 48 h before and during the experiment. Unexposed, control fish remained in the acclimation tank. Fish were sampled from each experimental tank after 24 and 48 h post exposure, killed by a blow to the head, pithed and their gills were dissected and immediately fixed in 2% glutaraldehyde solution, buffered with 0.1 M sodium cacodylate pH 7.2 at 4 °C for 2 h. The study complied with Italian national guidelines governing the use of experimental animals and related procedures (Legislative Decree 116/1992, according to 86/609/EEC Directive). In particular, the experimental part was performed in the facilities of the Ferrara section of the Regional Environmental Protection Agency of Emilia-Romagna Region, as an institutional mission on behalf of the Italian Environmental Ministry. Actually, the gills and other organs of European sea bass were sampled from fish institutionally tested for acute and subacute toxicity against "priority substances", according to Italian Legislative Decree 106/1999 (Belli et al., 2003).

2.2. Tissue processing and histological observation

After glutaraldehyde fixation tissue was post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate at pH 7.2 for 2 h, dehydrated in a graded series of ethanol, transferred to propylene oxide and embedded in an Epon-Araldite mixture. Semithin sections (1.5 μm) were cut with a Reichert Om U2 (Reichert Optische Werke A.G., Wien, Austria) ultramicrotome with glass knives and stained with toluidine blue. Semithin tissue section were observed and photographed with a microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan) equipped with a digital color camera (DS-5M; Nikon, Tokyo, Japan) manually set to ensure the same exposure parameters, light intensity, and white balancing. Selected images were saved in TIFF (Tagged Image File Format; RGB-Red, Green, Blue method) uncompressed file format.

In order to characterize the best gill lesions, ultrastructural observation was performed on selected resin embedded samples. Ultrathin sections were contrasted in a 50% alcohol-uranyl acetate solution and lead citrate, and examined in a Hitachi H-800 (Hitachi