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 hours. 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 analysed 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.

Keywords: canonical correspondence analysis; linear discriminant analysis; guided quantitative expert analysis; toxicologic pathology; *Dicentrarchus labrax*.

1. **INTRODUCTION**

Fish gills are the primary site of gas exchange, osmoregulation, acid-base regulation, and excretion (Evans 1987; Evans *et al.* 2005; Brauner & 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 & 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; Dezfuli *et al.* 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.*2015). 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.*2015).

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 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.*2015). 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.*2015; 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 bio-medical 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 & Šmilauer 2003). ter Braaka & 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 & Lemaire, 1992; Battaglini et al., 1993; Thophon et al., 2003; Giari et al., 2007; Manera et al., 2016a-b).

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; Salminen 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-b). 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-b).

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%, fibre 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**

144 After glutaraldehyde fixation tissue was post-fixed in 1% osmium tetroxide in 0.1 M sodium
145 cacodylate at pH 7.2 for 2 h, dehydrated in a graded series of ethanol, transferred to propylene oxide
146 and embedded in an Epon-Araldite mixture. Semithin sections (1.5 μm) were cut with a Reichert
147 Om U2 (Reichert Optische Werke A.G., Wien, Austria) ultramicrotome with glass knives and
148 stained with toluidine blue. Semithin tissue section were observed and photographed with a
149 microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan) equipped with a digital color camera (DS-
150 5M; Nikon, Tokyo, Japan) manually set to ensure the same exposure parameters, light intensity, and
151 white balancing. Selected images were saved in TIFF (Tagged Image File Format; RGB-Red,
152 Green, Blue method) uncompressed file format.
153 In order to characterize the best gill lesions, ultrastructural observation was performed on selected
154 resin embedded samples. Ultrathin sections were contrasted in a 50% alcohol-uranyl acetate
155 solution and lead citrate, and examined in a Hitachi H-800 (Hitachi Ltd., Tokyo, Japan) electron
156 microscope operated at 80 kV. Detailed results of microscopic and ultrastructural patterns have been
157 previously reported elsewhere (Dezfuli *et al.* 2006; Giari *et al.* 2007).
158 2.3. **Guided expert analysis**
159 Representative histopathological patterns at the level of the secondary lamellae were selected,
160 images acquired, and the TIFF images submitted, with no indication of exposure group, to a trained
161 fish pathologist. He/she quantitatively described lesions according to a precompiled table, and to
162 the relative spatial occurrence of some selected elementary pathological findings, in accordance
163 with previous studies (Mallatt 1985; Dezfuli *et al.* 2006; Giari *et al.*2007; Wolf *et al.* 2015) and a
164 previously developed categorization (Manera et al. 2016a). Table pathological entries were:
165 “epithelial lifting”= detachment and lifting of the epithelial cells lining the secondary lamella due to
166 fluid accumulation beneath them; “epithelial shrinkage”= shrinkage/curling of epithelial cells due to
167 the acute coagulation of cellular protoplasm as a result of the exposure to denaturant toxicants;
168 “epithelial swelling”= degenerative swelling/enlargement of epithelial cells due to the failure of
169 membrane-associated ionic pumps; “pillar cells coarctation”= active or passive contraction

(shrinkage) of pillar cells, resulting in the reduction of the lumen of the blood channel/sinuses; "pillar cells detachment"= detachment of the cellular body of pillar cells from the basement membrane, which separate them from the overlying epithelial cells; "channels fusion"= blood channels/sinuses fusion caused by pillar cells rarefaction; "chloride cells swelling"= degenerative swelling/enlargement of chloride cells due to the failure of membrane-associated ionic pumps; "chloride cells invasion"= hyperplastic chloride cells response resulting in the occurrence of the latter on the secondary lamella. The relative spatial extension was determined for each of the above lesions using Image J (v1.50b; Rasband W., National Institute of Health, USA) and expressed as cover classes (percentages referred to overall tissue area): 0, absent; 1, 0< cover < 0.1 %; 2, 0.1 % ≤ cover < 1 %; 3, 1 % ≤ cover < 10 %; 4, 10 % ≤ cover < 50 %; 5, cover ≥ 50 %. Therefore, expert analysis should be considered effectively as a "guided quantitative expert analysis" as previously proposed by Manera *et al.*(2015).

2.4. **Multivariate exploratory data analysis**

Canonical Correspondence Analysis (CCA) was performed on the obtained data. Cover classes of each pathological finding were introduced as main (primary) matrix data, exposure time (in hours) and exposure dose (as mM), the latter two log-transformed [log (n+1)], as secondary, environmental matrix. Exposure class data were introduced as categorical variable. The null hypothesis of no structure in the main matrix (elementary pathological findings) and therefore non relationship between matrices [namely main matrix and secondary matrix (i.e. exposure dose and time)] was specifically tested. Accordingly, the correlations between each histopathological scoring and exposure dose and time was tested by means of Monte Carlo test. PC-ORD was used as multivariate analysis software. Furthermore, linear discriminant analysis was performed on numerical data in order to evaluate the discriminant power of elementary pathological findings in exposure class detection. In particular, stepwise analysis, Mahalanobis distance and both pooled covariance matrix and separate covariance matrices were adopted in the analysis as previously reported (Manera *et*

195 *al.*2015). ANOVA has also been employed to test significant differences among exposure classes.
196 SPSS® 14.0.2 (SPSS Inc., Chicago, IL, USA) was the statistical package for data analysis.
197
198 3. **RESULTS**
199 Figures 1b and c show the pathology of the secondary lamellae resulting, respectively, from
200 cadmium and terbuthylazine exposure compared with unexposed lamellae (Fig. 1a). In general,
201 cellular shrinkage/coarctation predominated in cadmium exposed lamellae. Cellular swelling and
202 epithelial lifting were both widespread in terbuthylazine exposed lamellae, and mixed patterns were
203 common. The ultrastructure of a cadmium exposed secondary lamellae is shown in Figure 1d and is
204 a representative example of the elementary pathological findings recorded during the study.
205 The CCA triplot is shown in Figure 2. Elementary pathological findings points (centroids) are
206 reported as asterisks. The closer a single case is to a centroid, the greater the probability that
207 lamellae displayed the related elementary pathological finding. An approximate ordering of each
208 elementary pathological finding with respect to exposure dose and time, can be obtained by
209 projecting centroids onto the respective vector. The same holds true for each sampling point.
210 Accordingly, epithelial swelling and epithelial shrinkage were comparatively associated to higher
211 exposure time than the other elementary pathological findings, whereas epithelial shrinkage and
212 pillar cells coarctation were comparatively associated to higher exposure dose than the others.
213 Sampling to exposure parameters (species to environment) correlation was 0.663 (randomized
214 0.516; p= 0.060) for Axis 1 and 0.632 (randomized 0.344; p= 0.006) for Axis 2. Considering the
215 correlation of the main matrix variables with the ordination axes, “epithelial swelling” shows the
216 highest R^2 (0.705, with Axis 1), followed by “epithelial shrinkage” (0.689, with Axis 2) and “pillar
217 cells coarctation” (0.519, with Axis 2). With regard to the correlation of the second matrix variables
218 with the ordination axes, both experimental parameters show the best correlation with Axis 2
219 (exposure dose and time, respectively R^2: 0.378 and 0.369), though exposure time correlates also
220 with Axis 1 (R^2: 0.281). Graphically, the convex hull polygons for each exposure class are

adequately separated according to exposure class, although partial superimposition appears between cadmium and terbuthylazine exposed samples. In particular, cases n. 5, 12, 16 are enclosed in the convex hull polygon of the terbuthylazine exposed cases. Interestingly, and with particular regard to case 12 and 16, such imperfect ordination result (in term of exposure class separation) corresponds to relatively lower exposure doses.

The classification table (Table 1), presents correct classification percent, test sensitivity and specificity (according to linear discriminant, stepwise analysis – Mahalanobis distance) with the following elementary pathological features retained in the model: “epithelial shrinkage”, “epithelial swelling” and “pillar cells coarctation”. As previously reported these features showed the highest R^2 with the ordination axes and are the most distant centroids with respect to the convex hull polygon of unexposed samples. Case number 22 appears misclassified both with original and with cross-validated cases. Specifically, it is misclassified as a terbuthylazine exposed case, resulting in a false positive, type I error. Furthermore, case number 14, with original cases, and cases number 14 and 17 (terbuthylazine exposed), with cross-validated cases, are misclassified as unexposed, resulting in false negative, type II errors. When only an exposure category is considered (unexposed vs. both cadmium and terbuthylazine exposed) only one false positive case – number 22 – is detected (95.8 % of both original and cross-validated cases are correctly classified), yielding 100 % sensitivity and 75 % specificity, and only “epithelial shrinkage” is retained in this stepwise analysis. Interestingly, cadmium exposed cases are never misclassified either as unexposed or terbuthylazine exposed by means of the linear discriminant, stepwise analysis – Mahalanobis distance.

Figure 3 displays the canonical discriminant function plot with all the discriminant variables in the model. Convex hull polygons are clearly separated and the discriminant variables are reported as vectors. The total length of the vectors accounts for the related discriminant power and their orthogonal projection with respect to the ordination axis for the related contribution to ordination. Interestingly, “epithelial shrinkage” appeared to be the most discriminant pathological feature and it lays exactly opposite to the unexposed convex hull polygon and its major axis, and, as a vector,

247 approximately equally orthogonally projected with respect to the two ordination axes. It is followed
248 first by “pillar cells coarctation”, mainly orthogonally projected with respect to Axis 1, along the
249 major axis of the convex hull polygon of cadmium exposed cases, and then by “epithelial swelling”,
250 mainly orthogonally projected with respect to Axis 2, along the minor axis of the convex hull
251 polygon of terbuthylazine exposed cases. The misclassified cases 22 and 14 are effectively
252 neighbours (Fig. 3). This also holds true for the cross-validated misclassified case 17, but only in
253 the canonical discriminant function plot with only the three previously mentioned discriminant
254 variables (not shown). A clear overlap was found between unexposed cases convex hull polygon
255 and terbuthylazine exposed cases convex hull polygon in the canonical discriminant function plot
256 calculated with only three of the previously mentioned discriminant variables. In contrast cadmium
257 exposed cases convex hull polygon showed no overlapping with both of the ordination methods.
258 Figure 4 reports the mean and the 95% confidence interval values of the selected discriminant
259 elementary pathological findings, according to exposure class. The related ANOVA contrast table in
260 reported in Table 2.
261
262 4. **DISCUSSION**
263 Guided quantitative expert analysis and multivariate exploratory analysis approaches are reliable
264 methods to objectively describe fish gill lesions and discriminate with confidence among
265 terbuthylazine, cadmium exposed and unexposed fish gill. Guided quantitative expert analysis has
266 previously been used by the authors, but only discriminant analysis was performed in that study
267 (Manera *et al.*2015). A multivariate graphical approach was utilized in the present study, resulting in
268 a more reliable and visibly appreciable ordination/classification method.
269 In the present study the following multivariate exploratory data analysis techniques were adopted:
270 CCA and LDA/CVA. As in other ordination methods CCA arranges points originally distributed in
271 a multidimensional space, into groups in an artificially reduced space. However ordination is not
272 entirely based upon the “species” (main matrix) since the axes are also influenced by the

"environmental" variables (secondary matrix), in order to detect possible species to environment relationships within the ordination of objects (Podani 2000, ter Braaka & Šmilauerb 2014). Interestingly, because a permutation test may be performed within CCA, it is possible to test species to environment correlation for significance. Indeed only the correlation referred to Axis 2 was fully significant ($p < 0.01$), though the correlation referred to Axis 1 was close to significance ($p= 0.06$). Nevertheless this fact reflects the good ordination and separation of the exposure groups along the Axis 2 and the partial convex hull polygon superimposing along Axis 1.

LDA is a special ordination technique in which axes are derived in order to maximize the separation of *a priori* defined groups. This terminology is mainly used when the objective is to determine which variable is the most discriminant one (i.e. enhances group separation) and to assign new data into the defined groups. On the other hand, "canonical variates analysis" (CVA) is used when the main goal is the reduction of dimensionality as in the other ordination techniques. In the previous case, possibly the most diffuse bio-medical application, ordination plot is not even performed (Podani 2000). In the present study LDA was applied in both the above mentioned procedures with particular attention to the biplot, namely the projection, as vectors, of the discriminant variables in order to visibly evaluate their relative contribution to ordination/classification.

The occurrence of both type I and type II errors in the evaluation of fish gill structural changes has been extensively reviewed and discussed by Mallatt (1985). In particular, false positive (type I) errors were ascribed to artifacts of fixation and tissue processing, unsuitable control selection, incorrect and uneven sectioning, and post-mortem alterations. Interestingly, provided proper controls are used, type I errors are not critical, while type II errors cannot be readily dismissed. The need for both a guide to correctly assess fish gill pathology and of morphometry in order to avoid false negatives has been stressed previously (Mallatt 1985). Type II errors impact test sensitivity and are a serious concern from a clinical perspective, as compared to false positive errors (Mallatt 1985; Manera 2013; Szczypinski *et al.* 2014; Manera et al. 2016a,).

298 The authors have recently assessed fish gill pathology due to cadmium and terbuthylazine exposure
299 by means of two replicable methods: guided quantitative expert analysis, which was compared to
300 fractal analysis (Manera *et al.*2015). Despite the presence of significant differences in the analysed
301 features according to exposure groups with both methods, only expert analysis was shown to
302 discriminate among treatment groups, which drastically reduced classification errors. Nevertheless,
303 LDA was performed without the CVA and the ordination plot analysis performed here (Manera *et*
304 *al.*2015).
305 The dramatic improvement and usefulness of image analysis techniques in bio-medicine has been
306 recently stressed in several studies (e.g., Sertel et al. 2009, Manera 2013, Szczypinski et al. 2014,
307 Manera et al. 2016b). Pathology is a somewhat conservative diagnostic discipline, relying on
308 descriptive and qualitative analysis of tissue samples by trained individuals. Thus the need of
309 objective, quantitative diagnostic methods has been advocated to assist, rather than substitute for
310 trained pathologists (Al-Janabi et al. 2012, Manera et al. 2016b).
311 Although fish gills are extensively used in environmental and ecotoxicological studies, there is to
312 date no standardized method to quantify their pathological patterns and thus gills pathology is
313 mainly approached by means of qualitative or semi-quantitative analysis (Mallatt 1985, Pawert et
314 al. 1998, Pandey et al. 2008, Gomes et al., 2012; Nascimento et al., 2012), with the notable
315 exception of the quantitative studies of Manera et al. (2016a, b) which like the present study also
316 assessed the sensitivity and the specificity of the adopted methods. Methods relying on cell
317 counting, evaluation of tissue elements and/or reaction pattern recognition are rather time
318 consuming and potentially prone to human bias unless adequately assisted (Manera et al. 2016b).
319 The topic of misclassification in fish histopathology has recently been reviewed by Wolf and
320 collaborators (2015), where the authors stress that with regard to gill pathology, the limited
321 repertoire of pathological response to multitudes of physico-chemical injuries is a significant cause
322 of mis-interpretation. Thus fish gills are a very sensitive but general and nonspecific biomarker
323 (Mallatt 1985; Takashima & Hibiya 1995; Pawert *et al.* 1998; De Oliveira Ribeiro *et al.* 2002;

Dezfuli *et al.* 2003; Dezfuli *et al.* 2006; Giari *et al.* 2006, 2007, 2008; Pandey *et al.* 2008; Gomes *et al.*2012; Nascimento *et al.* 2012; Wolf *et al.*2015). Chloride cell hyperplasia, epithelial lifting and lamellar edema were reported as possible sources of error caused by incorrect positioning during tissue processing, suboptimal/improper fixation, and poor water quality (Mallatt 1985; Wolf *et al.* 2015). The use of a guided expert analysis, limiting the possible pathological entries, the area extension measurement of the recorded elementary pathological features, and the multivariate approach enables concentration only on the really discriminant lesions. In particular, “epithelial shrinkage” was able to discriminate exposed from unexposed fish, while “epithelial swelling” and “pillar cells coarctation” were able to characterize, respectively, terbuthylazine and cadmium exposed fish. False positive and false negative classification errors occurred only between unexposed and terbuthylazine exposed fish. In other words, swelling artifacts were more frequently encountered compared to coarctation artifacts. Moreover, possible suggestions could be inferred with regard to the pathogenesis induced by each toxic condition. Although not toxicant-specific, some lesions are more frequently associated with some irritants and/or environmental conditions. For example, heavy metals frequently induce epithelial necrosis, whereas epithelial lifting was observed less frequently in saltwater than in freshwater fish, possibly due to an osmolarity driven pathogenesis (Mallatt, 1985). The authors have emphasized the need to evaluate the fine pathology occurring at the level of secondary lamellae where diagnoses are reliable, such as in Epon-Araldite semithin tissue sections, while excluding prominent and frequent pathology cues such as gill telangectasia which are easily detectable in paraffin-embedded sections (Manera et al. 2016a). Fish gill pathology resulting from cadmium and terbuthylazine experimental exposure has been previously reported by Dezfuli *et al.*2006, Giari *et al.* 2007 and Manera *et al.*2015. Both toxics were shown to induce epithelial lifting, and hyperplasia and swelling of chloride cells, with necrosis and inflammatory reactions occurring at the highest concentrations and exposure time.

Cadmium toxicity is known to be dependent on protein denaturation, oxidative stress and the interference with the homeostasis of essential metals (Stohs 1995; Stohs & Bagchi 1995; Suzuki *et*

350 *al.* 2001; Moulis 2010). Cadmium interferes with the integrity of the actin cytoskeleton and the
351 function of cadherin-based cell-cell adhesion (Prozialeck and Edwards 2012; Choong *et al.* 2013a)
352 and has also been shown to disrupt vinculin- and focal adhesion kinase-rich focal contacts through a
353 Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II) pathway (Choong *et al.* 2013b). Physical
354 disruption of cell-cell and cell-matrix adhesions might well explain the cell coarctation and curling
355 observed here and in a previous study (Manera et al. 2016a). Furthermore, cadmium is known to
356 have a concentration-dependent effect on vasoreactivity (Takahashi *et al.* 2004) and has been
357 reported to produce changes of the α-actin cytoskeleton in cultured mesangial cells, inducing their
358 contraction (L'Azou *et al.* 2002). Pillar cells contain contractile microfilaments involved in their
359 contraction and in the regulation of the blood flow through the lamellae (Evans *et al.* 2005).
360 Consequently, the reported known effects of cadmium on cytoskeleton contractility suggest a
361 pathogenetic basis for the high discriminant power of pillar cells coarctation/contraction with regard
362 to cadmium exposed fish and dose-related action.
363 Terbuthylazine [IUPAC name is 6-chloro-N-(1,1-dimethylethyl)-N'-ethyl-1,3,5-triazine-2,4-
364 diamine] is a relatively widespread and selective chloro-triazine pre- and post-emergence herbicide,
365 affecting photosynthesis. In particular, Hill reaction in the photosynthetic electron transport is
366 inhibited, whereas mitochondrial electron transport is not affected (Hostovsky *et al.* 2014). The
367 mechanism of action of terbuthylazine and other triazines in animal cell has not yet been fully
368 elucidated, although non-polar narcosis (non-specific toxicity) was suggested by Bermúdez-Saldaña
369 *et al.* (2005).
370 Narcosis mode of action is based on hydrophobic chemicals accumulating within cell membranes,
371 disrupting the native hydrophobic protein-protein or protein-membrane interactions within them,
372 and ultimately disturbing their function (Schultz *et al.* 2003). Interestingly, a proton-pump leak
373 mechanism for narcosis has been proposed (Bangham & Hill 1986). This hypothesis assumes that
374 concentration gradients in living beings are in a steady-state, where passive leaks across membranes
375 are balanced by temperature, pressure, and energy dependent ion pumps. The alteration of the

chemical potential of the hydrophobic membrane phase by hyperbaric, hypothermic, anoxic or toxic conditions causes the resetting of the steady-state parameters (Bangham & Hill 1986). The possible impairment of active/passive ions flux across the membrane, as a consequence of the disruption of the hydrophobic interactions within the latter, may explain why epithelial swelling, as a result of vesiculation, vacuolization of the lamellar epithelial cells, due to intracellular aqueous solution accumulation, resulted to be discriminant for terbuthylazine exposure.

5. CONCLUSIONS

The combined use of semithin sections, which enhanced the appreciation of the overall lamellar structure evaluation; of guided quantitative expert analysis, which minimizes the risk of histopathological misinterpretation and permits an objective evaluation of lesions extension; and of multivariate exploratory data analysis, which allows a graphic, intuitive data ordination/classification, was shown to be a robust, sensitive and sufficiently specific approach to study fish gill lamellar pathology and to discriminate among exposure classes in experimental surveys, isolating the effectively and really discriminant elementary pathological findings from misdiagnosis and artifacts. Furthermore, the identification of the really discriminant elementary lesions establishes a firm foundation upon which to conduct further study into the mechanisms of action of each toxic at the cellular level.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the results reported.

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543

Table 1. Classification table according to linear discriminant, stepwise analysis – Mahalanobis distance, with the following elementary pathological features: epithelial shrinkage, epithelial swelling and pillar cells coarctation.

	Case number	Given group	Classified group	Error type
Original[a]	1	Cd exposed	Cd exposed	-
	2	Cd exposed	Cd exposed	-
	3	Cd exposed	Cd exposed	-
	4	Cd exposed	Cd exposed	-
	5	Cd exposed	Cd exposed	-
	6	Cd exposed	Cd exposed	-
	7	Cd exposed	Cd exposed	-
	8	Cd exposed	Cd exposed	-
	9	Terbuthylazine exposed	Terbuthylazine exposed	-
	10	Cd exposed	Cd exposed	-
	11	Cd exposed	Cd exposed	-
	12	Cd exposed	Cd exposed	-
	13	Terbuthylazine exposed	Terbuthylazine exposed	-
	14	Terbuthylazine exposed	Unexposed *	False negative – Type II
	15	Cd exposed	Cd exposed	-
	16	Cd exposed	Cd exposed	-
	17	Terbuthylazine exposed	Terbuthylazine exposed	-
	18	Terbuthylazine exposed	Terbuthylazine exposed	-
	19	Terbuthylazine exposed	Terbuthylazine exposed	-

	20	Terbuthylazine exposed	Terbuthylazine exposed	-
	21	Terbuthylazine exposed	Terbuthylazine exposed	-
	22	Unexposed	Terbuthylazine exposed*	False positive – Type I
	23	Unexposed	Unexposed	-
	24	Unexposed	Unexposed	-
Cross-validated [b]	1	Cd exposed	Cd exposed	-
	2	Cd exposed	Cd exposed	-
	3	Cd exposed	Cd exposed	-
	4	Cd exposed	Cd exposed	-
	5	Cd exposed	Cd exposed	-
	6	Cd exposed	Cd exposed	-
	7	Cd exposed	Cd exposed	-
	8	Cd exposed	Cd exposed	-
	9	Terbuthylazine exposed	Terbuthylazine exposed	-
	10	Cd exposed	Cd exposed	-
	11	Cd exposed	Cd exposed	-
	12	Cd exposed	Cd exposed	-
	13	Terbuthylazine exposed	Terbuthylazine exposed	-
	14	Terbuthylazine exposed	Unexposed *	False negative – Type II
	15	Cd exposed	Cd exposed	-
	16	Cd exposed	Cd exposed	-
	17	Terbuthylazine exposed	Unexposed *	False negative – Type II

	18	Terbuthylazine exposed	Terbuthylazine exposed	-
	19	Terbuthylazine exposed	Terbuthylazine exposed	-
	20	Terbuthylazine exposed	Terbuthylazine exposed	-
	21	Terbuthylazine exposed	Terbuthylazine exposed	-
	22	Unexposed	Terbuthylazine exposed*	False positive – Type I
	23	Unexposed	Unexposed	-
	24	Unexposed	Unexposed	-

*Misclassified data. [a] 91.7 % of cases correctly classified; sensitivity of 95.45 %, specificity of 75 %. [b] 87.5 % of cases correctly classified; sensitivity of 91.3 %, specificity of 75 %.

Table 2. ANOVA contrast table related to Figure 4.

	Contrast	Unexposed	Cd exposed	Terbuthylazine exposed	Significance[1]
Epithelial shrinkage	1	2	-1	-1	*p< 0.01*
	2	0	1	-1	p> 0.05[2]
	3	1	-1	0	*p< 0.01*
	4	1	0	-1	*p< 0.01*
Epithelial swelling	1	2	-1	-1	*p< 0.05*
	2	0	1	-1	p> 0.05
	3	1	-1	0	p> 0.05
	4	1	0	-1	*p< 0.05*
Pillar cells coarctation	1	2	-1	-1	p> 0.05
	2	0	1	-1	*p< 0.01*
	3	1	-1	0	p> 0.05
	4	1	0	-1	p> 0.05

[1]Significant contrasts are reported in italic. [2]p= 0.056.

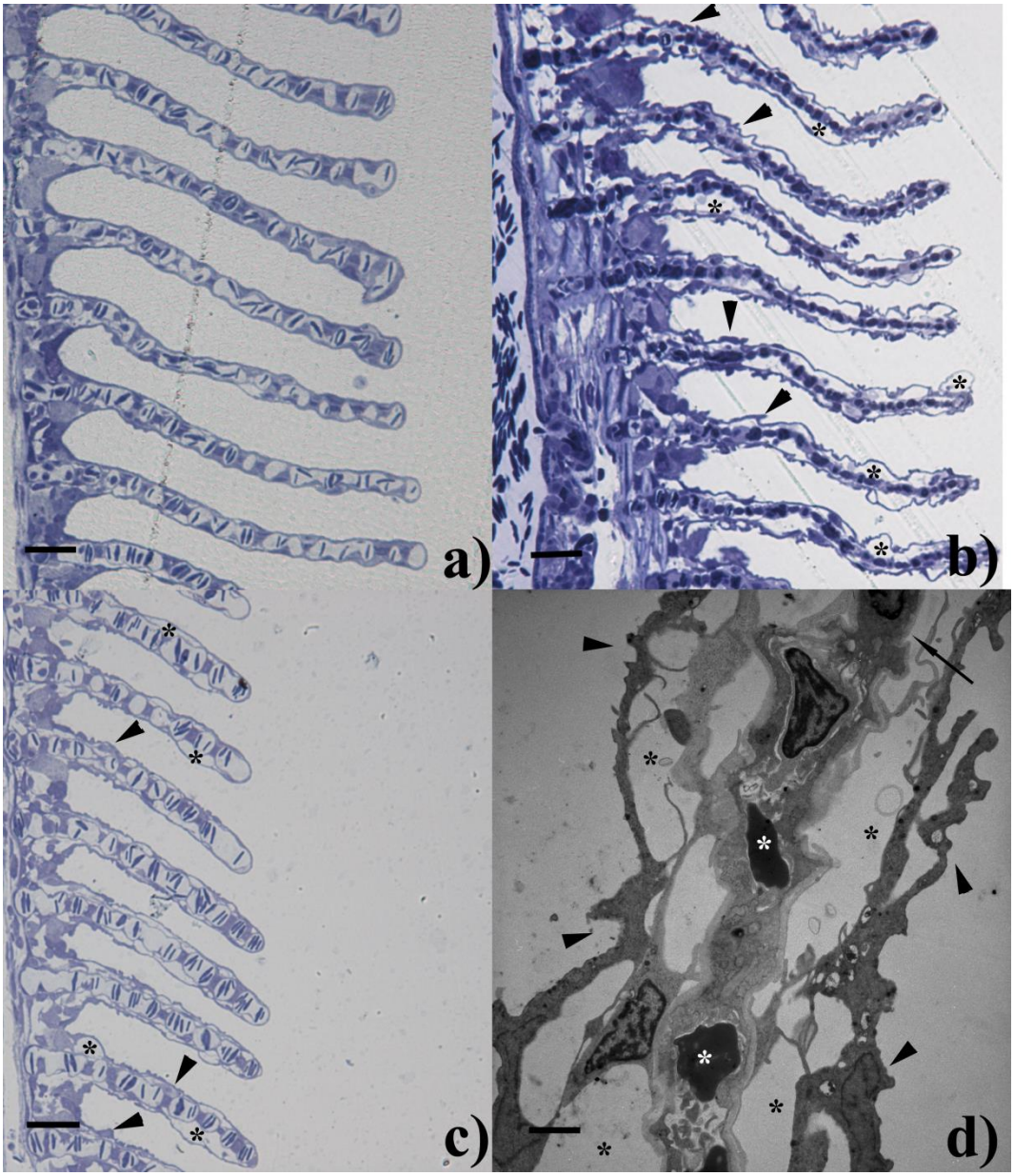
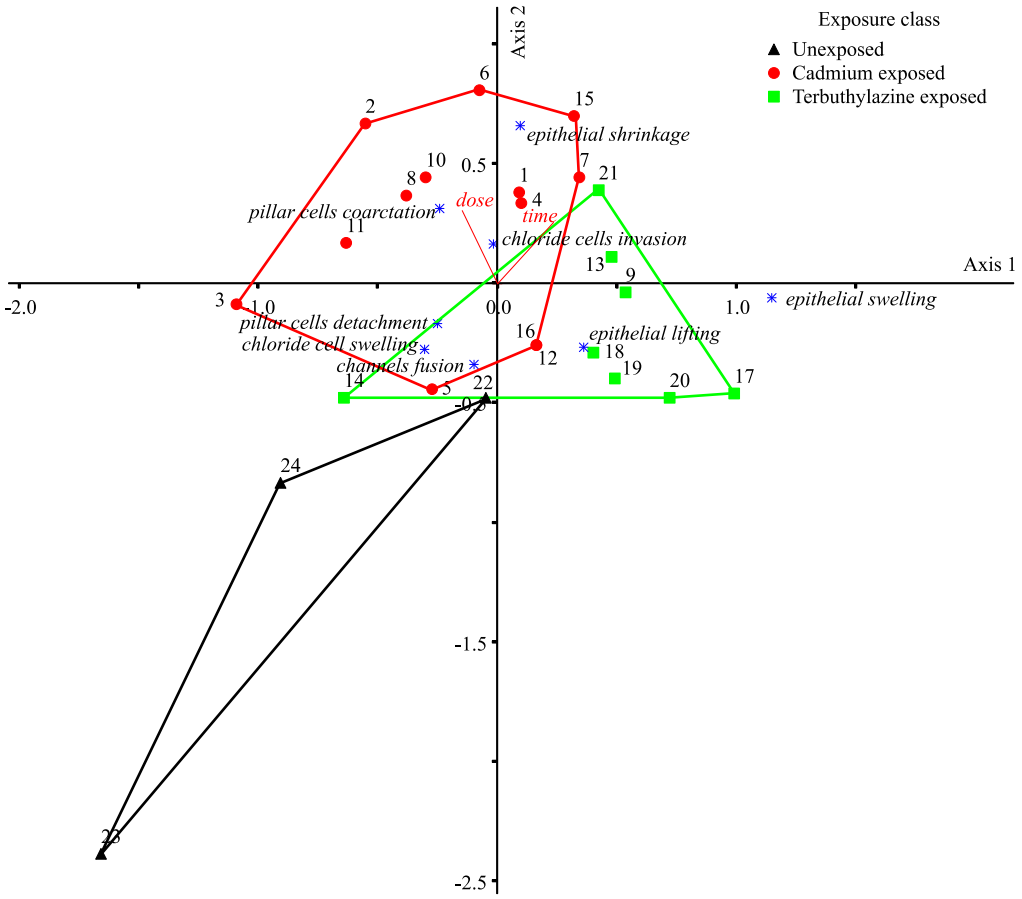


Figure 1. Sections of European sea bass secondary gill lamellae. (a). Normal lamellar architecture is appreciable in the unexposed lamellae. Scale bar = 20 μm. (b). Epithelial lifting (black asterisks), shrinkage/curling of epithelial cells (arrow heads) are appreciable in cadmium exposed lamellae. Scale bar = 20 μm. (c). Epithelial lifting (black asterisks), epithelial swelling (arrow heads) are visible in terbuthylazine exposed lamellae. Scale bar = 20 μm. (d) Ultrastructural appearance of a secondary gill lamella exposed to cadmium. Epithelial lifting (black asterisks) and shrinkage/curling of epithelial cells (arrow heads) are shown. Pillar cells coarctation/contraction is also appreciable (thin arrow). Erythrocytes in blood sinuses are visible (white asterisks). Scale bar = 2.33 μm.

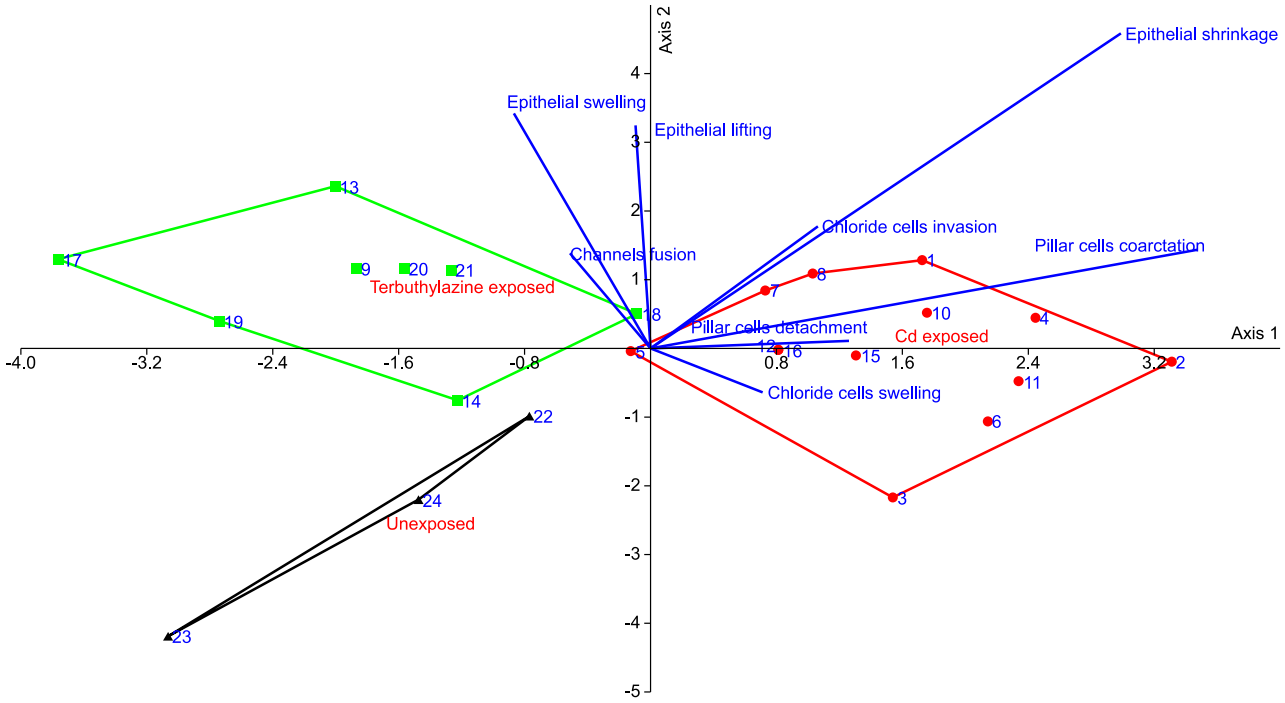


564

565 **Figure 2**. Canonical Corresponding Analysis ordination tri-plot of expert analysis data and
566 exposure parameters. Centroids of the elementary pathological findings are reported as asterisks
567 and second matrix variables (exposure time and dose) as vectors.

568

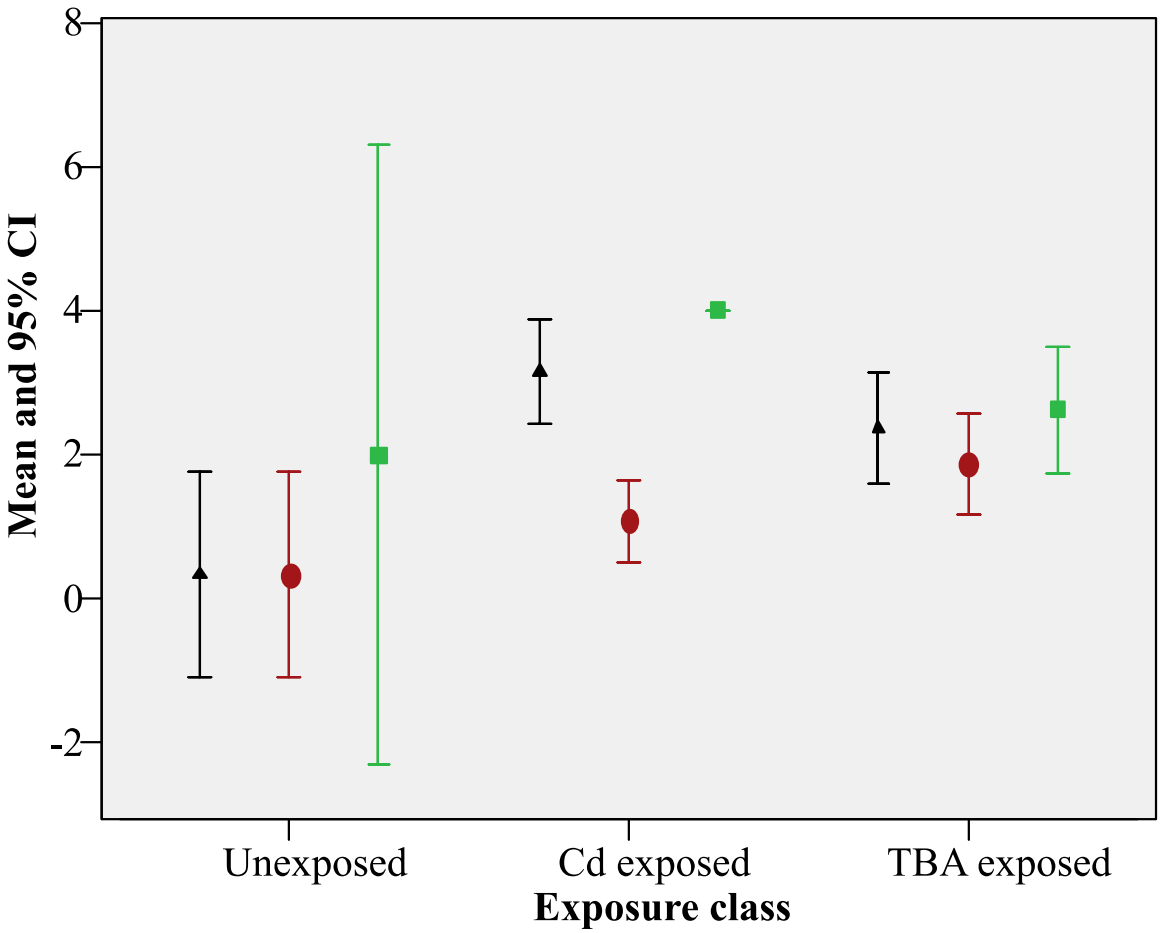
569



570

571 **Figure 3**. Canonical discriminant function plot. Discriminant variables are reported as vectors. The
572 length of the vector is proportional to the discriminant power of the ordination variables and their
573 orthogonal projection with respect to the ordination axis is related to the respective contribution
574 to ordination. Convex hull polygons are clearly separated according to exposure group.

575



576

577 **Figure 4.** Mean and the 95% confidence interval values of the selected discriminant elementary
578 pathological findings (▲: epithelial shrinkage, ●: epithelial swelling; ■: pillar cells coarctation),
579 according to exposure class. Table 2 is the related ANOVA contrast table.

580

581