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Exposure of rainbow trout *(Oncorhynchus mykiss)* to nonylphenol is associated with an increased chloride cell fractional surface area

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Summary

Nonylphenol is a biodegradation product of a widely used group of non-ionic detergents. Because of its ubiquitous distribution and persistence, nonylphenol is present in surface waters as a pollutant. Little is known about its biological effects at environmentally relevant concentrations other than its action as a xenoestrogen. The goal of the present paper was to study the trout gill surface epithelium as the major interface between fish and water in view of possible morphological alterations due to exposure to nonylphenol. Rainbow trout were intermittently exposed to 10 µg/l nonylphenol and gill samples from experimental and control animals were investigated by scanning electron microscopy. Gill surface epithelium was scrutinised for changes in chloride cell density and their status regarding cell surface modifications. In addition, chloride cell fractional surface area (CCFA) was determined by morphometrical methods. Statistical analysis revealed a highly significant increase of CCFA in animals exposed to nonylphenol as compared to control animals ($P = 0.0001$). Semi-quantitative assessment of the other parameters suggested a higher chloride cell density and a larger proportion of chloride cells bearing microvilli. Taken together, these results provide evidence that exposure of trout to nonylphenol is associated with a substantial increase in the active interface of chloride cells with water. We interpret these findings as being a means to further the fish's capacity for calcium exchange.

Key words: fish – gill epithelium – water pollution – endocrine disrupters

Les truites *(Oncorhynchus mykiss)* exposées au nonylphénol présentent une augmentation de la surface apicale des cellules à chlorure

Le nonylphénol est un produit de dégradation final de tout un groupe de détergents non-ioniques très répandus. De par sa distribution ubiquitaire et sa résistance à la biodégradation, le nonylphénol pollue un nombre important d'eaux de surface. Ses effets biologiques aux concentrations constatées dans l'environnement sont peu connus, si ce n'est son activité en tant que xénoestrogène. Le but de la présente publication était donc d'étudier l'épithélium revêtant les branchies de truites en vue d'altérations morphologiques dûes à l'exposition au nonylphénol. Des truites arc-en-ciel ont été exposées à plusieurs reprises à des concentrations de 10 µg/l de nonylphénol. Des échantillons ont ensuite été prélevés sur les branchies de truites exposées ainsi que de truites de contrôle pour être examinés au microscope électronique à balayage. Les spécialisations de surface ainsi que la densité des cellules à chlorure ont été étudiées au niveau de l'épithélium des branchies. De plus, l'aire apicale du plasmalemme des cellules à chlorure (AACC) a été déterminée par des méthodes morphométriques. Une analyse statistique a révélé une augmentation hautement significative de la AACC chez les truites exposées ($P = 0.0001$). Les autres paramètres examinés de manière semi-quantitative ont indiqué une augmentation de la densité des cellules à chlorure ainsi qu'un pourcentage plus élevé de cellules à chlorure portant des microvillosités. Les résultats démontrent que l'exposition au nonylphénol est associée à une augmentation marquée de l'aire apicale des cellules à chlorure, cette surface représentant une zone de contact importante entre le poisson et l'eau environnante. Nous interprétons ces résultats comme étant un moyen permettant aux truites d'accroître l'échange de calcium.

Mots-clé: poisson – épithélium des branchises – pollution des eaux – dysfonctionnement endocrinien

Introduction

Nonylphenol is an ubiquitous and persistent biodegradation product of the alkylphenol ethoxylates, a major group of non-ionic surfactants used in the manufacturing of plastics, textiles, agricultural chemicals and paper. Although contamination levels of open waters are improving in Switzerland, concentration of nonylphenolic compounds still exceeds 3 µg/l in the effluents of some Swiss sewage treatment plants (Ahel et al., 1999). Because of its effects on reproduction and development in vertebrates including mammals, nonylphenol has attracted considerable attention over the last years (for literature see Talmage, 1994). Besides these interactions with the endocrine system, however, little is known about other reactions at environmentally relevant concentrations. In trout species though, nonylphenol was found to accumulate in the liver, gill, skin, gut, kidney and fat tissue (Coldham et al., 1998; Lewis and Lech, 1996).

In addition, biochemical studies have demonstrated that nonylphenol influences ATPases and affects the calcium metabolism (Michelangeli et al., 1990). This raised our interest since both epithelial cell types lining the fish gill, i.e. the respiratory pavement cell and the ion-regulatory chloride cell, harbour high amounts of ATPases, the latter cell being responsible for calcium uptake in freshwater fish (Laurent and Perry, 1995; Lock et al., 1996). Whereas the pavement cells exhibit a species-specific pattern of microridges at their apical surface, the apex of chloride cells is rather smooth or bears small microvilli. Surface area as well as the number and size of chloride cells, however, are known to depend on environmental factors (for review see Laurent and Perry, 1995). The goal of the present study, therefore, was to scrutinise the trout gill surface epithelium for morphological alterations resulting from exposure to water contaminated with nonylphenol.

Animals, material and methods

Animal care and treatments

Within a 4 months period, three year old (3+) male rainbow trout, *Oncorhynchus mykiss,* were intermittently exposed to nonylphenol as described previously (Burkhardt-Holm, in press). Nonylphenol was added at a final concentration of 10 µg/l. This experimental procedure was repeated four times for 10 days each. In between, fish were kept in spring water.

Control animals of the same batch were held under similar conditions in the absence of nonylphenol. The fish density was the same for all groups. Five animals each of the exposed and of the control groups were investigated.

Scanning electron microscopy and morphological analysis

Fish were killed by an overdose of ethylenglycolmonophenylether (Merck, Darmstadt, Germany). Small pieces of gill of all investigated animals were fixed and processed immediately after sampling as described earlier (Burkhardt-Holm et al., 1997). Fixed samples were washed in buffer, postfixed with 1% OsO_4 in 0.1 M cacodylate buffer, dehydrated through an ascending ethanol series and critical point dried in a Bal-Tec CPD 030 (Balzers, Liechtenstein). Immediately thereafter, samples were mounted onto stubs by means of double adhesive conductive tabs (Provac, Liechtenstein), sputtered with approximately 10 nm of gold in a Bal-Tec SCD 004 (Balzers, Liechtenstein), and stored in an exsiccator until examination at 10 to 15 kV accelerating voltage at a working distance of 5 mm in a Zeiss digital scanning electron microscope DSM 982 (Leo, Oberkochen, Germany).

Chloride cell fractional surface area (CCFA) was determined by the point counting method (Weibel, 1979). Micrographs showing frontal views of chloride cells were printed out at a final magnification of 16,400×. Micrographs were overlaid with a square grid of test points 1 cm apart. Number of test points counted per chloride cell were used for statistical analysis of relative CCFA in control animals (50 cells) versus animals exposed to nonylphenol (56 cells). The frequency distribution of obtained values was compiled using the class intervals 1–19, 20–39, 40–59, 60–79, 80–99, 100–119, and 120–139. Furthermore, an estimate of the absolute surface area was computed as $A = n \times d^2/M^2$ where n denotes the median number of points counted per chloride cell, d the width of the square grid and M the magnification. Statistical analysis of collected data included determination of median values with corresponding quartiles, and testing of a hypothetical increase in CCFA after exposure to nonylphenol using the Wilcoxon rank sum test. SEM micrographs were further scrutinised for the presence of cell surface modifications of chloride cells.

Results

Quantitative aspects

Results obtained for the chloride cell fractional surface area (CCFA) in exposed and control animals are compiled in Table 1. Histograms and box plots of relative surface areas (number of test points per cell) are shown in Figure 1. Parting from the

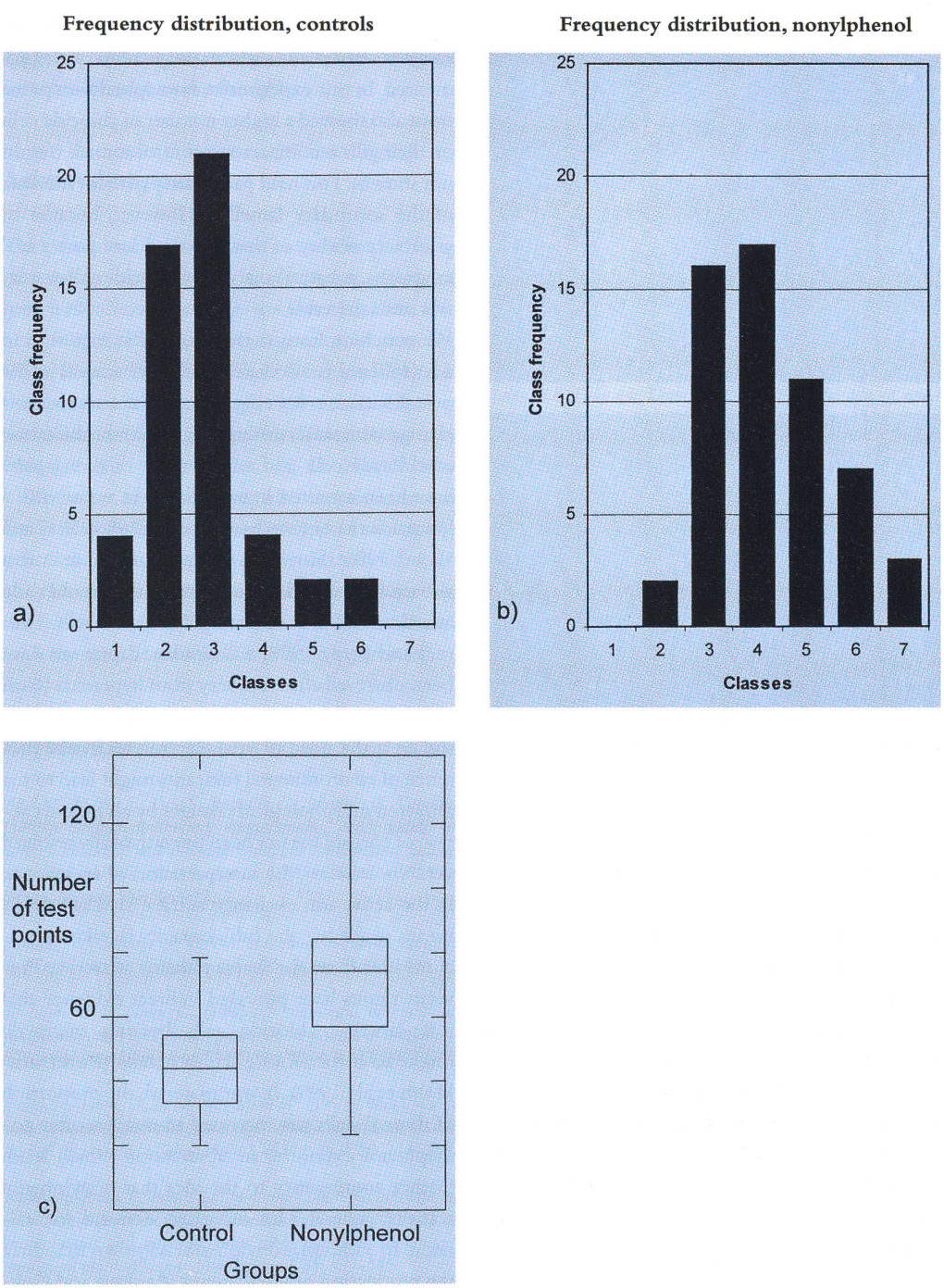

Figure 1: Relative chloride cell fractional surface area (number of test points per cell). Frequency distribution from controls (a) and experimental animals (b), respectively; (c) box plots with median values, 25% and 75% quartiles as well as minimum and maximum values.

median values, average absolute CCFA in exposed trout versus control animals were computed as 28 μm^2 and 16 μm^2, respectively, the ratio being 1.74. According to the Wilcoxon scores (rank sums), the difference in CCFA was highly significant ($P = 0.0001$).

Qualitative and semi-quantitative aspects

Nonylphenol-exposed trout showed an increased number of chloride cells in the interlamellar space. Scanning electron microscopy also allowed two

Table 1: Statistical analysis of relative chloride cell fractional surface area (number of test points per cell).

	Controls	Nonylphenol
Number of observations	50	56
Maximum value	119	131
75% Q3	54	85
50% Median value	43	75
25% Q1	31	57
Minimum value	16	20
Range	103	111
Q3–Q1	23	28

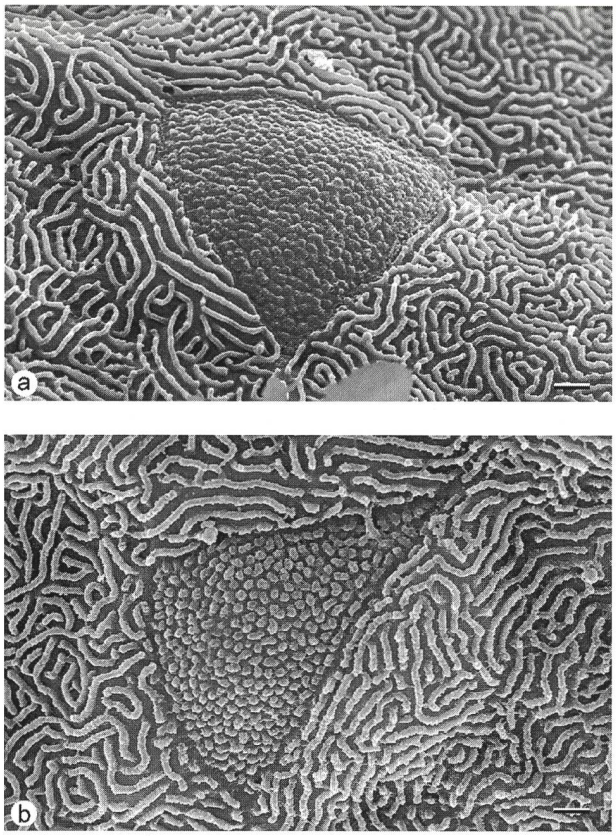

Figure 2: Chloride cells of the smooth type (a) and of the microvilli-bearing type (b) surrounded by pavement cells with typical microridges. Scale bar = 1 µm.

phenotypically distinct varieties of chloride cells to be distinguished. One type exhibited an almost smooth apical surface (Fig. 2a) whereas the second type displayed short but dense microvilli (Fig. 2b). Although both cell types were observed in control as well as in exposed animals, the ratio of smooth cells to cells bearing microvilli was approximately 6 : 1 in control animals whereas the corresponding value in trout exposed to nonylphenol was 1 : 1. No statistical analysis was performed on these results since distinction of the two cell types does not rely on absolute criteria.

Discussion

Enlargement of the overall apical surface of chloride cells basically can be achieved in three ways: by increasing the cell fractional surface area (CCFA), by adding cell surface projections, and by multiplying the number of cells. The present study provides highly significant evidence that exposure of trout to nonylphenol-contaminated water is linked to a dramatic rise in the average CCFA. This observation is also illustrated by the frequency distribution which revealed that no cells pertaining to the largest size class were observed in control animals whereas representatives of the smallest class were lacking in exposed trout. The increased proportion of chloride cells bearing tangible microvilli further suggests that cell surface projections are augmented. In our experiment, nonylphenol-exposed trout also showed a higher number of chloride cells on their gills as compared to control animals (results not shown). This held particularly true for the base of the secondary lamellae. However, because of poor accessibility of this region to low power micrographs, statistical analysis of chloride cell density was not achievable.

We conclude from these results, that exposure to nonylphenol is associated with an increase in the overall surface area being available for active ion exchange with water. Interestingly, both proliferation of chloride cells and enlargement of their apices have been reported in trout living in water with a low calcium content (Laurent et al., 1985; Perry and Wood, 1985). However, chronic cortisol treatment also causes a significant increase in the number and apical surface area of trout gill chloride cells (Laurent and Perry, 1990) and similar adaptations have been observed after recovery from hyperoxia (Goss et al., 1994). Thus, the question whether any stressor, be it the need of vital electrolytes or the presence of environmental toxicants might lead to the reported morphological changes by an indirect action of cortisol has not been unequivocally resolved yet. Nonetheless, the interpretation of an increase in the active ion exchange surface area as being a means to further the fish's capacity to take up calcium ions from the water remains attractive. Previous results have provided indirect evidence that inter-lamellar chloride cells bearing microvilli might be involved in calcium uptake (Burkhardt-Holm et al., 1999). In our material, the proportion of these ß-cells was increased in number after nonylphenol exposure, an observation which lends further contingency to the idea that nonylphenol actually generates an increased demand for calcium. In addition, nonylphenol is known to induce the production of vitellogenin (Arukwe and Goksoyr, 1998). This calcium-rich yolk protein is normally secreted by the liver of adult female oviparous vertebrates. Production of vitellogenin, however, can be induced in males and juveniles by several xenoestrogens including nonylphenol (Arukwe and Goksoyr, 1998). Synthesis of vitellogenin in its turn is highly dependent upon adequate calcium levels and thus promotes the mobilization of calcium from all available sources (Björnsson and Haux, 1985; Carragher and Sumpter, 1991).

In summary, the present study provides unequivocal evidence for a highly significant correlation between exposure of trout to nonylphenol-contaminated water and a substantial increase in the active interface of chloride cells with water. These data do not warrant a causal connection *per se*, but taken to-

gether with reports in the literature, they strongly suggest a repercussion of exposure to nonylphenol on ion exchange in the fish gill.

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