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Biochemical and Physiological Responses in Atlantic Salmon (*Salmo salar*) Following Dietary Exposure to Copper and Cadmium

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Three experiments were conducted with Atlantic salmon (*Salmo salar*) to assess the effects of dietary exposure to copper and cadmium. The results presented here provide an overview, details of each experiment will be published in full elsewhere. In the first experiment, salmon parr exposed for four weeks to 35 and 700 mg Cu kg^{-1} diet had significantly elevated intestinal copper concentrations, cell proliferation (PCNA) and apoptosis rates compared to control fish. No differences were observed in gill or plasma copper concentrations among the groups. In contrast to the controls, the Cu exposed groups did not grow significantly during the exposure period. The second experiment (three months exposure) was conducted to assess the effects of dietary copper (control, 35, 500, 700, 900 or 1750 mg Cu kg^{-1} diet) on growth and feed utilization in salmon fingerlings. Growth was significantly reduced after three months exposure to dietary Cu concentrations above 500 mg kg^{-1}. Similarly, copper body burdens were significantly higher in fish exposed to elevated dietary copper concentrations (above 35 mg Cu kg^{-1} diet). In the third experiment, salmon parr were exposed to one of six dietary cadmium concentrations (0, 0.5, 5, 25, 125 or 250 mg Cd kg^{-1} diet) for four months. Cadmium accumulated in the liver > intestine > gills of exposed fish. Rates of apoptosis and cell proliferation in the intestine increased following exposure to dietary cadmium. Exposure to elevated concentrations of dietary cadmium had no effect on growth in salmon parr. Results from these studies indicate that cellular biomarkers have potential as early warning signs of negative effects on the overall fitness of an organism. © **1999 Elsevier Science Ltd. All rights reserved.**

Keywords: Atlantic salmon (Salmo salar); copper; cadmium; diet; toxicity; biomarkers.

Whereas toxic responses in fish exposed to elevated waterborne concentrations of copper and cadmium are well documented, relatively little research has been done on dietary metal toxicity despite diet being a significant route of contamination in wild fish (Dallinger and Kautzky, 1985). Dietary exposure to copper (Lanno *et al*., 1985) and cadmium is considerably less toxic than waterborne exposure. This is attributable to the mucosal layer in the gut which represents a formidable barrier to toxic metals (Handy, 1993), although both copper and cadmium are eventually absorbed across the gut. Hence the bioavailability of Cu or Cd is much lower in contaminated feed than the equivalent dose presented in aqueous form (Miller *et al*., 1993).

Fish feed regulations have been implemented to protect fish health and consumer safety. The European Union (EU) has set maximum permitted concentrations of potentially toxic substances that can legally be present in fish feed. These include essential trace nutrients (e.g. Cu) which are supplemented to fish feed, and non-essential substances which are present in raw materials or contaminate the feed during processing (e.g. Cd). Maximum permitted concentrations for copper and cadmium in fish feed are currently 35 mg kg^{-1} dry weight and 0.5 mg kg^{-1} dry weight, respectively. However, EU fish feed regulations are currently based primarily on research done on cattle and poultry. Data from fish experiments are required in order to set appropriate limits in fish feed to guarantee fish health and consumer safety. A further consideration is protection of the environment from excessive inputs of potential toxicants from aquaculture, hence maximum permitted feed concentrations should be kept to a minimum.

The present studies were conducted to assess the effects of chronic dietary exposure to an essential and a non-essential element (copper and cadmium) on Atlantic salmon. This species was chosen because it is

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of great economic importance to Norwegian aquaculture.

Materials and Methods

Diet preparation

Formulations of the diets used in the three experiments are given in Table 1. Diets for the first and second experiments were supplemented with 0, 35, or 700 mg Cu kg^{-1} diet, and 5, 35, 500, 700, 900 or 1750 mg Cu kg^{-1} diet, respectively. Diets for the third experiment were supplemented with 0, 0.5, 5, 25, 125 or 250 mg Cd kg^{-1} diet. Metals were dissolved in 500 ml acidified water and mixed well with the other feed ingredients prior to pelleting. The diets were cold pelleted after adding approximately 12% water and were subsequently dried. All diets were stored at −20°C until they were fed to the fish.

Experimental protocol

Initial weight of Atlantic salmon used in the three experiments were 72.2 ± 2.1, 0.9 ± 0.2 and 26.5 ± 3.9 g (mean ± SD), respectively. In the first experiment 540 fish were distributed equally into nine experimental tanks (1.5×1.5×0.5 m), with 60 fish per tank (three triplicate groups). In the second and third experiments 18 experimental tanks (three triplicate groups) were each stocked with approximately 1000 fry and 100 parr, respectively. Prior to each experiment, fish were fed unsupplemented diets for at least a two week acclimation period. Fish were reared under constant light during the exposure period and fed by automatic feeders (for 5 s, every 7 min, 24 h per day) according to the growth tables of Austreng *et al*. (1987). For the last two weeks of the second experiment the fish were fed experimental diets supplemented with Cr_2O_3 (1%) as an external indicator to estimate apparent availability of the different feed components (Lied *et al*., 1982).

Biological sampling

In experiments one and three, fish were starved for 24 h prior to sampling to allow all feed to be excreted. Tissue samples were removed from six fish from each tank (after being sacrificed by a blow to the head) at 0, 7, 14, and 28 days and week 0, 4, 8, 12 and 16 in the first copper experiment and the cadmium experiment, respectively. Weight and fork length were recorded for each individual. Gill, liver and intestine samples were removed from each fish and the latter two tissue samples were immediately frozen in liquid nitrogen. Gill samples were frozen at −20°C. In Experiment 1, tissue samples from three fish from each tank were pooled for Cu analysis (hence $n = 2$ per tank). In Experiment 3, tissue samples from two fish from each tank were pooled for Cd analysis ($n = 3$ per tank except at the initial sampling time where tissue weight made it necessary to pool three fish per tank i.e., $n = 2$). Fat and veins were removed from the intestine prior to analysis, and the intestine was rinsed three times with an ice-cold saline solution (0.9% NaCl) to remove food remnants.

In the second copper experiment, fish were bulk weighed initially, and after 6, 10, and 12 weeks of exposure. At each sampling time 100 fish from each experimental container were transferred to aerated 30 l containers with static water (changed daily) and starved for four days to remove their intestinal content. These fish were subsequently killed in tricaine methanesulfonate (MS-222). Fifty fish sampled after 0 and 6 weeks of exposure and 20 fish sampled after 10 and 12 weeks of exposure were pooled and frozen for subsequent whole body analyses. Length and weight were recorded for 50 individual fish after 0, 6 and 12 weeks of exposure. At the end of the experiment, 12 un-starved fish from each tank were sacrificed and faeces samples were stripped by pressing the abdomen from the ventral fins to the anus (Austreng, 1978). Faeces from 6 fish per tank were pooled. The gastrointestinal tract (from the oesophagus to the rectum) was removed from the remaining 6 fish from each tank. These fish were pooled and frozen for subsequent analysis of whole body copper minus the gastrointestinal tract. The pooled faeces and whole body samples were freeze-dried, homogenised and analysed for Cu.

TABLE 1

Basal composition (g kg^{-1}) of the fish diets used in the three experiments.

Ingredient	1st copper study	2nd copper study	Cadmium study
Fish meal (Norse LT94, Norway)	573	573	580
Capelin oil (Norsalmoil, SSF, Norway)	119	119	119
Wheat meal (CODRICO LTD, Netherlands)	167	167	160
Gelatin (TORO A/S, Norway)	28	28	28
Squid	95	95	95
Mineral mix[a]	9	9	9
Vitamin mix[b]	9	9	9

[a] The mineral mix for the first and second experiments provided the following (mg kg^{-1} diet): Zn (as $ZnSO_4 7H_2O$):68; Fe (as $FeSO_4\ 7H_2O$):34; and Mn (as $MnSO_4 H_2O$):13. The mineral mix for the third experiment was as for the first two experiments except it also provided Cu (as $CuSO_4 5H_2O$):5.
[b] The vitamin mix provided the following (mg kg^{-1} diet): Retinyl palminta: 5; Cholecalciferol: 4.8; Alphatocopheryl acetate: 100; Menadione: 5; Thiamin: 10; Riboflavin: 20; Pyridoxin: 10; Pantothenic acid: 40; Niacin: 170; Biotin: 1.2; Vitamin B_{12}: 0.02; Inositol: 450; Ascorbic acid (coated): 1000; and Folic acid: 5.

Metal analysis

Freeze-dried tissue samples (0.2 g) were microwave digested with 2 ml nitric acid (65%) and 0.5 ml hydrogen peroxide (30%). Copper was analysed by flame atomic absorption spectrophotometry (AAS) and cadmium was analysed by graphite furnace AAS. Analysing standard reference material with each set of samples controlled accuracy and precision of the analyses.

Histology and tissue processing

In the first experiment, apoptosis (regulated cell death) was examined by transmission electron microscopy after osmium fixation according to Wendelaar Bonga and Van der Meij (1989). In the cadmium experiment apoptosis was assessed using confocal laser microscopy following TUNEL staining.

Intestinal cell proliferation was examined immunohistochemically with a monoclonal antibody raised against proliferating cell nuclear antigen (PCNA). This method is described in detail by Ortego *et al.* (1994). The proportion of PCNA positive crypt cells to PCNA negative crypt cells was subsequently quantified (6 crypts of 6 cross sections were examined per fish, and 3 fish per tank (three tanks per concentration i.e. $n = 9$ fish) were examined at random).

Statistical analysis

Normality of the data and homogeneity of variance were checked using Kolmogrov–Smirnov's test and Levene's *F*-test, respectively. Data, which complied with the criteria, were analysed by two way (nested) analysis of variance (ANOVA), with data from experimental tanks nested in dietary exposure groups. Significance was tested using Tukey's test ($p < 0.01$ and $p < 0.05$). Data, which were not normally distributed, were log transformed. Transformed data from the cadmium experiment did not comply with the criteria for ANOVA hence these data were tested non-parametrically. All tests were performed using STATISTICA^{tm} (Statsoft Inc., USA, 1993).

Results

Exposure to metal-enriched diets did not lead to mortality, and moribund fish were not observed in either the first-or the third experiment. The mortality rate in

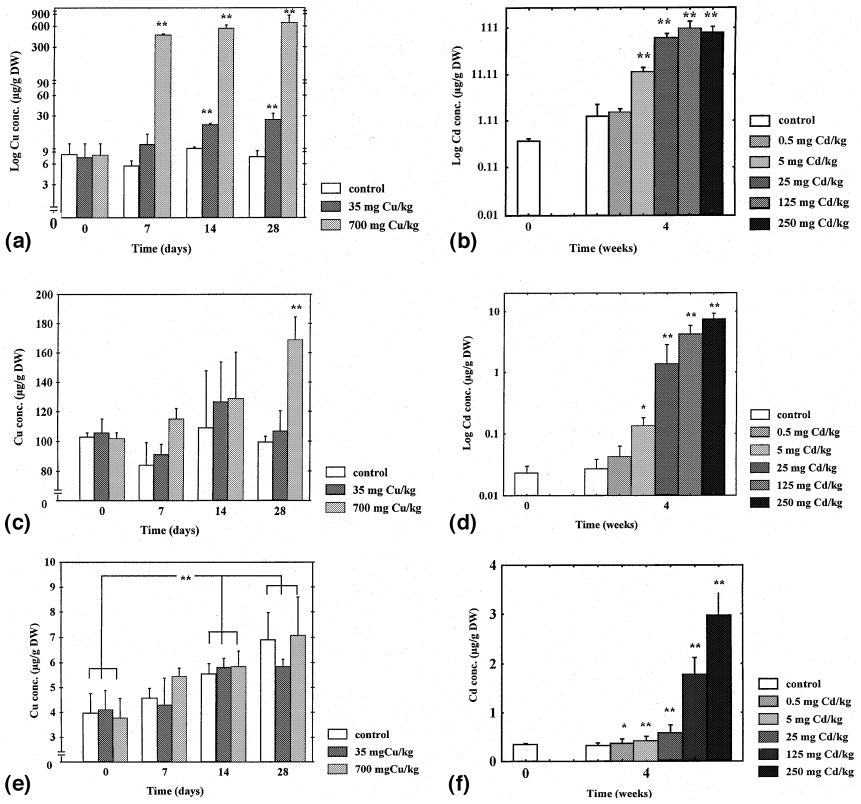

Fig. 1 Mean copper and cadmium concentrations (µg g^{-1} dry weight) with standard deviations (SD) in intestine (A and B), liver (C and D), and gills (D and E) of Atlantic salmon parr exposed to dietary copper and cadmium, respectively. Significant differences from controls at each time interval are indicated with asterisks (* for $p < 0.05$ and ** for $p < 0.01$, $n = 9$ except for Cu intestine $n = 6$). The *Y*-axes in Fig. 1(A), (B) and (D) have logarithmic scales due to the large differences in metal concentrations among treatments (from Berntssen *et al.*, 1999a).

the second experiment was low (2%), and did not differ among the dietary treatments. Routine daily observations did not show a decrease in appetite or swimming activity in any of the exposure groups. The amount of uneaten feed did not increase with increasing exposure concentration in any of the studies.

The three studies were conducted in flow through systems, and metal concentrations were measured in water samples taken at each sampling point. They did not differ from the controls in any of the experiments.

Copper concentrations

Intestinal copper concentrations were significantly elevated ($p < 0.01$) in the 35 and 700 mg Cu kg^{-1} diet groups compared to the control group, after 14 days of exposure. By the end of the exposure period, the groups exposed to 35 and 700 mg Cu kg^{-1} diet had 3.5 and 89-fold increases in intestinal Cu concentrations, respectively (Fig. 1a).

In contrast to the intestine, relatively little copper accumulated in the liver. Only the group exposed to the highest dietary Cu concentration (700 mg Cu kg^{-1} diet) had a significant (1.6-fold) increase in liver Cu concentration after 28 days of exposure (Fig. 1c). In the gills, there were no significant differences in copper concentrations among the treatments, however all groups had a slight but significant increase ($p < 0.01$) in gill copper content over time (Fig.1e).

Cadmium concentrations

For comparative purposes only data from four weeks dietary cadmium exposure have been included in this paper. All exposure groups except the lowest (0.5 mg Cd kg^{-1} diet) had significantly higher intestinal cadmium concentrations than the controls after four weeks exposure (Fig. 1b). Mean intestinal cadmium concentrations were 1.4 μg g^{-1} dry weight (DW) and 108.1 μg g^{-1} DW for the control and 125 mg Cd kg^{-1} diet groups, respectively. In contrast to the slight increase in liver concentration seen in the copper experiment, cadmium accumulated approximately 300-fold from 0.02 to 7.5 μg g^{-1} DW in the control fish and fish exposed to 250 mg Cd kg^{-1} diet, respectively (Fig. 1d).

Differences in gill cadmium concentrations were observed among treatments (Fig. 1f). All exposure concentrations had significantly elevated cadmium concentrations compared to the controls, which had a mean concentration of 0.3 μg Cd g^{-1} DW while the

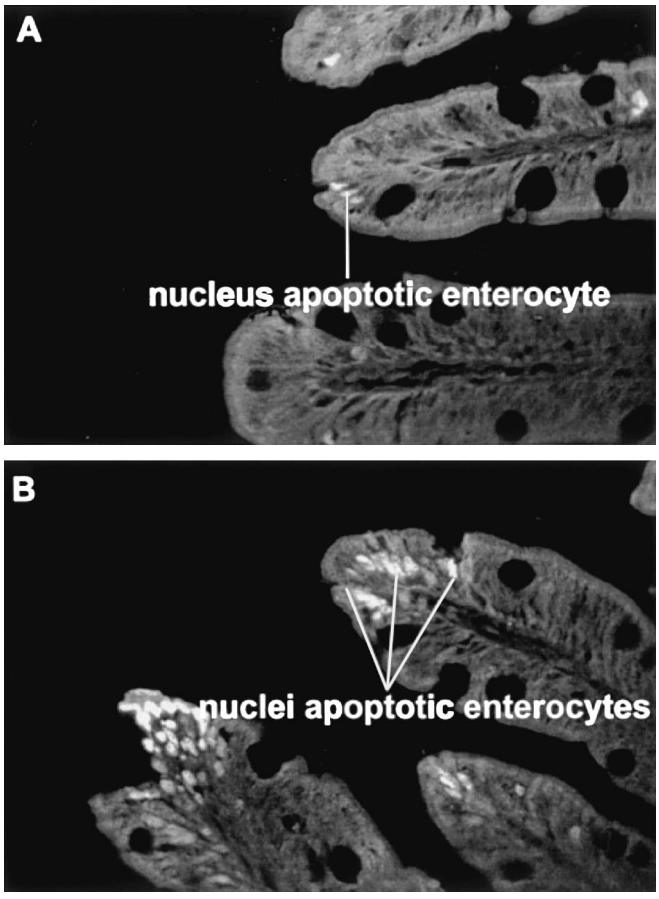

Fig. 2 Confocal laser scanning illustrations of apoptosis at the apex of intestinal folds in Atlantic salmon parr exposed to (A) zero (control) and (B) 5 mg kg^{-1} dietary cadmium for eight weeks (fluorescent TUNEL stained apoptotic nuclei, magnification × 400), after Lundebye *et al.*, in preparation.

highest exposure group had a 10-fold higher concentration; 3 μg Cd g^{-1} DW.

Apoptosis and cell proliferation

Electron microscopy of the intestine revealed an increase in the number of enterocytes with condensed cellular components, and shrunken and dense nuclei in both of the groups exposed to dietary copper for 28 days (results not shown). Similarly, confocal laser microscopy revealed an increase in the number of apoptotic cells in the intestines of fish exposed to dietary cadmium compared to the control fish (Fig. 2a and b). These apoptotic enterocytes were limited to the tips of the intestinal folds. Few necrotic cells were observed in the control fish or the fish exposed to dietary copper or cadmium.

PCNA-positive epithelial cells were limited to the intestinal crypts (Fig. 3a and b). Both of the copper treatments had significantly ($p < 0.05$) increased ratios of PCNA-positive cells to PCNA-negative cells compared to the controls after 28 days of exposure (Table 2: 46.0% and 96.8% increases in the 35 and 700 mg Cu kg^{-1} diet groups, respectively).

A similar increase in intestinal cell proliferation was observed in fish exposed to dietary cadmium (5 mg Cd kg^{-1} diet, data not shown).

Copper availability

Sixty four percent of the copper in the diet was digested from the control diet (Table 2). Copper availability was significantly lower in copper-exposed fish than in the control fish at dietary copper concentrations up to 900 mg Cu kg^{-1} diet. However, copper availability increased to approximately 50% at the highest copper concentration (1750 mg Cu kg^{-1} diet).

Whole body burdens of copper and whole body burden minus the intestine are given in Table 2. Significant increases in whole body copper burdens were apparent in fish exposed to 500 mg Cu kg^{-1} diet and above. In whole body minus intestine, significant increases in copper concentrations were seen in fish exposed to 900 and 1750 mg Cu kg^{-1} diet.

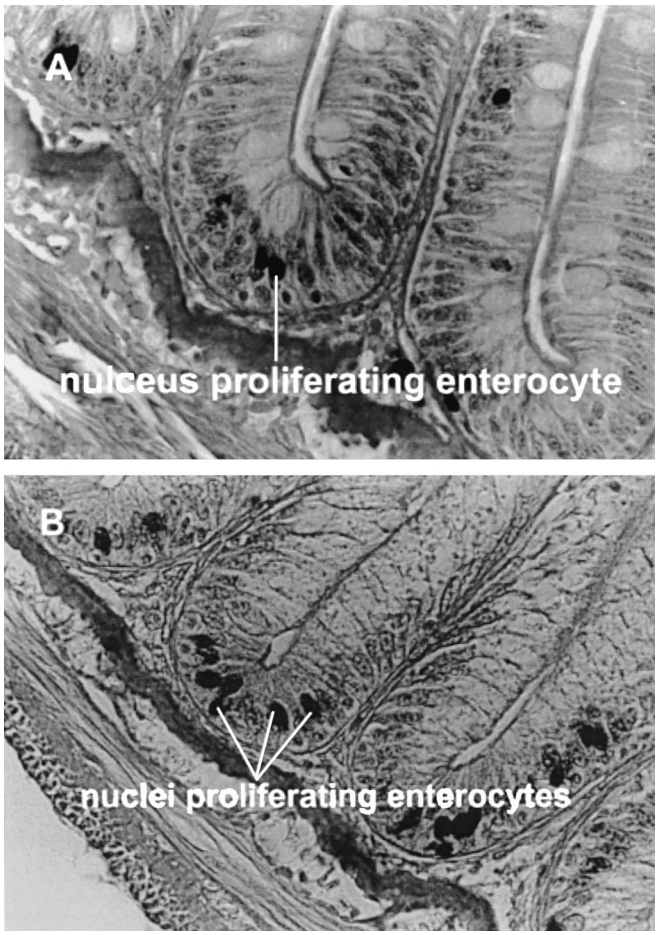

Fig. 3 Light microscopical illustrations of cell proliferation (PCNA) in intestinal crypts of Atlantic salmon parr exposed to (A) zero (control) and (B) 35 mg kg^{-1} dietary copper for 28 days (immunohistologically stained (DAB) against PCNA (proliferating nuclei), magnification × 600), from Berntssen et al., 1999a.

TABLE 2

Experiment 1: intestinal cell proliferation (% PCNA positive crypt cells/ PCNA negative crypt cells) in Atlantic salmon parr exposed to elevated dietary Cu for 28 days (mean ± SD, $n = 9$). Experiment 2: Whole body Cu content and whole body Cu content minus intestine (mg/kgDW), and apparent Cu digestibility (%) in whole Atlantic salmon fry exposed for 12 weeks to elevated dietary Cu levels (mean ± SD, pooled samples of six fish, $n = 3$).

Cu added to feed (mg kg^{-1})[1]	0 (control)	5 (control)	35	500	700	900	1750
Experiment 1							
PCNA (%)	10.2 ± 2.2[a]		17.2 ± 2.1[b]		21.5 ± 5.7[b]		
Experiment 2							
Cu content (mg/kg)		2.4 ± 0.2[a]	2.3 ± 0.6[a]	13.6 ± 0.2[b]	15.6 ± 0.9[b]	27.5 ± 2.0[c]	35.3 ± 3.9[d]
Cu content –intestine (mg/kg)		2.5 ± 0.1[a]	2.6 ± 0.2[a]	6.2 ± 5.4[a]	9.3 ± 4.9[a]	20.8 ± 4.7[b]	25.5 ± 2.2[b]
Cu digestibility (%)		64.7 ± 8.6[a]	23.0 ± 9.3[b]	26.8 ± 4.3[b]	24.2 ± 3.7[b]	39.0 ± 4.2[c]	52.0 ± 4.1[a]

[a] Cu concentration in unsupplemented feed is 2.2 ± 0.3 mg kg^{-1}.
[b] Values in rows with the same superscripts are not significantly different ($p > 0.05$).

Growth

No significant difference in growth was observed between the two Cu-exposed groups and the control group after 28 days exposure to dietary copper. However, mean weight of the control fish increased significantly ($p < 0.05$) from initial to final weight, whereas there was no apparent weight increase in the fish exposed to dietary copper.

In the second copper experiment, relative growth was significantly reduced in fish after 10 weeks exposure to 900 and 1750 mg Cu kg^{-1} diet. After 12 weeks exposure to 700, 900 and 1750 mg Cu kg^{-1} diet, relative growth of fish was reduced (Fig. 4a).

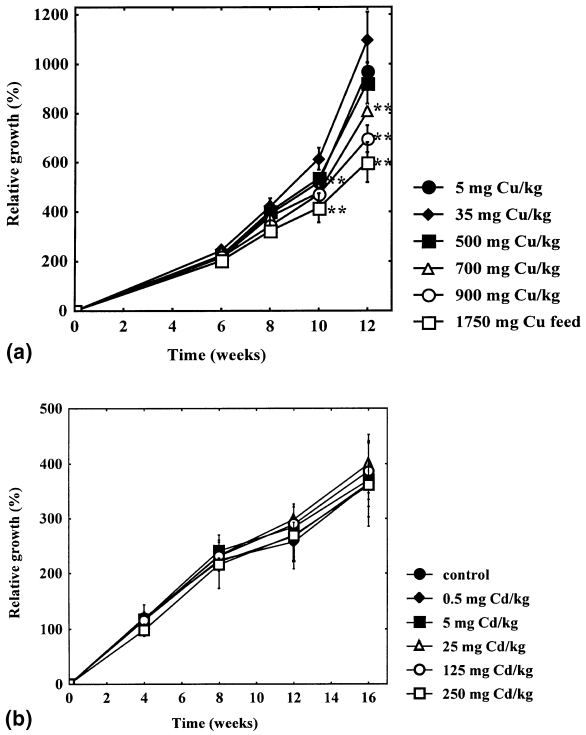

Fig. 4 Relative growth rate (%) of Atlantic salmon exposed to dietary (A) copper (Experiment 2) and (B) cadmium (Experiment 3). After Berntssen et al., 1999b and Lundebye *et al.*, in preparation.

In the cadmium experiment, no effects on growth were evident at any of the dietary exposure concentrations over the 4 month exposure period (Fig. 4b).

Discussion

Cu and Cd accumulation

The pattern of trace metal distribution among internal organs in fish is dependent on the route of uptake. The gills contain up to 40% of the body burden in fish exposed to either waterborne cadmium or copper (Handy, 1992a; Harrrison and Klaverkamp, 1989). In contrast, the gut supports approximately 75% of the cadmium (Handy, 1992b; Harrrison and Klaverkamp, 1989) and 53% of the copper burden during dietary exposure (Handy, 1992b). Furthermore, Handy (1992b) found no increases in liver copper concentrations in rainbow trout exposed to 200 mg Cu kg^{-1} diet.

In the first copper experiment, considerable increases were observed in intestinal copper concentrations in dietary copper-exposed fish, whereas liver copper concentrations were only slightly elevated. In the second copper experiment, approximately 50% of the whole body burden in fish exposed to 500 and 700 mg Cu kg^{-1} diet was present in the intestine (Berntssen *et al.*, 1999b). These results indicate that the intestine plays an important role in regulating the uptake of dietary copper.

There were no differences in gill copper concentrations among the treatments however, there was a slight but significant increase in gill copper content over time. This may be attributable to an increase in Cu-rich components, such as red blood cells containing enzymes with Cu as a co-factor (Berntssen *et al.*, 1999a). In contrast, Handy (1992b) reported copper accumulation in the gills of rainbow trout exposed to dietary copper (200 mg Cu kg^{-1} dry weight (DW) of feed).

In the present study, cadmium was found to accumulate in the intestine, liver and gills of fish exposed to elevated dietary cadmium concentrations (Lundebye *et al.*, in preparation). This is in agreement with results from studies on rainbow trout exposed to elevated

dietary cadmium levels: 150 mg and 10 g Cd kg^{-1} DW of feed (Handy, 1992b, 1993, respectively).

Intestinal cellular responses

In contrast to apoptotic cells which were only observed at the apex of the intestinal folds of both copper- and cadmium exposed fish, PCNA-positive cells were mainly detected in the intestinal crypts (Berntssen *et al*., 1999a). This supports the widely accepted notion that intestinal cell turn-over is mediated by the formation of new cells in the crypts of intestinal folds which migrate upwards and disappear at the tips (Eckert *et al*., 1988). Little information is available in the literature regarding dietary metal exposure and apoptotic and/or cell proliferating responses in fish. Pratap and Wendelaar Bonga (1993) observed increased apoptosis in the gills of tilapia (*Oreochromis mossambicus*) exposed to dietary cadmium.

The increased rates of intestinal mitosis and apoptosis in fish exposed to elevated dietary copper and cadmium concentrations seen in the present studies indicate increased intestinal cell-turnover. Greene and Moran (1994) suggested that increased cell sloughing is a possible route of excretion for metals bound to metallothionein. Hence, increased intestinal cell turn-over and induction of metallothionein synthesis may be involved in regulating the dietary uptake of metals (Berntssen *et al*., 1999a).

Copper availability

Sugiura *et al*. (1998) found that coho salmon (*Oncorhynchus kitsutch*) absorbed more copper from a range of diets than did rainbow trout (*Oncorhynchus mykiss*). This may reflect differences between these two species in dietary requirements or ability to utilise dietary copper. In the second copper experiment, approximately 60% of the copper was absorbed by Atlantic salmon from the control diet, however availability decreased with increasing dietary copper concentration suggesting that copper uptake is restricted when present at excessive concentrations. At the highest dietary copper concentration (1750 mg Cu kg^{-1} diet) apparent copper availability increased, indicating that regulatory mechanisms were overloaded (Berntssen et al., 1999b).

Growth

Four weeks exposure to elevated dietary copper concentrations did not affect growth in the first copper experiment. In the second copper experiment 12 weeks exposure to dietary copper concentrations above 500 mg Cu kg^{-1} diet resulted in reduced growth in Atlantic salmon (Berntssen *et al*., 1999b). Lanno *et al*. (1985) attributed reduced growth in rainbow trout exposed to 700 mg Cu kg^{-1} diet to loss of appetite. However, no feed refusal was observed in Atlantic salmon exposed to dietary copper in the present studies. Several authors have suggested that the stress responses (including tissue repair, development of the defence system and copper excretion) observed in fish following exposure to elevated water copper concentrations may cause increased metabolic activity, which in turn will reduce growth (De Boeck *et al*., 1997; Marr *et al*., 1995; Buckley *et al*., 1982). The decreased growth seen in the second copper experiment may be attributable to increased metabolic costs of intestinal cellular changes and copper excretion in fish exposed to elevated dietary copper concentrations.

In contrast to dietary copper exposure, elevated dietary cadmium concentrations had no effect on growth in Atlantic salmon (Lundebye *et al*., in preparation). To the authors' knowledge, no information is available in the literature regarding growth inhibition in fish exposed to dietary cadmium.

Conclusion

Previous studies on the dietary toxicity of copper with salmonids established toxic levels (deduced from reduced growth, feed conversion and increased mortality) at 700 mg Cu kg^{-1} diet (Lanno *et al*., 1985). However, the first copper experiment revealed stress responses (including induction of intestinal apoptosis and cell proliferation coupled with retarded growth) at a dietary copper concentration well below the previously established toxic concentration (35 vs 700 mg Cu kg^{-1} diet). Results from the present studies indicate that cellular biomarkers have potential as early warning signs of negative effects on the overall fitness of an organism.

There is a need for further information regarding the availability and toxicity of trace metals in fish feed in order to optimise fish health, avoid excessive levels of available nutrients and toxicants in diets and thereby reduce the environmental impact of waste feed from aquaculture systems.

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