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# Effects of Waterborne Copper, Cyanide, Ammonia, and Nitrite on Stress Parameters and Changes in Susceptibility to Saprolegniosis in Rainbow Trout (*Oncorhynchus mykiss*)

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**The effects of toxic exposures on the susceptibility of rainbow trout (*Oncorhynchus mykiss*) to saprolegniosis were evaluated. Fish were exposed to sublethal concentrations of copper (0.25 mg/liter), cyanide (0.07 mg/liter), ammonia (0.5 mg/liter), and nitrite (0.24 mg/liter) for 24 h. After exposure, the fish were challenged by *Saprolegnia parasitica* ($3.6 \times 10^6$ zoospores per liter) for 10 min. Cortisol and cholesterol were used to indicate stress response. Similar increases of cortisol were found for the four tested chemicals. All fish with cortisol levels higher than 370 ng/ml developed the disease, while only 24% of the fish with cortisol levels lower than 370 ng/ml were infected. Cholesterol levels remained unchanged after toxic exposure. Increased susceptibilities to the pathogen were observed for ammonia (71%), copper (57%), nitrite (50%), and cyanide (33%). The increases in susceptibility as a result of cyanide and nitrite exposure could be explained by the stress response. For copper and ammonia, the combination of two different effects, the stress response and specific impairments of the defense mechanism of trout against saprolegniosis, should be considered.**

The presence of sublethal concentrations of noxious chemicals in freshwater environments can promote the emergence and development of infectious diseases in fish (6, 18, 32). This effect can be explained by two different mechanisms. The first includes direct effects on immunological function (19, 38); the second is indirect and related to stress (26, 46). The stress hormone cortisol affects the immune response (17, 29). It has been suggested that only chronic stress is responsible for impairment of immunity compatible with the development of disease, while acute stress would not exert a significant effect on immunocompetence (29). However, different studies reveal the importance of acute stress in the increased susceptibility to diseases (9, 15, 18).

Ammonia, nitrite, copper, and cyanide are common pollutants in aquatic environments, and their lethal and sublethal toxicities for salmonids are well documented (1, 16, 37, 40). While data on the effect of cyanide and nitrite on the fish immune system are scarce, the effects of copper and ammonia have been reported (7, 12, 18, 32).

Mycotic dermal infections in salmonid fish are caused by *Saprolegnia parasitica* (syn. *S. diclina* type 1) and other *Saprolegnia* spp., characterized by groups of long hooked hairs on the secondary zoospore cyst (31). Some predisposing factors increasing the susceptibility of salmonids to *Saprolegnia* infection have been described previously (30). These factors included sexual maturation, integument damage, stress, and the presence of other pathogenic agents. Toor et al. (39) found high levels of organic manure to be a predisposing environmental factor to outbreaks of hemorrhagic septicemia and saprolegniosis, but additional data suggested that this effect could be related to the specific pollutant usually found at toxic concentrations in such situations (6).

The objective of this study was to determine the influence of acute sublethal toxic exposures on saprolegniosis susceptibility in rainbow trout (*Oncorhynchus mykiss*). In addition, the study was designed to establish whether increased susceptibility can be explained by a stress response or is related to direct effects on defense mechanisms. Cortisol was measured as a primary stress response; cholesterol levels were used as a measure of metabolic disturbance.

## MATERIALS AND METHODS

**Experimental conditions. (i) Fish.** *O. mykiss* (weight, 25 to 35 g) were obtained from a fish farm in northeastern Spain. The fish were acclimatized to laboratory conditions for 1 week in 350-liter glass aquaria. Aquaria were supplied with a continuous flow of dechlorinated tap water. Water quality characteristics were analyzed by standard procedures (2, 34). The average water quality conditions were as follows: pH, 7.5; temperature, 10°C; dissolved oxygen concentration, 7.4 mg of $O_2$ per liter; total hardness, 28.4 mg of $CaCO_3$ per liter; alkalinity, 32.6 mg of $CaCO_3$ per liter; unionized ammonia, 0.004 mg/liter; nitrite, 0.007 mg/liter. Variation coefficients were lower than 10% for all parameters. Fish were marked with cotton color fiber.

**(ii) Chemical exposure.** Fifty fish were used. Groups of 10 fish each were exposed for 24 h to the 96-h 50% lethal concentration ($LC_{50}$) of each test pollutant, corresponding to the following nominal concentrations: copper, 0.25 mg/liter; cyanide, 0.07 mg/liter; ammonia, 0.5 mg/liter; nitrite, 0.24 mg/liter. Another group of 10 fish maintained under the same conditions but free of toxin exposure was used as a control. Exposures were run in 80-liter glass aquaria under static conditions. Real concentrations were analytically verified before introduction of fish, and deviations higher than 5% from the nominal values were not allowed.

Copper was added as copper nitrate (Merck, Darmstadt, Germany) solution and was analyzed with a model 3030B atomic absorption spectrophotometer (Perkin-Elmer, Überlingen, Germany) equipped with a model HG-300 graphite furnace. Cyanide was added as potassium cyanide (Merck) solution and was analyzed with a cyanide-selective electrode (Orion, Cambridge, Mass.) calibrated with a potassium cyanide solution. Ammonia was added as ammonium chloride (Merck) solution, and total ammonia ($NH_3$-N) was analyzed with an ammonia-selective electrode (Orion) calibrated with ammonium chloride solution. The $NH_3$ concentration was calculated from published equations (1, 10). Nitrite was added as potassium nitrite (Merck) solution and was analyzed by a standard method (34).

**Pathogen challenge. (i) Infective agent.** *S. parasitica* CM-2101a (Commonwealth Mycological Institute, Kew, England) isolated from infected trout was used in this study. Stock cultures were maintained on slants of glucose-peptone agar (45), kept at 8 to 12°C, and subcultured every 3 to 6 months.

Secondary zoospore suspensions, used for the experimental infection of fish, were obtained by the method of Willoughby and Pickering (45) and Willoughby et al. (44). Hemp seeds colonized by the fungi were incubated in sterile tap water at 20 ± 1°C for 2 days. Water surrounding the seeds contained motile secondary

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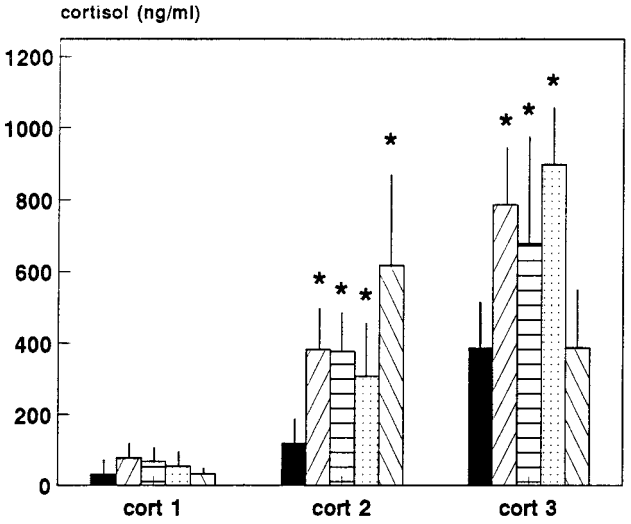


FIG. 1. Changes in cortisol levels in plasma in *O. mykiss* before toxin exposure (cort 1), after chemical exposure for 24 h (cort 2), and after pathogen challenge (cort 3). Values are arithmetic means ± standard errors of the means ($n = 10$). *, significant differences ($P < 0.01$) from control group. Symbols: ■, control; ▨, copper; ▤, cyanide; ▥, ammonia; ▧, nitrite.

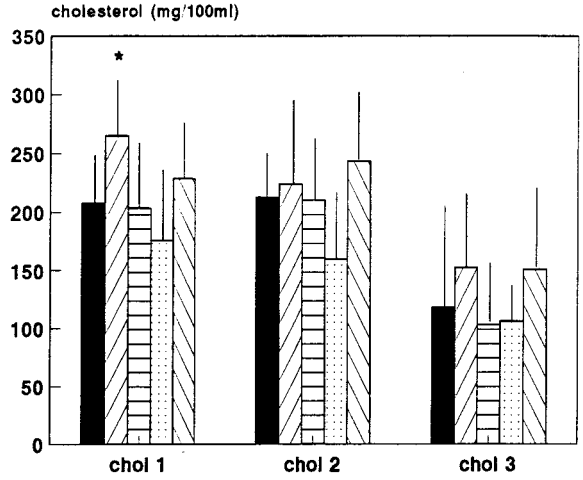


FIG. 2. Changes in cholesterol levels in plasma in *O. mykiss* before toxin exposure (chol 1), after chemical exposure for 24 h (chol 2), and after pathogen challenge (chol 3). Values are arithmetic means ± standard errors of the means ($n = 10$). *, significant difference ($P < 0.01$) from control group. Symbols as in Fig. 1.

zoospores. Zoospore suspensions were obtained by filtering this water through double layers of Whatman 541 filter paper. Concentrations were estimated by inoculating 0.1 ml of different dilutions of spore suspension on glucose-peptone agar plates and incubating at 20 ± 1°C for 8 to 12 h. For each dilution, an average count was made from four plates.

**(ii) Infection.** The challenge method has been described elsewhere (6). After toxin exposure, fish were anesthetized in 0.03% 2-phenoxyethanol and scale removal from each side above the lateral line and near the peduncle caudal was made before challenge to *S. parasitica* as recommended by Singhal et al. (36) and Nolard-Tintigner (25). Animals were immersed for 10 min in the secondary-zoospore suspension (25, 36). The zoospore concentration was estimated at $3.6 \times 10^6$ zoospores per liter. The control group was also challenged.

Five 120-liter glass flowthrough aquaria were used to transfer each group of pathogen-exposed fish. The fish were observed daily. Infection was estimated by the presence of cottony white patches on the surface of the fish and diagnosed on GY-PS agar plates by the method on Willoughby and Pickering (45). Susceptibility was estimated by the percent morbidity (11).

**Sampling and analysis of blood.** Blood samples from each fish, including controls, were obtained at three different times: before toxin exposure, immediately after toxin exposure, and at the end of the experiment. The end of the experiment was either after diagnosis of saprolegniosis in infected fish or 15 days after challenge in noninfected fish. Fish were anesthetized with 0.03% 2-phenoxyethanol and bled by venipuncture of the tail. Blood samples (0.5 ml) were collected in heparinized tubes. Tubes were centrifuged at 1,200 × *g* for 5 min, and plasma was removed and stored at −70°C until analysis.

Cortisol levels in plasma were measured by an immunological method (Sibar, Perugia, Italy) (4). Cholesterol levels in plasma were analyzed spectrophotometrically (at 365 nm) with a test kit (Merckotest; Merck) by the CHOD-iodide method (33).

**Statistical analysis.** Data were statistically analyzed by one-way analysis of variance. Significant differences were established at the $P < 0.05$ level. Plasma measurements in treated and control groups of fish were compared by Student's *t* test ($P < 0.05$). Analyses were made with Microstat and BMDP software.

## RESULTS

**Stress parameters.** The stress parameter levels are indicated in Fig. 1 and 2. The first set of values correspond to samples collected before the chemical exposure (cort 1 and chol 1). The analysis of variance revealed no significant differences between groups as regards cortisol values. However, significant differences ($P < 0.05$) were observed for cholesterol. Fish used for copper exposure had higher initial values than did those in the control group ($t$, −2.01; 14 degrees of freedom; $P < 0.05$).

After exposure, a large increase in plasma cortisol levels (cort 2) was observed for all groups. The control group had a mean level of 120 ng/ml, while all exposed groups had mean values above 300 ng/ml. Significant differences ($P < 0.001$) were observed for both the analysis of variance ($P < 0.001$) and Student's *t* test between control and each exposed group. No significant differences were observed for cholesterol values.

At the end of the experiment (2 to 4 days after challenge for infected fish, 15 days after challenge for noninfected fish), the analysis of variance showed significant differences among groups ($P < 0.01$) for cortisol (cort 3) levels. Student's *t* test showed significant differences versus control for copper-, cyanide-, and ammonia-exposed fish but not for the nitrite-exposed group. No significant differences were observed for cholesterol values.

**Infection.** The results of the *Saprolegnia* challenge are as follows. Infection occurred in all groups, with the percentage of infected fish being highest in the group of fish exposed to ammonia (71%), followed by copper (57%), nitrite (50%), and cyanide (33%). The infection incidence in control fish was 22%.

Infection was clearly evident as cottony white mycelial masses which appeared 2 to 4 days after challenge on the descaled areas of the fish. Cultures of fungi isolated from the fish were analyzed, and typical *S. parasitica* characteristics were recorded.

**Stress parameters and challenge response.** Figures 3 and 4 show the evolution of cortisol and cholesterol levels, respectively, in plasma of fish grouped by their final response to the challenge (infected or noninfected). The evolution in both groups is quite similar, with increases in the cortisol level after the toxic exposure, additional increases in the cortisol level at the end of the experiment, and reductions in the cholesterol level at this last point. However, not all these changes were statistically significant.

The differences in cort 3 values can be explained by different reasons, but what is really important is the significant difference in cort 2 values between infected and noninfected fish, i.e., the values measured between the exposure to a toxic chemical and the challenge with the infection agent. These data can be observed at the individual level.

Figure 5 shows the frequency distribution histogram of cortisol levels after chemical exposure (immediately before chal-

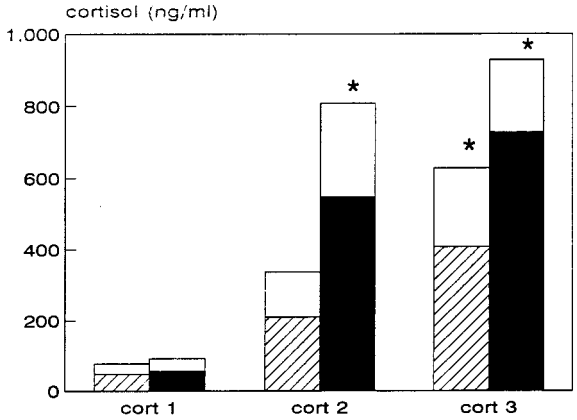


FIG. 3. Cortisol levels in *O. mykiss*, grouped in infected (■) and noninfected (▨) fish, before toxin exposure (cort 1), after toxin exposure (cort 2), and after pathogen challenge (cort 3). Values are arithmetic means ± standard errors of the means (□) (II). *, significant differences ($P < 0.01$) between groups.

lenge). It can be observed that all fish with prechallenge cortisol levels higher than 370 ng/ml were infected by *S. parasitica*, while a large percentage of fish with cortisol levels below 370 ng/ml were not infected.

Grouping by the chemical, all infected fish in the cyanide- and nitrite-exposed groups had prechallenge cortisol levels above 370 ng/ml. For ammonia and copper exposures, the percentages of infected fish with prechallenge cortisol levels above 370 ng/ml were 40% and 75%, respectively.

## DISCUSSION

Exposure to the four tested chemicals, ammonia, copper, nitrite, and cyanide, led to significant increases in cortisol levels in plasma and, after the challenge, to increases in the susceptibility of rainbow trout to saprolegniosis. Data are in agreement with previous reports. Ammonia, nitrite, and copper have been related to increases in susceptibility to different parasitic, bacterial, and viral fish diseases (6, 12, 14, 15, 18, 32), while data on the role of cyanide are scarce.

The concentrations were chosen to have a similar toxicological significance, i.e., 24 h of exposure to the 96-h $LC_{50}$, and in fact they led to similar cortisol levels in plasma: 383 ± 179 ng/ml for copper, 378 ± 107 ng/ml for cyanide, 308 ± 174 ng/ml for ammonia, and 610 ± 306 ng/ml for nitrite. The higher value observed for nitrite is not significantly different, because of the higher variability observed for this chemical than for the others. However, the percentage of infected fish was different depending on the toxic material, ranging between 71 and 33% (the highest percentage was for ammonia exposure, followed by copper, nitrite, and cyanide). Differences between the mechanisms responsible for lethality and those responsible for sublethal effects have been reported for copper and ammonia (7, 20, 37). This also seems to be the case for saprolegniosis susceptibility.

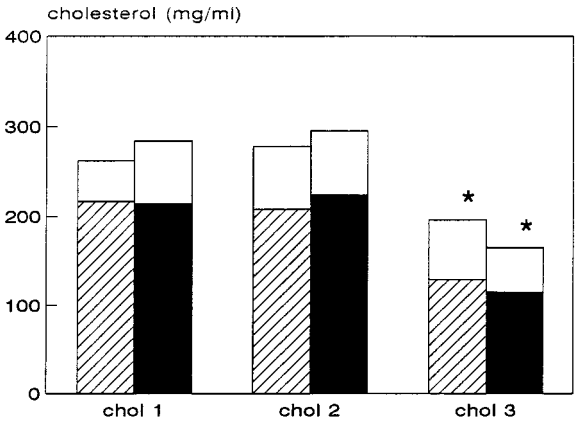


FIG. 4. Cholesterol levels in *O. mykiss*, grouped in infected (■) and noninfected (▨) fish, before toxin exposure (chol 1), after toxin exposure (chol 2), and after pathogen challenge (chol 3). Values are arithmetic means ± standard errors of the mean (□) (II). *, significant differences ($P < 0.01$) between groups.

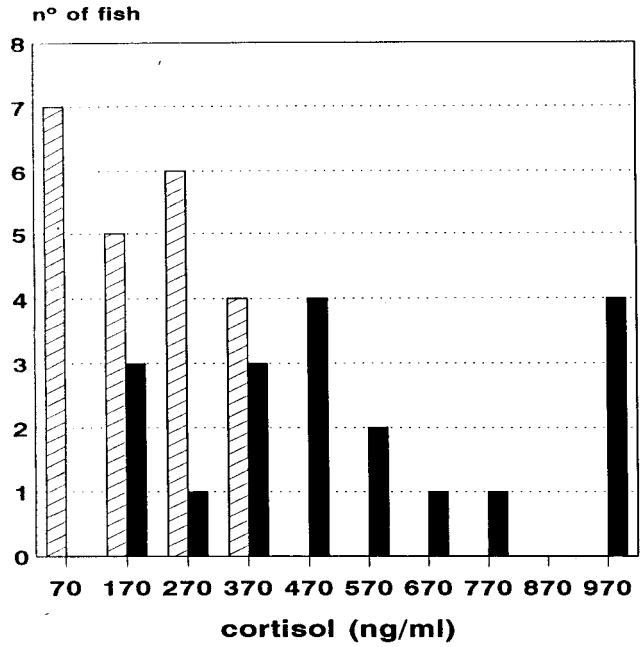


FIG. 5. Frequency distribution histogram of cortisol levels in *O. mykiss* before chemical exposure. Fish are grouped in infected (■) and noninfected (▨) groups.

The challenge conditions were close to the limit of infection, as reflected by the lack of mortality and the low percentage of infection reported for the control group. This situation has been previously reported as highly suitable for the estimation of susceptibility increases related to exposure to toxins (11). The conditions were achieved by using a concentration of fungal inoculum 100 times higher than the infective concentration for mature fish (45) and the scale removal procedure recommended by several author (25, 36). The observations are in agreement with the high resistance of immature trout to saprolegniosis (30).

The levels of cortisol in plasma prior to exposure, 30 to 77 ng/ml, were similar to that usually observed in nonstressed salmonids (5, 27, 41) and did not show differences among groups. Regarding cholesterol levels in plasma, the single set of data found for rainbow trout, published by our own group (23), was slightly lower than that observed in this experiment. Nevertheless, the differences can be explained by the role of nutritional status, including cholesterol levels in food, in the values obtained (21).

The evolution of cortisol and cholesterol levels in plasma confirmed that challenged fish were under acute but not chronic stress and corroborated previous data indicating that

acute stress can have a significant role in the increase of disease susceptibility in fish (9, 15, 18). The absence of chronic stress was checked by using cholesterol levels (5). Unlike to physical stressors, toxic chemicals, even after short-term exposure, can lead to chronic stress situations as a result of the effects of the chemical that remains in the system after exposure.

The set of individual data presented in the histogram (Fig. 5) clearly shows that the development of saprolegniosis was related mainly to cortisol levels in plasma. A cortisol level of 370 ng/ml was the borderline value between infected and noninfected groups of fish. Nevertheless, 25% of fish with cortisol levels below this limit were infected, suggesting individual variations or the role of additional factors. Wiik et al. (42) reported that levels of 275 ng of cortisol per ml in plasma increase the susceptibility of Atlantic salmon to *Vibrio salmonicida*, while Pickering and Duston (28) found increased susceptibility of brown trout (*Salmo trutta*) to saprolegniosis and furunculosis at cortisol levels of 130 ng/ml.

All infected fish from the groups exposed to either cyanide or nitrite but only 75% exposed to copper and 40% exposed to ammonia had cortisol levels higher than the 370-ng/ml limit when challenged. It must be considered that ammonia and copper exposures led to the highest increases in the incidence of infection.

These data suggest a dual explanation for the increase in susceptibility observed for the tested chemicals. The stress response is responsible for the susceptibility increase observed for cyanide and nitrite but not for the other toxins. Part of the effects observed for copper and ammonia can be explained by toxicological mechanisms different from the stress responses. This hypothesis is supported by specific toxic effects that could be related to the defensive mechanisms of fish against saprolegniosis. Three different defense mechanisms has been reported: (i) elimination of attached spores by mucus renewal (24), (ii) fungistatic activity in mucus (43), and (iii) a cellular immune response (35, 43, 47). Ammonia interferes with mucus renewal by retarding mucus production by the mucous cells (20) and reducing the presence of defensive substances in the mucus (13, 22). Copper has been reported to be immunotoxic and to decrease humoral (3) and cellular (8) immune responses, including the level of circulating lymphocytes (8) and the phagocytic response (9).

As preliminary conclusions, this work shows that toxicologically equivalent exposures to ammonia, copper, cyanide, and nitrite produce a similar acute stress response in rainbow trout. When these stressed fish were challenged with *S. parasitica*, different increases in the percentage of infected fish were observed; these percentages were highest for ammonia, followed by copper, nitrite, and cyanide. The acute stress response provoked by the toxin exposure accounts for the main contribution to the increase in saprolegniosis susceptibility, representing approximately a 100% increase in the percentage of infected fish when compared with the control group. The higher responses observed for copper and ammonia can be explained by the additional contribution of specific toxic mechanisms that are able to impair the protective mechanisms of trout against saprolegniosis. The discovery of different sublethal effects previously described for ammonia and copper support this suggestion.

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