Cadmium disrupts behavioural and physiological responses to alarm substance in juvenile rainbow trout (Oncorhynchus mykiss)

Graham R. Scott1,*, Katherine A. Sloman1, Claude Rouleau2 and Chris M. Wood1

1Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada and
2National Water Research Institute, PO Box 5050, 867 Lakeshore Road, Burlington, Ontario L7R 4A6, Canada

*Author for correspondence (e-mail: scott@zoology.ubc.ca)

Accepted 5 March 2003

Summary

Alarm substance is a chemical signal released from fish skin epithelial cells after a predator causes skin damage. When other prey fish detect alarm substance by olfaction, they perform stereotypical predator-avoidance behaviours to decrease predation risk. The objective of this study was to explore the effect of sublethal cadmium (Cd) exposure on the behavioural and physiological responses of juvenile rainbow trout (Oncorhynchus mykiss) to alarm substance. Waterborne exposure to 2 μg Cd l⁻¹ for 7 days eliminated normal antipredator behaviours exhibited in response to alarm substance, whereas exposures of shorter duration or lower concentration had no effect on normal behaviour. Furthermore, dietary exposure to 3 μg Cd g⁻¹ in the food for 7 days, which produced the same whole-body Cd accumulation as waterborne exposure to 2 μg l⁻¹, did not alter normal behaviour, indicating that an effect specific to waterborne exposure alone (i.e. Cd accumulation in the olfactory system) results in behavioural alteration. Whole-body phosphor screen autoradiography of fish exposed to ¹⁰⁹Cd demonstrated that Cd deposition in the olfactory system (rosette, nerve and bulb) during waterborne exposure was greater than in all other organs of accumulation except the gill. However, Cd could not be detected in the brain. A short-term elevation in plasma cortisol occurred in response to alarm substance under control conditions. Cd exposures of 2 μg l⁻¹ waterborne and 3 μg g⁻¹ dietary for 7 days both inhibited this plasma cortisol elevation but did not alter baseline cortisol levels. Our results suggest that exposure to waterborne Cd at environmentally realistic levels (2 μg l⁻¹) can disrupt the normal behavioural and physiological responses of fish to alarm substance and can thereby alter predator-avoidance strategies, with potential impacts on aquatic fish communities.

Key words: quantitative autoradiography, cortisol, fish, Oncorhynchus mykiss, behaviour, metal, olfaction, predator avoidance, alarm pheromone.

Introduction

Chemical alarm signalling systems were first described by von Frisch (1938) and have since been extensively studied (see review by Smith, 1992). Chemical alarm signals have been traditionally ascribed to the superorder Ostariophysi, which includes the minnows, characins and catfishes. This system is characterized by a chemical signal called alarm substance, which is released from specialized epidermal cells in fish skin when attack by a predator causes sufficient skin damage. Other prey fish detect alarm substance by olfaction (Chivers and Smith, 1993) and exhibit stereotypical predator avoidance behaviours that decrease their predation risk (see reviews by Smith, 1992; Chivers and Smith, 1998). Responses to alarm substance by individuals or groups of fish may include several component behaviours (e.g. dashing, freezing, schooling or hiding), the nature of which depends on the species in question and the environmental conditions. Recent evidence has suggested that chemical alarm signalling systems are present in salmonid fish (Brown and Smith, 1997, 1998; Mirza and Chivers, 2001). Indeed, salmonids respond behaviourally to alarm substance by decreasing swimming and feeding activities when observed under laboratory conditions. The adaptive significance of these behaviours to prey fish in natural environments presumably involves being inconspicuous to predators. Importantly, the behavioural responses to alarm substance increase survival during encounters with predators (Chivers et al., 2001).

As well as the immediate behavioural responses to alarm substance, there also exist physiological responses that enable prey fish to cope with predation stress. The stress response can be divided into two general routes of action (see review by Wendelaar Bonga, 1997). The hypothalamo–sympathetic–chromaffin cell axis mediates the immediate release of catecholamines into the circulation, which increases cardiac output, blood flow to muscle and gills, respiration rate and mobilization of energy reserves. The hypothalamo–pituitary–interrenal cell axis mediates the release of cortisol into the circulation, which similarly

1779
mobilizes energy during periods of stress (reviewed by Wendelaar Bonga, 1997). Many different aspects of the integrated stress response have been observed in fish after detection of alarm substance, including elevated plasma cortisol and glucose (Rehnerg et al., 1987), increased respiration rate (Lebedeva et al., 1993) and sharpened optical alertness (indicated by dorsal light responsiveness; Pfeiffer and Riegelbauer, 1978).

Several studies have illustrated the sensitivity of olfaction to toxicants, including cadmium (Brown et al., 1982; Stromberg et al., 1983), copper (Hara et al., 1976; Brown et al., 1982; Rehnerg and Schreck, 1986; Juliard et al., 1995; Hansen et al., 1999), diazinon (Moore and Waring, 1996) and mercury (Hara et al., 1976; Brown et al., 1982; Rehnerg and Schreck, 1986). It has recently become apparent that olfactory disruption by sublethal toxicant exposure may consequently disturb olfaction-mediated predator avoidance behaviours of fish. Examples include copper (Beyers and Farmer, 2001), diazinon (Scholz et al., 2000), atrazine and diuron (Saglio and Triage, 1998). Due to the importance of olfaction in the predator avoidance strategy of numerous fish species, any toxicant that disrupts behavioural or physiological responses to alarm substance could impair the success of prey fish populations. Cadmium (Cd) is an anthropogenic trace metal pollutant of surface waters, occurring primarily as a result of industrial activity. Cd is extremely toxic to aquatic animals, with concentrations producing lethality that are lower than for many other metals (Canadian Council of Ministers of the Environment, 1999). The acute toxicity of Cd is due to its actions as a calcium antagonist, and its pathological effects thus tend to be less severe at higher water calcium levels (i.e. water hardness; Wood, 2001). Uptake of Cd during waterborne exposure occurs primarily at the gill, where it enters through La³⁺-sensitive apical calcium channels in chloride cells and subsequently inhibits basolateral high affinity Ca²⁺-ATPase (Verboest et al., 1987, 1989; Wicklund Glynn et al., 1994; Craig et al., 1999). By contrast, uptake of Cd during dietary exposures occurs primarily by the gastrointestinal tract (Szebedinsky et al., 2001), although its mechanism of action at this tissue appears to be similar to that at the gill (Schoenmakers et al., 1992). Cd can remain and accumulate in the respective uptake tissue during waterborne or dietary exposure but has also been shown to enter the circulation and accumulate to a significant extent in the liver and kidney (McGeer et al., 2000; Szebedinsky et al., 2001).

An additional uptake route of Cd during waterborne exposure in fish is the olfactory rosette, as demonstrated by autoradiography. Cd readily crosses the olfactory epithelium and accumulates in the olfactory bulb after anterograde axonal transport along the olfactory nerve (Tjälve and Gottofre, 1986; Gottofre and Tjälve, 1991; Tjälve and Henriksson, 1999). This transport is facilitated by metallothionein complexation (Tallkvist et al., 2002). However, Cd does not accumulate in other regions of the brain and does not enter central nervous tissue from the circulation, indicating that it cannot cross the blood–brain barrier or synapses in the olfactory bulb (Evans and Hastings, 1992; Szebedinsky et al., 2001). Therefore, if Cd accumulation in the olfactory rosette, nerve or bulb impairs olfactory function, then detection of alarm substance will be inhibited by waterborne but not dietary Cd exposure.

Previous studies have shown Cd exposure to decrease prey fish survival when subjected to an unexposed predator (Sullivan et al., 1978). The objectives of this study were to examine the effects of both waterborne and dietary sublethal Cd exposure on the behavioural and physiological responses of juvenile rainbow trout to skin extract (a skin homogenate preparation from ruptured skin cells). In doing so, possible behavioural and physiological mechanisms through which cadmium increases prey susceptibility to predation were explored. Three separate sets of experiments were conducted. In the first, the effect of different Cd exposure regimes, at concentrations of environmental relevance, on the behavioural responses to skin extract (swimming activity, feeding activity and use of shelter) was determined. In the second, Cd accumulation in the olfactory system was visualized and quantified using phosphor screen autoradiography. Finally, the normal plasma cortisol and ion responses to skin extract in rainbow trout and the effect of sublethal Cd exposure on these responses were explored. Our specific hypothesis was that waterborne Cd inhibits the detection of alarm substance by inhibiting olfaction, thus interfering with the ability of juvenile rainbow trout to respond properly to alarm substance.

Materials and methods

Experimental animals

Juvenile rainbow trout Oncorhynchus mykiss Walbaum were obtained from Humber Springs trout hatchery (Orangeville, ON, Canada) and held in 300-litre flow-through tanks supplied with dechlorinated Hamilton City tapwater (hardness, 120 mg l⁻¹ as CaCO₃; Na⁺, 13.8 mg l⁻¹; Cl⁻, 24.8 mg l⁻¹; Ca²⁺, 40 mg l⁻¹; temperature, 12°C; pH, 8.0; dissolved organic carbon, 3 mg l⁻¹; natural background Cd concentration, 0.02 μg Cd l⁻¹), using a 12 h:12 h L:D photoperiod. All experiments were carried out in dechlorinated Hamilton tapwater. Fish were held for at least two weeks before experiments were performed and were fed commercial trout pellets (Martin’s Trout Feed: 42% crude protein, 16% crude fat, 40% crude carbohydrate, 0.35% sodium, 1% calcium, 0.65% phosphorus; measured background Cd content, 0.184±0.001 μg Cd g⁻¹, mean ± s.e.m., N=3; Martin Feed Mill, Elmira, ON, Canada) at a 1% daily ration (food mass/wet body mass).

Skin extract preparation

Skin extract was prepared according to the method of Brown and Smith (1998). For each of 11 skin extract preparations, 20 juvenile rainbow trout (2.4±0.1 g, mean ± s.e.m., N=220) were selected and sacrificed immediately with a sharp blow to the head. Skin was removed from both sides of each fish and rinsed with distilled deionized water (DDW), then placed in 50 ml
DDW on ice. A total of 4.3±0.7 g (N=11) of skin was collected for each preparation. The skin–water mixture was homogenized and filtered through glass wool. The filtrate was then brought to a final volume of 400 ml by adding DDW. Skin extract preparations were stored in either 30-ml or 200-ml samples at −20°C until use. 30-ml and 200-ml DDW samples were also frozen at −20°C to be used as control stimulus.

Cadmium exposures

To achieve nominal flow-through waterborne Cd exposure concentrations, a 3.7-litre header tank was fed with control water at a flow rate of 1.5 l min−1. Cd stock [of appropriate Cd(NO₃)₂·4H₂O concentration for each exposure regime; Fisher Scientific, Nepean, ON, Canada] acidified to 0.1% with nitric acid (approximately 0.02 mol l−1 HNO₃; trace metal analysis grade; Fisher Scientific) was added drop-wise at a rate of 0.5 ml min−1 to the header tank using a piston pump (Fluid Metering, Syosset, NY, USA). The header tank outlet then fed two exposure tanks at a flow rate of 0.75 l min−1. Water samples were taken regularly (approximately every day) to verify the nominal water Cd concentrations, and fish were fed control diets (1% daily ration) during waterborne exposure periods.

Dietary Cd exposures were performed in the same exposure tanks as waterborne exposures, but tanks were fed with control water. A 7-day 3 µg g⁻¹ dietary Cd exposure period (at 1% daily ration) was chosen based on preliminary experiments, which showed that this exposure achieved the same whole-body Cd burden as a 7-day exposure to 2 µg l⁻¹ waterborne Cd in 2.5 g rainbow trout (2 µg l⁻¹; 52.6±5.0 ng Cd g⁻¹ fish wet mass, N=14; 3 µg g⁻¹; 64.8±9.9 ng Cd g⁻¹ fish wet mass, N=8; P=0.297). Cd-containing food was prepared according to Szebedinsky et al. (2001) by mixing appropriate amounts of Cd(NO₃)₂·4H₂O into commercial trout food. Trout pellets were ground in a blender and hydrated with approximately 50% (water volume/food mass) DDW. Cd was dissolved in DDW, added to the hydrated food, and the paste was then mixed for at least 1 h. Food paste was extruded to desired thickness (same as control food) using a commercial pasta maker (Popiel Ronco, Chatsworth, CA, USA) into long strings. Food was dried at room temperature for 48 h and broken into small pellets, and the nominal Cd content was verified using atomic absorption spectrophotometry (see below). Control food was prepared in the same manner without the addition of Cd. Water samples were collected daily during dietary Cd exposures to verify that the fish received negligible waterborne exposure. Both flow-through waterborne and dietary exposures were always followed by 2 days depuration in control water (allowing fish time to settle after tank transfer before behavioural observations began; see below). For simplicity, the term ‘exposure’ is used throughout to indicate Cd exposure only, and not exposure of fish to skin extract.

Experiment 1: effect of cadmium on behavioural responses to skin extract

Experimental rainbow trout (2.5±0.1 g, mean ± S.E.M., N=96) were either subjected to control conditions (unexposed to Cd) or exposed four at a time to sublethal concentrations of Cd. A total of 16 fish were subjected to each of the following Cd exposures (summarized in Table 1): (1) 1-day waterborne exposure to 2 µg Cd l⁻¹ (measured concentration, 2.3±0.06 µg Cd l⁻¹; N=24); (2) 7-day waterborne exposure to 0.5 µg Cd l⁻¹ (0.56±0.01 µg Cd l⁻¹; N=40); (3) 7-day waterborne exposure to 2 µg Cd l⁻¹ (2.06±0.08 µg Cd l⁻¹; N=34); and (4) 7-day dietary exposure to 3 µg Cd g⁻¹ food (3.18±0.15 µg Cd g⁻¹; N=6) at 1% daily ration (measured waterborne [Cd], 0.05±0.02 µg Cd l⁻¹; N=15). Less than 5% mortality occurred for all exposures.

At the end of the exposure period, trout were transferred individually to 7-litre flow-through glass observation tanks (Fig. 1). Tanks contained a commercial pebble substrate, approximately 2 cm deep, and a shelter consisting of a ceramic tile (10 cm×10 cm) mounted on four ceramic legs (10 cm long). An air stone and inlet water tube were located at the end of the tank containing the shelter. The water outlet and introduction point for food and alarm substance were located at the opposite end of the tank, and the entire tank was surrounded with black plastic to minimize disturbance of the fish.

Fish were allowed to settle for 48 h in the observation tanks after tank transfer (depuration period; see above) and were fed to satiation with control feed 20–24 h before the 20-min observation period began. During the observation period, inlet water flow was shut off. Observations were conducted in a similar fashion to those of Brown and Smith (1997) and Mirza and Chivers (2001). Trials consisted of a 10-min pre-stimulus and a 10-min post-stimulus observation period. One 30-ml stimulus sample (either skin extract or DDW) was added after the pre-stimulus period using a glass funnel. Juvenile rainbow trout tested were of three main categories (see Table 1): (1) unexposed to Cd, DDW stimulus (DDW control); (2) unexposed to Cd, skin extract stimulus (skin extract control); and (3) exposed to Cd, skin extract stimulus (experimental, four different Cd exposures). During the pre-stimulus and post-stimulus periods, one control food pellet was added every

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cd exposure</th>
<th>Durationa</th>
<th>Stimulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (control)</td>
<td>–</td>
<td>–</td>
<td>DDW</td>
</tr>
<tr>
<td>2 (control)</td>
<td>–</td>
<td>–</td>
<td>SE</td>
</tr>
<tr>
<td>3</td>
<td>2 µg l⁻¹ waterborne</td>
<td>1 day</td>
<td>SE</td>
</tr>
<tr>
<td>4</td>
<td>0.5 µg l⁻¹ waterborne</td>
<td>7 days</td>
<td>SE</td>
</tr>
<tr>
<td>5</td>
<td>2 µg l⁻¹ waterborne</td>
<td>7 days</td>
<td>SE</td>
</tr>
<tr>
<td>6</td>
<td>3 µg g⁻¹ dietaryb</td>
<td>7 days</td>
<td>SE</td>
</tr>
</tbody>
</table>

DDW, distilled deionized water; SE, skin extract.

aEach exposure was followed by a 2-day depuration period in control water.
b3 µg Cd g⁻¹ dietary exposure for 7 days achieves the same whole-body cadmium burden as 2 µg l⁻¹ waterborne cadmium exposure for 7 days (for both 2.5 g and 30 g rainbow trout; see Materials and methods and Results, respectively).

Table 1. Experimental combinations of cadmium (Cd) exposure and stimulus in Experiment 1
through exposures, see above) to a nominal concentration of were exposed in a 26-litre static exposure tank (unlike flow-

Experiment 2: determination of olfactory accumulation of $^{109}$Cd by autoradiography

Fourteen juvenile rainbow trout (18.3±1.0 g, mean ± S.E.M.) were exposed in a 26-litre static exposure tank (unlike flow-

Experiment 3: physiological response to skin extract and the effect of cadmium

A time-course study was conducted to determine the physiological responses of juvenile rainbow trout to skin
Cadmium and responses to alarm substance

Unexposed fish significantly increased performance of predator avoidance behaviours in response to skin extract compared with DDW controls. The change in the number of line crossings and food pellets consumed decreased when compared with DDW controls, while the change in the latency to first feeding and shelter use were unchanged (Fig. 2). However, the change in latency to first feeding significantly increased in response to skin extract compared with DDW controls, when considered alone using an unpaired t-test (P=0.001).

To determine the effect of Cd alone on quantified behaviours (before introduction of skin extract), pre-stimulus behaviours were compared between controls and all Cd exposures. Cd exposure did not significantly alter pre-stimulus midline crossings (overall mean ± S.E.M., 45±3; P=0.153; data not shown), feeding bites (overall, 6.7±0.3; P=0.183), latency to first feeding (overall, 115±18 s; P=0.429) or shelter use (overall, 108±17 s; P=0.231). Therefore, Cd exposure had no effect on the behavioural parameters quantified before introduction of stimulus.

Waterborne exposure to 2 µg Cd l⁻¹ for one week was the only Cd treatment that eliminated the normal behavioural response to alarm substance. The change in the number of line crossings, number of feeding bites and latency to first feeding...
between post-stimulus and pre-stimulus observation periods were all statistically indistinguishable from DDW controls (P=0.837, 0.969 and 0.860, respectively), indicating the elimination of a response to skin extract (Fig. 2). Furthermore, the changes in the number of line crossings and feeding bites were significantly higher than those of skin extract controls (P=0.001 and 0.028, respectively). Since all exposure periods were immediately followed by a 2-day settling period in control water, the effect of Cd observed on normal behaviour is present after two days of depuration.

For 2 μg Cd l⁻¹ exposure for one day, line crossings, feeding bites and latency changes in response to skin extract were all statistically different from DDW controls (P=0.006, 0.009 and 0.003, respectively) and statistically indistinguishable from skin extract controls (P=0.985, 0.973 and 0.779, respectively), indicating that this exposure regime did not inhibit the normal behavioural response to skin extract (Fig. 2). For 7-day exposure to 0.5 μg Cd l⁻¹, all behavioural responses were intermediate between DDW and skin extract controls, being statistically indistinguishable from both (DDW, P=0.112, 0.139 and 0.330; skin extract, P=0.999, 0.481 and 0.996) (Fig. 2). Dietary Cd exposure of 3 μg g⁻¹ for 7 days also had no effect on the performance of predator-avoidance behaviours. Changes in the number of line crossings, feeding bites and latency in response to skin extract were statistically different from those of DDW controls (P=0.023, 0.003 and 0.001, respectively) and statistically indistinguishable from those of skin extract controls (P=0.999, 0.981 and 0.567) (Fig. 2).

**Determination of olfactory accumulation of cadmium by autoradiography**

After 7 days of waterborne exposure, Cd accumulation was seen in gill, liver and kidney despite 2 days of depuration in control water, as indicated by qualitative autoradiogram inspection (Fig. 3). Cd accumulation was also observed in the olfactory rosette, nerve and anterior bulb but not in the rest of the brain. Unexposed fish ¹⁰⁹Cd activities were indiscernible from background and were assumed to be zero in all tissues. Cd accumulation was quantified in the liver and olfactory...
system for all exposures. Accumulation within the olfactory system was greatest in the rosette, followed by the nerve, then the bulb for every exposure duration (Fig. 4A). Accumulation in the rest of the brain was indistinguishable from background levels. Cd accumulation increased progressively with exposure for all three olfactory tissues ($P < 0.001$). After 2 days of
Fig. 4. (A) Accumulation of cadmium (Cd) after 0, 3, 5 and 7 days exposure, as well as 7 days exposure followed by a 2-day depuration period in control water, in the olfactory rosette, olfactory nerve and olfactory bulb. Calculated Cd accumulation (right-hand y-axis) was determined as described in Materials and methods. All points within each tissue are statistically distinct (P<0.05) before transfer to control water (depuration period). (B) Concentration index relative to mean fish liver concentrations for the olfactory rosette, olfactory nerve and olfactory bulb. * represents a significant difference between fish exposed for 7 days and those exposed for 7 days followed by 2 days of depuration (P<0.05).

depuration, Cd accumulation declined in the olfactory rosette (Fig. 4A), being significantly lower than the accumulation after 7 days of exposure (P=0.020). However, Cd accumulation in the olfactory nerve or bulb did not decrease significantly after 2 days of depuration (P=0.290 and 0.999, respectively). Saturation did not appear to occur in any of the three tissues before transfer to control water, as indicated by the continuing upward trend.

To compare Cd accumulation in the olfactory system with other body tissues, $^{109}$Cd activity in each tissue of the olfactory system was divided by the mean fish liver $^{109}$Cd activity to determine the concentration index (I; see Materials and methods). The ratio of Cd accumulation in the olfactory rosette to the liver changed throughout the exposure period (ANOVA, P=0.001; Fig. 4B). For 3- and 5-day exposures, the olfactory rosette accumulated approximately seven times more $^{109}$Cd than did the liver, whereas after 7 days of exposure the rosette only accumulated approximately five times more $^{109}$Cd than did the liver. Furthermore, this did not change after 2 days of depuration. By contrast, the ratios of $^{109}$Cd accumulation in the olfactory nerve and bulb (Fig. 4B) to the liver (four and three times higher, respectively) did not change with exposure duration since neither Ic changed with exposure time (P=0.132 and 0.722). These results indicate that Cd accumulation in olfactory tissue is significant when compared with other target organs. Indeed, when $^{109}$Cd accumulation was used to determine a calculated Cd accumulation for all exposure durations at 2 $\mu$g/l cold Cd concentration (right-hand y-axis in Fig. 4A), accumulation in olfactory tissues (approximately 65–110 ng Cd g$^{-1}$) was only exceeded by the gill (259 ng Cd g$^{-1}$) (Fig. 5), the major Cd uptake organ during waterborne exposure.

Physiological response to skin extract and the effect of cadmium

There was a significant effect of skin extract on plasma cortisol levels in fish not exposed to Cd (Fig. 6A). Cortisol levels were significantly elevated in response to skin extract, both 15 min and 30 min after stimulus introduction (P=0.045 and 0.010, respectively), rising to nearly four times resting cortisol levels. Sixty minutes after skin extract was introduced, plasma cortisol had returned to resting (control) levels (P=0.899). No effect of sampling order within each tank was observed (P≥0.261). Plasma sodium concentration increased throughout the sampling period (P=0.033), while plasma calcium concentration remained unchanged up to 60 min after introduction of alarm substance (P=0.429) (Fig. 6B).

There were significant inhibitory effects of both waterborne and dietary Cd on the plasma cortisol response to skin extract (Fig. 7). Plasma cortisol was significantly elevated in unexposed rainbow trout 15 min after introduction of skin
Cadmium and responses to alarm substance

Fig. 7. Plasma cortisol concentrations for rainbow trout exposed to alarm substance and control treatments. * represents a significant difference between fish given DDW stimuli and those given skin extract stimuli. † represents a significant difference from the control (P<0.05).

Discussion

The results of previous studies examining the behavioural response of juvenile rainbow trout to alarm substance were similar to the control trials in this experiment. Unexposed rainbow trout have been shown to decrease their swimming and feeding activities and increase their latencies to first feed and time spent under shelter in response to alarm substance (Brown and Smith, 1997; Mirza and Chivers, 2001). Although time spent under shelter in response to alarm substance also appeared to increase slightly in this study, this was not statistically significant. Brown and Smith (1997) have observed similar results, indicating that there exists a large degree of variability among individuals in the use of shelter for predator avoidance in juvenile rainbow trout.

Exposure to 2 μg Cd l⁻¹ for 7 days eliminated the behavioural response to alarm substance, and there appeared to be a small but statistically insignificant disruption of the behavioural response when fish were exposed to 0.5 μg Cd l⁻¹ for 7 days. One-day exposure to 2 μg Cd l⁻¹ appeared to have no effect on the performance of normal olfaction-mediated behaviours, showing that the observed behavioural effects were dependent on exposure duration. Seven-day dietary...
Exposure to 3 μg Cd g\(^{-1}\) did not alter normal alarm response behaviour. Since 2 μg l\(^{-1}\) waterborne and 3 μg g\(^{-1}\) dietary exposures both resulted in similar whole-body Cd accumulation after 7 days of exposure in 2.5 g rainbow trout (see Materials and methods), the difference between exposure routes may explain the different behavioural effects of each. During waterborne exposure, Cd uptake occurs primarily at the gills and olfactory system, both organs contacting the surrounding media. During dietary exposure, Cd uptake is across the intestinal wall, and Cd does not subsequently enter the brain or olfactory system from the circulation (Evans and Hastings, 1992). No changes in plasma ion concentrations resulted from either waterborne or dietary exposure regimes, and Cd is unlikely to cause respiratory toxicity at the concentrations used in this experiment (Hughes et al., 1979; Majewski and Giles, 1981), so behavioural alteration is not likely to be a result of Cd actions at the gills. The results of the present study therefore suggest that Cd inhibits the performance of predator avoidance behaviours through accumulation in the olfactory system. Disruption of olfactory function may explain previous observations that Cd exposure increases susceptibility of prey fish to predation (Sullivan et al., 1978). Cd has recently been shown to disrupt olfaction-mediated migratory behaviours in banded kokopu (Galaxias fasciatus; Baker and Montgomery, 2001) and to alter dominance behaviours in juvenile rainbow trout (Sloman et al., 2003) at similar waterborne Cd concentrations.

Using quantitative autoradiography, the calculated Cd accumulations in the olfactory rosette, nerve and bulb were all equal or greater than in either the kidney or liver, two major target organs of Cd accumulation (McGeer et al., 2000). Therefore, Cd accumulation in the olfactory system is substantial, supporting its possible role as an olfactory toxicant. Furthermore, two days of depuration in clean water did not remove significant amounts of Cd from the olfactory nerve or bulb. Therefore, if Cd does indeed inhibit olfaction, disruptive effects on olfaction-mediated fish behaviour may persist well after exposure has ceased. This is consistent with the behavioural observations discussed above, whereby 2 μg Cd l\(^{-1}\) waterborne exposure disrupted normal responses to alarm substance after two days of depuration in control water.

Low concentrations of Cd for relatively short exposure periods can therefore inhibit the normal behavioural responses of juvenile rainbow trout to alarm substance. Although the effects of Cd exposure between lab and field data may not be directly comparable due to possible differences in water chemistry, the waterborne Cd concentrations chosen for this set of experiments are similar to those measured in polluted waters of Canada, USA and Europe (Jensen and Bro-Rasmussen, 1992; Cabrera et al., 1998; Norris et al., 1999). Furthermore, concentrations chosen are in the range of current water quality guidelines for Cd in surface waters [2.5 μg l\(^{-1}\) and 0.29 μg l\(^{-1}\) for acute and chronic exposure, respectively, at 120 mg l\(^{-1}\) water hardness (US Environmental Protection Agency, 2001); 0.24 μg l\(^{-1}\) for chronic exposure (Canadian Council of Ministers of the Environment, 1999)]. Therefore, the ability of fish to respond to alarm substance with appropriate behaviours that reduce predation risk may be disrupted after similar Cd exposures in natural ecosystems.

To the best of our knowledge, the present study is the first to use phosphor screen autoradiography as a tool for quantification and description of differential tissue distribution of toxicants in fish. This technique is extremely effective when more traditional means of tissue toxicant burden determination are impractical (e.g. due to the size or inaccessibility of the tissue). Previous studies using similar waterborne Cd concentrations (1.0 μg l\(^{-1}\) and 10.0 μg l\(^{-1}\) for one week at approximately 40 mg l\(^{-1}\) water hardness as CaCO\(_3\)) have shown qualitatively that cadmium accumulates in the olfactory system of brown trout (Salmo trutta; Tjalve and Gottofrey, 1986). Furthermore, several other metals have been shown to accumulate in the olfactory system of fish (Rouleau et al., 1995, 1999; Tjalve and Henriksson, 1999). However, unlike some other metals, cadmium does not cross the blood–brain barrier or synapses in the olfactory bulb (Evans and Hastings, 1992), so does not accumulate in higher centres of the brain. It is evident in the autoradiograms of \(^{109}\)Cd accumulation (Fig. 3B) that Cd does not leave pre-synaptic neurons in the olfactory bulb. This possibly explains why past studies have suggested that Cd does not alter normal fish behaviour by disrupting brain function (e.g. neurotransmitter levels; Beauvais et al., 2001).

Previously, waterborne Cd has been shown to inhibit the bulbar electrical responses of adult rainbow trout to L-serine after 1- and 2-week waterborne exposures to 150 μg Cd l\(^{-1}\) but not after 2-week exposure to 50 μg Cd l\(^{-1}\), both at a water hardness of 90 mg l\(^{-1}\) as CaCO\(_3\) (Brown et al., 1982). Bearing in mind possible differences between our experimental design and the study by Brown et al. (1982), our results suggest that Cd may disrupt olfactory function at much lower waterborne concentrations when fish encounter natural odourants. This may be explained by differential activation of olfactory neuron regeneration after different toxicant exposures (see reviews by Hara, 1986; Laberge and Hara, 2001), which could influence the range of Cd concentrations that cause olfactory dysfunction. However, this suggestion remains to be studied.

Since plasma cortisol and ion changes in response to alarm substance have not previously been explored in rainbow trout, a time-course experiment was conducted. Plasma cortisol levels were significantly elevated compared with those of controls at 15 min and 30 min after stimulus introduction but returned to basal concentrations after 60 min. Rehnberg et al. (1987) observed a significant elevation of plasma cortisol levels 15 min after introduction of alarm substance to pearl dace (Semotilus margarita), and this response was also insignificant compared with controls 1 h after alarm substance introduction. Cortisol is an important hormone in the integrated stress response, functioning as both a glucocorticoid and mineralocorticoid (Wendelaar Bonga, 1997). Since predation is generally an acute stressor, it is not surprising that cortisol is rapidly elevated and then returns to basal levels 1 h after...
alarm substance detection. Changes in plasma sodium after 60 min further support the presence of a physiological response to alarm substance. However, these results are in contrast to the generally expected decrease in plasma sodium concentration after stress in freshwater environments (Wendelaar Bonga, 1997). This may be explained by specific alarm substance-induced changes in gill and/or kidney function, although the mechanisms remain unknown. It is of interest to note that alarm substance-induced changes in plasma cortisol and ion levels in this and other studies are relatively minor compared with those frequently observed after major physiological stress (e.g. handling and seawater transfer; Norris et al., 1999; Evans, 2002). This may reflect differences in the nature of the stressors and the energetic requirements of the physiological response to each stressor.

The cortisol response to alarm substance was inhibited when fish were exposed for one week to sublethal Cd; plasma cortisol levels became statistically indistinguishable from those of controls. This supports previous research that has suggested Cd exposure disrupts cortisol mobilization in response to handling stress in vivo or adrenocorticotropic hormone (ACTH) challenge in vitro (Brodeur et al., 1997; Leblond and Hontela, 1999). However, unlike previous studies (Hontela et al., 1995; Norris et al., 1999), resting plasma cortisol levels were not affected by Cd exposure to any significant extent. This previous work was carried out in the field, where fish were presumably exposed to sublethal waterborne Cd for much longer durations, so this result is perhaps not surprising. Levels of plasma ions did not change as a result of alarm substance in the Cd exposure experiment. However, this result was expected, as changes in plasma ions were insignificant 15 min after alarm substance introduction in the time-course experiment.

It is likely that altered detection of alarm substance due to olfactory impairment contributed to a reduction in the plasma cortisol response in waterborne-exposed fish. However, unlike the effects of Cd exposure on behaviour, both waterborne and dietary Cd exposure inhibited the cortisol response to alarm substance. Since dietary Cd was equally effective as waterborne Cd in this regard, and dietary exposure does not result in olfactory Cd accumulation (Evans and Hastings, 1992), we interpret these results to mean that reduced mobilization of cortisol is not due to reduced olfactory detection of predation threat alone. This suggests that Cd has either a direct inhibitory effect on the interrenal cells responsible for cortisol synthesis and secretion or disrupts an intermediate step in the control pathway between olfactory detection and interrenal stimulation. Previous in vitro studies suggest that Cd may cause a direct inhibition of cortisol release from interrenal tissue (Leblond and Hontela, 1999).

In conclusion, the results of the present study demonstrate that Cd exposure at low concentrations for relatively short periods can alter the olfaction-mediated behavioural and physiological responses of juvenile rainbow trout to alarm substance. As a result, sublethal Cd effects could have important implications for the predator avoidance strategies and possibly the population success of prey fish species. Mechanisms of toxicity at such low concentrations are as yet unclear, but, clearly, disturbance of olfactory function may be one of them. Future studies should strive to better understand mechanisms of behavioural toxicity, as well as other physiological effects that occur at low toxicant exposures.

This work was supported by a Natural Sciences and Engineering Research Council of Canada Strategic Grant with co-funding from the International Lead Zinc Research Organization, the International Copper Association, the Nickel Producers Environmental Research Association, Noranda Inc., Cominco Ltd and Falconbridge Ltd. The authors would like to thank Dr Jeff Richards, Carrie Ho, Tammy Morgan and Grazina Pacevicius for invaluable help and support. The authors also gratefully acknowledge Dr Chris Glover, Dr Jim McGeer, Jason Bystriansky and Nann Fangue for comments on an earlier draft of the manuscript.

References


