The Journal of Experimental Biology 206, 1779-1790

doi:10.1242/jeb.00353

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

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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 ^{109}Cd 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 (Mirza and Chivers, 2000) and have been shown to occur in the wild (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

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 (Rehnberg 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; Rehnberg and Schreck, 1986; Julliard et al., 1995; Hansen et al., 1999), diazinon (Moore and Waring, 1996) and mercury (Hara et al., 1976; Brown et al., 1982; Rehnberg 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 Trijasse, 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 La^{3+}-sensitive apical calcium channels in chloride cells and subsequently inhibits basolateral high affinity Ca^{2+}-ATPase (Verbost 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 Gottofrey, 1986; Gottofrey 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 $CaCO_3$; Na^+, 13.8 mg l^{-1}; Cl^-, 24.8 mg l^{-1}; Ca^{2+}, 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 $Cd(NO_3)_2.4H_2O$ concentration for each exposure regime; Fisher Scientific, Nepean, ON, Canada] acidified to 0.1% with nitric acid (approximately 0.02 mol l^{-1} HNO_3; 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 $Cd(NO_3)_2.4H_2O$ 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, Chastworth, 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, 2.33±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.

Table 1. *Experimental combinations of cadmium (Cd) exposure and stimulus in Experiment 1*

Condition	Cd exposure	Duration[a]	Stimulus
1 (control)	–	–	DDW
2 (control)	–	–	SE
3	2 μg l^{-1} waterborne	1 day	SE
4	0.5 μg l^{-1} waterborne	7 days	SE
5	2 μg l^{-1} waterborne	7 days	SE
6	3 μg g^{-1} dietary[b]	7 days	SE

DDW, distilled deionized water; SE, skin extract.

[a]Each exposure was followed by a 2-day depuration period in control water.

[b]3 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).

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

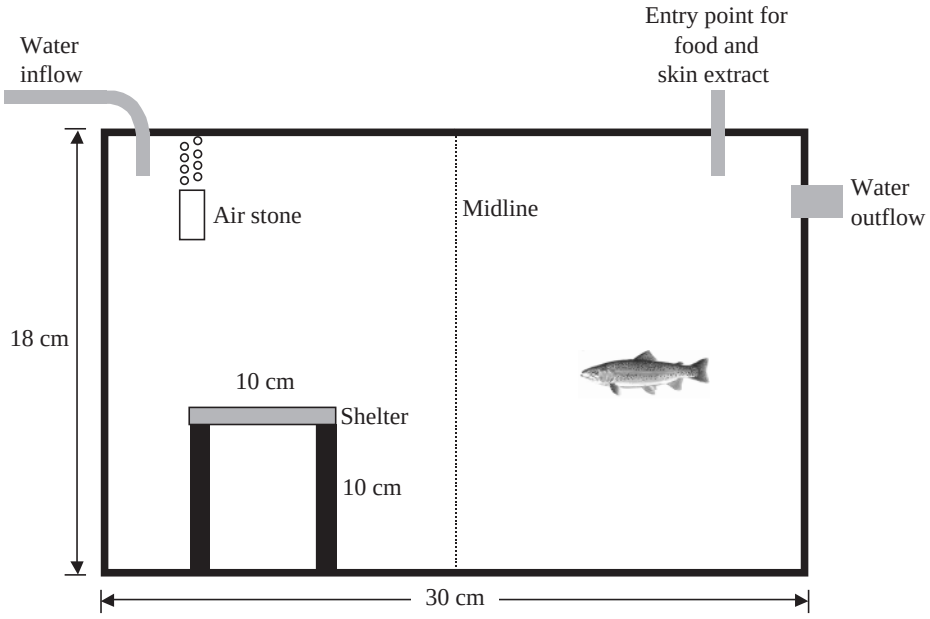

Fig. 1. Diagrammatic representation of the observation tank. Fine sewing thread was placed on the outside of each tank to indicate the tank midline (dotted line). A fish scored one midline crossing each time its head (from snout to end of operculum) passed the midline. Tanks also contained a shelter and air stone and a point of introduction for food and alarm substance. Diagram not to scale.

minute. During both periods, the number of midline crossings (Fig. 1), the number of food items consumed (feeding bites), the time elapsed until the first food item added during either period was taken (latency) and the amount of time spent under shelter were recorded. Observations were made live through a viewing window in the black plastic so as not to disturb the fish.

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-through exposures, see above) to a nominal concentration of 5 μg Cd l^{-1} [measured concentration, 5.30±0.28 μg l^{-1}, N=10; added as $Cd(NO_3)_2.4H_2O$] containing 1.7 kBq ^{109}Cd l^{-1} (measured concentration, 1.70±0.07 kBq l^{-1}, N=10; added as $^{109}CdCl_2$; Perkin Elmer, Boston, MA, USA). A Cd concentration of 5 μg l^{-1} rather than 2 μg l^{-1} was used in this experiment as ^{109}Cd could not be used on a flow-through basis, and Cd bioavailability is generally reduced in static exposures (Wood, 2001). Water was replaced after 3 days and 5 days of exposure with freshly prepared water of the same Cd and ^{109}Cd concentration. Water samples were taken regularly. Two, three and four fish were sampled after 3 days, 5 days and 7 days of exposure, respectively. After 7 days, the remaining five fish were moved to flow-through control water and sampled two days later (i.e. a 2-day depuration period). Fish were sacrificed and immediately freeze clamped in liquid nitrogen. Whole-body samples were stored at –20°C until radioactive Cd accumulation could be determined by autoradiography. Fish were not fed throughout the experiment to minimize Cd complexation with food and thus maximize Cd bioavailability while also maintaining water clarity under static exposure conditions.

Sampled fish were embedded in carboxymethylcellulose gel and frozen in hexane-dry ice slurry. The blocks produced were sectioned sagittally (whole body, vertical plane) on tape with a specially designed cryomicrotome (Leica CM3600, Nussloch, Germany) to a thickness of 20 μm. At least 10 sections were taken of each fish at the level of the olfactory system; each section was then freeze-dried. Sections were selected at random from each exposure condition, representing various levels within each tissue, and were mounted on phosphor screens (Canberra-Packard, Mississauga, ON, Canada) for whole-body autoradiography. After exposure of the phosphor screens, ^{109}Cd activities in liver and olfactory tissues were quantified using a Cyclone Storage Phosphor Imager and Optiquant© software (Canberra-Packard), with activities then being corrected for 1-week screen exposure time. Surface area was quantified for each tissue analyzed using the same software. Activity in olfactory tissues was expressed in digital light units per mm^2 (DLU mm^{-2}) and as a concentration index (I_c) relative to the mean liver value of each fish using the following equation: I_c = (DLU mm^{-2} tissue) / (DLU mm^{-2} liver). By multiplying the liver I_c and liver Cd accumulation [(Cd burden, 2 μg l^{-1} exposure) – (Cd burden, 0 μg l^{-1} exposure)] from the 7-day exposure followed by 2 days depuration in Experiment 3, a calculated Cd accumulation in olfactory tissues was also determined for hypothetical 2 μg l^{-1} cold Cd exposures. This calculation assumes that there is no difference between uptake patterns for 2 μg l^{-1} ‘cold’ Cd flow-through waterborne exposure and those for 5 μg l^{-1} ^{109}Cd-labelled static-renewal exposures. Additional representative whole-body sections were selected and applied to X-ray autoradiography film (Kodak ^{3}H-Hyperfilm; Amersham, Uppsala, Sweden) for 3 months at –20°C to visualize site-specific ^{109}Cd accumulation qualitatively.

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

extract (preparation described above) and the optimum sampling time for the Cd exposure experiment outlined below. Plasma cortisol, sodium and calcium responses were determined at rest (control) and 15 min, 30 min and 60 min after the introduction of skin extract. Ten juvenile rainbow trout (25.0±0.6 g, mean ± S.E.M.) were placed in each of four 50-litre flow-through tanks. Fish were allowed to settle for 9 days before sampling began, to reduce effects of initial handling on plasma cortisol, and were fed control diet (1% body mass) once each day. All sampling was conducted between 11.00 h and 13.00 h to control for diurnal variation in plasma cortisol levels (Pavlidis et al., 1999). After the settling period, flows to all tanks were stopped, and a 200-ml skin extract sample was added to each experimental tank. Fish were rapidly sacrificed by adding a lethal dose of tricaine methanesulfonate anaesthetic (0.8 g l^{-1} MS-222; Syndel Laboratories, Vancouver, BC, Canada) neutralized with NaOH. Fish were removed and placed on ice immediately after opercular movement had ceased. Blood samples were withdrawn by caudal venipuncture, centrifuged at 13 000 ***g*** for 2 min, and the plasma samples removed and immediately frozen in liquid nitrogen. Samples were stored at –80°C until later analysis of plasma cortisol and ions. Based on the cortisol results from this time-course study, a sampling time of 15 min after introduction of alarm substance was chosen for the remainder of this experiment.

To determine the effect of Cd exposure on the physiological responses to alarm substance, 7–10 juvenile rainbow trout (31.8±1.4 g, N=57) were placed in each of six 50-litre flow-through experimental tanks. After a 7-day acclimation period, fish were subjected to either another week in Cd-free water (control fish), one week exposure to 2 μg l^{-1} waterborne Cd (Cd-exposed fish; measured concentration, 2.08±0.02 μg l^{-1}, N=14) or one week exposure to 3 μg g^{-1} dietary Cd (measured concentration, 3.18±0.15 μg g^{-1}, N=6; waterborne [Cd], 0.09±0.02 μg l^{-1}, N=16). After 7 days of exposure, dietary and waterborne Cd exposure was stopped. Two days later (i.e. a 2-day depuration period), flow to the experimental tanks was stopped and a 200-ml sample of either DDW or skin extract stimulus was introduced to the tanks. Therefore, there were six exposure regimes: (1) control + DDW; (2) control + skin extract; (3) waterborne Cd + DDW; (4) waterborne Cd + skin extract; (5) dietary Cd + DDW and (6) dietary Cd + skin extract. Fifteen minutes after the stimuli were added, blood samples were taken for analysis of cortisol and plasma ion levels. All sampling was conducted between 11.00 h and 13.00 h. Gill, liver, kidney and carcass tissues were dissected, placed in pre-weighed containers and frozen at –20°C for later determination of tissue Cd burdens. Liver Cd burdens from control and waterborne Cd exposures were used to determine calculated Cd accumulations in the olfactory system (see Experiment 2).

Measurements and calculations

Water samples (10 ml) were acidified (to approximately 1% nitric acid) and stored in plastic scintillation vials. Food pellets, gill, liver, kidney and carcass samples were weighed and then digested in approximately four volumes of 1 mol l^{-1} HNO_3 for 48 h at 60°C. Samples were then centrifuged at 13 000 ***g*** for one minute. Cd contents were determined for tissue and food supernatants, as well as water samples, *via* graphite furnace atomic absorption spectrophotometry (SpectrAA-220, GTA 110; Varian, Walnut Creek, CA, USA) using certified standards (Inorganic Ventures, Lakewood, NJ, USA). Plasma sodium and calcium levels were determined using flame atomic absorption spectrophotometry (SpectrAA-220FS; Varian) with certified standards (Fisher Scientific). Water ^{109}Cd activity was determined using a Minaxi 8 cm-well NaI crystal gamma counter (Canberra Packard Instrument Company, Meriden, CT, USA). Plasma cortisol was determined by radioimmunoassay (ICN Biomedicals, Costa Mesa, CA, USA).

Data are expressed as means ± S.E.M. One-way analysis of variance (ANOVA) was used throughout to ascertain overall differences when more than two sets of data were being compared. *Post-hoc* Tukey tests were used to determine which pairs of experimental conditions differed. Unpaired *t*-tests were also used to compare DDW and skin extract controls for the change in latency in Experiment 1 and to compare whole-body Cd burden after either waterborne or dietary exposure in 2.5 g fish. Within-tank sampling order effects for plasma cortisol in Experiment 3 were examined using linear regression and the results analyzed using ANOVA. All statistical analyses were conducted using SPSS version 10.0, and a significance level of P<0.05 was used throughout.

Results

Effect of cadmium on behavioural responses to skin extract

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 (P=0.041, 0.001, 0.128 and 0.978, respectively; 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

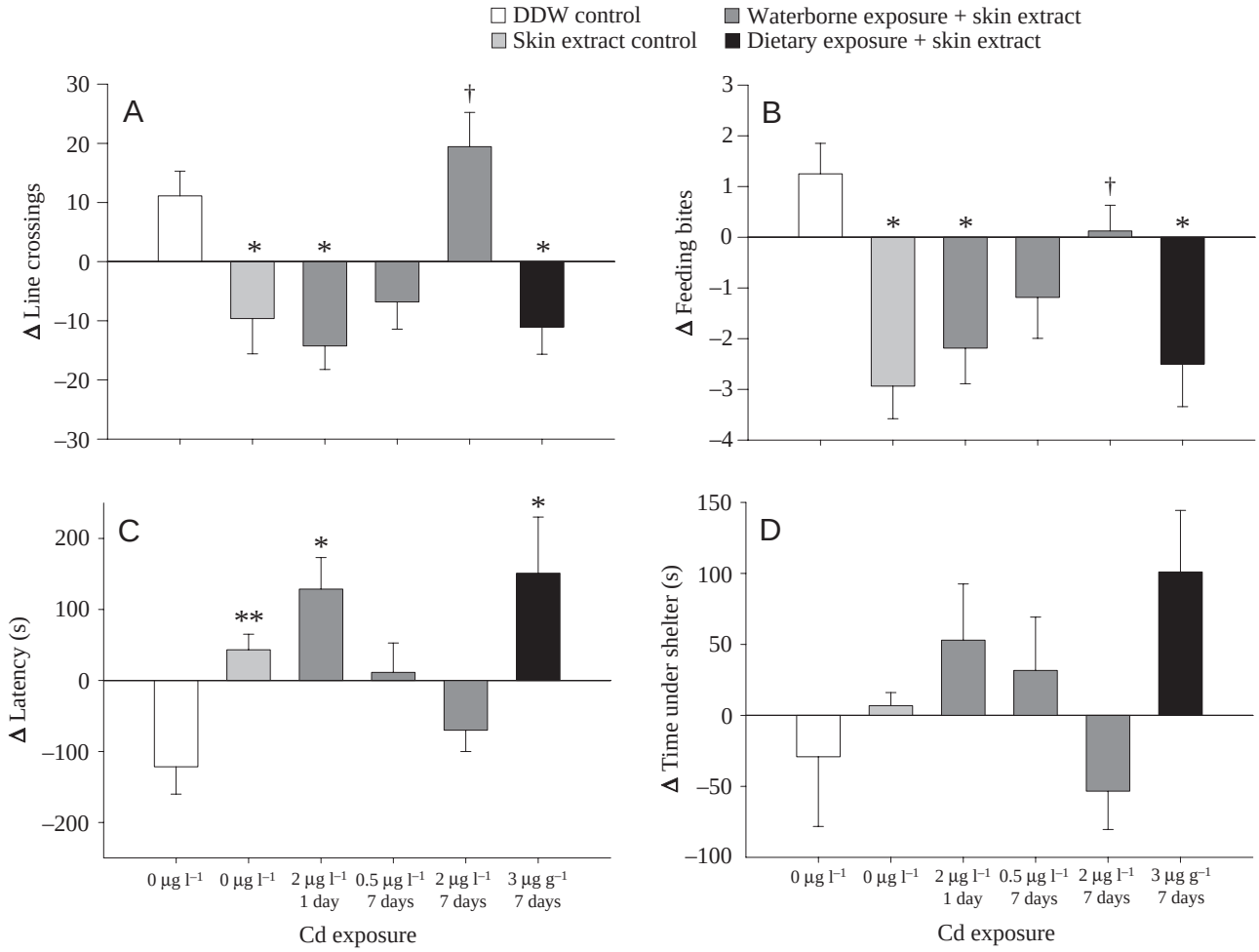

Fig. 2. Mean change in (A) the number of midline crossings, (B) the number of feeding bites, (C) the latency to first feed and (D) the time spent under shelter before and after stimulus for different cadmium (Cd) exposures (N=16 for each group). * represents a significant difference from the DDW (distilled deionized water) control (P<0.05). † represents a significant difference from the skin extract control (P<0.05); ** represents a significant difference from the DDW control (P=0.001) using an unpaired t-test only.

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, 0.998 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 ^{109}Cd activities were indiscernible from background and were assumed to be zero in all tissues. Cd accumulation was quantified in the liver and olfactory

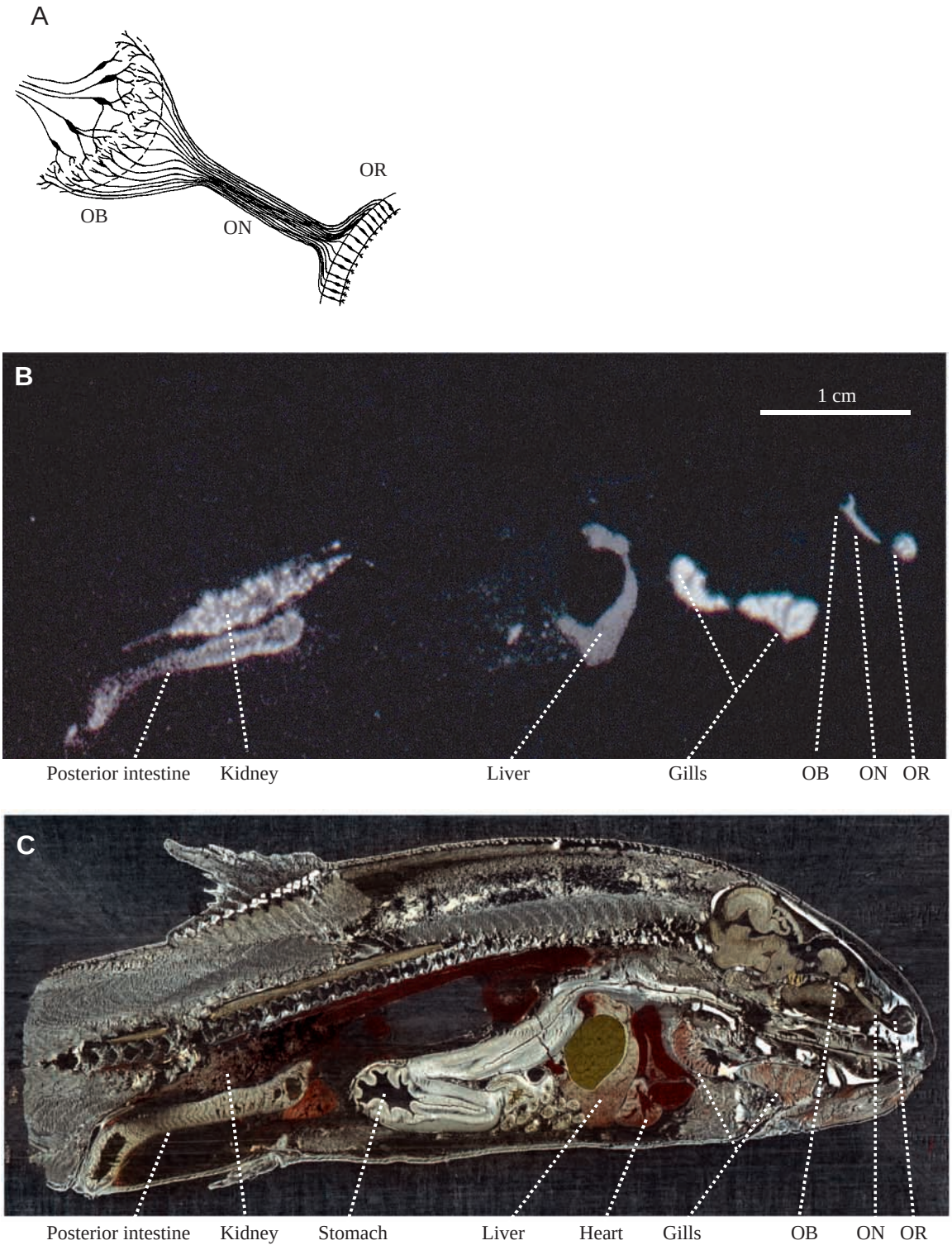

Fig. 3. (A) Schematic representation of the fish olfactory system, portraying cell bodies and dendrites of olfactory neurons within the olfactory epithelium. Axons of olfactory neurons extend *via* the olfactory nerve to the olfactory bulb where they synapse with post-synaptic bulbar neurons (after Hara, 1986). The broken line indicates the theoretical boundary separating the olfactory bulb from the olfactory nerve. (B) Sagittal whole-body autoradiogram showing ^{109}Cd accumulation after 7-day Cd exposure followed by 2-day depuration in control water. (C) Corresponding whole-body tissue section. OR, olfactory rosette; ON, olfactory nerve; OB, olfactory bulb.

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

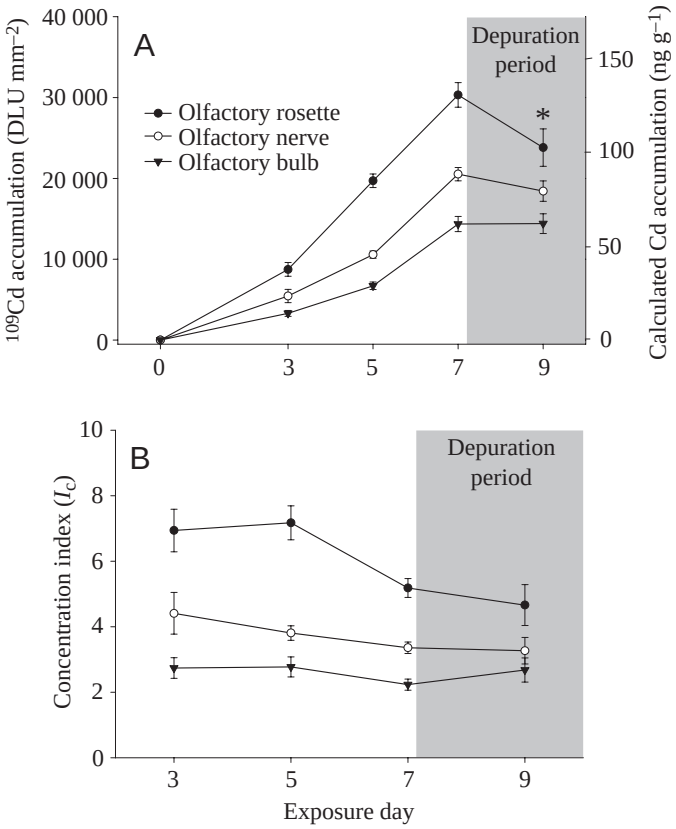

Fig. 4. (A) Accumulation of cadmium (Cd) after 0, 3, 5 and 7 days exposure, as well as 7 days exposure followed by a 2-day depuration period in control water, in the olfactory rosette, olfactory nerve and olfactory bulb. Calculated Cd accumulation (right-hand *y*-axis) was determined as described in 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_c; 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

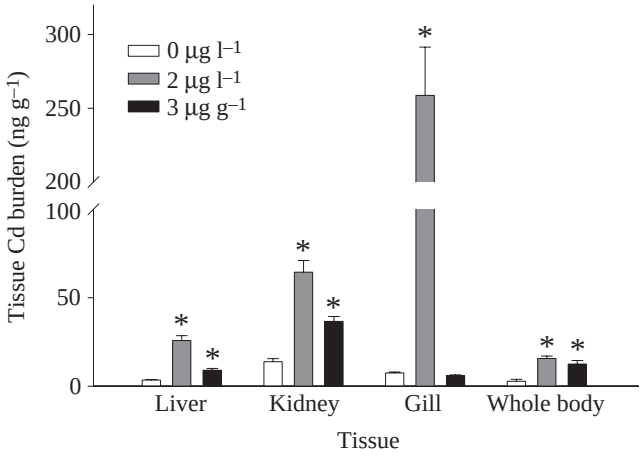

Fig. 5. Liver, kidney, gill and whole-body cadmium burdens for 0 µg Cd l^{-1} (control), 2 µg Cd l^{-1} (7-day waterborne exposure) and 3 µg Cd g^{-1} (7-day dietary exposure at 1% daily ration). Each exposure was followed by a 2-day depuration period in control water (N≥17 for each group). * represents a significant difference from the control (P<0.05).

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 I_c 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 µg l^{-1} 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

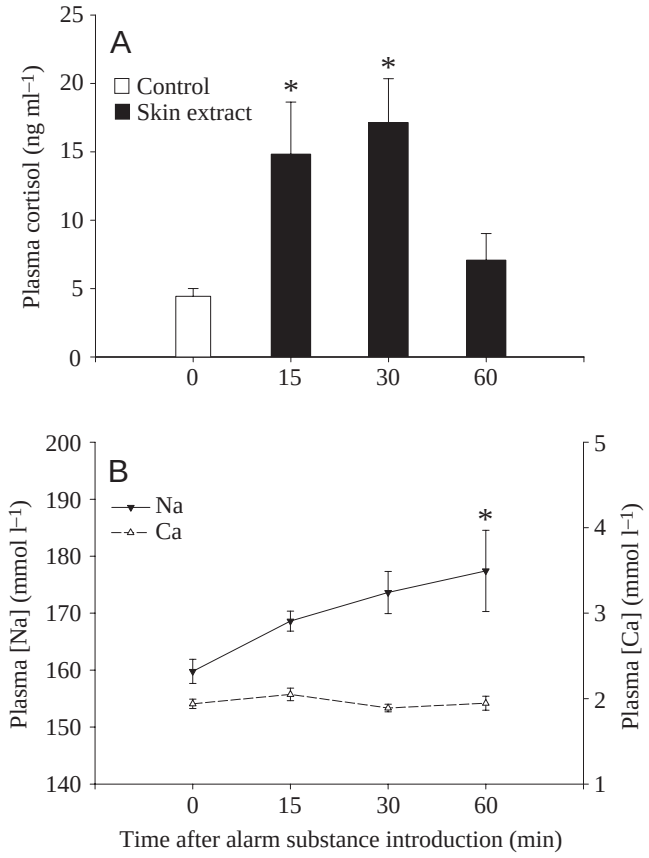

Fig. 6. (A) Plasma cortisol and (B) plasma ion concentrations before (control) and at increasing time points after introduction of skin extract into tanks (N=10 for each group). * represents a significant difference from the control (P<0.05).

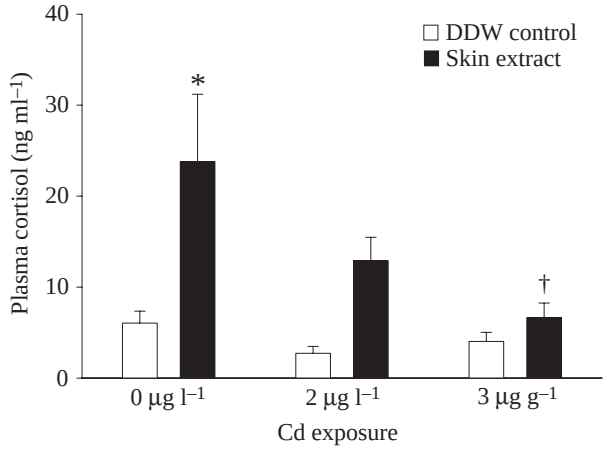

Fig. 7. Plasma cortisol concentrations for rainbow trout exposed to control or cadmium exposures of either 2 μg Cd l^{-1} waterborne or 3 μg Cd g^{-1} dietary (at 1% daily ration) for 7 days, followed by a 2-day depuration period in control water (N≥7 for each group). Plasma samples were taken 15 min after introduction of either distilled deionized water (DDW control) or skin extract stimuli. * represents a significant difference between fish given DDW stimulus and those given skin extract stimulus within each cadmium (Cd) treatment group (P<0.05). † represents a significant difference from Cd-unexposed skin extract control (P<0.05).

extract compared with DDW controls (P=0.007). However, there was no significant increase in the cortisol response to skin extract compared with DDW controls for fish exposed to either 2 μg l^{-1} waterborne Cd or 3 μg g^{-1} dietary Cd for 7 days (after a 2-day period in Cd-free water; P=0.341 and 0.994, respectively; Fig. 7). No statistical differences in resting cortisol (i.e. cortisol response to DDW) existed between control fish (unexposed to Cd) and fish that had been exposed to either waterborne or dietary Cd (P≥0.975). The plasma cortisol response to skin extract was significantly depressed in dietary Cd-exposed fish compared with unexposed skin extract controls (P=0.010). No effect of sampling order was observed within each tank (P≥0.464). No significant effects on plasma sodium or calcium existed as a result of cadmium exposure or alarm substance introduction (P≥0.154; data not shown).

After waterborne and dietary exposures, liver, kidney and whole-body Cd burdens were significantly elevated relative to controls (Fig. 5). Furthermore, 2 μg Cd l^{-1} waterborne exposure resulted in the greatest liver, kidney and gill Cd burdens (P<0.001 for all comparisons with unexposed or dietary exposed fish). However, both 2 μg Cd l^{-1} waterborne and 3 μg Cd g^{-1} dietary exposures resulted in equal whole-body Cd burdens (P=0.337), as was also the case for the smaller sized fish used in Experiment 1 (results from a preliminary experiment; see Materials and methods). Importantly, dietary exposure did not result in a greater gill Cd burden than in controls (P=0.997), indicating that exposure for this treatment group was not by a waterborne mechanism (i.e. from foodborne Cd leaking into surrounding water).

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 gill. 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*; Tjälve and Gottofrey, 1986). Furthermore, several other metals have been shown to accumulate in the olfactory system of fish (Rouleau et al., 1995, 1999; Tjälve 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, Tammie Morgan and Grazina Pacepavicius 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.

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