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Impaired behavioural response to alarm substance in rainbow trout exposed to copper nanoparticles

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ABSTRACT

To date, studies of the toxicity of engineered nanoparticles (NPs) in fish have not fully considered effects on olfactory-mediated behaviours, despite their ecological importance. In this study the effects of copper NPs (Cu NPs) on the anti-predator behavioural responses of juvenile rainbow trout (*Oncorhynchus mykiss*) to trout alarm substance was investigated. Individual fish were exposed for 12 h to a control (no added Cu), 50 $\mu g\,l^{-1}$ of Cu as Cu NPs, or 50 $\mu g\,l^{-1}$ Cu as $CuSO_4$, after which fish behaviours were analyzed in 10 min periods before and after the addition of the alarm substance stimulus. The response of control fish to deionised water (negative control, no alarm substance stimulus) was also analyzed. The alarm substance elicited a behavioural response in the control fish characterized by an immediate freeze response and the slower resumption of swimming activity compared to negative controls exposed to the sham deionised water stimuli. In fish exposed to Cu NPs, the behavioural response to alarm substance was eliminated, with no significant difference in behaviours compared to negative controls. In comparison, exposure to 50 $\mu g\,l^{-1}$ Cu as $CuSO_4$ decreased, but did not eliminate the response of fish to alarm substance, which indicated a significantly greater effect of Cu NPs on olfactory mediated behaviours than of the equivalent concentration of Cu as $CuSO_4$. Measurement of total Cu concentrations in the tissues of fish demonstrated no significant accumulation of Cu from any treatment in gill, liver or brain, confirming the effects of Cu NPs, and to a lesser extent $CuSO_4$, on behavioural responses were mostly associated with the interaction of the materials with the external surfaces of the fish. Scanning electron microscopy revealed that Cu as $CuSO_4$ caused a pronounced depletion of ciliated sensory and non-sensory cells in the olfactory rosette surrounding the midline raphe, whereas Cu NPs had no impact on the structure of the rosette. However, exposure to Cu NPs caused a significant increase in the ratio of oxidized to reduced glutathione in brains of fish, indicating some systemic oxidative stress that was not observed in either controls or fish exposed to $CuSO_4$. Overall, the study showed that the olfactory mediated behaviours of fish were potentially more sensitive to Cu NPs than $CuSO_4$ and NPs elicited effects via a mechanism that is distinct from that of the metal salt.

1. Introduction

The toxicity of traditional dissolved forms of copper (Cu) to fishes is relatively well known (see reviews by Bury and Handy, 2010; Grosell, 2012; Handy, 2003; Kamunde and Wood, 2004). The toxic effects of waterborne exposure to dissolved Cu include gill injury (Campbell et al., 1999), osmoregulatory disturbances (Laurén and McDonald, 1985; McGeer et al., 2000) and oxidative stress (Eyckmans et al., 2011; Lushchak, 2011). Interference with sodium homeostasis in the animal and inhibition of Na^+,K^+-ATPase (Grosell et al., 2002; Handy, 2003) is a particular feature of Cu toxicity and raises the concern that Cu is also neurotoxic to fishes. Indeed, waterborne exposure to $CuSO_4$ causes brain pathology in rainbow trout (Al-Bairuty et al., 2013) and chronic dietary exposure can cause vacuole formation in the mid-brain of fishes close to the hypothalamus (Handy, 2003).

This neurotoxicity also indicates that the sensory processing and/or motor functions of the brain will be affected by Cu. Rainbow trout (*Oncorhynchus mykiss*) fed diets containing elevated levels of

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Cu do show changes in swimming speed distributions and a loss of circadian rhythms that is explained by changes in the neurosecretion of serotonin/melatonin, as well as interference with the brain Cu-ATPase which is involved in controlling the biological clock (Campbell et al., 2002; Handy, 2003). Early electrophysiology experiments also showed that $CuSO_4$ can reduce the responsiveness of the olfactory bulb in salmonids (Hara et al., 1976). All these effects suggest the environmental sensing or perception may be altered in fishes during exposure to traditional dissolved forms of Cu or dietary metal salts.

Fishes are able to sense the chemical composition of the surrounding water through olfactory sensory neurons arrayed in rosettes inside the nasal cavities. To facilitate rapid detection of chemical odours, cilia in the nasal cavity create a unidirectional water current across the sensory receptors (Døving et al., 1977). However, this also accentuates exposure of olfactory rosettes to pollutants in ambient water and olfaction in fish is a sensitive target of pollutants, including Cu (Baldwin et al., 2011; Hansen et al., 1999; Kolmakov et al., 2009; McIntyre et al., 2008; Sandahl et al., 2006; see review by Tierney et al., 2010). Concentrations of 2.3–3.0 $\mu g\,l^{-1}$ of Cu above background in the olfactory chamber have been shown to decrease electrophysiological responses to odorants in coho salmon [*Oncorhynchus kisutch* (Baldwin et al., 2003)]. Copper exposure has also been demonstrated to affect olfactory-mediated fish behaviours. For example, avoidance of L-histidine, a natural fish repellent, was inhibited by 50% in Chinook salmon (*Oncorhynchus tshawytscha*) exposed to 45 $\mu g\,l^{-1}$ Cu (calculated IC_{50}) for 4 h (Kennedy et al., 2012). Olfaction is essential to integrating environmental stimuli and modifying the behaviours of fishes, including searching for food, finding mates, or avoiding predators (Beyers and Farmer, 2001; McIntyre et al., 2012; Scott et al., 2003).

In comparison, knowledge of the effects of engineered nanomaterials on fish behaviours and sensory physiology is very sparse. Recent reports show that 14 d waterborne exposure to 1 $mg\,l^{-1}$ TiO_2 NPs can alter the swimming behaviours of rainbow trout (Boyle et al., 2013). Chronic dietary exposure of trout to TiO_2 NPs also causes some inhibition of Na^+,K^+-ATPase activity in the brain (Ramsden et al., 2009). Waterborne exposure to 20 $\mu g\,l^{-1}$ Cu NPs for 10 days in rainbow trout caused inhibition of Na^+,K^+-ATPase in both the gill and the brain (Shaw et al., 2012) with subsequent brain pathology, including necrotic nerve cells in the forebrain and damage to the stratum periventriculare of the midbrain, indicative of osmotic swelling of the ventricles in the brain (Al-Bairuty et al., 2013). However, whether or not such injuries in Cu NP exposed fish translate into changes in sensory or motor functions is unknown.

To our knowledge, only one study has investigated olfactory toxicity of metallic NPs in fish (Ag NPs, Bilberg et al., 2011) and some disruption of olfaction was observed, but alteration of olfactory-mediated behaviours have yet to be considered for nanomaterials. The main objective of the present study, therefore, was to examine the effects of Cu NPs on olfaction of juvenile rainbow trout using a behavioural response assay to trout alarm substance. Alarm substance is an as yet unidentified mixture of chemicals localized in the epidermis of fishes, including rainbow trout (Brown and Smith, 1997; reviewed by Chivers and Smith, 1998). When the skin is damaged by a predator, alarm substance is released and nearby fish typically exhibit behavioural responses which decrease the risk of predation (McIntyre et al., 2012; Mirza and Chivers, 2003). In rainbow trout, this includes decreased swimming and feeding activity (Brown and Smith, 1997; Scott et al., 2003). In addition, and to gain a better understanding of the mechanistic basis of behavioural toxicity of Cu NPs, we used scanning electron microscopy to examine the morphology of olfactory rosettes in the fish and measured concentrations of reduced (GSH) and oxidized (GSSG) glutathione in brain tissue as an indicator of cellular redox status in response to Cu NP exposure. A $CuSO_4$ treatment was included in the study design to enable some direct comparison of the sensitivity of trout to the metal salt and nanoparticulate forms.

2. Materials and methods

2.1. Experimental animals

Juvenile rainbow trout *O. mykiss* weighing 3.00 ± 0.08 g (mean ± S.E.M., $n = 200$) were obtained from a local supplier (Torre Fishery, Watchet, Somerset, UK) and maintained for two weeks before the start of the experiments in continuously aerated, dechlorinated, recirculating Plymouth city tap water (aquarium water). The chemical composition of the water was (in $mmol\,l^{-1}$): Ca^{2+} 0.26 ± 0.01; K^+ 0.02 ± 0.01; Mg^{2+} 0.04 ± 0.01; Na^+ 0.28 ± 0.01; pH 6.59 ± 0.04; dissolved oxygen 10.02 ± 0.02 $mg\,l^{-1}$; total ammonia 0.05 ± 0.04 $mg\,l^{-1}$; temperature 14 ± 1 °C (means ± standard error of the mean (S.E.M.), $n = 23$–40). Fish were held in a 14 h light:10 h dark photoperiod and were fed twice daily with a commercial trout diet (EWOS, Westfield, UK). The background concentration of Cu in aquarium water used in all experiments was below the detection limit (<5.44 $\mu g\,l^{-1}$) of the ICP-OES instrument used for element analyses (see Section 2.6). The entire experiment was under ethical approval and fish were subject to independent health checks during the work.

2.2. Experimental design and exposure series

To investigate the effects of Cu NPs and Cu salt (as $CuSO_4$) on the behavioural responses of juvenile rainbow trout to a rainbow trout skin preparation containing alarm substance (skin extract, SE), two exposure series were used. In the first, trout behavioural responses to SE were assessed after 12 h exposure to 50 $\mu g\,l^{-1}$ Cu (as $CuSO_4$) and 50 $\mu g\,l^{-1}$ Cu NPs (nominal total Cu concentrations), i.e. in the presence of the test materials. In the second exposure series, the behavioural responses of fish to SE were measured after 30 min in clean water following 12 h Cu exposure, i.e. without $CuSO_4$ or Cu NPs in suspension. Nanoparticles have been shown to adsorb organic material (e.g. Hu et al., 2011) and could hypothetically bind the SE and alter its availability at sensory epithelia. To test this, trout were transferred to clean water following 12 h exposure to $CuSO_4$ and Cu NPs in separate aquaria, allowed to recover from handling stress and the alarm response of fish to SE measured. Preliminary experiments revealed a 30 min recovery period was sufficient for fish to resume normal behaviours (data not shown). The 50 $\mu g\,l^{-1}$ concentration for both $CuSO_4$ and Cu NPs were chosen after considering the finding of our previous work on the same materials by Shaw et al. (2012) and Al-Bairuty et al. (2013). Briefly, in Plymouth water Shaw et al. (2012) showed the time to 50% lethality for exposure to a nominal total concentration of 100 $\mu g\,l^{-1}$ Cu as $CuSO_4$ was 70 h, and derived sub-lethal (physiological) effects at a concentration of 20 $\mu g\,l^{-1}$ of Cu as $CuSO_4$ over at least 4 days without mortality. However, at 20 $\mu g\,l^{-1}$ of Cu some subtle effects on brain morphology were reported (Al-Bairuty et al., 2013). To avoid the latter and to ensure a sub-lethal physiological exposure in Plymouth water, the present study used a nominal total concentration of 50 $\mu g\,l^{-1}$ of Cu over 12 h, which equates to one third of the sub-lethal dose used in our previous work. Thus providing a physiological dose in Plymouth water, but also minimizing the risk of the subtle systemic neurological injury seen previously. In keeping with Shaw et al. (2012) and Al-Bairuty et al. (2013), an equal mass concentration of total Cu as $CuSO_4$ and Cu NPs was used. The alternative experimental approach of using a Cu NP concentration that equates to an equivalent apparent "dissolved metal" fraction released from the NPs (e.g. Griffitt et al., 2009) was not used

because recent information suggests that the free metal ion toxicity model does not adequately explain the toxicity of several metal-containing NPs, including Cu NPs; and a dissolved metal paradigm should not be assumed (review, Shaw and Handy, 2011). In addition, the concept of steady-state equilibria used for the uptake of soluble substances such as metals does not apply readily to engineered nanomaterials (Handy et al., 2012). Consequently, the $CuSO_4$ treatment used here should be regarded as a metal salt for bench marking the relative hazard of Cu NPs, and is not intended as a free metal ion toxicity control for the NP. Nonetheless, dialysis experiments were performed (see below) to estimate apparent release of a dissolved Cu fraction from the Cu NPs used here.

All fish exposures in both experimental series were performed in individual experimental glass aquaria (46 cm × 25 cm × 26 cm, $n = 1$ fish per aquarium) containing 20 l water (water chemistry as above). Trout are aggressive to conspecifics and having only one fish/aquaria eliminated the possibility of aggressive or social behaviours that could interfere with the alarm substance response. Aquaria were covered on three sides to minimize disturbance to the fish and a stationary digital video camera placed in front of aquaria to remotely record behaviours. After allowing the fish to acclimate to aquaria conditions for 10 h, the tanks were spiked to 50 $\mu g\,l^{-1}$ of Cu as $CuSO_4$ or Cu NPs (see Section 2.3 for details on copper stocks) and control tanks spiked with an equal volume of ultrapure deionised water (DW, vehicle control, MilliQ®, Millipore Company) from the rear of the tanks and fish exposed for 12 h prior to behavioural responses to SE being assessed (see Section 2.5). Following behavioural analyses, the fish were terminally euthanized (0.5 $g\,l^{-1}$ MS222, buffered to pH 7), and tissues collected for Cu analyses (see Section 2.6), morphological observations of the olfactory rosettes (Section 2.7) and measurements of glutathione (Section 2.8).

2.3. Copper stock suspensions and characterization of Cu NPs

The stock solution of Cu as $CuSO_4$ (analytical grade, Fisher Scientific, UK) was prepared at a concentration of 1 $g\,l^{-1}$ in DW and stored at 4 °C until required. Copper NPs were purchased from Sigma–Aldrich UK (manufacturer's information; particles < 50 nm; purity < 99.9%) and were the same batch as used by Shaw et al. (2012). Stock suspensions of Cu NPs were prepared fresh at concentrations of 1 $g\,l^{-1}$ in DW in 10 ml glass volumetric flasks and suspensions were then dispersed by sonication (35 kHz frequency, Fisherbrand FB 11010, Germany) for 1 h at room temperature immediately prior to dosing of experimental tanks and particle characterization. Primary particle sizes of the Cu NPs were measured manually from micrographs obtained using transmission electron microscopy (TEM, JEOL 12000EXII, Japan). The primary particle sizes (diameter) of Cu NPs in stock suspensions were 20.3 ± 0.9 nm (mean ± S.E.M., $n = 100$ particles). The particle size distributions of Cu NPs in stock suspensions prepared as described above were measured by nanoparticle tracking analysis (NTA, NanoSight LM 10, NanoSight, Salisbury, UK) in 20 $mg\,l^{-1}$ dilutions (in DW) to avoid saturating the instrument. Dilutions of stock suspensions to 20 $mg\,l^{-1}$ gave sufficient particle tracks (>100 tracks per sample) to provide reproducible data of particle size distributions in stock suspensions. Stock suspensions were observed to contain a normal distribution of particle sizes, ranging from individual Cu NPs of 0–20 nm to larger particles, almost certainly aggregates of Cu NPs, of >100 nm. The mean calculated hydrodynamic diameter of aggregates in the suspension immediately prior to dosing was 153 ± 36 nm (mean ± S.E.M., $n = 3$).

To investigate the potential role of the release of dissolved Cu ions from Cu NPs on any observed effects in trout, the dissolution of a dissolved fraction of Cu from Cu NPs was determined by dialysis using a modification of Besinis et al. (2012). Briefly, the appearance of a total dissolved Cu fraction from dialyzing Cu NPs was measured in DW over 12 h to inform on the release of Cu during fish exposures. Sections of dialysis tubing (15 cm long × 3.2 cm wide, $n = 3$; cellulose membrane, pore size 2.5 nm, Sigma–Aldrich, UK) containing 3 ml of 100 $mg\,l^{-1}$ Cu NPs (prepared as described above and then diluted) and sealed at both ends, were rinsed with DW and placed in beakers containing 297 ml DW. The DW was stirred continuously with magnetic stirrers at room temperature. After 10 min equilibration ($t = 0$) and at 3, 6 and 12 h thereafter, water samples (5 ml) were removed from beakers, acidified with 1 ml of HNO_3 (68% nitric acid, trace element grade, Fisher Scientific, UK) and water total Cu concentrations measured by ICP-OES (Varian 725-ES). A higher concentration of Cu NPs was spiked into the dialysis tubing than was used in exposures with fish to provide reliable measurements of Cu in DW that were consistently higher than detection limits of the ICP-OES instrument (5.44 $\mu g\,l^{-1}$). Release of Cu from NPs was linear during 12 h ($R^2 > 0.95$), occurring at a rate of 2.23 ± 0.07 $\mu g\,mg^{-1}\,h^{-1}$ and corresponded to 25.7 ± 0.3 $\mu g\,l^{-1}$ in suspension (2.57 ± 0.05% total dissolution of Cu metal from Cu NPs) at the final sampling point during the 12 h test period (data are means ± S.E.M.).

2.4. Preparation of skin extract

The SE containing alarm substance was prepared from skin of stock rainbow trout (weighing 97.1 ± 3.3 g, mean ± S.E.M., $n = 65$) maintained in aquarium water (see details of water chemistry in Section 2.1) according to a method adapted from Scott et al. (2003). Trout were terminally anaesthetized (see Section 2.2) and the skin was removed from fish with careful incisions to avoid drawing blood and stored at −20 °C until required. To prepare the SE, skin was thawed on ice, rinsed in DW to remove any residual mucus and other tissue debris, macerated by hand into small pieces and homogenized at 4 °C in DW. The homogenate was filtered through Whatman number 1 filter paper (11 μm pore size, Whatman, UK) overnight at 4 °C and the filtrate diluted with DW to a concentration of 85 g of the initial skin weight l^{-1}. The SE was then stored in 35 ml aliquots at −20 °C. Aliquots of filtered DW were also frozen at −20 °C for use in controls. Before behavioural analyses, aliquots of SE and DW were thawed on ice, diluted 1:3 in aquarium water and used immediately.

2.5. Behavioural responses of rainbow trout to skin extract

The approaches used to measure the fish responses to the SE and the DW control ($n = 10$–12 fish per treatment) were adapted from Scott et al. (2003). Control, $CuSO_4$ and Cu NPs exposed fish were exposed to SE for behavioural analyses. Additional control fish were exposed to DW instead of SE to control for the disturbance in the tank environment caused by introducing a solution. The fronts of aquaria were delineated with fine black permanent marker pen to indicate the vertical midline (separating left and right halves of the tanks) and the lower, middle and upper thirds of the aquaria (horizontal lines). All behavioural analyses were performed between 9 and 10 a.m. to minimize effects of diurnal rhythms on activity levels of fish (Campbell et al., 2002). The behavioural assays consisted of 10 min pre- and 10 min post-stimulus periods. Preliminary experiments indicated the behavioural responses of trout to SE were greatest in the first 10 min and declined thereafter (data not shown). The pre-stimulus period was used to normalize behavioural responses of each individual fish to correct for variability in individual fish activity levels (i.e. using each fish as its own behavioural control). However, the raw data of behaviours of fish in the pre-stimulus period were also directly compared between treatment groups to ascertain if exposure to the Cu salt or Cu NPs may have affected normal swimming behaviours in

these fish and this information could aid subsequent interpretation of the response of fish to SE. After the 10 min pre-stimulus period, 30 ml of diluted SE or DW was introduced into tanks using a syringe, washed through with 30 ml of aquarium water, and then behaviours recorded for a further 10 min.

Videos of fish behaviours were analyzed using Observer software (Observer XT 7.0, Noldus Information Technology, The Netherlands). Behaviours in individual fish were scored using the following metrics: (1) the total number of horizontal lines crossings (i.e. movements between upper and middle, and middle and lower, areas of the aquarium); (2) the number of vertical midline crossings (i.e. movements between left and right sides of the aquarium); (3) the total time spent immobile during the 10 min periods (in seconds); (4) latency to first movement after addition of SE or DW (in seconds). The total time spent inactive is a useful indicator of fish behaviour in aquaria, but may not give a clear indication of the rate of swimming or exploratory behaviours of fish. For this reason, horizontal and vertical line crossings were also scored.

Several different approaches were used to normalize the behaviours of fish to pre-stimulus activity levels to avoid skewing datasets. Change (Δ) in time (T) spent inactive was analyzed by expressing each as net % change relative to pre-stimulus values, i.e. Δ = % $T_{inactive}$ post-stimulus − % $T_{inactive}$ pre-stimulus. Since individual fish often exhibited marked differences in numbers (#) of vertical and horizontal line crossings in the pre-stimulus period (roughly comparable to baseline activity levels), changes in these metrics from the pre- to the post-stimulus period were expressed as relative % changes, i.e. $\Delta = (\#_{crossings}$ post-stimulus $- \#_{crossings}$ pre-stimulus$)/\#_{crossings}$ pre-stimulus. The latency to first movement was not assessed in the pre-stimulus period and absolute values (times) recorded are presented. Several fish were removed from the dataset after initial behavioural analyses as their lack of activity (>75% time inactive) in the pre-stimulus period prevented reliable comparisons of activity levels between pre- and post-stimulus periods being calculated. In total, $n = 6$ fish were removed from the dataset for the main experiment, including at least one fish from each treatment group, out of a combined total of $n = 50$ fish analyzed.

2.6. Copper analyses

Copper was analyzed in brain, gills, and livers that were excised on ice from whole fish terminally euthanized (see Section 2.2) and snap-frozen at −80 °C immediately after behavioural analyses. Following the methods of Shaw et al. (2012), excised organs were weighed into 5 ml tubes and dried to constant weight at 60 °C for 48 h. Dry tissues were digested in 1 ml HNO_3 (68% nitric acid, trace element grade, Fisher Scientific, UK) at room temperature for 48 h followed by 2 h at 60 °C. Digests were then diluted to 2 ml with DW and Cu concentration measured by ICP-OES (Varian 725-ES) and compared to matrix matched element standards (Fisher Scientific, UK). Procedural blanks (acid digest protocol performed as above but without tissue) were also included in each run. The measured Cu concentrations in procedural blanks were below the limits of detection (<5.44 μg l^{-1}, calculated from $n = 7$ samples). The accuracy of the instrument was assessed by measuring the concentration of Cu from digests of dogfish liver (DOLT 4) reference material (National Research Council Canada); the recovery of Cu was 106.2 ± 7.5% of the reported Cu concentration (mean ± S.E.M., $n = 3$). In the absence of certified reference materials for Cu NPs, spike recovery tests were performed using trout gill and liver digests spiked with Cu NPs to a nominal total concentration of 100 μg l^{-1}. Recovery of Cu NPs from digests of gill and liver were 90.82 ± 0.81 and 91.11 ± 1.10% of nominal Cu concentrations, respectively (means ± S.E.M., $n = 6$). Analysis of water Cu concentrations from exposure tanks and dialysis experiments were performed as described above and compared to matrix matched acidified element standards.

2.7. Microscopic analysis of the olfactory rosette

Following the exposures, $n = 4$ or 5 fish per treatment were collected for electron microscopy. Olfactory rosettes were carefully excised from terminally euthanized fish (see Section 2.2) and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 4 °C. Following fixation, samples were rinsed twice (15 min each) in sodium cacodylate buffer, dehydrated in a graded ethanol series (30, 50, 70, 90, 100, 100%) and dried using a CO_2 critical point dryer (Emitech K850, UK). Dried rosettes were placed on metal studs covered in silver loaded adhesive, coated with gold (Emitech K550, UK) and gross morphology observed by scanning electron microscopy (JEOL JSM-7001F, Japan).

2.8. Brain glutathione analysis

Concentrations of oxidized and reduced glutathione were analyzed in brains of fish ($n = 5$ per treatment). Fish were terminally anaesthetized (see Section 2.2) and the brains immediately excised, snap frozen in liquid nitrogen, and stored at −80 °C until analyses. Glutathione was measured according to an original method of Griffith (1980), and with modifications and adapted to the microplate as according to Baker et al. (1990). In brief, brains were homogenized on ice for 30 s in 5% 5-sulfosalicylic acid (1:5 volumes of tissue:buffer) and centrifuged for 2 min at 13,000 rpm to deproteinate the samples. Total glutathione (TG) and oxidized glutathione (GSSG) were then measured in separate aliquots of the obtained supernatant. Aliquots used for measurement of GSSG were treated immediately with 97% 2-vinylpyridine at a dilution of 1:22, 2-vinylpyridine:tissue supernatant, and incubated for 1 h at room temperature to derivatise GSH in the sample. Total glutathione and GSSG were then measured in a final assay mixture containing: 0.15 mmol l^{-1} 5,5′-dithiobis-(2-nitrobenzoic acid), 0.2 mmol l^{-1} NADPH, and 1 U ml^{-1} glutathione reductase in 100 mmol l^{-1} phosphate buffer containing 0.5 mmol l^{-1} EDTA at pH 7.5. Concentrations of total (TG), oxidized (GSSG) and reduced glutathione (GSH; TG − GSSG) were normalized to the original wet weight of the tissue. The ratio of GSH:GSSG was also calculated.

2.9. Data handling and statistical analyses

All data in the results are presented as means ± S.E.M. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., *v.* 6). All data were tested for normality (Shapiro–Wilk test) and if appropriate data were $\log_{10}$- or arcsine-transformed. Statistically significant differences in behavioural responses of fish to SE and the measured Cu and glutathione concentrations in tissues of fish from different treatment groups were determined by ANOVA with *a posteriori* Tukey's test. If $\log_{10}$ or arcsine transformation had failed to normalize data, the Kruskal–Wallis test with *a posteriori* Dunn's multiple comparisons test was used. Differences were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Water and tissue Cu measurements

Exposures of trout to $CuSO_4$ and Cu NPs were verified using ICP-OES. Measured concentrations of Cu in tanks after 12 h were below the limits of detection of the instrument in the control tanks (<5.44 μg l^{-1}), and 46.38 ± 0.31, and 21.75 ± 0.49 μg l^{-1} in tanks dosed with Cu as $CuSO_4$, and Cu NPs, respectively. These measured

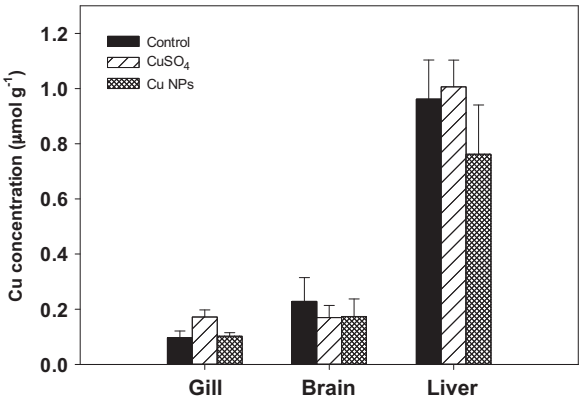

Fig. 1. Concentrations of Cu (μmol g^{-1} dry weight) in gill, brain and liver of rainbow trout exposed to control, 50 μg l^{-1} Cu (as $CuSO_4$), and 50 μg l^{-1} Cu NPs for 12 h. Data are means ± S.E.M. ($n = 7$). There were no significant differences between treatment groups ($p > 0.05$).

concentrations of Cu NPs were 43.50 ± 0.98% of the nominal concentration and likely reflect settling of Cu NPs to the bottom of the aquaria.

Twelve hour exposure to Cu salt and Cu NPs did not result in significant increases in Cu levels in brains or livers of fish (ANOVAs, $p > 0.05$, Fig. 1). There was, however, a trend towards increased Cu in gills of fish exposed to Cu (as $CuSO_4$) compared to both Cu NPs exposed fish and controls, although the increase was not statistically significant (ANOVA, $p = 0.064$). Measured Cu concentrations in gills were 0.10 ± 0.02, 0.17 ± 0.03 and 0.10 ± 0.03 μmol g^{-1} in controls, Cu salt and Cu NP exposed fish, respectively.

3.2. Fish behavioural analyses

3.2.1. Fish behaviour during the pre-stimulus period

There were no statistically significant differences ($p > 0.05$) between unexposed fish from the two control groups (i.e. fish subsequently used to assay responses to DW and SE) in the pre-stimulus period, and therefore the data were combined ($n = 20$ fish combined total) to improve the robustness of the analyses (Fig. 2). Overall, exposure to $CuSO_4$ ($n = 12$ fish) but not Cu NPs ($n = 12$ fish) had minor effects on general swimming behaviours of trout. There were significant differences between treatment groups in the numbers of midline (Cu NPs vs $CuSO_4$; ANOVA, $p = 0.020$) and horizontal line (control vs $CuSO_4$; Kruskal–Wallis test, $p = 0.025$) crossings; however, neither treatment had significant effects on the % time that fish were inactive (Kruskal–Wallis test, $p > 0.05$). Fish exposed to the control, $CuSO_4$ and Cu NPs, were inactive for 11.0 ± 3.6, 19.5 ± 6.8 and 13.3 ± 5.9% time during the pre-stimulus period, respectively.

3.2.2. Fish behavioural responses to skin extract

Fish from all treatment groups exhibited changes in behaviours in response to the introduction of solutions (both DW and SE) into aquaria, as indicated by the deviation of Δ values from 0 (Fig. 3). However, there were also clear and significant differences in the behavioural responses of control fish (no added Cu) to the SE compared to control fish to DW, the sham stimulus. Compared to DW controls, the response to the SE in control fish was characterized by a significantly greater reduction in the relative numbers of horizontal line crossings, a greater increase in the % total time fish were inactive, and an increased latency to first movements immediately following the introduction of SE into the aquaria (Fig. 3).

In contrast to control fish, the SE elicited a weaker response in fish exposed to $CuSO_4$, and in fish exposed to Cu NPs this behavioural response was eliminated to the extent that the response of Cu NPs exposed fish to SE was not significantly different from the response of control fish to DW (Fig. 3). The introduction of SE into aquaria caused a significantly greater decrease (Δ change) in horizontal crossings in control fish compared to fish exposed to Cu NPs (Kruskal–Wallis test, $p = 0.0101$, Fig. 3A). The relative decrease in horizontal line crossings by $CuSO_4$ exposed fish in response to SE was not significantly different from any other treatment group. A similar pattern of effects of $CuSO_4$ and Cu NPs was also evident in the relative decrease in numbers of midline crossings by fish from different treatment groups in response to the SE; however although the overall trend was significant (Kruskal-Wallis test, $p = 0.05$, Fig. 3A), there were no statistically significant differences between individual treatment groups ($p > 0.05$). Control fish exposed to SE were significantly less active than fish exposed to SE following Cu NP exposure or control fish exposed to DW (ANOVA, $p = 0.005$, Fig. 3B). The increase in time that $CuSO_4$ exposed fish were inactive was not significantly different from any other treatment group. The response of control fish to the SE was typically immediate, and fish ceased all swimming activity for a period of time (latency) before slowly resuming activity (Fig. 3C). This response to SE was also observed in fish exposed to $CuSO_4$. In contrast to controls and $CuSO_4$, but similar to the response of control fish to DW, fish exposed to Cu NPs rapidly resumed swimming following the introduction of the SE into aquaria (Kruskal–Wallis test, $p < 0.001$). Latencies to first movement following introduction of SE or DW were 2.0 ± 1.2 and 280.3 ± 81.1 s, in controls in response

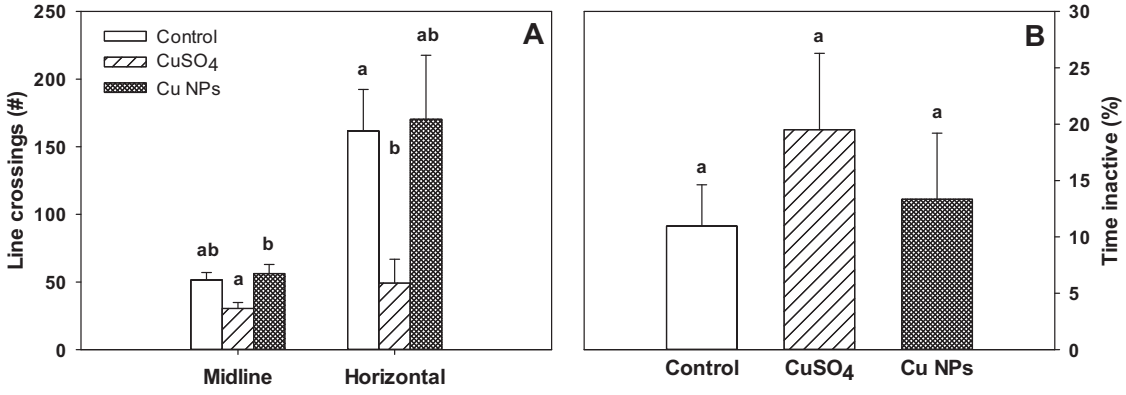

Fig. 2. Behaviours of rainbow trout during the pre-stimulus period of observation prior to the introduction of skin extract into aquaria. The numbers of line crossings (A), and the time (%) the fish were inactive (B) were measured in 10 min periods following exposure to control, 50 μg l^{-1} Cu (as $CuSO_4$), and 50 μg l^{-1} Cu NPs for 12 h. Data are means ± S.E.M. ($n = 20$ controls, $n = 12$ $CuSO_4$, and $n = 12$ Cu NPs). Different lower case letters indicate significant differences between treatments within each parameter ($p \leq 0.05$).

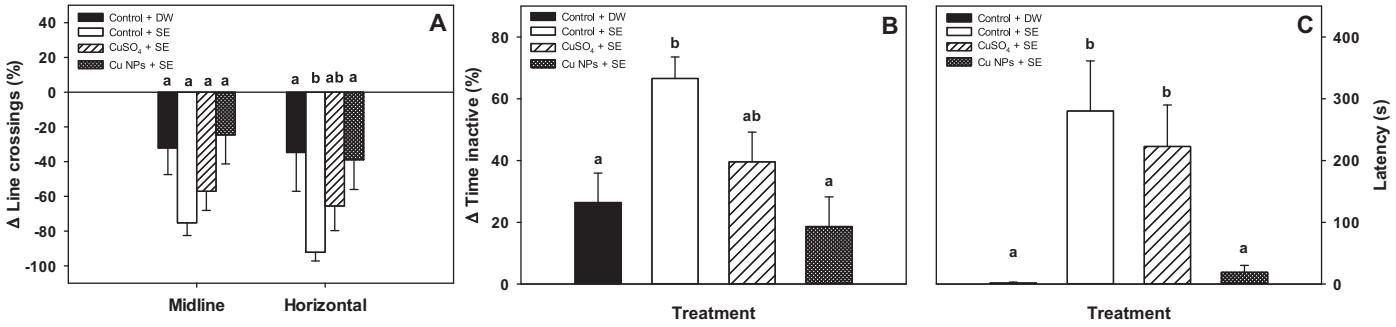

Fig. 3. Behaviours of trout in response to deionised water (DW) and skin extract (SE). Data shown are: mean changes in midline and horizontal line crossings (A), mean change in % time fish were inactive (B), and latency to first movements (C). Behaviours were assayed in 10 min periods before and after the introduction of DW and SE and following exposure to control, 50 μg l^{-1} Cu (as $CuSO_4$), and 50 μg l^{-1} Cu NPs for 12 h. Behaviours are expressed as % change from the pre-stimulus periods of individual fish (Δ change), with the exception of latency to first movement which was assessed in the post-stimulus period only (see main text for details of calculations). Data are means ± S.E.M. (n = 10–12). Different lower case letters within each parameter indicate significant differences between treatments ($p \leq 0.05$).

to DW and SE, and 222.8 ± 67.0 and 19.1 ± 11.2 s in response to SE for fish exposed to $CuSO_4$ and Cu NPs, respectively.

3.2.3. Fish behavioural responses to skin extract in clean water

Following exposure to control, $CuSO_4$ or Cu NPs for 12 h, a subset of trout were removed to individual aquaria containing clean water (no Cu) to assay behavioural responses of fish to DW (control fish only) and SE (all treatment groups) without $CuSO_4$ or Cu NPs in suspension (Fig. 4). Overall, trout retained the characteristic pattern of behavioural responses to SE (and DW) that were observed in fish assayed in water containing Cu (see Section 3.2.2). Fish from all treatment groups, including both controls, exhibited a behavioural response to the introduction of DW and SE compared to the respective pre-stimulus responses, as indicated by the deviation from 0 in several of the behavioural metrics measured. However, there were significant differences between treatment groups in the magnitude and duration of several of the observed behavioural responses. In fish exposed to Cu NPs, the relative (Δ) change in numbers of midline and horizontal line crossings were significantly different compared to control fish in response to SE (Kruskal–Wallis tests, p = 0.034 and p = 0.030, respectively, Fig. 4A). Specifically, a relative decrease in midline and horizontal line crossings in response to the SE that were observed in control fish were not observed in clean water with the Cu NPs treated fish. The introduction of SE into control aquaria also caused a Δ (%) increase in time control fish were inactive which was not observed in fish that had been exposed to Cu NPs; these fish responded to SE in a manner not significantly different from the response of control fish to the sham DW stimuli (ANOVA, p = 0.017, Fig. 4B). The latency to first movement following the introduction of SE was also significantly less in fish exposed to Cu NPs compared to controls; $CuSO_4$ fish exhibited an intermediate response (Kruskal–Wallis test, p = 0.0155, Fig. 4C). Latencies to first movement were 4.4 ± 4.4 and 265.0 ± 90.2 s in controls in response to DW and SE, and 133.1 ± 57.4 and 9.9 ± 7.9 s in response to SE for fish exposed to $CuSO_4$ and Cu NPs, respectively.

3.3. Morphology of the olfactory rosette

In rosettes excised from control fish (Fig. 5B), the epithelia immediately adjacent to the midline raphe was densely covered with cilia of both sensory and non-sensory cells. A similar abundance of ciliated cells was also apparent in all Cu NPs exposed fish examined and there were no overt differences between controls and NP exposed fish in the structure of the cilia (Fig. 5C). In contrast, in $CuSO_4$ exposed fish (Fig. 5D and E) there was a clear and pronounced degeneration of cilia. However, the cilia present in crypts between lamellae did not appear damaged (Fig. 5F) and the abundance was similar to cilia abundance observed in crypts in control fish and fish exposed to Cu NPs (images not shown).

3.4. Brain glutathione

There were no significant differences in measured concentrations of TG in brains of fish (ANOVA, p = 0.117). However, there were clear trends towards decreased GSH (ANOVA, p = 0.062), and increased GSSG (overall ANOVA, p = 0.060) in fish exposed to Cu NPs and taken together amounts to a significant decrease in the ratio of GSH to GSSG in fish exposed to Cu NPs compared to both controls

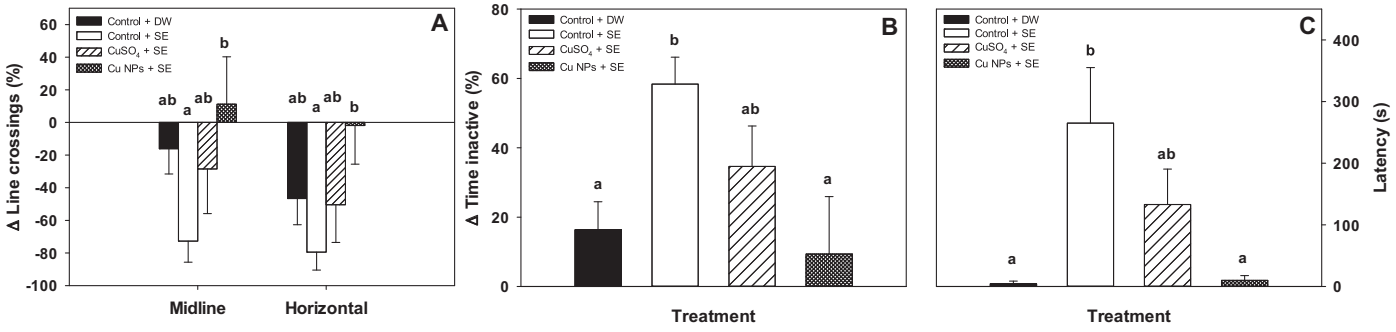

Fig. 4. Behaviours of trout in response to deionised water (DW) and skin extract (SE) in clean water (no Cu) following 12 h exposure to control, 50 μg l^{-1} Cu (as $CuSO_4$), and 50 μg l^{-1} Cu NPs. Data shown are: mean changes in midline and horizontal line crossings (A), mean change in % time fish were inactive (B), and latency to first movements (C). Behaviours were assayed in 10 min periods before and after the introduction of DW and SE and are expressed as % change from the pre-stimulus periods of individual fish (Δ change), with the exception of latency to first movement which was assessed in the post-stimulus period only (see main text for details of calculations). Data are means ± S.E.M. (n = 7–10). Different lower case letters within each parameter indicate significant differences between treatments ($p \leq 0.05$).

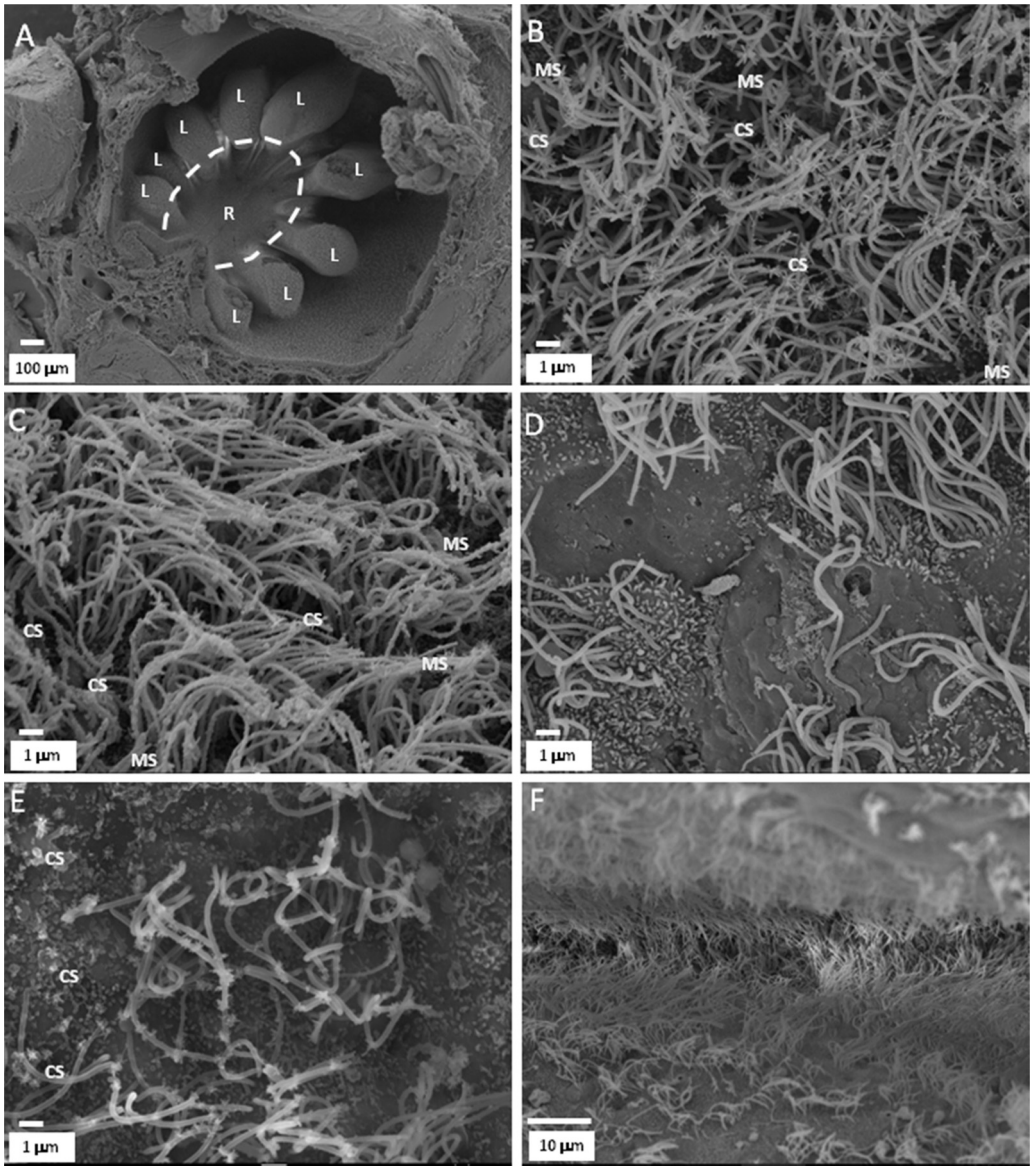

Fig. 5. Representative micrographs of the olfactory rosettes of rainbow trout exposed to control, 50 µg l^{-1} Cu (as $CuSO_4$), and 50 µg l^{-1} Cu NPs for 12 h. An example micrograph showing the morphological features of the olfactory rosette in trout is shown in panel A; the gross structure, including the lamellae (L) and midline raphe (R), was normal in fish from all treatment groups. The epithelia immediately surrounding the midline raphe is shown in micrographs from fish exposed to control (panel B), Cu NPs (panel C) and $CuSO_4$ (two separate fish, panels D and E). The epithelia in controls and fish exposed to Cu NPs was densely covered with ciliated sensory (CS), ciliated non-sensory, and microvillous cells (MS). In contrast, CS and MS were less abundant with clear degradation of cilia surrounding the midline raphe in fish exposed to $CuSO_4$. However, cilia were still abundant in crypts between lamellae (region indicated by dashed line in panel A) in fish exposed to $CuSO_4$ (panel F).

and fish exposed to $CuSO_4$ ($p = 0.015$, Fig. 6B). Ratios of GSH to GSSG were 6.82 ± 0.51, 6.69 ± 0.36 and 4.92 ± 0.24 for control, Cu salt and Cu NP exposed fish, respectively.

4. Discussion

This study demonstrated that Cu NPs had an adverse effect on olfactory-mediated behaviours of rainbow trout, and this new finding is significant because the effects on behaviour appeared to be more pronounced than with the equivalent nominal total concentration of Cu in the form of a metal salt ($CuSO_4$). Exposure to Cu NPs caused a significant inhibition of the anti-predator behavioural response of juvenile rainbow trout compared to control trout and fish exposed to the Cu salt which is consistent with an impaired ability to detect alarm substance in the SE. Tissue Cu analyses indicated this was not associated with accumulation of Cu in tissues of fish and may have been a surface mediated effect of Cu NPs. Observation of the structure of the fish sensory organ, the olfactory rosette, revealed no overt morphological injury in fish exposed to Cu NPs, unlike in fish exposed to the Cu salt. However, Cu NPs, but not $CuSO_4$, did cause elevated oxidative stress in brains of fish as evidenced by a significant reduction in the GSH/GSSG ratio as a consequence of GSH depletion. Together, these findings indicate olfactory-mediated behaviours in trout may be more sensitive to Cu NPs at equal nominal concentrations to Cu salts; and nanoparticulate Cu may exert effects on fish behaviours via a distinct mechanism that is different to dissolved copper salts.

Exposure to $CuSO_4$ did not lead to appreciable tissue accumulation of Cu (Fig. 1), tentatively implying that any behavioural effect of $CuSO_4$ may have occurred directly via the external medium rather than from systemic Cu toxicity. Although trout exposed to $CuSO_4$ showed a close to significant increase of Cu concentration in or on the gills, neither the livers or brains of these fish showed measurable increases in total Cu concentrations confirming that

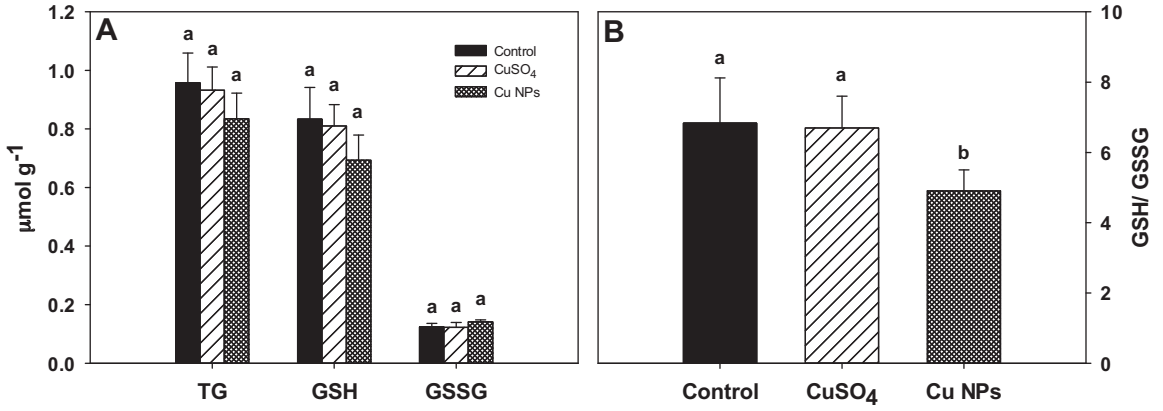

Fig. 6. Concentrations of glutathione in the brains of rainbow trout exposed to control, 50 μg l^{-1} Cu (as $CuSO_4$) and 50 μg l^{-1} of Cu NPs for 12 h. Measured concentrations (μmol g^{-1} wet weight tissue) of total (TG), reduced (GSH) and oxidized (GSSG) glutathione are shown in panel A. Ratios of GSH/GSSG are shown in panel B. All data are means ± S.E.M. ($n = 5$). Different lower case letters within parameter indicate significant differences between treatments ($p \leq 0.05$).

the nominal concentration and short exposure time in the present study was insufficient to cause significant Cu accumulation in the internal organs. Waterborne exposure to 15 μg l^{-1} of total dissolved Cu for 15 days resulted in Cu accumulation in the melanosomes of melanophores in the lamina propria of the olfactory epithelium; but not in the olfactory nerve, bulb or brain (Julliard et al., 1995) indicating that sensory and/or neurological effects of $CuSO_4$ are possible without appreciable internalization of the metal into the CNS. Similar to $CuSO_4$, the present study showed no measurable accumulation of total Cu from exposure to Cu NPs in trout. The bioavailability and behaviour of Cu NPs in fish is unclear, although in common with the present study, recent data of 10 d exposure of juvenile rainbow trout to a total nominal Cu concentration of 100 μg l^{-1} as Cu NPs also indicated NPs may not accumulate in the liver or brain of fish in longer term waterborne exposures (Shaw et al., 2012).

The introduction of the SE into aquaria induced behaviours in control rainbow trout consistent with an anti-predatory response (Fig. 3). Similar to previous studies of anti-predator behaviours in fish (Brown and Smith, 1997; Poulin et al., 1999; Scott et al., 2003), trout in this study exhibited a general decrease in activity levels (as measured by decreased horizontal line crossings, increased time immobile and increased latency to first movement) following the introduction of SE into aquaria when compared to the response of fish to DW. The rainbow trout in the present study did respond to the introduction of DW with decreased activity which was most likely due to the injection of 30 ml of water into an otherwise undisturbed environment, but the response was small compared to the introduction of SE, and fish rapidly resumed activity (<10 s).

Exposures to both forms of Cu (salt and NPs) caused changes in the behavioural responses of trout to SE compared to controls, but these effects appeared more pronounced in fish exposed to the NPs. Following the introduction of SE into aquaria, fish exposed to $CuSO_4$ showed a moderate decrease in activity (~40%). This decrease was not statistically significant compared to that of unexposed controls (~70% decrease in activity), but the effect of $CuSO_4$ on the alarm response to SE may have been confounded by the significant inactivity of fish exposed to $CuSO_4$ during the pre-stimulus period (~50 horizontal line crossings vs >150 in controls and Cu NP exposed fish). Previously, Sandahl et al. (2007) also saw lower pre-stimulus activity levels in coho salmon (*O. kisutch*) exposed to 20 μg l^{-1} Cu as $CuCl_2$. Nevertheless, in this study the elevated latency to first movement characteristic of the control fish response to SE was retained in these fish. This suggests that fish exposed to $CuSO_4$ were able to detect alarm substance in the SE, but the magnitude of the overall behavioural response elicited in fish was decreased. These observations are consistent with previous studies that have reported Cu exposure may decrease behavioural responses of fish to odours (e.g. Beyers and Farmer, 2001; Kennedy et al., 2012). Moreover, Cu (as $CuCl_2$) exposure has been shown to decrease the amplitude of odour-evoked electro-olfactograms in salmonids in a dose-dependent manner (Baldwin et al., 2003) and the diminished, but not eliminated, response to SE of $CuSO_4$ exposed fish observed in the present study may be linked to a decrease in sensitivity of the olfactory apparatus.

In contrast to controls and to a lesser extent $CuSO_4$, the alarm response of trout to SE was eliminated by exposure to Cu NPs and fish behaviours were not significantly different from responses of control fish to DW. This strongly suggests that fish exposed to Cu NPs did not detect the alarm substance present in the SE. Furthermore, the greater effect of Cu NPs compared to $CuSO_4$ in fish exposed at equal gravimetric concentrations of Cu also indicates that some effects of the NPs were likely caused by specific properties of Cu at the nanoscale and unrelated to NP dissolution (dialysis of NPs indicated dissolution would contribute < 2 μg l^{-1} Cu above background to the exposure tanks). Recent studies have demonstrated NPs can adsorb chemical and biological material in suspension (Baun et al., 2008; Hu et al., 2011; Zhang et al., 2007) and it is possible that adsorption of the SE to Cu NPs in the present study could lower bioavailability and/or also the bioreactivity of alarm substance by catalyzing the hydrolysis of the active component(s) of the SE, at the olfactory sensory neurons. Alternatively, the Cu NPs may precipitate or complex in the mucous environment of the nasal pits, thus not reaching the olfactory cells, and act indirectly by blocking water flow to the olfactory sensory cells. In a recent study of the effects of Ag NPs on olfaction in Crucian carp (*Carassius carassius*), irrigation of the olfactory rosette with freshwater immediately restored the electro-olfactory response (Bilberg et al., 2011). However, the diminished responses of Cu NP exposed trout to SE were also evident in fish transferred to clean water following Cu exposure (Fig. 4). Whilst the possibility of Cu NPs still being present in the nasal pits of fish removed to clean water, with subsequent physical effects on water flow and olfaction cannot be excluded; these data could also imply the effects of Cu NPs on the behavioural responses of trout were due to changes in the sensory physiology of the animal. Contaminants may alter the behavioural responses of fish to olfactory stimuli by causing toxicity at multiple sites along the olfactory-neuromuscular axis. However, in the present study, effects on neuromuscular function appear unlikely. Swimming behaviours of fish exposed to Cu NPs during the pre-stimulus period were not different to controls and this would suggest neuromuscular function of these fish was intact.

Examination of the olfactory rosettes of fish using scanning electron microscopy revealed 12 h exposure to 50 $\mu g\,l^{-1}$ Cu (as $CuSO_4$), but not Cu NPs caused injury to the olfactory epithelium. This observation is consistent with the decreased behavioural responsiveness of Cu salt exposed fish to SE. A loss of cilia from olfactory rosettes has previously been reported in rainbow trout exposed to Cu salts (Hansen et al., 1999; Julliard et al., 1996). For example, Hansen et al. (1999) reported a loss (~60%) of ciliated and microvillar sensory receptors due to cellular necrosis from the rosettes of rainbow trout during short term (4 h) exposure to 50 $\mu g\,l^{-1}$ Cu that was concomitant with reduced electro-responsiveness of olfactory epithelia to natural odours. A loss of olfactory responsiveness has previously been shown to correlate closely with loss of alarm response to SE (Sandahl et al., 2007). This effect also likely explains the decreased behavioural responses to SE in $CuSO_4$ exposed fish seen in the present study. In contrast, exposure to Cu NPs in the present study did not alter the morphology of the olfactory rosette or affect the density of ciliated cells. The observed effects on behaviour might therefore simply arise from blockage of the nasal passage with NPs (no exposure, so no effect on the olfactory rosette), or there may be a distinct physiological mechanism for Cu NPs compared to $CuSO_4$. Gene expression profiles in Cu NP-exposed fish appear to be different to that of animals exposed to $CuSO_4$ (e.g. zebrafish gills, Griffitt et al., 2009), including the expression of Na^+,K^+-ATPase sub-units and genes involved in signal transduction. Interestingly, Tilton et al. (2008) advocated the use of molecular biology to understand the mechanistic aspects of olfactory injury in zebrafish and found that 40 $\mu g\,l^{-1}$ Cu as $CuCl_2$ caused a down-regulation of genes in the olfactory signal transduction (OST) pathway including those for calcium channels, g-proteins, and olfactory receptors. A similar approach could provide insight into the cause of behavioural impairments in fish exposed to Cu NPs. From the perspective of electrophysiology, recent data have indicated that Ag NPs can hyperpolarize the olfactory epithelium in Crucian carp, an effect which was not seen in fish upon exposure to Ag^+ (Bilberg et al., 2011) and could be independent of olfactory rosette injury. Furthermore, an intact olfactory epithelium may not necessarily indicate a fully functioning sensory apparatus. In yellow perch (*Perca flavescens*) native to metal-contaminated lakes, Mirza et al. (2009) reported an intact and electrophysiologically responsive olfactory epithelium with no significant loss of olfactory sensory neurons, however this did not correspond to a SE response in fish, leading the authors to suggest other brain centres may have been impacted by metal exposure.

Exposure to Cu NPs caused a significant decrease in the ratio of reduced to oxidized glutathione in brains of trout compared to controls and, to a lesser extent, $CuSO_4$ exposed fish. This raises the possibility that areas of the brain involved in the processing of sensory information were affected by oxidative stress. The separation of different regions of the brain and also analysis of glutathione in the olfactory rosette was not possible in the present study due to the small size of the fish used. Copper NPs have been shown to alter expression of biomarkers of oxidative stress in tissues in trout (Shaw et al., 2012) and cause pathologies in brains of fish (Al-Bairuty et al., 2013). These effects have also been reported to occur without concomitant tissue accumulation of Cu, which suggests effects of NPs at external epithelia, including gill and possibly also the olfactory rosette, may lead to toxicities in internal tissues. The mechanism by which Cu NPs caused a change in oxidative status in brains of trout and its significance to the observed impairment of olfactory-mediated behaviours is unclear, and requires further study. Nevertheless, the data presented are a further example that NPs can cause effects in internal organs, including brain, via mechanisms different from the equivalent metal salts.

In conclusion, Cu NPs caused a significantly greater disruption of the olfactory-mediated behavioural response of trout to SE compared to Cu salt at an equal gravimetric concentration. The mechanism of olfactory toxicity of Cu NPs was unclear, but was distinct from $CuSO_4$ and warrants further investigation. Olfactory impairment may have profound effects on the behaviours of fish and a decreased response to alarm substance has been demonstrated to increase predation risk with the potential to alter population dynamics of exposed fish (Mirza and Chivers, 2003; McIntyre et al., 2012). Recent data has shown that environmental metals legislation for Cu is protective of olfactory competence in salmonids (DeForest et al., 2011). The results of this study suggest effects of Cu NPs on olfaction of fish should be considered, with perhaps the current legislation for Cu being extended to include NPs.

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