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The effects of copper on the morphological and functional development of zebrafish embryos

A. Johnson, E. Carew, K.A. Sloman *

School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth, Devon PL4 8AA, United Kingdom

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Abstract

Waterborne copper exposure can exert a variety of physiological effects in fish, including the disruption of sensory system function, which has wide-reaching implications for fish behaviour. In developing fish larvae, copper is known to affect key parameters, such as survival and growth and more recently has been shown to interfere with the octavolateral system. The present study aimed to take a combined view of morphological (*e.g.* length, yolk sac area) and functional (*e.g.* heart beat, behaviour) processes to understand the complex effect of copper on fish development. In the first of two experiments, zebrafish embryos were exposed to a range of copper concentrations (11–1000 $\mu g\,l^{-1}$) from fertilisation for a 72 h period. The greatest mortality was seen between 5 and 24 h post-fertilisation (hpf) and was more pronounced at the higher copper concentrations. Copper also had an inhibitory effect on hatching. Length and yolk sac area of individuals were recorded across treatments at 72 hpf and elevated copper was found to slow development. Individuals from the higher copper treatments had the fastest heart rates at 28 hpf suggesting that stress responses were induced in the embryos during copper exposure. In the second experiment, embryos were exposed in a similar manner to two copper concentrations, based on those from Experiment 1 that resulted in <50% mortality. At 120 hpf, embryos exposed to both copper concentrations possessed significantly fewer functional neuromasts, an effect which was associated with a reduced ability to orientate in a current. Therefore, although mortality at these copper concentrations was low initially, and then almost non-existent after 24 hpf, the inability of copper-exposed larvae to orientate in a water current as a result of lateral line dysfunction is likely to seriously compromise survival.

Keywords: Copper; Development; Early life stage; Embryo; Zebrafish (*Danio rerio*)

1. Introduction

Copper is an abundant trace element found in a variety of rocks and minerals and has received much attention due to its widespread use, persistent nature, tendency to accumulate and toxicity to aquatic organisms (Mount, 1968). Xenobiotic sources of copper include the wear of tyres and break linings, components of electrical generators, radio and television sets, heating systems, water pipes and wiring (Sloman and Wilson, 2006). Although toxic in excess (Fleming and Trevors, 1989; Weis and Weis, 1991; Sorensen, 1991), copper is also an essential micronutrient for vertebrate function. It forms part of approximately 30 enzymes and glycoproteins, is important in the gastro-intestinal tract and nervous system and is necessary for haemoglobin synthesis (Sorensen, 1991).

There are many documented physiological effects of waterborne copper exposure in a variety of fish species. In excess, copper will target the gills, gut and sensory systems (*e.g.* Grosell et al., 2003; De Boeck et al., 2006; Shaw and Handy, 2006). Disruption of sensory systems has far-reaching implications for many fish behaviours, such as escape from predation and finding a mate, due to the reliance on information about the surrounding environment which the sensory system provides. Hara et al. (1976) found a significant reduction in the olfactory response to the amino acid, L-serine, when rainbow trout were exposed to 8 $\mu g\,l^{-1}$ of copper for 2 h, and while Hansen et al. (1999) demonstrated that damage to the olfactory system by copper may be reversible, brief disruption in behaviours such as predator avoidance or food location can prove fatal.

The auditory, vestibular and lateral line systems make up the octavolateral system of fish and are often considered together as they possess a common sensory cell type (Schellart and Wubbels, 1998). The octavolateral system of fish may also be targeted by trace metal exposure (Hernández et al., 2006; Linbo et al., 2006), although disruption to this system by metals is

* Corresponding author at: 402 Davy Building, School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, United Kingdom. Tel.: +44 1752 238340; fax: +44 1752 232970.
E-mail address: katherine.sloman@plymouth.ac.uk (K.A. Sloman).

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less understood. Exposure of fish to non-metal ototoxins, chemicals which damage the hair cells in the ear and lateral line, has demonstrated a potential link between damage to the lateral line and disruption in rheotaxis behaviours (Wiersinga-Post and van Netten, 1998; Baker and Montgomery, 2001). Therefore, through changes in behavioural ability, the implications of lateral line disruption for fish survival are likely to be as severe as for olfactory disruptions. While a myriad of studies have considered the physiological or behavioural effects of toxicants, the number of studies that consider the combined effects remains small (see review by Scott and Sloman, 2004). Distinguishing the physiological mechanisms by which behaviours vital for survival are affected is, therefore, particularly difficult. Additionally, there is evidence that in fish toxic exposure early on in development has crucial implications for behavioural pathways later in life (Dave and Xiu, 1991; Weber, 2006).

During development, larval stages appear to have greater sensitivity to copper toxicity than embryonic and adult stages (McKim et al., 1978; Shazili and Pascoe, 1986; Scudder et al., 1988; von Westernhagen, 1988), presumably due to a protective action of the chorion preventing free passage of pollutants to the embryo once hardened by exposure to water (von Westernhagen, 1988; Weis and Weis, 1991). The ability of fish eggs and larvae to accumulate heavy metals is well documented (Finn, 2007) and it is likely that exposure to toxicants before hardening of the protective chorion can have significant implications for embryo health. As eggs laid in a contaminated environment are likely to be exposed to contaminants very early in life, it is important that toxicological studies reflect this scenario. Gellert and Heinrichsdorff (2001) clearly demonstrated a decrease in susceptibility of zebrafish embryos to wastewater effluent during the first 4 h of life as the chorion became less permeable to external contaminants. Their results suggest that when early life stages of zebrafish are used in ecological risk estimations, that they should be exposed within an hour of being laid. Therefore, in the present study, zebrafish embryos were exposed to copper from <1 hpf, before complete hardening of the protective chorion.

Although numerous developmental studies have considered copper effects on mortality and hatching success, few have considered how combined aspects of functional development, *e.g.* growth, organ function and behaviour are affected by copper and in some areas the literature is conflicting. For example, brook trout larvae (*Salvelinus fontinalis*) exposed to copper ($32.5\,\mu g\,l^{-1}$) showed a decreased length and an increased time for complete yolk sac absorption compared to control larvae (McKim and Benoit, 1971), whereas fathead minnow embryos and larvae (*Pimephales promelas*) exposed to copper ($621\,\mu g\,l^{-1}$) had a smaller mass and length but no concomitant decrease in yolk sac absorption (Scudder et al., 1988). The small size and mass of zebrafish embryos makes it difficult to determine more traditional measures of contaminant exposure, such as tissue accumulation, at the individual level. Although measurements on samples of pooled individuals can be of interest, parameters that can be measured at the individual level allow consideration of between-individual variation in response when it occurs (Sloman et al., 2002).

The aim of the present study was, therefore, to take a multidisciplinary approach to consider the effects of waterborne copper exposure on zebrafish development. Where previous studies have focussed on simple indicators of fish development, the present study aimed to take a combined view of morphological and functional processes to understand the complex effect of copper on individual fish development.

2. Materials and methods

2.1. Experimental animals

Adult zebrafish were obtained from an existing breeding stock at the University of Plymouth and were maintained in 50-l glass aquaria at 28 °C with a 14 h light:10 h dark photoperiod. All parent fish were approximately 3 months old and fed three times daily *ad libitum* (twice on flake and once on *Artemia* sp. nauplii). To maintain similar ages of parental fish, Experiments 1 and 2 were carried out on different breeding fish from the same strain. Males and females were kept separate until the night before spawning, at which time they were placed together in breeding aquaria and eggs were collected the next morning, after spawning had been induced at first light.

2.2. Copper exposures

Test solutions were made up to the correct concentrations using a weakly acidified, concentrated stock of $CuSO_4{\cdot}5H_2O$ ($100\,mg\,Cu\,l^{-1}$) with city of Plymouth tapwater ($Ca^{2+} = 20.40 \pm 1.86\,mg\,l^{-1}$; $Na^{+} = 18.03 \pm 0.80\,mg\,l^{-1}$; total hardness $= 32.7 \pm 0.8\,mg\,l^{-1}$; natural background copper concentration of $10.7 \pm 0.98\,\mu g\,l^{-1}$ (mean ± S.E.M.)) used as a diluent and control. The weak acidification of the stock concentration did not cause measurable changes in pH at the final exposure concentration. Prior to each test, all equipment was acid-washed in 3% HNO_3. Fifty percent of each test solution was renewed at 24 h intervals and copper concentrations of acidified samples (10 μl 1N HNO_3) of all test concentrations were determined every 24 h (Experiment 1) or 48 h (Experiment 2) by flame atomic absorption spectrophotometry (Varian, Spectra AA 600, Varian Walnut Creek, CA, USA) and graphite furnace atomic absorption spectrophotometry (Perkin-Elmer Simultaneous Multi-Element AA). Appropriate dilutions of a $10{,}000\,mg\,l^{-1}$ stock standard from Fisher Scientific (Loughborough, Leicestershire, UK) were used as standards.

2.2.1. Experiment 1—Growth and development to 72 hpf

Embryos were exposed to six copper concentrations (and a control) ranging from 50 to $1000\,\mu g\,Cu\,l^{-1}$ based on previous studies (Scott and Sloman, 2004). Six replicates for each concentration were used and each replicate consisted of a glass beaker containing 300 ml of the respective treatment solutions and 20 viable embryos. Viable embryos were placed into these solutions at <1 hpf. Beakers were continuously aerated and maintained at 28 °C (±1 °C). Actual concentrations were 53.13 ± 2.50, 92.98 ± 4.87, 190.43 ± 5.82, 326.71 ± 10.70, 463.85 ± 20.21, $1099.67 \pm 33.80\,\mu g\,Cu\,l^{-1}$. During the 72 h test period, mor-

tality and hatching in each beaker for each concentration was recorded at 2, 3, 5, 24, 26, 48 and 72 hpf, where time points are based on preliminary studies and known developmental stages (Kimmel et al., 1995). Heart rate was also measured in three embryos (selected at random) from each beaker (n = 18) at 28 hpf. Heart rate was measured at a separate time point to other parameters to ensure minimal time between measurements on different individuals and minimal disturbance to the embryos during this measurement. During all recordings, any dead eggs/larvae, detritus and faecal material were removed from the test chambers and discarded. Death in early embryos was classified as those exhibiting a marked loss in translucency, change in colouration or degree of fungal infection (Ozoh, 1979; Scudder et al., 1988). A cessation in heartbeat was the criteria for more developed embryos and larvae (Shazili and Pascoe, 1986).

At 72 hpf, any remaining unhatched individuals were removed from their chorions using a light microscope and finely mounted needle to allow more accurate measurement of the embryos. Three individuals from each beaker ($n = 18$) were randomly chosen and placed in a lethal dose of buffered MS-222 (tricaine methane-sulfonate; 0.8 $\mu g\,l^{-1}$) and the lengths and yolk sac areas measured using a Leica MZFIII photo-microscope and Leica Image Manager 50 in conjunction with Scion Image (calibrated using a 1 mm slide graticule). Dechorionation of all remaining unhatched individuals ensured random selection from both hatched and unhatched embryos. The larvae were measured for length (the distance from the most anterior part of the head to the tip of the tail, following the path of the developing spinal cord) and yolk sac area (which encompassed the whole visible outline of the yolk sac, including the yolk sac extension).

2.2.2. *Experiment 2—Development of sensory system and rheotaxis*

Embryos were exposed to two copper concentrations (and a control), which were 68.35 ± 4.27 and $244.36 \pm 17.40\ \mu g\,Cu\,l^{-1}$ (based on concentrations from Experiment 1 that resulted in <50% mortality). Six replicates for each concentration were used and each replicate consisted of a glass beaker containing 300 ml of the respective treatment solutions and 30 viable embryos. Viable eggs were placed into the beakers at <1 hpf as in Experiment 1. At 120 hpf, five larvae were removed from each replicate beaker ($n = 30$) in each treatment, terminally anaesthetised with MS222 and placed in a 0.05% concentration of DASPEI (4-(4-diethylaminostryryl)-*N*-methylpyridinum iodide; Sigma) for 15 min followed by a brief (30 s) rinse in distilled water. The larvae were then photographed under fluorescent light using a Leica MZFIII photo-microscope and Leica Image Manager 50 and the number of neuromasts, which fluoresced due to the DASPEI stain (Linbo et al., 2006; Fig. 1) were counted. To determine at what point during development neuromasts become visible using this method in unexposed zebrafish larvae, a control experiment was carried out where five embryos held in control water were sampled at 48, 72 and 96 hpf (replicated three times) and analysed for number of neuromasts visible and length using the same method.

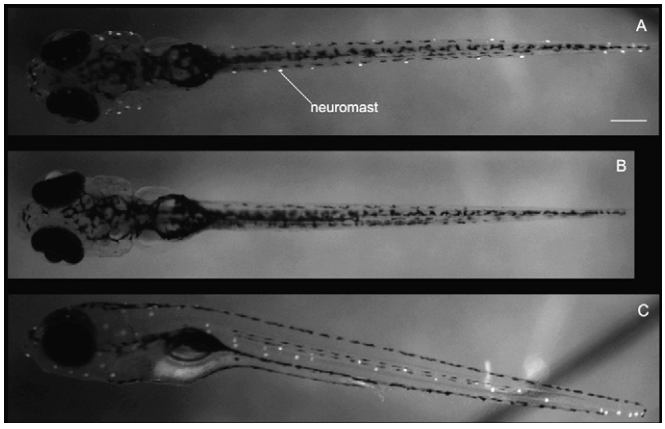

Fig. 1. Control (A) and copper-exposed (B) zebrafish larvae stained with DASPEI illustrating the presence (A) and absence (B) of DASPEI-stained neuromasts. Neuromasts were counted along the side view (C) of zebrafish; note that the transparent nature of the zebrafish means that the neuromasts on both sides of the fish can be seen in panel C. Scale bar = 0.25 mm.

From controls and experimental copper exposures, five more larvae were tested from each replicate for ability to orientate in a water current by placing each one individually into a 50-ml beaker containing 30 ml of appropriate test water. A unidirectional current was then generated within the beaker using a magnetic flea and a magnetic stirrer (Stuart Scientific) set to a constant speed. The larvae were allowed to acclimate to the water current for 2 min and then the proportion of time within a three minute period that the larvae could remain at equilibrium was observed. Equilibrium was defined as the fish remaining in a forward facing position or actively swimming against the water flow.

2.3. *Statistical analysis*

All data were tested for a normal distribution using a Kolmogorov–Smirnov test. Data are presented as mean ± S.E.M. Significance for mortality and hatch data was determined using a repeated-measures two-way analysis of variance (ANOVA) post hoc: least significant difference (LSD) comparison of means ($P < 0.05$), for exposure time and concentration. No significant differences between replicate batches were found for either experiment and one-way ANOVAs were used to assess differences between treatments regarding yolk sac area and body length. The number of neuromasts visible was recorded as a percentage of the maximum number seen in any fish and percentage/proportion data were all arc-sin transformed before statistical analysis; neuromast and behavioural data were not normally distributed and so non-parametric statistics were used. All statistical analyses were undertaken using SPSS 11.5 for Windows (2003).

3. Results

3.1. *Experiment 1—Growth and development to 72 hpf*

Mortality increased significantly and was more pronounced with increasing copper concentration and hours post-fertilisation

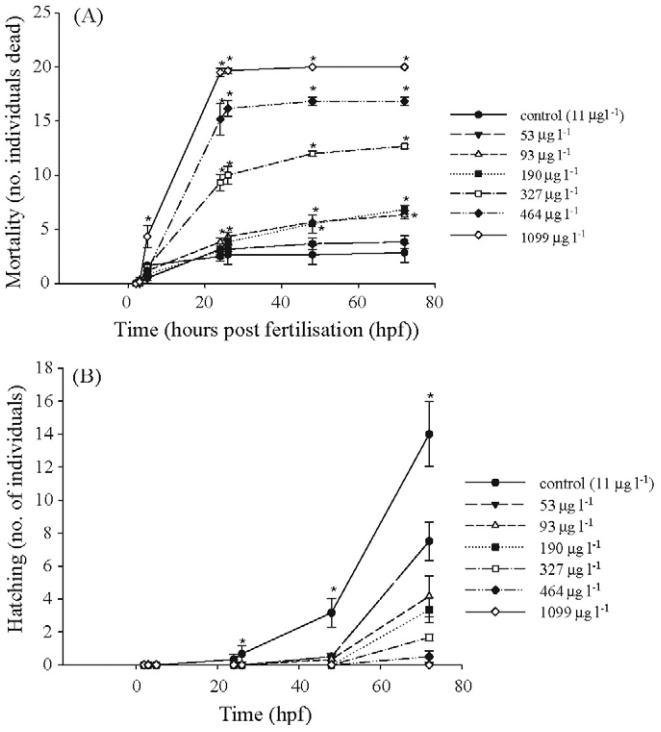

Fig. 2. (A) Mortality and (B) hatching of zebrafish embryos exposed to each copper treatment (mean ± S.E.M.; $n = 6$). Asterisks denote a significant difference (post hoc: LSD) between treatments and the control (11 µg Cu l^{-1}) for a given time period (repeated-measures ANOVA post hoc: mortality: $F_{6,35} = 138.98$, $P < 0.001$) or between the control and other treatments (repeated-measures ANOVA post hoc: hatching: $F_{6,35} = 23.38$, $P < 0.001$).

(repeated-measures ANOVA, $P < 0.05$; Fig. 2A). At 2, 3 and 5 hpf, there was no significant difference in the number of dead individuals compared to the control, except for 1099 µg l^{-1}, which showed a large increase in mortality at 5 hpf and 100% mortality within 26 hpf (hence there are no data for this treatment regarding weight, heart rate, length and yolk sac area).

Increasing copper concentrations had an inhibitory effect on hatching (Fig. 2B). After 26 hpf, embryos in the control treatment had a significantly higher hatching rate than embryos in the copper solutions (repeated-measures ANOVA, $P < 0.05$).

At 72 hpf, copper exposure had exerted a significant negative effect on the length of individuals (one-way ANOVA, $P < 0.05$; Fig. 3A) with all embryos exposed to concentrations of 93 µg l^{-1} and above showing significantly shorter body lengths than control larvae. Exposure to increasing copper concentrations resulted in a larger yolk sac area of individuals at 72 hpf (one-way ANOVA, $P < 0.05$; Fig. 3B).

Higher copper concentrations resulted in significantly higher heart rates (Fig. 4; ANOVA, $P < 0.05$) with embryos exposed to 93, 327 and 464 µg Cu l^{-1} showing higher heart rates at 28 hpf compared to embryos exposed to 53, 190 µg Cu l^{-1} and the control embryos.

3.2. Experiment 2—Development of sensory system and rheotaxis

Neuromasts were visible in control larvae at 72 hpf (Table 1). At 120 hpf, embryos exposed to both copper concentrations pos-

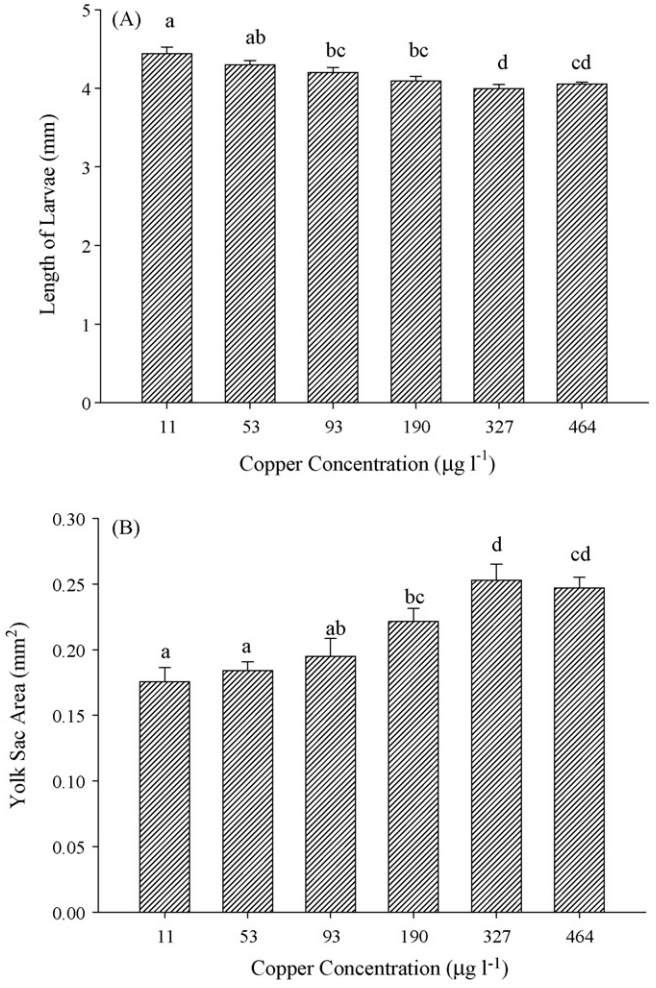

Fig. 3. (A) Body length and (B) yolk sac areas of zebrafish larvae from each copper treatment at 72 hpf (11 µg Cu l^{-1} represents the control). Data are presented as mean ± S.E.M. ($n = 18$). Points sharing a letter are not significantly different from one another (ANOVA post hoc: LSD: length: $F_{5,30} = 5.551$, $P < 0.05$; yolk sac area: $F_{5,30} = 9.631$, $P < 0.05$).

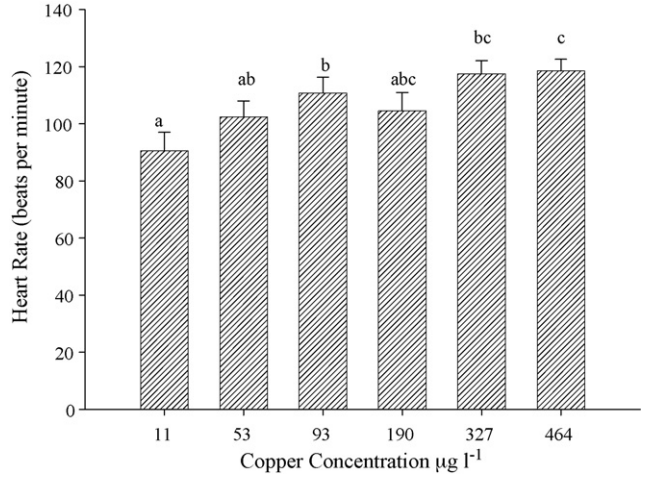

Fig. 4. Heart rates of zebrafish embryos from each treatment at 28 hpf (11 µg Cu l^{-1} represents the control; $n = 18$). Data are presented as mean ± S.E.M. Bars sharing a letter are not significantly different from one another (ANOVA post hoc: LSD $F_{5,30} = 3.616$, $P < 0.05$).

Table 1
Length (mm) and percentage of neuromasts visible (after arc-sin transformation) in control zebrafish embryos at 48, 72, 96 ($n = 15$) and 120 hpf ($n = 30$)

Hours post-fertilisation	Length (mm)	Neuromasts visible (%)
48	3.03 ± 0.04	0
72	3.55 ± 0.04	59.11 ± 5.14
96	3.73 ± 0.05	84.55 ± 2.47
120	4.17 ± 0.03	88.93 ± 1.07

Data are presented as means ± S.E.M.

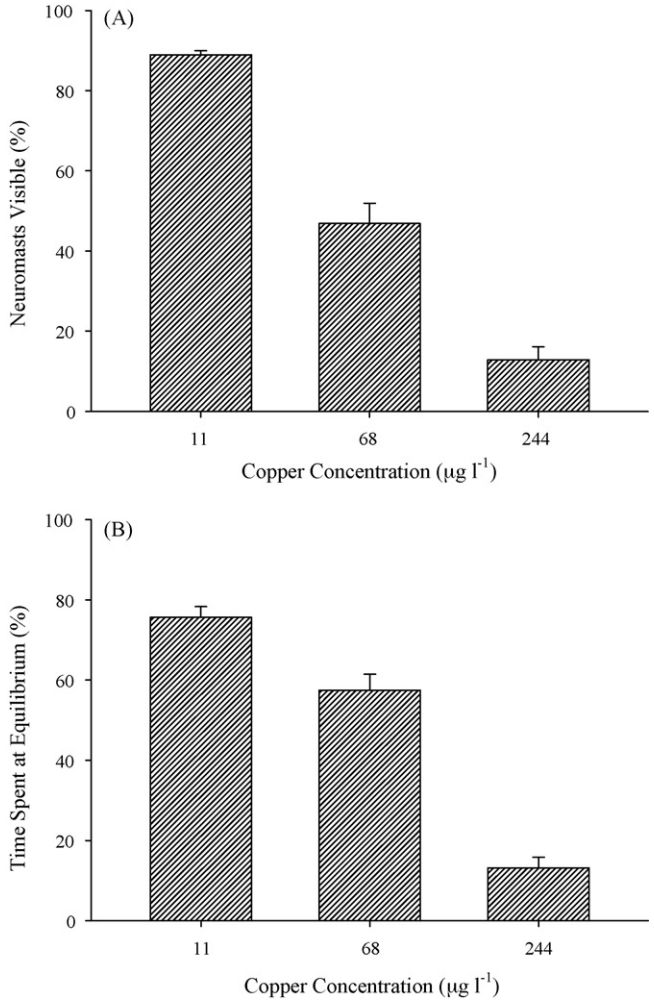

Fig. 5. (A) Percentage (following arc-sin transformation) of neuromasts found and (B) proportion of time spent at an equilibrium state in the different copper concentrations (11 μg Cu l^{-1} represents the control). Data are presented as mean (following arc-sin transformation) ± S.E.M. ($n = 30$) where all bars are statistically different from one another. (Kruskal–Wallis with Mann–Whitney post hoc: $P < 0.001$).

sessed significantly fewer neuromasts (Fig. 5A; Kruskal–Wallis: $P < 0.001$) than controls, which was additionally associated with a reduced ability to orientate in a current (Fig. 5B; Kruskal–Wallis: $P < 0.001$).

4. Discussion

Copper significantly increased the mortality of zebrafish, beyond 24 hpf, above a threshold concentration between 50 and 100 μg l^{-1}. The results presented show that the majority of mortalities occurred during the gastrulation and segmentation periods (occurring between 5 and 24 hpf (Schilling, 2002)), which have previously been termed 'critical windows' in the development of fish (Weis and Weis, 1991; Chow and Cheng, 2003). In the present study, zebrafish embryos were exposed from <1 hpf. Eggs laid by females in a contaminated environment would be exposed to contaminants as soon as they were laid and so toxicology studies exposing zebrafish embryos to contaminants after hardening of the protective chorion may not be truly representative. Indeed, the high sensitivity of embryos to copper in the present study supports the idea that when using early stages of zebrafish for ecological risk estimations, the eggs should be exposed as soon after fertilisation as possible (Gellert and Heinrichsdorff, 2001). Other studies in zebrafish have supported that very early life stages in which the chorion has not yet hardened are particularly susceptible to waterborne contaminants (Herrmann, 1993).

Decreased hatching success was observed in embryos exposed to concentrations of 50 μg Cu l^{-1} and above. The inhibition of hatching caused by copper is supported elsewhere in the literature, both in zebrafish and other teleost species (Ozoh, 1979; Dave and Xiu, 1991; Palmer et al., 1998). Interference with or inhibition of the secretion or activity of the hatching enzyme, chorionase, which degrades the zona interna of the chorion (Yamamoto and Yamagami, 1975), leaving the softer zona externa to be broken down by osmotic and mechanical processes (Schoots et al., 1982), can result in delayed hatching. Indeed, copper has been found to inhibit the proteolytic function of chorionase extracted from rainbow trout eggs (Hagenmaier, 1974). Hatching is often the result of a combination of biochemical (enzymatic), biophysical (mechanical) and osmotic mechanisms (Yamagami, 1981) and so the observed inhibition may result from the action of copper on more than one or all of these mechanisms. Alternatively delayed hatching may be attributed to a slower developmental rate.

Yolk sac edema in embryonic and larval fish, following metal exposure, has commonly been identified by changes in the shape of the yolk sac and space between the yolk and yolk sac itself (Ozoh, 1979, 1980; von Westernhagen, 1988; Cheng et al., 2000). No such abnormalities or deformations were noted in any of the individuals analysed, which, therefore, suggests that the increased yolk sac area, with increasing copper concentration, was not caused by edema, but a reduction in yolk utilisation. The increased yolk sac area and the concomitant decrease in length of individuals are commonly seen in metal toxicity tests and suggest that copper impaired the embryonic rate of development (McKim and Benoit, 1971; Rosenthal and Alderdice, 1976; Peterson et al., 1983). Furthermore, the results for hatching success may support this suggestion. In zebrafish, hatching is not as useful as a staging index compared with other types of embryos (Kimmel et al., 1995) as individuals within a single developing clutch may hatch sporadically over a period of approximately 24 h. However, the marked difference in hatching rates of embryos in the higher concentrations compared to controls is likely to be a function of their slower development.

The decreased length and yolk sac absorption in those fish exposed to copper support the idea of delayed development. Perhaps surprisingly, larvae from the copper-exposed treatments showed higher heart rates at 28 hpf. Heart rate in control zebrafish is seen to increase dramatically during the first few days post-fertilisation (Jacob et al., 2002; Bagatto, 2005) and so a fish earlier in development should display a lower heartbeat. It is likely that exposure to copper is mediating a stress response in the larvae, which is resulting in an increased heartbeat and it is possible that the energy requirements of responding to a toxic stress are diverting some energy away from growth (Barton, 1997). One of the main toxic mechanisms of copper is the disruption of ionoregulatory processes, for example, the inhibition of Na^+K^+ATPase (Wood, 2001). It is possible that a stress response, mediated *via* increases in stress hormones, such as cortisol, is mounted in response to ionoregulatory disruption. Determination of Na^+K^+ATPase levels in individuals is difficult in very early life stages of zebrafish due to their small size, but in salmonid embryos, which represent some of the largest eggs shed by broadcast spawners (Finn, 2007), silver exposure has been found to increase Na^+K^+ATPase activity at 24 days post-fertilisation (dpf), with a subsequent decrease seen in larvae at 37 dpf (Brauner and Wood, 2002).

In control fish, neuromasts were visible at 72 hpf, when the larvae reached around 3.5 mm in length. The number of neuromasts in control individuals had increased by 96 hpf but remained similar at 120 hpf. Exposure to both copper concentrations caused a significant reduction in neuromasts, as supported by Linbo et al. (2006) and Hernández et al. (2006). Here, we measured the presence or absence of neuromasts stained with DASPEI as an indirect measure of neuromast function. Using electron microscope techniques, Linbo et al. (2006) have determined that the absence of DASPEI stain from the individual mechanoreceptor neurons is related to a loss of both kinocilia and stereocilia from the neurons. The requirement of these structures for mechanical signal transduction strongly suggests that the absence of DASPEI staining in neuromasts is directly related to loss of function. Both Linbo et al. (2006) and Hernández et al. (2006) exposed larvae to copper later in development in contrast to the present study that exposed developing embryos from just after fertilisation up to 120 hpf. The individuals in the present study showed no ability to acclimate to the presence of copper during development and both copper concentrations resulted in a decreased rheotaxis ability at 120 hpf (Fig. 5). While mortality at these concentrations in Experiment 1 was very low after 24 hpf, the ability of any of the fish exposed to these concentrations to survive in their natural environment with this impaired behavioural function is debatable.

Toxicity of copper to neuromast hair cells is very rapid (Linbo et al., 2006) and it is likely that copper accumulates in neuromast hair cells *via* the Ctr1 copper transporter, which is widely expressed in the early life stages of zebrafish. During early life stages, distribution of Ctr1 appears relatively ubiquitous but becomes restricted to the brain and ventral tissue by 24 hpf and from 72 hpf is found mainly in the developing intestine (Mackenzie et al., 2004). Hernández et al. (2006) clearly demonstrated that copper is quite unusual in its toxicity to hair cells, being considerably more toxic than many other trace metals (including Cd, Co, Mn, Zn, Sn, Fe). Interestingly, they found that silver behaved most similarly to copper, which may be related to the fact that Ctr1 can also transport silver (Lee et al., 2002). Other studies considering trace metals interactions with fish behaviour and physiology have also found similarities between copper and silver toxicity that may be related to transport mechanisms (Sloman et al., 2003). Future studies should explore whether it is possible to predict metal toxicity effects at the behaviour/physiology interface based on transport mechanisms of specific toxicants.

Many chemical contaminants target specific physiological systems and through these effects influence a myriad of essential behaviours. For a complete understanding of the toxicity of aquatic contaminants, both the physiological and behavioural effects of toxicants need to be considered simultaneously. Here, we show a link between loss of neuromast function and disruption in rheotaxis. The cellular damage that copper induces in hair cells is likely to be specific to copper or metals that utilise similar transport mechanisms (Hernández et al., 2006) and it is possible that other studies demonstrating links between changes in orientation behaviour in fish and exposure to metals other than copper may not be a direct result of lateral line disruption. For example, Baker and Montgomery (2001) found that waterborne cadmium exposure affected both the ability of banded kokopu, *Galaxias fasciatus*, to orientate in a water current and their attraction towards adult pheromones. They concluded that cadmium interferes with the olfactory and lateral line sensory systems. Certainly, cadmium is known to accumulate in the olfactory system of fish and a direct link between olfactory accumulation and behavioural response to alarm substance has been demonstrated (Scott et al., 2003). However, whether the loss of orientation in the study of Baker and Montgomery (2001) is a direct result of lateral line dysfunction or a more general neurotoxic response to cadmium (Sorensen, 1991) remains unknown.

In conclusion, exposure to copper significantly decreased the survival of zebrafish embryos, exerting the greatest effect during the sensitive stages of gastrulation and segmentation. Decreased body length and enlarged yolk sac sizes at 72 hpf in larvae exposed to higher copper concentrations suggested that copper retards the development of zebrafish embryos, and increased heart rates suggests that this may be mediated *via* a stress response. Although mortality was highest before 24 hpf, the inability of zebrafish exposed to copper to orientate in a water current as a result of lateral line dysfunction is likely to seriously compromise survival of the remaining larvae under natural conditions.

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