

The relative sensitivity of sperm, eggs and embryos to copper in the blue mussel (*Mytilus trossulus*)

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Received 5 December 2007; received in revised form 23 January 2008; accepted 24 January 2008

Available online 5 February 2008

Abstract

Copper, an essential element, is toxic at elevated concentrations, and as a result of anthropogenic activities is becoming increasingly prevalent in marine environments. In this study, we examined the effects of copper on early life stages of the blue mussel, *Mytilus trossulus*. We assessed the impacts of increasing copper concentrations on embryo development, egg viability, sperm fertilization capacity and, in particular, on sperm swimming speed using computer-assisted sperm analysis. Sensitivity to copper followed the pattern: embryos > sperm > eggs. A dramatic increase in abnormal embryo development was observed following exposure to copper concentrations exceeding 10 µg/L. Sperm swimming speeds decreased significantly when exposed to 100 µg/L of copper, but lower doses did not influence sperm swimming speed. Copper exposure (at any tested concentration) did not affect sperm flagellum length, or alter egg viability. Based on our results, we suggest that exposure of sperm to copper may interfere with mitochondrial activity, which reduces sperm swimming speed during the extended duration of sperm motility in blue mussel.

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Keywords: Ecotoxicology; Fertilization; Sperm; Eggs; Spermiotoxicity; Computer-assisted sperm analysis; Development

1. Introduction

Anthropogenic activities, such as the release of industrial effluent and the growing use of copper as an antifoulant (Apte and Day, 1998; Paulson et al., 1989), have resulted in copper becoming increasingly prevalent in coastal ecosystems. Natural copper concentrations in marine environments are typically <5 µg/L (Soegianto et al., 1999; Hall and Anderson, 1999; Davenport and Redpath, 1984), but in polluted areas, copper levels can be orders of magnitude higher than natural levels, reaching concentrations of >50 µg/L in heavily-polluted

harbours (Haynes and Loong, 2002; Soegianto et al., 1999). The increasing copper concentrations in marine ecosystems are of particular concern because, although copper is a naturally-occurring trace element that is essential for some metabolic processes, it can be toxic to marine organisms at elevated concentrations (White and Rainbow, 1985; Viarengo, 1989). A comprehensive examination of the potential impacts of copper toxicity on a variety of life stages of various marine biota can assist in informing regulatory decisions regarding acceptable water quality in the marine environment.

The early life stages of marine invertebrates are usually more susceptible to environmental toxicants than are the adult forms (see review by His et al., 1999). Copper exposure has been demonstrated to reduce fertilization success, larval development and attachment, impair embryogenesis, and alter enzymatic activity (Katranitsas et al., 2003; Xie et al., 2005; Bellas et al., 2001; Bellas et al., 2003; Bellas et al., 2004;

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Novelli et al., 2003; Fernandez and Beiras, 2001; Reichelt-Brushett and Harrison, 2005; Ringwood, 1992; Dinnel et al., 1989). However, despite the extensive documentation of copper's adverse effects on early developmental stages, few studies have examined precisely how copper (or indeed any metal) decreases fertilization success by assessing how metals influence gamete function and morphology (see Earnshaw et al., 1986; Au et al., 2000, 2001a,b). Typically, in spermio-toxicity tests, metal toxicity is assessed by exposing sperm to the toxic agent for a short time period, then adding exposed sperm to eggs to assess fertilization success and allowing embryos to develop in polluted water. In echinoderms (i.e., sea urchins and sand dollars), bivalves and ascidians, such short-term exposure of sperm to a variety of metals, including copper, has been demonstrated to reduce fertilization success (Novelli et al., 2003; Reichelt-Brushett and Harrison, 2005; Ringwood, 1992; Dinnel et al., 1989; Au et al., 2001a; Pagano et al., 1982; Bowen and Engel, 1996; Eyster and Morse, 1984; Ringwood, 1992; Bellas et al., 2004; but see Bellas et al., 2001). However, because fertilization trials in spermio-toxicity tests are performed with sperm that are swimming in metal-polluted water, this factor may also influence embryo development. Therefore it is not clear whether the reduced fertilization rates observed in these tests are the result of impaired sperm function or due to exposure of the developing embryos to metals.

Blue mussels (*Mytilus trossulus*), which are semi-sessile, filter-feeding marine bivalves found in all temperate and polar waters worldwide, were used in this study because ambient water quality guidelines are often based on toxicity tests with early life stages of blue mussels (US EPA, 1995a, summarized by Arnold, 2004; Arnold et al., 2005). Furthermore, there is ecological relevance in using this species as seasonal increases in copper concentrations (in spring and summer; Alliot and Frenet-Piron, 1990) often coincide with blue mussel gametogenesis (spring) and spawning (summer) (Thompson, 1984). Ripe male and female mussels release gametes through their exhalant siphon directly into the water column, where fertilization takes place (Thompson, 1984; Thorarinsdottir and Gunnarsson, 2003). Therefore, copper may influence recruitment by impairing sperm function (Earnshaw et al., 1986) and by disrupting embryogenesis (see references above). Current copper water quality guidelines are based on 48-h EC50 embryo-larval development chronic toxicity tests, in which developing embryos are exposed to a toxicant and effects are measured after 48-h exposure (US EPA, 1995a, summarized by Arnold, 2004; Arnold et al., 2005). Consequently, efforts aimed at improving environmental regulations focus solely on embryo survival, rather than examining the effects of toxicants on several early life stages.

Our study had two main objectives. First, we investigated whether or not the decreased fertilization success of blue mussels reported in other copper exposure studies was a result of decreased gamete function. Second, because it is unlikely that copper has effects on only one life stage, we examined effects at multiple early life stages, to better understand the adverse effects of copper exposure to blue mussels.

2. Materials and methods

2.1. Study Species

Between June 20–July 6, 2006, ripe blue mussels (*M. trossulus*) were collected from natural intertidal populations in a pristine area, the Broken Island Group, near Bamfield, British Columbia (48.45N, 125.10W). Adults were cleaned, separated and transferred to 11–13 °C aerated, flowing seawater baths where they were held for up to 24 h prior to experimental treatment. Adults were then placed in a 10 L seawater bath (15–20 adults/bath) maintained at 22–25 °C, constituting a thermal shock, which induced spawning. Adult gender cannot be determined externally; therefore, gender could only be determined subsequent to gamete release. Once gamete release was observed, the mussels were placed in isolated 250 mL beakers, on ice, containing filtered seawater (water volume varied by experiment) where the gametes were released into the water column. Adults were only used if they continued to release gametes after transfer to the beaker.

2.2. Copper solutions and chemical analyses

Copper solutions were prepared from a copper chloride (CuCl_2 ; Sigma-Aldrich) stock solution, using 0.20 μm -filtered seawater, serially diluted to make six copper solutions (nominally, 0.32, 1.0, 3.2, 10.0, 32.0, and 100.0 $\mu\text{g/L}$ copper above the seawater background). In all experiments, gametes (both eggs and sperm) or developing embryos were exposed to these six copper solutions, as well as a control solution of filtered seawater.

Copper in seawater was analyzed based on methods modified from those described in Toyota et al. (1982). Briefly, 1 μL of 10 mg/mL lanthanum oxide, 7.5 μL of 1 M sodium carbonate, and 7.5 μL of a 1 N nitric acid were added to 1 mL of seawater. The solutions were placed in an 80 °C heat bath for 30 min, forming a precipitate, then centrifuged at 3000 G for 15 min. The supernatant was decanted and the precipitates were dissolved in 1 mL 1 N nitric acid. Copper concentrations were determined from this solution using a graphite furnace atomic absorption spectroscopy (GFAAS; Varian SpectrAA-220 with graphite tube atomizer [GTA-110], Mulgrave, Australia). Fisher Scientific calibration standards were used for every run. The method detection limit was 0.2 $\mu\text{g/L}$ and copper recovery was $\pm 10\%$, as determined from the Analytical Reference Material TM15 (Environment Canada, National Water Research Institute). Copper concentrations were determined for the seawater and six copper exposure solutions used in this experiment (Exposure Concentrations, Table 1).

2.3. Experiment 1: Effects of copper on sperm motility and fertilization success

Following sperm release, males ($n=11$) were placed in individual beakers (on ice) containing 70 mL of filtered seawater. They were left undisturbed for 3 min, as sperm release

Table 1
Copper concentrations (in $\mu\text{g/l}$) determined for exposure solutions at each of the nominal copper concentrations used in experiments during this study

Nominal concentration	Exposure concentrations (measured)
0 (control sea water)	1.47 \pm 0.12
0.32	1.15 \pm 0.12
1.0	2.37 \pm 0.31
3.2	4.63 \pm 0.61
10	11.67 \pm 0.29
32	27.40 \pm 0.0
100	71.00 \pm 6.90

All data are presented as mean \pm standard error (SE) ($n=4$).

continued, and were then removed from the beaker, leaving a 70 mL sperm/water mixture. Sperm concentration varied among males as some males released more sperm than others. As sperm concentration influences fertilization success (e.g. Hollows et al., 2007), we controlled for sperm density within each male by adding a homogenized mixture of sperm from a single male to a homogenized mixture of eggs across all treatments, thereby maintaining a roughly equal sperm:egg ratio in all treatments for each male. Thus, by using a repeated measures design we ensured that any observed effects are due to the effects of copper, rather than resulting from variation in sperm concentrations. The sperm/water mixture was homogenized by gently stirring, and 9 mL from each male's beaker was added to each of seven 15 mL plastic scintillation vials (on ice), representing the seawater control and the six copper solutions. Immediately after the sperm/water mixture was added to the scintillation vials, 1 mL of filtered seawater was added to the control while a 1 mL copper solution, at a concentration 10 times higher than the desired concentration, was added to each of the six copper treatments (9 mL), yielding the appropriate concentration.

After copper was added to the sample, sperm motility was recorded by placing a 60 μL subsample from the scintillation vial on a 1-mm deep well microscope slide, with a cover slip covering half of the depression (see Liley et al., 2002 for similar methods). Videos of sperm motility were captured at 60 frames/s using a PixelINK Megapixel PL-A662 digital video camera (PixelINK, Ottawa, Ontario, Canada) mounted on a Leica DME light microscope (Leica Microsystems Inc., Buffalo, New York, USA), with a phase contrast filter in place, at 200X magnification. Images were recorded using PixelINK PL-A600 Series Camera Software (v. 3.1, PixelINK, Ottawa, Ontario, Canada). Sperm motility from each male was assessed at approximately 3.2, 32, and 100 min subsequent to the copper spike, for each of the seven treatments.

Sperm velocity was measured using a CEROS (v.12) video sperm analysis system (Hamilton-Thorne Research, Beverly, Maine, USA). Only those spermatozoa whose forward movement was recorded for at least 0.33 s (≥ 20 frames) were analyzed (see Lahnsteiner et al., 1998; Burness et al., 2004 for a similar criterion). The median sperm smooth path velocity (VAP) was calculated for all spermatozoa recorded at each time period (number of sperm recorded, mean \pm SE: 23.9 \pm 1.6 cells per time period; range: 1–222).

In addition, seven 1 mL samples of the sperm/water mixture from each male, representing each of the seven treatments, were placed in a 1.5 mL Eppendorf[®] tube and preserved by adding 500 μL of buffered formalin. A 10 μL sub-sample from each treatment was placed under a cover slip on a glass slide, and sperm morphology was examined under 400 \times magnification. The first ten clear spermatozoa observed from each male were photographed using a PixelINK Megapixel PL-A662 digital camera mounted on the microscope. Tail lengths were measured using NIH ImageJ software (available at <http://rsb.info.nih.gov/ij/>), by tracing a freehand line over the tail using an Intuos graphic tablet (Wacom Co. Ltd., Japan), measuring from the centre of the sperm head to the end of the tail to the nearest 0.1 μm (see Balshine et al., 2001 for additional details). For all seven treatments, tail lengths were analyzed using a mean value from the 10 photos for each male.

The same experimental treatments were used to assess fertilization success. In this phase of the experiment, our aim was to assess the fertilizing ability of sperm that had been exposed to varying copper concentrations, and not to assess how fertilization proceeds in copper-polluted seawater. Therefore, prior to fertilization trials, we removed aqueous copper from the sperm/water mixture by performing two water changes. Following the 100-min recording of sperm velocity, 1 mL of the sperm/water solution from each of the seven treatments per male was placed in 1.5 mL Eppendorf[®] tubes and centrifuged for 10 min at 14,000G, forming a pellet of sperm at the bottom of the tube. A 900 μL aliquot of the supernatant was decanted and replaced with filtered seawater. This procedure was repeated twice. The sperm/water solution was then used in the fertilization success trials (see below). Centrifuging the sperm/water solution did not adversely effect sperm viability, as preliminary trials demonstrated that sperm exhibited comparable motility after centrifuging, and were able to successfully fertilize eggs (see Results; Earnshaw et al., 1986).

To control for variability between and among males and females used in the experiments, fertilization success trials were conducted by mixing sperm from a single male (from each of the seven treatments) with eggs from a single female. The experiment was repeated 7 times with different individuals at each concentration. The female was placed in an isolated container of 250 mL filtered seawater once she began to release eggs (as was done for the males). Once egg release was complete (approximately 1–2 h), the female was removed, and 125 mL of water was decanted from the container, leaving a 125 mL egg/water solution. The egg/water mixture was gently stirred, and 9 mL of this mixture was added to 7 scintillation vials, representing the control and six copper treatments. One milliliter of the centrifuged sperm/water solution from a single male was added to the egg solution and gametes were allowed to interact in an aerated vial for 1 h, at which point 0.5 mL of buffered formalin was added to arrest fertilization and preserve developing embryos. Fertilization rate was determined by counting the ratio of fertilized/unfertilized eggs from a randomly selected subsample of at least 100 eggs (range 100–152). Fertilized eggs, which exhibit a characteristic polar body, are clearly visible under 200 \times magnification.

2.4. Experiment 2: Effects of copper on egg viability

To assess the impact of copper on egg viability, we exposed eggs to varying copper concentrations and then added sperm. Following the onset of gamete release in the water bath, females ($n=8$) were placed in a beaker containing 70 mL of filtered seawater and allowed to release eggs for approximately 15 min. The female was then removed from the beaker, and the egg/water solution was gently stirred to homogenize the solution. Nine milliliter of the egg/water solution were added to each of seven 15 mL scintillation vials, and the egg/water solution was spiked with 1 mL of seawater (control) or a copper solution at a concentration 10-fold higher than the desired concentration, to yield the six copper concentrations used in the experiment. To facilitate comparison with the results from Experiment 1, in each treatment (i.e., control and six copper solutions) the egg/water solutions were held on ice for 100 min prior to fertilization success trials.

As in Experiment 1, our aim was to assess the fertilizing ability of eggs that had been exposed to varying copper concentrations. Following 100 min of copper exposure, in each scintillation vial, 8 mL of the egg/water solution was decanted and replaced with 7 mL filtered seawater (~ 11 °C) and a 1 mL sperm/water solution (which was previously kept on ice). The sperm used in this study were kept in filtered seawater held on ice and were not previously exposed to any of the copper treatments. In all cases, sperm from a single male was used to fertilize the eggs from a single female, to control for variability between and among males and females used in the experiments. The experiment was repeated 8 times with different individuals at each concentration. The sperm/egg mixtures were allowed to interact for 1 h prior to the addition of 0.5 mL buffered formalin. Fertilization rates were determined as described above.

2.5. Experiment 3: Effects of copper on embryo development

Blue mussel embryo survival was assessed using a well-established protocol (NIWA, 2005). As described above, once the mussels began releasing gametes, they were removed from the water bath and placed in individual 250 mL beakers with 200 mL filtered seawater. Gamete quality was assessed visually under a microscope at 200 \times magnification; individuals were only used if females were shedding round, non-transparent eggs and males were releasing motile sperm. Eggs from 3–4 females were pooled, mixed by gentle stirring, and sieved through a 250- μ m mesh into a beaker. Sperm from 2–3 males were pooled in a 1 L beaker and homogenized by gentle stirring. Eggs were placed in filtered, aerated seawater in a 1 L beaker and a 10 mL sperm/water solution was added to achieve fertilization. A random 100 μ L subsample of egg/sperm mixture was assessed periodically until the total number of fertilized eggs (as evident by the existence of a polar body) equaled or exceeded 80% of the eggs counted.

The effects of copper toxicity on development were assessed by placing 100 μ L of the egg solution into seven 15 mL scintillation vials containing 10 mL of filtered seawater of each treatment (i.e., control and six copper solutions). This was

repeated, resulting in five replicates. Developing embryos in the vials were incubated at 20 °C for 48 h, after which time 1 mL buffered formalin was added to arrest development and preserve the embryos. After incubation and preservation, 8 mL of solution was decanted off and the eggs in the remaining 2 mL were transferred to a Sedgewick-Rafter counting chamber, where embryo development was scored on the first 100 embryos randomly observed, under 100 \times magnification. Normal embryo development results in the formation of a characteristic ‘D’ shaped mantle; these embryos were considered normal. Embryos not characterized by a clear ‘D’ shaped mantle were considered abnormal.

2.6. Data analyses

Data are expressed as means \pm 1 standard error of the mean (SEM) (n) where n represents the number of individual mussels tested. Statistical analyses were performed using the programs JMP (version 5.1, SAS Institute Inc., 2004) and StatView 4.0 (SAS Institute Inc., 1992–1998). Data were first tested for normality using a Shapiro–Wilk test, transformed when necessary to improve the fit to normality, and analyzed using parametric statistics. In cases where data were not normally distributed, non-parametric statistics were used, with all rank data corrected for ties. Analyses of variance (ANOVAs) were followed by a Tukey’s HSD post-hoc test. Non-parametric post-hoc tests were calculated without using computer software (Siegel and Castellan, 1989).

Sperm swimming speed (VAP=smooth path velocity) data were \log_{10} transformed and examined with repeated-measures ANOVA. To avoid pseudoreplication, all statistical analyses of sperm characteristics were performed by collapsing data collected from each male into a single median (smooth path velocity) or mean value (other parameters) for each male at each time period sampled. As percentage data commonly form a binomial, rather than a normal, distribution, all percentage data (e.g., fertilization success and the percentage of abnormally-developed embryos) were arcsine-square root transformed prior to statistical analysis (see Zar, 1999). The influence of copper on fertilization success (using sperm and eggs, respectively) was examined using Friedman’s tests (a non-parametric repeated-measures ANOVA; Zar, 1999).

3. Results

3.1. Copper analyses

Background copper levels measured in seawater were less than 1.5 μ g/L, confirming that tests were done in a pristine area. Nominal concentrations have been used to describe our results. Measured copper concentrations for each of the nominal exposure values are presented in Table 1; the first two experimental concentrations were not significantly different from this background. Following the washing and centrifugation of eggs and sperm, water samples from all treatments yielded copper concentrations less than 2.5 μ g/L, indicating very little carry-over into the fertilization trials.

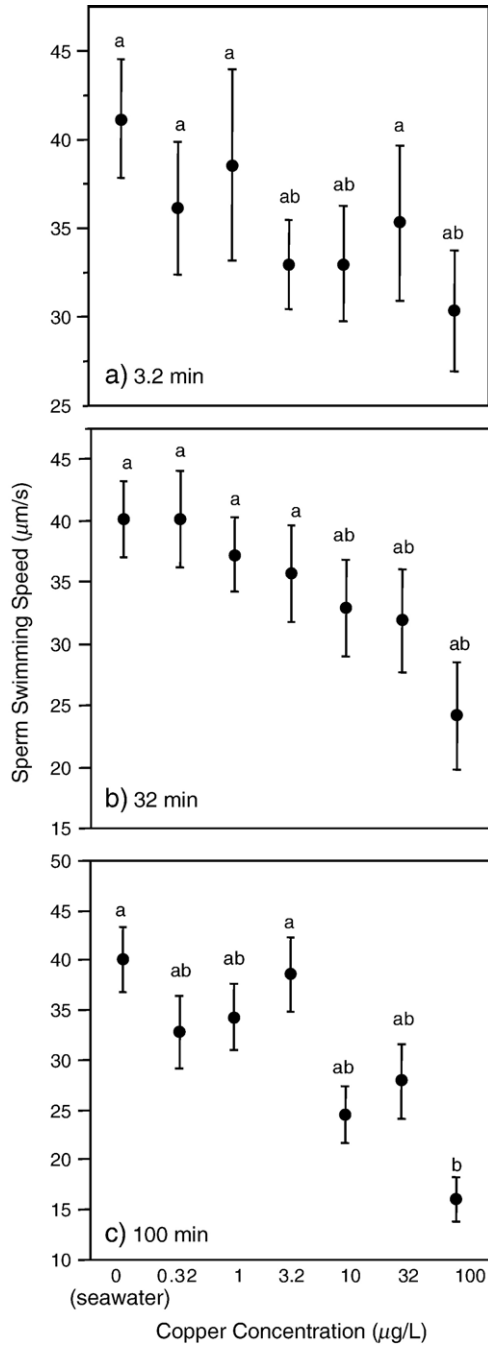


Fig. 1. Medians±SE ($n=11$) sperm swimming speed after exposure to increasing copper concentrations for a) 3.2, b) 32 and c) 100 min. Significant differences are indicated with different letters as determined by a Tukey's HSD test following a repeated-measures ANOVA.

3.2. Experiment 1: Effects of copper on sperm motility and fertilization success

Sperm swimming speeds varied significantly among treatments, specifically, swimming more slowly when exposed to higher concentrations of copper (repeated-measures ANOVA; $F=4.72$, $p=0.003$). The effects of copper on sperm were only obvious when both the copper concentration and the sperm exposure duration increased. At 3.2 and 32 min after copper

exposure, sperm swimming speeds were similar to those observed in controls (Fig. 1a, b). However, sperm exposed to 100 µg/L copper for 100 min swam significantly slower than sperm swimming in unamended seawater (Fig. 1c).

There was little variation in sperm length (i.e., mean sperm length = 21.81 ± 0.08 , range: 20.35–23.37 µm) among treatments of varying copper concentrations (ANOVA; $F=0.67$, $p=0.67$). This result suggests that copper does not alter sperm tail morphology and that the effects of copper observed on sperm velocity (see above) were not caused by morphological changes. Furthermore, incorporating mean sperm length as a covariate into the repeated-measures ANOVA examining sperm velocity did not reveal a correlation between sperm length and sperm swimming speed ($F=0.07$, $p=0.27$).

Sperm fertilizing capabilities were impaired by exposure to high levels of copper (Freidman's test; $\chi^2=19.3$, $p=0.003$; Fig. 2a). Exposure to the 100 µg/L copper treatment significantly reduced the fertilizing ability of sperm compared to

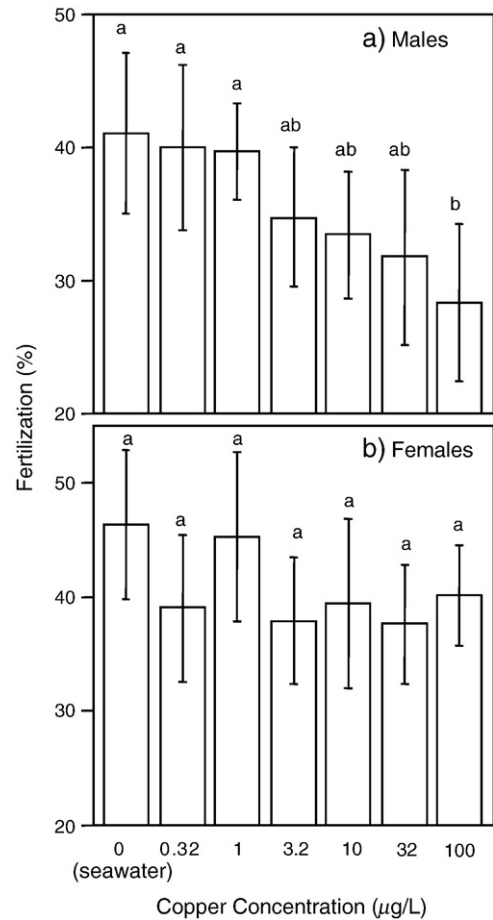


Fig. 2. Fertilization rate of a) males whose sperm were exposed to increasing copper concentrations for 100 min prior to mixing with unexposed eggs and b) females whose eggs were exposed to increasing copper concentrations for 100 min prior to mixing with unexposed sperm. Gametes were allowed to mix in aerated vials for 1 h before fertilization was arrested. The percentage of eggs fertilized in a) and b) were determined by counting approximately 100 randomly-sampled eggs. Data are presented as means±SE (males $n=7$, females $n=8$) and significant differences in mean values are indicated with different letters.

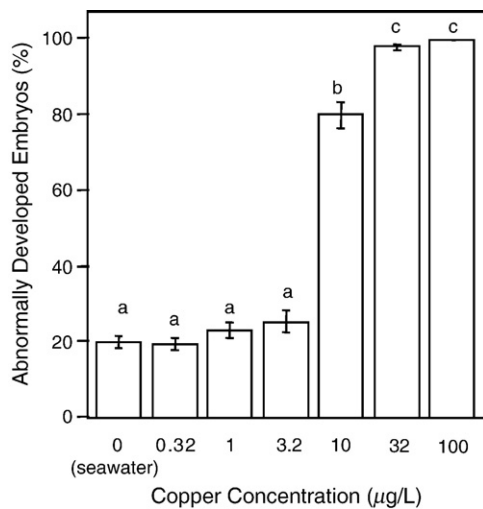


Fig. 3. The percentage of abnormally-developed embryos following exposure to increasing copper concentrations for 48 h. Means \pm SE ($n=5$) are presented and different letters indicates differences between copper concentrations.

fertilization capabilities of sperm kept in unamended seawater, or in 0.32 $\mu\text{g/L}$ and 1 $\mu\text{g/L}$ copper treatments.

3.3. Experiment 2: effects of copper on egg viability

Increasing concentrations of copper did not influence egg viability, as there were no differences in the percentage of eggs fertilized among the various treatments ($\chi^2=8.8$, $p=0.18$, Fig. 2b).

3.4. Experiment 3: effects of copper on embryo development

Embryos exposed to high copper concentrations exhibited significantly greater abnormalities than embryos exposed to low concentrations ($F=277.8$, $p<0.001$; Fig. 3). Embryos developed in unamended seawater (i.e., controls) exhibited $20\pm 3.5\%$ abnormal embryos, and a similar proportion of abnormal embryos were observed in the 0.32, 1.0, and 3.2 $\mu\text{g/L}$ copper solutions. The number of embryos with abnormal development was dramatically higher (i.e., a 4-fold increase relative to seawater controls) in the 10 $\mu\text{g/L}$ copper treatment. The number of abnormally-developed embryos was highest at concentrations of 32 and 100 $\mu\text{g/L}$ copper; in the latter concentration (i.e., 100 $\mu\text{g/L}$ copper), all embryos exhibited abnormal development (Fig. 3).

4. Discussion

The results of this study demonstrate that exposure of blue mussel sperm and embryos to copper reduce fertilization success at concentrations consistent with those observed at heavily-polluted harbours (e.g., Haynes and Loong, 2002; Soegianto et al., 1999). Thus, by using video tracking and computer-assisted analysis we found that sperm were considerably more sensitive to the adverse effects of copper in this study than in previous work where sperm motility was measured spectrophotometrically (Earnshaw et al., 1986). Notably, Earnshaw et al. (1986)

demonstrated a reduction in sperm motility at copper concentrations that exceeded environmentally relevant levels by approximately 100–3000 times (range 6300–208,000 $\mu\text{g/L}$). However, the observed adverse effects of copper on blue mussel sperm in the present study occurred only at concentrations approximately an order of magnitude higher than those causing adverse effects in developing embryos. Embryos demonstrated a dramatic increase in abnormal development between the 3.2 and 10 $\mu\text{g/L}$ copper treatments, considerably lower than the 100 $\mu\text{g/L}$ that results in a decrease in sperm swimming speed and fertilization success. Therefore, as demonstrated in previous studies with other species of marine invertebrates (Ringwood, 1992; Dinnel et al., 1987; Geffard et al., 2001; Novelli et al., 2003), blue mussel embryotoxicity tests were more sensitive in measuring the effects of copper toxicity than blue mussel spermotoxicity tests. Developing embryos are metabolically active, and slight alterations in enzyme activity can reduce survival. Embryos of species from the genus *Mytilus* are more sensitive to copper exposure than other marine invertebrates (Arnold et al., 2005). Present US EPA guidelines for copper exposures in seawater are calculated by factoring a “Final Acute Value” of 9.625 $\mu\text{g/L}$ (by 2 for the acute criterion, and by 3.127 for the chronic criterion, US EPA, 1995a). This “Final Acute Value” is based on the genus mean acute value for *Mytilus*, using EC50 values from 48-hr embryo-larval tests (US EPA, 1995b). The data from this study (i.e., Fig. 3) are in good agreement with this value. Indeed the ecotoxicological results from this study confirm the importance of the 48-hr EC50 embryo-larval development test (US EPA, 1995a) in assessing the effects of copper on recruitment after 48-hr exposure.

Sperm swimming speed and fertilization rates of copper-exposed sperm were more sensitive endpoints than egg viability endpoints. In this study, exposing eggs to increasing copper concentrations did not influence fertilization success. This is consistent with previous studies on other marine invertebrates, in which acute exposure of eggs to copper and silver had no effect on fertility (surf clam, *Spisula solidissima*, Eyster and Morse, 1984; polychaete, *Galeolaria caespitosa*, Hollows et al., 2007). Therefore, prior to fertilization, eggs are generally relatively insensitive to acute exposures to metals. These results suggest that temporary increases in metal concentrations, that may result from, for example, the introduction of a pollutant from a point source, will not reduce the fertilization capacity of eggs prior to fertilization. However, immediately following fertilization, eggs become metabolically active, exhibiting dramatic changes in membrane ion permeability (Franchet et al., 1997). Copper accumulation into the egg may not be possible prior to fertilization, and therefore, the adverse effects of copper may only be possible subsequent to fertilization.

The sensitivity of blue mussel sperm to copper increased with both copper concentration and exposure time. Sperm exposed to the highest copper concentrations (nominally, 100 $\mu\text{g/L}$), for the longest time period measured (i.e., 100 min), swam more slowly and exhibited reduced fertilization capacity than control sperm swimming in seawater. Our results are consistent with the conclusions of previous studies examining how metals influence sperm functions, which demonstrated a reduction in sperm

swimming speed following exposure of sperm to 1–1000 µg/L mercury (goldfish, *Carassius auratus*, Van Look and Kime, 2003; African catfish, *Clarias gariepinus*, Rurangwa et al., 1998; sea bass, *Dicentrarchus labrax*, Abascal et al., 2007), 1000–100,000 µg/L cadmium (sea urchin, *Anthocardis crassis-pina*, Au et al., 2000; African catfish, Kime et al., 1996), and 2000 mg/L zinc (African catfish, Kime et al., 1996). Yet by demonstrating that copper reduces sperm swimming speed in blue mussels, our results are at odds with those of other externally-fertilizing fishes. For example, a number of previous studies have indicated that copper exposure does not influence sperm velocity (sea bass, Abascal et al., 2007; African catfish, *Mocholkiella paynei*; chub, *Leuciscus cephalus*, and burbot, *Lota lota*, Lahnsteiner et al., 2004) and sperm even swam faster than corresponding control treatments in the brown trout (*Salmo trutta fario*, Lahnsteiner et al., 2004). Blue mussel sperm continues swimming for several hours (>4 h, personal obs.) while in fishes, particularly freshwater species, sperm are generally short lived (e.g. Ginsberg, 1963). Given the time-dependent nature of the effects of copper on sperm swimming speed these differences in the duration of sperm motility likely account for the opposing results observed when examining how copper influences sperm swimming speed in the freshwater fish species studied to date and the marine invertebrate described in this study.

Reduced sperm swimming speeds are often associated with alterations of sperm morphology. For example, exposure of blue mussel (*Mytilus edulis*) sperm to much higher copper levels (6300–208,000 µg/L) caused mitochondrial damage (Earnshaw et al., 1986), while in sea urchins, sperm exhibited swelling of the midpiece, mitochondrial damage, and breakage of the flagella subsequent to exposure to >5 mg/L cadmium (Au et al., 2000, 2001b), and in goldfish, exposure to 100 mg/L mercury reduced sperm flagellar length (Van Look and Kime, 2003). However, the reduction in sperm swimming speed observed in this study occurred at much lower metal levels and does not appear to be the result of copper-induced changes in sperm tail length. Similarly, previous studies did not detect dramatic alterations in sperm morphology following cadmium exposure in the green-lipped mussel (Au et al., 2000). Therefore, it is possible that the impaired sperm function in mussels may be due to physiological, rather than morphological, damage. In particular, we think it likely that exposure of blue mussel sperm to copper interferes with mitochondrial activity, as copper exposure, albeit at higher concentrations, has been earlier shown to reduce the respiration rates of sperm in mussels (Akberali et al., 1985; Earnshaw et al., 1986), which, in turn, reduces sperm swimming speed.

In marine invertebrates, sperm motility is initiated following dilution in seawater, which results in the activation of metabolic pathways necessary to drive the flagella (Rothschild, 1948). Prior to sperm release and activation, sperm are immotile as a result of uncoupled sulfhydryl groups suppressing the activity of cytochrome C, a key enzyme of the electron transport chain (ETC) in the mitochondria that drives energy production (Mohri, 1956). Following sperm release, cytochrome C is released from this inhibition allowing for respiration, which ultimately produces the energy required for the sperm's

motility. Once taken up into a cell, copper is reduced to cuprous ions that will readily bind with sulfhydryl groups (Viarengo et al., 1996), and ultimately inhibit ATP production by interfering with electron transport along the ETC (Ay et al., 1999). Furthermore, copper accumulates in sperm mitochondria (Earnshaw et al., 1986), which may decrease mitochondrial membrane potential while causing the formation of reactive oxygen species (ROS), leading to oxidative damage (Krumsh-nabel et al., 2005). In trout hepatocytes the extent of ROS formation following incubation in 10 µM Cu increased over time, with ROS in exposed cells more than doubling that of controls after 1 h incubation (Krumsh-nabel et al., 2005). Therefore, exposure to copper likely interferes with mitochondrial activity, reducing sperm swimming speed. As in the time dependent formations of ROS in trout hepatocytes, the delay in an observed response of sperm swimming speed following copper exposure in this study suggests that spermatozoa are actively respiring, and that copper uptake into the cell (in this case, the gamete cell), and subsequent oxidative damage, are time-dependent. If this hypothesis is correct, male blue mussels may suffer greater reproductive impairment when maintained in environments with chronically-elevated copper concentrations, such as marine harbors, where copper can accumulate in the testes (Kumari et al., 2006) and potentially impair sperm mitochondrial function prior to sperm release.

Chronic effects of metals on reproductive impairment and gametogenesis in marine invertebrates have not been well characterized. However, in polluted marine environments, sexually-mature adults may be chronically exposed to elevated metal concentrations, which can lead to a bioaccumulation of metals in adult tissues. For example, in the short-neck clam (*Paphia malabarica*) adults living in a polluted estuary exhibited an accumulation of copper in their gonads (Kumari et al., 2006). Furthermore, while Au et al. (2000) demonstrated a time-dependent adverse effect of cadmium on sperm motility, subsequent studies by the same authors demonstrated that exposure of mature adults to cadmium for a period of 4 weeks greatly reduced the concentration of cadmium required to impair sperm motility (Au et al., 2001a,b). It remains to be seen if adult blue mussels exposed to chronically-elevated copper concentrations bioaccumulate copper in gonadal tissue. If so, we might expect an intensification of the toxic effects of copper on impairing reproductive function, likely by interfering with mitochondrial activity, as reported by Au et al. (2001a,b) following chronic cadmium exposure. In particular, reductions in sperm swimming speeds have direct fitness consequences as sperm speed is positively correlated with fertilization success in marine invertebrates (Leviton, 2000), as well as vertebrates (e.g. Gage et al., 2004). Consequently, impaired sperm function subsequent to exposure to metals, together with reduced embryo survival, may magnify any deleterious impacts that metals exert on recruitment in marine environments.

5. Summary and conclusions

Acute exposure of blue mussel gametes and embryos to copper impaired sperm motility and reduced fertilization

success. Eggs were unaffected by copper exposure at all exposure concentrations of copper tested in this study, suggesting that the impaired fertilization rates observed were likely the result of poor sperm function alone. To our knowledge, this study is the first of its kind to demonstrate a reduction in sperm swimming speed in an externally-fertilizing species acutely exposed to copper at environmentally relevant concentrations. This result may be due to the extended duration of sperm motility in blue mussels, which may facilitate time-dependent impacts of copper on mitochondrial function.

Acknowledgments

We thank the Director and Staff of Bamfield Marine Sciences Centre for their help and cooperation during this study. We also want to especially thank the Research Co-ordinator, Dr. Bruce Cameron, for his assistance during this study and Dr. Robert Montgomerie for kindly providing access to his sperm tracker. We also thank Susan Marsh-Rollo and Julie Desjardins for providing logistical support while in the field, Frédéric Chain for his powerful assistance during data analysis, and Drs. Peter Chapman, Astrid Voigt, and Guy Gilron for constructive criticism. This work was funded by an NSERC (Canada) CRD Grant to CMW, in co-operation with the International Copper Association, the Copper Development Association, the Nickel Producers Environmental Research Association, the International Lead Zinc Research Organization, the International Zinc Association, Noranda-Falconbridge (now Xstrata), Teck Cominco, and Inco, and by a Canadian Foundation for Innovation and Ontario Innovation Trust awarded to SB. JLF and CB are supported by NSERC postgraduate scholarships, and CMW and SB are supported by the Canada Research Chair Program.

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