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Subchronic and chronic developmental effects of copper oxide (CuO) nanoparticles on *Xenopus laevis*

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HIGHLIGHTS

- Moving from acute (4 d) to subacute (14 d) to chronic (50 d) nano-CuO exposure times increase Xenopus mortality.
- NanoCuO subacute LC50s are below LOECs for Xenopus.
- At low concentrations nano-CuO has marginally beneficial effects on Xenopus growth and development.
- At 0.3 mg L^{-1}, less than 40% of tadpoles had completed metamorphosis.

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ABSTRACT

Metal oxide nanoparticles, such as copper oxide (CuO), are mass produced for use in a variety of products like coatings and ceramics. Acute exposure to CuO nanoparticles has caused toxicity to many aquatic organisms, yet there is no information on the effect of prolonged CuO nanomaterial exposures. This study examined effects of chronic exposure to CuO nanoparticles on *Xenopus laevis* growth and development. Experiments included a 14 d subchronic exposure and a 47 d chronic exposure throughout metamorphosis. The subchronic exposure caused mortality in all tested CuO concentrations, and significant growth effects occurred after exposure to 2.5 mg L^{-1} CuO. Chronic exposure to 0.3 mg L^{-1} CuO elicited significant mortality and affected the rate of metamorphosis. Exposure to lower concentrations of CuO stimulated metamorphosis and growth, indicating that low dose exposure can have hormetic effects.

1. Introduction

Metal oxide nanoparticles (NP) are among the most widely produced nanoparticles (Kumar, 2006), and are used in many applications. Metal oxide nanoparticles can be used to remediate a wide variety of hazardous materials due to high surface area, enhanced interfacial reactivity, increased dispersibility, and facile sorption kinetics (Kumar, 2006). Some metal oxide nanoparticles, such as zinc oxide (ZnO) and CuO, have antimicrobial and antifungal properties, which make them ideal for a variety of coating applications. NanoArc® CuO antimicrobial properties are active for extended periods of time, even in harsh conditions (Society of Manufacturing Engineers, 2008; Copper Development Association, 2009). NanoArc® CuO is utilized in wood preservation, textile fibers, marine antifouling, coatings, and thermoplastics to inhibit microbial and fungal growth (Society of Manufacturing Engineers, 2008). These CuO nanoparticles are also used in optical glass polishing, additives for ceramics processing and colorants, and pigments for other materials (Nanophase Technologies Corporation, 2009).

Because commercial production capabilities of nanoparticles such as CuO are on the order of metric tons (Society of Manufacturing Engineers, 2008), it is important to investigate the toxicity of these materials, especially to aquatic organisms, because products and wastes containing CuO nanoparticles can enter aquatic ecosystems. For example, copper oxide nanoparticles have exhibited toxicity to aquatic organisms such as the algae *Pseudokirchneriella subcapitata* growth (Aruoja et al., 2009), the bacterium *Vibrio fischeri*, and the crustaceans *Daphnia magna* and *Thamnocephalus platyurus* (Heinlaan et al., 2008; Mortimer et al., 2008). Copper oxide nanoparticles were relatively more toxic than bulk CuO, and CuO NP toxicity was attributed to soluble Cu ions (Heinlaan et al., 2008; Mortimer et al., 2008).

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There are several reasons to evaluate CuO nanoparticle effects in *X. laevis*. First, copper salts decrease embryo hatching, survival, and body length, and increase occurrence of malformations in this species (Luo et al., 1993; Haywood et al., 2004; USEPA, 2007). Because soluble copper ions may contribute to CuO NP toxicity (Heinlaan et al., 2008; Aruoja et al., 2009), it was hypothesized that similar effects would be seen in *X. laevis* chronically exposed to CuO NP. Acute exposure of *X. laevis* tadpoles to CuO nanoparticles has also been investigated in our lab in previous studies utilizing the Frog Embryo Teratogenesis Assay-Xenopus (FETAX; 18). In that study, CuO NP did not decrease embryo hatching or survival, but did significantly decrease snout vent length at 10 mg L^{-1} and total body length at 1000 mg L^{-1}. Increased malformation incidence occurred at 1000 mg L^{-1} with 33.3 ± 8.8% malformation (Kumar, 2006). We further hypothesize that similar effects will occur at lower concentrations during chronic exposures.

Therefore, the purpose of this study was to determine the effects of CuO nanoparticle exposure to *Xenopus laevis* tadpoles throughout metamorphosis. To assess effects of exposure on metamorphosis, endpoints of this study included mortality, growth (body measurements), and time to complete metamorphosis. The combination of results from this study, our acute (Nations et al., 2011) and other chronic studies (Nations et al., 2010), as well as other toxicological studies evaluating manufactured nanoparticles (Lovern and Klaper, 2006; Franklin et al., 2007; Heinlaan et al., 2008; Mortimer et al., 2008; Zhu et al., 2008; Aruoja et al., 2009) is important for determination of NP risks on environmental and human health.

2. Methods and materials

Copper oxide nanoparticles were purchased from Alfa Aesar (Ward Hill, MA). Average particle size (APS) and surface area of the NanoArc® CuO nanoparticles purchased for this project were 23–37 nm, 25–40 m^2 g^{-1}, respectively. All salts for making FETAX medium were obtained from VWR (West Chester, PA): NaCl (100% purity), $NaHCO_3$ (99–100% purity), KCl (100% purity), $CaCl_2$ (99–100% purity), $CaSO_4 2H_2O$ (98–100% purity), and $MgSO_4$ (99–100% purity). For chemical determinations, trace metal grade nitric acid (70%) and hydrogen peroxide (30%) were obtained from Fisher Scientific (Fisher, Waltham, MA). Human chorionic gonadotropin (HCG) and L-cysteine (⩾98%), from non-animal source, cell culture) were obtained from Sigma–Aldrich (St. Louis, MO).

2.1. Nanoparticle solutions

Nanoparticle solutions were prepared in FETAX medium – a medium used to culture *X. laevis* larvae (Bantle et al., 1989) – hereafter referred to as FETAX (625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 2H_2O$, and 75 mg $MgSO_4$ per liter of deionized water). Test solutions were sonicated with Fisher Scientific Model 500 Sonic Dismembrator (Fisher, Waltham, MA), until stably suspended in the medium, as described in Nations et al. (2010). Before tests began, SEM showed particles ranging from 18 to 285 nm with an average of 57 nm.

For subchronic exposures, each test solution was prepared in a 208 L drum at the appropriate concentration. For the chronic CuO test solutions, a 500 mg L^{-1} stock solution was prepared, and individual test solutions were diluted to a specified concentration with FETAX medium. Exposure solutions were prepared fresh every week during the studies. Differential light scattering (Nano-ZS, Malvern) was used to determine the CuO hydrodynamic particle size in the test solutions. Test solutions for subchronic exposure included the following concentrations: control (FETAX only), 0.156, 0.313, 0.625, 1.25, and 2.5 mg L^{-1}. Concentrations utilized in the chronic exposures were control (FETAX only), 0.01875, 0.0375, 0.075, 0.150, and 0.3 mg L^{-1}. The highest subchronic exposure concentration (2.5 mg L^{-1}) was derived from the malformation EC_{10} (2.1 mg L^{-1}) in the acute study (Nations et al., 2011). Results from the subchronic study were used to determine the highest test solution concentration for the chronic study. The highest subchronic exposure without significantly greater mortality than controls (i.e. NOEC) was 0.313 mg L^{-1}, thus the highest test solution concentration of 0.3 mg L^{-1} was selected for the chronic exposure.

2.2. Breeding and embryo collection

Breeding procedures followed a modified ASTM E1439-98 method and previously established methods utilized within our lab (Nations et al., 2010, 2011). Eggs were obtained from four *X. laevis* mating pairs per test, to increase the possibility that at least one pair of frogs produced an adequate supply of viable embryos. Males were injected with 250 IU of human chorionic gonadotropin (HCG) in the dorsal lymph sac, and females were injected with 750 IU HCG in the dorsal lymph sac to induce reproduction. Embryo collection began approximately 24 h after HCG injection and was conducted as described in previous studies (Nations et al., 2010, 2011).

2.3. Exposure procedures

Each concentration was tested in triplicate with 15 tadpoles in each replicate. Small 9.5 L glass aquaria were used as exposure chambers. To begin the exposure, 6 L of test solution was placed in every tank. After the solution was added to each tank, at least 20 tadpoles were added with plastic transfer pipettes. The number of tadpoles within each tank was reduced to 15 on day 5 of the exposure.

2.4. Test chamber and preparation

Both subchronic and chronic exposures utilized a preparation similar to a previously conducted chronic ZnO nanoparticle exposure (Nations et al., 2010). Both the aquaria and the 208 L drums (from which the test solutions were dispensed) were aerated using commercially-available aquarium air pumps and air stones. A Living Stream reservoir (Fridgid Units, Toledo, OH, USA) was used as a water bath. The water in the reservoir was heated to 23 ± 2 °C using three commercially-available glass aquarium heaters. Exposure tanks were randomly arranged within the reservoir.

A flow-through design for chronic ZnO nanoparticle exposure was utilized for both subchronic and chronic CuO exposures. One drum was designated for each exposure concentration. Each drum was aerated to maintain water quality and mixing of solution. A peristaltic pump provided a flow rate of 12.5 mL min^{-1} to each tank; which pumped at approximately 18 L d^{-1} through each tank (Nations et al., 2010). Each tank had a standpipe that allowed FETAX medium to drain when the volume exceeded 6 L, so this was equivalent to 3 water exchanges per day.

Tadpoles in this exposure were fed the same diet, which contained a combination of Nutrafin®, trout chow, and Frog Brittle (Nasco, Inc., Fort Atkinson, WI, USA), according to Nations et al. (2010), Koss and Wakeford (2000). Tadpoles were fed *ad libitum* each day, beginning on day 5 post hatch.

2.5. Observations

The following water quality parameters were monitored every other day: ammonia, temperature, conductivity, salinity, dissolved

oxygen, and pH. Soluble Cu concentration was determined in subchronic and chronic exposures with flame atomic absorption spectroscopy and graphite furnace absorption spectroscopy respectively (M Series AA Spectrometer, Thermo, Waltham, MA). Water samples were acidified to pH 2 by adding 150 μL concentrated nitric acid to a 30 mL sample.

Mortality and reservoir temperature were recorded on a daily basis. Mortality was determined by lack of movement or response from external stimulus, such as touching the side of a tadpole with a transfer pipette. During the 14-d subchronic study, measurements were taken at day 5, 10, and 14, and included Nieuwkoop and Faber (NF) stage Nieuwkoop and Faber, 1975, snout vent length (SVL), and total body length (TBL). Measurements during the 47-d chronic exposure were taken every five days and at the conclusion of the study to the nearest mm. These measurements included SVL, TBL, hind-limb length (HLL), and NF stage. SVL was measured from the tip of the nose to the anal vent, while TBL was measured from the tip of the nose to the tip of the tail. HLL was measured from the apex of the leg to the end of the toes. Time to metamorphic climax (NF stage 66) was also evaluated as a growth endpoint for the chronic exposure and was recorded from the day the first tadpoles reached stage 66 to the completion of the study. Malformations were also noted following published guidelines (Bantle et al., 1989). Malformations and staging were identified using a Motic K series stereoscope. Measurements of Stage, TBL, SVL, and HLL were taken on day 40, 45, and 47 of the chronic exposure. Measurements were also taken for any juveniles that reached stage 66 before the aforementioned collection days.

2.6. Euthanization and storage

During both subchronic and chronic exposures, dead tadpoles were removed from aquaria and stored in 10% buffered formalin. At the conclusion of the subchronic and chronic study, all surviving tadpoles were euthanized with MS-222 and stored in 10% formalin. When tadpoles reached NF stage 66, they were removed from the tank, euthanized with MS-222, and stored in 10% buffered formalin. Stage 66 juveniles were measured for TBL and HLL at the time of euthanization. Because *X. laevis* can be cannibalistic, removing tadpoles when they reach stage 66 ensured survival of smaller, less developed tadpoles. The study was concluded when 90% of the controls reached stage 66 (ASTM, 1999; Nations et al., 2010).

2.7. Determination of Cu concentration in tissue

Whole organisms were processed following the chronic exposure without depuration of GI tract content. Individual tadpoles were weighed (±1 mg) before and after drying. Tadpoles were freeze-dried (FreeZone 2.5, Labconco, Kansas City, MO). Each tested concentration contained 15 samples, five from each replicate. Samples were acid digested and prepared in batches of 30 plus one standard reference material sample (DOLT-4, National Research Board of Canada), and sample blank as described in a previous study (Gale et al., 2003). Dry tissue samples were digested with nitric acid and H_2O_2 additions as described previously (Gale et al., 2003). Tissue digests were cooled and diluted to 25 mL (control and all NP treatments up to 0.5 mg L^{-1}) or 50 mL (1 and 2 mg L^{-1} treatments) with 3% nitric acid.

2.8. Statistical analyses

Growth responses, mortality, Cu concentration in tissue, and Cu concentration in solution were analyzed utilizing nested analysis of variance (ANOVA) followed by Tukey Honestly Significant Differences test when significance was determined, $p \leqslant 0.05$ ANOVA. Growth measurements were tested for variance at every measure day and the conclusion of the study. Mortality in the subchronic test was also analyzed to determine LC_{50}s using probit analysis. Statistical analyses used nominal CuO concentrations and measured Cu concentrations are provided to allow the reader to translate our results to measured Cu terms.

3. Results

3.1. Subchronic

The initial trial of chronic study produced high mortality in several treatment groups, so this trial was shortened in duration and completed as a subchronic exposure. The lowest observed effect concentration (LOEC) for mortality during this CuO nanoparticle exposure was 0.625 mg L^{-1} CuO and the no observed effect concentration (NOEC) for mortality was 0.313 mg L^{-1} CuO.

3.1.1. Water quality and metal cation concentration

Temperature and pH were within acceptable ranges, according to ASTM E1439-98 (24 ± 2° C and pH = 6.5–9.0), throughout the entire study with averages of 23.7 °C and 8.01 respectively (Table 1). Conductivity, ammonia, and dissolved oxygen were within ranges previously reported by other similar studies (Bernardini, 1999; Carr et al., 2003; Coady et al., 2005; Tietge et al., 2005).

Copper concentration in solution was lower than nominal calculations (amount of Cu in solution resulting from 100% dissolution of CuO). The overall percent nominal determined in test solutions ranged from 17.7% to 53.0%, with the lowest percentages found in the highest exposure groups (Table 2). With the exception of the lowest concentration test solutions (0.156 mg L^{-1}), copper concentrations were significantly higher than Cu in FETAX control solution ($p \leqslant 0.04$). FETAX solution, prepared with deionized distilled water, has been reported to contain less than 0.04 mg L^{-1} Cu, as determined by atomic absorption analysis (Bantle et al., 1989). Our FETAX control contained 0.009 ± 0.002 mg L^{-1} Cu.

3.1.2. Mortality

During these tests, CuO nanoparticle exposures induced mortality in all test groups. A 14-d LC_{50} was calculated to be 0.44 mg L^{-1}. The control treatment caused 2.2 ± 2.2% mortality, and all exposures to CuO nanoparticles induced a minimum of 11.1% mortality (Fig. 1A). Tadpoles exposed to 0.313 mg L^{-1} CuO experienced the lowest mortality incidence among CuO exposed tadpoles. Three doses (0.625, 1.25, and 2.5 mg L^{-1}) induced significantly higher mortality compared to controls ($p = 0.001$, Fig. 1A). Mortality increased with increasing concentrations of CuO nanoparticles.

Mortality was also affected by duration of exposure (Fig. 2). Mortality increased following day 4. On day 5, the highest exposures induced at least 40% mortality and, mortality increased over time for these doses. The LC_{50} on day 5 was 1.25 mg L^{-1} and decreased steadily over time to produce an LC_{50} of 0.44 mg L^{-1} CuO by day 13 (Fig. 2).

Table 1
Water quality parameters for subchronic and chronic CuO nanoparticle exposure.

Water quality parameter[a]	Subchronic	Chronic
Temperature (°C)	23.69 ± 0.04	23.3 ± 0.11
pH	8.01 ± 0.04	7.71 ± 0.04
Conductivity (μs cm^{-1})	1867 ± 29	1779 ± 37
Salinity (ppt)	0.96 ± 0.02	0.90 ± 0.00
NH (mg L^{-1})	0.1 ± 0.04	0.60 ± 0.11
Un-ionized NH_3 (mg L^{-1})	0.01 ± 0.00	0.03 ± 0.01
Dissolved Oxygen (mg L^{-1})	4.93 ± 0.07	4.34 ± 0.08

[a] Values reported as mean ± SE.

Table 2
Concentration of Cu in subchronic test solutions and mortality induced by 14 d exposure to CuO nanoparticles.

CuO dose (mg L^{-1})	Calculated Cu (mg L^{-1})	Actual Cu (mg L^{-1})[a] (% Nominal)
Control	>0.04	0.009 ± 0.002
0.156	0.125	0.053 ± 0.005 (42.3)
0.313	0.25	0.071 ± 0.006 (28.3)
0.625	0.499	0.144 ± 0.012* (28.8)
1.25	0.999	0.203 ± 0.016* (20.3)
2.5	1.997	0.280 ± 0.033* (14.0)

[a] Values reported as mean ± SE.
* ANOVA and TukeyHSD p-value ⩽ 0.05 compared to controls.

3.1.3. Growth and development

The LOEC for developmental stage was the lowest tested concentration; therefore a true LOEC of CuO nanoparticle was not determined. The LOEC for SVL and TBL during this CuO nanoparticle exposure was 0.313 mg L^{-1}, and the NOEC for SVL and TBL was 0.156 mg L^{-1}.

3.1.3.1. Nieuwkoop and faber stage. Tadpole stage increased in a dose dependent fashion over time (Fig. 3A). On day 5, the average stage was less than control for all CuO concentrations above 0.3125 mg L^{-1} ($p < 0.05$; Fig. 3A). By day 10, all treatments except the 0.156 mg L^{-1} treatment demonstrated less stage progression than controls, and by day 14, all treatments had a significantly lower average stage of development than controls ($p < 0.05$; Fig. 3A).

3.1.3.2. Snout vent length (SVL). At five days of exposure, no treatments had a statistically-significantly different average SVL from controls. At days 10 and 14, the 1.25 and 2.5 mg L^{-1} treatments were significantly different from controls ($p < 0.05$; Fig. 3B).

3.1.3.3. Total body length. At five days of exposure, no treatments had a statistically-significantly different average TBL from controls.

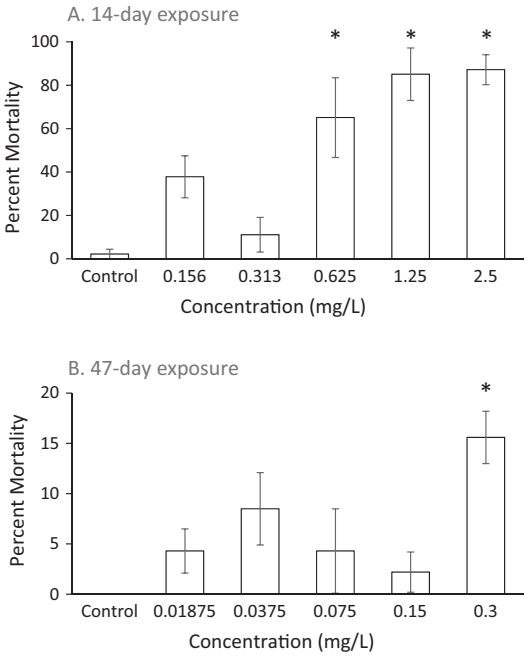

Fig. 1. Average (±SE) percent mortality in *Xenopus laevis* exposed to various concentrations of CuO nanoparticles for 14 (A) or 47 (B) days. * Treatment significantly different from control ($p < 0.05$; ANOVA).

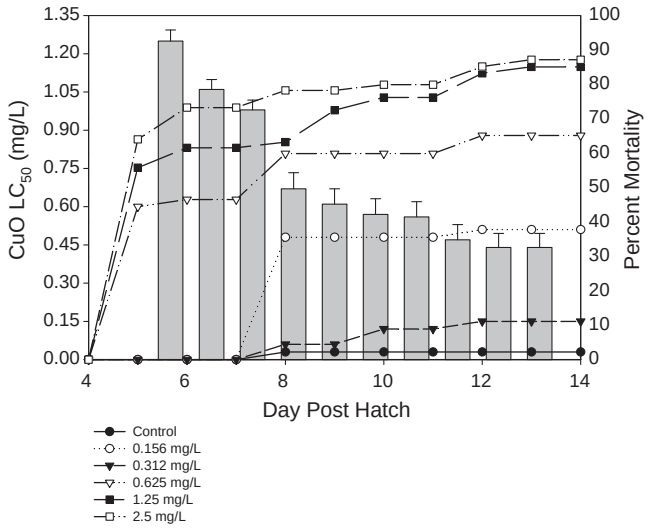

Fig. 2. *Xenopus laevis* mortality over time represented by the bar graph of LC_{50} values with 95% error bars and the increasing percent mortality represented by the line graph from days 4 to 14 with exposure to CuO nanoparticles. Data for the line graph are reported as mean per nominal CuO concentration per day from day 4 to day 14.

At 10 d of exposure, treatments with CuO NP concentrations of 0.625 mg L^{-1} or less had significantly greater TBL than controls, while tadpoles in the two highest treatments had significantly lower TBL than controls ($p < 0.05$; Fig. 3C).

3.2. Chronic

3.2.1. Water quality and copper characterization

Temperature and pH were within acceptable ranges, according to ASTM E1439-98 (24 ± 2 °C and pH = 6.5–9.0), throughout the entire study with averages of 23.1 °C and 7.71 respectively (Table 1). Conductivity, ammonia, and dissolved oxygen are within ranges previously reported (Bernardini, 1999; Carr et al., 2003; Coady et al., 2005; Tietge et al., 2005). At the initiation of exposures, copper oxide size distributions showed concentration-dependent maxima in light scattering that ranged from 310 to 1060 nm. Just before stock solution changes (d7) maxima distributions were 400–4850 nm, with the larger particles occurring in solutions with low overall concentrations. In solutions with the larger particle sizes at the later time point, particle distributions were strongly bimodal with large particle counts in the secondary peak (25–40% of the total). Copper concentrations were lower than nominal predictions or the amount of Cu in solution resulting from 100% dissolution of CuO (Table 2). The three highest test solutions (0.075, 0.150, and 0.3 mg L^{-1}) had significantly higher soluble Cu concentration (0.013, 0.022, and 0.045 mg L^{-1} Cu respectively) than control test solutions ($p \leqslant 0.001$). All solutions contained significantly lower Cu concentration than 0.15 and 0.3 mg L^{-1} CuO ($p \leqslant 0.002$). Concentrations of Cu in solution remained relatively constant throughout the exposure. FETAX solution has been reported to contain less than 0.04 mg L^{-1} Cu, as determined by atomic absorption analysis, when made with deionized distilled water (Bantle et al., 1989). Our FETAX control sample contained 0.0008 ± 0.0005 mg L^{-1} Cu.

3.2.2. Mortality

Control tanks had no mortality throughout the exposure. All CuO nanoparticle exposures caused mortality, with only the highest concentration, 0.3 mg L^{-1}, inducing significantly higher mortality than controls ($p < 0.05$, Fig. 1B).

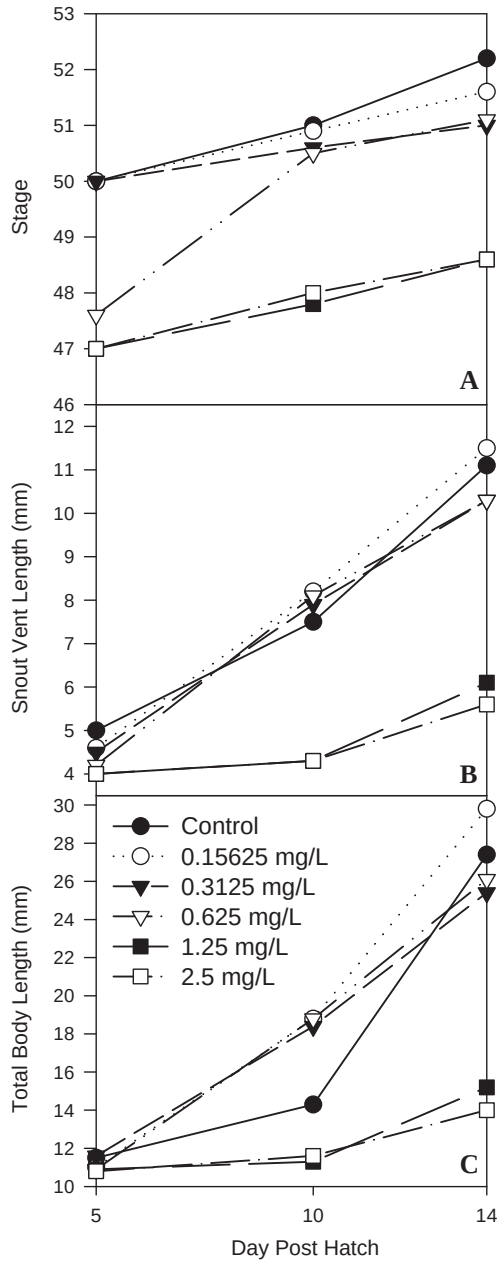

Fig. 3. Subchronic growth endpoint trends of *Xenopus laevis* tadpoles throughout 14 d exposure to CuO nanoparticles: A – Stage, B – Snout Vent Length, and C – Total Body Length. Data are reported as mean of each respective measurement from tadpoles in each nominal CuO concentration per measure day.

3.2.3. Growth

3.2.3.1. Nieuwkoop and faber stage. Tadpoles exposed to 0.3 mg L^{-1} CuO had significantly lower NF stages than controls on each day of measurement ($p < 0.05$; Fig. 4A). Tadpoles in the control and the 0.15 mg L^{-1} treatment experienced similar stage progression for most of the study. Tadpoles in the 0.075 mg L^{-1} treatment group achieved significantly higher staging, with a statistically-significantly greater average stage than control for 70% of the sampling days ($p \leqslant 0.016$; Fig. 4A).

3.2.3.2. Snout vent length (SVL). SVL generally increased steadily to approximately stage 58 and then decreased slightly for a short time before platueaing (Fig. 4B). Tadpoles in all treatments, excluding the 0.3 mg L^{-1} treatment, reached a maximum SVL on day 30, and tadpoles in the 0.3 mg L^{-1} treatment reached their maximum SVL on day 35. On days 5 through 30, tadpoles exposed to 0.3 mg L^{-1} had significantly shorter SVL than those exposed to all other treatments ($p \leqslant 0.001$). After day 20, tadpoles dosed with 0.15 mg L^{-1} CuO had SVLs that were not significantly different from control ($p > 0.05$). Tadpoles exposed to 0.0375 and 0.075 mg L^{-1} CuO had significantly longer SVLs than control tadpoles on days 20 and 25 ($p \leqslant 0.010$; Fig. 4B).

3.2.3.3. Hind limb length. HLL increased throughout the exposure, beginning with day 15 (Fig. 3C). On day 15, tadpoles exposed to 0.3 mg L^{-1} had not developed measurable hind limbs, and tadpoles exposed to 0.150 mg L^{-1} had significantly shorter hind limbs than all other concentrations ($p < 0.001$). From day 20 to day 35, tadpoles in treatment groups with NP concentrations less than 0.15 had larger average hind limb lengths than controls ($p < 0.05$).

3.2.3.4. Total body length. Tadpoles in all concentrations excluding 0.3 mg L^{-1} reached the maximum TBL on day 30, and 0.3 mg L^{-1} tadpoles reached their maximum TBL on day 35 (Fig. 4D). On days 10 through 30, tadpoles exposed to 0.3 mg L^{-1} attained significantly shorter TBLs than all other tadpoles ($p < 0.001$), and on days 40 through 47, tadpoles in the 0.3 mg L^{-1} treatment were longer than all other tadpoles ($p < 0.001$; Fig. 4D). This disparity in TBL at the conclusion of the study can be explained by the difference in staging; as tadpoles complete metamorphosis the tail resorbs, thereby shortening TBL.

3.2.3.5. Time to metamorphosis. Exposure to low concentrations of CuO seemed to stimulate metamorphosis. Control tadpoles did not start reaching metamorphic climax (stage 66) until day 38, in contrast to tadpoles exposed to NPs at concentrations below 0.15 mg L^{-1} (Fig. 5). Also, tadpoles exposed to 0.01875 or 0.075 mg L^{-1} were comprised of over 90% stage 66 individuals by day 45, while control individuals did not reach 90% stage 66 individuals until day 47. At the conclusion of the study, over 90% of the tadpoles in the control, 0.01875, 0.075, and 0.150 mg L^{-1} treatments completed metamorphosis. Significantly fewer tadpoles completed metamorphosis (39%) in 0.3 mg L^{-1} exposures as compared to controls and all other doses as well ($p \leqslant 0.001$).

3.2.3.6. Stage 66 juveniles. The TBL of stage 66 juveniles dosed with CuO nanoparticles were significantly different from control TBL (Fig. 6). All tadpoles in solutions containing less than 0.15 mg L^{-1} CuO achieved significantly longer TBL than control tadpoles ($p \leqslant 0.04$), while tadpoles in the 0.3 mg L^{-1} treatment were significantly shorter than control juveniles ($p = 0.001$; Fig. 6). Tadpoles exposed to 0.3 mg L^{-1} CuO had significantly lower TBL and HLLs than controls ($p < 0.001$). Juveniles exposed to 0.0375 and 0.075 mg L^{-1} CuO achieved significantly longer HLL than controls ($p \leqslant 0.02$).

3.2.3.7. Cu concentration in tissue. Average dry weight for all tissues was 0.254 g, ranging from 0.099 to 0.428 g. A standard reference material, DOLT-4, was analyzed with every 30 samples. Copper concentration in DOLT-4 is 31.2 ± 1.1 μg g^{-1} ($n = 3$), and the average Cu concentration recovered from DOLT-4 was 32.95 ± 0.96 μg g^{-1}. This represents 106 ± 2% recovery and is well within an acceptable recovery range with better precision than guaranteed by the supplier. Copper concentration in whole bodies increased with increasing concentration of CuO nanoparticles (Table 3). Significant copper uptake (214.98 ± 38.87 μg g^{-1}) was observed in frogs exposed to 0.3 mg L^{-1} CuO compared to control tissue concentration ($p < 0.001$). The highest exposure also had a significantly higher Cu concentration in tissue when compared to

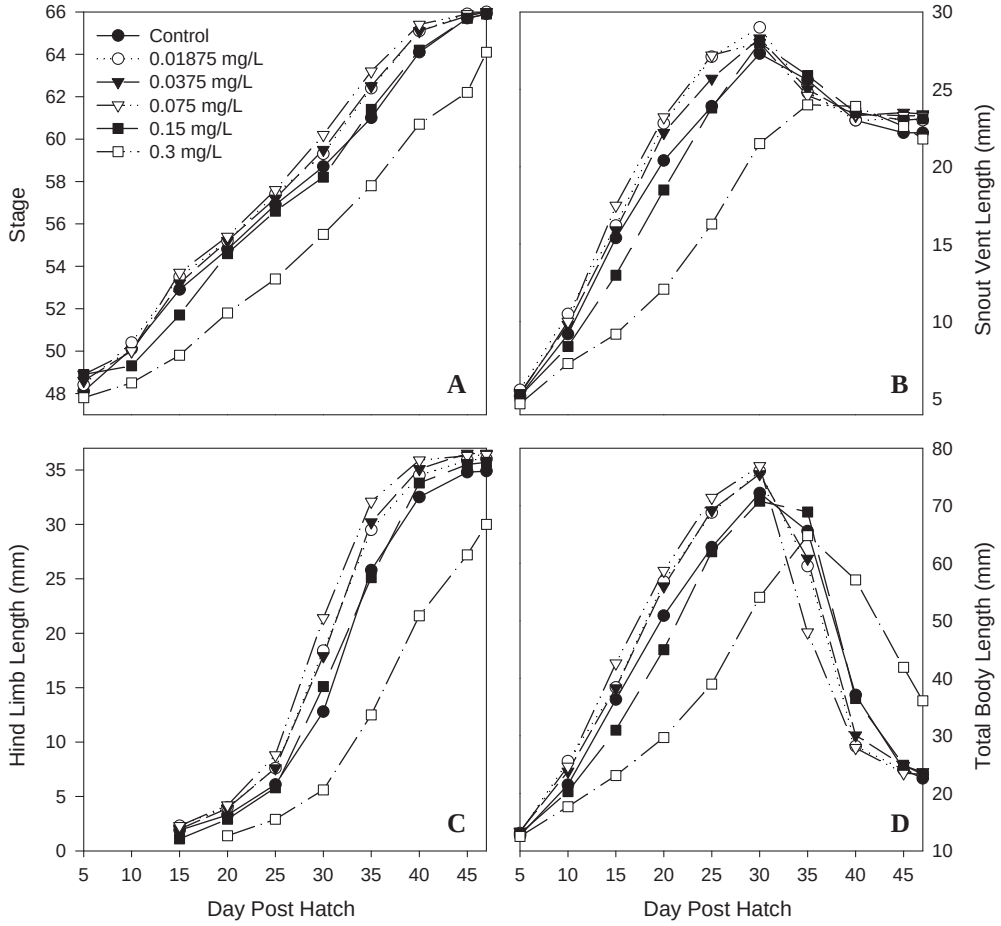

Fig. 4. Growth endpoint trends of *Xenopus laevis* tadpoles throughout 47 d exposure to CuO nanoparticles: A – Stage, B – Snout Vent Length, C – Hind Limb Length, and D – Total Body Length. Data are reported as mean of each respective measurement from tadpoles in each exposure group.

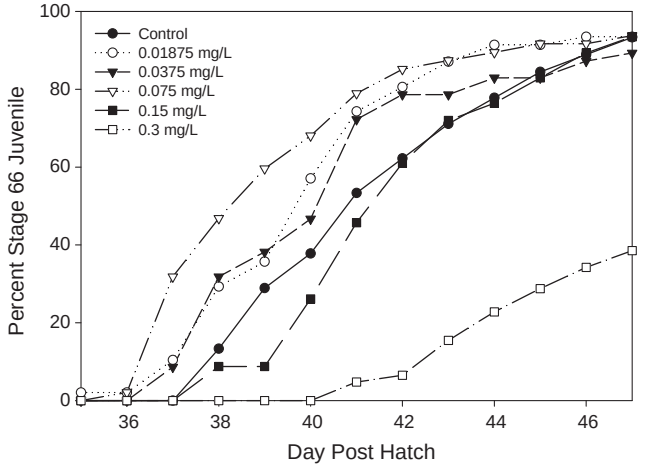

Fig. 5. Percentage of *Xenopus laevis* tadpoles completing metamorphosis from day 35 to day 47 that were exposed to five CuO nanoparticle concentrations. CuO concentrations are nominal.

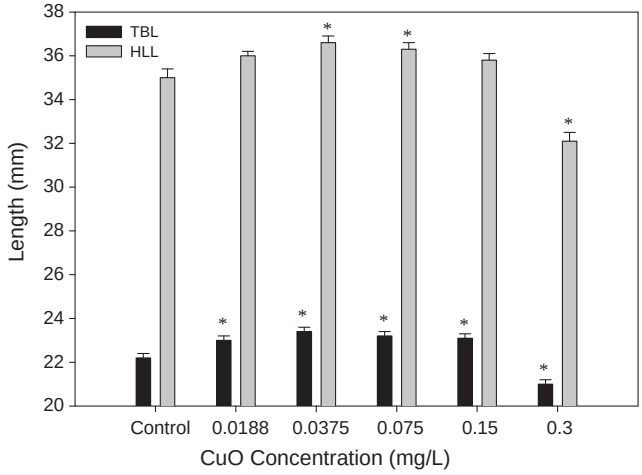

Fig. 6. Body measurements (mean ± standard error), total body length (TBL) and hind limb length (HLL), of stage 66 *Xenopus laevis* juveniles exposed to CuO nanoparticles from embryo until metamorphic completion for each nominal CuO concentration. *: ANOVA and TukeyHSD p-value $\leqslant 0.05$ compared to controls.

all other tested concentrations ($p < 0.001$). Bioconcentration factors (based on total copper) ranged from 685 to 3630 (Table 3).

4. Discussion

Results from our study supported hypotheses that CuO NP exposure would induce mortality, growth inhibition, and metamorphic inhibition, and would do so at effective concentrations lower than those seen during acute studies (Nations et al., 2011). Copper oxide nanoparticle exposure induced significant

Table 3
Concentration of Cu in chronic CuO test solutions, Cu concentration in *Xenopus laevis* whole body tadpoles/juvenile, and mortality induced by exposure to CuO nanoparticles.

CuO dose (mg L^{-1})	Calculated Cu (mg L^{-1})	Actual Cu (mg L^{-1})[a] (% Nominal)	Tissue Cu dry weight (μg g^{-1})[a]	Tissue Cu wet weight (μg g^{-1})[a]	BCF[b]
Control	<0.004	0.001 ± 0.000	17.31 ± 0.71	3.63 ± 0.12	3630
0.01875	0.01498	0.005 ± 0.000 (30.8)	29.93 ± 1.23	6.40 ± 0.32	1280
0.0375	0.02996	0.007 ± 0.000 (24.3)	45.61 ± 2.37	9.63 ± 0.49	1375
0.075	0.05992	0.013 ± 0.001* (22.0)	50.67 ± 4.78	10.64 ± 0.98	818
0.15	0.11984	0.022 ± 0.001* (18.4)	70.98 ± 5.12	15.07 ± 0.98	685
0.3	0.23967	0.045 ± 0.003* (18.9)	218.18 ± 38.87*	36.27 ± 5.28*	806

[a] Values reported as mean ± SE.
[b] Bioconcentration Factor.
* ANOVA and TukeyHSD p-value $\leqslant 0.05$ compared to controls.

mortality in both subchronic and chronic exposures, while acute 4-d exposure to CuO nanoparticles produced little to no mortality (Nations et al., 2011). The greatest mortality with acute CuO nanoparticle exposure was 3.33% in 0.1 and 1 mg L^{-1} CuO. Subchronic exposure to 1.25 mg L^{-1} CuO nanoparticle induced 85% mortality (Nieuwkoop and Faber, 1975). The 14-d subchronic exposure of 1.25 mg L^{-1} produced 25 times more mortality than the acute exposure of 1 mg L^{-1}. The increase in mortality after day 4 could be attributed to the fact that *X. laevis* tadpoles feed from their yolk for the first 3–4 d post-hatch (Raising Tadpoles: Tadpole Care, 2007; Herkovits and Silvia Perez-Coll, 2007). It appears that CuO nanoparticles become lethal to *X. laevis* tadpoles when they begin to actively filter-feed. This is further supported by mortality results from chronic CuO nanoparticle exposure. From day 5 to the conclusion of the study (day 47), 94.7% of total mortality occurred. The LC_{50} for subchronic exposure to CuO nanoparticles decreased with increased duration of exposure (Fig. 4). Our results are similar to the mortality pattern observed for another amphibian species, *Bufo arenarum*, exposed to Cu^{2+} solutions. *B. arenarum* embryos had increased mortality over a 7 d exposure to Cu^{2+}, and the LC50 also decreased over time (Herkovits and Helguero, 1998). The *B. arenarum* data indicate that longer exposure durations increase the toxicity of copper.

In the present study, the percent mortality from comparable concentrations in subchronic (0.313 mg L^{-1}) and chronic (0.3 mg L^{-1}) exposures were similar (11.1 ± 8.0% and 15.6 ± 2.6%, respectively). Thus, it would appear that within two weeks of exposure to ~0.3 mg L^{-1} CuO nanoparticles, resilliant *X. laevis* tadpoles acclimate to nanoparticle suspensions. Although the mechanisms for any such NP acclimation are unknown, acclimation or tolerance to metals in general is achieved with three possible mechanisms: modifications to uptake and elimination rates, ability to bind or sequester metals, and decreased enzyme sensitivity (Herkovits and Helguero, 1998; Alsop et al., 1999; Peterson and Boughton, 2000; Hansen et al., 2002, 2007). *X. laevis* tadpoles exposed to CuO NPs accumulated Cu (Table 3), so it is possible that they have a sequestration mechanism for nanoparticles.

There was, however, Cu detected in the control tadpoles as well as the exposed individuals, and was probably due to background copper in solutions or in food. The background copper concentration in the *X. laevis* tissue were similar to background Cu concentrations in tissues from a variety of other species. Fish tissue generally contains a background level of 1–12.3 μg g^{-1} of Cu (Authman and Abbas, 2007; Environmental Health Criteria, 2009). Our control tadpole whole body tissue samples contained 3.63 ± 0.13 μg g^{-1} wet weight, which is near Cu concentrations in whole fish tissue. Copper concentrations in fish liver in the Yellowstone River Basin (generally regarded as a reference site) ranged from 7.4 to 229 μg g^{-1} dry weight at different sites along the river basin (Gale et al., 2003).

Copper accumulation followed a dose dependent fashion, as seen for other species (Zhao et al., 2011; Shaw and Handy, 2011; Ates et al., 2015). Accumulation of CuO nanoparticles in organ tissues has been demonstrated in aquatic vertebrates (Ates et al., 2015). Given the ability of CuO to dissolve in aqueous solution and the essential nature of Cu, the tissues undoubtedly contain a mixture of copper ions and CuO nanomaterials. Studies of fish species indicate that copper accumulates in the intestine, gill, liver, and muscle. There is also some evidence of accumulation in the brain.

In the exposed treatments, growth and metamorphosis were also affected by CuO nanoparticles. In both Cu subchronic and chronic exposures, tadpoles exposed to 0.3 mg L^{-1} and higher had slower stage progression and smaller body measurements than control tadpoles. The LOEC of CuO nanoparticles for stage progression was 0.15 mg L^{-1}. This is slightly higher than previous reports of a NOEC of 0.050 mg Cu L^{-1} (derived from copper salts) for tail resorption (USEPA, 2007). Other studies have found that exposure to metals, such as Cu, negatively affected tadpole development by increasing the amount of time to complete metamorphosis and by reducing size and weight of metamorphs (Lefcort et al., 1999; Lavolpe et al., 2004). While SVL is considered the most standard measure of body size, TBL also provides a clear maximum (Fig. 4B and D) that can be used to differentiate progress through metamorphosis. Our data demonstrate a clear difference in time to maximum body length and difference in absolute maximum body length. Other investigators have argued that the increased time to complete metamorphosis could be attributed to an increase in energy consumption for metal metabolism, leaving less energy for growth (Lefcort et al., 1999; Haywood et al., 2004). This may be ecologically-significant, as smaller tadpoles may experience reduced survival because of an inability to compete for food or increased predation. Growth inhibition has also been observed for fish species (Raising Tadpoles: Tadpole Care, 2007).

In contrast to CuO effects on growth, it appears that exposure to low concentrations of CuO nanoparticles can positively affect metamorphosis and growth. A previous study found that low dose exposure to ZnO nanoparticles (0.125 mg L^{-1}) accelerated stage progression and larger body measurements for *X. laevis* (Nations et al., 2010). This type of overcompensation (or possibly hormesis) is not uncommon in toxicity studies involving nanoparticles (Wu et al., 2010) or toxicants in general (Xia et al., 2013).

As discussed previously, environmental CuO NP in aquatic environments can negatively impact aquatic ecosystems. Copper exposure can affect aquatic organisms at concentrations as low as 0.00258 mg L^{-1}, which is lower than the fresh water quality criteria continuous exposure of 0.009 mg L^{-1} (Lavolpe et al., 2004). Release of CuO nanoparticles to the environment could increase Cu concentrations to levels that adversely affect aquatic flora and fauna. In the present study, the lowest adverse effects concentration (mortality and decreased stage 66 TBL; Figs. 2 and 6, respectively) occurred at 0.3 mg L^{-1} nominal concentration, which corresponds to a measured concentration of 0.045 mg L^{-1} in the chronic exposure (Table 3). Although there is no information as

to the actual percentage of CuO NP that is liberated as free Cu^{2+}, the fresh water quality criterion of 0.009 mg L^{-1} for free copper would still be protective of aquatic life exposed to CuO nanoparticles.

Copper oxide nanoparticle exposure also increased the amount of time required to complete metamorphosis, which can be detrimental on survivorship of tadpoles (Haywood et al., 2004). In contrast, tadpoles that were exposed to low CuO nanoparticle concentrations completed metamorphosis faster and were larger than control organisms, as measured by TBL and HLL, indicating that low doses of CuO nanoparticles provided an optimal amount of Cu in solution to aid in growth. Completing metamorphosis faster and with a larger body size is beneficial because small body size can increase the risk of predation, inability to compete for food, and reduced survival rates (Haywood et al., 2004).

Previous work in our group (Nations et al., 2010, 2011) indicated *X. laevis* sensitivity to acute metal oxide exposure followed the pattern ZnO > CuO>>TiO_2 > Fe_2O_3. Toxicities were reversed for ZnO and CuO during chronic exposures, wherein mortality increased when CuO and ZnO reached 0.3 mg L^{-1} and 1 mg L^{-1}, respectively. SVL was impeded at 0.15 mg L^{-1} for CuO and at 0.50 mg L^{-1} for ZnO. Exposures to low concentrations of ZnO (<0.125 mg L^{-1}) or CuO (<0.075 mg L^{-1}) increase growth and reduced time to metamorphosis. During exposure to CuO NP, the transition of *X. laevis* from more rapid growth to a toxic response is extremely steep (0.075–0.15 mg L^{-1}), and ZnO demonstrates the transition across a slightly wider range (0.125–0.50 mg L^{-1}). Although UV light increases the sub-chronic NOAEL of 10 and 32 nm TiO_2 nanoparticles (Zhang et al., 2012), that toxicity (9.5–77 mg L^{-1}) is still far lower than subchronic NOAELs for ZnO (0.5 mg L^{-1}) or CuO (0.625 mg L^{-1}) without UV exposure. These comparisons indicate that ZnO is the most acutely toxic of the four nanomaterials tested and that CuO is more toxic in subchronic and chronic cases. Furthermore even though TiO_2 may be photoactivated, the combined subchronic toxicity is still lower than CuO or ZnO without such activation.

In conclusion, CuO NP exposure induced mortality, and produced toxic and beneficial effects on growth and metamorphosis. Therefore, it is important to examine effects of CuO nanoparticles on a variety of organisms due to variable toxicity of Cu exposure to different species to determine the effect that CuO nanoparticles will have on an ecosystem. This study demonstrates that exposure to high concentrations of CuO nanoparticles (0.3–2.5 mg L^{-1}) can negatively affect amphibians by decreasing survivability as well as inducing physiological stresses and lower growth rates. Although environmentally-relevant CuO concentrations have not been determined empirically or through fate modeling, the lowest adverse effect concentration (0.3 mg L^{-1} nominal = 0.045 mg L^{-1} measured concentration) is within the range of expected environmental concentrations predicted for other metal oxide nanoparticles, according to results from simulation models (Boxall et al., 2007). Measured concentrations in our study are far lower than the nominal concentrations (Table 2), which is presumably due to agglomeration and settling of the nanoparticles. However, the amount of Cu^{2+} ions released from these nanoparticles is not known. Thus, future research is needed to determine the relative contributions of particle versus dissolved copper to the responses seen here. Also, because dissolved copper has been found to affect environmentally-relevant sublethal responses such as sensory and antipredator behavior in amphibians at concentrations lower than those required to elicit effects on fitness components (Lefcort et al., 1998, 1999; Haywood et al., 2004; Lavolpe et al., 2004), it would be of interest to determine if these endpoints are affected by CuO nanoparticles as well. Such information would be vital in formulating environmentally-realistic risk assessments of nanoparticles.

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