RESEARCH ARTICLE

Detrimental Effects of Zinc Oxide Nanoparticles on Amphibian Life Stages

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ABSTRACT

While the use of nanoparticles has dramatically increased in recent years, the ecological consequences are not well known. In particular, little research has been done to investigate the potentially detrimental effects of nanoparticles on amphibians, especially across all life-history stages of salamanders and newts (caudates). To address this dearth in knowledge, we examined the effects of zinc oxide (ZnO) nanoparticles on egg, larval, and adult Rough-skinned Newts (*Taricha granulosa)*. Chronic toxicity was tested on eggs and larvae, and acute toxicity was tested on eggs, larvae, and adults. For eggs, chronic exposure to ZnO nanoparticles caused higher mortality at 10.0 and 100.0 mg L^{-1} compared to 0.0, 0.1, and 1.0 mg L^{-1}. When given an acute exposure (24 hr) to 10.0 mg L^{-1} nanoparticles at a late developmental stage, larvae hatched 5 days early, at a decreased developmental stage, and smaller size compared to the control. Chronic and acute exposure of larvae increased mortality up to 75% at both 10.0 and 100.0 mg L^{-1} and exhibited sublethal effects, most dramatically, severe gill degradation. These results suggest nanoparticles can have lethal and sublethal effects on all life stages of amphibians. *J. Exp. Zool. 00A:1–10, 2016.*

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INTRODUCTION

Metal oxide nanoparticles, a unique and new type of pollutant, are increasing in usage (Oberdörster et al., 2007) in products that range from cosmetics to pesticides (Liu et al., 2008; Panácek et al., 2009; Mu et al. 2010). Nanoparticles, including zinc oxide nanoparticles (hereafter ZnO), are defined as particles less than 100 nm in length (Williams et al., 2005) and have many applications in chemistry, agriculture, medicine, and material science due to their large surface area to mass ratios (Choi, '95; Daniel and Astruc, 2004; Salata, 2004; Dimkpa et al., 2012.). These qualities also raise new questions in toxicology because compounds smaller than 100 nm have been shown to be more toxic than

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the same compound in bulk form (Borm, 2002) due to increased surface area.

Aquatic environments often act as sinks for environmental contaminants, and current research suggests nanoparticles act in a similar way to large particle pollutants in these habitats (Moore, 2006; Petosa et al., 2010). It is imperative to understand the effects of nanoparticles on the organisms that will come into contact with them in the environment. Current research shows that nanoparticles can have deleterious effects on aquatic organisms, including fish, macroinvertebrates, and algae (Lovern et al., 2006; Navarro et al., 2008; Zhu et al., 2008). Current evidence as to the effects of nanoparticles on amphibians has demonstrated negative effects on frog development, hormone signaling, and survival (Mouchet et al., 2008; Nations et al., 2011a,b, Bacchetta et al., 2012, 2015; Carew et al. 2015). Nanoparticles have been shown to negatively affect larvae of the Spanish newt *Pleurodeles waltl* (Bour et al., 2015), but other life stages of caudates have not been studied. Amphibian declines have been observed since the 1950s, and worldwide declines are occurring rapidly in the present day (Houlahan et al., 2000; Alford et al. 2001; Sodhi, 2008; Wake and Vrendenburg, 2008); among many causes, including habitat destruction and disease (Daszak et al., '99; Collins et al., 2004; Stuart et al., 2004), pollution is particularly apparent (Hayes et al., 2002; Blaustein et al., 2003; Collins and Storfer, 2003; Davidson, 2004).

Our goal in the present study was to examine the effects of ZnO nanoparticles on a common caudate amphibian on the west coast of North America, the Rough-skinned Newt (*Taricha granulosa* Skilton, Caudata: Salamandridae; Nussbaum et al., '83). To accomplish this, two types of experiments were performed: chronic exposure and acute exposure. Chronic exposure experiments were conducted with eggs and larvae, whereas acute exposure experiments were conducted with eggs, larvae, and adults. Chronic experiments give a greater understanding of the potential impacts of nanoparticles on *T. granulosa* in general, whereas the acute experiments give information about pulses of nanoparticle pollution, and particularly critical exposure periods during development. For all egg and larval studies, mortality, larval length, and developmental stage were measured because these have been shown to be important indicators of future fitness (Cooke, '79; Beebee, '86; Warkentin, '95, '99; Boone et al., 2002; Gall et al., 2011; Hopkins et al., 2014). For adults, corticosterone, an energy-mobilizing glucocorticoid that is often used as an indicator of physiological stress (Moore and Miller, '84; Sapolsky, '92), was measured, along with immunocompetence and oxidative stress (Sies, '86), all critical components of adult health (Lochmiller and Deerenberg, 2000).

We addressed the following questions: (1) How do ZnO nanoparticles affect embryos and larvae of newts exposed chronically at multiple concentration levels?, (2) How do ZnO nanoparticles affect embryonic and larval newts exposed acutely at different developmental periods?, and (3) How do ZnO nanoparticles affect adult newts exposed acutely? We hypothesized that ZnO nanoparticles would adversely affect newts at all life history stages, with chronic exposure to ZnO nanoparticles causing higher mortality than acute exposure, and that negative sublethal effects would include impeded development and smaller size at hatching, and an increased frequency in developmental deformities relative to controls.

METHODS AND MATERIALS

Experimental Animals

Ten gravid adult female *T. granulosa* were collected from Hunter Creek, Curry County, Oregon (42°23′19.60″ N, 124°25′21.54″W) in May 2014 and transported to Utah State University, where they were housed individually in plastic containers with 2.0 L of filtered dechlorinated tap water and a styrofoam perch in an environmental controlled chamber at 14°C on a 12-hr light to 12-hr dark cycle.

After 48 hr acclimation, females were injected with 10 μL luteinizing-hormone-releasing-hormone ([des-Gly10,D = His(Bzl)6]-LHRH ethylamide; Sigma #L2761; St. Louis, Missouri, USA) to induce oviposition onto provided polyester fiber. All eggs were deposited between May 20, 2014 and June 10, 2014 and were collected and separated from the oviposition site within 24 hr. All experiments were conducted with approval from the Utah State University Institutional Animal Care and Use Committee (Protocol #2332).

Solution Preparation

Treatment solutions included filtered dechlorinated tap water (control; 0.0 mg L^{-1} ZnO), 0.1, 1.0, 10.0, and 100.0 mg L^{-1} of ZnO nanoparticles (Sigma Alridch, #721077; St. Louis, Missouri, USA), made by mixing ZnO nanoparticles with control solution. ZnO nanoparticles were all below 100 nm in diameter, and the average particle size was below 40 nm in diameter. Nanoparticle concentrations were selected from previous work on tadpoles (Nations et al., 2011a), and are similar to concentrations used in studies investigating their effects for agricultural use (Dimkpa et al., 2011). All solutions were stored in sealed glass jars at 14°C in the same environmental chamber as the newts. Solutions were not sonicated but rather mixed before being dispensed to simulate a more natural environmental setting, similar to other experiments (Nations et al., 2011a). Once in Petri dishes, test solutions were not stirred, as this could be stressful to the animal. ZnO nanoparticles visibly precipitated out of solution at 10.0 and 100.0 mg L^{-1} ZnO.

Nanoparticle Experiments

Newt eggs were reared based on a previous protocol (Hopkins et al., 2012). Briefly, eggs were reared individually in Petri dishes in 14°C environmental chambers. Petri dishes contained 4 mL of

Table 1. *T. granulosa* egg and larvae zinc oxide nanoparticle experiment designs (Experiments 1–5)

Experiment number	Life stage	Chronic vs. acute	Treatments (mg L^{-1} ZnO)	Exposure timing	Length of exposure	N	Number of females (eggs or larvae per female)
1	Egg	Chronic	Control, 0.1, 1.0, 10.0, 100.0	From oviposition	Entire embryonic period + 2 weeks after hatching	537	9 (10–15)
2	Egg	Acute	Control, 10.0	1, 9, 17, or 25 d after oviposition	24 hr	399	5 (79–80)
3	Larvae	Chronic	Control, 0.1, 1.0, 10.0, 100.0	From hatching	2 weeks	297	5 (57–63)
4	Larvae	Acute	Control, 10.0	1, 4, 7, or 10 days after hatching	24 hr	394	5 (77–80)
5	Eggs + Larvae	Chronic	Control, 0.1, 1.0, 10.0, 100.0	Eggs: From oviposition Larvae: Placed in either control or new treatment solution at hatching	Entire embryonic period + 2 weeks after hatching	91	9

treatment solution at all times to maintain solution concentrations. For each experiment, larval developmental stages (Harrison, '69) and size (total length) were recorded with a stereomicroscope (Olympus, Shinjuku, Tokyo, Japan) at hatching and 2 weeks after hatching at which point the larvae were sacrificed in 5% MS-222. To reduce bias, we had the same person measure and stage larvae at hatching and 2 weeks later. It is at this point in their development that larval mouths open (Harrison, '69), so this end point prevents any ingestion of nanoparticles. All eggs and larvae were checked daily for hatching and mortality. In all experiments, eggs and larvae were randomly assigned treatment. For chronic experiments, eggs and larvae were placed in treatment solution within 24 hr of oviposition or hatching, respectively. For acute experiments, eggs and larvae were kept in a control solution composed of filtered dechlorinated water at all times except during the 24-hr treatment. After 24 hr of treatment, eggs or larvae were returned to a Petri dish of control water. For larval specific experiments, eggs were reared in control water until larvae hatched; see Table 1 for a detailed overview of egg and larval experiments (Experiments 1–5).

Adult Experiment

Experiment 6 examined the acute exposure of adults. Adult newts ($N = 36$, 18 male and 18 female) were obtained from Hunter Creek, Curry County, Oregon (42°23′19.60″ N, 124°25′21.54″ W) in May 2014. All newts were housed in individual plastic containers of control solution at 14°C with 12 hr light to 12 hr dark cycle. Nine female newts were used to provide eggs for previous experiments, but they were given at least 7 weeks to acclimate in control water after being induced to ovulate before the start of adult experiments. Newts were randomly assigned a treatment group, 0.0 or 100.0 mg L^{-1} ZnO nanoparticle solution, and their entire body immersed in 0.5 L of solution for 24 hr. At 24 hr, newt tail tips were removed and blood was collected from the caudal vein using a capillary tube (Neuman-Lee et al., 2015). Blood was centrifuged, and the plasma was collected and frozen at −80°C until ready to be assayed.

Corticosterone concentrations were determined using a radioimmunoassay protocol adapted from a previous protocol (French et al., 2008; Neuman-Lee et al., 2015). In brief, samples were extracted using a solution of 30% ethyl acetate: isooctane in duplicate for corticosterone (MP Biomedicals, Lot #3R3PB-19E). Final concentrations were adjusted using individual recoveries. Microbiocidal assays were performed following previous protocol (French and Neuman-Lee, 2012). Briefly, 1:5 plasma dilution with CO_2-independent media and L-glutamine were combined with 10^4 colony-producing units of *Escherichia coli* (EPowerTM Microorganisms #483-237-1, ATCC 8739, MicroBioLogics, St. Cloud, Minnesota, USA) and agar broth. Samples were incubated in a 96-well microplate for 12 hr, and absorbance was calculated using a microplate reader (300 nm, BioRad Benchmark, Hercules, California, USA). Reactive oxygen metabolites (ROMs) in the plasma were measured to determine whether there were elevated levels of oxidative stress. We followed the protocol included with the d-ROMs Test kit (Diacron, Grosseto, Italy). Briefly, we mixed the provided R1 and R2 reagents in a

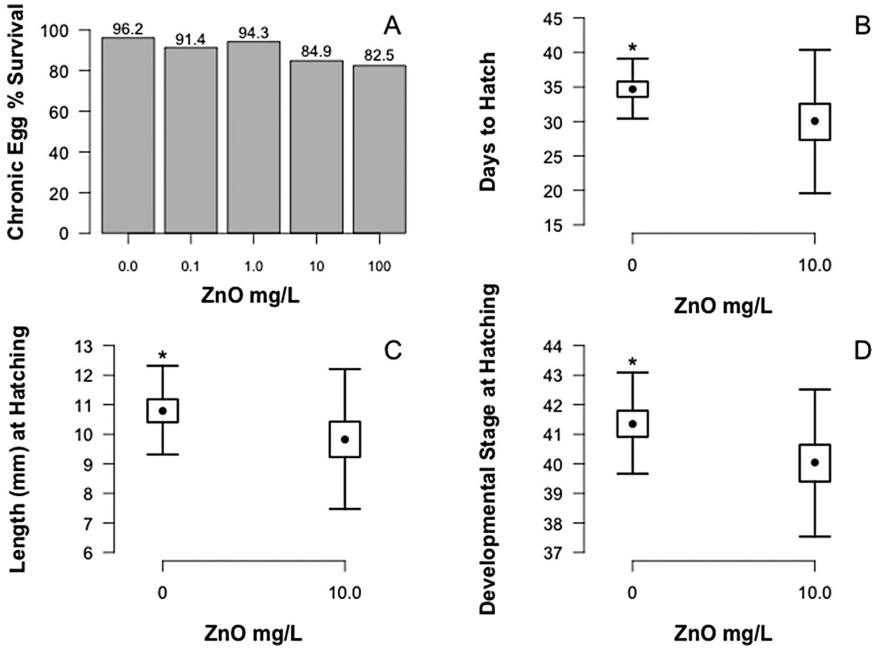

Figure 1. Effects of chronic and acute exposure of *T. granulosa* eggs to nanoparticles. (A) Percent survival at multiple ZnO concentrations ($N = 537$). Eggs ($N = 399$) treated on day 25 hatched significantly earlier (B), shorter (C), and less developed (D). Asterisks denote significance differences between treatments, dots denote median, and error bars denote interquartile range.

1:100 dilution to create an acidic buffered solution with a chromogen. This resulting solution was kept in the dark until 5 μL of sample plasma was added into separate wells of a 96-well microplate and 100 μL of the R1/R2 solution was added to each well. Nanopure water was used as sample blanks and the provided serum was used as a calibrator solution. We followed the "end-point mode" from the manufacturer's protocol and measured absorbance at 505 nm after a 90-m incubation at 37°C. The resulting units are in mg H_2O_2/dL.

Statistical Analyses

Mixed effect generalized linear models were used to predict mortality rate and sublethal effects on eggs and larvae at different nanoparticle concentrations for all experiments. ZnO nanoparticle concentration was treated as a fixed effect, and individual female (mother) was included as a random effect. Bayesian Information Criterion was used to choose which model incorporated the random effect as an intercept or slope. *P* values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question.

Continuous variables in the adult experiments, including corticosterone, bacterial killing ability, and oxidative stress, were analyzed in *R* using a Student's *t* test comparing treatments. Owing to differences in response, adult data were analyzed separately by sex. All statistical analyses were completed in R 3.1.1 with the packages "lme4" and "stats," and figures were made with "plyr" (Wickham, 2011; Bates et al., 2014; R Core Team, 2014). Significance was set at $\alpha = 0.05$.

RESULTS

Experiment 1: Chronic Exposure of Eggs

Egg mortality significantly increased as the concentration of ZnO nanoparticles increased ($\chi^2_1 = 13.431$, $P < 0.001$) (Fig. 1A). The effect of nanoparticle concentration on egg mortality was highly significant.

Significant sublethal effects included nanoparticles decreasing the number of days to hatching ($\chi^2_1 = 42.99$, $P < 0.001$), length at hatching ($\chi^2_1 = 46.288$, $P < 0.001$), and developmental stage at hatching ($\chi^2_1 = 101.27$, $P < 0.001$), as well as developmental stage ($\chi^2_1 = 13.431$, $P < 0.001$), and length ($\chi^2_1 = 4.479$, $P < 0.034$) of larvae 2 weeks posthatching. Eggs in 100.0 mg L^{-1} ZnO were covered with a white substance not found on control eggs (Fig. 2A, B).

Experiment 2: Acute Exposure of Eggs

All eggs survived 24 hr of 10.0 mg L^{-1} ZnO exposure. Generalized linear mixed effects models were created with fixed effects of treatment, and adding treatment day and their interaction if

significant. Experimental eggs hatched significantly earlier ($\chi^2_1 = 44.062$, $P < 0.001$) with a model with treatment, treatment day, and their interaction. The developmental stage at hatching was significantly decreased ($\chi^2_1 = 17.412$, $P < 0.001$), and the length at hatching was significantly decreased ($\chi^2_1 = 16.797$, $P < 0.001$) with treatment being the only significant model. Eggs treated on day 25 hatched earlier (Fig. 1B), shorter (Fig. 1C), and less developed (Fig. 1D) than control eggs.

Experiment 3: Chronic Exposure of Larvae

Larval survival significantly decreased with ZnO nanoparticle concentration ($\chi^2_1 = 170.94$, $P < 0.001$). The effect of ZnO nanoparticle concentration was highly significant. There was greater mortality at 10.0 and 100.0 mg L^{-1} than at control, 0.1, or 1.0 mg L^{-1} (Fig. 3B).

Seventy-five percent of all newt larvae exposed to 10 or 100.0 mg L^{-1} ZnO nanoparticle concentration exhibited severe gill degradation. Gill degradation occurred within 24–72 hr of exposure and reduced the gills from long and filamentous to short and simple, essentially destroying the gills completely (Fig. 2D). No control larvae experienced visible gill degradation (Fig. 2C).

Experiment 4: Acute Exposure of Larvae

No larvae died in the control groups at any day treated. Larval mortality was greater than the control when exposed to ZnO nanoparticles for 24 hr at all time periods, with increasing mortality at later time periods (Fig. 3C). Generalized linear mixed effects models showed significant fixed effects of treatment and treatment day on developmental stage 2 weeks posthatching ($\chi^2_1 = 8.233$, $P < 0.004$) Body length 2 weeks posthatching was also significantly decreased ($\chi^2_1 = 25.833$, $P < 0.001$) by treatment and treatment day. For each experimental day, 74–84% of larvae exposed to 10.0 mg L^{-1} ZnO nanoparticle concentration exhibited gill degradation (Fig. 2B) within 24 hr of exposure.

Experiment 5: Chronic Exposure of Eggs and Secondary Exposure of Larvae

Eggs were randomized to treatment groups: control, 0.1, 1.0, 10.0, or 100.0 mg L^{-1} of ZnO nanoparticles. Larvae were then placed into control solution or new corresponding treatment solution after hatching. All larvae reared in control solution survived, regardless of the solution in which they were reared embryonically. The new concentration of nanoparticles in which larvae were placed was a significant indicator of mortality ($\chi^2_1 = 27.163$, $P < 0.001$). Larvae that were reared embryonically in 10.0 or 100.0 mg L^{-1} ZnO nanoparticle solution had 78% and 30% survival, respectively, when transferred to a Petri dish with new 10.0 or 100.0 mg L^{-1} ZnO solution (Fig. 3A) and showed degraded gills.

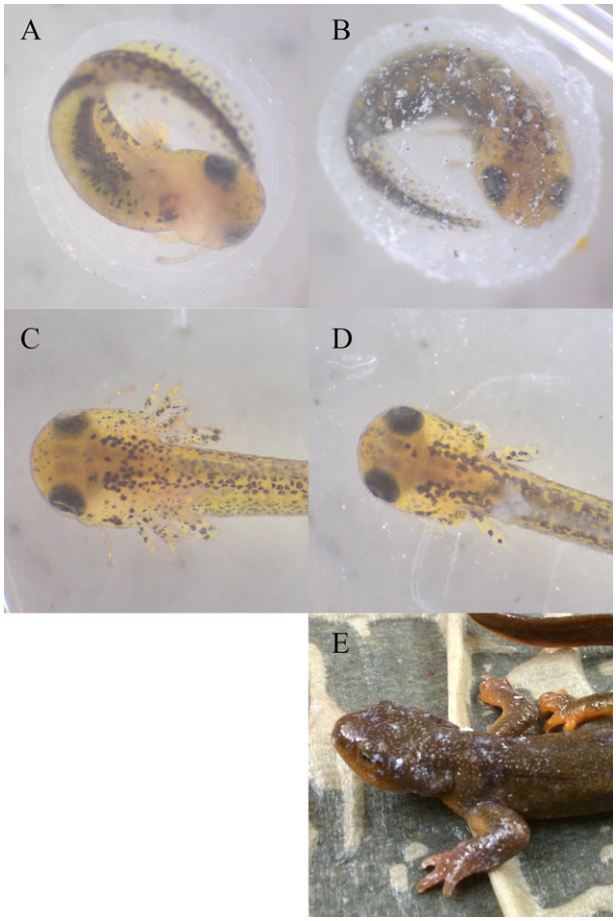

Figure 2. Photographs of healthy (left column) and treated (right column) *T. granulosa* across all life stages. (A) Egg in control solution with healthy egg layers. (B) Egg treated with 100.0 mg L^{-1} ZnO nanoparticles. There is a white substance and visible degradation on the outer egg layer. (C) Newly hatched larva with healthy gills. (D) Larva after 1 day of treatment in 100.0 mg L^{-1} ZnO nanoparticles. (E) Adult newt treated for 24 hr in 100.0 mg L^{-1}. White material has accumulated on the head and back.

Experiment 6: Acute Exposure of Adults

Adult newts exposed to ZnO nanoparticles had significantly higher corticosterone levels than controls (females, $t_{12} = 2.248$, $P = 0.044$; males, $t_{12} = 2.021$, $P = 0.066$) (Fig. 4). Neither females nor males showed differences in bacterial killing ability (females: $t_{16} = 0.053$, $P = 0.959$; males: $t_{16} = 0.067$, $P = 0.947$) nor oxidative stress (females: $t_{13} = 0.053$, $P = 0.959$; males: $t_{16} = 0.564$, $P = 0.581$) with treatment. Adult newts showed an accumulation of white substance on their bodies after exposure to ZnO nanoparticles (Fig. 2E).

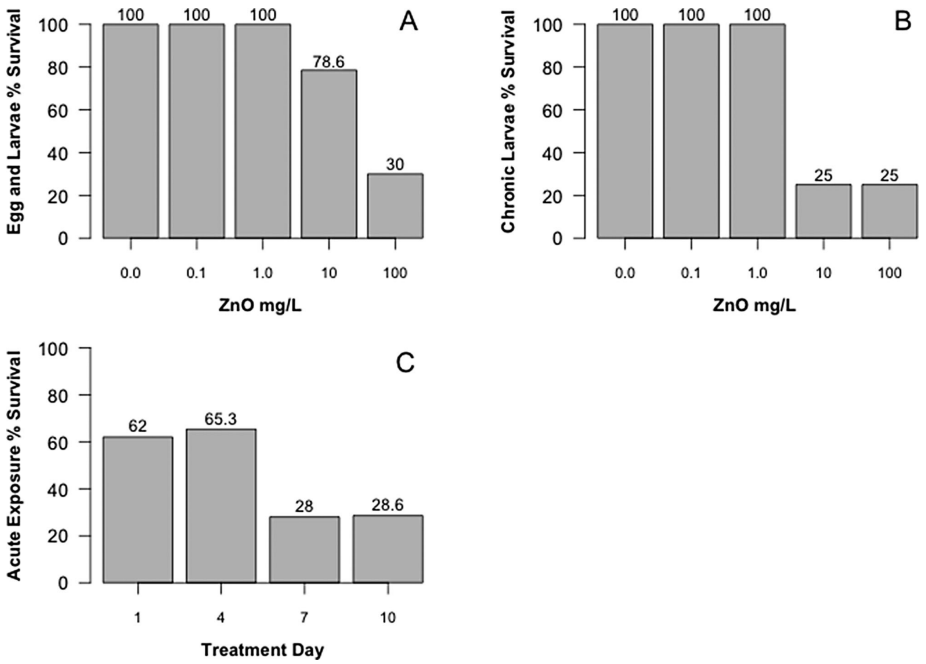

Figure 3. Effects of chronic and acute exposure of *T. granulosa* larvae to nanoparticles. (A) Percent survival of larvae ($N = 91$) previously exposed to nanoparticles as eggs. (B) Percent survival of larvae ($N = 297$) chronically exposed to different concentrations of nanoparticles. (C) Percent survival of larvae ($N = 394$) exposed to nanoparticles at different days from hatching.

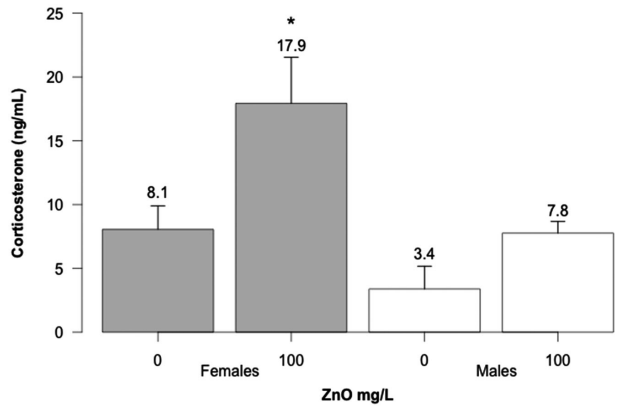

Figure 4. Mean corticosterone levels of female (gray, $N = 18$) and male (white, $N = 18$) *T. granulosa* in both the control and treatment groups. Asterisk denotes significance and error bars denote 95% confidence interval.

DISCUSSION

Our results demonstrate that ZnO nanoparticles had significant negative effects on all life stages of newts. Chronic exposure of eggs dramatically increased mortality. Acute exposure of eggs decreased time to hatching and subsequent size and developmental stage at hatching. Chronic and acute exposure of larvae significantly increased mortality and caused severe gill degradation (Fig. 4). Acute exposure of adult newts increased corticosterone (physiological stress). Our results provide critical data on the effects of chronic and acute exposure of this emerging pollutant across all life-history stages of an amphibian.

Exposure to nanoparticles during embryonic development has previously been shown to cause increased deformities and mortality in other aquatic species, including *Xenopus laevis* (Bacchetta et al., 2012) and *Danio rerio* (Zhu et al., 2008; Bar-Ilan et al., 2009). Although salamanders have been shown to be phenotypically plastic in their hatch time and can sense both predators and chemicals given off by predators (Sih and Moore, '93), in this experiment, it appeared that nanoparticles physically degraded the egg capsule. This caused the eggs to open and forced larvae to hatch earlier. Not being able to alter hatching timing restricts larvae from developing in the egg shorter or longer depending on the current environment of the egg (Moore et al., '96; Chivers et al., 2001; Warkentin et al., 2001). This early hatching can also significantly affect survival to adulthood because small, less developed larvae are more susceptible to pollution (Cooke, '79; Beebee, '86; Hopkins et al., 2014),

predation, and competition (Boone et al., 2002; Gall et al., 2011), as well as reduced learning of feeding behaviors (Warkentin, '95, '99).

Chronic exposure of larvae significantly increased mortality at 10.0 and 100.0 mg L^{-1}. Acute exposure of larvae resulted in significantly higher mortality at all developmental stages, with mortality increasing as the larvae were older and larger. Overall, chronic exposure of ZnO nanoparticles affected mortality more significantly than acute exposure. Similar results of longer exposure times increasing mortality were seen in a previous study testing zinc pollution on *X. laevis* embryos (Haywood et al., 2004). Newt larvae exposed in our experiment to 10.0 mg L^{-1} nanoparticles exhibited severe gill degradation in both chronic and acute experiments. Larval mortality of newts was correlated with gill destruction and may be due to nanoparticles having an affinity for ciliated or filamentous ectoderm cells. Nanoparticles have been shown to adhere to those portions of tadpoles based on scanning electron microscopy of *X. laevis* skin (Nations et al., 2011a). Older larval newts have larger, more filamentous gills to better meet a larger metabolic demand (Feder, '77), and are consequently more susceptible to gill destruction.

Eggs appeared to be providing protection to developing larvae by accumulating ZnO nanoparticles out of solution, as seen by the white precipitate which formed on the outside of eggs (Fig. 2B). This is further demonstrated when the results of Experiments 1, 3, and 5 are combined and compared. All larvae survived in their original solution (Experiment 1) after hatching from eggs in the solution for over 1 month, yet when larvae were exposed to 10.0 and 100.0 mg L^{-1} ZnO (Experiment 3) they quickly showed high mortality. This was corroborated by Experiment 5 in which eggs exposed to ZnO solutions were also exposed to new ZnO solutions as larvae. Larvae experienced mortality at 10.0 and 100.0 mg L^{-1}, showing that the larvae were still susceptible to ZnO nanoparticles even if they experienced them as eggs, and that the eggs were taking ZnO nanoparticles out of solution. The environmental implications of this egg protection mean that nanoparticles may be most harmful at certain times of the year, such as when the newts are in their larval stage. Given that nanoparticles also cause newts to hatch earlier, and thus potentially are in their most vulnerable larval stage longer, this increases the potential impacts of pollution.

When exposed to 100.0 mg L^{-1} ZnO solution, adult female newts showed significantly higher corticosterone concentrations and males showed a trend for higher corticosterone. These data follow previous research showing pollutants can cause increased corticosterone (Hopkins et al., '97; Sorg et al., 2001; Hayes et al., 2006). Physiologically, this is important because an organism has a finite amount of energy and individuals try to maintain a dynamic equilibrium where energy consumption is equal to energy expenditure (McCue, 2010). When stressors, such as nanoparticles, are present, more energy might be needed than is available (Wingfield, 2005). Previous research has shown high levels of corticosterone suppress the reproductive and immune systems (Berger et al., 2005; French et al., 2007), although it does not appear that the innate immune system was impacted in our study. Similarly, adult newts did not show additional oxidative stress when exposed to nanoparticles. However, previous research has shown the opposite in different organisms (Huang et al., 2010; Xiong et al, 2011). This may be due to the short duration of exposure in our study, and only sampling the blood once after 24 hr exposure rather than sampling multiple times after exposure.

By examining multiple life-history stages of this amphibian, we have provided a clearer understanding of the effects that nanoparticles may have on an individual at any specific time over its complex life cycle. As nanoparticles appear to particularly affect larval newts, which are reliant on their gills during this fully aquatic life stage, there may be serious impacts at the population level due to the importance of larval survival for amphibian population persistence (Vonesh and De la Cruz, 2002). The negative effects seen in our study at all life stages illustrate the need for continued study and monitoring of nanoparticles, including the mechanism behind toxicity seen in newts and other organisms. Conservation efforts for amphibians and other aquatic organisms should regard nanoparticles as an emerging and potentially dangerous new pollutant.

CONCLUSION

ZnO nanoparticles cause adverse effects to amphibians at all life-history stages. *T. granulosa* egg and larva survival significantly decreased at levels of 10 mg L^{-1} ZnO nanoparticles and above. ZnO nanoparticles cause severe deformations, including complete gill destruction, in larvae at all developmental stages in as little as 24 hr of exposure. Adult newts exposed to ZnO nanoparticles caused increased levels of corticosterone (a glucocorticoid associated with stress).

AUTHOR CONTRIBUTIONS

ARS conceived the study. ARS, GRH, and EDB created the study design. SSF sponsored ARS to secure funding. GRH collected the study animals. ARS performed the experiments and collected the data. ARS, GRH, and EDB analyzed and interpreted the data. LNL and GDS performed hormone assays. ARS wrote a first draft of the manuscript, and all authors contributed to revisions.

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