SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 36, No. 6, pp. 1606–1613, 2017

Printed in the USA

EFFECT OF NANOSILVER ON CORTISOL RELEASE AND MORPHOMETRICS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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(Submitted 24 May 2016; Returned for Revision 2 August 2016; Accepted 14 November 2016)

Abstract: Nanosilver (nAg) is a nanoparticle commonly incorporated into consumer products for its antimicrobial properties that has been detected in aquatic environments. Toxic effects of nAg on fish have been observed, and nAg may induce a stress response in fish in the form of increased blood plasma cortisol. Effects of nAg exposure on rainbow trout (*Oncorhynchus mykiss*) were investigated over a 28-d period using blood plasma cortisol concentrations as an indicator of stress. Several morphometric measures (growth, Fulton's condition factor, and hepatosomatic index [HSI]) were also taken during the experiment to investigate potential whole-body effects of exposure, and concentrations of nAg in fish muscle tissue were measured. Fish were exposed to environmentally relevant (average 0.28 μg/L) and higher (average 47.60 μg/L) exposure concentrations of nAg. The results showed a significant increase in blood plasma cortisol for both exposure treatments. A significant effect on HSI by treatment dependent on exposure time was also observed, although no obvious trend was detected, whereas other morphometric measures were not affected by nAg exposure. In addition, Ag was detected in fish muscle tissue. The results indicate that although nAg did engage the stress response in fish, it did not affect growth or condition under the experimental conditions and time frame investigated. *Environ Toxicol Chem* 2017;36:1606–1613. © 2016 SETAC

Keywords: Fish Salmonid Stress Nanotoxicology Silver Nanosilver Morphometrics Cortisol

INTRODUCTION

Nanosilver (nAg) is a nanoparticle that has been widely incorporated into many consumer products (especially textiles) for its antimicrobial properties [1] and commonly enters wastewater from the washing of these products. It may also be released from industrial activities and solid waste leaching [2]. Concentrations of nAg in the aquatic environment are not well known; however, most studies estimate environmental concentrations in the nanograms per liter range [3,4].

Once in the aquatic environment, nAg becomes chemically active and may dissolve to silver ions (Ag^+), which may in turn complex with various ligands, such as Cl^-, S_2^-, or S_2O_3, or aggregate with other nAg particles or with dissolved organic carbon (DOC) [5]. These actions depend on the nature of receiving waters, including pH, ionic strength, temperature, and type of ligands present, as well as size of the nAg particles released and the capping agent used to promote particle stability [6]. Nanosilver may also settle from the water column to the sediment and be taken up by invertebrates and fish [7].

Toxicity from nAg exposure has been widely reported and is thought to originate from a combination of the effects of both the nAg itself and the Ag^+ it releases [8]. The main mode of toxicity appears to be through oxidative stress, whereas toxicity from Ag ions appears mainly to be through inhibition of the sodium/potassium ion–activated adenosine triphosphatase (Na^+/K^+-ATPase) pump in the gill cells, eventually leading to plasma ion loss and osmoregulatory failure [9]. Median lethal concentrations of nAg to fish range from micrograms per liter to milligrams per liter, depending on fish species, life stage, and particle size (Supplemental Data, Table S2). Sublethal effects include altered gene expression at 0.6 μg/L [10], thickening of gill tissue at 10 μg/L [11], and impaired osmoregulation via inhibition of Na^+/K^+-ATPase at 20 μg/L [12]. Gill necrosis has been observed at a concentration of 100 μg/L [12] and impaired gas exchange at 300 μg/L [13]. However, most of what is known regarding the effects of nAg on fish comes from relatively short acute exposures (median exposure time 4 d; Supplemental Data, Table S2) and at exposure levels much higher than are likely to be environmentally relevant (median exposure concentration 200 μg/L; Supplemental Data, Table S2).

Cortisol is one of the most common stress indicators in fish and may therefore be well suited to measuring stress caused by nAg exposure [14]. Its release may be caused by nAg either through oxidative or osmoregulatory stress [15] and has been demonstrated in several studies [16,17]. After its release, cortisol causes a cascade of changes to blood and tissue chemistry, allowing the fish to react to the stress [18], including a rise in the plasma glucose levels to provide increased energy. Cortisol is usually released quickly after stress is detected and often only released for the first 6 h to 48 h of experiencing the stressor [19].

If nAg exposure causes changes in fish energetics because of its toxic effects (via a stress response or other mechanisms), chronic exposure to nAg may result in morphological changes in the fish that reflect a decline in whole-body performance. Growth and Fulton's condition factor (K) are 2 morphometric measures that can provide insight into the overall health of a fish. Growth is often measured as the percentage of body weight gained over a certain time period, whereas K estimates

This article includes online-only Supplemental Data.
* Address correspondence to lamurray12@gmail.com
Published online 17 November 2016 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.3691

the condition of a fish under the assumption of isometric growth [20].

The liver plays an important role in the metabolism and excretion of xenobiotic compounds and may therefore also respond to nAg exposure. The liver helps process toxins by excreting them through bile secretions [21]. Because of its role in detoxification, the ratio of liver weight to fish body weight (i.e., the hepatosomatic index [HSI]) is commonly examined in toxicological studies. A decrease in HSI is indicative of stress and a loss of energy stores such as liver glycogen, and an increase in HSI may be indicative of exposure to contaminants because of the necessary increase in capacity to metabolize xenobiotics [22].

The present study investigated the effects of chronic nAg exposure on fish, with the objective of providing insight into how nAg affects both the stress response and whole-body performance of fish over a chronic (28 d) exposure period at environmentally relevant concentrations and higher. The stress response of rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) exposed to nAg was investigated through plasma cortisol release and 3 whole-body morphometric measures (growth, *K*, and HSI). Measurements were taken at various time points throughout the experiment. In addition, the stability of nAg in the water was monitored to determine the actual exposure of fish to nAg in comparison with the target concentrations. Total Ag concentrations in fish tissue were also analyzed to determine uptake during exposures.

MATERIALS AND METHODS

Experimental animals

Juvenile *O. mykiss* were obtained from Lyndon Fish Hatcheries on 11 February 2014 ($n = 200$) and 29 April 2014 ($n = 200$). On arrival at Fisheries and Oceans Canada's Freshwater Institute (Winnipeg, Manitoba), fish weighed approximately 1 g and were kept in quarantine tanks for 1 wk. All individuals were female diploid fish.

During both holding and experimental trials, fish were exposed to a 12:12 diurnal cycle and fed commercial sinking trout pellets (Aqua Pride Trout 42:15) at 1% to 2% body weight/d. Food and fish waste were removed daily, and algae were manually removed weekly from tank walls. Water quality parameters (pH, conductivity, specific conductivity, and nitrates) in tanks were checked weekly and found to be within acceptable ranges (L. Murray, 2015, Master's thesis, University of Manitoba, Winnipeg, Canada). All tanks were on flow-through systems with oxygenated, dechlorinated Winnipeg City tap water ($14 \pm 2\ ^{\circ}C$) at rates of replacement allowing maintenance of optimal water quality (Supplemental Data, Table S1).

Exposure experiment

A stock nAg solution was prepared at Trent University by adding powdered nAg (30–50 nm, polyvinylpyrrolidone-capped) received from Nanostructured and Amorphous Materials to distilled water (1 mg/mL), with an organic stabilizer (0.025% [w/v] gum arabic; Sigma-Aldrich) and milling with a rotor-stator dispersion mill (Kady International) for 15 min. Volumes of 2.5 L were mixed at 1 time. The solution was then refrigerated and stored for up to 2 wk before use; tests showed the solution to be stable under these storage conditions and for this duration (J.D. Martin et al., unpublished data). The prepared solution was then shipped to the Freshwater Institute, where it was diluted to the concentrations required for the experimental exposures. Stock solution was sonicated for 30 min and then vortexed prior to dilution. The required amount of stock solution was then added to 20-L plastic carboys with a measured amount of reverse-osmosis water to make exposure solutions at the appropriate nominal concentrations (Supplemental Data, Table S1). To counteract settling of nAg out of the exposure solution, carboys were placed on a stir plate with a stir bar and continuously stirred. The exposure solution was constituted and replaced every 2 d. The solution was delivered to exposure tanks through a peristaltic pump with a delivery rate set to achieve the desired concentrations of nAg in the tanks given the water flow rates through experimental tanks (Supplemental Data, Table S1). For control treatments, an exposure solution containing only reverse-osmosis water was used.

Exposures were conducted over 2 trials, with trial 1 consisting of control (C1), 1 μg/L (L1), and 200 μg/L (H1) nominal exposures and trial 2 of control (C2), 300 μg/L (L2), and 600 μg/L (H2) nominal exposures, for a total of 5 exposure levels over both trials. Fish were fin-clipped for individual identification, and 11 fish were added to each of the 12-L to 40-L exposure trial tanks (4 tanks/treatment). Fish were acclimated to trial tanks for 1 wk before nAg exposure began. At time 0 of the experimental trial, a measured amount of nAg stock solution was added (Supplemental Data, Table S1) to bring the tank up to the required initial concentration, after which the tube from the peristaltic pump system was introduced to the tank to maintain concentrations through the continued flow of nAg to the tank.

Nanosilver exposures were conducted for a total of 28 d. Total Ag concentrations in randomly selected exposure tanks were measured every 4 d, and stock and carboy total Ag concentrations were measured once during trial 2.

To determine whether nAg stability under static conditions differed from the flow-through setup used for the exposure trials, 2 additional exposure tanks with no flow-through were run for 4 d. These static exposure tanks were set up with an initial dose of nAg to achieve 300 μg/L and 600 μg/L concentrations and were identical to trial tanks with the exception of flow-through water delivery (and only 5 fish in each tank vs 11). Water samples were taken at 0 h, 1 d, and 4 d from both the top and bottom of the tanks for total Ag analysis.

Measurements of Ag in water

Water samples (10 mL) were immediately acidified (4% trace metal–grade HNO_3), shipped on ice packs to Trent University, and stored at 4 °C until analysis. Indium (5 ppb, PlasmaCal; SCP Science) was added as an internal standard, and samples were analyzed for total Ag by inductively coupled plasma–mass spectrometry (ICP-MS; XSeries 2; Thermo Scientific). Instrument operating conditions were optimized daily for maximum Ag intensity. Silver concentrations in water samples were determined against an external calibration curve of Ag^+ standards (0.78–200 ppb) and the internal indium standard.

The hydrodynamic diameters of nanoparticles were determined in stock samples diluted with MilliQ water using dynamic light scattering with a Nicomp 380 instrument (Particle Sizing Systems). Stock suspensions were sonicated for 30 s and vortexed immediately before pipetting into 5-mL cryogenic vials. Suspensions were then diluted with MilliQ water and compared against a standard curve of polystyrene spheres (certified mean diameter 96 ± 3.1 nm) supplied by Agar Scientific.

Particulate and dissolved Ag were determined by separation of dissolved and colloidal fractions by ultracentrifugation followed by analysis of total Ag of each fraction by ICP-MS.

Samples were collected in centrifugal filter tubes (Amicon® Ultra-3K; EMD Millipore), stored at –80 °C, and then thawed and centrifuged at 10 000 *g* for 1.5 h until all of the filtrate (containing dissolved Ag <3 kDa, corresponding to a size of <1 nm) had passed through the filter. Trace metal–grade HNO_3 (20%) was added to the filter containing the retentate (colloidal Ag fraction >3 kDa), and samples were heated to 80 °C for 3 h and centrifuged as described above to obtain the colloidal Ag fraction. Dissolved Ag fractions were acidified (4% HNO_3) and heated to 80 °C for 2 h. Colloidal Ag fractions were diluted to 4% HNO_3 using Milli-Q water (EMD Millipore). Analysis of total Ag of each fraction by ICP-MS proceeded as described above for total Ag in water samples.

Biological sampling

During nAg exposure, 153 fish were euthanized over 0 h, 2 h, 7 h, 7 d, 14 d, and 28 d of the experiment. Individual fish were removed from the tank; fork length, weight, and the identifying fin clip were noted; and fish were then placed in an overdose of a pH buffered solution of tricaine methanesulfonate (Syndel Laboratories; 300 mg/L) until opercular movement ceased (<3 min). The tail was cut off, and blood was drained from the caudal vein into a heparinized hematocrit tube. The fish was then euthanized by cervical dislocation. Blood was centrifuged for 6 min at 3000 *g* to separate plasma from red blood cells, and plasma was pipetted into another vessel and then frozen at –80 °C. Muscle tissue was sampled, and liver weights were taken.

Measurement of total Ag in fish

A minimum of 250 mg of tissue was needed for tissue total Ag analysis. Therefore, muscle tissues from 2 fish from the same treatment were pooled for each sample. Pooled fish muscle (250–500 mg wet wt) samples were placed in 70% trace metal–grade HNO_3 and spiked with indium (5 ng/mL) as a recovery standard. Samples were digested at 120 °C for 2 h and then evaporated to 1 mL at 150 °C. Digests were filtered through a 0.45-μm filter and diluted to 4% HNO_3 using Milli-Q water. Total Ag in fish muscle samples was measured by ICP-MS as described for the water samples, with the exception that rhodium (5 ng/mL) was added as an internal standard.

Blood plasma cortisol sample analysis

Cortisol sample analysis methods followed previously described methods (L.L. Bestvater, 2014, Master's thesis, University of Manitoba, Winnipeg, Canada). In brief, plasma samples were spiked with 5 ng of d_4-cortisol (10 μL of 0.5 ng/μL solution). Then, 3 mL of 9:1 hexane:ethyl acetate was added, and samples were vortexed (60 s), centrifuged (5 min at 4000 *g*), and frozen (5 min at –80 °C). Subsequently, the top layer was decanted into a clean glass tube (extract 1). These steps were repeated using 3:2 hexane:ethyl acetate, followed by 100% acetonitrile, and the upper layer was decanted and combined with extract 1. Samples were then blown to dryness using nitrogen (N_2) and rinsed with methanol multiple times to increase transfer efficiency. Samples were analyzed by high-performance liquid chromatography (HPLC)–MS/MS.

HPLC

An Agilent 1100 series HPLC system (Agilent Technologies) was used to analyze samples. It was equipped with a vacuum degasser, a binary pump, and an autosampler. A C18 analytical column (Grace; 50 mm × 2.1 mm inner diameter, 4 μm particle size; Chromatographic Specialties) was used to separate the cortisol. The mobile phase consisted of a gradient of (A) water and (B) methanol at a flow rate of 300 μL min^{-1}. The initial composition was 80:20 (A:B), held for 1 min, ramped linearly to 100% methanol in 9 min, and held for 6 min. The column was equilibrated between runs for 7 min.

MS

A Sciex 2000 triple quadrupole MS (Applied Biosystems) was used for identification and quantification of the compounds.

Cortisol was monitored in the negative ion mode under multiple reaction monitoring conditions. Quantitation for cortisol was based on the ion signal from the mass to charge (*m/z*) transition of 361.1 → 331.0 multiple reaction monitoring. d_4-Cortisol was based on the *m/z* 365.0 → *m/z* 335.0 multiple reaction monitoring transition.

Quality assurance/quality control

Procedural and instrumental (3 μL methanol injection) blanks were analyzed every 10 samples. Native hormones were not detected in the blanks. The recovery percentage for d_4-cortisol was found to average 24% (± 0.91 standard error of the mean, $n = 253$), a level of recovery common for this type of analysis because of matrix effects [23]. Quantitation of native cortisol in the samples was achieved by isotope dilution (Equation 1), a method used to compensate for matrix effects [23,24]

$$\text{Cortisol} = \frac{C_{sample} \times \text{average}\left(\frac{d_{4\ standard}}{c_{standard}}\right)}{d_{4\ sample}} \quad (1)$$

where C_{sample} and $d_{4\ sample}$ are the areas of cortisol and d_4-cortisol detected in the sample and $d_{4\ standard}$ and $c_{standard}$ are the areas detected in the standard, respectively.

The analytical detection limit for cortisol was found to be 1.2 pg, and the method detection limit was 0.005 ng/mL. In cases where cortisol was below the method detection limit, a concentration of one-half the method detection limit was assumed.

Data analysis

Growth rates (grams per day) were calculated as the percentage of body weight (grams) gained per day by Equation 2 [25]

$$\text{Growth} = \frac{\ln(\text{final wt}) - \ln(\text{initial wt})}{\text{time}} \times 100 \quad (2)$$

If the initial weight of the fish was not known because the identifying fin mark could not be clearly identified at the end of the experiment (38% of fish), the initial weight was taken as the average initial weight of all fish for that tank.

Hepatosomatic index was calculated (weight in grams) by Equation 3 [26]

$$\text{Hepatosomatic index} = \frac{\text{liver wt}}{\text{body wt}} \times 100 \quad (3)$$

Fulton's condition factor (*K*) was calculated (weight in grams, length in centimeters) by Equation 4 [20]

$$K = \frac{\text{body wt}}{\text{fork length}^3} \times 100 \quad (4)$$

Statistical analysis

Statistical analyses were run in R Ver 3.1.3 [27]. Measured water Ag concentrations between treatments were determined with an analysis of variance (ANOVA). Fish Ag concentrations were also analyzed by treatment and time with an ANOVA. Cortisol, growth, HSI, and *K* data were analyzed by treatment and time using a mixed-model ANOVA design (lmer in package lme4), taking into account tank as a random effect with the general equation (Equation 5).

$$\text{Cortisol} = \text{Treatment} + \text{Time} + (\text{Treatment} \times \text{Time}) + \text{Tank} \quad (5)$$

The significance of the interaction term was evaluated by comparing the full model (Equation 5) to a reduced model lacking the term using a log-likelihood test. In cases where the interaction term was shown to be nonsignificant, the reduced model was used as the full model (Equation 6)

$$\text{Cortisol} = \text{Treatment} + \text{Time} + \text{Tank} \quad (6)$$

In this case, the significance of the treatment effect was evaluated by comparing the full model to a reduced model lacking the treatment effect, again using a log-likelihood test.

When testing additive models for significance (only after interaction terms were determined to be nonsignificant), we excluded time 0 observations from the analysis from all treatments as expected similarities of treatments at time 0 (prior to any nAg exposure) violate the assumption of additivity.

For cortisol, growth, and condition, no significant differences were found for variables between the controls of each trial (ANOVA, $p > 0.05$); therefore, controls were combined, and trial was not considered a factor in the analysis. For the HSI, a significant difference was found between the trial controls, and trial was included in the models as a random effect. Cortisol data were $\log_{10}$-transformed to meet the assumptions of normality and homogeneity of residual distributions. Post hoc analyses were conducted with a Tukey's honestly significant difference test where appropriate.

RESULTS

Exposure concentrations of total Ag

Most of the Ag detected in the experimental tanks was in particulate form (nAg), with only 0% to 0.18% in ionic form (Ag^+; average 0.05% ionic). Hydrodynamic diameters of nAg particles in stock were approximately 34 nm (90–92% of particles), with some agglomeration (~200 nm, 8–10% of particles). Overall, concentrations of total Ag measured in the experimental tanks were much lower than nominal and quite variable (Table 1). Results suggest that this was attributable in part to the stability of the prepared stock solution despite efforts to keep the solution from settling. Carboys containing exposure Ag solution that was pumped into tanks were measured once in trial 2, and concentrations were found to be much lower than nominal (Table 1). Test static tanks were well below nominal concentrations (lower than flow-through exposure tanks) and consistently low over the 4-d exposure in both treatments. In addition, concentrations in static exposures did not significantly differ between the tank top and tank bottom measurements, suggesting no evidence of gradients within the static treatments (L. Murray, 2015, Master's thesis, University of Manitoba, Winnipeg, Canada).

Table 1. Total silver concentrations measured in exposure tanks, static experiment tanks, and carboys containing silver solution[a]

Tank	Nominal concentrations (μg/L)	Average measured concentrations (μg/L)	Range (μg/L)	Percentage of nominal (%)
C1	0	0.00	0.00–0.03	—
L1	1	0.28	0.18–0.38	28
H1	200	85.37	22.32–159.00	43
C2	0	0.06	0.00–0.12	—
L2	300	44.45	12.07–119.30	15
H2	600	40.73	4.46–71.80	7
Static L	300	4.87	1.87–11.82	2
Static H	600	15.72	9.83–20.27	3
Carboy L	450 300	24 517.07	—	5
Carboy H	900 600	150 503.98	—	17
% Dissolved Ag		0.05	0.00–0.18	

[a]Percentage of total silver that was measured as dissolved is also included. C1 = trial 1, control; L1 = trial 1, low; H1 = trial 1, high; C2 = trial 2, control; L2 = trial 2, low; H2 = trial 2, high; Carboy L = carboy containing low stock solution; Carboy H = carboy containing high stock solution; Static L = static low; Static H = static high.

Measured concentrations of total Ag within the 200 μg/L, 300 μg/L, and 600 μg/L nominal exposure tanks had a large degree of overlap with each other and did not differ statistically from each another (ANOVA, $F_{(5,85)} = 15.23$, $p > 0.05$). Because of this observation, the fish total Ag concentration, cortisol, and morphometric data for these higher concentration exposures were combined into 1 exposure treatment (called "high," [H], mean exposure concentration 47.61 ± 5.13 μg/L) and compared to the control (C1 and C2 combined, where appropriate) and the low (mean exposure concentration 0.28 ± 0.02 μg/L) exposures.

Fish tissue total Ag concentrations

Uptake of total Ag into fish muscle tissue was observed with a high degree of variation in the tissue concentrations, both within treatments (Figure 1A) and across time (Figure 1B). Between 7 d and 28 d, total Ag concentrations in the high-exposure fish differed significantly from those in control and low treatments (Tukey's honestly significant difference, $p_{C\text{-}H} = 0.006$, $p_{L\text{-}H} = 0.02$), whereas no significant difference was found between control and low treatments (Tukey's honestly significant difference, $p = 0.87$). The interaction between time of exposure and total Ag concentration was not significant (ANOVA, $F_{(df=4,16)} = 0.757$, $p = 0.57$), though the pattern in the data suggested continued accumulation of total Ag in fish as exposures continued, whereas fish in the low treatment appeared to be similar to or only slightly elevated relative to control fish (Figure 1B).

Cortisol

We found no significant effect of the treatment by time interaction on the cortisol response of fish to nAg exposure (log-likelihood test, $\chi^2_{(df=10)} = 8.522$, $p = 0.578$). However, there was a significant effect of treatment on cortisol concentrations (Table 2 and Figure 2; log-likelihood test, $\chi^2_{(df=2)} = 16.577$, $p < 0.001$). Significant differences in cortisol concentrations were found between the high treatment and the control (Tukey's honestly significant difference, $p < 0.001$), and the low and the control treatment

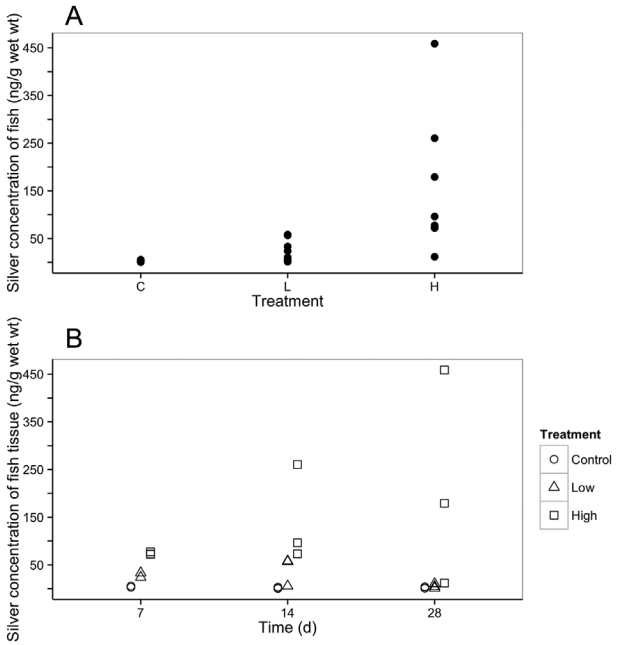

Figure 1. Total silver concentrations in fish muscle tissue (**A**) in exposure treatments and (**B**) over time at 7 d, 14 d, and 28 d. C = control; H = high; L = low.

Table 2. Summary of cortisol, growth, and Fulton's condition (*K*) results for nanosilver exposure treatments[a]

	Cortisol (ng/mL)	Growth (% body weight gain/d)	*K*
C	4.41 ± 1.30	1.93 ± 0.13	2.21 ± 0.07
L	15.92 ± 5.56	1.86 ± 0.18	2.35 ± 0.09
H	17.85 ± 3.14	1.75 ± 0.11	2.29 ± 0.06
Overall	12.84 ± 1.92	1.83 ± 0.08	2.27 ± 0.04

[a]Results are described as means ± standard errors.
C = control; L = low; H = high.

(Tukey's honestly significant difference, $p = 0.04$). Visually comparing the means in the experiment over time (Figure 3), cortisol levels of nAg exposed fish tended to be elevated relative to cortisol in control fish earlier in the experiment between 0.1 d, 0.3 d, and 7 d.

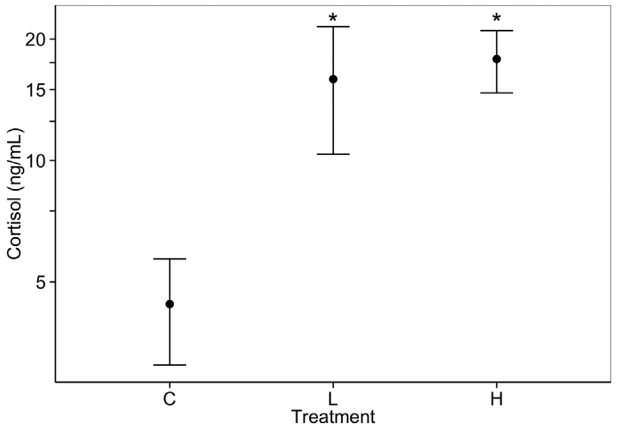

Figure 2. Blood plasma cortisol concentrations by nanosilver treatment, with means ± standard error. Note log scale on *y* axis. *Significant difference from the control group ($p < 0.05$). C = control; H = high; L = low.

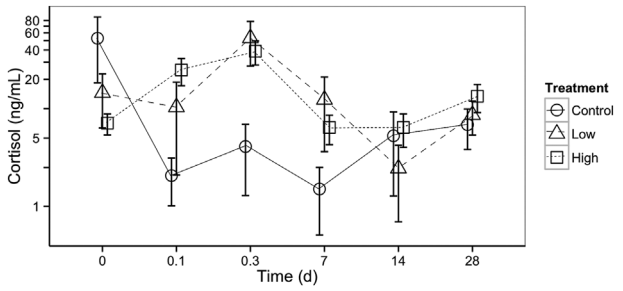

Figure 3. Blood plasma cortisol concentrations over time by nanosilver treatment, with means ± standard error. Note log scale on the *y* axis.

Growth, Fulton's condition factor, and HSI

Body weights were similar among all treatments for all fish between trial 1 (mean 2.94 ± 0.99 g) and trial 2 (mean 2.87 ± 1.28 g; *t* test: $t = 0.46$, degrees of freedom [*df*] = 231.32, $p = 0.646$), whereas relative liver weights between trial 1 (mean 0.07 ± 0.04 g) and trial 2 (mean 0.04 ± 0.03 g) differed (*t* test: $t = 6.88$, $df = 226.67$, $p < 0.001$). Growth tended to decrease with nAg exposure (Figure 4A) but did not differ significantly across treatments (Table 2; log-likelihood test, $\chi^2_{(df=2)} = 1.053$, $p = 0.591$), and neither did *K* (Table 2 and Figure 4B; log-likelihood test, $\chi^2_{(df=2)} = 1.448$, $p = 0.485$; Figure 4B). The interaction of time and treatment was significant for HSI (log-likelihood test, $\chi^2_{(df=10)} = 23.666$, $p = 0.009$; Table 2). However, no obvious trend of treatment over time within either trial was observed (Table 3 and Figure 5). Overall, fish in trial 1

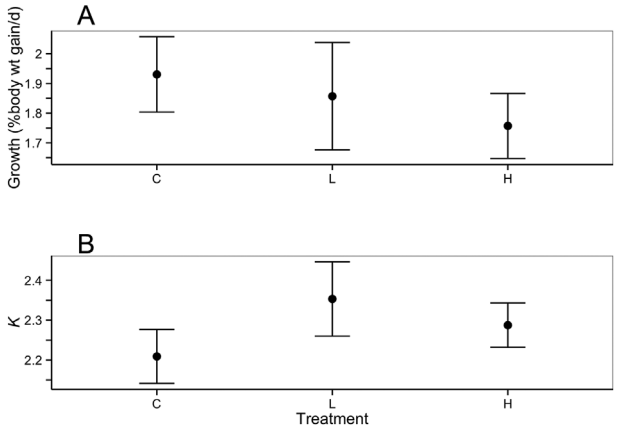

Figure 4. Effects of nanosilver treatment on growth and condition (*K*) with means ± standard error. (**A**) Growth, estimated as percentage of body weight gained per day, and (**B**) condition, estimated as body weight divided by fork length^3 multiplied by 100. C = control; H = high; L = low.

Table 3. Summary of hepatosomatic index results for nanosilver exposure treatments[a]

Treatment	Trial	Hepatosomatic index
C	1	2.34 ± 0.17
	2	1.47 ± 0.09
L	1	2.64 ± 0.19
H	1	2.43 ± 0.14
	2	1.34 ± 0.07
Overall		1.94 ± 0.07

[a]Results are described as means ± standard errors. Results are separated by trial due to differences in trial controls.
C = control; L = low; H = high.

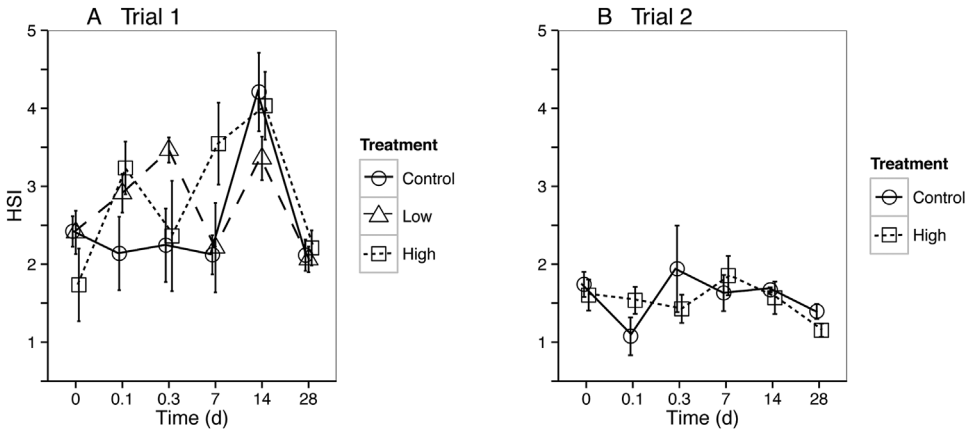

Figure 5. Hepatosomatic index (HSI) over time for nanosilver treatments in (A) trial 1 and (B) trial 2.

had higher HSI values compared with fish in trial 2, and there appeared to be a peak in HSI at 14 d for all treatments in trial 1, whereas trial 2 had more stable values across time.

DISCUSSION

In the present study, juvenile rainbow trout demonstrated a stress response to nAg exposure for 28 d, as indicated by significantly increased blood cortisol levels in fish exposed to nAg compared with control fish; in contrast, this did not translate into meaningful whole-body responses (growth, *K*, or HSI) over a similar time frame. Overall, cortisol concentrations in each of the treatment groups were increased by approximately 74% on average in comparison to controls. These findings are supported by research reporting elevated plasma cortisol in silver carp (*Hypophthalmichthys molitrix*) exposed to 20 μg/L and 40 μg/L of nAg for 3 d, 7 d, and 14 d [17]. In addition, juvenile rainbow trout have had increased plasma cortisol levels after exposure to extremely high concentrations of nAg (1000–8000 μg/L) for 3 h [18]. Both of these studies found an approximate 6-fold increase in cortisol, whereas the present study found an approximate 4-fold increase in cortisol over a longer exposure period of 28 d, possibly a result of the longer exposure duration conducted in the present study. Other toxic effects of nAg have been observed to decrease over time, including gill damage [11] and altered gene expression [28]. As an indication of this pattern, time dependence can also be observed in the cortisol data of the present study, with nAg-exposed fish having noticeably higher cortisol concentrations than controls at earlier time points (several hours to 7 d) but less of a difference between groups later in the exposure (14 d and 28 d). In contrast to these results, no effect on whole-body cortisol was detected when zebrafish (*Danio rerio*) embryos were exposed from 2 h post fertilization (hpf) to 96 hpf to 500 μg/L [29], potentially indicating that the cortisol response to nAg in fishes is dependent on life stage. However, this lack of cortisol response from nAg in embryos may also be because the cortisol response may not be functional in *D. rerio* before 97 hpf [30] or before hatching in *O. mykiss* [31].

The observed stress response of fish in the present study is most likely a result of the nAg exposure as opposed to Ag^+ exposure released from nAg. Only 0.05% of the Ag measured in our exposure water was in the dissolved fraction, suggesting that Ag^+ concentrations in our exposures were minimal. Although both nAg and Ag^+ can increase plasma cortisol in response to exposure in fish, published studies suggest that the effect of Ag^+ exposure may be delayed relative to the rapid response to nAg. Rainbow trout exposed to 9.2 μg/L Ag^+ have shown no significant increase in plasma cortisol levels until 4 d [32]. Similarly, starry flounder (*Platichthys stellatus*) had increased cortisol after exposure to 1000 μg/L Ag^+ after 6 d in saltwater [33] but not before. Based on these studies, it appears that Ag^+ may not cause a significant increase in cortisol release until 4 d to 6 d after exposure, in contrast with nAg, which caused an increase within hours of exposure in the present study (when effect of $\chi^2_{(df\ =2)} = 9.730$, $p = 0.008$), as well as others [16].

Hepatosomatic index was variable in the present study, with higher values overall for trial 1 than for trial 2 (caused by higher liver weights in trial 1). It is unknown why this difference was seen, although it is possible that it may have been caused by the small 14-d age difference in fish used between trials (trial 2 fish were 14 d older). Other studies on juvenile rainbow trout observed values for HSI more similar to trial 2 than trial 1 [34–36]. Overall, no obvious pattern of the effect of nAg exposure on HSI could be discerned from the data. In contrast, HSI has been observed to increase in rainbow trout when fish were exposed to 32 μg/L to 32 000 μg/L nAg for 14 d [37]. However, rainbow trout exposed to 3000 μg/L nAg for 8 wk have shown a decrease in HSI and significant damage to liver tissue (i.e., decreased size of hepatocytes, increased liver enzymes aspartate transaminase and alanine transaminase, congestion in liver parenchyma, massive destination in hepatic sinusoids sizes) as well as decreased levels of protein in serum [38]. These changes indicate that the liver activity to metabolize nAg and the decreased serum protein may result from the liver being metabolized as an alternate source of energy. The difference in the direction of change in HSI observed in these 2 studies may be another example of the time-dependent nature of nAg effects as a shorter exposure (2 wk) increased HSI and a longer exposure (8 wk) decreased it. Additional studies are needed to clarify these effects of nAg on HSI. Other contaminants have also been observed to affect HSI in opposing directions: copper has been observed to both increase [39] and decrease [40] HSI. In general, a decrease in HSI is indicative of stress and a subsequent loss of energy stores such as liver glycogen, and an increase in HSI may be indicative of exposure to contaminants from the needed increased capacity to metabolize xenobiotics [22].

Despite a significant response of cortisol to nAg exposure, this did not translate into a significant response for growth or *K* in the present study. Nanosilver has not been observed to have an effect on fish growth in other studies [11], similar to other

nanoparticles [41]. However, it is possible that studies conducted over longer time periods than previously tested may demonstrate an effect on growth, especially in light of the apparent tendency for decreased growth as a result of exposure in the present study; no effect on growth after 28-d exposure in Griffitt et al. [11] is perhaps not surprising, whereas no effect following an 8-wk exposure in Ramsden et al. [41] on a different nanoparticle (titanium dioxide) might be considered a more thorough assessment of contaminant exposure on growth rates. In contrast, Ag^+ has been shown to decrease growth in rainbow trout but over an 18-mo exposure study [42]. In general, it is known that metal exposure can inhibit growth. However, fish are often capable of adopting bioenergetic strategies that preserve growth at the expense of other aspects of metabolism [43]. For example, rainbow trout exposed to copper reduced their time spent swimming, which decreased metabolic costs [44]. Common carp (*Cyprinus carpio*) had decreased K after exposure to high concentrations of nAg over 8 wk [45], and similarly other metals have been found to decrease K [40].

Uptake of total Ag into fish muscle tissues was observed at all treatment levels. Other studies also report total Ag uptake into fish tissues from nAg exposure [12,37,46–48]. These studies observed uptake into various tissues, with uptake concentration in the order liver $>$ kidneys $\sim$ gills $>$ muscle. These studies also observed low levels of total Ag in control fish as the present study did, and it has been suggested that these are background levels that fish possess from total Ag uptake from the natural environment [33]. It was also observed that citrate-capped particles were taken up by fish more readily than polyvinylpyrrolidone-capped particles [48], the type used in the present study. It could be hypothesized that muscle tissue would have lower concentrations than gill or liver tissue because muscle is neither directly exposed to the water nor used for blood detoxification but instead represents a longer-term signature of exposure to nAg versus other tissues (e.g., liver) that have higher turnover rates [49]. Note that liver and gill tissues were not analyzed for total Ag in the present study because of the small size of fish.

Total Ag concentrations in the experiment were much lower than nominal at all times, both within carboys containing stock solution and in exposure tanks, and consisted of mostly particulate Ag with very low levels of ionic Ag. Static exposure tanks that were tested showed lower than nominal concentrations as well, indicating that it was not the flow-through system of water replacement in the exposure tanks that generated these low concentrations but rather an issue with the stability of the stock solution used. Other studies have found flow-through methods to be an appropriate method of nAg exposure [50]. In most nAg exposure experiments reported in the literature, actual concentrations of nAg were much lower than nominal, averaging 52% of nominal, presumably because of nAg agglomeration, sedimentation, or adsorption to tanks walls and other equipment, and consisted mainly of particulate Ag [11–13,45,47] (Supplemental Data, Table S3). Also consistent with the present experiment were studies showing that nAg came out of solution quickly and that higher concentrations of nAg were difficult to maintain in solution, particularly in studies using dechlorinated tap water, as in the present study. One study conducted using lake water reported much better nAg stability (80–100% stability over 24 h), potentially from the stabilizing effect of naturally occurring DOC [51]. Based on these reports and present study results, it is highly likely that other studies that report biological effects at nominal concentrations but fail to report actual concentrations [14,16,52] are likely significant overestimates of actual exposures. Importantly, this implies that studies reporting toxicological effects at nominal concentrations should be interpreted to have generated biological effects at concentrations nearly half of those reported. It is recommend that toxicological studies of nAg report measured total Ag (including percentages of particulate and ionic Ag) when assessing toxicity.

In conclusion, the present study found nAg to be taken up by fish and to activate a stress response in fish by increasing blood cortisol concentrations. However, these effects were not strong enough to translate into significant responses to growth or K, and effects on HSI were inconclusive. There was an average increase in cortisol at the environmentally relevant concentration of 0.28 μg/L nominal in comparison to controls, which indicates that nAg has the potential to cause stress to fish in the environment at concentrations that are currently reported for some aquatic habitats [2,53].

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3691.

Acknowledgment—Funding for this research was provided by the Natural Sciences and Engineering Research Council of Canada, the Experimental Lakes Area, the University of Manitoba Faculty of Science, and Fish Futures Inc. Thanks to K. Wautier, C. Rodgers, A. Chapelsky, and C. Kyle-Ottenson at Fisheries and Oceans Canada and E. Sotiri at the University of Manitoba for their technical assistance.

Data Availability—Data, associated metadata, and calculation tools are available from the corresponding author (lamurray12@gmail.com).

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