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Acute embryonic exposure to nanosilver or silver ion does not disrupt the stress response in zebrafish (*Danio rerio*) larvae and adults

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HIGHLIGHTS

- AgNP/Ag^+-exposed zebrafish larvae increased cortisol when stressed.
- Embryonic exposure to AgNP/Ag^+ did not affect adults' ability to elevate cortisol.
- AgNPs/Ag^+ do not impede the development of the zebrafish stress response.
- Ag^+ exposure may impact CRF-related processes other than the stress response.

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ABSTRACT

The antibacterial properties of silver nanoparticles (AgNPs) are widely exploited in a variety of medical and consumer products. AgNPs in these products can be released into the aquatic environment, however, the potential toxicity of AgNPs to organisms, including fish, is yet to be fully understood. The present study aimed to investigate the effects of the early life exposure to AgNPs on the hypothalamic–pituitary–interrenal (HPI) axis-mediated stress response in zebrafish (*Danio rerio*) larvae and adults. Zebrafish embryos were treated with AgNPs (0.5 μg/mL) or Ag^+ (0.05 μg/mL) starting at 2 h post fertilization (hpf). At 96 hpf the larvae were either subjected to a swirling stress and euthanized, or raised to adulthood (10 months) in silver-free water and then net-stressed, euthanized, and sampled. Whole-body basal or stress-induced cortisol levels in larvae were not affected by either AgNPs or Ag^+; however, the transcript levels of corticotropin releasing factor (CRF), CRF-binding protein (CRF-BP), CRF-receptor 2 (CRF-R2), and pro-opiomelanocortin (POMCb) were significantly decreased by Ag^+. The ability of the adult fish to release cortisol in response to a stressor was also not affected, although the transcript levels of CRF, CRF-BP, and CRF-R1 in the telencephalon were differentially affected in fish exposed to Ag^+ as embryos. This is the first study that investigated the potential endocrine-disrupting effects of AgNPs during the early life stages and although AgNPs or Ag^+ did not affect the ability of zebrafish to elevate cortisol levels in response to a stressor, the effects on transcript levels by Ag^+ should be investigated further since CRF does not solely regulate the HPI axis but is also implicated in other physiological processes.

1. Introduction

The advent of nanotechnology is considered the largest engineering innovation since the Industrial Revolution (Roco, 2005). Engineered nanomaterials (ENMs) are particles, tubes, rods, or fibers of less than 100 nm in at least one dimension (Niemeyer, 2001). ENMs are applicable in many fields, including healthcare, electronics, cosmetics, and clothing due to their unique physicochemical properties (Niemeyer, 2001). However, the same properties that make ENMs beneficial may also contribute to their toxicity, especially in relation to their ability to generate reactive oxygen species (ROS) and induce oxidative stress (Oberdörster et al., 2005; Lynch et al., 2007; Aillon et al., 2009; Prencipe et al., 2009).

The most common ENM in consumer products is silver nanoparticles (AgNPs) (Nanotechproject, 2013). The antimicrobial properties of silver (Ag) have been exploited since the 1800s (Chernousova and Epple, 2013), and in the mid-20th century Ag was introduced to treat burn wounds (Fong and Wood, 2006). Today Ag is commonly used in wound dressings and coatings of medical products. In recent years nanotechnology has improved the use of Ag for antimicrobial purposes through the synthesis and the subsequent incorporation of the AgNPs into various household and consumer products, including clothing, children toys, air and water purifiers (Nanotechproject, 2013).

ENMs have the potential to improve the quality of life, but they also raise health and safety concerns, especially in relation to the aquatic environment (Moore, 2006). Their application in clothing and personal care and household products may lead to their introduction into sewage

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treatment plants and ultimately into the aquatic environment. For example, studies demonstrated that various AgNP-textiles released 4.5–575 μg Ag/g textile under various washing conditions (Benn and Westerhoff, 2008; Geranio et al., 2009; Lorenz et al., 2012), and a recent study reported that physical activity resulting in sweating can release even more Ag than normal washing (von Goetz et al., 2013). Although the environmental concentrations of AgNPs are unknown, Blaser et al. (2008) predicted concentrations of 40–320 ng/L, whereas Gottschalk et al. (2009) estimated 0.088–2.63 ng/L AgNPs in surface water.

A number of studies investigated the toxicity of AgNPs (concentrations ranged from 0.004 ng/mL to 0.1 mg/mL) in zebrafish (*Danio rerio*). These studies demonstrated that exposure to AgNPs altered gill filament morphology and global gene expression in adult zebrafish (ZF) (Griffitt et al., 2009) and increased physical deformities in ZF embryos, including bent and twisted notochord, pericardial edema, reduced heart rate, and degeneration of body parts (Lee et al., 2007; Asharani et al., 2008; Bar-Ilan et al., 2009). Moreover, AgNPs delayed ZF embryo hatching (Yeo and Kang, 2008; George et al., 2011; Asharani et al., 2011; Powers et al., 2011) and led to oxidative stress (Massarsky et al., 2013).

Although a variety of effects are reported, the potential of AgNPs to act as endocrine disruptors has not been sufficiently addressed. To the best of our knowledge this is the first study that attempted to examine the potential of AgNPs to disrupt ZF endocrine function, specifically the ability to elevate cortisol levels in response to stress. Cortisol is the principal corticosteroid in teleost fish secreted in response to stress and plays a key role in the regulation of the endocrine stress response (Mommsen et al., 1999), and pioneering studies by Hontela and colleagues demonstrated that environmental contaminants including metals could disrupt the ability of fish to elevate cortisol levels when stressed (e.g. Hontela et al., 1995).

Fish display a typical vertebrate stress response, including an immediate response mediated by the sympathetic nervous system and specifically epinephrine/norepinephrine ('fight-or-flight' response), and a delayed hypothalamic–pituitary–interrenal (HPI) axis-mediated response. The HPI axis involves the corticotropin releasing factor (CRF), which is produced in the preoptic area of the teleost brain located within the telencephalic stalk region (Folgueira et al., 2004) in response to hypothalamic stimulation (Alderman and Vijayan, 2012). In turn, CRF stimulates the release of the adrenocorticotropic hormone (ACTH) from the pituitary by specific G-protein coupled receptors (CRF-R1 and CRF-R2) and its further regulation by a shared binding protein (CRF-BP). Consequently, ACTH, which is synthesized from pre-pro-opiomelanocortin (pre-POMC), stimulates cortisol synthesis and release by interrenal cells of the fish head kidney (To et al., 2007). The HPI axis in ZF is fully developed by 4 days post fertilization (dpf) (Alsop and Vijayan, 2009); potentially exposure to a toxicant during these early stages may impede its proper development. Therefore, this study aimed to investigate whether the early life exposure to AgNPs or Ag^+ can disrupt the formation of the HPI axis thus affecting the ability of ZF larvae and/or adults to respond to a standardized stressor. To this end we examined whole-body (larvae) and plasma (adults) cortisol levels, as well as the abundance of CRF-related transcripts in larvae and in the adult brain. Cholesterol (cortisol precursor) and triglycerides (potential energy source) were also assessed in ZF larvae.

2. Materials and methods

2.1. Silver nanoparticles (AgNPs) and soluble silver (Ag^+)

The AgNPs used in this study were carboxy-functionalized, stabilized by sodium polyacrylate (31% total Ag) (Vive Nano, 13010 L), and dispersed in water. These AgNPs were prepared in egg water (see below) and were characterized previously (Massarsky et al., 2013) using dynamic light scattering (8.39 ± 0.98 nm; average polydispersity index of 0.156 ± 0.025) and transmission electron microscopy (~10–20 nm). The amount of dissolved silver in the stock solution was estimated at 0.5% (Massarsky et al., 2013). $AgNO_3$ (63.5% total Ag; source of Ag^+) was purchased from Sigma-Aldrich (204390) and a 100 μg/mL stock solution was prepared in MilliQ water. All Ag concentrations presented here are total Ag concentrations based upon the % Ag content of both AgNPs and $AgNO_3$.

2.2. Zebrafish embryo collection

Adult wild type ZF obtained from a local supplier (Big Al's Aquarium, Ottawa, ON) were maintained in holding tanks at 28 °C on a 14:10 h light-dark cycle in a flow-through system (Aquatic Habitats, Apopka, FL) using aerated, dechloraminated City of Ottawa tap water. Embryo collection traps were set between 4 and 6 PM the evening before, and embryos were collected the following morning within 1 h of spawning between 9 and 10 AM. Collected embryos from the spawning of several wild type parents were mixed in a Petri dish containing egg water [containing an anti-fungal (3 mL/L 0.01% methylene blue) and salts (16 mL/L 60× E3 embryo medium—in g/L: 17.2 NaCl, 0.76 KCl, 4.9 $MgSO_4 \cdot H_2O$, 2.9 $CaCl_2$)] and incubated at 28 °C until separated into experimental groups. All procedures used were approved by the University of Ottawa Animal Care Protocol Review Committee and conform to the guidelines of the Canadian Council for Animal Care for the use of animals in research and teaching (www.ccac.ca/en_/standards/guidelines).

2.3. Experimental set-up

At 2 h post fertilization (hpf), 20 embryos were randomly assigned to 5.3 cm plastic Petri dishes, containing a total volume of 14 mL egg water supplemented with 1 mL of AgNPs, $AgNO_3$ (Ag^+), or MilliQ water (for control embryos). The nominal exposure concentrations were 0.5 and 0.05 μg/mL total Ag for AgNPs and Ag^+, respectively. These concentrations were used here as they led to similar mortality rates in our previous study (Massarsky et al., 2013). The Ag^+ chelator cysteine that also binds to and induces aggregation of AgNPs (Hajizadeh et al., 2012) was used in rescue experiments. Cysteine (Cys; 5 μg/mL final concentration) was prepared in water and added to the egg water prior to the addition of each Ag treatment. Static exposures were conducted in an incubator at 28 °C until 4 dpf, similarly to our previous study (Massarsky et al., 2013). The embryos were not fed as the yolk sac provides sufficient nutrients until depleted at approximately 6 dpf (Holmberg et al., 2004). Throughout the exposure period heart rate (at 48 hpf), hatching success (at 56 hpf), and survival (every 24 h) were evaluated. At 4 dpf the surviving larvae from control and treatment groups were either 1) stressed using swirling as per Alsop and Vijayan (2008), left for 5 min, then euthanized on ice, rinsed with ice-cold distilled water, frozen on dry ice, and stored at −80 °C until further analysis, or 2) raised to adulthood (10 months) in clean water without Ag to assess the adult stress response (the experimental set-up is summarized in Fig. 1). These fish were housed at a maximum density of 5 fish/L in 3 L tanks and fed approximately 1% body weight twice a day; the food consisted of 2:1:1 mixture of Adult Zebrafish Complete Diet (Zeigler®), Spirulina Aquarium Flake Food (Ocean Star International), and Golden Pearls 300–500 μm (Artemia International LLC). All experiments were performed on embryo batches generated from at least four ($n \geq 4$) separate 'mating events' with each treatment having two replicate Petri dishes. Embryos from four batches ($n = 4$) were raised to adulthood.

2.4. Adult zebrafish tissue collection

Ten months old adult ZF were stressed using the standard netting stress of Ramsay et al. (2009) and euthanized in ice-cold water. The fish were weighed and blood (5–15 μL) from the amputated-tail region was carefully collected into a 0.2 mL PCR tube using a heparinized

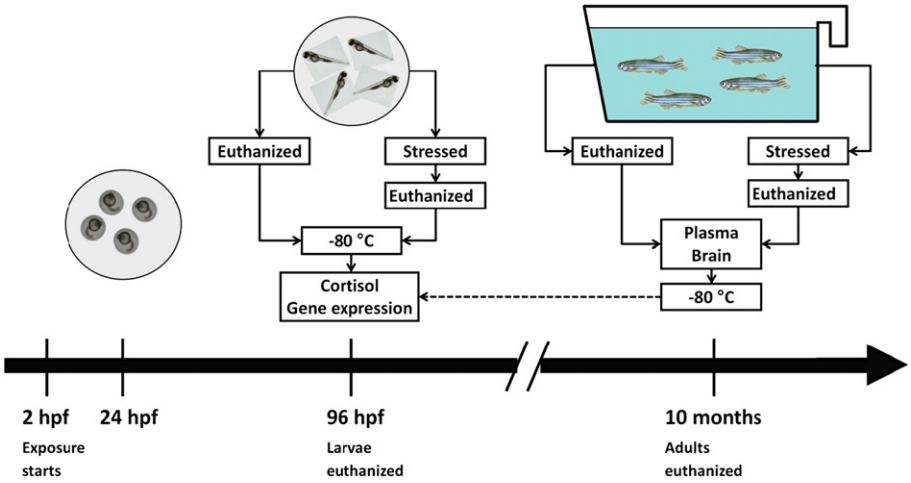

Fig. 1. Zebrafish stress response experimental set-up. Embryos were exposed to AgNPs or Ag^{+} until 4 dpf. The larvae were then 1) euthanized, or 2) stressed and euthanized, or 3) raised to adulthood in Ag-free water and then 1) euthanized, or 2) stressed and euthanized. Whole larvae and adult plasma and brain samples were used for cortisol and gene expression analyses.

capillary tube. The blood was centrifuged at 7000 *g* for 2 min and the resulting plasma was collected into a fresh 0.2 mL PCR tube (plasma from two fish of same sex was pooled to ensure sufficient volume for cortisol analysis). The cranial cap was removed and the telencephalon (Fig. 2) was carefully excised, placed into a 1.5 mL tube, and frozen on dry ice; at least two telencephalons were combined to ensure sufficient RNA. Both CRF and CRF-BP genes are primarily expressed in the ZF telencephalon (Alderman and Bernier, 2007), and to minimize the sampling time only the telencephalon was collected. All samples were frozen as quickly as possible and stored at −80 °C until analyzed. Fish were euthanized between 10 am and 1 pm.

2.5. Lipid extraction

Total lipid extraction was performed using a modification of the Folch method (Folch et al., 1957). Briefly, frozen ZF larvae were transferred to 15 mL Falcon tubes to which 7.5 mL 2:1 chloroform–methanol (v/v) was added. The contents were homogenized for 10 s with a tissue homogenizer (Polytron, Kinematica GmbH Kriens, Luzern Brinkmann Instruments). The samples were incubated for 15 min at room temperature to ensure an optimal extraction and extracts were washed with 2 M KCl containing 5 mM EDTA (1 part KCl to 5 parts extract). The tubes were shaken for 10 s and incubated at room temperature for 30 min to ensure layer separation. The bottom organic layer was collected into glass tubes and evaporated to dryness under a stream of nitrogen gas. Following drying, 0.2 mL 2-methoxy-ethanol was added to each tube to dissolve the lipid fraction. The tubes were vortexed for 10 s and contents were transferred to fresh 1.5 mL conical centrifuge tubes.

2.6. Cortisol, cholesterol, and triglycerides assays

Cortisol content in larval lipid extracts and adult plasma was assessed using a radioimmunoassay (RIA) I^{125} kit as per the manufacturer's

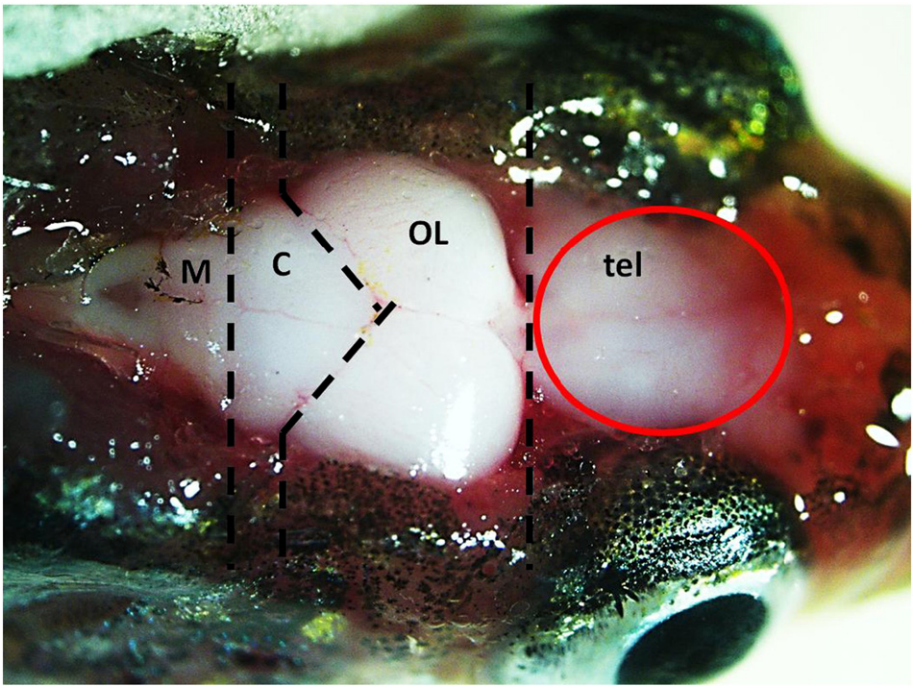

Fig. 2. Photomicrograph of the adult zebrafish brain regions: telencephalon (tel), optic lobe (OL), cerebellum (C), and medulla (M). The tel was excised (red oval) and used in these experiments.

protocol (MP Biomedicals, Orangeburg, NY). Cholesterol and triglycerides contents were assessed in larval lipid extracts using the cholesterol and triglycerides liquid reagent kits (TECO Diagnostic, Anaheim, CA) following the manufacturer's protocols.

2.7. Total RNA extraction and cDNA synthesis

Total RNA from frozen ZF larvae was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA from the ZF telencephalon was isolated using a commercially available RNeasy mini-prep kit (Qiagen) following the manufacturer's protocol. Total RNA concentration and purity were determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). The RNA quality was further confirmed using gel electrophoresis. Samples were stored at −80 °C until cDNA synthesis using a QuantiTect Reverse Transcription kit (Qiagen) following the manufacturer's protocol.

2.8. Quantitative RT-PCR analysis

2.8.1. Zebrafish larvae

Primers for CRF, CRF-BP, CRF-R2, POMCb, and β-actin (see Alderman and Bernier, 2009) were acquired from Invitrogen (Table 1). Amplicon sizes were confirmed using PCR and gel electrophoresis. Quantitative RT-PCR was performed using a CFX96 Real-Time PCR detection system (BIORAD) with SYBR green fluorescent master mix (Agilent Technologies, Santa Clara, CA). Final sample volume was 15 μL and samples were run in duplicate. The PCR cycling conditions were as follows: initial denaturation at 95 °C for 3 min, 40 cycles at 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s. A melt curve analysis was used at the end of each run to validate the amplification of only one product. Standard curves were constructed for each target gene using serial dilutions of a reference pool of representative cDNA from all experiments. Both RNase/DNase-free H_2O and non-reverse transcribed RNA were assayed to ensure no contamination was present in the reagents or in the primers used. To account for cDNA production and loading differences, all samples were normalized to the abundance of the housekeeping gene β-actin, which did not change significantly over the experimental treatments. The abundance of each transcript was further normalized to the control group.

Table 1
Primer sequences and amplicon sizes for the genes of interest used for larval and adult zebrafish gene expression analysis.

Gene	Accession no.	Sequence 5′-3′	Size (bp)
CRF	BC085458	F[a]: CGAGACATCCCAGTATCCAA	465
		R[a]: GATGACAGTGTTGCGCTTCT	
		F[b]: GCCGATTTCCCTAGATCTGAC	147
		R[b]: TCTTTGGCTGATGGGTTCG	
CRF-BP	NM001003459	F[a]: GCTGTGCTTCCTCCTGTTG	483
		R[a]: CCTGATTGGTGGAGCCTGA	
		F[b]: CTAAAGCGAGAGTTACCAGAGG	150
		R[b]: GATAACGTCAGTAGGTTCGCC	
CRF-R1	XM691254.2	F[b]: CTGGGCTAAGAAAGGGAACTAC	152
		R[b]: TGAAGAGGATGAATGCGACC	
CRF-R2	XM681362	F1: GAATCGCTTACAGAGAGTGT	465
		R1: ACCATCCAATGAAGAGGAAG	
POMCb	NM001083051	F1: GTTCTGTCCGTCTTGGCTTT	639
		R1: GTGAACTGCTGTCCATTGCC	
β-actin	AF057040	F[a]: GGTATTGTGATGGACTCTGG	583
		R[a]: AGCCACCGATCCAGACGGA	
		F[b]: TGAATCCCAAAGCCAACAGAG	139
		R[b]: CCAGAGTCCATCACAATACCAG	

[a] Sequences used for zebrafish larvae QPCR analysis.
[b] Sequences used for zebrafish adult QPCR analysis.

2.8.2. Zebrafish adults

The mRNA abundance in adult zebrafish telencephalon was quantified using a Rotor-Gene Q real-time PCR machine (Qiagen). A new set of primers was designed using Integrated DNA Technology software (www.idtdna.com; Table 1) to generate smaller amplicon sizes to account for the shorter cycling durations. Each reaction contained 5 μL Rotor-Gene SYBR green PCR master mix (Qiagen), 1 μL of each the forward and reverse gene specific primers for a final concentration of 1 μM, 2 μL RNase/DNase-free purified water (Roche), and 1 μL diluted cDNA. Cycling conditions were: 3 min initial denaturation at 95 °C, 40 cycles of 95 °C for 10 s, and 60 °C for 15 s. Relative quantification of target transcript abundance was determined using the Rotor-Gene Q software package (Qiagen). Standard curve analysis and contamination controls were performed as above. The transcript abundance was normalized to β-actin as above. The abundance of each transcript was further normalized to the control group.

2.9. Statistical analysis

Statistical analyses were conducted using SigmaPlot (SPW 11; Systat Software, Inc., San Jose, CA). One-way analysis of variance (ANOVA) with a post hoc Holm–Sidak method was used to assess the effect of treatment (i.e. control, AgNPs, or Ag^+) on gene expression in larval and adult ZF. Two-way ANOVA with a post hoc Holm–Sidak method was used to assess the effects of treatment and Cys on mortality, hatching, cortisol, cholesterol, and triglycerides levels in ZF larvae. Three-way ANOVA with a post hoc Holm–Sidak method was used to assess the effects of treatment, sex, and stress on plasma cortisol levels in adult ZF. Significant differences from one- and two-way ANOVA tests are indicated as letters and asterisks on the corresponding graphs, whereas the results from the three-way ANOVA are summarized in Table 2. Linear regression analysis was used to assess the relationship between fish mass and tank density. In all cases P-value < 0.050 was considered significant.

3. Results

3.1. Embryo and adult zebrafish parameters

Both AgNPs (0.5 μg/mL total Ag) and Ag^+ (0.05 μg/mL total Ag) significantly increased embryo mortality. The average mortalities at 96 hpf for AgNPs and Ag^+ were 40% and 30%, respectively (Fig. 3A); most mortalities occurred by 24 hpf. Hatching at 56 hpf was also significantly delayed by both Ag-types, with only 40–50% of the embryos hatching by 56 hpf (Fig. 3B). Cysteine (Cys) significantly reduced mortality and increased hatching success in Ag-treated embryos. There was no effect of treatment on heart rate at 48 hpf between the treated and control embryos, and with or without Cys; ZF larvae had heart rates of ~110 beats/min (data not shown).

Table 2
Three-way ANOVA with post hoc Holm–Sidak method was used to assess the statistical differences in plasma cortisol levels in adult zebrafish (see Fig. 6). The three factors are treatment (control, AgNPs, or Ag^+), sex, and stress. $P < 0.050$ is considered significant. 'NS' indicates 'not significant'.

Factor/interaction	P-value
Treatment (A)	NS
Sex (B)	0.047
Stress (C)	<0.001
AxB	NS
AxC	NS
BxC	0.037
AxBxC	NS

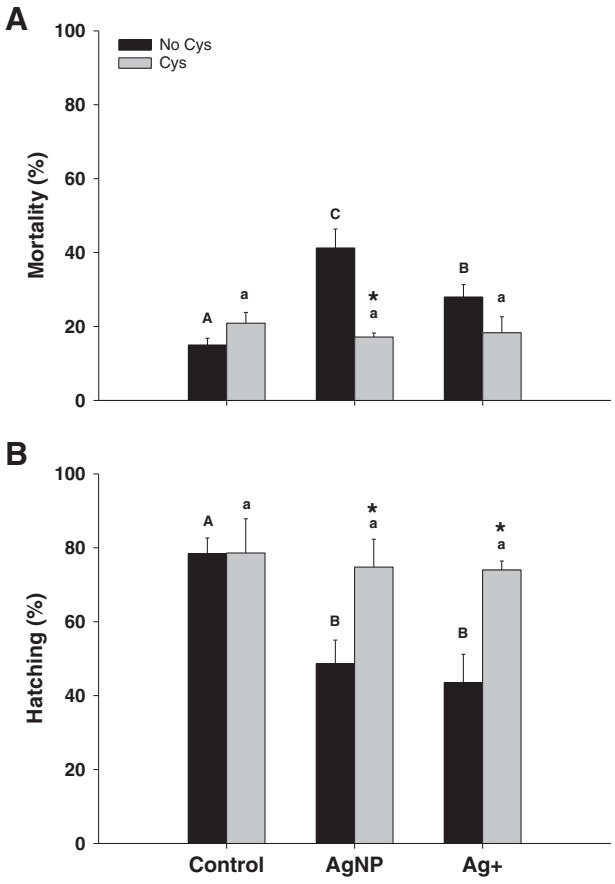

Fig. 3. Zebrafish embryo mortality at 96 hpf (A) and hatching at 56 hpf (B) in control, AgNP (0.5 μg/mL total Ag), and Ag^+ (0.05 μg/mL total Ag) treated embryos. Values are means + SEM (n = 5–17 and 4–16 for mortality and hatching, respectively). Capital and small letters indicate differences between the treatment groups in the absence and presence of cysteine (Cys), respectively. The asterisks indicate differences between Cys-treated and untreated embryos within the same treatment. Two-way ANOVA with post hoc Holm–Sidak method was used to assess statistical differences ($P < 0.050$).

Interestingly, adult ZF mass at 10 months was significantly increased in fish exposed to AgNPs as embryos (Fig. 4); however, this may reflect the number of fish in a tank as there was a significant relationship between fish mass and the average number of fish per tank (Fig. 4, inset), with the least populated tanks (AgNPs-exposed fish) containing the larger fish. Housing the same number of fish per tank was not possible due to space limitations.

3.2. Cortisol, cholesterol, and triglycerides

The cortisol levels in ZF larvae were not affected by AgNPs or Ag^+. Stressed larvae (4 dpf) had significantly higher cortisol levels by 2–3 times than the unstressed larvae (Fig. 5). Similarly adult ZF exposed to Ag as embryos/larvae and raised in Ag-free water for 10 months, responded to a netting stress by significantly increasing plasma cortisol levels (Fig. 6). In fact stressed fish had 20–40 times higher levels than their unstressed counterparts regardless of treatment (Table 2). Moreover, the unstressed female fish had significantly higher cortisol levels than the males, but the males had higher cortisol levels than the females when stressed (Fig. 6); there were significant effects of sex and a significant interaction between sex and stress (Table 2). There was no effect of Cys on cortisol levels in any treatment (data not shown). The cholesterol and triglycerides levels were not significantly affected by Ag-type or Cys in ZF larvae (data not shown); adults were not assessed.

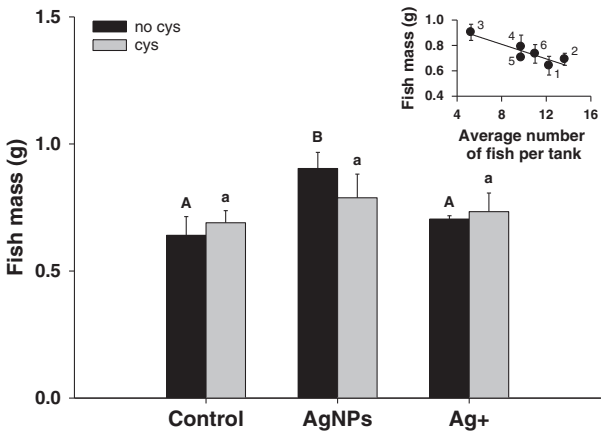

Fig. 4. Adult zebrafish mass at the end of the experiment (10 months) after treating embryos until 4 dpf in presence or absence of AgNPs or Ag^+ (see Fig. 3 for details). Values are means + SEM (n = 4). Capital and small letters indicate differences between treatment groups in the absence and presence of cysteine (Cys), respectively. Two-way ANOVA with post hoc Holm–Sidak method was used to assess statistical differences ($P < 0.050$). Inset: linear regression analysis of the fish mass (means ± SEM, n = 4) as a function of the average number of fish per tank: 1. control; 2. control (+Cys); 3. AgNPs; 4. AgNPs (+Cys); 5. Ag^+; 6. Ag^+ (+Cys). The r^2 and P values are 0.81 and 0.015, respectively; the equation of the line is $y = -0.0287x + 1.0382$.

3.3. Transcript abundance

As Cys did not impact cortisol levels, transcript abundance was assessed only in non-Cys treated larvae/adults. Moreover, we did not predict that stress would induce differences in transcript abundance given the short timeframe of the stressor (30 s for larvae and 6 min for adults), so only the non-stressed larvae/adults were assessed. All four transcripts (CRF, CRF-BP, CRF-R2, and POMCb) assessed were significantly down-regulated by Ag^+ in ZF larvae (Fig. 7). CRF-R1 transcripts were not detected in ZF larvae. Abundance of these transcripts in the adult ZF telencephalon was differentially affected by Ag^+ and sex of the fish. The abundance of CRF-BP and CRF-R1 transcripts in males was down- and up-regulated, respectively (Fig. 8A), whereas the abundance of CRF and CRF-R1 in females was down-regulated (Fig. 8B). Transcripts for CRF-R2 and POMCb were not detected in the telencephalon region. Abundance of CRF and CRF-related transcripts was not significantly affected by AgNPs; however, there were trends for decreased abundance of CRF-BP and increased abundance of CRF-

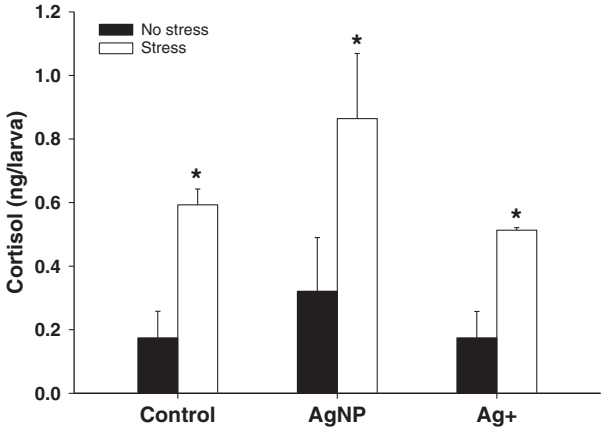

Fig. 5. Whole-body cortisol levels in stressed and unstressed zebrafish larvae (4 dpf) following exposure to AgNPs or Ag^+ (see Fig. 3 for details). Values are means + SEM (n = 4–9). There were no differences between treatment groups in unstressed or stressed larvae, but there was a significant effect of stress within each treatment indicated by the asterisk. Two-way ANOVA with post hoc Holm–Sidak method was used to assess statistical differences ($P < 0.050$).

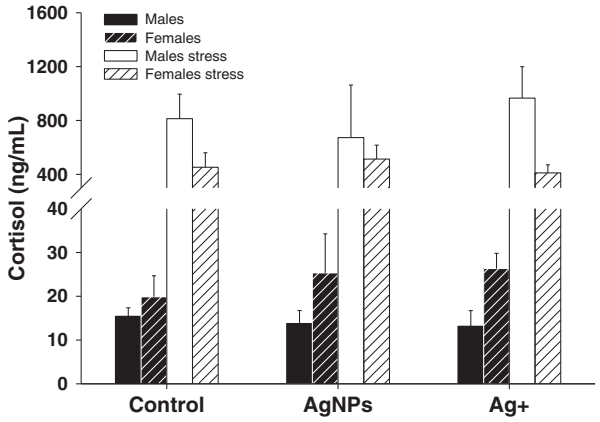

Fig. 6. Plasma cortisol levels in stressed and unstressed male and female adult zebrafish (10 months) that were treated as embryos with AgNPs or Ag^+ until 4 dpf (see Fig. 3 for details). Values are means + SEM (n = 4). There were no significant differences between treatment groups, but there was an overall significant effect of sex and stress. Three-way ANOVA with post hoc Holm–Sidak method was used to assess statistical differences ($P < 0.050$) (see text and Table 2 for details).

R1 in males, while in females the abundance of CRF and CRF-R1 was decreased.

4. Discussion

We demonstrate for the first time that an acute exposure to AgNPs or Ag^+ during the early life stages did not impede the ability of larval and adult zebrafish (ZF) to elevate cortisol levels in response to a stressor, at least under the conditions described in this study. The nominal concentrations used here were 0.5 and 0.05 μg/mL total Ag for AgNPs and Ag^+, respectively. These concentrations were chosen since the relatively low mortality ensured sufficient larval survival, and the viability and hatching values at these concentrations were similar to our previous study (Massarsky et al., 2013). It is important to note that the AgNP concentration used in this study is similar to previous studies, although higher than the environmental concentrations of 0.088–2.63 and 40–320 ng/L in surface water predicted by Gottschalk et al. (2009) and Blaser et al. (2008), respectively. As reported previously (Massarsky et al., 2013), environmentally relevant concentrations of AgNPs did not affect ZF embryos, at least with respect to mortality, hatching, or oxidative stress.

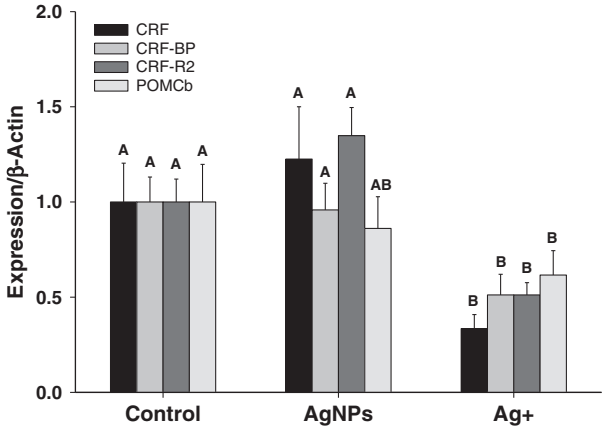

Fig. 7. Transcript abundance within the HPI axis in 4 dpf zebrafish larvae treated with AgNPs or Ag^+ (see Fig. 3 for details): CRF, CRF-BP, CRF-R2, and POMCb. Transcript abundance was normalized to the control group. Values are means + SEM (n = 7). The letters indicate differences in gene expression between treatments for a specific gene. One way-ANOVA with post hoc Holm–Sidak method was used to assess statistical differences ($P < 0.050$).

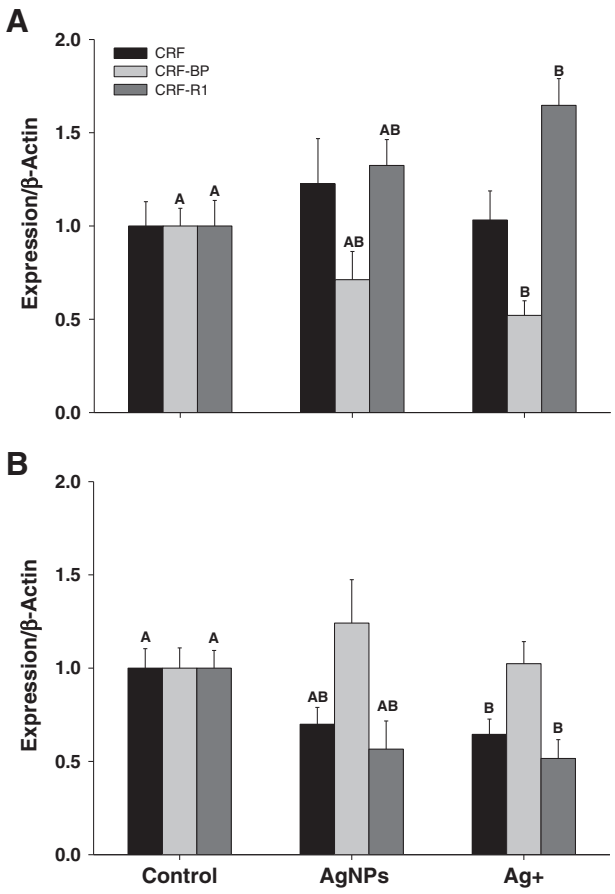

Fig. 8. Transcript abundance within the HPI axis in 10 month old male (A) and female (B) zebrafish telencephalon following treatment with AgNP or Ag^+ until 4 dpf (see Fig. 3 for details): CRF, CRF-BP, and CRF-R1. Transcript abundance was normalized to the control group. Values are means + SEM (n = 4). The letters indicate differences in gene expression between treatments for a specific gene. One way-ANOVA with post hoc Holm–Sidak method was used to assess statistical differences ($P < 0.050$).

The ability of environmental pollutants, especially metals, to impact the HPI axis and/or cortisol levels in fish was addressed in several studies with variable results. Cadmium disrupted the biosynthesis of cortisol through the suppression of corticosteroidogenic gene transcripts in rainbow trout (*Oncorhynchus mykiss*) without affecting cortisol production (Sandhu and Vijayan, 2011). Basal cortisol levels were not affected in the sabalo (*Prochilus lineatus*) exposed to aluminum (Camargo et al., 2009) or in the round goby (*Neogobius melanostomus*) from contaminated areas (Marentette et al., 2013). In contrast, basal cortisol levels were increased in ZF exposed to copper (Craig et al., 2009) and selenomethionine (Thomas and Janz, 2011), Nile tilapia (*Oreochromis niloticus*) exposed to mercury (Cogun et al., 2011) and heavy metals (Firat and Kargin, 2011), and carp (*Cyprinus carpio*) exposed to a variety of pollutants (Firat and Alici, 2012). Lastly, chromium exposure in snakehead (*Channa punctatus*) decreased basal cortisol levels (Mishra and Mohanty, 2009). Overall, these studies indicate the importance of pollutant-type and fish species in eliciting a cortisol-mediated stress response.

In contrast, in this study we hypothesized that an acute exposure to AgNPs or Ag^+ during the early stages of ZF development would alter the proper formation and functioning of the HPI axis in ZF embryos and that these changes would persist in the adult fish. This hypothesis was not supported by the cortisol data, which clearly showed that both the larvae and the adult ZF were able to elevate cortisol levels in response to a stressor equal to that of the control fish. Two issues should be raised with respect to plasma cortisol levels in adult ZF. First, it is feasible to obtain enough plasma to assess cortisol levels when combining 2–3

fish and perhaps future studies should assess plasma cortisol instead of whole-body cortisol as generally done. Only a few studies report plasma cortisol levels for ZF. Filby et al. (2010) reported that subordinate (or 'naturally' stressed) ZF had higher cortisol levels than their dominant counterparts (50–100 vs 75–175 ng/mL); this difference was especially apparent in males. Félix et al. (2013) reported similar plasma cortisol levels in unstressed ZF such that males had slightly higher concentrations than females (57 vs 46 ng/mL). In contrast, the plasma cortisol levels for unstressed ZF adults reported here ranged from 15 to 25 ng/mL and were similar to the 20 ng/mL levels reported by Ziv et al. (2013) for unstressed male ZF. The cortisol levels for stressed fish reported here ranged from 400 to 800 ng/mL, which probably reflects the maximal cortisol levels elicited by the netting stress since the acute confinement method mentioned in Ziv et al. (2013) elevated cortisol levels by only 1.5 fold.

Second, female fish had higher cortisol levels when unstressed while males had higher levels when stressed. These differences could have been potentially missed if whole-body cortisol levels were assessed. For example, Fuzzen et al. (2011) found no sex differences in whole-body cortisol levels in unstressed or stressed (vortex stress) adult ZF. In contrast, Filby et al. (2010) reported higher plasma cortisol levels in subordinate (and supposedly stressed) males and to a lesser extent in females. Other studies reporting sex differences in plasma cortisol levels in ZF were not found. Sex differences in cortisol levels and their physiological importance should be investigated further (using more natural stressors) since it appears that the ability to cope with stress may be affected by sex.

Although Ag-treatments did not affect larval cortisol levels, whole-body transcript levels of CRF, CRF-R2, CRF-BP, and POMCb were significantly down-regulated in Ag^+-exposed larvae, suggesting that the HPI axis-mediated stress response could be impaired. This was unexpected since no changes in cortisol levels were observed in Ag^+-exposed larvae. Also, Ag^+ (0.05 μg/mL) was less toxic than AgNPs (0.5 μg/mL) based on the mortality data and one would have predicted that changes should be observed in the AgNPs-exposed larvae; given this did not happen, the toxicity mechanisms of AgNPs and Ag^+ may be quite different. Although CRF and CRF-related genes are most commonly known to be involved with the regulation of the HPI axis-mediate stress response, these genes also influence most if not all physiological functions in vertebrates, including nervous, cardiovascular, immune, muscular, and reproductive systems, as well as behavior and food intake (Yao and Denver, 2007; Alderman and Bernier, 2009). Additionally, the CRF system is thought to play an important role during early development of ZF embryos (Alderman and Bernier, 2009). Thus, it is possible that other systems, which were not considered here, could be affected in ZF larvae in response to Ag^+ exposure. This is especially important since the whole-body transcript levels were measured in the larvae, which may have masked even larger changes.

The transcript abundance for CRF, CRF-R1, and CRF-BP was also affected in the adult ZF telencephalon. Significant changes were only observed in fish that were exposed to Ag^+ as embryos. In males the abundance of CRF transcripts was unaffected, while that of CRF-BP and CRF-R1 was down- and up-regulated, respectively. In females the abundance of CRF-BP transcripts was unaffected, while that of CRF and CRF-R1 was down-regulated. Precisely what these changes indicate is not clear, but these changes did not affect the ability of fish to elevate cortisol levels in response to a stressor. Alternatively, CRF-related peptides such as urotensin I could also elevate cortisol levels during stress (Alderman and Bernier, 2009); therefore, if the function of HPI axis was impaired, the urotensin I system could potentially compensate and increase cortisol levels. This could explain why differences were observed for CRF-related transcript levels but not cortisol levels. Finally, transcript abundance differed between males and females which could lead to the observed sex differences in cortisol levels in unstressed and stressed fish.

Previous studies investigating the effects on the transcript levels of CRF and CRF-related genes in larval and adult ZF emphasize the complexity of CRF regulation and how it is impacted under various conditions. Embryos exposed to perfluorooctane sulfonate (Shi et al., 2009), polybrominated diphenyl ethers (PBDE; Yu et al., 2010; Chen et al., 2012), and microcystin (Yan et al., 2012) increased CRF transcript levels. These studies, however, focused on the hypothalamic–pituitary–thyroid (HPT) axis, which emphasizes the important role of CRF outside the HPI axis. In ZF adults, prochloraz exposure down-regulated the transcript levels of brain CRF, CRF-BP, and CRF-R2 and decreased plasma cortisol levels (Liu et al., 2011), while PBDE exposure down-regulated brain CRF transcript levels (Yu et al., 2011). Moreover, adult ZF subjected to a restraint stress decreased the transcript levels of brain CRF and CRF-related genes and increased whole-body cortisol levels (Ghisleni et al., 2012). In contrast, unpredictable chronic stress elevated brain CRF transcript levels (Piato et al., 2011; Chakravarty et al., 2013) and increased whole-body cortisol levels (Piato et al., 2011). Lastly, transport stress did not change the transcript levels of brain CRF, but increased whole-body cortisol levels (Dhanasiri et al., 2013). This further supports the complexity of the CRF and its involvement in physiological processes.

In conclusion, we demonstrate that even though the acute exposure to AgNPs and Ag^+ until 4 dpf influenced embryo viability and hatching, these Ag compounds did not impact the ability of ZF larvae or adults to elevate cortisol when stressed. Furthermore, we demonstrate that embryonic exposure to Ag^+ down-regulated CRF and CRF-related genes in larvae, and although these changes did not impair the cortisol-mediated stress response, other systems not considered in this study, could have been affected. The abundance of these transcripts was also differentially affected in the telencephalon of the male and female adults exposed to Ag^+ as embryos, suggesting potential sex-differences in response to Ag^+. Currently there are no studies on the impact of either AgNPs or Ag^+ on the fish HPI axis; our transcript results suggest that Ag^+ may influence the CRF signaling, which should be pursued in future studies.

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