Effect of nanosilver particles on metabolism and cortisol release in rainbow trout (*Oncorhynchus mykiss*)

by

Laura Murray

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Biological Sciences

University of Manitoba

Winnipeg

Abstract

Nanosilver (nAg) is a nanoparticle incorporated into products for its antimicrobial properties that causes diverse toxic effects. This study investigated the effects of nAg on metabolic rate and stress response in rainbow trout (Oncorhynchus mykiss Walbaum 1792). Fish were exposed to environmentally-relevant concentrations of nAg (average 0.28 µg l^{-1}) and higher (average 47.60 µg l^{-1}) for 28 days (d). Measurements of cortisol, growth, condition (*K*), and hepatosomatic index (HSI) were analyzed, as well as metabolic measurements. Cortisol concentrations were higher in all nAg exposures, and were significantly elevated in high exposures. No significant effects of nAg exposure were observed on metabolic variables, growth or condition. There was a significant effect of time and treatment on HSI, but no discernible trend. There was also a significant increase in cortisol in fish measured for metabolic variables. Overall nAg was seen to engage the stress response, but not affect metabolism or morphometric measures.

Acknowledgements

I would like to thank my advisor Mike Rennie, as well as everyone on my committee (Eva Enders, Jim Roth, and Paul Blanchfield) for helping me every step of the way with my project. There were a lot of twists and turns, but good guidance got me through. I'd also like to thank Kerri Pleskach and Greg Tomy for helping me with everything cortisol-related, and Kerry Wautier for helping me with everything fish husbandry related. Jon Svendsen guided me with the respirometry set-up, and Jon Martin guided me with the nanosilver exposures, made up nanosilver solutions, and processed samples. I also had many people assisting me with my sampling: AJ Chapelsky, Chandra Rodgers, Emianka Sotiri, and Carissa Kyle-Ottenson. I would also like to thank my Dad for accompanying me on a very long drive out to Ontario to pick up my first batch of unfortunately ill-fated fish.

Thanks also to the Natural Sciences and Engineering Research Council of Canada (NSERC), the Experimental Lakes Area (ELA), the University of Manitoba Faculty of Science, and Fish Futures for funding my project and making it all possible.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Nanosilver

Nanosilver (nAg) is a commonly used type of nanoparticle – small particles between the sizes of 1-100 nm (ASTM 2006). Particles of nanosilver are usually made of only 20-15000 silver atoms per particle, and they are incorporated into many consumer products for their antimicrobial properties such as clothing and other textile products, medical devices, electronics, paints, baby products, food storage containers, washing machines, contraceptive devices, and water purificants (Wijnhoven et al. 2009). Silver has also been used for its antimicrobial properties; however nAg due to its increased surface area is more reactive in the environment, and has been shown to have deviating physico-chemical properties and biological activities to silver (Pulit-Prociak et al. 2014).

Nanosilver is widely used; at least 1628 products are known to contain nanomaterials, and of those 383 contain nanosilver (Woodrow Wilson 2013), with its use increasing every year. It is often coated with a substance or capping agent (citrate, polyvinylpyrrolidone) in an attempt to stabilize it, but nAg does enter wastewater from the washing of these consumer products, as well as from other sources such as industrial activity, atmospheric emissions, accidental spills, and leaching from landfills (Pulit-Prociak et al. 2014).

Amounts of nAg currently in the environment are not well known, as nAg is difficult to test for and a standard method of testing does not yet exist. In the few studies that have measured levels in surface waters concentrations have been reported at approximately 1.5 µg l^{-1} (Liu et al. 2009), while modeled levels are

lower: anywhere from the low ng l^{-1} range (Gottschalk et al. 2009), to 0.32 µg l^{-1} (Blaser et al. 2008). Levels in wastewater influent have been measured from as low as 0.012 µg l^{-1} (Chao et al. 2011) to 2.49 µg l^{-1} (Liu et al. 2009), and estimated based on models at higher levels, from 2-18 µg l^{-1} (Blaser et al. 2008). Levels in wastewater effluent have been measured at 2.8 µg l^{-1} (Liu et al. 2009), while modeled levels were lower: from 21 - 42.4 ng l^{-1} (Gottschalk et al. 2009).

Nanosilver regulations

Regulating nAg is challenging, as it is a relatively new substance and there is an incomplete knowledge of its toxicity, with no standard, effective method to monitor its levels in the environment (Faunce and Watal 2015). Also it is open for debate if nAg should be regulated as a separate substance from silver. The precautionary principle would suggest that nAg should be regulated separately, until we know it does not differ significantly from silver in its toxicological effects (Fairbrother and Fairbrother 2009); however many regulations at present treat nAg as silver.

In Canada under the Canadian Environmental Protection Act (1999) there is mandatory reporting of any use of manufactured nanomaterials. This information is then used by Health Canada and Environment Canada to make risk assessments and to establish more specific regulations in the future (Sanderson 2009). Silver has a guideline level of 0.1 µg l^{-1} in freshwater under the guidelines for protection of aquatic life (Environment Canada 1999) and guideline levels for sewer discharge are 2 mg l^{-1} (Federation of Canadian Municipalities and NRC 2003).

Internationally, groups such as the OECD WPN (Organization for Economic Co-operation and Development Working Party of Nanotechnology) advise on policy issues regarding nanomaterial (Government of Canada 2014). In the European Union nanomaterial regulation is controlled by REACH (Registration, Evaluation, Authorization, and Restriction of Chemical program), which like Canada's regulations requires companies to report nanomaterial use and provide toxicology information (Faunce and Watal 2015). In the United States, nanomaterial regulation is controlled by the US Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) (Faunce and Watal 2015). Under the United States EPA, nAg may be considered a pesticide due to its antimicrobial effects, and there have been several products banned under these regulations. There has also been a lawsuit brought against the EPA recently by several non-governmental organizations (NGO's) for not regulating all nAg-containing products as pesticides (Seltenrich 2013).

nAg in the environment

The release of nAg from consumer products into the aquatic environment is mainly through domestic wastewater (as well as to a smaller extent leaching from landfills, industrial activity, accidental spills, and atmospheric emissions) (Pulit-Prociak et al. 2014). For example, nAg has been shown to be released by various consumer products when washed such as socks, towels, and teddy bears at levels up to 650ug in 500ml water (Benn and Westerhoff 2008; Benn et al. 2010). In addition, there are washing machines on the market that release nAg into the wastewater stream at levels on average of 11ug l^{-1} (Farkas et al. 2011a). It has been estimated

that the average North American consumer contributes approximately 470 ug l^{-1} per day of nAg into wastewater (Benn et al. 2010).

When nAg in wastewater is transported through sewers, it is likely complexed with ligands (especially sulfide), which decreases its toxicity (Choi et al. 2009). When it reaches a wastewater treatment plant, most is removed with the activated sludge and very little likely reaches surface waters (Kaegi et al. 2013). In the aquatic environment, nAg may dissolve to Ag^+ (which may complex with various ligands such as Cl^-, S_2^-, or S_2O_3), aggregate with other nAg particles, or aggregate with dissolved organic carbon (DOC) (Choi et al. 2009). The extent to which each of these actions occurs depends on factors of the water chemistry such as pH, ionic strength, temperature, types of ligands present, as well as characteristics of the nAg particles released such as presence/type of capping agent and size of particle (Stebounova et al. 2011).

The effects of water chemistry on nanoparticles is influenced by DLVO theory (Derjaguin and Landau, Verwey and Overbek theory), which describes how increased ionic strength of an aquatic medium may decrease the diffuse layer size around particles, and allow nanoparticles to come into closer contact to allow for more aggregation. This explains how in higher ionic strength water (such as saltwater), nAg is less stable and more likely to come out of solution (Stebounova et al. 2011). In contrast, DOC appears to bind to nAg and Ag^+ and stabilize it, maintaining it in the water column at higher concentrations (Cumberland and Lead 2009).

Nanosilver has been observed to move from the water column mostly to the sediment, as well as to be taken up into invertebrates and fish (Lowry et al. 2012). It also likely moves up the food chain (Pulit-Prociak et al. 2014). Once inside an organism the form nAg will take is controlled by the chemistry of body fluids such as pH and ionic strength (Stebounova et al. 2011).

nAg toxicity

In fish, silver ion (Ag^+) uptake occurs primarily at the gills, while nAg uptake may occur both at the gills and via digestion (Scown et al. 2010). Silver can either interact with the membrane of cells, or enter the cell. Interactions with the cell membrane may be from electrostatic attraction or binding with membrane proteins (especially thiol groups), which will destabilize the membrane. Silver may enter the cell through phagocytosis, passive diffusion, or protein-mediated transport. Toxicity of nAg seems to be a combination of the effects of the nAg and the Ag^+ it releases (Lapresta-Fernández et al. 2012).

Nanosilver's main mode of toxicity appears to be through oxidative stress, via destabilizing the electron transport chain in the cell's mitochondria. This causes the creation of an excess of reactive oxygen species (ROS), which may cause numerous types of damage in the cell such as lipid peroxidation, protein modification, and DNA damage. Antioxidant enzymes are produced by the cell in response to ROS, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), glutathione transferase (GST) or a high GSH/GSSG (total glutathione/oxidized glutathione) ratio, and can therefore be used as indicators of this oxidative stress (Lapresta-Fernández et al. 2012).

Silver ions' main mode of toxicity appears to be through the inhibition of the Na^+/K^+ ATPase pump in fish gill cells, eventually leading to osmoregulatory failure (Farkas et al. 2010). The inhibition of Na^+/K^+ ATPase dissipates the proton-motive force of the cell membrane and therefore interferes with energy-dependent reactions. One of the main effects of this inhibition is gill Na^+, Cl^- loss (in freshwater fish). This leads to plasma Na^+ and Cl^- loss, which in turn leads to increased blood viscosity, which can in extreme cases cause circulatory failure. This loss of plasma ions also activates the stress response through the sympathetic nervous system, which causes the release of both catecholamines and cortisol (Wood et al. 1996).

Fish LC50s (lethal concentration, 50%) for nAg range from the $\mu g\,l^{-1}$ to the $mg\,l^{-1}$ range, depending on fish species, life stage and particle size (Appendix A). Non-lethal effects include altered gene expression (such as induction of a heat shock protein and metallothionein) at $1\,\mu g\,l^{-1}$ (Pham et al. 2012), thickening of gill tissue at $10\,\mu g\,l^{-1}$ (Griffitt et al. 2012), and impaired osmoregulation via inhibition of $Na+/K+ATPase$ at $20\,\mu g\,l^{-1}$ (Farmen et al. 2012). Gill necrosis has been observed at a concentration of $100\,\mu g\,l^{-1}$ (Farmen et al. 2012) and impaired gas exchange at $300\,\mu g\,l^{-1}$ (Bilberg et al. 2010). High concentrations ($600\,\mu g\,l^{-1}$) have caused embryonic abnormalities such as absence of the air sac, pericardial and yolk sac edema, hemorrhages to head and pericardial area, lordosis, and craniofacial abnormalities (Laban et al. 2010). Overall, smaller nAg particles were seen to be more toxic than large (Scown et al. 2010), and citrate-capped particles more toxic than PVP-capped (polyvinylpyrrolidone) (Farkas et al. 2011b). By contrast, silver ion exposure is typically more toxic to fish than comparable concentrations of nAg.

Cortisol response

Cortisol, along with glucose, is one of the most common stress indicators in fish (Martinez-Porchas et al. 2009). Cortisol release can be caused by nAg either through oxidative stress or plasma ion loss effects (Wood et al. 1996), and has been seen to do so in several studies (Johari et al. 2013; Shaluei et al. 2013). Its release is controlled by the HPI axis (hypothalamus – pituitary – interrenal axis). Stress is sensed by the central nervous system, which signals the hypothalamus to release corticotropin-releasing hormone (CRH), which travels to the pituitary, which is in turn signaled to release adrenocorticotropin (ACTH). ACTH travels to the interrenal cells, which release corticosteroids, the primary one of which is cortisol. Cortisol then causes a cascade of changes to blood and tissue chemistry that allow the fish to better deal with the stress, such as increased plasma glucose levels to provide increased energy (Barton 2002).

Cortisol is released quickly after stress is detected, and often only released for the first 6-48 h of experiencing a stressor. After this acute response a fish's general adaptation response may sets in, where cortisol-mediated responses are no longer needed. There may also be an increase in plasma cortisol after a longer time period of being exposed to a stressor, once the fish has reached exhaustion due to energy depletion and can no longer adjust to the stressor. Aside from time other factors that may affect cortisol release are age, genotype/phenotype, position in dominance hierarchy, crowding stress, thermal acclimation, domestic vs. wild rearing of fish, and nutritional status (Martinez-Porchas et al. 2009).

Growth and Fulton's condition factor (*K*)

Growth and Fulton's condition factor (*K*) are both measures that may provide insight into the overall health of a fish. Nanosilver exposure may affect these measures through toxic effects affecting fish energetics. Growth is a change in the size of the fish, measured in unit of length, weight or energy. It is often considered the percentage of body weight gained over a certain time period. It may be affected by age of the fish, season, food type and availability, temperature, oxygen availability, place in dominance hierarchy, genotype, and physiological condition (Wootton 1990). Examples of contaminant effects on growth can be seen in Davies et al. (1978), where exposure to silver was seen to decrease growth in rainbow trout (*Oncorhynchus mykiss*) fry, and in Sherwood et al (2000), where growth was estimated to be decreased by 3 times from heavy metal (Cd, Cu, and Zn) exposure.

Condition (*K*) describes the relationship between length and weight under the assumption of isometric growth (Sutton et al. 2000). Fish with a high value of *K* are heavy for their length, while fish with a low value are light for their length. Effects of contaminants on *K* were seen in Pyle et al. (2008), where lower condition values were seen in metal-contaminated lakes.

Hepatosomatic index (HSI)

The liver is sensitive to contaminant exposure and may therefore be affected by nAg. These effects may be quantified by measuring the hepatosomatic index (HSI) of a fish; the ratio of liver weight to body weight of a fish. The liver plays a large role in metabolism and excretion of xenobiotic compounds; a toxicant may enter the liver through blood circulation, where it will be excreted through bile

secretion or transferred to the kidney for filtration and transformation (Sadekarpawar and Parikh 2013). The increase in needed detoxification function of the liver may cause increases in liver weight. Conversely, liver weight may decrease with the stress of contaminant exposure due to a loss of liver energy stores such as glycogen (Heath 1995). In addition to changes in HSI, changes to the liver from contaminants include histological changes such as increased vacuoles in the cytoplasm, enlarged lysosomes, changes to nuclear shapes, changes to the endoplasmic reticulum, and mitochondrial changes (Heath 1995). An example of a contaminant's effect on HSI was observed in Gagnon et al (2006), where copper exposure decreased HSI in rainbow trout.

Metabolism

General

The term metabolism encompasses all energy costs of a fish, such as costs arising from basic bodily functions (respiration), activity, and digestion/absorption/processing of food (specific dynamic action). It can be measured directly through oxygen consumption (Jobling 1994). Standard metabolic rate (SMR) defines the minimal energy requirement of ectothermic animals at a specific temperature, and is made up of all of the basic functions of cellular metabolism such as protein synthesis and ATP turnover that keep a fish alive (Sokolova and Lannig 2008). Maximum metabolic rate (MMR) defines the maximal aerobic metabolic rate attainable (Fry 1971). These two rates encompass the aerobic scope, which describes the amount a fish can increase its metabolic activity above maintenance levels. This amount represents the capacity of a fish to support

oxygen-consuming functions (activity, digestion, response to stressors), and therefore is an important constraint. For example, a decrease in aerobic scope may cause decreased activity, decreased fitness, and potentially even decreased survival in severely constrained individuals (Killen et al. 2007).

Metabolic measurements

Metabolism is quantified through respirometry methods, during which oxygen consumption is measured. SMR is measured in inactive fish that are post-absorptive (but not starving) and have no oxygen debt (Brett, 1964). Also routine metabolic rates can be measured that are similar to SMR, but fish have some level of normal or 'routine' activity (McKenzie et al. 2007). MMR measurements are obtained by maximally exercising a fish (through forced swimming in a swim tunnel or manually chasing) and measuring oxygen consumption (MO_2) during or directly afterwards (Clark et al. 2013). In addition, a spontaneous MMR may be obtained by measuring the highest MO_2 in volitionally performing fish (Svendsen et al. 2014). As a measure of hypoxia tolerance the critical O_2 saturation may be obtained by gradually decreasing the surrounding O_2 levels around the fish, and then noting when MO_2 is no longer independent of the ambient O_2 level (decreasing MO_2) (Svendsen et al. 2012).

Effects of contaminants on metabolism

Contaminants may affect MMR and/or SMR depending on their concentration, pathway of uptake or other natural factors such as temperature (Cannas et al. 2013). MMR may be affected by contaminants through damage to the

gills such as hyperplasia/hypertrophy of cells, degeneration of cilia, vacuolization,

necrosis as well as increased mucus production. All of these changes can decrease

gas exchange and therefore the amount of O_2 being transported to tissues, which in

turn can impact MMR (Sokolova and Lannig 2008). An example of this effect has

been reported for aluminum exposure, which has been observed to damage the gills

of rainbow trout and decrease their MMR (Wilson et al. 1994). SMR may not be

affected by this gill damage as MMR is, as fish may not require the entire gill surface

to be functional to maintain basal metabolic rates (Bilberg et al. 2010). However,

SMR may be affected by increased activity of cellular defense and detoxification

actions in response to contaminants, such as increased expression of antioxidant

enzymes (glutathione, metallothionein), increased expression of heat-shock protein

(HSP), up-regulation of other defense mechanisms as well as any increased

metabolic costs from repairing cellular damage done by the contaminant. An

example of contaminant effects has been reported in oysters and clams that had

increased SMR when exposed to cadmium (Sokolova and Lannig 2008). Due to the

importance of metabolism to fish ecology, measures of metabolic rate provide a

useful link between the physiological effects of contaminants and their effects on the

fish as a whole (Handy and Depledge 1999; McKenzie et al. 2007).

Study Objectives

Objectives of this study were to investigate potential effects of nAg chronic

exposure on rainbow trout, specifically cortisol release, metabolism, and

morphometric measures (growth, condition (K), and hepatosomatic index (HSI)).

Fish were exposed for 28 d to environmentally relevant concentrations of nAg and higher. Cortisol samples and morphometric information were collected at various time points, while metabolic measurements were taken at the end of the exposure. In addition, fish tissue silver concentrations were analyzed at various time points to observe their uptake of silver from the water, and the stability of nAg in the water was monitored to observe the actual amount of silver fish were being exposed to in comparison to nominal concentrations. Through these observations knowledge was gained of how nAg affects fish at various levels of stress response. By investigating these diverse effects (cortisol, metabolism, morphometric indices) a broader sense of how nAg may affect fish will be gained.

References

American Society for Testing and Materials. (2006). *Standard terminology - relating to nanotechnology.* E 2456-06. West Conshohocken. PA.

Barton, B. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology* **42**, 517–525.

Benn, T., Cavanagh, B., Hristovski, K., Posner, J. D. & Westerhoff, P. (2010). The release of nanosilver from consumer products used in the home. *Journal of Environmental Quality* **39**, 1875–1882. doi:10.2134/jeq2009.0363

Benn, T. M. & Westerhoff, P. (2008). Nanoparticle silver released into water from commercially available sock fabrics. *Environmental Science and Technology* **42**, 4133–4139. doi : 0.1021/es7032718

Bilberg, K., Malte, H., Wang, T. & Baatrup, E. (2010). Silver nanoparticles and silver nitrate cause respiratory stress in Eurasian perch (*Perca fluviatilis*). *Aquatic Toxicology* **96**, 159–65. doi:10.1016/j.aquatox.2009.10.019

Blaser, S., Scheringer, M., Macleod, M. & Hungerbühler, K. (2008). Estimation of cumulative aquatic exposure and risk due to silver: contribution of nano-functionalized plastics and textiles. *Science of the Total Environment* **390**, 396–409. doi :10.1016/j.scitotenv.2007.10.010

Brett, J.R. (1964). The respiratory metabolism and swimming performace of young Sockeye salmon. *Journal of the Fisheries Research Board of Canada* **21**, 1183-1226.

Cannas, M., Atzori, F., Rupsard, F., Bustamante, P., Loizeau, V. & Lefrançois, C. (2013). PCBs contamination does not alter aerobic metabolism and tolerance to hypoxia of juvenile sole (*Solea solea* L. 1758). Aquat. Toxicol. **127**, 54–60. doi:10.1016/j.aquatox.2012.04.017

Chao, J., Liu, J., Yu, S., Feng, Y., Tan, Z., Liu, R. & Yin, Y. (2011). Speciation analysis of silver nanoparticles and silver ions in antibacterial products and environmental waters via cloud point extraction-based separation. *Analytical Chemistry* **83**, 6875–82. doi:10.1021/ac201086a

Choi, O., Clevenger, T. E., Deng, B., Surampalli, R. Y., Ross, L. & Hu, Z. (2009). Role of sulfide and ligand strength in controlling nanosilver toxicity. *Water Research* **43**, 1879–1886. doi:10.1016/j.watres.2009.01.029

Clark, T. D., Sandblom, E., Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *The Journal of Experimental Biology* **216**, 2771–2782. doi:10.1242/jeb.084251

Cumberland, S. & Lead, J. R. (2009). Particle size distributions of silver nanoparticles at environmentally relevant conditions. *Journal of Chromatography A* **1216**, 9099–9105. doi:10.1016/j.chroma.2009.07.021

Davies, P. H., Goettl. J. P. & Stnlev, J. R. (1978). Toxicity of silver to Rainbow Trout (*Salmo Gairdneri*)*. *Water Research* **12**, 113–117.

Environment Canada. (1999). Canadian water quality guidelines for the protection of aquatic life.

Fairbrother, A. & Fairbrother, J. R. (2009). Are environmental regulations keeping up with innovation? A case study of the nanotechnology industry. *Ecotoxicology and Environmental Safety* **72**, 1327–1330. doi:10.1016/jecoenv.2009.04.003

Farkas, J., Christian, P., Urrea, J. A. G., Roos, N., Hassellöv, M., Tollefsen, K. E. & Thomas, K. V. (2010). Effects of silver and gold nanoparticles on rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic Toxicology* **96**, 44–52. doi:10.1016/j.aquatox.2009.09.016

Farkas, J., Peter, H., Christian, P., Gallego Urrea, J. A., Hassellöv, M., Tuoriniemi, J., Gustafsson, S., Olsson, E., Hylland, K., Thomas, K. V. (2011a). Characterization of the effluent from a nanosilver producing washing machine. *Environment International* **37**, 1057–1062. doi:10.1016/j.envint.2011.03.006

Farkas, J., Christian, P., Gallego-Urrea, J. A., Roos, N., Hassellöv, M., Tollefsen, K. E. & Thomas, K. V. (2011b). Uptake and effects of manufactured silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*) gill cells. *Aquatic Toxicology* **101**, 117–25. doi:10.1016/j.aquatox.2010.09.01

Farmen, E., Mikkelsen, H. N., Evensen, O., Einset, J., Heier, L. S., Rosseland, B. O., Salbu, B., Tollefsen, K. E. & Oughton, D. H. (2012). Acute and sub-lethal effects in juvenile Atlantic salmon exposed to low μg/L concentrations of Ag nanoparticles. *Aquatic Toxicology* **108**, 78–84. doi:10.1016/j.aquatox.2011.07.007

Faunce, T. & Watal, A. (2015). Nanosilver and global public health: international regulatory issues. *Nanomedicine* **5.4**, 617-639. doi:10.2217/nnm.10.33

Federation of Canadian Municipalities and National Research Council. (2003). *Wastewater source control: a best practice by the national guide to sustainable municipal infrastructure*. **1**, 21pp.

Fry, F. E. J. (1971). The effect of environmental factors on the physiology of fish. In: Hoar. W. S., Randall, D. J. (Eds.), *Environmentla Relations and Behavior (VI)*. Academic Press, New York, 1-98.

Gagnon, A., Jumarie, C. & Hontela, A. (2006). Effects of Cu on plasma cortisol and cortisol secretion by adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* **78**, 59–65. doi:10.1016/j.aquatox.2006.02.004

Gottschalk, F., Sonderer, T., Scholz, R. W. & Nowack, B. (2009). Modeled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag, CNT, fullerenes) for different regions. *Enviromental Science & Technology* **43**, 9216–9222. doi:10.1021/es9015553

Griffitt, R. J., Brown-Peterson, N. J., Savin, D. A., Manning, C. S., Boube, I., Ryan, R. A., Brouwer, M. (2012). Effects of chronic nanoparticulate silver exposure to adult and juvenile sheepshead minnows (*Cyprinodon variegatus*). *Enviromental Toxicology and Chemistry* **31(1)**, 160–167. doi:10.1002/etc.709

Handy, R. D., Depledge, M. H. (1999). Physiological responses: their measurement and use as environmental biomarkers in ecotoxicology. *Ecotoxicology* **8**, 329–349.

Heath, A. G. (1995). *Water Pollution and Fish Physiology*. Boca Raton, FL: CRC Press.

Jobling, M. (1994). *Fish Bioenergetics*, Chapman and Hall Fish and Fisheries Series 13. 2-6 Boundary Row, London: Chapman and Hall.

Johari, S. A., Kalbassi, M. R., Soltani, M. & Yu, I. J. (2013). Toxicity comparison of colloidal silver nanoparticles in various life stages of rainbow trout (*Oncorhynchus mykiss*). Iranian Journal of Fisheries Sciences **12**, 76–95.

Kaegi, R., Voegelin, A., Ort, C., Sinnet, B., Thalmann, B., Krismer, J., Hagendorfer, H., Elumelu, M. & Mueller, E. (2013). Fate and transformation of silver nanoparticles in urban wastewater systems. Water Research **47**, 3866–3877. doi:10.1016/j.watres.2012.11.060

Killen, S. S., Costa, I., Brown, J. A. & Gamperl, A. K. (2007). Little left in the tank: metabolic scaling in marine teleosts and its implications for aerobic scope.

Proceedings of the Royal Society of London B. **274**, 431–438.
doi:10.1098/rspb.2006.3741

Laban, G., Nies, L. F., Turco, R. F., Bickham, J. W. & Sepúlveda, M. S. (2010).
The effects of silver nanoparticles on fathead minnow (*Pimephales promelas*)
embryos. *Ecotoxicology* **19**, 185–95. doi:10.1007/s10646-009-0404-4

Lapresta-Fernández, A., Fernández, A. & Blasco, J. (2012). Nanoecotoxicity
effects of engineered silver and gold nanoparticles in aquatic organisms. *Trends in
Analytical Chemistry* **32**, 40–59. doi:10.1016/j.trac.2011.09.007

Liu, J., Chao, J., Liu, R., Tan, Z., Yin, Y. & Wu, Y. (2009). Cloud point extraction
as an advantageous preconcentration approach for analysis of trace silver
nanoparticles in environmental waters. *Analytical Chemistry* **81**, 6496–6502.
doi:10.1021/ac900918e

Lowry, G. V., Espinasse, B. P., Badireddy, A. R., Richardson, C. J., Reinsch, B. C.,
Bryant, L. D., Bone, A. J., Deonarine, A., Chae, S., Therezien, M., Colman, B. P., Hsu-
Kim, H., Bernhardt, E. S., Matson, C. W., and Wiesner, M. R. (2012). Long-term
transformation and fate of manufactured ag nanoparticles in a simulated large scale
freshwater emergent wetland. *Environmental Science & Technology* **46**, 7027–36.
doi:10.1021/es294608d

Martinez-Porchas, M., Martinez-Cordova, L. R. & Ramos-Enriquez, R. (2009).
Cortisol and glucose : reliable indicators of fish stress ? *Pan-American Journal of
Aquatic Sciences* **4(2)**, 158–178.

McKenzie, D. J., Garofalo, E., Winter, M. J., Ceradini, S., Verweij, F., Day, N., Hayes, R., van der Oost, R., Butler, P. J., Chipman, J. K., & Taylor, E.W. (2007). Complex physiological traits as biomarkers of the sub-lethal toxicological effects of pollutant exposure in fishes. *Philosophical Transactions of The Royal Society B* **362**, 2043–2059. doi:10.1098/rstb.2007.2100

Minister of Justice. (1999). Canadian Environmental Protection Act, 1999.

Norin, T. & Malte, H. (2012). Intraspecific variation in aerobic metabolic rate of fish: relations with organ size and enzyme activity in brown trout. *Physiological and Biochemical Zoology* **85(6)**, 645–656. doi:10.1086/665982

Pham, C. H., Yi, J. & Gu, M. B. (2012). Biomarker gene response in male Medaka (*Oryzias latipes*) chronically exposed to silver nanoparticle. *Ecotoxicology and Environmental Safety* **78**, 239–245. doi:10.1016/j.ecoenv.2011.11.034

Pulit-Prociak, J., Stokłosa, K. & Banach, M. (2014). Nanosilver products and toxicity. *Environmental Chemistry Letters* **13**, 59–68. doi:10.1007/s10311-014-0490-2

Pyle, G., Busby, P., Gauthier, C., Rajotte, J. & Couture, P. (2008). Seasonal and regional variations in metal contamination and condition indicators in Yellow perch (*Perca flavescens*) along two polymetallic gradients. II. growth patterns, longevity, and condition. Human and Ecological Risk Assessment **14**, 126–145. doi:10.1080/10807030701790504

Sadekarpawar, S. & Parikh, P. (2013). Gonadosomatic and hepatosomatic indices of freshwater fish *Oreochromis mossambicus* in response to a plant nutrient. *World Journal of Zoology* **8(1)**, 110–118. doi:10.5829/idosi.wjz.2013.8.1.7268

Sanderson, K. (2009). Tighter nanotech regulations touted. *Nature* **457(5)**, 647.

Scown, T. M., Santos, E. M., Johnston, B. D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J. R., Stone, V., Fernandes, T. F., Jepson, M., Aerle, R. V., & Tyler, C. R. (2010). Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. *Toxicological Sciences* **115(2)**, 521–534. doi:10.1093/toxsci/kfq076

Seltenrich, N. (2013). Nanosilver: weighing the risks and benefits. *Environmental Health Perspectives* **121(7)**, 220–225.

Shaluei, F., Hedayati, A., Jahanbakhshi, A., Kolangi, H. & Fotovat, M. (2013). Effect of subacute exposure to silver nanoparticle on some hematological and plasma biochemical indices in silver carp (*Hypophthalmichthys molitrix*). *Human and Experimental Toxicology* **32(12)**, 1270–1277. doi:10.1177/0960327113485258

Sherwood, G. D., Rasmussen, J. B., Rowan, D. J., Brodeur, J. & Hontela, A. (2000). Bioenergetic costs of heavy metal exposure in yellow perch (*Perca flavescens*): in situ estimates with a radiotracer (^{137}Cs) technique. *Canadian Journal of Fisheries and Aquatic Sciences* **57**, 441–450.

Sokolova, I. & Lannig, G. (2008). Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. *Climate Research* **37**, 181–201.

Stebounova, L. V., Guio, E. & Grassian, V. H. (2011). Silver nanoparticles in simulated biological media: a study of aggregation, sedimentation, and dissolution. *Journal of Nanoparticle Research* **13**, 233–244. doi:10.1007/s11051-010-0022-3

Sutton, S. G., Bult, T. P. & Haedrich, R. L. (2000). Relationships among fat weight, body weight, water weight, and condition factors in wild Atlantic salmon parr. *Transactions of the American Fisheries Society* **129**, 527–538. Doi: 10.1577/1548-8659(2000)129<0527:RAFWBW>2.0.CO;2

Svendsen, J. C., Genz, J., Anderson G. W., Stol, J. A., Watkinson, D. A. & Enders, E. C. (2014). Evidence of circadian rhythm, oxygen regulation capacity, metabolic repeatability and positive correlations between forced and spontaneous maximal metabolic rates in lake sturgeon *Acipenser fulvescens PLoS ONE* **9(4)**, e94693. doi: 10.1371/journal.pone.0094693

Svendsen, J. C., Steffensen, J. F., Aarestrup, K., Frisk, M., Etzerodt, A. & Jyde, M. (2012). Excess posthypoxic oxygen consumption in rainbow trout (*Oncorhynchus mykiss*): recovery in normoxia and hypoxia. *Canadian Journal of Zoology* **11**, 1–11. doi:10.1139/Z11-095

Wijnhoven, S. W. P., Peijnenburg, W. J. G. M., Herberts, C. A., Hagens, W.I., Oomen, A. G., Heugens, E. H. W., Roszek, B., Bisschops, J., Gosens, I., Van De Meent, D.,

Dekkers, S., De Jong, W. H., Zijverden, M. V., Sips, A. J. A. M., & Geertsma, R. E. (2009). Nano-silver – a review of available data and knowledge gaps in human and environmental risk assessment. *Nanotoxicology* **3(2)**, 109–138. doi:10.1080/17435390902725914

Wilson, R. W., Bergman, H. L. & Wood, C. M. (1994). Metabolic costs and physiological consequences of acclimation to aluminum in juvenile Rainbow trout (*Oncorhynchus mykiss*). 2: Gill morphology, swimming performance, and aerobic scope. *Canadian Journal of Fisheries and Aquatic Sciences* **51**, 536–544.

Wood, C. M., Hogstrand, C., Galvez, F. & Munger, R. S. (1996). The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) 1. The effects of ionic Ag+. Aquatic Toxicology **35**, 93–109.

Wootton, R. J. (1990). *Ecology of teleost fishes*. Fish and fisheries series: 1. 11 New Fetter Lane, London: Chapman and Hall Ltd.

Electronic References

Government of Canada (2014). Nanoportal - Home. Available at Nanoportal.gc.ca (last accessed 1 April 2015).

Woodrow Wilson International Centre for Scholars (2013). The nanotechnology consumer inventory of the Woodrow Wilson International Centre for Scholars. Available at: http://www.nanotechproject.org (last accessed 10 April 2015).

Chapter 2: Effect of nanosilver on cortisol release and morphometrics in rainbow trout (*Oncorhynchus mykiss*)

Abstract

Nanosilver (nAg), a type of nanoparticle commonly incorporated into consumer products for its antimicrobial properties, is being discharged into and detected in aquatic environments but lacks specific environmental regulation. Toxic effects of nAg on fish have been observed and from these effects it is possible that nAg may induce a stress response in fish in the form of increased blood plasma cortisol concentrations. In this study the effects of nAg exposure on rainbow trout (*Oncorhynchus mykiss*) were investigated using blood plasma cortisol concentrations as an indicator of stress. Several morphometric measures (growth, Fulton's condition factor, hepatosomatic index) were measured to investigate potential whole-body effects of exposure, and in addition concentrations of nAg within fish muscle tissue were measured to observe if nAg was taken up into tissue. Fish were exposed to environmentally-relevant (average 0.28 µg l^{-1}) and higher (average 47.60 µg l^{-1}) concentrations of nAg for 28 d. At various time-points (0.1, 0.3, 7, 14, and 28 d) data were collected on cortisol and morphometric measures. A significant difference between the control and high exposure treatments was found for blood plasma cortisol concentration as well as increased concentrations in comparison to the control in the low exposure. A significant effect on HSI by treatment dependent on exposure time was observed, although no obvious trend in the data was detected, while other morphometric measures were not affected by exposure. In addition nAg was seen to be taken up into fish muscle tissue. These

results indicate that nAg does engage the stress response of fish, while not affecting

growth or condition under the experimental conditions and timeframe.

Introduction

Nanosilver (nAg) is a nanoparticle that has been widely incorporated into many consumer products for its antimicrobial properties. These products include many textile products, medical devices, electronics, paints, baby products, food storage containers, washing machines, contraceptive devices, and water purificants (Wijnhoven et al. 2009). Nanosilver commonly enters wastewater mainly from the washing of consumer products (it has been estimated that the average North American consumer contributes approximately 470 μg l^{-1} per day of nAg into wastewater) (Benn et al. 2010) as well as from other sources such as industrial activity, atmospheric emissions, accidental spills, and leaching from landfills (Pulit-Prociak et al. 2014). It is difficult to test for and there is no standard testing method. Consequently, amounts currently in the aquatic environment are not well known. Studies that have measured concentrations in surface waters have reported levels of approximately 1.5 μg l^{-1} (Liu et al. 2009), while predicted concentrations from models are lower, ranging from the low ng l^{-1} range (Gottschalk et al. 2009) to 0.32 μg l^{-1} (Blaser et al. 2008).

Nanosilver is chemically active in water, which can affect its toxicity. Once in the aquatic environment, nAg may dissolve to silver ions (Ag$^+$) (which may in turn complex with various ligands such as Cl$^-$, S$_2^-$ or S$_2$O$_{3)}$, aggregate with other nAg particles or aggregate with dissolved organic carbon (DOC) (Cumberland and Lead 2009). The extent to which each of these actions occurs depends on factors of the receiving water chemistry such as pH, ionic strength, temperature, type of ligands present, as well as characteristics of the nAg particles released such as

presence/type of capping agent (nAg particles may be coated or 'capped' with substances such as polyvinylpyrrolidone or citrate in an attempt to increase their stability) and size of particle (Stebounova et al. 2011). Nanosilver has been observed to settle from the water column to the sediment and it has also been found to be taken up by invertebrates and fishes (Lowry et al. 2012).

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 (Lapresta-Fernández et al. 2012). The main mode of nAg's toxicity appears to be through oxidative stress, while toxicity from silver ions' main appears to be through the inhibition of the Na^+/K^+ ATPase pump in the gill cells, eventually leading to plasma ion loss and osmoregulatory failure (Farkas et al. 2010). Fish LC50s (lethal concentration, 50 %) for nAg range from the $\mu g\ l^{-1}$ to the mg l^{-1} range, depending on fish species, life stage and particle size. Other effects include altered gene expression at 1 $\mu g\ l^{-1}$ (Pham et al. 2012), thickening of gill tissue at 10 $\mu g\ l^{-1}$ (Griffitt et al. 2012), and impaired osmoregulation via inhibition of Na+/K+ATPase at 20 $\mu g\ l^{-1}$ (Farmen et al. 2012). Gill necrosis has been observed at a concentration of 100 $\mu g\ l^{-1}$ (Farmen et al. 2012) and impaired gas exchange at 300 $\mu g\ l^{-1}$ (Bilberg et al. 2010) (Appendix A).

Cortisol, along with glucose, is one of the most common stress indicators in fish, and may therefore be well-suited to measuring stress due to nAg exposure in fish (Katuli et al. 2014). Its release can be caused by nAg either through oxidative or osmoregulatory stress (Wood et al. 1996) and has been seen to do so in several studies (Johari et al. 2013; Shaluei et al. 2013). After its release cortisol causes a

cascade of changes to blood and tissue chemistry that allow the fish to better deal with the stress, such as causing increased plasma glucose levels to provide increased energy (Barton 2002). Cortisol is usually released quickly after stress is detected, and is often only released for the first 6-48 h of experiencing a stressor. Aside from exposure time, other factors that may affect cortisol release are age, genotype/phenotype, position in dominance hierarchy, crowding stress, thermal acclimation, domestic versus wild rearing of fish, and nutritional status (Martinez-Porchas et al. 2009).

If nAg exposure causes changes in fish energetics via its toxic effects, chronic exposure resulting in energy depletion may result in morphological changes in fish. Growth and Fulton's condition factor (*K*) are both morphometric measures that may provide insight into the overall health of a fish. Growth is often measured as percentage of body weight gained over a certain time period, while *K* estimates the condition of a fish under the assumption of isometric growth (Sutton et al. 2000). These measures may respond to nAg's toxic effects if they are intense enough to affect the whole-body performance of fish. Overall toxicants have been seen to affect these measures in some cases; however, fish may be able to adopt a bioenergetic strategy that preserves their overall condition (Shaw and Handy 2011).

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 processing and excreting them through bile secretions (Sadekarpawar and Parikh 2013). Because of this role in detoxification, the hepatosomatic index (HSI), i.e., the ratio of liver weight to body weight of a fish, is

commonly used in toxicological studies. Overall 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 (from the needed increased capacity to metabolize xenobiotics) (Heath 1995). In past studies HSI has been seen to both increase and decrease from nAg exposure (Gagnon et al. 2006; Joo et al. 2013).

Study Objectives

To provide insight into how nAg affects both the stress response and whole-body responses of fish, the stress response of rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) exposed to nAg was investigated through plasma cortisol release as well as various whole-body morphometric measures (growth, *K*, HSI). Measurements were taken at various time points throughout a chronic exposure period, at environmentally relevant levels and higher. In addition, the stability of nAg in the water was monitored to determine the actual exposure of fish to nAg in comparison to target concentrations. Fish tissue silver concentrations were also analyzed throughout the exposure to determine how much silver the fish were taking up from their environment.

Materials and Methods

Experimental animals

Juvenile *O. mykiss* were obtained from Lyndon Fish Hatcheries (New Dundee, Ontario) on Feb 11, 2014 (n=200) and April 29, 2014 (n=200). Upon arrival at the DFO Freshwater Institute (Winnipeg, Manitoba) they weighed approximately 1 g. All individuals were female diploid fish.

After arrival, fish were kept in large quarantine tanks for one week. During both holding and experimental trials, fish were exposed to a 12:12 diurnal cycle, and were fed commercial trout pellets (Aqua Pride Trout 42:15(sinking)) at 1-2% body weight per day. Any leftover food or fish waste that built up on the bottom of tanks was removed daily and tanks were manually cleaned weekly to remove any algae that built up on the sides of the tank. Water quality parameters in tanks were checked weekly (Table I). All tanks were on flow-through systems of water replacement with dechlorinated Winnipeg City tap water (14 ± 2 °C) at rates of replacement to maintain optimal water quality (Table II). Fish also received supplemental oxygenation from air stones.

Table I.   **Average water quality parameter values ± standard errors (Temperature (T), pH, Conductivity (Cond), Specific Conductivity (SC), Oxygen saturation (O_2), and Nitrate concentration) throughout quarantine and exposure experiments for all tanks.**

T(°C)	pH	Cond (µS)	SC (µS)	O_2 (%)	Nitrate (ppm)
14.7 ± 0.1	7.94 ± 0.03	261.8 ± 1.5	341.3 ± 20.0	94.9 ± 0.3	0-0.25

Exposure experiment

A stock nanosilver (nAg) solution was prepared at Trent University (Peterborough, Ontario) by adding powdered nAg (30-50 nm, polyvinylpyrrolidone (PVP)-capped) received from Nanostructured and Amorphous Materials (NanoAmor) to distilled water and mixing it at a high velocity for 15 min. Gum arabic was also added to this mixture as a stabilizer at a concentration of 0.025 %. Volumes of 2.5 l were mixed at one time. This solution was then refrigerated and

stored for up to 2 weeks before use (tests showed the solution to be stable for up to

this time) (J. Martin unpublished data, Trent University, 2014).

The prepared solution was then shipped to the Freshwater Institute

(Winnipeg, Manitoba) where it was diluted to concentrations required for

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 plastic

20 l carboys with a measured amount of reverse osmosis water to make exposure

solutions at the appropriate nominal concentrations (Table II). To counteract

settling of nAg out of exposure solution, carboys were placed on a stir plate with a

stir bar and continuously stirred, and the exposure solution was constituted and

replaced every 2 d. The solution was delivered to exposure tanks through a

peristaltic pump. The rate of the peristaltic pump was set to achieve the desired

concentrations of nAg in the tanks, given the water flow rates through experimental

tanks (Table II). For control treatments an exposure solution containing only

reverse osmosis water was used.

Table II.   Flow rates of peristaltic pump, water flow to tanks, nominal tank nAg concentrations, exposure solution concentrations, and time 0 stock additions for exposure experiment tanks.

Peristaltic pump rate (l min^{-1})	Tank water flow rate (l min^{-1})	Nominal tank nAg concentration (µg l^{-1})	Stock concentration (g l^{-1})	Exposure solution concentration (µg l^{-1})	Amount of stock added at time 0 (l)
0.00018	0.27	1	2.5	1501	0.000016
0.00018	0.27	200	2.5	300200	0.0032
0.00018	0.27	300	5	450300	0.00006
0.00018	0.27	600	5	900600	0.00012

Exposures were conducted over 2 trials, with Trial 1 consisting of control, 1

µg L^{-1}, and 200 µg l^{-1} nominal exposures, and Trial 2 consisting of control, 300 µg l^{-1}

and 600 µg l⁻¹ nominal exposures, for a total of 5 exposure levels over both trials. After the 1-week quarantine, 11 fish were added to each 40 l exposure trial tank. There were 12 exposure tanks in total, with 4 tanks for each treatment level. At addition fish were fin-clipped for individual identification. Fish were acclimated to trial tanks for one week before nAg exposure began. At time 0 of the experimental trial, a measured amount of nAg stock solution was added (Table II) 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. Experimental fish were cared for as in the acclimation tanks. In addition total silver concentrations in randomly selected exposure tanks were measured every 4 d. Stock and carboy total silver concentrations were measured once during Trial 2, and not during Trial 1.

To determine if nAg stability under static conditions was different than in the flow-through set-up used for the exposure trials, two additional exposure tanks with no flow-through were set up for 4 d. These static exposure tanks were set up with an initial dose of nAg to achieve 300 µg l⁻¹ and 600 µg l⁻¹ concentrations and were identical to trial tanks with the exception of flow-through water delivery. Water samples were taken at 0 h, 1 d, and 4 d from both the top and bottom of the tanks for total silver analysis.

Biological sampling

During the nAg exposure, individual fish were sacrificed to provide data on fork length, weight, liver condition, and blood plasma cortisol at 0 h, 2 h, 7 h, 7 d, 14

d, and 28 d during the nAg exposure. Fish were gently netted out of the tank, fork

length and weight were recorded, and the identifying fin clip was noted. The fish

was then placed in a bucket with an overdose of a pH buffered solution of tricaine

methanesulfonate (MS-222, Syndel Laboratories Ltd.) (300 mg l^{-1}) until opercular

movement ceased (< 3 min). To obtain blood samples for cortisol determination 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 x g to separate plasma from red blood cells. Plasma

was pipetted into another vessel, and then frozen at -80 °C. Muscle tissue was

sampled for total silver concentration analysis and liver weights were taken for

hepatosomatic index measurements.

Blood plasma cortisol sample analysis

Cortisol sample analysis methods followed Bestvater (2014) (Fig. 1). Plasma

samples were thawed and pipetted into 15 ml disposable glass tubes. All samples

were spiked with 5 ng of d4-cortisol (10 μl of 0.5ng $μl^{-1}$ solution). To each sample 3

mL of 9:1 hexane:ethyl acetate was added, and they were then vortexed for 60 s,

centrifuged for 5 min at 4000 x g, and frozen for 5 min at -80°C. The top layer was

then removed and transferred to another glass tube. The steps were repeated using

3:2 hexane:ethyl acetate, followed by 100% acetonitrile (ACN) and the upper layer

was removed and combined with the first extract (Figure 1). The samples were then

blown to dryness using nitrogen (N$_2$) and rinsed with 1.5 ml methanol (MeOH) into

a gas chromatography (GC) vial. The sample was then evaporated with N$_2$, rinsed

with 200 μl MeOH into a GC vial with glass insert, evaporated with N$_2$, and then

rinsed with 22.5 μl MeOH into another GC vial with glass insert. The samples were then analyzed by High Performance Liquid Chromatography Tandem Mass Spectrometry HPLC-MS/MS.
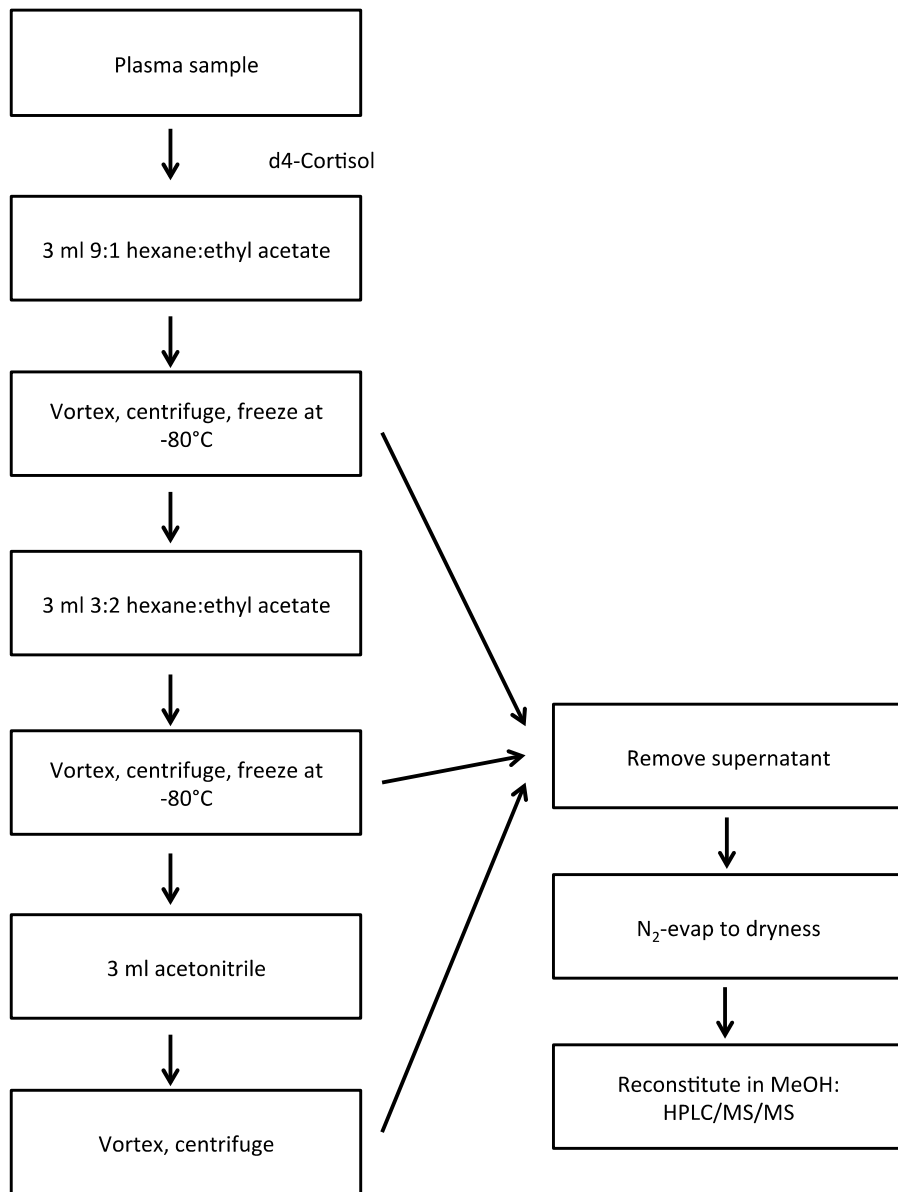
Figure 1 **Schematic of extraction method of cortisol from blood plasma.**

High Performance Liquid Chromatography Tandem Mass Spectrometry

An Agilent 1100 series HPLC system (Agilent Technologies, Palo, CA, US) was used. It was equipped with a vacuum degasser, a binary pump, and an autosampler. A C18 analytical column (Grace; 50 mm x 2.1 mm i.d., 4um particle size; Chromatographic Specialties Inc, Brockville, ON, Canada) was used to separate the cortisol. The mobile phase consisted of a gradient of water and methanol at a flow rate of 300 μL min^{-1}. The initial composition was 80:20 water:MeOH (v/v), held for 1 min, ramped linearly to 100% MeOH in 9 min, and held for 6 min. The column was equilibrated between runs for 7 min. A Sciex 2000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) was used for identification and quantification of the compounds.

Cortisol was monitored in the negative ion mode under multiple reaction monitoring (MRM) conditions. The mass to charge (M/z) values, product ion transitions for quantification and confirmation for compounds are listed in Table III. The optimized MS/MS parameters in electrospray ionization (ESI) negative ion MRM mode are shown in Appendix B.2.

Table III.   Multiple Reaction Monitoring (MRM) and ions monitored for cortisol and d4-cortisol.

Hormone	Mass to charge (m/z)	Transitions monitored		Ion Mode
		Quantification	Confirmation	
Cortisol	361.1	331.0	282.0	-
d4-cortisol	365.0	335.0	301.0	-

Quality Assurance/Quality Control

Procedural blanks were analyzed every 10 samples. The native hormones were not detected in the blanks, so blank correction was not necessary. Injections of MeOH (3 µl) were used as instrument injection blanks for HPLC/MS/MS, and were run every 10 samples. Percent recovery for d_4-cortisol was found to average 24 % (± 0.91 SEM, n = 253). This level of recovery was lower than ideal, and likely due to matrix effects, a common issue to this type of analysis (Gosetti et al. 2010). However, quantitation of native cortisol in the samples was achieved by isotope dilution, a method used to compensate for matrix effects (Gosetti et al. 2010; Trufelli et al. 2011). In isotope dilution, the response of d_4-cortisol (an isotope that is known to behave in a similar fashion to native cortisol) that was purposely added to samples as an internal standard and taken through the entire extraction and work-up procedures was compared to the measured response of native cortisol in the samples, with the equation:

$$Cortisol = \frac{C_d * average \ (\frac{d_4}{C_{d \ native}})}{d_4}$$

where C_d is the amount of cortisol detected, and the average ratio of d_4-cortisol (d_4) to the native cortisol ($C_{d \ native}$) standard detected is taken over each round of samples analyzed. In this way lower recovery values were effectively compensated for (Trufelli et al. 2011).

The analytical detection limit (ADL) for cortisol was found to be 1.2 pg, and the method detection limit (MDL) was 0.005 ng ml^{-1}. ADL was determined by injecting a known amount of compound and suppressing the signal to noise (S/N)

ratio to a 5:1 value. MDL is defined as the amount of analyte in the procedural blanks plus 3 x standard deviation and were normalized to mass or volume of sample extracted (Long and Winefordner 1983). The signals of the analytes in the blanks were adjusted to estimate concentrations that would give a S/N ratio of 5:1. For those samples that had undetectable amounts of the cortisol in the blanks, the MDL was determined by adding a known amount of each cortisol to the blank extract and suppressing the S/N value to 5:1. In cases where cortisol was below the MDL, a concentration of ½ MDL was assumed.

Data analysis

Growth rates were calculated as percent body weight (wt) gained per day by the equation:

$$Growth = \frac{ln(final\ wt\ (g)) - ln(initial\ wt\ (g))}{time\ (d)} \cdot 100 \quad \textit{(Cook et al. 2000).}$$

If the initial weight of the fish was not known (due to the identifying fin mark not being clear at the end of the experiment – approximately one third of fish), then the initial weight was taken as the average initial weight of all fish for that tank. HSI was calculated by the equation:

$$HSI = \frac{liver\ wt\ (g)}{body\ wt\ (g)} \cdot 100 \quad \textit{(Cobo et al 2013).}$$

Fulton's condition factor (K) was calculated by the equation:

$$K = \frac{body\ wt\ (g)}{fork\ length\ (cm)^3} \cdot 100 \quad \textit{(Sutton et al. 2000).}$$

Statistical analysis

Statistical analyses were run in R version 3.1.3 (R Foundation for Statistical Computing 2015). Measured water silver concentrations between treatments were analyzed with an ANOVA. Fish silver concentrations were also analyzed by treatment and time with an ANOVA, and Tukey's HSD was used for post-hoc analysis where appropriate. . Cortisol, growth, HSI, and K (Fulton's condition factor) data were analyzed by treatment and time using a mixed-model ANOVA design (lmer in package lme4), taking into account tank as random effects with the general equation:

$$Cortisol = Treatment + Time + Treatment * Time + Tank$$

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

$$Cortisol = Treatment + Time + Tank$$

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.

For cortisol, growth, and condition, no significant differences were found for variables between the controls of each trial (ANOVA, $p > 0.05$), so controls were combined and trial was not considered a factor in the analysis. For HSI, a significant difference was found between the trial controls, and trial was included in the

38

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 HSD test where appropriate.

Results

Exposure concentrations of silver

Measurements of silver in experimental tanks were of total silver. Most of the silver detected was in particulate form, with only 0-0.18% in ionic form (average 0.05% ionic). Overall, concentrations of total silver measured in the experiment were much lower than nominal, and quite variable (Table IV, Figure 2). The carboys containing exposure silver solution that was pumped into tanks were measured once in Trial 2, and concentrations were found to be much lower than nominal. Test static tanks (tanks with silver solution added directly, and no inflow/outflow of water) had lower concentrations of silver than trial tanks, levels stayed relatively constant over the 4 d, and concentrations did not significantly differ between the tank top and tank bottom measurements.

Measured concentrations within the 200, 300, and 600 µg l^{-1} exposure tanks had a large degree of overlap with each other, and did not differ statistically from one another (ANOVA, $F_{(5,85)}$ = 15.23, $p > 0.05$). As such, the cortisol and morphometric data for these higher concentration exposures were combined into one exposure treatment (called 'high', H), and were compared to the control (C1 and C2 combined, where appropriate) and the low (L, 1 µg l^{-1}) exposures.

Table IV. Total silver concentrations measured in exposure experiment tanks, static experiment tanks, and carboys containing silver solution as well as percentage of total silver that was measured as dissolved.

Tank	Nominal concentrations (μg l^{-1})	Average measured concentrations (μg l^{-1})	Range (μg l^{-1})	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	0.00-0.04	-
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	450300	24517.07	-	5
Carboy H	900600	150503.98	-	17
% dissolved Ag		0.05	0.00-0.18	
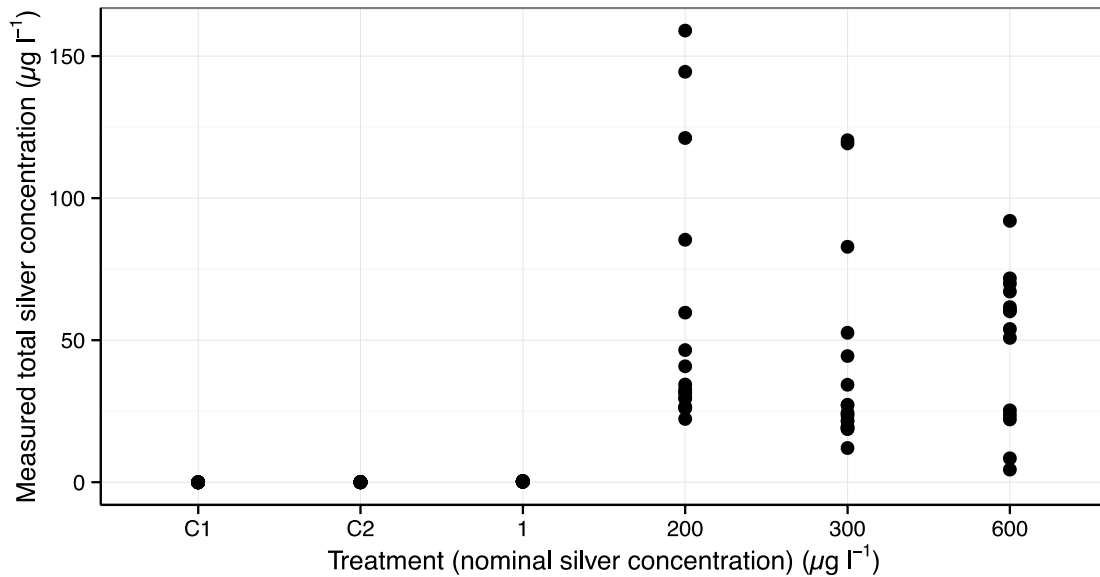
Figure 2. Measured concentrations of total silver for nominal nAg treatments in exposure experiment tanks over the course of experimental exposures.

Fish tissue silver concentration results

Uptake of silver into fish muscle tissue was observed, with a high degree of variation in the tissue concentrations, both within treatments and across time. (Note: only 7, 14, and 28 d time points included). Between days 7-28, the nAg concentrations in high treatment fish differed significantly from those in control and low treatments (Tukey's HSD; p_{C-H} = 0.006, p_{L-H} = 0.02), while no significant difference was found between control and low treatments (Tukey's HSD; p = 0.87) likely due to the high level of variability and low sample sizes. Similarly, no significant differences were found for the interaction between time and treatments (ANOVA, $F_{(df=8)}$=2.86, p = 0.07).
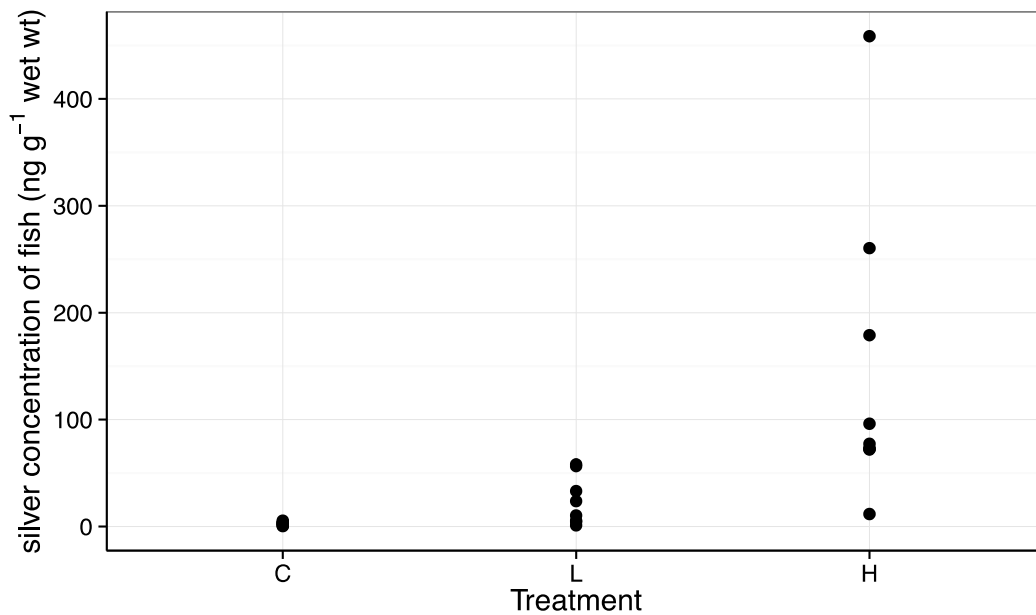
Figure 3. Total silver concentrations of fish muscle tissue in exposure treatments. C = control, L = low, H = high.
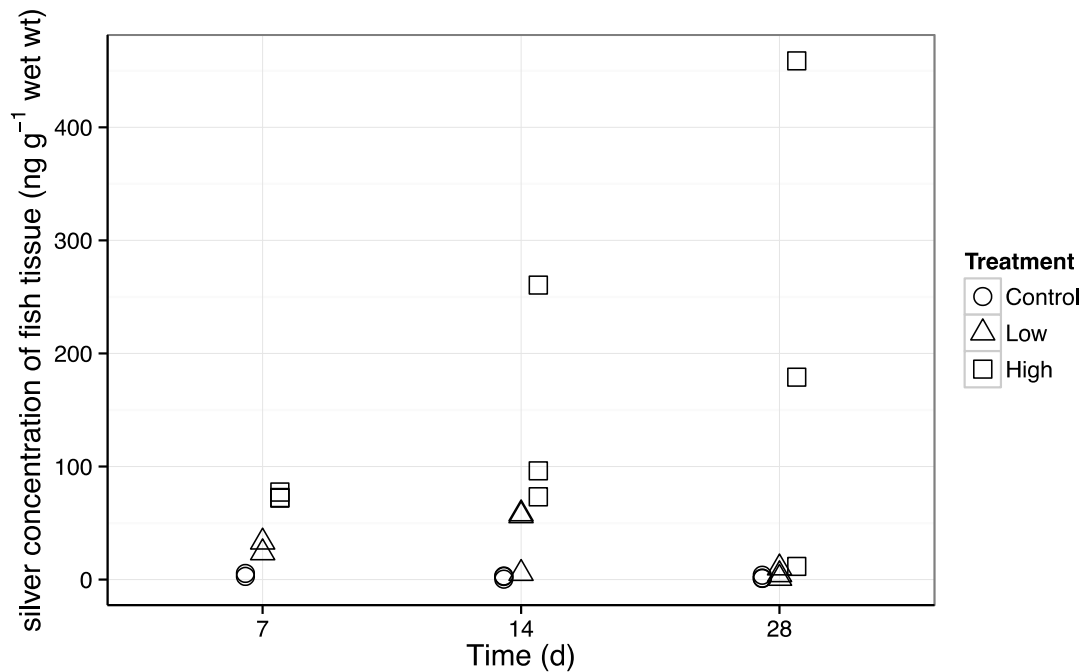
Figure 4. Silver concentrations in fish muscle tissue at 7, 14, and 28 d in exposure treatments.

Cortisol Results

There was a significant effect of treatment on cortisol concentrations (Table V; Figure 5; Log-likelihood test, $\chi^2(df = 2) = 14.906$ $p = 0.0006$). Significant differences in cortisol concentrations were found between the H treatment and the C (Tukey's HSD, $p < .001$). The difference between the L and C treatment was only marginally insignificant (Tukey's HSD, $p = 0.060$).

Table V. Summary of cortisol, growth, hepatosomatic index (HSI), and condition (K) results for nAg exposure treatments (means ± standard errors). HSI was separated by trial due to differences in trial controls.

	Cortisol (ng ml^{-1})		Growth (% body wt gain day^{-1})		HSI		K	
	mean	SE	mean	SE	mean	SE	mean	SE
C	11.70	5.55	1.91	0.13			2.19	0.06
C1					2.35	0.15		
C2					1.49	0.09		
L	15.72	4.86	1.91	0.17	2.56	0.18	2.29	0.09
H	16.08	2.68	1.74	0.11			2.26	0.05
H1					2.43	0.13		

	Cortisol (ng ml^{-1})		Growth (% body wt gain day^{-1})		HSI		K	
	mean	SE	mean	SE	mean	SE	mean	SE
H2					1.36	0.06		
Overall	14.51		1.83		1.94		2.24	
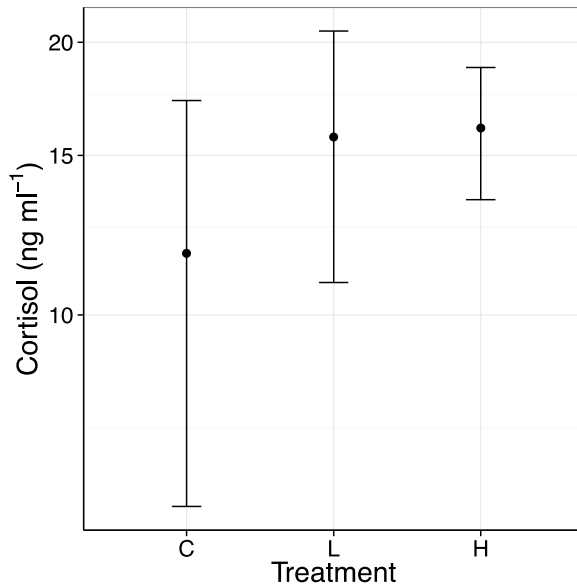
Figure 5. **Blood plasma cortisol concentrations by nAg treatment, with means ± standard error.**

We found no significant effect of the Treatment by time interaction on the cortisol response of fish to nAg exposure (Log-likelihood test, $\chi^2(df = 10) = 8.522$ $p = 0.578$). Visually comparing the means in the experiment (Figure 6), cortisol levels of nAg exposed fish tended to be elevated relative to cortisol in control fish earlier in the experiment, between 0.1, 0.3, and 7d.
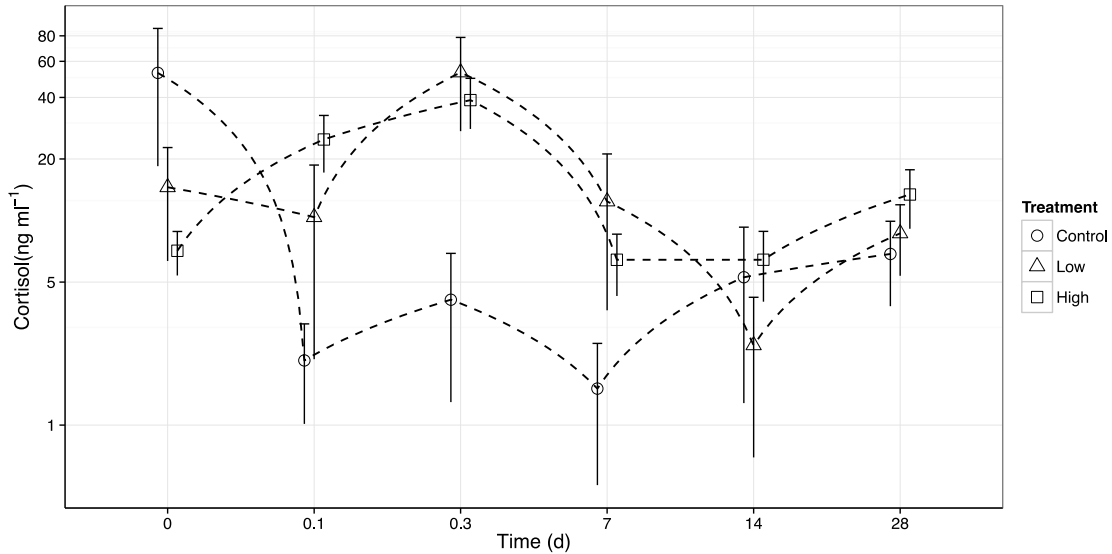
Figure 6. Blood plasma cortisol concentrations over time by nAg treatment, with means ± standard error. Note log scale on the y-axis.

Growth, Condition Factor (K), and HSI

HSI did have a significant difference by the interaction of time and treatment (Log-likelihood test, $\chi^2(df = 10) = 23.666$ $p = 0.009$) (Table V; Figure 7). No obvious trend by treatment could be seen; however, fish in Trial 1 did have higher values than fish in Trial 2. Also, there was a peak at 14 d for all treatments in Trial 1, while Trial 2 had more stable values across time. Body weights were similar between Trial 1 (mean 2.94 ± 0.99 g) and Trial 2 (mean 2.87 ± 1.28 g) (t test: t = 0.46, $df = 231.32$, $p = 0.646$), while 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 exposure (Figure 8a), but did not differ significantly across treatments (Table V: Log-likelihood test, $\chi^2(df = 2) = 0.801$ $p = 0.670$), nor did K (Table V: Log-likelihood test, $\chi^2(df = 2) = 0.834$ $p = 0.659$) (Table V; Figure 8).

44
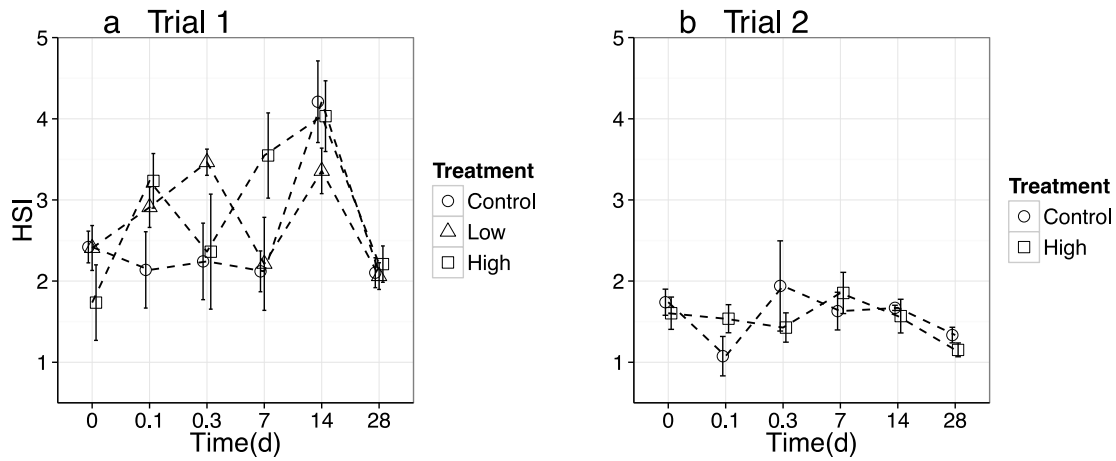
Figure 7. Hepatosomatic index over time for nAg Treatments in Trial 1 (Panel a) and Trial 2 (Panel b).
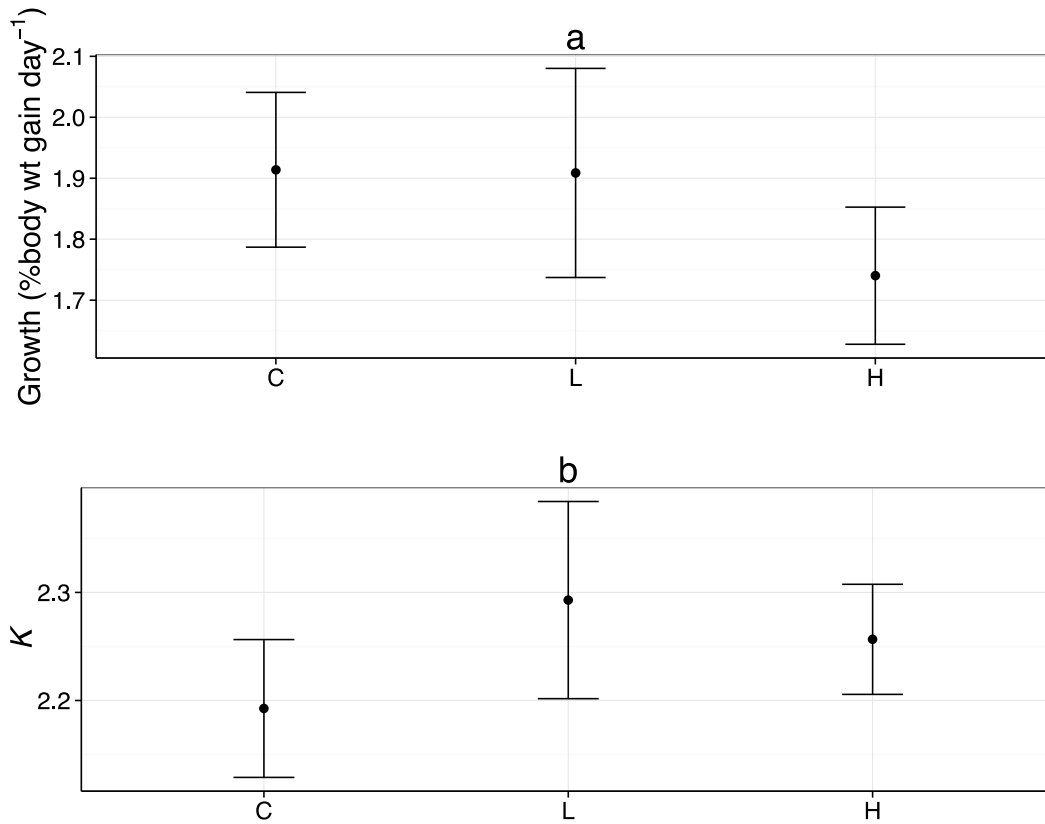
Figure 8. Effects of nAg treatment on growth and condition (*K*) with means ± standard error. Panel a; growth, estimated as percent body weight gained per day. Panel b: condition, estimated as body weight divided by fork length[3], multiplied by 100.

Discussion

In this study juvenile rainbow trout demonstrated a clear response to elevated nAg exposure for 28 days, as indicated by significantly increased blood cortisol levels in fish exposed to high concentrations of nAg compared with control fish. Cortisol levels of fish exposed to environmentally relevant levels of nAg were also increased, although not significantly. Cortisol concentrations in each of the treatment groups were increased by approximately 25% in comparison to controls. Plasma cortisol release has been observed in other nAg exposure studies as well. In a study of comparable concentrations and duration of exposure as this study (Shaluei et al. 2013) silver carp (*Hypophthalmichthys molitrix*) were exposed to 20 and 40 µg l^{-1} of nAg for 3, 7, and 14 d, and plasma cortisol was observed to be increased at all time points at both concentrations. In addition, juvenile rainbow trout also had increased plasma cortisol levels after exposure to extremely high concentrations of nAg (1000-8000 µg l^{-1}) for 3 h (Johari et al 2013). Both of these studies found approximately a 6 - fold increase in cortisol, while the present study found an approximately 3 - fold increase in cortisol over a much longer exposure period of 28 days. This difference in magnitude of cortisol response among studies may be due to the difference in exposure duration, as the current study was conducted for a longer time period than past studies; some toxic effects of nAg have been observed to decrease over time, such as gill damage (Griffit et al. 2012), and altered gene expression (Pham et al. 2012). An indication of this pattern time dependence can also be seen in the cortisol data in this 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 on in the exposure (14 and 28 d). In contrast to these results, Massarsky et al. (2014) found no effect on whole-body cortisol when embryos were exposed from 2 hpf (hours post-fertilization) to 96 hpf to 500 μg l^{-1}, potentially indicating that the cortisol response to nAg is dependent on life stage in fishes. Fish larvae and embryos have otherwise been seen to be sensitive to nAg exposure (Appendix A). However this lack of cortisol response from nAg in embryos may also be because the cortisol response may not be functional before hatching in *O. mykiss* (Auperin and Geslin 2008).

Silver ions have also been seen to increase plasma cortisol in fish. In Hogstrand et al. (1999) starry flounder (*Platichthys stellatus*) were exposed to 250 and 1000 μg l^{-1} Ag for 0, 12, 25, 38, 96, and 144 h, and there was an increase in cortisol for the 1000 ug l^{-1} exposure at 144 h (6 d). Web and Wood (1998) exposed rainbow trout to 9.2 μg l^{-1} Ag, and observed a trend of increased plasma cortisol at 4, 24, and 48 h, and a significantly higher plasma cortisol level at 96 and 144 h. Based on this comparison of studies, it appears that Ag may cause a more delayed cortisol release than nAg, with significant increases not appearing for 4-6 d after exposure versus nAg which caused an increase within hours in the current study.

Chemicals in general may incite or inhibit the cortisol response, with metals appearing to typically increase cortisol. For example, aluminum is known to increase cortisol in Atlantic salmon (*Salmo salar*) (Martinez-Porchas et al. 2009) and cadmium was found to increase cortisol the African sharptooth catfish (*Clarias gariepinus*) (Karaytug et al. 2010). However, in contrast copper was found to have

no effect on cortisol when rainbow trout were exposed solely to the contaminant, and it was found to inhibit the response when fish were exposed to copper along with the stressor of being exposed to air (Gagnon et al. 2006).

HSI was variable in this study, with higher values for Trial 1 than Trial 2 (caused by higher liver weights in Trial 1), and more stable values for Trial 2. It is unknown why this difference was seen, although it may have been affected by the 14 d age difference between Trials (Trial 2 fish were 14 d older). Other studies on juvenile rainbow trout observed lower values for HSI closer to Trial 2 than Trial 1, (Bayir et al. 2014; Hernandez et al. 2013, Kose and Yildiz 2013). Overall no obvious pattern of the effect of nAg exposure on HSI could be discerned from the data. This is in contrast to other experiments where an effect was observed. In Joo et al. (2013), HSI was observed to increase in rainbow trout when fish were exposed to anywhere from 32 to 32000 ug l^{-1} nAg for 14 d. In contrast, Monfared and Soltani (2013) exposed rainbow trout to 3000 ug l^{-1} nAg for 8 weeks (4 times longer than the exposure used by Joo et al. 2013). In their study, HSI was observed to decrease, and other damage to the liver was seen (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. These changes indicate 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 two studies may be another example of the time-dependent nature of nAg effects, as a shorter exposure (14 d) increased HSI, and a longer exposure (8 weeks)

decreased it. More studies would need to be done to clarify the effects though. Other contaminants have been observed to affect HSI as well, and in opposing directions. Copper has been observed to both increase HSI (Figueiredo-Fernandes et al. 2007) and decrease HSI (Gagnon et al. 2006). In general, 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 (from the needed increased capacity to metabolize xenobiotics) (Heath 1995).

Despite a significant response of cortisol to nAg exposure, this did not translate into a significant response for growth or Fulton's condition factor (K) in the study, although there was a tendency for decreased growth from control to low to high treatments. Nanosilver has not been observed to have an effect on growth in other studies (Griffitt et al. 2012), similar to other nanoparticles (Ramsden et al. 2009). However, it is possible that studies conducted over longer time periods than previously tested (48 h in Griffit et al. (2012), 8 weeks in Ramsden et al. (2009)) may show an effect to growth, especially in light of the apparent tendency for decreased growth due to exposure in this study. For instance, Ag^+ has been seen to decrease growth in rainbow trout, but over an 18-month exposure study (Davies et al. 1978). In general, it is known that metal exposure can inhibit growth; however, fish are usually capable of adopting bioenergetic strategies that preserve growth at the expense of other aspects of metabolism (Shaw and Handy 2011). For example, rainbow trout exposed to copper reduced their time spent swimming (which decreased metabolic costs) (Handy et al. 1999). No other studies on nAg or other

nanoparticles analyzed the effects on condition (K); however, other metals have been found to decrease K (Gagnon et al. 2006).

Uptake of nAg into fish muscle tissues was observed at all treatment levels. Other studies also saw nAg uptake into fish tissues (Griffitt et al. 2009; Scown et al. 2010; Farkas et al. 2011; Farmen et al. 2012; Joo et al. 2013). These studies observed uptake into various tissues, with uptake concentration in the order of: liver > kidneys ~ gills > muscle. Other studies also observed low levels of Ag in control fish as we did, and it has been suggested that these are background levels that fish possess from uptake from the natural environment (Webb and Wood 2000). It was also observed that citrate-capped particles were taken up more readily than PVP-capped (Farkas et al. 2011), the type used in our study. It could be hypothesized that muscle tissue would have lower concentrations than gill or liver tissue because muscle is not directly exposed to the water, nor is it a site of blood detoxification.

Nanosilver concentrations in the experiment were much lower than nominal at all points (both within carboys containing stock solution and in exposure tanks), and consisted of mostly particulate Ag, with low levels of ionic silver. 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. This is similar to what has been seen in other experiments (Appendix D). In most nAg exposure experiments, actual concentrations of nAg were much lower than nominal, averaging 45% of nominal

(presumably due to nAg agglomeration, sedimentation or adsorption to tanks walls and other equipment) and consisted mainly of particulate silver. Also consistent with this experiment were other studies showing that nAg came out of solution quickly and that higher concentrations of nAg were difficult to maintain in solution. Most of these studies were done with dechlorinated tap water. One study was done in lake water, which had much better nAg stability (80% to 100% stability over 24 h), potentially from the stabilizing effect of naturally-occurring DOC (Choi et al. 2009). Other studies had no measurement of actual nAg concentrations, and only reported nominal concentrations (Wu et al. 2010; Johari et al. 2013; Katuli et al. 2014), which this study and review of the published evidence suggests are likely overestimates of actual exposures.

In conclusion, this study found nAg to be taken up by fish and to activate a stress response in fish (increased cortisol). However, these effects were not strong enough to translate into significant responses to growth or K, and effects to HSI were unclear. There was an increase in cortisol at the environmentally relevant concentration of on average 0.28 µg l^{-1} 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 (Liu et al. 2009).

References

Auperin, A. & Geslin, M. (2008). Plasma cortisol response to stress in juvenile rainbow trout is influenced by their life history during early development and by egg cortisol content. *General and Comparative Endocrinology* **158**, 234-239. doi:10.1016/j.ygcen.2008.07.002

Barton, B. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology* **42**,517–525.

Bayir, M., Bayir, A. & Aras, N. M. (2014). A comparison of the effect of long-term starvation on responses to low-temperature stress by juvenile rainbow (*Oncorhynchus mykiss*) and brown (*Salmo trutta*) trout reveal different responses in the two species. *Marine and Freshwater Behaviour and Physiology* **47:4**, 239-251. doi: 10.1080/10236244.2014.910901

Benn, T., Cavanagh, B., Hristovski, K., Posner, J. D. & Westerhoff, P. (2010). The release of nanosilver from consumer products used in the home. *Journal of Environmental Quality* **39**,1875–1882. doi: 10.2134/je2009.0363

Bestvater, L. (2014). The persistence, bioaccumulation, and inherent toxicity of two organophosphate flame retardants tris (2-butoxyethyl) phosphate and tris (1-chloro-2-propyl) phosphate in juvenile rainbow trout (*Oncorhynchus mykiss*). University of Manitoba.

Bilberg, K., Malte, H., Wang, T. & Baatrup, E. (2010). Silver nanoparticles and silver nitrate cause respiratory stress in Eurasian perch (*Perca fluviatilis*). *Aquatic Toxicology* **96**, 159–65. doi: 10.1016/j.aquatox.2009.10.019

Blaser, S., Scheringer, M., Macleod, M. & Hungerbühler, K. (2008). Estimation of cumulative aquatic exposure and risk due to silver: contribution of nano-functionalized plastics and textiles. *Science of the Total Environment.* **390**, 396–409. doi: 10.1016/j.scitotenv.2007.10.010

Choi, O., Clevenger, T. E., Deng, B., Surampalli, R. Y., Ross, L. & Hu, Z. (2009). Role of sulfide and ligand strength in controlling nanosilver toxicity. *Water Research.* **43**,1879–1886. doi: 10.1016/j.watres.2009.01.029

Cobo, F., Sánchez-Hernández, J., Vieira-Lanero, R. & Servia, M. J. (2013). Organic pollution induces domestication-like characteristics in feral populations of brown trout (*Salmo trutta*). *Hydrobiologia* **705**,119–134. doi: 10.1007/s10750-012-1386-4

Cook, J., McNiven, M., Richardson, G. & Sutterlin, A. (2000). Growth rate, body composition and feed digestibility/conversion of growth-enhanced transgenic Atlantic salmon (*Salmo salar*). *Aquaculture* **188**, 15–32.

Cumberland, S., Lead, J. R. (2009). Particle size distributions of silver nanoparticles at environmentally relevant conditions. *Journal of Chromatography* A. **1216**, 9099–105. doi: 10.1016/j.chroma.2009.07.021

Davies, P. H., Goettl, J.P. & Sinley, J. R. (1978). Toxicity of silver to rainbow trout (*Salmo Gairdneri*)*. *Water Research* **12**,113–117.

Farkas, J., Christian, P., Gallego-Urrea, J. A., Roos, N., Hassellöv, M., Tollefsen, K. E. & Thomas, K. V. (2010). Effects of silver and gold nanoparticles on rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic Toxicology*. **96**, 44-52. doi: 10.1016/j.aquatox.2009.09.016

Farkas, J., Christian, P., Gallego-Urrea, J. A., Roos, N., Hassellöv, M., Tollefsen, K. E. & Thomas, K. V. (2011). Uptake and effects of manufactured silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*) gill cells. *Environment International* **101**, 117–25. doi: 10.1016/j.envint.2011.03.006

Farmen, E., Mikkelsen, H. N., Evensen, O., Einset, J., Heier, L. S., Rosseland, B. O., Salbu, B., Tollefsen, K. E. & Oughton, D. H. (2012). Acute and sub-lethal effects in juvenile Atlantic salmon exposed to low μg/L concentrations of Ag nanoparticles. *Aquatic Toxicology* **108**, 78–84. doi: 10.1016/j.aquatox.2011.07.007

Figueiredo-Fernandes, A., Ferreira-Cardoso, J. V., Garcia-Santos, S., Monteiro, S. M., Carrola, J., Matos, P. & Fontaínhas-Fernandes, A. (2007). Histopathological changes in liver and gill epithelium of nile tilapia, *Oreochromis niloticus*, exposed to waterborne copper. *Pesquisa Veterinária Brasileira* **27**, 103–109.

Gagnon, A., Jumarie, C. & Hontela, A. (2006). Effects of Cu on plasma cortisol and cortisol secretion by adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* **78**, 59–65. doi:10.1016/j.aquatox.2006.02.004

Gosetti, F., Mazzucco, E., Zampieri, D. & Gennaro, M. C. (2010). Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A* **1217**, 3929-3937. doi:10.1016/j.chroma.2009.11.060

Gottschalk, F., Sonderer, T., Scholz, R. W. & Nowack, B. (2009). Modeled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag, CNT, fullerenes) for different regions. *Environmental Science & Technology* **43**, 9216–9222.

Griffitt, R. J., Brown-Peterson, N. J., Savin, D. A., Manning, C. S., Boube, I., Ryan, R. A. & Brouwer, M. (2012). Effects of chronic nanoparticulate silver exposure to adult and juvenile sheepshead minnows (*Cyprinodon variegatus*). Environmental Toxicology and Chemistry **31**:1, 160–167. doi: 10.1002/etc.709

Griffitt, R. J., Hyndman, K., Denslow, N. D.& Barber, D. S. (2009). Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanoparticles. *Toxicological Sciences* **107**:2, 404–15. doi: 10.1093/toxsci/kfn256

Handy, R. D., Sims, D. W., Giles, A., Campbell, H. A. & Musonda, M. M. (1999). Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (*Oncorhynchus mykiss*) during chronic dietary exposure to copper. *Aquatic Toxicology* **47**, 23-41

Heath, A. G. (1995). *Water pollution and fish physiology*. Boca Raton, FL: CRC Press.

Hernandez, A. J., Roman, D., Hooft, J., Cofre, C., Cepeda, V. & Vidal, R. (2013). Growth performance and expression of immune-regulatory genes in rainbow trout (*Oncorhynchus mykiss*) juveniles fed extruded diets with varying levels of lupin (*Lupinus albus*), peas (*Pisum sativum*) and rapeseed (*Brassica napus*). *Aquaculture Nutrition* **19**, 321-332. doi: 10.1111/j.1365-2095.2012.00961.x

Hogstrand, C., Ferguson, E. A., Galvez, F., Shaw, J. R., Webb, N. A. & Wood, C. M. (1999). Physiology of acute silver toxicity in the starry flounder (*Platichthys stellatus*) in seawater. *Journal of Comparative Physiology* B. **169**, 461–73.

Johari, S. A., Kalbassi, M. R., Soltani, M. & Yu, I. J. (2013). Toxicity comparison of colloidal silver nanoparticles in various life stages of rainbow trout (*Oncorhynchus mykiss*). *Iranian Journal of Fisheries Sciences* **12**, 76–95.

Joo, H. S., Kalbassi, M. R., Yu, I. J., Lee, J. H. & Johari S. A. (2013). Bioaccumulation of silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*): influence of concentration and salinity. *Aquatic Toxicology* **140(141)**, 398–406. doi: 10.1016/j.aquatox.2013.07.003

Karaytug, S., Karayakar, F., Ciftci, N., Cicik, B., Ay, O. & Erdem, C. (2010). Effects of cadmium on sera glucose and cortisol levels in *Clarias gariepinus* (Burchell, 1822). *Journal of Animal and Veterinary Advances* **9(16)**, 2159–2162.

Katuli, K. K., Massarsky, A., Hadadi, A. & Pourmehran, Z. (2014). Silver nanoparticles inhibit the gill Na+/K+-ATPase and erythrocyte AChE activities and

induce the stress response in adult zebrafish (*Danio rerio*). *Ecotoxicology and Environmental Safety* **106**, 173–180. doi: 10.1016/j.ecoenv.2014.04.001

Kose, I. & Yildiz, M. (2013). Effects of diets containing sesame oil on growth and fatty acid composition on rainbow trout (*Oncorhynchus mykiss*). **29**, 1318-1324. doi:10.1111/jai.12184

Lapresta-Fernández, A., Fernández, A. & Blasco, J. (2012). Nanoecotoxicity effects of engineered silver and gold nanoparticles in aquatic organisms. *Trends in Analytical Chemistry* **32**, 40–59. doi: 10.1016/j.trac.2011.09.007

Liu J., Chao, J., Liu, R., Tan, Z., Yin, Y. & Wu, Y. (2009). Cloud point extraction as an advantageous preconcentration approach for analysis of trace silver nanoparticles in environmental waters. *Analytical Chemistry* **81**, 6496–6502. doi: 10.1021/ac900918e

Long, G. L., & Winefordner, J. D. (1983) Limit of detection: a closer look at the IUPAC definition. *Analytical Chemistry* **55(07)**, 712A-724A.

Lowry, G. V., Espinasse, B. P., Badireddy, A. R., Richardson, C. J., Reinsch, B. C., Bryant, L. D., Bone, A. J., Deonarine, A., Chae, S. & Therezien, M., (2012). Long-term transformation and fate of manufactured Ag nanoparticles in a simulated large scale freshwater emergent wetland. *Environmental Science & Technology* **46**, 7027–7036. doi :10.1021/es204608d

Martinez-Porchas, M., Martinez-Cordova, L. R. & Ramos-Enriquez, R. (2009). Cortisol and glucose : reliable indicators of fish stress ? *Pan-American Journal of Aquatic Sciences* **4(2)**, 158–178.

Martin, J. (2014). Nanosilver solution stability data. Unpublished raw data. Trent University, Peterborough, ON.

Massarsky, A., Strek, L., Craig, P. M., Eisa-Beygi, S., Trudeau, V. L. & Moon, T. W. (2014). Acute embryonic exposure to nanosilver or silver ion does not disrupt the stress response in zebrafish (*Danio rerio*) larvae and adults. *Science of the Total Environment.* **478**, 133–140. doi: 10.1016/j.scitotenv.2014.01.077

Monfared, A. L., Soltani, S. & Louei, A. (2013). Effects of silver nanoparticles administration on the liver of rainbow trout (*Oncorhynchus mykiss*): histological and biochemical studies. *European Journal of Experimental Biology* **3(2)**, 285–289.

Pham, C. H., Yi, J. & Gu, M. B. (2012). Biomarker gene response in male medaka (*Oryzias latipes*) chronically exposed to silver nanoparticle. *Ecotoxicology and Environmental Safety*. **78**, 239–45. doi: 10.1016/j.ecoenv.2011.11.034

Pulit-Prociak, J., Stokłosa, K. & Banach, M. (2014). Nanosilver products and toxicity. Environmental Chemistry Letters. **13**, 59–68.

Ramsden, C. S., Smith, T. J., Shaw, B. J. & Handy, R. D. (2009). Dietary exposure to titanium dioxide nanoparticles in rainbow trout (*Oncorhynchus mykiss*): no effect

on growth, but subtle biochemical disturbances in the brain. *Ecotoxicology* **18**, 939-951. doi: 10.1007/s10646-009-0357-7

Sadekarpawar, S. & Parikh, P. (2013). Gonadosomatic and hepatosomatic indices of freshwater fish *Oreochromis mossambicus* in response to a plant nutrient. *World Journal of Zoology* **8(1)**, 110–118. doi: 10.5829/idosi.wjz.2013.8.1.7268

Scown, T. M., Santos, E. M., Johnston, B. D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J. R., Stone, V., Fernandes, T. F. & Jepson, M. (2010). Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. *Toxicological Sciences* **115(2)**, 521–34. doi: 10.1093.toxsci/kfq076

Shaluei, F., Hedayati, A, Jahanbakhshi, A, Kolangi, H. & Fotovat, M. (2013). Effect of subacute exposure to silver nanoparticle on some hematological and plasma biochemical indices in silver carp (*Hypophthalmichthys molitrix*). *Human and Experimental Toxicology* **32(12)**, 1270–1277. doi: 10.1177/0960327113485258

Shaw, B. J. & Handy, R. D. (2011). Physiological effects of nanoparticles on fish: a comparison of nanometals versus metal ions. *Environment International* **37**, 1083–97. doi: 10.1016/j.envint.2011.03.009

Stebounova, L. V., Guio, E. & Grassian, V. H. (2011). Silver nanoparticles in simulated biological media: a study of aggregation, sedimentation, and dissolution. *Journal of Nanoparticle Research* **13**, 233–244. doi: 10.1007/s11051-010-0022-3

Sutton, S. G., Bult, T. P. & Haedrich, R. L. (2000). Relationships among fat weight, body weight, water weight, and condition factors in wild atlantic salmon parr. *Transactions of the American Fisheries Society* **129(2)**, 527–538. doi: 10.1577/1548-8659(2000)129<0527:RAFWBW>2.0.CO;2

Trufelli, H., Palma, P., Famiglini, G. & Cappiello, A. (2011). An overview of matrix effects in liquid chromatography – mass spectrometry. *Mass Spectrometry Reviews* **30**, 491-509.

Webb, N. A. & Wood, C. M. (1998). Physiological analysis of the stress response associated with acute silver nitrate exposure in freshwater rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry* **17(4)**, 579-588.

Webb, N. A. & Wood, C. M. (2000). Bioaccumulation and distribution of silver in four marine teleosts and two marine elasmobranchs: influence of exposure duration, concentration, and salinity. *Aquatic Toxicology* **49**, 111–129. doi: 10.1016/SO166-445X(99)00063-6

Wijnhoven, S. W. P., Peijnenburg, W.J. G. M., Herberts, C. A., Hagens, W. I., Oomen, A. G., Heugens, E. H. W., Roszek, B., Bisschops, J., Gosens, I. & Van De Meent, D. (2009). Nano-silver – a review of available data and knowledge gaps in human and environmental risk assessment. *Nanotoxicology* **3(2)**, 109–138. doi: 10.1080/17435390902725914

Wood, C. M., Hogstrand, C., Galvez, F. & Munger, R. S. (1996). The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) 1. The effects of ionic Ag^{+}. *Aquatic Toxicology* **35**, 93–109.

Wu, Y., Zhou, Q., Li, H., Liu, W., Wang, T. & Jiang, G. (2010) Effects of silver nanoparticles on the development and histopathology biomarkers of Japanese medaka (*Oryzias latipes*) using the partial-life test. *Aquatic Toxicology* **100**, 160–167. doi: 10.1016/j.aquatox.2009.11.014

Chapter 3: Effect of nanosilver on metabolism in rainbow trout (*Oncorhynchus mykiss*)

Abstract

Nanosilver (nAg) is a type of nanoparticle incorporated into many consumer products for its antimicrobial properties. It is known to enter the aquatic environment and past studies have observed toxic effects to fish such as altered gene expression, gill damage, impaired gas exchange as well as mortality at high concentrations. Through these effects, it is possible that nAg may affect the energy costs of fish. This study investigated the effects of nAg on the metabolism of rainbow trout (*Oncorhynchus mykiss*). Fish were exposed to environmentally-relevant concentrations (0.28 ± 0.02 µg l^{-1}) and higher (47.60 ± 5.13 µg l^{-1}) for a chronic time period (28 d) and their standard metabolic rate (SMR), forced maximal metabolic rate (MMR$_f$), and spontaneous maximal metabolic rate (MMR$_s$) were measured. MMR$_s$ was significantly depressed in the high concentration exposure of nAg, but no significant differences in metabolic variables were observed between the different treatments when random effects were accounted for. MMR$_f$ and MMR$_s$ were found to be significantly positively correlated, but MMR$_s$ tended to be greater than MMR$_f$ on average. In conclusion, chronic nAg exposure for 28 d at these exposure concentrations had limited significant effects on the metabolism of *O. mykiss*, and suggests that exposures at environmentally-relevant concentrations are unlikely to directly affect fish metabolism.

Introduction

Nanosilver is a type of nanoparticle commonly used in many consumer products for its antimicrobial properties (Wijnhoven et al. 2009). From the washing of these products nAg has been shown to enter wastewater and affect the aquatic environment. Once in the aquatic environment nAg may dissolve to silver ions (Ag^+) or aggregate with compounds or other nAg particles (Choi et al. 2009).

Toxicity from nAg is thought to result from a combination of the effects of both the nAg and the Ag^+ it releases (Lapresta-Fernández et al. 2012), with nAg's main mode of toxicity being through oxidative stress, and silver ion's main mode being through inhibition of the Na^+/K^+ ATPase pump in gill cells leading to osmoregulatory impairment (Farkas et al. 2010). Toxic effects from nAg include altered gene expression, gill damage, impaired gas exchange as well as mortality at high concentrations (Bilberg et al. 2010; Farmen et al. 2012; Pham et al. 2012) (Appendix A).

Through these toxic effects nAg may affect the metabolism of fish. The term metabolism encompasses all energy costs of a fish, such as costs arising from basic bodily functions (respiration), activity, and digestion/absorption/processing of food (specific dynamic action). Standard metabolic rate (SMR) defines the minimal energy requirement of ectothermic animals at a specific temperature, and is made up of all of the basic functions of cellular metabolism such as protein synthesis and ATP turnover that keep a fish alive (Sokolova and Lannig 2008). Maximum metabolic rate (MMR) defines the maximal aerobic metabolic rate attainable (Norin and Malte 2012). These two rates encompass the aerobic scope (AS), which

describes the amount a fish can increase its metabolic activity above maintenance levels.

Nanosilver exposure could potentially affect SMR through its toxic effects on fish of altered gene expression and oxidative stress increasing the basic energy demands of a fish (Griffitt et al. 2012; Pham et al. 2012). Additionally, nAg exposure could affect MMR through it damage to the gills decreasing the ability of fish to take up oxygen under intense activity (Bilberg et al. 2010; Farmen et al. 2012; Griffitt et al. 2012). Due to the importance of metabolism to fish ecology, measures of metabolic rate provide a useful link between the physiological effects of contaminants and their effects on fish as a whole (Handy and Depledge 1999; McKenzie et al. 2007)

Objectives of this study were to investigate the effects of nAg on metabolism in rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) through chronic nAg exposure at environmentally relevant levels and higher. The toxic effects of nAg may potentially increase SMR or decrease MMR, and therefore may decrease AS. These potential metabolic changes caused by nAg exposure would decrease energy available for other integral processes such as activity, growth, and reproduction and therefore potentially decrease fish fitness or even fish survival.

Materials and Methods

Experimental animals

O. mykiss were obtained from Lyndon Fish Hatcheries (New Dundee, Ontario) on Feb 11, 2014 (n=200), and April 29, 2014 (n=200). All fish were female diploid fish and weighed approximately 1 g at arrival. Fish were quarantined for one week

in large (160-200 l) tanks, and throughout the experiment were kept at a water temperature of 14 °C. Fish were exposed to a 12:12 diurnal lighting regime with gradual light changes (lights turned on and off in three stages to mimic dawn and dusk) throughout the quarantine and experiment. After quarantine, 11 fish were placed in each of the 40 l exposure tanks, and acclimated for one week. At the time that respirometric experiments were initiated, fish weighed on average 2.91 ± 1.14 g.

Exposure experiment

Nanosilver exposure was achieved through a flow-through set-up, where a peristaltic pump transported nAg solution to the tanks. There were two exposure trials, with treatments comprising of 1) a control, and exposure concentrations of 1μg l^{-1}, and a 200 μg l^{-1} in the first trial, and 2) a control, and exposure concentrations of 300 μg l^{-1}, and a 600 μg l^{-1} in the second trial. However it was observed that measured nAg concentrations were much lower than nominal concentrations and quite variable (7-43% of nominal), and concentrations within the higher exposure tanks (200, 300, and 600 μg l^{-1} nominal) overlapped with each other, and did not differ statistically (ANOVA, $F_{5,85}$ = 15.228 p > 0.05). Due to this observation it was assumed that fish in the higher exposure treatments were all likely exposed to similar amounts of waterborne nAg, and therefore respirometry data for these groups was combined into a single group (called "high"). Therefore for analysis, we considered three treatment levels over both trials: the controls (from both trials), a low (1μg l^{-1}), and a high exposure (200, 300, and 600 μg l^{-1} nominal concentrations). Total silver measured in respirometry tanks was also

lower than nominal (Appendix D). Exposures were conducted for 28 d prior to experimentation. Further details on the nAg exposure trials can be found in Chapter 2.

Respirometry

Four cylindrical chambers (with length= 12.3 cm, diameter= 3.3 cm, and volume = 92 ml each) were installed in a 400 l tank with recirculating water. Each chamber was sealed and monitored for O_2 using an O_2 probe. Temperature was kept at 14 ± 0.5 °C with water baths, and water was kept oxygenated and mixed with air stones and pumps. Fish were exposed to the same 12:12 diurnal cycle as for the exposure experiment (with gradual light changes), and shielded from any external stimuli with a dark curtain draped around all edges of the tank. Activity in the room where experiments were conducted was also minimized. Equipment in the tank was kept to a minimum to reduce bacterial respiration and adhesion of nanoparticles. Oxygen probes were calibrated every week or when inconsistencies in oxygen measurements were observed. Before each trial nAg stock solution was added to the tank to match nominal exposure concentrations, and water samples were taken near the end of the experiment to ascertain actual Ag concentrations (Appendix D).

Oxygen consumption was measured by automatic intermittent-flow respirometry using a LoliResp system (Loligo Systems, Tjele, Denmark). In the respirometry system, water was pumped continuously by a flow pump (Eheim Universal Hobby Pump 1046, Germany) from each chamber to the OxyGuard oxygen electrode and back into the chamber. Each respirometry cycle lasted for 8.5 - 9 min and consisted of a flushing period of 3 min where water in the respirometer was

renewed with water from the holding tank, a 1 min wait period where the respirometer was closed but no oxygen concentration measurements were taken, and a 5.5-6 min measure period when oxygen concentrations were measured (the measurement period was shortened to 5.5 min due to low O_2 values during the course of the experiment). The rate of decline in oxygen concentration during this period permitted estimation of consumption rate (MO_2; see data analysis below).

Respirometry experiments

Respirometric experiments were conducted at the end of the exposures on four fish from each exposure tank that had not been fed for at least 24 h. Three respirometric rates were measured: standard metabolic rate (SMR), forced maximal metabolic rate (MMR_f), and spontaneous maximal metabolic rate (MMR_s). Estimation of SMR and MMR_s were estimated from fish held in respirometry chambers for a 24 h period (see data analysis below).

To estimate MMR_f, one fish at a time was placed in a circular bucket, and exhausted through a standardized 5 min manual chase method; 1 min chase, 3 min turning the fish over, and 1 min holding the fish out of water (similar to Roche et al. 2013). At the end of this 5 min chase, fish would not be capable of burst swimming, a sign of exhaustion (Milligan 1996). Immediately after chasing, fish would be placed into a respirometry chamber to measure maximal metabolic rate. For a subset of fish (the first 3 tanks tested out of 24), MMR_f was conducted at the end of the SMR measurements. The protocol was then changed to test MMR_f at the beginning of the respirometry trial, in an attempt to obtain higher MMR_f measurements by potentially capturing the immediate stress of transfer to a novel

environment (the respirometry chamber) in addition to the chase methods. The influence of the timing of MMR_f estimation on other respirometric variables was evaluated to ensure this change in methods did not influence results.

To minimize background bacterial respiration and contamination of nAg between experiments, the tank was drained and all surfaces cleaned and flushed with water between experiments. After respirometry experiments were completed the fish were measured, weighed, and euthanized by cervical dislocation. Muscle tissue was also taken for determination of total silver concentration.

Estimation of mass-specific metabolic rates

In order to mass-adjust metabolic rates (SMR, MMR_f, MMR_s) for the size of fish appropriately (e.g., convert mass-relative to mass-specific rates), we first needed to determine whether our rates were isometric with body size (Rennie et al. 2010). To do this, we examined $\log_{10}$-transformed relationships between each rate with $\log_{10}$-transformed body mass, including Treatments (C, L, H) as a grouping variable. For each rate, we first examined differences in slope among treatments. In all but one case, slopes were not significantly different among treatments (test for heterogeneity of slopes, all $p > 0.05$). In the case of MMR_f, a single outlier in the L treatment with high leverage resulted in a more elevated slope compared with the other two treatments (Test for Heterogeneity of slopes, $p = 0.045$) (Appendix E.1). Upon removal of this single outlier value, slopes were not significantly different among treatments ($p = 0.055$), and analysis proceeded without the outlier.

Next, for each metabolic rate, ANCOVA was used to determine a common slope value for instances where differences existed among treatments in the

relationship between $\log_{10}$(metabolic rate) and $\log_{10}$(weight). For SMR and MMR$_f$

the effect of treatment was not significant (ANCOVA, SMR: $F_{1,2} = 0.34$, $p = 0.71$,

MMRf: $F_{1,2} = 0.20$, $p = 0.82$). In the case of MMR$_s$, treatment was a significant effect

in the model (ANCOVA: $F_{1,2} = 3.61$, $p = 0.03$), with the high treatment significantly

lower than the control (Tukey's HSD, $p = 0.04$) (Appendix E.2). In this case, we used

the common slope from the ANCOVA, and evaluated its difference from a value

representing isometry (slope of 1) using a t-test.

Where there was no significant treatment effect in the ANCOVA models, we

ignored treatment and estimated the slope of each metabolic rate with body mass

for all observations (each $\log_{10}$-transformed). In all but one case, the slope of each

rate was not significantly different from 1 (t-test, SMR $t_{78} = 1.501$ $p = 0.544$; MMR$_s$ $t_{86} = -0.557$, $p = 0.491$). For these rates, we concluded that our metabolic rates were

isometric with a mass exponent equal to 1. In the case of MMR$_f$, the slope of the log-

log relationship of mass-relative rates against body size resulted in a slope of 0.88,

which was significantly different from a slope of 1 (t-test, $t_{90} = -1.717$, p= 0.045)

(Appendix E.2).

Data analysis

The basic equation for mass-specific metabolic rates was:

$$MO_2 = \frac{\Delta P_W O_2}{\Delta t} \cdot \beta \cdot \frac{(V_R - V_F)}{M^b}$$

where MO_2 is the oxygen consumption rate (mg O_2 kg^{-1} h^{-1}), $\Delta P_W O_2$ is the decline in

the partial oxygen pressure (kPa), Δt is the time elapsed during the closed

measuring period (h), consequently $\frac{\Delta P_W O_2}{\Delta t}$ is the oxygen depletion slope, β is the

oxygen solubility in water (= 0.4959 mg O_2 l^{-1} kPa^{-1} at 14°C) (Green and Carrit

1967), V_R the water volume in the respirometer system (l), V_F the fish volume (l),

and M the fish body mass (kg), and b is the mass exponent adjustment. For MMR$_f$,

X=0.88, but was 1 for all other respirometric rates.

Background MO$_2$ measurements from before and after each respirometry

trial were measured and averaged for each respirometer, and subtracted from all

fish MO$_2$ measurements. Individual MO$_2$ measurements with an r2 lower than 0.9

were determined to reflect poor linear estimates of O$_2$ consumption rates and were

eliminated. Estimates of MO$_2$ in the respirometers with low O$_2$ levels in the

respirometers were also eliminated to ensure fish were not oxygen-limited (<73.1%

or 7.6 mg L^{-1} as per Tang et al. 2000). This data processing resulted in the removal

of a small amount of MO$_2$ measurements (0.08% of measurements not used due to

low O$_2$, 0.16% of measurements not used due to low r2, for a total of 0.24% of the

data being removed from estimation of respirometric rates, out of approximately

13120 measurements).

SMR was estimated as the mean of the 10 lowest observed MO$_2$

measurements during the 24 h experiment (Bilberg et al. 2010). MMR$_f$ was

calculated as the highest single MO$_2$ measurement of the 3 measurements observed

immediately after the chase procedure (Svendsen et al. 2012). MMR$_s$ was calculated

as the highest single MO$_2$ measurement observed during the 24 h experiment

(excluding MMR$_f$ measurements)(Svendsen et al. 2014). Aerobic scopes between

both SMR and MMR$_f$ (AS$_f$), and SMR and MMR$_s$ (AS$_s$) were calculated by subtracting the SMR from the MMR$_f$ and MMR$_s$, respectively (Clark et al. 2013).

Statistical analysis

Analyses were conducted in R version 3.1.3 (R Foundation for Statistical Computing, 2015). Respirometry data (SMR, MMR$_f$, MMR$_s$, AS$_f$, AS$_s$) were analyzed using a mixed-effect model (lmer). The full model tested for effects was:

$$Respirometry\ variable = Treatment + Tank + Chamber + Time\ of\ MMR_f$$

where treatment was a fixed effect, and tank, chamber, and timing of MMR$_f$ (conducting MMR$_f$ procedure before or after SMR experiments) were random effects. In order to determine the significance of nanosilver exposure on rainbow trout metabolic rates, we used log-likelihood tests comparing the full model (above) to a reduced model (the full model without the Treatment effect).

The relationship between MMR$_f$ and MMR$_s$ was also tested using a mixed model approach with the full model:

$$MMR_s = MMR_f + Tank + Chamber + Time\ of\ MMR_f$$

To determine the significance of the relationship between MMR$_s$ and MMR$_f$, the model was compared to a reduced model with the MMR$_f$ variable absent using a log-likelihood test. The effect of timing of MMR$_f$ chase procedure on respirometry variables was analyzed using a Welch two sample t-test. No significant differences were found for most respirometric variables between the controls of each trial (ANOVA, $p > 0.05$), so controls were combined into a single treatment and trial was

not considered a factor in the analysis. For MMR$_f$, a significant difference was found between trial controls (likely due to the change in timing of MMR$_f$ procedure), and trial was included in the models as a random effect. Residual plots of the models were examined for normality and homogeneity among treatments and in all cases were found to meet assumptions. Where appropriate, post-hoc analyses were conducted with Tukey's HSD test.

Results

Most of the total silver detected in the exposure tanks was in particulate form, with on average only 0.05% as ionic silver. Silver was taken up into fish muscle tissue, with a high degree of variation in the concentrations both within treatments and across time. Nanosilver concentrations in high treatment fish differed significantly from those in control and low treatments (Tukey's HSD; p_{C-H} = 0.006, p_{L-H} = 0.02), while no significant difference was found between control and low treatments (Tukey's HSD; p = 0.87). Similarly, no significant differences were found for the interaction between time and treatments (ANOVA, $F_{(df=8)}$=2.86, p = 0.07). Additional details of water and fish silver concentrations can be found in Chapter 2.

Respirometry

No difference by nAg treatment was found for SMR, which had an overall mean of 119 mg O$_2$ kg^{-1} h^{-1} (Figure 9a, Table VI; log-likelihood test, $\chi^2(df = 2) = 2.682$ $p = 0.262$). There was also no difference between treatments for MMR$_f$, which had

an overall mean of 470 mg O_2 kg^{-1} h^{-1} (Figure 9b, Table VI; log-likelihood test, χ^2(df =

2) = 0.202 p = 0.904), or MMR$_s$, which had an overall mean of 493 mg O_2 kg^{-1} h^{-1},

although the High treatment was lower than other treatments. (Figure 9c, Table VI;

log-likelihood test, χ^2(df = 2) = 5.279 p = 0.071). Finally, no difference by treatment

was found for either AS$_f$, with an overall mean of 357 mg O_2 kg^{-1} h^{-1} (Table VI; log-

likelihood test, χ^2(df = 2) = 0.081 p = 0.960), or AS$_s$ with an overall mean of 376 mg

O_2 kg^{-1} h^{-1} (Table VI; log-likelihood test, χ^2(df = 2) = 3.409 p = 0.182)

Table VI.   **Mean and standard error values for metabolic variables in different nAg exposure treatments.**

Exposure levels	SMR (mg O_2 kg^{-1} h^{-1})		MMR$_f$ (mg O_2 kg^{-1} h^{-1})		MMR$_s$ (mg O_2 kg^{-1} h^{-1})		AS$_f$ (mg O_2 kg^{-1} h^{-1})		AS$_s$ (mg O_2 kg^{-1} h^{-1})	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Control	119.66	5.33	474.82	17.58	515.79	15.26	366.70	19.31	389.17	13.82
Low	128.35	6.95	455.88	26.06	525.43	14.94	344.06	22.30	401.79	18.59
High	114.84	3.97	471.92	15.52	467.23	12.55	356.04	19.02	358.05	11.82
Overall	118.88		470.25		492.58		357.29		375.65	
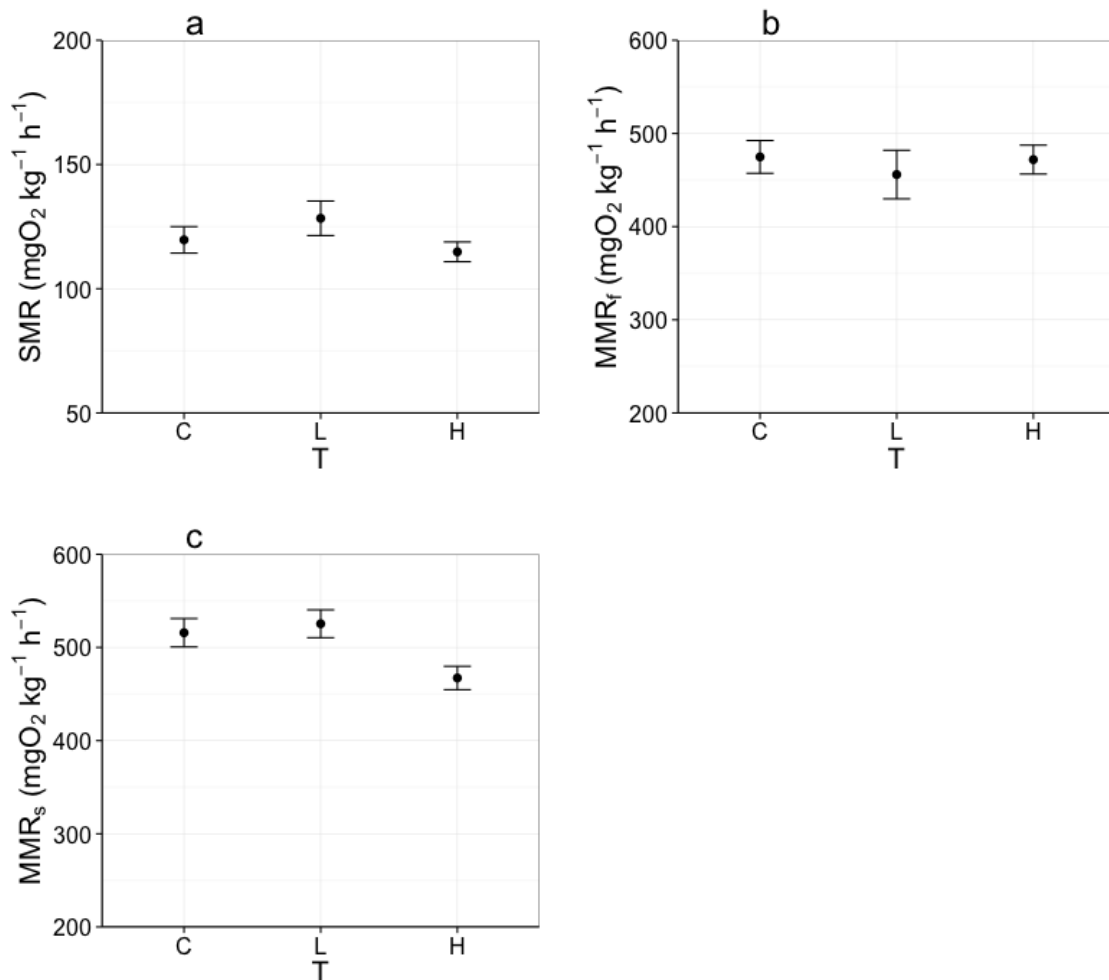
Figure 9. Metabolic variables for different treatment (T) levels. Data expressed as means ± standard errors. Panel a: standard metabolic rate (SMR). Panel b: forced maximal metabolic rate (MMR_f). Panel c: spontaneous maximal metabolic rate (MMR_s).

MMR_f and MMR_s

MMR_s was higher than MMR_f 58% of the time (out of 88 measurements). Controlling for random effects, the two variables were significantly correlated (log-likelihood test, $\chi^2(df = 1) = 52.988$, $p < 0.001$) with MMR_f being on average 98% of MMR_s (Fig 10). Interestingly, MMR_s most often occurred at the moment when the room lighting changed, particularly when the lights turned on in the room the

respirometers were in (63.3% of MMR_s measurements). An example of the typical

pattern of MO_2 in the experiments can be seen in Figure 11, with the timing of lights

going on and off marked.
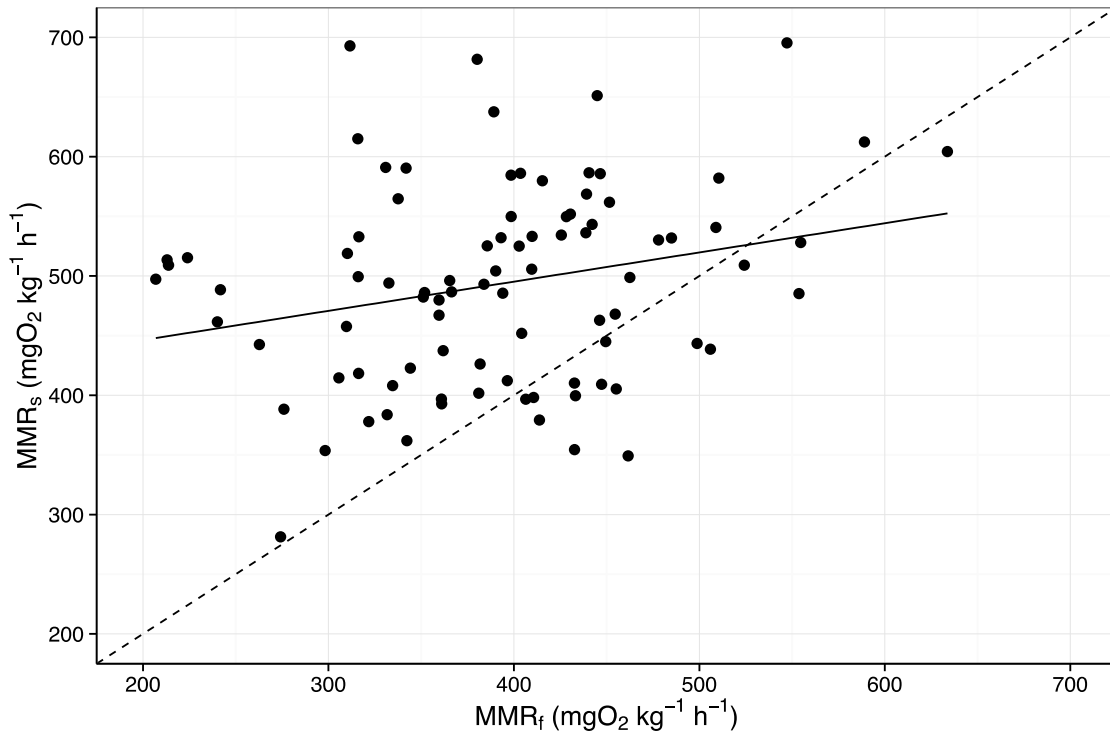
Figure 10. **Relationship between spontaneous maximal metabolic rate (MMR_s) and forced maximal metabolic rate (MMR_f). Dashed line is 1:1 line, solid line is regression line between metabolic rates.**
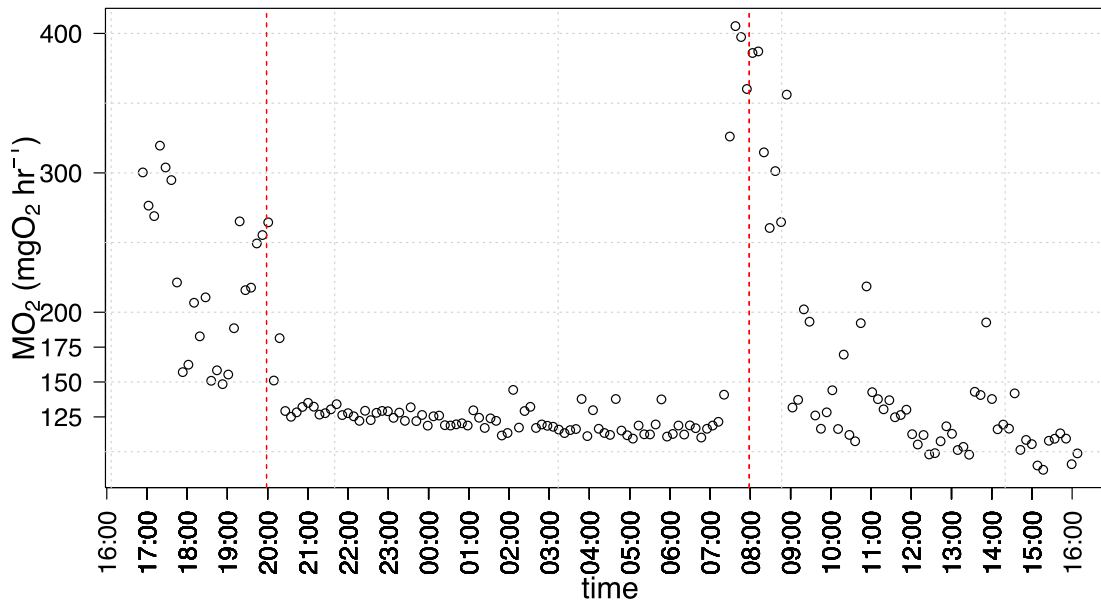
Figure 11. SMR experiment for a sample trial fish (L7-CH4), showing MO_2 over 24 h. Timing of changes in lighting (lights turned on at 8:00 h, and off at 20:00 h) are marked with hashed red lines.

Effect of the timing of MMR_f procedure

Metabolic variables were also analyzed for the effect of timing of MMR_f procedure (manually chasing the fish either before or after the 24 h SMR experiment) in trial 1. MMR_f and AS_f were found to be higher if the MMR_f procedure was conducted before the respirometric experiment (Figure 12; t-test; MMR_f, $t_{11.01}$ = -3.015, $p < 0.012$; AS_f $t_{8.029}$ = -5.738, $p < 0.001$). The mean for MMR_f for MMR_f before was 481.44 ± 10.60 (n = 82), and 378.49 ± 32.45 (n = 10) after. The mean for AS_f for MMR_f before was 368.54 ± 11.91 (n = 73), and 220.36 ± 22.91 (n = 6) after. SMR was found to be lower if the MMR_f procedure was conducted before the respirometric experiments (Figure 11; t-test; $t_{77.812}$ = 3.117, $p < 0.003$). The mean for SMR before was 116.43 ± 2.88 (n = 73), and 144.44 ± 11.22 (n = 7) for MMR_f

after. No significant difference was found for the other metabolic variables (MMR_s and AS_s) based on the timing of MMR_f procedure.
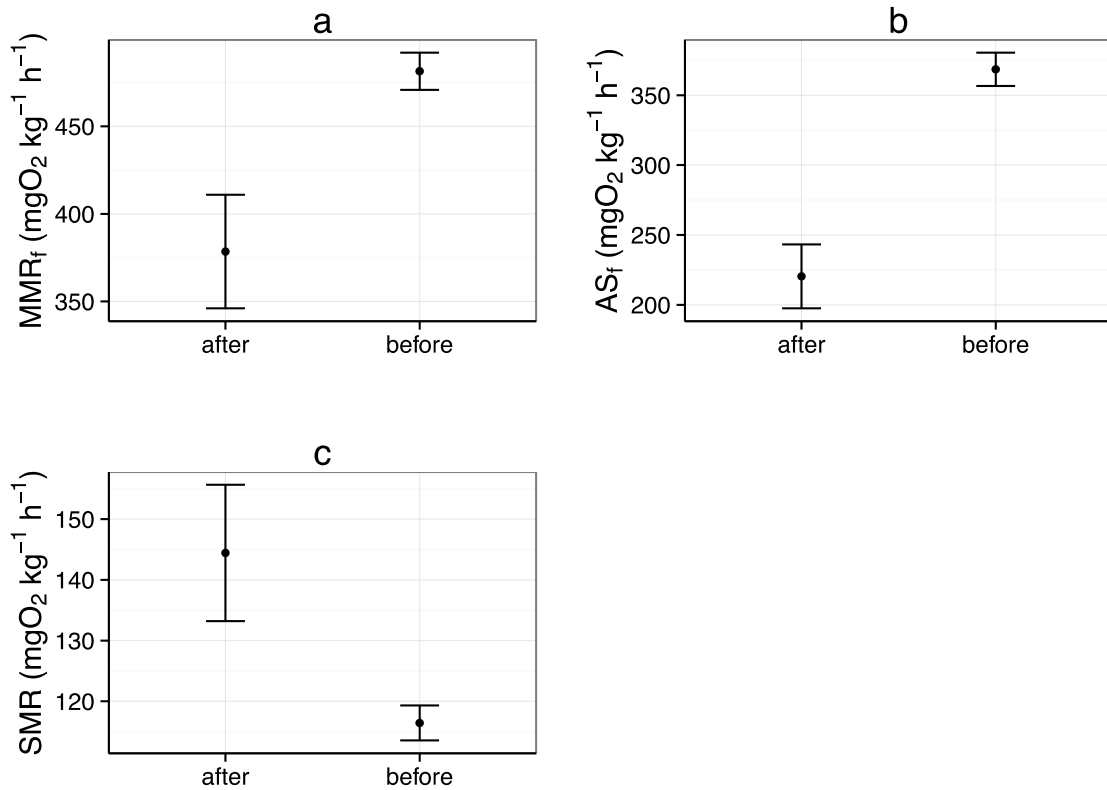
Figure 12. Differences in metabolic variables by timing of MMR_f procedure (before or after SMR experiment). Panel a: forced maximal metabolic rate (MMR_f). Panel b: forced aerobic scope (AS_f). Panel c: standard metabolic rate (SMR).

Discussion

nAg effects on metabolism

Once random effects were accounted for, results showed no significant effect of nAg on metabolic variables of SMR, MMR_f of rainbow trout. Effects on MMR_s were significantly depressed relative to controls using body size as a covariate, but were only marginally insignificant once random effects were accounted for. Similar to

our results, Bilberg et al. (2010) did not observe an effect of nAg on SMR of Eurasian perch (*Perca fluviatilis*) at concentrations from 63-300 μg l^{-1} over 24 h, but nAg did decrease hypoxia tolerance at 300 μg l^{-1} exposure. In the same study, bulk silver (in the form of silver nitrate) increased SMR and decreased hypoxia tolerance. Cowart et al. (2011) also found no effect of nAg on the metabolic rate of zebrafish (*Danio rerio*) embryos exposed for 24-48 h to high levels of nAg (20,000 – 140,000 μg l^{-1}). From these studies as well as the present study's results it appears that nAg has no significant effect on SMR. This is perhaps surprising, since one would predict SMR to be elevated as a consequence of higher blood plasma cortisol levels in nAg exposed fish (Chapter 2), as well as the other toxic effects of nAg. Other studies showed an effect on metabolism when fish were under additional stress (hypoxia); which one might expect due to the gill damage nAg has been observed to cause in fish (Farmen et al. 2012; Griffit et al. 2012). This study did find some evidence that MMR$_s$ may be depressed at a high concentration of nAg, which may indicate some effect of nAg on maximal metabolic rates, though this effect was muted once random effects were accounted for.

To our knowledge, no other studies have been conducted on the effects of other nanoparticles on fish metabolism. However, a variety of studies have analyzed the effect of ionic metals on metabolic rate, and generally indicate that SMR is usually increased and MMR is usually decreased, but appears to depend on exposure time (chronic versus acute). In chronic exposures to low but environmentally-relevant concentrations metals usually either have no effect to metabolism or

increase it, while acute exposures to higher concentrations may decrease

metabolism (Sokolova and Lannig 2008).

For example, the effect of aluminum (Al) on rainbow trout in acidic water

exposed for 36 d was found to decrease MMR_f and AS_f by approximately half, and

there was a trend for increased SMR (Wilson et al. 1994). Lead (Pb) decreased MMR_f

during acute exposure (24 h), but not in chronic exposures of 33-57 d in fathead

minnow (*Pimphales promelas*), indicating that fish were able to recover from the

effects of Pb on metabolism (Mager and Grosell 2011). It is possible that this same

phenomenon of recovery with chronic exposure to a contaminant may be at work in

this study, and may be why no significant effect on metabolic rates was seen after 28

d, while Bilberg et al. (2010) did see a significant effect on metabolism when fish

were under additional stress (hypoxia) following their shorter (24 h) exposure. A

pattern of recovery of other toxic effects from nAg over time also supports this. For

example Griffit et al. (2012) observed that gill epithelial proliferation was more

distinct at 14 d exposure in comparison to 35 d, and in Pham et al. (2012), altered

gene expression was often greater at 7 or 21 d exposure versus 28 d.

Maximal metabolic rates

MMR_f was typically lower than MMR_s in most cases. This indicates that the

chase protocol of 5 min (consisting of 1 min chase, 3 min turning fish over, and 1

min air exposure) was not sufficient to obtain as high of a maximal metabolic rate

compared with spontaneous rates observed in the chambers. While critical

swimming speed trials can also be used to obtain MMR, chase protocols are widely

used as well. Roche et al. (2013) found that swim trials did obtain the highest MMR,

with chase trials reflecting 36% lower MMR than swim trials, and only 23% lower when an air exposure element was added to the chase trial (Roche et al. 2013). They therefore suggested that a chase with an air exposure element should be used if swim trials were not possible, as was the methodology followed in this study. Other studies have suggested that chase and swim trials give similar results (Cutts et al. 2002). Our results support the idea that chase trials may not obtain true MMR (as our MMR_s were higher than MMR_f in most cases). Comparisons between swimming trials and MMR_s should be conducted in future studies.

While MMR_s is less often used as a metabolic metric than MMR_f, it has been used in other studies and there is evidence that it is ecologically relevant (Svendsen et al. 2014). Animals may not exercise at maximal intensity very often under natural conditions and spontaneous maximal performance is likely used more frequently. Therefore spontaneous performance may be more indicative of how the animal will behave in nature, and how the stressor under study will affect it (Husak 2006).

MMR_f and MMR_s in this study were correlated, supporting similar findings reported elsewhere (Svendsen et al. 2014). This suggests that while MMR_f was typically lower than MMR_s, they do appear to reflect similar mechanisms. In our study, MMR_s was usually caused by light disturbances in the room. The effect of light changes on respirometric rates has been observed before, and because of this many studies expose fish to only dim lighting during respirometry trials, or otherwise shield fish from large changes in light intensity (Morgan and Iwama 1996; Norin and Malte 2011; Svendsen et al. 2012). This was not done in this study; fish were exposed to the same lighting regime as they were throughout the 28 d exposure

trials. This lighting regime did employ gradual light changes, but appears to have still been sufficient to cause a reaction in fish. However, this may also simply reflect natural patterns of metabolic expenditure, since fish are traditionally known to be most active during the crepuscular periods of sunrise and sunset (Helfman 1981).

MMR_f was approximately 21% higher when fish were chased before the SMR experiments, likely due to the increased stress fish were experiencing from being in the new environment of the respirometry tubes, combining with exercise stress when the chase protocol was conducted first. This suggests that in general it may be beneficial to conduct chase protocols before SMR trials, to increase the metabolic rate that is achieved. Conducting chase protocols was also found to decrease SMR measurements by approximately 20 %. The reason for this difference was unknown, as it was expected that the timing of the chase protocol would either have no effect or potentially increase SMR if done before (due to residual stress or post-exercise recovery). Regardless, any differences were accounted for in analysis through inclusion of the variable in the mixed-model design used.

Overall, no significant effects of chronic nAg exposure to rainbow trout metabolism were found in our study. This is despite the facts that fish took up nAg into muscle and other stress responses were observed from nAg exposure in this study, such as increased cortisol response (Chapter 2). This may indicate that fish were able to put into place adaptive mechanisms to counteract the toxic effects of nAg exposure without any significant additional metabolic costs over the chronic exposure. Lack of metabolic effects may also be due to the masking effect of the stress caused by the respirometry methods themselves (Chapter 4), making any

toxic/stress effects of nAg undetectable. Future studies could focus on investigating

different exposure periods on fish to capture any shorter-term effects (potentially

before adaptive mechanisms are initiated).

References

Bilberg, K., Malte, H., Wang, T. & Baatrup, E. (2010). Silver nanoparticles and silver nitrate cause respiratory stress in Eurasian perch (*Perca fluviatilis*). *Aquatic Toxicology* **96**,159–65. doi: 10.1016/j.aquatox.2009.10.019

Choi, O., Clevenger, T. E., Deng, B., Surampalli, R. Y., Ross, L. & Hu, Z. (2009). Role of sulfide and ligand strength in controlling nanosilver toxicity. *Water Research* **43**, 1879–1886. doi: 10.1016.j.waters.2009.1.029

Clark, T. D., Sandblom, E. & Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *The Journal of Experimental Biology* **216**, 2771–2782. doi: 10.1242/jeb.084251

Cowart, D. A., Guida, S. M., Shah, S. I., & Marsh, A. G. (2011). Effects of Ag nanoparticles on survival and oxygen consumption of zebra fish embryos, *Danio rerio. Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering* **46(10)**, 1122–1128. doi: 10.1080/10934529.2011.590726

Cutts, C. J., Metcalfe, N. B., & Taylor, A. C. (2002). Juvenile Atlantic salmon (*Salmo salar*) with relatively high standard metabolic rates have small metabolic scopes. *Functional Ecology* **16**, 73–78.

Farkas, J., Christian, P., Urrea, J. A. G., Roos, N., Hassellöv, M., Tollefsen, K. E., & Thomas, K. V. (2010). Effects of silver and gold nanoparticles on rainbow trout

(*Oncorhynchus mykiss*) hepatocytes. Aquatic Toxicology **96**, 44–52. doi: 10.1016/j.aquatox.2009.09.016

Farmen, E., Mikkelsen, H. N., Evensen, O., Einset, J., Heier, L. S., Rosseland, B. O., Salbu, B., Tollefsen, K. E., & Oughton, D. H. (2012). Acute and sub-lethal effects in juvenile Atlantic salmon exposed to low μg/L concentrations of Ag nanoparticles. *Aquatic Toxicology* **108**, 78–84. doi: 10.1016/j.aquatox.2011.07.007

Green, E. J. & Carrit, D. E. (1967). New tables for oxygen saturation of sea water. *Journal of Marine Biology* **25**, 140-147.

Griffitt, R. J., Brown-Peterson, N. J., Savin, D. A., Manning, C. S., Boube, I., Ryan, R. A., & Brouwer, M. 2012. Effects of chronic nanoparticulate silver exposure to adult and juvenile sheepshead minnows (*Cyprinodon variegatus*). *Environmental Toxicology and Chemistry* **31(1)**, 160–167. doi: 10.1002/etc.709

Handy, R. D., & Depledge, M.H. (1999). Physiological responses: their measurement and use as environmental biomarkers in ecotoxicology. *Ecotoxicology* **8**, 329–349.

Helfman, G. S. (1981). Twilight activities and temporal structure in a freshwater fish community. *Canadian Journal of Fisheries and Aquatic Sciences* **38**, 1405-1420.

Husak, J. F. (2006). Does survival depend on how fast you *can* run or how fast you *do* run? *Functional Ecology* **20**, 1080-1086. doi: 10.1111/j.1365-2435.2006.01195.x.

Lapresta-Fernández, A., Fernández, A., & Blasco, J. (2012). Nanoecotoxicity effects of engineered silver and gold nanoparticles in aquatic organisms. *Trends in Analytical Chemistry*. **32**, 40–59. doi: 10.1016/j.trac.2011.09.007

Mager, E. M., Grosell, M. (2011). Effects of acute and chronic waterborne lead exposure on the swimming performance and aerobic scope of fathead minnows (*Pimephales promelas*). *Comparative Biochemistry and Physiology. Part C* **154**, 7–13. doi: 10.1016/j.cbpc.2011.03.002

McKenzie, D. J., Garofalo, E., Winter, M. J., Ceradini, S., Verweij, F., Day, N., Hayes, R., van der Oost, R., Butler, P. J. & Chipman, J. K. (2007). Complex physiological traits as biomarkers of the sub-lethal toxicological effects of pollutant exposure in fishes. *Philosophical Transactions of the Royal Society B* **362**, 2043–2059. doi: 10.1098/rstb.2007.2100

Milligan, C. L. (1996). Metabolic recovery from exhaustive exercise in Rainbow trout. *Comparative Biochemistry and Physiology* **113A(1)**, 51-60.

Morgan, J. D. & Iwama, G. K. (1996). Cortisol-induced changes in oxygen consumption and ionic regulation in coastal cutthroat trout (*Oncorhynchus clarki clarki*) parr. *Fish Physiology and Biochemistry* **15(5)**, 385–394.

Norin, T. & Malte, H. (2011). Repeatability of standard metabolic rate, active metabolic rate and aerobic scope in young brown trout during a period of moderate food availability. *The Journal of Experimental Biology* **214**, 1668–75. doi: 10.1242/jeb.054205

Norin, T. & Malte, H. (2012). Intraspecific variation in aerobic metabolic rate of fish: relations with organ size and enzyme activity in brown trout. *Physiological and Biochemical Zoology* **85(6)**, 645–656. doi: 10.1086/665982

Pham, C. H., Yi, J. & Gu, M. B. (2012). Biomarker gene response in male Medaka (*Oryzias latipes*) chronically exposed to silver nanoparticle. *Ecotoxicology and Environmental Safety* **78**, 239–245. doi: 10.1016/j.ecoenv.2011.11.034

Rennie, M. D., Purchase, C. F., Shuter, B. J., Collins, N. C., Abrams, P. A. & Morgan, G. E. (2010). Prey life-history and bioenergetic responses across a predation gradient. *Journal of Fish Biology* **77**, 1230–1251. doi: 10.1111/j.1095-8649.2010.02735.x

Roche, D. G., Binning, S. A., Bosiger, Y., Johansen, J. L. & Rummer, J. L. (2013). Finding the best estimates of metabolic rates in a coral reef fish. *The Journal of Experimental Biology* **216**, 2103–2110. doi: 10.1242/jeb.082925

Sokolova, I. & Lannig, G. (2008). Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. *Climate Research* **37**, 181–201. doi: 10.3354/cr00764

Svendsen, J. C., Genz, J., Anderson, G. W., Stol, J. A., Watkinson, D. A., Enders, E. C. (2014). Evidence of circadian rhythm, oxygen regulation capacity, metabolic repeatability and positive correlations between forced and spontaneous maximal metabolic rates in lake sturgeon *Acipenser fulvescens*. *PLoS One* **9(4)**, e94693. doi: 10.1371/journal.pone.0094693

Svendsen, J. C., Steffensen, J. F., Aarestrup, K., Frisk, M., Etzerodt, A. & Jyde, M. (2012). Excess posthypoxic oxygen consumption in rainbow trout (*Oncorhynchus mykiss*): recovery in normoxia and hypoxia. *Canadian Journal of Zoology* **11**, 1–11. doi: 10.1139/Z11-095

Tang, M., Boisclar, D., Mènard, C. & Downing, J. (2000). Influence of body weight, swimming characteristics, and water temperature on the cost of swimming in brook trout (Salvelinus fontinalis). *Canadian Journal of Fisheries and Aquatic Sciences* **57**, 1482-1488.

Wijnhoven, S. W. P., Peijnenburg, W. J. G. M., Herberts, C. A., Hagens, W. I., Oomen, A. G., Heugens, E. H. W., Roszek, B., Bisschops, J., Gosens, I. & Van De Meent, D., (2009). Nano-silver – a review of available data and knowledge gaps in human and environmental risk assessment. *Nanotoxicology* **3(2)**, 109–138. doi: 10.1080/17435390902725914

Wilson, R. W., Bergman, H. L. & Wood, C. M. (1994). Metabolic costs and physiological consequences of acclimation to aluminum in juvenile Rainbow trout

(*Oncorhynchus mykiss*). 2: gill morphology, swimming performance, and aerobic scope. *Canadian Journal of Fisheries and Aquatic Sciences* **51**, 536–544.

Chapter 4: Effect of respirometric experiments on cortisol release in rainbow trout *Oncorhynchus mykiss*

Abstract

While respirometry is a key method to measure standard metabolic rate in fish, handling and confining the tested animal to a relatively small chamber during the respirometric experiment may be stressful for the animal, and could potentially affect the metabolic measurements. Using juvenile rainbow trout *Oncorhynchus mykiss*, this study observed increased cortisol levels in animals tested using intermittent-flow respirometry, suggesting that respirometric experiments may stress the animal, even after a 24 h exposure period to the respirometer chamber.

Introduction

Metabolism encompasses all energetic costs of a fish, such as costs arising from basic bodily functions, activity, and digestion/absorption/processing of food (Brett, 1964), and is therefore an important parameter in fish behaviour and survival (Killen et al. 2007). It can be measured through oxygen consumption rate (MO$_2$) using respirometric experiments (Jobling, 1994). Standard metabolic rate (SMR) is estimated in inactive fish that are post-absorptive and have no oxygen debt (Brett, 1964), usually by testing fish in a respirometry chamber in which MO$_2$ measurements are made over at least 24 h. Maximum metabolic rate (MMR) is obtained by exercising a fish intensively often to exhaustion through either forced swimming in a swim tunnel or manually chasing and transferring it to a static

respirometer chamber and measuring $M\text{O}_2$ during or directly after the exercise, respectively. The transfer of fish to the chamber and the confinement in the chamber representing a new and space-constrained environment may be stressful to the fish. Consequently, $M\text{O}_2$ may be affected if fish do not habituate to the chamber during the experiment (Clark et al. 2013).

Cortisol is one of the most common stress indicators in fish (Martinez-Porchas *et al.*, 2009). Therefore, measuring cortisol release in fish that undergo respirometric experiments is a useful method to determine stress levels in test animals. Several studies have shown a positive relationship between cortisol level and metabolism (Morgan & Iwama, 1996; O'Conner et al. 2011). To obtain reliable and relevant SMR estimates from respirometric experiments, it is therefore crucial that fish are not stressed as this could lead to elevated cortisol levels and consequently increased $M\text{O}_2$ during the experiment.

This study was part of a larger investigation on the effects of the contaminant nanosilver (nAg) on the stress responses of rainbow trout *Oncorhynchus mykiss* (Walbaum 1792). The objective of this article is to describe observed cortisol levels of fish from four procedural groups after a 28 d nAg exposure (Table I). The effect of the four different procedures on cortisol levels was analysed by comparing the four otherwise identical groups.

Methods

Juvenile O. *mykiss* (n = 109, body mass: 2.91 ± 1.14 g) were exposed to three concentrations of nAg for a period of 28 d over two consecutive trials. Concentrations of nAg consisted of a control of 0 µg l^{-1} nAg, a low concentration of

0.28 µg l⁻¹, and a high concentration of 47.60 µg l⁻¹. After 28 d, blood samples were immediately taken from Group 1 and analysed for blood plasma cortisol levels (hereafter referred as cortisol). Groups 2, 3 and 4 were not fed for 24 h before the respirometric experiment. Group 2 was manually chased for 5 min and then immediately placed in the respirometer to estimate MMR by using the highest of the three MO$_2$ following the chase protocol (Roche *et al.*, 2013). Subsequently, SMR was obtained by leaving the fish undisturbed in the chamber over 24 h, measuring MO$_2$ over this period every 8.5 – 9 min and averaging the ten lowest MO$_2$. After the respirometric experiment, blood plasma was immediately sampled for cortisol analysis. Group 3 underwent respirometric experiments for SMR first, and were then chased for the MMR experiment followed by cortisol sampling. Finally, Group 4 was treated identically to Group 2 except fish were exposed to hypoxia after the SMR experiment by shutting off the flush pump until the fish lost equilibrium in the chamber. Plasma samples were analysed for cortisol through High Performance Liquid Chromatography Tandem Mass Spectrometry.

Statistical analysis

Statistical analyses were conducted in R version 3.1.3 (R Foundation for Statistical Computing, 2015). Differences in cortisol were analysed using a mixed-effects model (lmer) with trial and tank as random effects, and procedural type as a fixed effect. The effect of the four procedures on cortisol was evaluated through the model:

Cortisol = Procedure + Tank + Trial

In order to determine the significance of the procedure on cortisol, log-likelihood tests comparing the full model (above) to a reduced model (the full model without the procedure effect).

As this study was conducted as part of a larger experiment on the effects of nAg exposure on the fish metabolism, differences in cortisol of different nAg concentrations in respirometry fish were also analysed through a mixed-effect model (with trial and tank as random effects), and the effect of nAg concentration as a fixed effect. The effect of nAg concentration was evaluated through the model:

Cortisol = Treatment + Tank + Trial

A log-likelihood test comparing the full model to a reduced model (the full model without the nAg concentration effect included) was used to determine the significance of nAg concentration on cortisol.

The relationship between SMR and cortisol in respirometry fish not immediately exposed to a stressor before cortisol sampling (Group 2) was analysed using the same mixed model approach with the full model:

SMR = Cortisol + Tank + Chamber

To determine the significance of the relationship between SMR and cortisol, the model was compared to a reduced model with the cortisol variable absent using a log-likelihood test. In all cases cortisol data was $\log_{10}$-transformed to meet the assumptions of normality and homogeneity of residual distributions. Post-hoc analyses were conducted with Tukey's HSD test when significant differences were found with the log-likelihood tests.

Results

Mean ± S.E. cortisol levels were 9.90 ± 2.21 ng ml^{-1} for Group 1, 138.89 ± 15.59 ng ml^{-1} for Group 2, 138.44 ± 24.89 ng ml^{-1} for Group 3, and 365.27 ± 35.95 ng ml^{-1} for Group 4. Groups 2 and 3 (that underwent respirometric experiments) had 14-times higher cortisol levels than Group 1 (that did not undergo respirometry), and Group 4 (that underwent respirometry and hypoxia exposure) had 37-times higher cortisol levels than Group 1 (Fig. 13; Log-likelihood test, χ^2= 84.71; $d.f.$ = 3; P < 0.001). Cortisol levels from Groups 2, 3 and 4 did not differ significantly (Tukey's HSD; P_{Grp2-3} = 0.99, P $_{Grp2-4}$ = 0·301, P_{Grp3-4} = 0.483). No difference by nAg concentration was found for the cortisol levels of fish (Log-likelihood test, χ^2= 2.49; $d.f.$ = 2; P = 0.29). Additionally, no significant relationship was found between SMR and cortisol levels in respirometry fish not exposed to a stressor (Group 2) (Log-likelihood test, χ^2= 0.09; $d.f.$ = 1; P < 0.76.

Table I. Descriptions of procedures for the four groups.

Group	n	Procedure
1	37	blood samples were taken immediately
2	37	manually chased for 5 min $\rightarrow$ MMR $\rightarrow$ SMR $\rightarrow$ blood samples
3	12	SMR $\rightarrow$ manually chased for 5 min $\rightarrow$ MMR $\rightarrow$ blood samples
4	23	manually chased for 5 min $\rightarrow$ MMR $\rightarrow$ SMR $\rightarrow$ hypoxia $\rightarrow$ blood samples
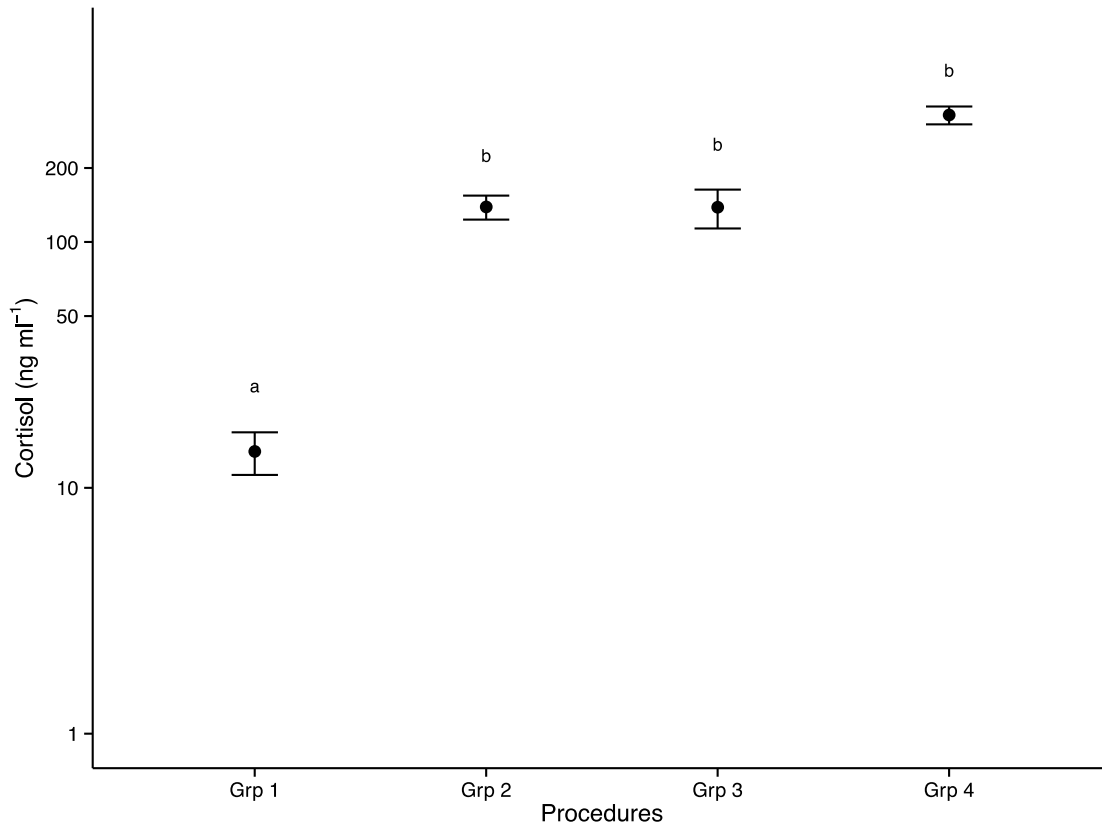
Figure 13. Blood plasma cortisol concentrations for different procedures (Grp 1 – immediately sampled for cortisol, Grp 2 – chased, respirometric experiments, sampled for cortisol, Grp 3 – respirometric experiments, chased, sampled for cortisol, Grp 4 – chased, respirometric experiments, exposed to hypoxia, sampled for cortisol). Data shown are means ± standard errors.

Discussion

A wide range of plasma cortisol levels have been described in the literature for unstressed *O. mykiss* ranging from 1.7 ng ml^{-1} (Barton, 2000) to 50.0 ng ml^{-1} (Webb & Wood 1998). Consequently, the result of 9.90 ng ml^{-1} for Group 1 from this study (fish not involved in respirometry) lies within this range, indicating that these fish were not stressed. Cortisol levels in stressed *O. mykiss* range from 43.3 (Auperin & Geslin, 2008) to 155.0 ng ml^{-1} (Woodward & Strange, 1987). Again, the observed cortisol level of 138.9 ng ml^{-1} for Group 2 falls in this range indicating that these fish were stressed. Group 3 had similar levels to Group 2, indicating a similar level of

stress in these fish. Group 4 had much higher cortisol levels, agreeing with other studies that have found that hypoxia causes high stress levels (Maxime et al. 1995).

After exposure to stress, cortisol levels usually return to baseline within 2 to 3 h (Woodward & Strange, 1987; Auperin & Geslin, 2008), indicating that the elevated levels seen in Group 2 were not residually elevated levels from chase methods performed at the beginning of the respirometric experiments, but stressed from the respirometry itself. SMR estimates from this study (mean = 118.88 mg O_2 kg^{-1} h^{-1}) were comparable to ones found by Blake & Chan (2006) for similarly sized *O. mykiss* (3.5 g).

Respirometry has been recognized to potentially cause stress (Martins *et al.*, 2011). Respirometry chambers are new environments where fish are constrained in a small area and isolated from its mates. It is usually assumed that SMR is obtained after the fish has acclimated to the chamber, several hours into the respirometric experiment. However, the results of this study suggest that fish did not habituate to the experimental setting, displaying increased cortisol level even after 24 h. If fish remain in a stressed state throughout the experiment MO_2 may be increased, impeding an accurate estimation of SMR.

In contrast to this study, Morgan & Iwama (1996) found only slightly (but not significantly) increased cortisol levels in juvenile (37.6 g) cutthroat trout *Oncorhynchus clarkii* (Richardson 1836) in fish submitted to respirometric experiments. However, Morgan & Iwama (1996) used swim tunnels and fish were swimming at low speeds during experiments as opposed to stationary respirometry used in this study. Swimming at low speeds has been shown to decrease cortisol

levels and hasten metabolic recovery (*i.e.* replenishment of muscle glycogen, clearance of lactate load) from exhaustive exercise in salmonids (Boesgaard et al. 1993). Swimming has also been shown to have a positive effect on growth (Skov et al. 2011), indicating that remaining stationary in itself may be stressful for these species and the reason for the observed elevated stress levels.

In conclusion, the stress response caused by exposure to a static respirometry method may interfere with metabolic measurements, artificially increasing estimates of SMR. Past studies have not found such an elevated stress response. Consequently, it would be useful to conduct additional research in this area to explore these differences in results and obtain a more informed knowledge of the amount of stress caused by respirometry. At a minimum, this study suggests that researchers should seek independent evaluation (*e.g.* via cortisol analysis) of the stress caused by respirometry to evaluate its effect on M_{O_2} measurements.

References

Auperin, B. & Geslin, M. (2008). Plasma cortisol response to stress in juvenile rainbow trout is influenced by their life history during early development and by egg cortisol content. *General and Comparative Endocrinology* **158**, 234–239. doi:10.1016/j.ygcen.2008.07.002

Barton, B. A. (2000). Salmonid fishes differ in their cortisol and glucose responses to handling and transport stress. *North American Journal of Aquaculture* **62**, 12–18. doi:10.1577/1548-8454(2000)062<0012:SFDITC>2.0.CO;2

Blake, R. W. & Chan, K. H. S. (2006). Cyclic feeding and subsequent compensatory growth do not significantly impact standard metabolic rate or critical swimming speed in rainbow trout. *Journal of Fish Biology* **69**, 818–827. doi:10.1111/j.1095-8649.2006.01152.x

Boesgaard, L., Nielsen, M. E. & Rosenkilde, P. (1993). Moderate exercise decreases plasma cortisol levels in Atlantic salmon (*Salmo salar*). *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* **106**, 641–643.

Brett, J.R. (1964). The respiratory metabolism and swimming performace of young Sockeye salmon. *Journal of the Fisheries Research Board of Canada* **21**, 1183-1226.

Clark, T. D., Sandblom. E. & Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *The Journal of Experimental Biology* **216**, 2771–2782. doi:10.1242/jeb/084251

Jobling, M. (1994). *Fish Bioenergetics*. Chapman and Hall Fish and Fisheries Series 13. 2-6 Boundary Row, London: Chapman and Hall.

Killen, S.S., Costa, I., Brown, J. A. & Gamperl, A. K. (2007). Little left in the tank: metabolic scaling in marine teleosts and its implications for aerobic scope. *Proceedings of the Royal Society B*. Sci. **274**, 431–438. doi:10.1098/rspd.2006.3741

Martinez-Porchas, M., Martinez-Cordova. L.R. & Ramos-Enriquez, R. (2009). Cortisol and glucose: Reliable indicators of fish stress? *Pan-American Journal of Aquatic Sciences* **4**, 158–178.

Martins, C. I. M., Castanheira, M. F., Engrola, S., Costas, B. & Conceição, L. E. C. (2011). Individual differences in metabolism predict coping styles in fish. *Applied Animal Behaviour Science* **130**, 135–143. doi:10.1016/j.applanim.2010.12.077

Maxime, V., Nonnotte, G., Peyraud, C., Williot, P. & Truchot, J.P. (1995). Circulatory and respiratory effects of an hypoxic stress in the Siberian sturgeon. *Respiration Physiology* **100**, 203-212.

Morgan, J.D. & Iwama. G. K. (1996). Cortisol-induced changes in oxygen consumption and ionic regulation in coastal cutthroat trout (*Oncorhynchus clarki clarki*) parr. *Fish Physiology and Biochemistry* **15**, 385–394.

O'Conner, C.M., Gilmour, K. M., Arlinghaus, R., Matsumura, S., Suski, CD., Philipp, D. P. & Cooke, S. J. (2011). The consequences of short-term cortisol elevation on individual physiology and growth rate in wild largemouth bass (*Micropterus salmoides*). *Canadian Journal of Fisheries and Aquatic Sciences* **68**, 693–705. doi: 10.1139/F11-009

Roche, D. G., Binning, S. A., Bosiger, Y., Johansen, J.L. & Rummer, J. L. (2013). Finding the best estimates of metabolic rates in a coral reef fish. *The Journal of Experimental Biology* **216**, 2103–2110. doi:10.1242/jeb.082925

Skov, P. V., Larsen, B. K., Frisk, M. & Jokumsen, A. (2011). Effects of rearing density and water current on the respiratory physiology and haematology in rainbow trout, *Oncorhynchus mykiss* at high temperature. *Aquaculture* **319**:446–452. doi:10.1016/j.aquaculture.2011.07.008

Webb, N. A. & Wood, C. M. (1998). Physiological analysis of the stress response associated with a silver nitrate exposure in freshwater rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry*. **17**, 579–588.

Woodward, C. C. & Strange, R. J. (1987). Physiological stress response in wild and hatchery-reared rainbow trout. *Transactions of the American Fisheries Society* **116**, 574–579. doi:10.1577/1548-8659(1987)116<574:PSRIWA>2.0.CO:2

Chapter 5: Summary

In summary, the results of this study suggest that chronic nAg exposure causes significant cortisol release in rainbow trout at high concentrations, as well as a trend of increased cortisol release at lower, environmentally-relevant concentrations, indicating that nAg does cause a stress response in fish. No effect from nAg to metabolic measurements of SMR, MMR_f, or MMR_s was found once random effects were accounted for, indicating that nAg exposure had little to no effect on the metabolism of fish. No effects were seen in morphometric indices of growth, K, or hepatosomatic index. Overall our study found effects of chronic nAg exposure on an endocrine level, but not on a metabolic or whole-body level.

Other studies have also found nAg exposure to cause increased blood plasma cortisol. However, this study found an increase at environmentally-relevant concentrations, which had not been tested previously. No effect to SMR has been seen from other studies as well; however, Bilberg et al. (2010) did observe decreased hypoxia tolerance, likely caused by direct interference of oxygen uptake at the gill, which would possibly also affect MMR, an effect that was not seen in this study. No effect was seen on growth in other studies (Griffit et al. 2012). And no clear effect was seen on HSI, unlike other studies that did see an effect to HSI from nanosilver exposure (Joo et al. 2013; Monfared and Soltani 2013).

Overall, research has shown a diverse number of toxic effects from nAg exposure, from altered gene expression (Farkas et al. 2011; Farmen et al. 2012; Pham et al. 2012; Scown et al. 2010), cortisol and glucose release (Johari et al. 2013;

Katuli et al. 2014; Shaluei et al. 2013), tissue damage in liver and gill tissues (Griffit et al. 2012; Joo et al. 2013; Monfared and Soltani 2013), embryonic abnormalities (Laban et al 2010; Wu et al 2010), and even death at high concentrations (Farmen et al. 2012; Shahbazzadeh et al. 2009; Shaluei et al. 2013). In most cases no effects to metabolism or growth were seen, indicating that the main toxic effects of nAg do not manifest as significant bioenergetic changes in fish. However, changes to growth may be seen over longer time periods of nAg exposure, as studies done to date have been short-term (Griffit et al. 2012). Also, there was only one other study (Bilberg et al. 2010) done on the effects of nAg to metabolism other than the present study, so additional research on this effect would also be beneficial to investigate potential bioenergetic effects of nAg. In addition there were trends of decreased growth and MMRs at high concentrations of nAg in this study, which, while not significant, do indicate potential effects, which would be valuable to investigate further.

Effects observed at environmentally-relevant concentrations (low ng l^{-1} range to 1.5 µg l^{-1}) (Gottschalk et al 2009; Liu et al 2009) of nAg exposure include altered gene expression (Pham et al 2012) and increased blood plasma cortisol concentrations (current study), but no whole-body effects such as liver or gill tissue damage or embryonic abnormalities. These results indicate that in the environment at this time fish should not experience serious toxicological effects from nAg exposure. However due to increasing nAg use environmental concentrations may increase in future, potentially causing other toxicological effects as observed in lab studies. In addition fish may be affected by nAg exposure indirectly in the aquatic environment as their food sources may decrease due to nAg's toxic effects on lower

trophic levels, such as bacteria (Das et al. 2012), algae (Dash et al. 2012), and aquatic invertebrates (Griffit et al. 2008).

Studies on the effects of nAg are important; as it is a new chemical that is entering the aquatic environment at ever-increasing levels due to its expanding use in consumer products. The effects of nAg in the aquatic environment are complex and differ based on the species studied, water chemistry and timing of exposure. Potential future areas of study are broad and include using natural waters in fish toxicological studies, increasing chronic time spans of exposure, or investigating different routes of exposure (e.g., exposure via diet). Studies of these types would better inform predictions on how nAg will affect fish in the aquatic environment.

In addition to investigating the effects of nAg on fish, our study also found that fish used to obtain metabolic measurements had increased cortisol levels in comparison to fish that had not, indicating that respirometric methods may be stressful, and thereby potentially affecting the metabolic measurements obtained by these methods. This effect may have caused a masking effect on any metabolic changes caused by the nAg – the level of stress caused by the respirometry methods overwhelming any effect seen from the contaminant; however there is no way to test this. This may also be a factor in why so few studies have seen an effect of contaminants on SMR in fish overall.

Common methods for measuring SMR in fish include directly measuring the lowest oxygen consumption rates of fish (as was done in this study), as well as using forced swimming trials to extrapolate SMR from the swimming speed-oxygen consumption relationship back to zero swimming speed (Enders and Scruton 2006).

Both these methods have been found to give similar estimates. However it is known that the extrapolation method may overestimate SMR due to fish exhibiting increased spontaneous activity at lower flow velocities (Enders and Scruton 2006). The increased stress experienced by fish in trials directly measuring SMR may also cause overestimation in this method. Accurate measurements of metabolic rates (including SMR) are important as these rates are used to inform bioenergetic models, which in turn are used to quantify consumption, growth, and activity rates of fish populations, and ultimately predict the carrying capacity of ecosystems (Enders and Scruton 2006).

References

Bilberg, K., Malte, H., Wang, T. & Baatrup, E. (2010). Silver nanoparticles and silver nitrate cause respiratory stress in Eurasian perch (*Perca fluviatilis*). Aquatic Toxicology **96**, 159–65. doi:10.1016/j.aquatox.2009.10.019

Das, P., Williams, C. J., Fulthorpe, R. R., Hoque, M. E., Metcalfe, C. D. & Xenopoulos, M. A. (2012). Changes in bacterial community structure after exposure to silver nanoparticles in natural waters. *Environmental Science & Technology* **46**, 9120–9128. doi:10.1021/es3019918

Dash, A., Singh, A. P., Chaudhary, B. R., Singh, S. K. & Dash, D. (2012). Effect of Silver Nanoparticles on Growth of Eukaryotic Green Algae. *Nano-Micro Letters* **4**, 158–165. doi:10.3786/nml.v4i3

Farkas, J., Christian, P., Gallego-Urrea, J. A., Roos, N., Hassellöv, M., Tollefsen, K. E. & Thomas, K. V. (2011). Uptake and effects of manufactured silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*) gill cells. *Aquatic Toxicology* **101**, 117–125. doi:10.1016/j.aquatox.2010.09.010

Farmen, E., Mikkelsen, H. N., Evensen, O., Einset, J., Heier, L. S., Rosseland, B. O., Salbu, B., Tollefsen, K. E. & Oughton, D. H. (2012). Acute and sub-lethal effects in juvenile Atlantic salmon exposed to low µg/L concentrations of Ag nanoparticles. Aquat. Toxicol. **108**, 78–84. doi:10.1016/j.aquatox.2011.07.007

Gottschalk, F., Sonderer, T., Scholz, R. W. & Nowack, B. (2009). Modeled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag, CNT,

fullerenes) for different regions. *Environmental Science & Technology* **43**, 9216–9222. doi:10.1012/es9015553

Griffitt, R. J., Brown-Peterson, N. J., Savin, D. A., Manning, C. S., Boube, I., Ryan, R. A. & Brouwer, M. (2012). Effects of chronic nanoparticulate silver exposure to adult and juvenile sheepshead minnows (*Cyprinodon variegatus*). *Environmental Toxicology and Chemistry* **31(1)**, 160–167. doi :10.1002/etc.709

Griffitt, R. J., Luo, J., Gao, J., Bonzongo, J. C. & Barber, D. S. (2008). Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. *Environmental Toxicology and Chemistry* **27(9)**, 1972–1978.

Johari, S. A., Kalbassi, M. R., Soltani, M. & Yu, I. J. (2013). Toxicity comparison of colloidal silver nanoparticles in various life stages of rainbow trout (*Oncorhynchus mykiss*). *Iranian Journal of Fisheries Sciences* **12(1)**, 76–95.

Joo, S. H., Kalbassi, M. R., Yu, I. J., Lee, J. H. & Johari, S. A. (2013). Bioaccumulation of silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*): influence of concentration and salinity. *Aquatic Toxicology* **140-141**, 398–406. doi:10.1016/j.aquatox.2013.07.003

Katuli, K. K., Massarsky, A., Hadadi, A. & Pourmehran, Z. (2014). Silver nanoparticles inhibit the gill Na+/K+-ATPase and erythrocyte AChE activities and induce the stress response in adult zebrafish (*Danio rerio*). *Ecotoxicology and Environmental Safety* **106**, 173–180. doi:10.1016/j.ecoenv.2014.04.001

Laban, G., Nies, L. F., Turco, R. F., Bickham, J. W. & Sepúlveda, M. S. (2010). The effects of silver nanoparticles on fathead minnow (*Pimephales promelas*) embryos. *Ecotoxicology* **19**, 185–195. doi:10.1007/s10646-009-0404-4

Liu, J., Chao, J., Liu, R., Tan, Z., Yin, Y., Wu, Y. & Jian, G. (2009). Cloud point extraction as an advantageous preconcentration approach for analysis of trace silver nanoparticles in environmental waters. *Analytical Chemistry* **81**, 6496–6502. doi:10.1021/ac900918e

Monfared, A. L., Soltani, S. & Louei, A. (2013). Effects of silver nanoparticles administration on the liver of rainbow trout (*Oncorhynchus mykiss*): histological and biochemical studies. *European Journal of Experimental Biology* **3(2)**, 285–289.

Pham, C. H., Yi, J. & Gu, M. B. (2012). Biomarker gene response in male Medaka (*Oryzias latipes*) chronically exposed to silver nanoparticle. *Ecotoxicology and Environmental Safety* **78**, 239–245. doi:10.1016/j.ecoenv.2011.11.034

Scown, T. M., Santos, E. M., Johnston, B. D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J. R., Stone, V., Fernandes, T. F., Jepson, M., Aerle, R. & Tyler, C. R. (2010). Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. Toxicological Sciences **115(2)**, 521–534. doi:10.1093/toxsci/kfq076

Enders, E. C. & Scruton, D. A. (2006). Potential application of bioenergetics models to habitat modeling and importance of appropriate metabolic rate estimates with special consideration for Atlantic salmon. *Canadian Technical Report of Fisheries and Aquatic Sciences* **2641**, v + 40pp.

Shahbazzadeh, D., Ahari, H., Rahimi, N. M., Dastmalchi, F., Sotani, M., Fotovat, M., Rahmannya, J. & Khorasani, N. (2009). The effects of nanosilver (Nanocid ®) on survival percentage of Rainbow trout (*Oncorhynchus mykiss*). *Pakistan Journal of Nutrition* **8(8)**, 1178–1179.

Shaluei, F., Hedayati, A., Jahanbakhshi, A., Kolangi, H. & Fotovat, M. (2013). Effect of subacute exposure to silver nanoparticle on some hematological and plasma biochemical indices in silver carp (*Hypophthalmichthys molitrix*). *Human and Experimental Toxicology* **32(12)**, 1270–1277. doi:10.1177/0960327113485258

Wu, Y., Zhou, Q., Li, H., Liu, W., Wang, T. & Jiang, G. (2010). Effects of silver nanoparticles on the development and histopathology biomarkers of Japanese medaka (*Oryzias latipes*) using the partial-life test. Aquatic Toxicology **100**, 160–167. doi:10.1016/j.aquatox.2000911.014

Appendix A:

Appendix A.   Summary of nanosilver toxicity in fish.

Substance	Concentration (µg l^{-1})	Exposure time	Particle Size (nm)	Capping agent[**]	Effects	Species	Life stage	Study
nAg	10	27d	25	-	thickening of epithelial gill tissue, Ag in tissue, altered gene expression (esp. in adult gonads), no effect to growth	sheepshead minnow (*Cyprinodon variegatus variegatus*)	juvenile – adult	Griffit et al. 2012
nAg	1-25	28d	24	-	general stress responses (gene expression in liver of MT, GST and others), induction of choriogenin and vitellogenin - indicating potential estrogenic activity of nAg	Japanese medaka (*Oryzias latipes*)	adult	Pham et al. 2012
nAg	10	10d	10	-	increased gill [Ag]	rainbow trout (*Oncorhynchus mykiss*)	adult	Scown et al. 2010
	100				increased gill and liver [Ag], increased stress gene expression (cyp1a)			
	10		35		increased gill and liver [Ag]			
	100		Bulk (600-1600)		increased gill and liver [Ag], with largest concentration in liver			
AgNO$_3$	0.1		-		lipid peroxidation, gill damage			
nAg	20	48h	30-40	citrate	accumulation of Ag in gills, impaired osmoregulation (inhibition of Na+/K+ ATPase), metallothionein induced	Atlantic salmon (*Salmo salar*)	adult	Farmen et al. 2012
	100				accumulation of Ag in gills, impaired osmoregulation (inhibition of Na+/K+ ATPase), metallothionein induced,heat shock protein induced, acute gill l amellae necrosis, 73% fish mortality			
nAg	100-1000	60d	~25	PVP	morphological defects (pericardial edema, eye abnormalities, finfold abnormalities, skeletal flexure and truncation), no significant effect on growth	Japanese medaka (*Oryzias latipes*)	embryo – juvenile	Wu et al. 2010

Substance	Concentration (µg l^{-1})	Exposure time	Particle Size (nm)	Capping agent**	Effects	Species	Life stage	Study
nAg	300		30-40	PVP	increased Pcrit*	Eurasian perch (*Perca fluviatilis*)	adult	Bilberg et al. 2010
AgNO₃	39	24h			increased Pcrit			
	386		-	-	increased Pcrit, increased BMR†			
nAg	100-10000	48h	3-40	citrate	oxidative stress, increased uptake into cells in comparison to PVP capped	Rainbow trout (*Oncorhynchus mykiss*)	cultured gill cells	Farkas et al. 2011
	1000-10000		5-15	PVP	oxidative stress			
nAg	32-32000, exposed to 0.4-12 ppt salinity	14d	16.6	-	concentration-dependant Ag uptake into tissues, uptake [] in order: liver > kidneys ~ gills > white muscle, uptake higher in high salinity, concentration-dependant increase in HIS	rainbow trout (*Oncorhynchus mykiss*)	juvenile	Joo et al. 2013
	100000, exposed to 6 and 12 ppt salinity				all fish died w/n 4 h			
nAg	600	96h	21-60	-	abnormalities (absence of air sac, pericardial and yolk sac edema, haemorrhages to head and pericardial area, lordosis, craniofacial abnormalities (small heads)	fathead minnow (*Pimephales promelas*)	embryo	Laban et al. 2010
	1250-10600				96h LC 50 (depending on type and solution preparation)			
nAg	1000	48h	27	-	Ag associated with gill (trapped in mucus, taken up into cells)	zebrafish (*Danio rerio*)	adult	Griffit et al. 2008
nAg	1000-8000	3h	16.6 nm	-	increased plasma cortisol	rainbow trout (*Oncorhynchus mykiss*)	juvenile	Johari et al. 2013
nAg	2000-4000	3 weeks	16.6	-	decreased gill Na/K ATPase activity after 14 d, decreased erythrocyte AChE activity (biomarker of neurotoxicity), decreased plasma electrolyte levels, plasma glucose and cortisol levels increased	zebrafish (*Danio rerio*)	adult	Katuli et al. 2014
	16760	4d			decreased erythrocyte AChE activity (biomarker of neurotoxicity), decreased plasma electrolytes levels, plasma glucose and cortisol levels			

Substance	Concentration (μg l^{-1})	Exposure time	Particle Size (nm)	Capping agent[**]	Effects	Species	Life stage	Study
					increased			
nAg	2300 - 3500	48 – 96 h	4.5	-	LC 50 48h – 3500ug/L, 72 h – 3000μg l^{-1}, 96 h – 2300 μg l^{-1}	rainbow trout (*Oncorhynchus mykiss*)	adult	Shahbazzadeh et al. 2009
nAg	3000	8 weeks	-	-	congestion in liver parenchyma, decreased size and diameter of hepatocytes, massive destination in hepatic sinusoids sizes; decreased levels of total protein in serum, AST and ALT levels (enzymes in liver - used to detect liver damage) in sera increased; decreased HSI	rainbow trout (*Oncorhynchus mykiss*)	adult	Monfared and Soltani 2013

*critical oxygen tension
†basal metabolic rate
** '-' indicates no capping agent

References

Bilberg, K., Malte, H., Wang, T. & Baatrup, E. (2010). Silver nanoparticles and silver nitrate cause respiratory stress in Eurasian perch (*Perca fluviatilis*). *Aquatic Toxicology* **96**, 159–165. doi:10.1016/j.aquatox.2009.10.019

Farkas, J., Christian, P., Gallego-Urrea, J. A., Roos, N., Hassellöv, M., Tollefsen, K. E, Thomas, K. V. (2011). Uptake and effects of manufactured silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*) gill cells. *Aquatic Toxicology* **101**, 117–125. doi:10.1016/j.aquatox.2010.09.010

Farmen, E., Mikkelsen, H. N., Evensen, O., Einset, J., Heier, L. S., Rosseland, B. O., Salbu, B., Tollefsen, K. E. & Oughton, D. H. (2012). Acute and sub-lethal effects in juvenile atlantic salmon exposed to low μg/L concentrations of Ag nanoparticles. *Aquatic Toxicology* **108**, 78–84. doi:10.1016/j.aquatox.2011.07.007

Griffitt, R. J., Brown-Peterson, N. J., Savin, D. A., Manning, C. S., Boube, I., Ryan, R. A. & Brouwer, M. (2012). Effects of chronic nanoparticulate silver exposure to adult and juvenile sheepshead minnows (*Cyprinodon variegatus*). *Environmental Toxicology and Chemistry* **31**, 160–167. doi:10.1002/etc.709

Griffitt, R. J., Hyndman, K., Denslow, N. D. & Barber, D. S. (2009). Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanoparticles. *Toxicological Sciences* **107(2)**, 404–415. doi:10.1093/toxsci/kfkn256

Johari, S. A., Kalbassi, M. R., Soltani, M. & Yu, I. J. (2013). Toxicity comparison of colloidal silver nanoparticles in various life stages of rainbow trout (*Oncorhynchus mykiss*). *Iranian Journal of Fisheries Sciences* **12(1)**, 76–95.

Katuli, K. K., Massarsky, A., Hadadi, A. & Pourmehran, Z. (2014). Silver nanoparticles inhibit the gill Na+/K+-ATPase and erythrocyte AChE activities and induce the stress response in adult zebrafish (*Danio rerio*). *Ecotoxicology and Environental Safety* **106**, 173–180. doi:10.1016/j.ecoenv.2014.04.001

Laban, G., Nies, L. F., Turco, R. F., Bickham, J. W. & Sepúlveda, M. S. (2010). The effects of silver nanoparticles on fathead minnow (*Pimephales promelas*) embryos. *Ecotoxicology* **19**, 185–95. doi:10.1007/s10646-009-0404-4

Monfared, A. L., Soltani, S. & Louei, A. (2013). Effects of silver nanoparticles administration on the liver of rainbow trout (*Oncorhynchus mykiss*): histological and biochemical studies. **3(2)**, 285–289.

Pham, C. H., Yi, J. & Gu, M. B. (2012). Biomarker gene response in male Medaka (*Oryzias latipes*) chronically exposed to silver nanoparticle. *Ecotoxicology and Environmental Safety* **78**, 239–45. doi:10.1016/j.ecoenv.2011.11.034

Joo, H. S., Kalbassi, M. R., Yu, I. J., Lee, J. H. & Johari, S. A. (2013). Bioaccumulation of silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*): influence of concentration and salinity. Aquatic Toxicology **140-141**, 398–406. doi:10.1016/j.aquatox.2013.07.003
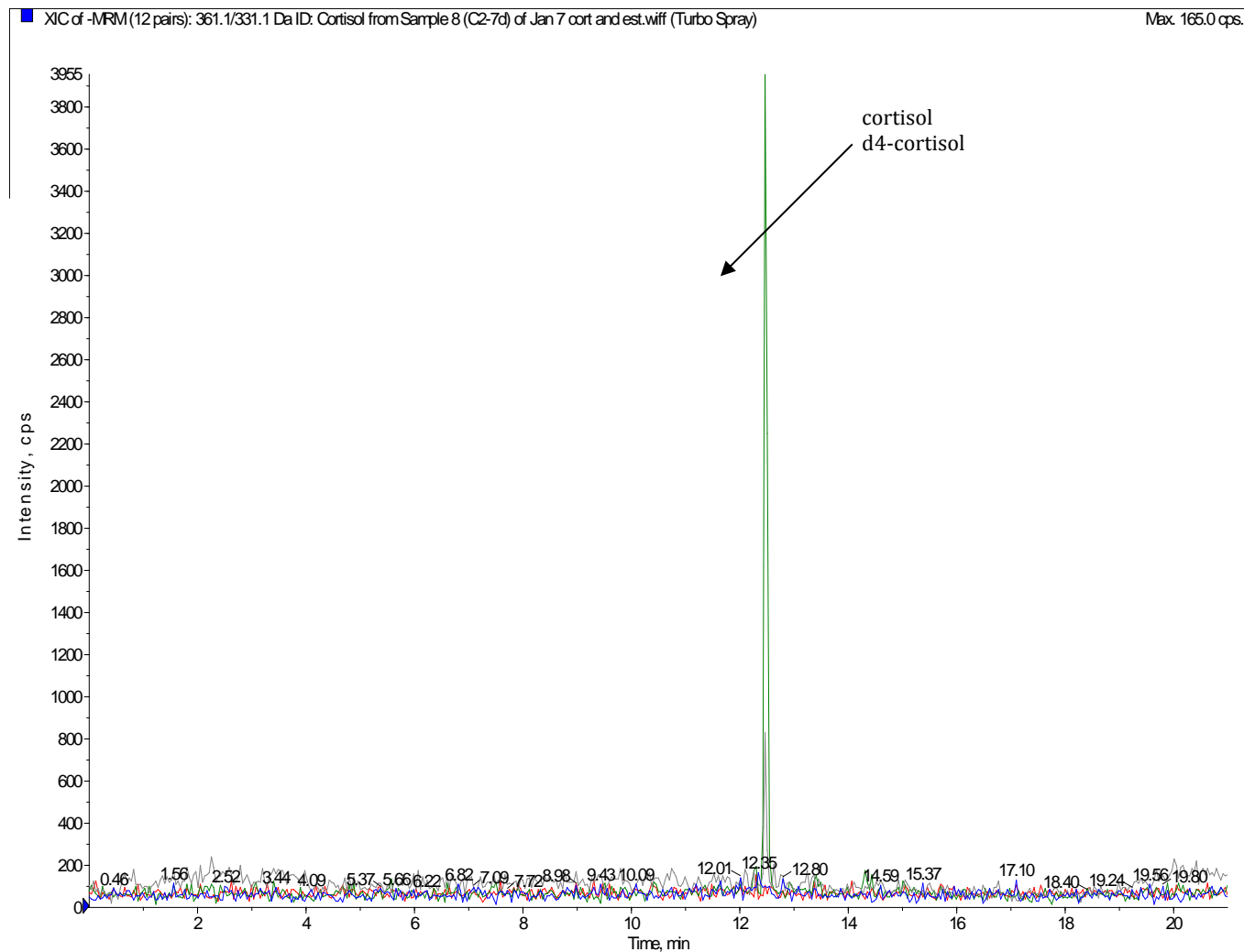
Scown, T. M., Santos, E. M., Johnston, B. D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J. R., Stone, V., Fernandes, T. F., Jepson, M., Aerle, R. & Tyler, C. R. (2010). Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. Toxicological Sciences **115(2)**, 521–534. doi:10.1093/toxsci/kfq076

Shahbazzadeh, D., Ahari, H., Rahimi, N. M., Dastmalchi, F., Sotani. M., Fotovat, M., Rahmannya, J., Khorasani, N. (2009). The effects of nanosilver (Nanocid ®) on survival percentage of rainbow trout (*Oncorhynchus mykiss*). *Pakistan Journal of Nutrition* **8(8)**, 1178–1179.

Wu, Y., Zhou, Q., Li, H., Liu, W., Wang, T., Jiang, G. (2010). Effects of silver nanoparticles on the development and histopathology biomarkers of Japanese medaka (*Oryzias latipes*) using the partial-life test. Aquatic Toxicology **100**, 160–167. doi:10.1016/j.aquatox.2009.11.014

Appendix B

XIC of -MRM (12 pairs): 361.1/331.1 Da ID: Cortisol from Sample 8 (C2-7d) of Jan 7 cort and est.wiff (Turbo Spray)   Max. 165.0 cps.

cortisol
d4-cortisol

Appendix B.   Chromatogram of cortisol in a representative sample (C2-7d) analyzed by HPLC-MS-MS.

Appendix B 2.   Parameters for negative ion mode of tandem mass spectrometry (MS/MS).

Parameter	Abbreviation	Cortisol	d4-Cortisol
Curtain gas	CUR	30	30
Sheath gas	GSI	20	20
Turbo gas	GS2	80	80
Ionspray voltage	IS (V)	-4400	-4400
Turbo-gas temperature	TEM (°C)	550	550
Declustering potential	DP (V)	-35	-21
Focusing potential	FP (V)	-330	-340
Entrance potential	EP (V)	-10	-10
Collision gas	CAD	8	8
Collision cell entrance potential	CEP (V)	-20	-20
Collision energy	CE (V)	-12/-28	-12/-28
Collision cell exit potential	CXP (V)	-18/-20	-18/-20

Appendix C

Appendix C.   Nominal and measured concentrations of nAg in various exposure studies.

| Nominal concentration (μg l^{-1})	Size (nm)	Time	Measured concentration (μg l^{-1}), and as % of nominal	Ag$^+$ concentration	Exposure water	Study
1000	~27	2, 24, 48 h	50 μg l^{-1} (4.9%)	5 μg l^{-1}	dechlorinated tap water	Griffitt et al. 2009
10	25n	Various time pnts throughout 27 d	2.9 μg l^{-1} ± 0.6 (30%)	0.38 ±0.05 μg l^{-1}	seawater	Griffitt et al. 2012
1	30-40	0 h	~1 μg l^{-1} (100%)	below detection	lake water	Farmen et al. 2012
1	30-40	24 h	~1 μg l^{-1} (100%)	below detection	lake water	Farmen et al. 2012
20	30-40	0 h	~20 μg l^{-1} (100%)	10-25% of total Ag	lake water	Farmen et al. 2012
20	30-40	24 h	~20 μg l^{-1} (100%)	0.2-1% of total Ag	lake water	Farmen et al. 2012
100	30-40	0 h	~100 μg l^{-1} (100%)	not measured	dechlorinated tap water	Bilberg et al. 2010
100	30-40	24 h	80 μg l^{-1} (80%)	not measured	dechlorinated tap water	Bilberg et al. 2010
63	30-40	5 min	43 μg l^{-1} (68%)	not measured	dechlorinated tap water	Bilberg et al. 2010
63	30-40	2 h	29 μg l^{-1} (46%)	not measured	dechlorinated tap water	Bilberg et al. 2010
63	30-40	20 h	17 μg l^{-1} (27%)	not measured	dechlorinated tap water	Bilberg et al. 2010
129	30-40	5 min	98 μg l^{-1} (76%)	not measured	dechlorinated tap water	Bilberg et al. 2010
129	30-40	2 h	73 μg l^{-1} (57%)	not measured	dechlorinated tap water	Bilberg et al. 2010
129	30-40	20 h	39 μg l^{-1} (30%)	not measured	dechlorinated tap water	Bilberg et al. 2010
300	30-40	5 min	150 μg l^{-1} (50%)	not measured	dechlorinated tap water	Bilberg et al. 2010
300	30-40	2 h	130 μg l^{-1} (43%)	not measured	dechlorinated tap water	Bilberg et al. 2010
300	30-40	20 h	50 μg l^{-1} (17%)	not measured	dechlorinated tap water	Bilberg et al. 2010
10	10	various time pnts throughout 10 d	below detection	not measured	dechlorinated tap water	Scown et al. 2010
100	10	various time pnts throughout 10 d	35.5 μg l^{-1} ± 2.44(SE) (35%)	not measured	dechlorinated tap water	Scown et al. 2010
10	35	various time pnts throughout 10 d	9.4 μg l^{-1} ± 4.62 (SE) (90%)	not measured	dechlorinated tap water	Scown et al. 2010
100	35	various time pnts throughout 10 d	35.3 μg l^{-1} ± 2.64 (SE) (35%)	not measured	dechlorinated tap water	Scown et al. 2010
100	Bulk (600-1600)	various time pnts throughout 10 d	46.6 μg L^{-1} ± 4.73 (SE) (46%)	not measured	dechlorinated tap water	Scown et al. 2010

Appendix D

Appendix D.   Total silver concentrations measured in respirometry tanks. Respirometry tanks were 140 l in volume.

Tank	Nominal concentrations (µg l⁻¹)	Stock concentration (g l⁻¹)	Amount of nAg stock added (l)	Average measured concentrations (µg l⁻¹)	Range (µg l⁻¹)	Percentage of nominal (%)
C1-resp	0	-	-	0.39	0 – 0.27	-
L1-resp	1	2.5	0.000056	0.45	0.08 – 0.93	45
H1-resp	200	2.5	0.0112	6.74	518 – 9.4	3
C2-resp	0	-	-	3.11	0.69 – 6.63	-
L2-resp	300	5	0.0084	7.14	2.97 – 11.43	2
H2-resp	600	5	0.0168	15.71	13.32 – 20.48	3

Appendix E
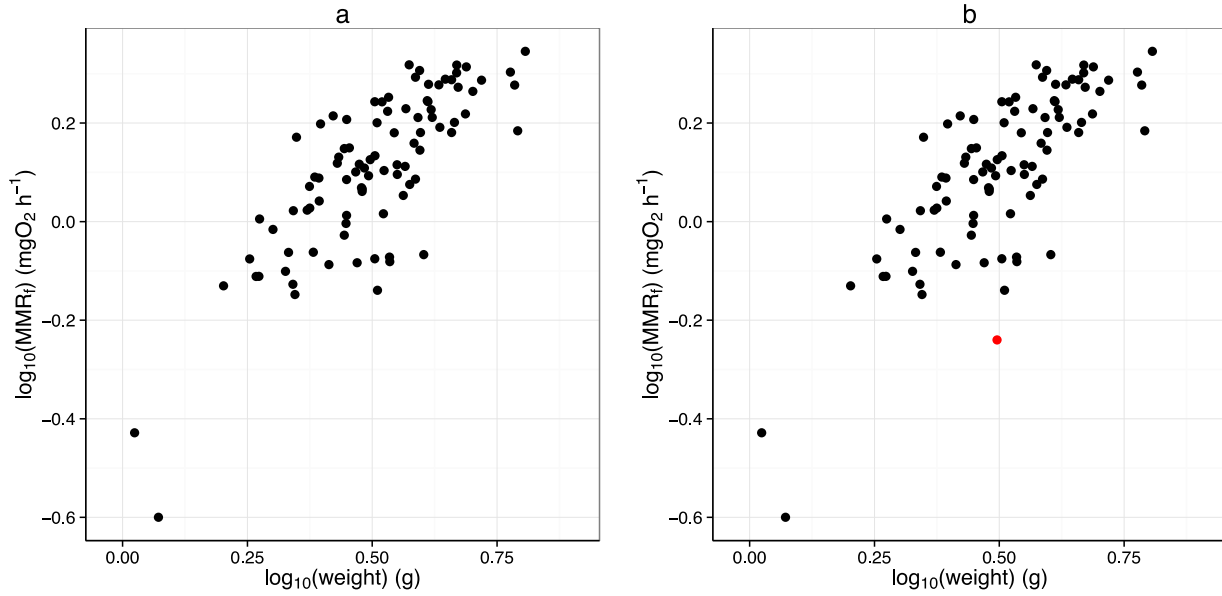
Appendix E.1. Relationship between forced maximal metabolic rate and weight, showing outlier removed for analysis. Panel a: no outlier. Panel b: including outlier (in red).
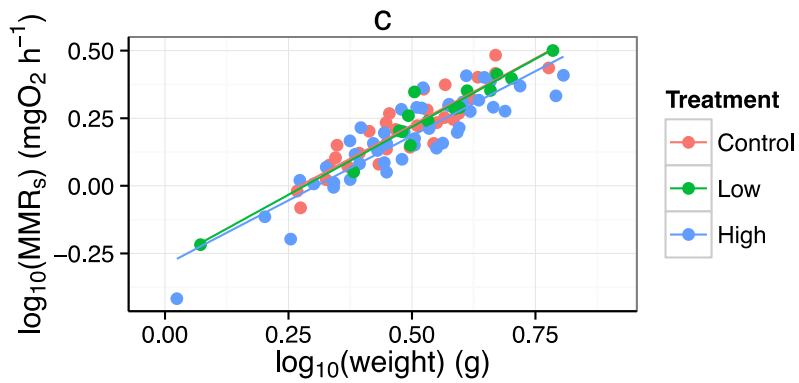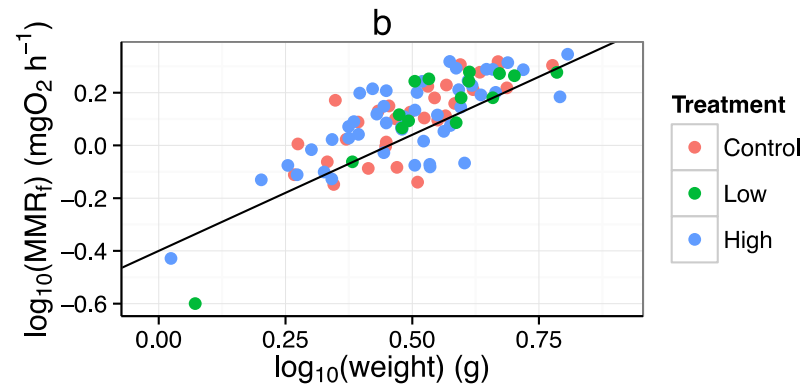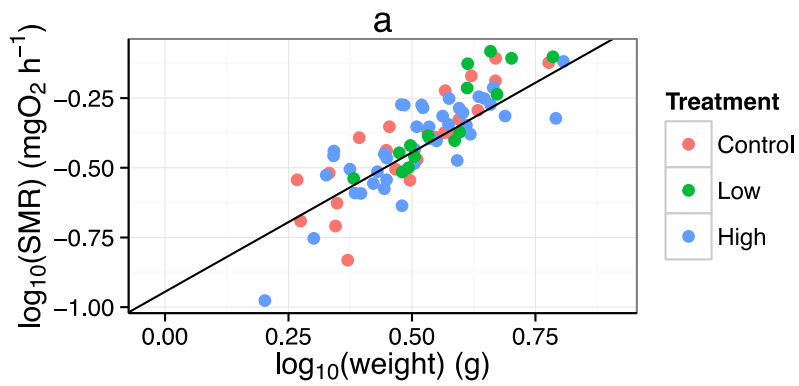
Appendix E.2. Relationship between metabolic variables and weight. Panel a: standard metabolic rate (SMR). Panel b: forced maximal metabolic rate (MMR$_f$). Panel c: spontaneous maximal metabolic rate (MMR$_s$), note differing relationship between variables by treatment.