Silver Nanoparticles have Protein-Specific Negative Impacts on Cardiac Function in Brook Trout (*Salvelinus fontinalis*)

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Abstract

Silver nanoparticles (nAg) have been increasingly prevalent in societal use due to their antimicrobial nature, highly diversifiable structure, and small size. Non-exhaustively, they are being used in textiles, toys, cleaning products, and other technologies before being released into the environment at various stages of production. With the increasing use of nAg in consumer products, potential long-term effects on humans and the aquatic environment are a serious concern. This study looked to characterize the cardiac effects of nAg in brook trout. The nAg exposure through intravascular (I.V.) injection, 700µg/kg, resulted in a significant increase in both heart rate and blood pressure. There was also a decrease in Na^+/K^+ ATPase function seen in heart tissue. Inhibition of Na^+/K^+ ATPase could have indirectly led to the decrease in Na^+/Ca^{2+} exchange, increasing localized Ca^{2+} and thereby increasing contractility. The total membrane ATPase was not affected, therefore the mechanism of this inhibition of Na^+/K^+ ATPase is likely enzyme specific. in the nAg treatment groups, propranolol and atropine were not able to induce an effect on heart rate. This could be due to nAg binding to β-adrenergic, or cholinergic receptor sites prior to pharmacological intervention. These results indicate that nAg have a negative impact on cardiac function in brook trout, however further research is required to characterize the mechanism of toxicity.

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Introduction

Nanoparticles are increasingly prevalent in societal use due to their antimicrobial properties, highly diversifiable structure, and small size. They are used in textiles, toys, cleaning products, technologies and released into the environment at various stages of production. The International Organization for Standardization (https://www.iso.org) has classified nanomaterials as "as a material having any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale" ranging from 1-100nm (ISO/TS 80004-1, 2015). The high surface area to volume ratio of nanoparticles allow them to have varying traits which are based on their surface features. This results in a wide variety of particles and correspondingly, a vast number of unanswered questions specific to each type of nanoparticle. During nanoparticle synthesis, product manufacturing, recycling, and product use there is run-off that can enter ecosystems (Callaghan and MacCormack, 2017). There are also a number of potential exposure routes directly to the body, through the skin, gut, or inhaled as an aerosolized product (Calderón-Jiménez *et al.,* 2017). With the increased use of nanoparticles in products, risk of exposure is proportionally increased with unknown long-term effects. Beyond the initial exposure risk to employees working in the associated industries, the environmental impact is felt in the aquatic ecosystems due to run-off. The long-term effects on those ecosystems and fishing industries are as yet unclear and under-researched.

Silver nanoparticles (nAg) have been increasingly used in manufactured products due to their antimicrobial properties especially in health care for use in textiles, wound dressings, and biomedical devices (Calderón-Jiménez *et al.,* 2017). Environmentally relevant silver nanoparticle exposures induced silver buildup in the liver of zebrafish resulting in measurable uptake (Bacchetta *et al.,* 2016). These data show that nAg are getting into animals in the environment. Nanoparticles are likely interacting with the cardiovascular system in some fashion before their accumulation in the liver, however the *in vivo* cardiac effects have yet to be quantified.

Effects of nanoparticles on fish

Many studies have been done to determine the *in vitro* effects of silver nanoparticles on tissues. In 2018, Callaghan *et al*. showed that 10 µg/mL nAg severely impaired force development in isolated rainbow trout cardiac muscle *in vitro*, but the study did not establish

the biological relevance of the response in the whole fish. Additionally, other studies have identified subtle *in vivo* cardiorespiratory effects of other metal nanoparticles (e.g., Bessemer *et al.,* 2015) but follow-up studies were unable to clearly characterize a mechanism of action (Callaghan *et al.,* 2016). Identifying potential mechanisms of toxicity such as this is important because the cardiovascular system is critical for performance and survival.

Silver ions are known to negatively impact the environment through their detrimental effects on fish, mainly through the binding of silver to thiols of Na^{+}/K^{+} ATPase, blocking osmoregulation over the gills. Silver ions have also been shown to accumulate in the blood stream and thence the liver. It is thought that the silver ions affect the sodium and chloride ion channels in the gills, thereby affecting osmolality in the blood and eventually leading to cardiac-related death (Wood *et al.,* 1996). Silver nanoparticles in the environment undergo many changes that affect how they interact with the environment and its inhabitants. Nanoparticles undergo aggregation and disaggregation as well as dissolution and degradation overtime (Handy *et al.,* 2008). However, polyvinylpyrrolidone (PVP) coating increases stability and prevents aggregation of the nanoparticles. Nanoparticles are diverse in terms of the surface proteins that associate with them, which heavily determines how they interact with their environment and each other (Bundschuh *et al.,* 2018). The known silver ion effects impacting fish health have been shown to be independent of nano-specific toxicity in relation to PVP functionalized nAg (nAgPVP). nAgPVP have been determined to insignificantly leach silver ions (Ollerhead *et al*., 2020). This would suggest that any effects of nAg exposure are due to the silver nanoparticles as opposed to the silver ions. This was shown in relation to sodium/potassium ATPase (Na^{+}/K^{+} ATPase) activity in heart strips *in vitro* (Callaghan *et al.,* 2018). *In vivo* selenium nanoparticles have a significant impact on the mortality, heart rate (*f*h), and cell death of exposed zebrafish embryos. The potential mechanism suggested was through angiogenesis inhibition during development which then led to cardiac impact (Duan *et al.,* 2013). Exposure to nAg *in vivo* likely would give a more ecologically relevant model of their cardiac effects.

nAg toxicity has been observed in whole animals such as killifish (Campbell *et al.,* 2019). In this paper, routine and maximum oxygen consumption rates were decreased by 53 and 30%,

respectively. This shows an effect on metabolic rate which is inextricably linked to cardiac function. However, in other whole animal studies, no significant effects were seen on metabolic rate. For example, rainbow trout exposed to 100μg/L nAg for 48 h showed no significant impacts on aerobic scope, critical swimming speed, and fractional rates of protein synthesis (Ollerhead *et al*., 2020). The effects of silver nanoparticles have not yet been studied in brook trout to determine whether they will be more or less sensitive to nAg than rainbow trout.

Nanoparticles have also been shown to have negative effects on gill function, but the mechanism is still uncertain. nAg could be taken up by the gill or get trapped in the protective mucus membrane layer (Smith *et al.,* 2007). After uptake, either through interaction, ingestion or respiration the nAg will enter the blood stream of the fish (Callaghan and MacCormack, 2017). Once nanoparticles enter the blood stream, they may have antioxidant properties in higher concentrations instead of generating the oxidative response shown *in vitro* (Akter *et al.,* 2018). The effects of nanoparticles themselves on plasma in blood are minimal and damage is likely only seen when a whole animal immune response is triggered (Gormley *et al.,* 2016). This implies that any oxidative response seen from nanoparticle exposure is the result of physiological responses instead of chemical reactions from the nanoparticles themselves. Nanoparticles when exposed to a large amount of proteins create a corona. This protects the cells from potential damage and allows for uptake by phagocytosis. Once inside the cell, they are degraded by lysosomes and the nanoparticle can now cause damage as they no longer have that protective corona. (Wang *et al.,* 2013)

Na^+/K^+ ATPase and Osmoregulation

The Na^+/K^+ ATPase pump is the most energetically important pump in the body of all animals working to maintain cellular electrochemical gradients. In fish, this responsibility increases to encompass homeostatic requirements for osmoregulation (Lingwood *et al.,* 2005). Osmolarity of the fish plasma must remain constant even when the fish has higher or lower concentration of solutes relative to its surrounding environment (Metz *et al.,* 2003). Brook trout have been observed in the wild going from freshwater to saltwater as juveniles and back to freshwater as adults making osmoregulation very important to this species. Brook trout osmoregulation is controlled mostly through Na^+/K^+ ATPase, which is regulated by cortisol

(Shaughnessy and McCormick, 2018). Silver ions have been shown to inhibit gill Na^+/K^+ ATPase causing an impact on osmoregulation in fish, specifically adult zebrafish by Katuli *et al.,* in 2019. Silver nanoparticles themselves also inhibit Na^+/K^+ ATPase in juvenile rainbow trout (Schultz *et al.,* 2012). nAg have been shown to bioaccumulate in the liver, plasma, and gill tissues and induce necrosis in all tissues (Johari *et al.* 2014). The inhibition of the Na^+/K^+ ATPase pump could cause some of the necrosis seen when nAg bioaccumulates in these tissues.

Background on Brook Trout

Brook Trout or *Salvelinus fontinalis* of the genus char are a cold freshwater species that are commonly used in aquaculture. Aquaculture has increased the use of triploid species in farming where fish contain 3N chromosomes as opposed to the usual diploid 2N species. The 3N are the same size as the 2N and have the same size organs but the 3N cells are larger due to their ploidy (Galbreath *et al.,* 2006). As a result, in the heart there is a decrease in cardiomyocyte surface area to volume ratio. Based on these anatomical differences, if nanoparticles impact membrane enzyme activity, it would be more pronounced in the 3N species. Therefore, ploidy differences would allow for a mechanism of action to be further supported or refuted. There are noticeable differences in survival rates of triploid versus their diploid counter parts, particularly when confronted with physiological stress. There has been evidence pointing to inconsistencies in hypoxia tolerance that may be the leading cause of inferiority (Scott *et al.,* 2014). The increased sensitivity to environmental stressors may cause 3N fish to have an increased susceptibility to anthropogenic stressors such as nanoparticles.

Regulation of Heart Rate and Blood Pressure

The blood flow of a fish flows linearly through the heart to the rest of the body before looping back to the heart. There are four compartments but the blood travels linearly through the heart from sinus venosus, to atrium, then ventricle and out the bulbus arteriosus to the gill (Farrell and Jones, 1992). The blood around the body is oxygenated in the gills and then slowly decreases in oxygen concentration throughout the circuit as it progresses through the body (Bushnell *et al.,* 1992). The overall metabolism is then limited in most peripheral tissues requiring oxygen. Heart Rate (*f*h) and blood pressure (BP) are excellent indicators of fish health. When exposed to a toxin or physiological stress, fish generally increase *f*h in order to increase

oxygen delivery to fuel the increases in demand associated with detoxification and repair processes. Quantitative values of cardiovascular parameters give an ecologically important idea of the overall health of the fish and can indicate the long-term health if exposed to a given toxin over the period of a lifetime (Sopinka *et al.,* 2016). There are chemoreceptors located inside blood vessels and on the first gill arch, the carotid arch, and the branchial branch of the cranial nerve that monitor oxygen levels (Burleson and Milson, 1995). Stimulation of the vagus nerve via these receptors will also lead to bradycardia or a decrease in *f*h. Different responses will result dependent on nAg interaction with the fish physiology. (Leite *et al.,* 2009). Another cardiovascular attempt to maintain homeostasis is alterations of BP. This is done through the adrenergic vasomotor tone and vasodilation to decrease BP (Sundell *et al.,* 2018). The renin-angiotensin system and osmoregulatory control of are interconnected in BP regulation (Greenwell *et al.,* 2003).

Heart rate and blood pressure are controlled by the sympathetic nervous system. During exercise the fish will release acetylcholine from the vagus nerve, then norepinephrine onto adrenergic receptors. The parasympathetic system can decrease *f*h in a similar fashion via muscarinic receptors (McCorry, 2007). Atropine and propranolol are common drug regulators of *f*h. Atropine is an acetylcholine receptor blocker and propranolol a beta-adrenergic receptor blocker (Oduleye and Evans, 1983). Any cardiac effects of the nanoparticles on the *f*h could potentially be controlled by input from the autonomic nervous system. However, using drugs such has atropine and propranolol, the signals are blocked resulting in the intrinsic *f*h. Documented effects of propranolol and atropine for brook trout are not yet available; however, in rainbow trout, atropine results in an increase in *f*h and then propranolol will decrease *f*h, offsetting the increase previously seen by the atropine (Miller *et al.,* 2011). The brain sends these signals to ensure the heart sends enough blood to the tissues. During time of exercise or stress the pacemaker activity is increased and adjusted accordingly to increase cardiac output (Wilson *et al.,* 2015). The catecholamines may be released in response to toxin stressors such as nAg. There are many possible responses ranging from stimulation to inhibition that could cause variance in the timing of the pacemaker from a given stressor (Burleson and Milsom, 1995). This will then in turn alter both the BP and *f*h of the fish. The stressor itself is not necessarily a

bad thing as it is the response to maintain homeostasis; however, over prolonged periods of time or with a high intensity, there are primary and secondary responses that can have detrimental effects on the fish health. (Barton, 2002)

Aim of Study

As we develop more uses for nAg, we need to fully understand how their inevitable disposal and introduction into aquatic ecosystems will impact aquatic wildlife. If suspended nanoparticles cause similar cardiorespiratory toxicity in fish as dissolved silver, it may hinder their ability to escape predators, find prey, and perform the normal activities necessary for survival and growth. With the increasing use of nAg in consumer products, potential long-term effects on humans are also a concern. The aim of this study is to determine the *in vivo* cardiac effects of nAg in brook trout and determine possible mechanistic pathways of toxicity. The results of this study will help us to characterize cardiac effects of nAg and therefore contribute to our understanding of risks to human health.

Methods

Animals

Salvelinus fontinalis, or brook trout, (body mass 561 ± 99 g) were obtained from broodstock held at the University of New Brunswick in Fredericton. Twelve fish, 2 years of age, male and female separated by diploid (2N) and triploid (3N), were held at 16ºC and fed 3 mm commercial trout chow twice daily. The fish were housed in 750 L partially recirculating freshwater tanks with like species. Fish selected for surgeries were drawn at random and food was withheld for 24 h prior to procedures. All experiments were approved by the Mount Allison University Animal Care committee (Animal Care Committee Protocol #102526).

Surgical Procedure

Fish were anesthetized in a solution of 3 g Tricaine mesylate (TMS 222) from AquaLife, 6 g sodium bicarbonate from Sigma-Aldrich (SA) in 10 L of water. The fish were knocked out in the higher dose, weighed, then moved onto a table with a constant flow of anesthetic dose over the gills. The surgery was performed under a lower dose of 1.5 g TMS 222 and 3g of sodium bicarbonate in 10 L of water. PE-50 tubing was filled with a heparinized saline solution (143.0 mM NaCl, 0.900 mM $MgSO_4$, 3.35 mM KCl, 2.30 mM NaH_2PO_4, 5.50 mM $NaHCO_3$, 10.0 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 2.93 mM $CaCl_2$. The pH was adjusted to 7.8 with 0.1 M NaOH, then brought up to a total volume of 1.0 L by diH_2O and stored at 4°C,). A sharpened guitar string (#17) in a P50 tube was used to cannulate the dorsal aorta because it allowed for a clean entry into the aorta, preventing blood clotting (Soivio et al., 1975). The guitar string was removed, and the presence of blood flow indicated successful cannulation leaving the PE-50 tube to allow for blood sampling or injection into the blood stream. This procedure is imaged in Figure 1.1. Following cannulation, 2 mL of saline was heparinized with heparin ammonium from porcine intestinal mucosa, then injected into the fish to push back any blood and prevent clotting at the surgical site. The cannula was then capped with crit-o-seal® and sutured to the roof of the mouth and through the nose to prevent the fish from pulling it out. The fish were awakened in freshwater and placed into a holding tube housed in an aerated 30L of water held at 16°C in a 40 L cooler to prevent the fish turning and potentially tangling the cannula (Fig. 1.2). Every 12 h, 250 µL of heparinized saline solution was injected into the cannula to prevent blood clotting.

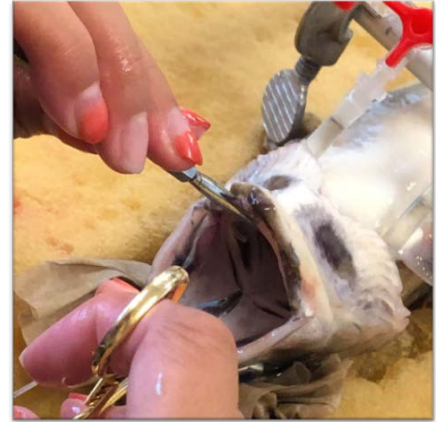

Figure 1.1: Cannulation of Brook Trout. The placement of the cannula was between the first and second gill ridge directly into the dorsal aorta with a sharpened guitar string and a PE-50 tube.

Experimental Set-up

Water pumps with cooling rings were to control water temperature in each cooler. Air bubbling and pump filters were used to keep the water clean. The fish were placed in holding tubes with holes for water flow on the sides and a grated cap on the front and back. A slit was made at the top of the tube for the cannula, to ensure it did not get tangled, caught or pulled out. Dissolved oxygen was checked daily with an oxygen probe.

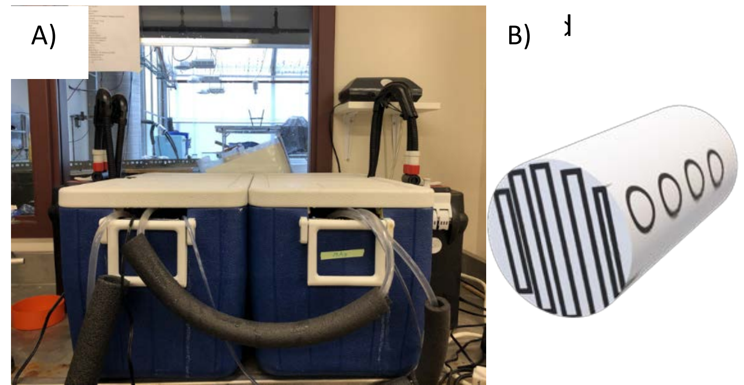

Figure 1.2: A) Cooler set up and B) Schematic of tube for fish holding. Holes were cut out of cooler lid for cooling, water pumps and air bubbling into the tank. 30L of water was aerated and held at 16°C in a 40L cooler.

Nanoparticles

Fully characterized silver nanospheres with a diameter of 5 nm and a polyvinylpyrrolidone surface functionalization from nanoComposix (5mg/mL Lot #ECP1598) were used. Characterization was reported in Ollerhead *et al.,* (2020): TEM analysis found nAgPVP core diameters between 5 and 25 nm, agreeing with the Z-average hydrodynamic diameter of 33.1 ± 11.0 nm, a polydispersity index (PDI) of 0.111, and a zeta potential of -12.5 ± 4.0 mV. To our knowledge, there has not been an injection of silver nanoparticles into fish before, so a dosage of 700 µg/kg was decided based on minimal dosage to elicit the stress response from injection of PVP functionalized nAg in Sprague Dawley rats (Vidanapathirana *et al.,* 2018).

Experimental Testing

The treatment group consisted of 6 fish(3-2N and 3-3N). The nanoparticles were sonicated prior to administration and diluted in up to 250 µL of saline solution. A dosage of 700 µg/kg silver nanoparticles were administered over 3 min, after a 24 h recovery period in the cooler set up. The control group also had 6 fish (3-2N and 3-3N). 250 µL of heparinized saline solution (heparin 50 µg/L ammonium from Porcine Intestinal Mucosa; (SA) was injected over 3 min into after a 24 h recovery period.

Heart rate (*f*h) and blood pressure (BP) were recorded continuously for 24 h after injections, after which propranolol (3 mg/1000 g) (SA) was injected and changes in *f*h and BP were recorded after 30 min. Subsequently, atropine (1.2 mg/1000 g) (SA) was injected and *f*h and BP were recorded again after 30 min.

A blood sample was taken from the cannula immediately after the atropine *f*h and BP were recorded. The blood sample was centrifuged for 5 min at 13000 rpm to separate the plasma, which was then stored in liquid nitrogen at -80°C. The fish was then exposed to a fatal dose of TMS222. Following 5 min without observed movement, spinal cord severance was used to confirm death. Samples of gill, heart, liver, and brain were harvested and immediately flash frozen in liquid nitrogen and then stored in a -80°C freezer.

Plasma Osmolality

Plasma osmolality was measured using a VAPRO 5520 vapour pressure osmometer (Wescor, Inc., Utah, USA) to determine the concentration of solutes in the plasma, or osmotic pressure (mosmols/kg). Ten µL of each sample was assayed in duplicate.

Na^+/K^+ ATPase Assay

The Na^+/K^+ ATPase assay was done on both the gill and heart tissues as gill ATPase has been shown to be affected by silver ions and nanoparticles (Yue *et al.,* 2017) and heart strips affected by silver nanoparticle exposure (Callaghan *et al.,* 2018). The Na^+/K^+ ATPase assay was performed as per McCormick (1993). Briefly, Sucrose, EDTA and Imidazole (SEI) buffer was first made using 250 mM sucrose, 10 mM NA_2EDTA (SA), 50 mM imidazole then the tissue was homogenized in SIE buffer with 0.5% sodium deoxycholic acid. The salt solution was made with 50 mM imidazole, 189 mM NaCl, 10.5 mM $MgCl_2x6H_2O$ and 42 mM KCl. ADP standards were made with 67 mM sodium acetate and 4 mM ADP. The assay mixture contained 50 mM imidazole buffer in water, 21 mM phosphoenolpyruvate (PEP) and 10.5 mM ouabain octahydrate (SA).

Immediately before the assay was performed, the following was added to the assay mixture: 0.22mM nicotinamide adenine dinucleotide reduced (NADH) from BioShop Canada Inc., 0.7mM adenosine triphosphate (ATP) from BioShop, 4.6U/mL lactic dehydrogenase (LDH)

from rabbit muscle (calzyme) and 5.1U/mL pyruvate kinase (PK) from rabbit (SA). In each well, 10µL of sample was added in triplicate twice followed by 200µL of ouabain assay mixture in half of the wells and imidazole assay mixture in the other half. A standard curve was made with the ADP standards. The samples were read using Spectra MAX 190 microplate spectrophotometer (Molecular Devices, California, USA) every 10 s for 10 min at an absorbance of 340 nm. The total membrane ATPase activity was obtained from the wells containing imidazole assay mixture and that value minus the activity in the ouabain assay mixture gave activity of Na^{+}/K^{+} ATPase.

Data Collection

The cannula was attached to a needle head connected to a pressure transducer (ADInstruments, Oxford, UK) to allow for continuous measurement of BP and *f*h through Lab Chart 8 (Fig. 1.3 and 1.4). TeamViewer (TeamViewer GmbH, Göppingen, Germany) allowed for continued observance of the fish's *f*h and BP overnight to ensure successful recovery before nanoparticle injection. The PowerLab pressure transducer was calibrated using a two-point calibration and a static column of water to units of cmH_2O. Heart rate was measured using cyclic measurement to determine the distance between peaks created by increases in pressure occurring from the fish heart beating. BP was measured by the mean value across a steady 1-minute interval.

Figure 1.3: PowerLab Pressure transducer. This sent the signal to the transformer box to the LabChart8 on the computer.

Statistical Analysis

Analysis of the data was performed using a two-way ANOVA with repeated measures using GraphPad Prism 6 (*GraphPad* Software Inc., SanDiego, California). Tests of homogeneity of variance (Bartlett) and normality (Shapiro-Wilks) were tested using R. To determine significant differences between treatment groups over time a Bonferroni Post-Hoc analysis was performed. Plasma osmolality, membrane ATPase and Na^{+}/K^{+} ATPase enzyme activity differences were assessed using an independent t-test between control and treatment groups. Significance was determined based on a p-value of 0.05.

The *f*h and BP were determined using lab chart and the pressure transducer and were taken as mean values over a steady one-minute period that had minimal fish movement to disrupt the signal. The values were constantly monitored over the 24h exposure but recorded at 1, 5 and 10h to show the effects nanoparticles had on the *f*h and BP over time. The *f*h was measured as a straight value but BP measurements were calculated as a change in BP after the initial 24hr recovery and converted from cmH_2O to mmHg.

The statistical differences in response to nanoparticle treatment between the diploid and triploid fish were assessed using a two-way ANOVA comparison. This was done for both the recorded mean *f*h, change in BP over time, plasma osmolality and Na^+/K^+ ATPase assay, total membrane ATPase assay. These were all found to be insignificantly different ($p>0.05$) allowing for amalgamation of these data sets for the subsequent testing of significance between nanoparticle treatment and control in brook trout.

Results

The aim of this study was to determine the *in vivo* cardiac effects of nAg in brook trout and determine possible mechanistic pathways of toxicity. This was done through observation of *f*h and BP to determine cardiac effects and then plasma osmolality, Na^+/K^+ ATPase assay, total membrane ATPase to elucidate a mechanism and characterize cardiac effects of nAg.

Diploid vs Triploid

Two-way ANOVA comparison showed the p-values for a diploid vs triploid comparison. The p-values for both *f*h and BP were found to be insignificant, $p=0.41$ and $p=0.54$, respectively (N=3 for each ploidy). The plasma osmolality was also insignificant with $p=0.44$. The p-values for both Na^+/K^+ ATPase and total membrane activity were found to be insignificant, $p=0.0546$ and $p=0.2603$ respectively (N=3). This insignificance allows for the amalgamation of the respective data sets for the subsequent testing of significance between nanoparticle treatment and control in brook trout.

Heart Rate and Blood Pressure

A significant effect was found on *f*h between treatments, with higher rates in nAg exposed fish from 5 h post treatment to the end of the experiment (Fig. 2.1, p = 0.0060). There was not a significant difference in regards to a factor of time

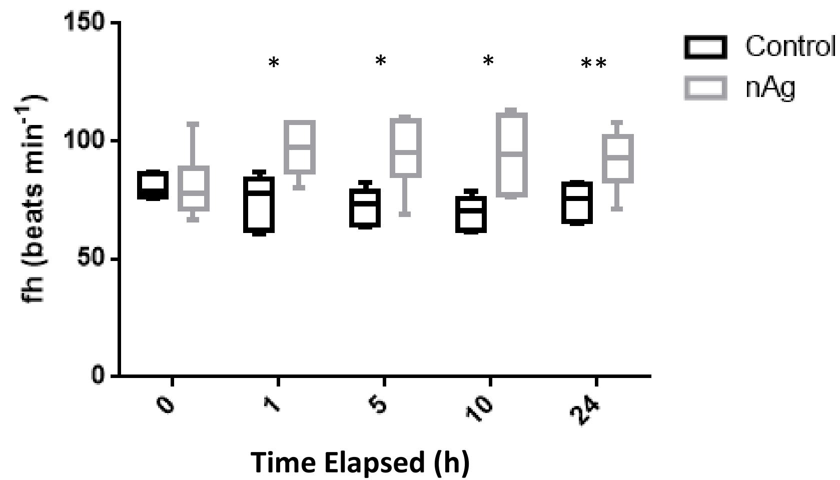

Figure 2.1: Effects of silver nanoparticle (nAg) injection on heart rate (*f*h). 700µg/kg nAg were injected and *f*h was measured in beats per minute (beats min^{-1}) over 24h compared to control brook trout. The effects between treatments were significant p=0.006, N=6, Bonferroni post hoc test determined significance between each time point (**p<0.05, **p <0.01)

BP measurements from the pressure transducer were calculated as a change in BP after the initial 24hr recovery with a mean BP of 17.57 mmHg. The change in BP was determined to be different between the control and treated group (Fig. 2.2, p = 0.005). The fish injected with nAg had a significantly higher BP than the control group at 5h. There was no significant effect of time on either treatment group.

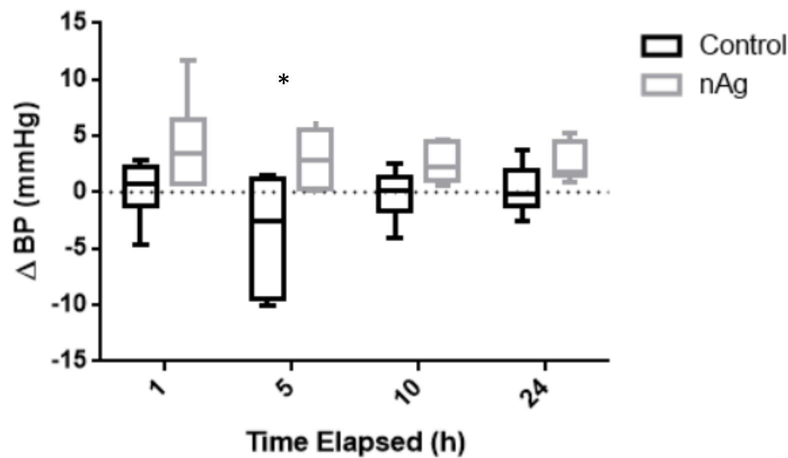

Figure 2.2: Effects of silver nanoparticle (nAg) injection on blood pressure (BP). 700µg/kg nAg were injected and BP was measured (in cmH2O then converted to mmHg), over 24h compared to control brook trout. p = 0.005, N=6. Bonferroni post hoc test determined significance between each time point (* p<0.05)

After a 24 h I.V. nAg exposure, the fish were injected with propranolol and then atropine to block β-adrenergic and acetylcholine receptors, respectively (Fig. 2.3). Propranolol caused a significant (25.5%) decrease in *f*h in the control fish (p= 0.0416) but not the treated fish (p=0.992). The atropine then caused a return to a non significant the resting *f*h in control and no change in *f*h in the nAg treated group. The difference between control and treated after the propranolol injection was significant (p = 0.0095). The change in *f*h over time is trending to significant (p=0.054) but there was variance in the control values following the propranolol injection (Fig. 2.3).

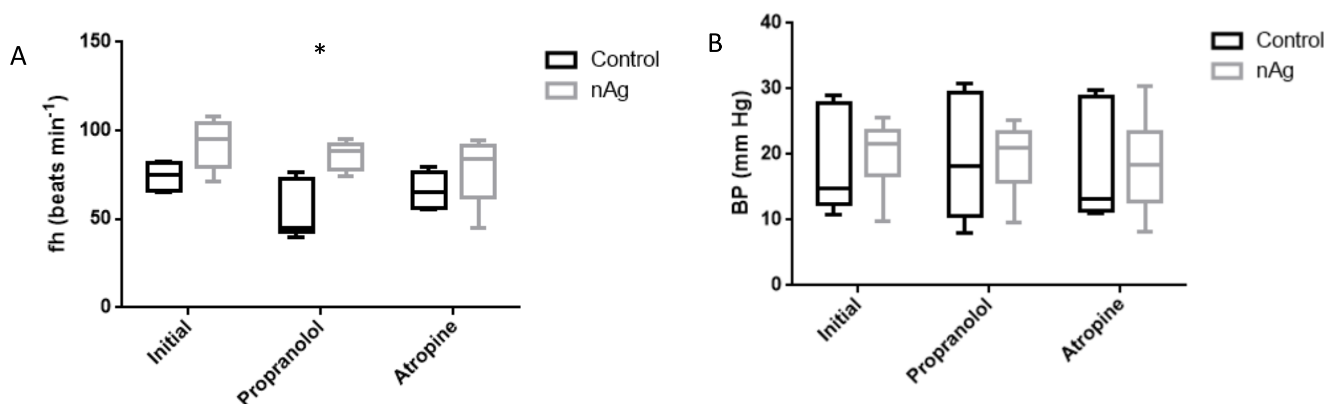

Figure 2.3: Effects of silver nanoparticle (nAg) injection on the brook trout with 700µg/kg of nAg. After injection with 3mg kg^{-1} propranolol and subsequent 1.3mg kg^{-1} atropine on A) Heart Rate (*f*h) and B) Blood Pressure (BP).

Plasma Osmolality and Sodium Potassium ATPase

Plasma osmolality did not differ between the control fish (263.7±54.8 mOsmols kg^{-1}) and the nAg-treated fish (262.3±21.2 mOsmols kg^{-1}, p = 0.96, Fig. 2.4).

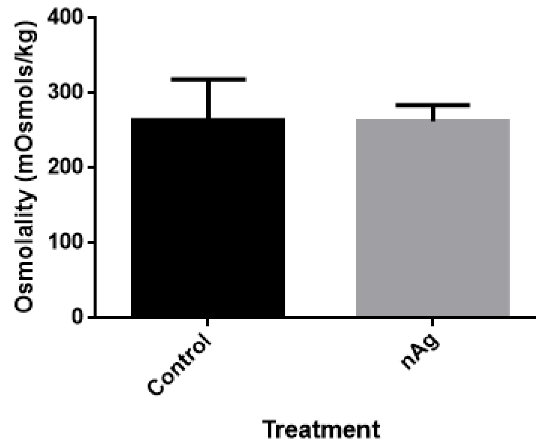

Figure 2.4: Plasma osmolality for control and nAg treatment groups of brook trout. Treatment was 700µg/kg injection of nAg. No significant difference was found between treatment groups p=0.957, N=6.

Brook trout gill tissue samples did not show a significant difference in Na^+/K^+ ATPase activity between the control and nAg injected fish (p=0.868). However, the heart tissue exposed to nAg injection exhibited a 74% decrease in activity of Na^+/K^+ ATPase compared to control (17.25±2.036 µmol mg^{-1} h^{-1} to 4.491±1.003 µmol mg^{-1} h^{-1}) (Fig. 2.5, p=0.0002).

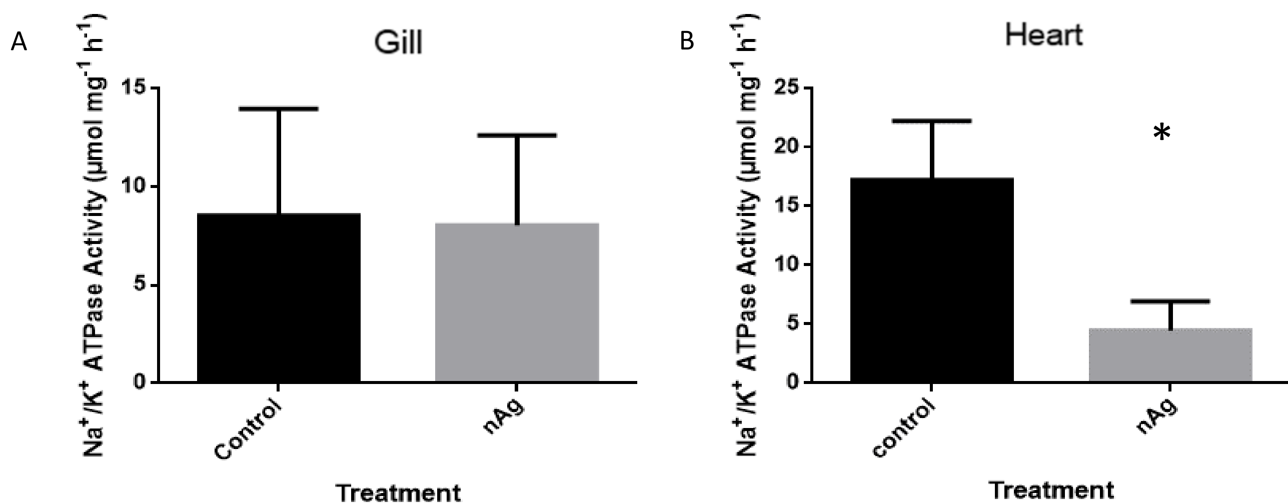

Figure 2.5: Effects of silver nanoparticle (nAg) injection on the maximal Na+/K+ ATPase activity in tissue samples of brook trout. Treatment was 700µg/kg injection of nAg. Samples were harvested 24h after exposure, flash frozen in liquid nitrogen and stored at -80°C. A) Gill tissue was not significantly different between treatment groups (p = 0.868) B) Heart tissue had a significant effect from treatment (p = 0.0002, N=6).

Total membrane ATPase enzymes were found to show no significant effects of nAg in both gill (p p=0.76) and heart tissues (Fig. 2.6, p=0.68,).

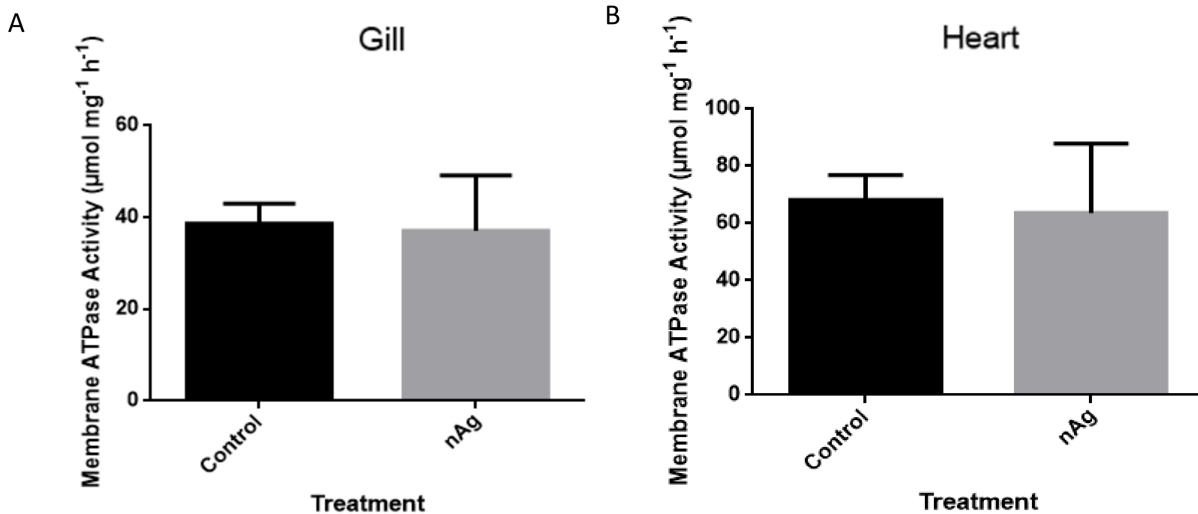

Figure 2.6: Effects of silver nanoparticle (nAg) injection on the maximal membrane ATPase activity in tissue samples of brook trout. Treatment was 700µg/kg injection of nAg. Samples were harvested 24h after exposure, flash frozen in liquid nitrogen and stored at -80°C. A) Gill tissue not significantly different between treatment groups p=0.763 N=6. B) Heart tissue not significantly different between treatment groups p=0.676 N=6.

Collectively these findings suggest that nAg impacts both fh and BP, as there were significant decreases in both after 24h I.V. nAg exposure. No impact was seen on plasma osmolality or total membrane ATPase activity in heart or gill, however there was a significant decrease in Na^+/K^+ ATPase activity in the heart.

Discussion:

The aim of this study was to determine the *in vivo* cardiac effects of I.V. nAg exposure in brook trout and determine possible mechanistic pathways of any associated toxicity. Through *in vivo* exposure to nAg and pharmacological approaches, the *in vivo* cardiotoxicity of nAg was characterized in brook trout and potential underlying mechanisms were further elucidated. I.V. exposure to nAg elicited changes in both *f*h and BP of brook trout and pharmacological and biochemical approaches highlighted several possible mechanisms to explain these effects.

I.V. nAg exposure impairs cardiovascular regulation

Brook trout exhibited a mean BP of approximately 17mmHg, which is within the normal range for related species like the rainbow trout (~20-30mmHg; Sundell *et al.,* 2018). The mean *f*h observed prior to injection of saline or nAg was 74.5 beats min^{-1} which also falls close to the normal range for rainbow trout (~55-65 beats min^{-1}; Sundell *et al.,* 2018). Figures 2.1 and 2.2 indicate that nAg significantly increased both BP and *f*h in brook trout. Sequential propranolol and atropine injections predictably affected *f*h in control brook trout but *f*h in nAg treated fish was unresponsive to either drug (Fig. 2.3). The control group showed a significant decrease in *f*h with propranolol that was restored to baseline by atropine, which is the normal response in other fish species (Miller *et al.,* 2011). The absence of a *f*h effect in the nAg treated fish could be due to nanoparticles blocking access to the respective receptor sites. If the nanoparticles are already bound, potentially irreversibly, or if they denature the receptors, neither adrenaline nor acetylcholine would be capable of regulating *f*h. The effect of nanoparticles on adrenergic signalling has not been studied but they can impact acetylcholinesterase activity at high doses (Katuli *et al.,* 2014; Callaghan *et al.* 2016). This could trigger increased cardiac acetylcholine levels and decrease *f*h (Callaghan *et al.* 2016), but only if acetylcholine receptors were functional. In white suckers exposed to zinc oxide nanoparticles, atropine injection triggered the expected increase in *f*h, indicating acetylcholine receptor function remained intact. nAg either acts on the heart via a different mechanism than zinc oxide nanoparticles or the route of exposure (I.V. vs. environmental) causes differential toxicity. Regardless of the mechanism, without the capacity to rapidly and effectively regulate *f*h via these pathways, physiological performance would be limited and survival in the wild would be unlikely.

Exposing rainbow trout to environmental nAg did not impact their aerobic metabolic performance in a previous study (Ollerhead *et al.,* 2020). This study was done via water

exposure, which is a more realistic example of how fish would be exposed to nAg in the wild. This suggests that the cardiovascular responses observed here with I.V. nAg injections may not be environmentally relevant. However, it has been shown that even closely related species have varied responses to the same stressor. For example, lake trout (*Salvelinus namaycush*) and brook trout (*Salvelinus fontinalis*) respond differently to transportation stressors (McDonald *et al.,* 1993). Further studies are required across species to confirm the mechanism and indeed the generalisability of the responses observed in this study.

Increases in *f*h and BP are expected when exposing the fish to a toxin or stressor (Barton, 2002) as increased blood flow is required to support energetically demanding processes like protein synthesis in an immune response. If nAg trigger an immune response, they may cause inflammation of the blood vessels that can then contribute to the observed increase in BP (Reyes-Cerpa *et al.,* 2012). nAgs are functionalized with PVP so they do not aggregate as much as other nAgs, but they will interact with particles in the blood that could lead to clumping. This does not cause increased clotting and aggregation but can increase platelet adhesion (Laloy *et al.,* 2014). If this then impairs blood flow, it may cause baroreceptors to signal the brain to increase *f*h and BP to compensate. However, this would normally be achieved by decreasing acetylcholine release onto the cardiac pacemaker and given the insensitivity of *f*h to atropine in nAg treated fish, this seems an unlikely mechanism.

The increase in BP and *f*h could also potentially be explained by the decrease in cardiac Na^+/K^+ ATPase activity (Fig. 2.5). Inhibition of Na^+/K^+ ATPase will indirectly lead to a decrease in cardiomyocyte Na^+/Ca^{2+} exchange, which is essential for contractile function (Pirahanchi and Aeddula, 2020). Such small changes in cardiac function may be amplified when considered over the lifetime of a fish, where they could negatively affect performance and impact survival.

I.V. nAg treatment did not impact osmoregulation

Silver ions have been reported to inhibit gill Na^+/K^+ ATPase and carbonic anhydrase activity to disrupt Na^+ uptake and osmoregulation in freshwater fish (McGeer and Wood, 1998). In contrast to heart, where nAg treatment inhibited Na^+/K^+ ATPase activity, there was no significant effect of nAg exposure on either total ATPase or Na^+/K^+ ATPase activity in brook trout gill (Figs. 2.5 and 2.6). It is unclear why heart Na^+/K^+ ATPase activity was affected but not

activity in the gill, since membrane ATPases are highly conserved (Serrano, 1989). In keeping with the lack of inhibition of gill Na^+/K^+ ATPase activity, no significant differences in plasma osmolality were noted in nAg treated fish (Fig. 2.4). Additional research is necessary to determine the mechanism(s) by which I.V. nAg selectively impacts the heart while leaving the gill, the stereotypical target of silver toxicity, intact. Understanding this mechanism may be valuable in clinical applications of nAg targeting to specific tissue types for therapeutic or diagnostic purposes.

Conclusions

The aim of this study was to characterize the cardiovascular effects of nAg in brook trout and more broadly gain insight into how nAg impact the cardiac system of vertebrates. Through *in vivo* analyses, nAgs were determined to significantly impact cardiovascular function by increasing *f*h and BP. Biochemical analyses revealed a specific decrease in cardiac Na^+/K^+ ATPase activity and blockade of both β-adrenergic and acetylcholinergic receptors. Given the potential importance of these findings, further research would be valuable to increase sample sizes and investigate responses in other species. Furthermore, the methods used for testing, while being minimally invasive, might be improved to increase the environmental relevance in the method of administration and a wider range of exposure doses. This proof-of-concept will hopefully give rise to further studies having demonstrated now the practical application of nAgPVP exposure in fish (Vidanapathirana *et al.,* 2018).

Future Directions:

The experience and data gained from this study suggest a number of avenues of future research. Having nanoparticles administered through the water instead of injection would increase the environmental relevance of the study as well as provide an opportunity to examine chronic vs. acute vs. staggered exposures. Chronic exposure to nAg might be predicted to show effects that would not be seen in an acute 24 or 48h study. Studies looking at the potential concentration of nAg up the food chain could prove beneficial as well. If nAg-exposed zebrafish fed to larger fish resulted in a cardiac response in the larger fish, there would be reason to believe the effects could be amplified in humans. Further biochemical analysis such as

inductively coupled plasma mass spectrometry could help to identify which tissues are accumulating nAg and indicate where to focus assays for determining tissue-specific toxicity and mechanisms of action. Transmission electron microscopy might also be able to visualize the potential mechanism of action of nAg on cardiac proteins. Immediate extraction of cardiac muscle and observation of contractility under various conditions could allow for quantification of the performance capacity of the heart muscle after an environmentally relevant nAg exposure. This *ex vivo* model would allow for expanded pharmacological testing with controlled exposure, to further elucidate the toxic mechanism of action glimpsed in this study.

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