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N-acetylcysteine manipulation fails to elicit an increase in glutathione in a teleost model

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Abstract Levels of oxidative stress can be affected by a range of compounds including toxins and pharmaceuticals. Antioxidants are important protective compounds which counteract the damaging effects of oxidative stress. Glutathione (GSH) is one of the main antioxidants for many organisms and can be synthesized from administered N-acetylcysteine (NAC). NAC has therefore often been used in a wide range of taxa to manipulate levels of GSH. Our objective was to validate this approach in a wild temperate teleost fish model, the brown trout (*Salmo trutta*). We used intracoelomic injections of NAC in saline and vegetable shortening, at two different concentrations (100 and 400 mg/kg), with the appropriate controls and shams, under controlled laboratory settings. We found that NAC failed to elicit an increase in GSH over three time periods and concluded that NAC is not an effective method to enhance GSH levels in teleost fish using the concentrations and vehicles tested here. We emphasize the importance of validation studies across all new species/taxa when possible and suggest that more investigation is required with regard to NAC manipulation in fish if this approach is to be used.

Keywords Glutathione · N-acetylcysteine · Teleost fish · Saline · Validation studies · Vegetable shortening

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Introduction

Antioxidants, and more generally oxidative stress, have received much attention in recent years. The field of oxidative ecology has emerged with the growing body of evidence that oxidative stress processes are linked with life history strategies, coping mechanisms associated with environmental alterations, and pathogenesis (Kehrer 1993; Beaulieu et al. 2013; Speakman et al. 2015). Reactive oxygen species (ROS) are continuously generated through mitochondrial respiration (Halliwell and Gutteridge 2015) as well as by the actions of various hormones and neurotransmitters (Finkel 1998). The presence of ROS, if unquenched, can be highly detrimental to cellular macromolecules and cause oxidative stress (Halliwell and Gutteridge 2015). Of all the forms of ROS, hydrogen peroxide (H_2O_2) is of particular interest as it is one of the most stable and long-lived ROS (Kress et al. 1995).

N-acetylcysteine (NAC) is a known thiolic antioxidant, and is a precursor to glutathione synthesis as it provides cysteine groups for γ-glutamylcysteine synthetase, an essential enzyme required for the generation of glutathione (Pena-Llopis et al. 2003; Gutiérrez-Praena et al. 2012). NAC also protects against cellular damage through its direct reaction with ROS and cannot be obtained from diet (Aruoma et al. 1989). NAC manipulation has been used in a wide range of taxa (mammals (Reid et al. 1994; Tomkiewicz et al. 1994); amphibians (Giniatullin and Giniatullin 2003); birds (Valvidia et al. 2001)) in an attempt to manipulate glutathione levels. All studies dedicated to NAC manipulation in fish thus far have focused on the investigation of its protective effects against pesticides and pathogens under controlled laboratory conditions (e.g., Pena-Llopis et al. 2003; Sevgiler et al. 2007; Puerto et al. 2009; Üner et al. 2009; Gutiérrez-Praena et al. 2012), but none have documented its potential effects on wild populations. Moreover, to our knowledge, no studies have tested vegetable shortening as a carrier. Vegetable shortening is commonly used when manipulating wild fish from temperate regions (such as the brown trout) because the vehicle will solidify and hence prolong the effects of the injected substance.

Given that studies in the wild involving NAC manipulation have yet to be performed, our aim was to determine the best method to administer NAC in a wild population of brown trout under a highly controlled laboratory setting in an attempt to induce an increase in glutathione. Our goal was to bring a more experimental approach to a body of literature that is dominated by correlations (i.e., this approach would provide us with a way of manipulating antioxidants in wild fish, which may help us understand the role of oxidative stress processes in an ecological context). We tested saline and vegetable shortening as vehicles for intracoelomic NAC injections. We hypothesized that saline injections containing NAC would be more readily absorbed given that NAC is highly soluble in water, and therefore predicted that glutathione (GSH) would become elevated more quickly than in the vegetable shortening-treated fish. Furthermore, we predicted that the increase in GSH using saline would be short-lived, given that NAC will be absorbed more rapidly than with the vegetable shortening. We also hypothesized that vegetable shortening injections containing NAC would take longer to be absorbed given that vegetable shortening solidifies after the injection, and thus predicted that elevated GSH may take longer to appear, but that its presence will be long-lasting in comparison to saline-injected fish.

Material and methods

On July 1, 2016, wild juvenile brown trout (n = 240) were captured from the Kastbjerg stream, Jutland, Denmark, using backpack electrofishing (Scubla ELT 60 II GI; 300 V). Fish were transported to the laboratory facilities in a 100-L tank of fresh oxygenated stream water and were randomly attributed to one of three identical 4000-L tanks (n = 80 per tank). The tanks had a constant circulating flow of fresh oxygenated water, held at a constant temperature of 13.5 ± 0.4 °C (average temperature in the wild typically fluctuates between 10 and 15 °C during the summer). All fish were kept at a 17:7 light to dark photoperiod (representative of daylight in Denmark during the summer months) and fed daily with mosquito larvae, starting 1 day after the manipulation.

Fish were left to acclimate for 24 h, prior to manipulation. Fish were anesthetized using a solution of benzocaine (0.03-g l^{-1} ethyl-*p*-aminobenzoate; Sigma) in water, then weighed (± 0.01 g), measured for total length (± 0.1 cm), and tagged using a 12-mm PIT tag (Texas Instruments, RI-TRP-RRHP, 134 Hz, 0.01-g mass in air, Plano, Texas, USA). Fish were randomly assigned to one of seven treatment groups: (1) control, (2) sham-saline, (3) sham-shortening, (4) 100-mg/kg NAC in saline (sal-low), (5) 100-mg/kg NAC in vegetable shortening (veg-low), (6) 400-mg/kg NAC in saline (sal-high), and (7) 400-mg/kg NAC in vegetable shortening (veg-high), each group containing 30 fish (10 fish from each tank). In addition, some fish were simply left in the tank (i.e., totally undisturbed), and remained untouched until sampling (i.e., not tagged, weighed, or measured) so as to detect tagging effects if necessary, despite evidence that tagging has minimal impacts on salmonids (Larsen et al. 2013). Control fish were recovered in a 60-L tank of fresh water following tagging. NAC-treated fish received an intracoelomic injection of a suspension of physiological saline (0.59% NaCl in pure water) or vegetable shortening (100% vegetable shortening, Crisco, OH, USA) mixed with N-acetylcysteine (NAC; Sigma-Aldrich, St. Louis, MO, USA, Product A7250) using a dosage of 0.01-mL vehicle (concentration of 0.01-g or 0.04 NAC per mL) per 1 g of fish (equivalent to 100 or 400 mg kg^{-1}, respectively). Sham fish were injected with only 0.01-mL g^{-1} saline or vegetable shortening. NAC-treated fish were recovered separately from control and sham fish to prevent cross-treatment contamination of NAC. Once recovered, all fish were returned to the tank.

After 3 days, all fish from tank 1 (10 fish from each treatment group) were anesthetized and weighed as per the above description. Fish were sampled for blood (0.1 ml) from the caudal vasculature using a 25-gauge heparinized needle. Fish were then immediately euthanized using a lethal percussion. All samples were immediately flash-frozen with liquid nitrogen, and then stored at – 80 °C until analyzed. The same sampling technique was used at 6 and 9 days post-treatment using fish from tank 2 and 3, respectively. This method was used to avoid disturbing fish until sampling. These standardized techniques were approved by the Danish Animal Experiments Inspectorate (License number: 2013-15-2934-00808).

Glutathione (GSH) was measured in red blood cells (RBCs) samples using a glutathione assay as described in Birnie-Gauvin et al. (2017). This assay measures total glutathione (TGSH) and oxidized glutathione (GSSG). The concentration of reduced glutathione (GSH), the antioxidant, can then be derived from these values. Final values of GSH were reported in micrometer.

Statistical analyses were conducted using JMP v12.0.1 (SAS Institute Inc., Buckinghamshire, UK). A two-way ANOVA followed by a Tukey post hoc was used to evaluate differences in mass changes among the seven treatment groups, as well as differences in glutathione concentration.

Results

Fish initially weighed between 22.8 and 28.0 g. Fish in each treatment and day did not differ in condition initially ($F_{6,179} = 0.94$, $p = 0.47$). An interaction between treatment and day was detected for change in mass ($F_{12,179} = 2.12$, $p = 0.0178$, Table 1) such that sal-high fish gained the most mass on day 3. Generally, fish progressively decreased in mass over the course of the study. Time had a significant effect on glutathione concentration, such that day 6 had significantly elevated glutathione in comparison to days 3 and 9 ($F_{2,206} = 20.01$, $p < 0.0001$, Fig. 1). This elevation was observed in all groups including the control fish and the shams. On day 5 of the study, 6 and 3 fish, from tanks 2 and 3, respectively, were found dead. These fish all belonged to the sal-high treatment group. Additionally, one fish from the veg-low group was found dead in tank 3 as a result of jumping out of the tank. No other mortality occurred and at sampling, all fish were vigorous.

Discussion

The objective of our study was to validate the use of N-acetylcysteine (NAC) as a method to increase glutathione (GSH) in a teleost model, the brown trout. Based on the literature, we anticipated that we would see an increase in GSH with NAC injections. However, our results fail to demonstrate that NAC elicited such a response, given that no differences were observed among treatments. These findings pose a set of important concerns with regard to the use of NAC in teleost fish.

While a number of studies have claimed the protective effects of NAC against oxidative stress processes via an increase in glutathione synthesis (Pena-Llopis et al. 2003; Gutiérrez-Praena et al. 2012), none that we know of have properly validated this in fish. A common caveat to these types of experimental studies is in fact the lack of appropriate controls, shams, or validations (Cooke et al. 2017). In the majority of studies currently in the literature, either shams or controls are missing, making it rather difficult to interpret results and draw conclusions. Many of the studies investigating the protective effects of NAC have been performed on human patients with various illnesses (e.g., Horowitz et al. 1988; Prescott et al. 1989) or other mammalian models such as the rat (e.g., Moussawi et al. 2009). It is therefore possible that the physiological mechanisms by which NAC acts in mammals differ from those in fish. Alternatively, it is possible that the effects of NAC take longer to appear in tissues in wild brown trout given that antioxidant capacity and glutathione concentrations are already high (Birnie-Gauvin et al. 2017). No differences were observed in the control, shams, and untagged fish, when compared to treated fish, suggesting that NAC injections had no effect on GSH at all. Additionally, the increase in GSH observed in all groups at day 6 is likely not the result of the NAC injections themselves, given that control, shams, and untagged fish showed the same

Table 1 Change in mass of treated brown trout. Average change in mass (g) for each treatment groups, across sampling days (±SEM). Sample sizes are shown in parentheses. Treatment and day had significant effects on changes in mass (Tukey post hoc, $p < 0.001$). Asterisk represents significant difference from control of the same day

	Sampling day		
Treatment	3	6	9
Control	− 0.38 ± 0.18 (10)	− 0.92 ± 0.20 (10)	− 0.83 ± 0.21 (10)
Veg	0.16 ± 0.14 (10)	− 0.25 ± 0.22 (10)	− 0.89 ± 0.19 (10)
Veg-low	0.22 ± 0.29 (10)	− 0.36 ± 0.17 (10)	− 1.13 ± 0.26 (9)
Veg-high	0.18 ± 0.20 (10)	− 0.55 ± 0.27 (10)	− 1.02 ± 0.30 (10)
Sal	− 0.28 ± 0.21 (10)	− 0.97 ± 0.23 (10)	− 1.56 ± 0.27 (10)
Sal-low	− 0.33 ± 0.17 (10)	− 1.04 ± 0.25 (10)	− 1.16 ± 0.17 (10)
Sal-high	1.54 ± 0.23 (10)*	0.10 ± 0.21 (4)	− 1.01 ± 0.28 (7)

increase. It is also highly unlikely that laboratory conditions caused the observed day 6 increase, given that temperature, lighting, flow, and overall fish conditions were monitored at least four times a day.

Though NAC injections failed to increase GSH, a number of fish from the sal-high group were found dead on day 6 (from both the day 6 and day 9 tanks), suggesting that high concentrations of NAC absorbed at a rapid rate may have lethal impacts in fish. Similar results were found in a study on rats where low doses of NAC had protective effects against lipopolysaccharide toxicity, but high doses had the opposite effect and even increased mortality (Sprong et al. 1998). While it is possible that keeping wild fish in captivity has caused unknown physiological alterations where glutathione synthesis could be affected, previous studies have demonstrated that after 24 h in captivity, wild salmonids are typically calm with normal baseline levels of cortisol in comparison to captive-bred counterparts (e.g., Lepage et al. 2000; Patterson et al. 2004; Portz et al. 2006). Alternatively, GSH may have increased prior to the first sampling period at 3 days. Pena-Llopis et al. (2003) detected an increase in GSH as early 12 h post-injection with saline, though only sham fish were used in this study (no controls), making it difficult to properly interpret the results. Another alternative hypothesis as to why GSH increased in all treatments on day 6 is coincidental fluctuations in normal GSH levels. Further investigation is required to better understand natural patterns of GSH as well as the mechanistic basis for NAC in fish.

Validation studies, such as the present one, are crucial components of proper experimental science. We therefore urge other groups to take a similar approach to test the fundamental concepts applied to their study, and for each new species, when possible. We conclude that

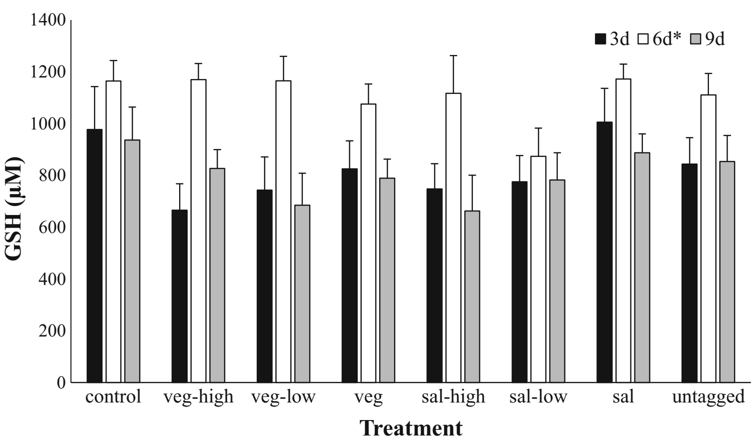

Fig. 1 Levels of glutathione in treated brown trout. Glutathione concentration (μM) ± SEM, across treatments and days. Time had a significant effect at day 6 on all treatments (Tukey post hoc, $p < 0.0001$)

further studies are required to investigate whether NAC injection is an adequate method to manipulate glutathione levels in teleost fish. We acknowledge that other vehicles or concentrations could have yielded different findings, but the ones used here are common carriers for other taxa. It may be worthwhile to explore other manipulation methods such of those that involve dietary manipulation (e.g., NAC infused in food items) or use of mini-osmotic pumps. Clearly, additional detailed validation work is needed before NAC is used to manipulate oxidative status in wild fish. Given the interest in bringing a more experimental approach to oxidative ecology, such validations are pressing. Until then, we caution against using NAC to manipulate oxidative status in teleosts.

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