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Comparative ploidy response to experimental hydrogen peroxide exposure in Atlantic salmon (*Salmo salar*)

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ABSTRACT

While research into the growth, survival, nutrition and, more recently, disease susceptibility of triploid Atlantic salmon has expanded, there remains an overall lack of studies assessing the response of triploids to chemical treatments. It is essential that the response of triploids to disease treatments be characterised to validate their suitability for commercial production. This study aimed to investigate and compare the stress and immune responses of triploid and diploid Atlantic salmon following an experimental treatment with hydrogen peroxide (H_2O_2). A dose response test was first undertaken to determine a suitable test dose for both diploid and triploid Atlantic salmon. Following this, diploids and triploids were exposed to H_2O_2 (1800 ppm) for 20 min, as per commercial practices, after which blood glucose and lactate, and plasma cortisol and lysozyme were measured, along with the expression of oxidative stress and immune-related genes. In the first 6 h post-exposure to H_2O_2, comparable mortalities occurred in both diploid and triploid Atlantic salmon. Cortisol, glucose and lactate were not significantly influenced by ploidy suggesting that, physiologically, triploid Atlantic salmon are able to cope with the stress associated with H_2O_2 exposure as well as their diploid counterparts. Exposure to H_2O_2 significantly elevated the expression of *cat* and *sod2* in diploid livers and *gr*, *il1β* and *crp/sap1b* in diploid gills, while it significantly decreased the expression of *saa5* and *crp/sap1a* in diploid gills. In triploids, the expression levels of *cat*, *hsp70*, *sod1*, *saa5*, *crp/sap1a* and *crp/sap1b* in liver was significantly higher in fish exposed to H_2O_2 compared to control fish. The expression of *gr*, *sod1* and *il1β* in triploid gills was also elevated in response to H_2O_2 exposure. This study represents the first experimental evidence of the effects of H_2O_2 exposure on triploid Atlantic salmon and continues to support their application into commercial production.

1. Introduction

As the global population continues to grow, so will the demand for aquaculture food products, with the industry aiming towards the intensification and expansion of production [1,2]. Increasing intensification, however, is not without risks as it is considered that fish in intensive aquaculture systems can experience higher pathogen infection pressures than their wild counterparts [3,4]. As a result, disease and resultant health and welfare issues are recognised as one of the largest single causes of economic losses for aquaculture, representing a significant constraint to the industry's continued development and success [5–7].

Over the years, bacterial and viral diseases have caused significant problems for the Atlantic salmon (*Salmo salar*) aquaculture industry including those caused by *Aeromonas salmonicida*, *Moritella viscosa* and *Flavobacterium psychrophilum*; infectious pancreatic necrosis virus (IPNV), salmon alphavirus (SAV) and infectious salmon anaemia virus (ISAV) [8]. Effective vaccines have been developed to prevent and control many of these diseases, particularly for those caused by bacterial pathogens [6,8–10]. Currently, the issues associated with parasitic diseases pose the most significant threat for Atlantic salmon aquaculture. In particular, sea lice (*Lepeophtheirus salmonis*; *Caligus elongatus*) and, more recently, amoebic gill disease (AGD; *Neoparamoeba perurans*) are considered two of the most damaging parasites for the salmonid industry, with losses equating up to 430 million and 80 million USD ($) worldwide per year, respectively [11–14]. With no vaccine available for the prevention of these parasites, a large proportion of the associated economic losses can be attributed to chemotherapeutic treatments [13]. One such treatment, now regularly employed by the aquaculture industry for the control of both sea lice and AGD, is hydrogen peroxide (H_2O_2) [15,16].

Hydrogen peroxide has long been used in aquaculture as a disinfectant for eggs [17]. Its use in the control for sea lice infections began in the early 1990's and it has been implemented to control AGD since

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2012 [16,18]. In current aquaculture practices, the recommended H_2O_2 concentration for the treatment of these parasites is 1500 ppm although it is recognised that concentrations above 2000 ppm are used [19–21]. Several factors make this product suitable for application in aquaculture. First of all, it has a highly reactive nature which makes it ideal for combatting external parasites [17]. Hydrogen peroxide is a strong oxidising agent that causes mechanical paralysis, peroxidation of lipid and cellular membranes, inactivation of enzymes and inhibition of DNA replication. In sea lice, this compound appears to induce mechanical paralysis when bubbles form in the gut and haemolymph, causing the parasite to release from the host and float to the surface [22]. Additionally, when in the aquatic environment H_2O_2 breaks down quickly (1–10 days) into water and oxygen, therefore leaving no toxic by-products and making it reasonably environmentally friendly [17,23,24]. However, concerns have been raised regarding fish welfare during exposure to this chemical and it has been reported to cause stress in Atlantic salmon in the first 24 h post-exposure [25,26].

Triploid Atlantic salmon have long been considered as a solution to address production issues associated with pre-harvest sexual maturation and escapees in the aquaculture industry [27]. While many similarities with diploid salmon have been reported, differences have also been documented, with variable growth and increased deformities reported in triploids [28–31]. With the expansion of triploid research over the last ten years, advances in triploid nutrition and rearing have now shown triploids performing equally or, in many cases, better than their diploid counterparts [32–38]. While recent research has continued to elucidate the response of triploid Atlantic salmon to disease [39–43], their response to disease treatments is still a relatively unexplored subject, particularly relating to chemical treatments such as H_2O_2. This is an important milestone given the increased environmental sensitivity reported in triploids when exposed to elevated temperatures and reduced oxygen levels [44,45]. Considering the potential to apply triploid salmon in full commercial production, it is crucial to understand their physiological response when exposed to aquaculture medicines in order to optimise health management strategies without compromising fish welfare. The aim of this study was to investigate the response of diploid and triploid Atlantic salmon to experimental exposure with H_2O_2 and assess susceptibility along with stress, immune and toxicological responses.

2. Materials and methods

2.1. Ethical approval

Experimental procedures were approved by the Animal Welfare and Ethical Review Body (AWERB) of the University of Stirling and were completed under UK Government Home Office project licence 60/4522. The euthanisation of fish for sampling was carried out according to the UK Government Home Office Schedule 1 regulations.

2.2. Fish stock and history

Eggs and milt were stripped from commercial Atlantic salmon broodstock (Landcatch Ltd.) and delivered to the Institute of Aquaculture, University of Stirling in December 2014. Following fertilisation, half of each egg batch was subjected to a pressure shock (655 bar for 6.25 min, 37.5 min post-fertilisation at 8 °C) to induce triploidy. Eggs were then incubated at 8.0 ± 0.1 °C in troughs until hatching (5th February 2015). At first feeding (2nd April 2015, 949 °D), fry were transferred into 300 L tanks and reared under constant light. Fry were fed a commercial diet (diploids - Inicio Plus; triploids – Inicio-TriX, BioMar UK), distributed by automatic feeders (Arvo-Tec Oy, Finland). When reaching 1 g (1738 °D), all fry were transferred to the Institute of Aquaculture freshwater unit at Buckieburn. They were maintained in 1.6 m^3 tanks (< 30 kg per m^3) under ambient water temperature (average: 8.3 ± 4.2 °C; range: winter 1.5 °C – summer 14.0 °C) and photoperiod to produce S1 + smolts. Specific feeding rates (% tank biomass per day) were adjusted automatically according to predicted growth (verified by sample weigh every 6 weeks) and daily temperature, and pellet size (0.5–3.0 mm) increased with fish size. To verify ploidy status in each stock, smears were prepared from blood collected by tail ablation from euthanised fish at 5 g (100/ploidy). After air drying, slides were fixed in 100% methanol and then placed into 6% Giemsa stain (6 ml Giemsa in 94 ml distilled water) for 10 min. Erythrocyte length and diameter were measured at 40 × magnification using image capture software (Image-Pro Premier, MediaCybernetics, Rockville, USA). All erythrocytes were numbered then selected using a random number generator. A total of 20 randomly chosen nuclei per slide were measured to the nearest 0.01 μm. Diploid control groups had significantly smaller erythrocyte nuclear lengths (two-sided T-test, $p < 0.05$) with no overlaps with the pressure shock triploid groups (2 N 6.8–7.7 μm; 3 N 9.0–10.2 μm) confirming that fish subjected to hydrostatic pressure shock were triploids.

Diploid and triploid Atlantic salmon were then transferred to the Institute of Aquaculture Temperate Aquarium Facilities on 18th February 2016. In preparation for sea water transfer, fish were vaccinated on 14th March 2016 with WINVIL® 3 micro (*Aeromonas salmonicida* subsp. *salmonicida*, *Moritella viscosa* & Infectious Pancreatic Necrosis Virus; Elanco Europe Ltd., United Kingdom). Mortality between first feeding and sea transfer was 4.8% and 5.1% for diploids and triploids, respectively. On 14th April 2016, 250 diploid (88.5 ± 2.2 g average body weight) and 250 triploid (78.2 ± 1.0 g average body weight) Atlantic salmon smolts were transferred to the Institute of Aquaculture seawater facilities at Machrihanish and stocked into two 2 m diameter stock tanks (3 m^3; 0.5 L kg biomass^{-1} min^{-1} flow rate). Tanks were maintained under ambient temperature (11.5 ± 1.8 °C) with aeration provided by air stones for 96 days until the trial commenced.

2.3. Hydrogen peroxide stress challenge

2.3.1. Dose-response toxicity test

A dose response test was first undertaken to determine an appropriate H_2O_2 dose for the stress challenge in diploid and triploid Atlantic salmon. This test was performed in July 2016 at ambient water temperature (14.0 °C) and simulated natural photoperiod set at 17 h light: 7 h darkness. For the dose response test, 21 diploid (183.0 ± 5.6 g body weight and 266.7 ± 2.4 mm body length) and 21 triploid (215.0 ± 4.9 g body weight and 282.4 ± 2.2 mm body length) Atlantic salmon were used. Experimental fish were randomly allocated into 14 × 397 L cylindrical tanks (n = 3 fish tank^{-1}; 7 tanks ploidy^{-1}). Following stocking, 1 tank ploidy^{-1} was allocated to each test concentration (1500, 1700, 1900, 2100, 2300, 2500 and 2700 ppm). Each concentration was then assessed separately. Fish were exposed to each concentration for 20 min before the tanks were flushed and the water refilled. During the H_2O_2 exposure, water was aerated ensuring that oxygen levels remained above 7 mg L^{-1}. After exposure, fish were monitored for 2 h for visual signs of stress *e.g.* flared opercula, increased ventilation, loss of equilibrium. Fish exposed to 1500 ppm H_2O_2 showed no change in behaviour, with fish exposed to 1700 and 1900 ppm exhibiting slightly increased ventilation. Rapid ventilation, loss of equilibrium and mortalities were observed following exposure to concentrations of 2100 ppm and above (total mortality for fish exposed to 2100–2700 ppm: diploids 25.0%; triploids 16.7%). As such, a nominal concentration of 1800 ppm was selected for the acute stress response trial.

Water samples were collected from each tank following the addition of H_2O_2 and the H_2O_2 concentration was immediately measured by cerium sulphate titration method [25]. To this end, 5 ml of 5 N H_2SO_4 and 7.5 ml of cerium IV sulphate solution were mixed in a conical flask. Then, a burette was filled with 50 ml of water sample and was slowly dispensed into the cerium IV sulphate solution, swirling to mix until the