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The effects of ozonation on select waterborne steroid hormones in recirculation aquaculture systems containing sexually mature Atlantic salmon *Salmo salar*

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ABSTRACT

Steroid hormones have been shown to accumulate in recirculation aquaculture system (RAS) water over time; however, their influence on the reproductive physiology of fish within RAS remains unknown. Whether ozonation impacts waterborne hormone levels in RAS has likewise not been fully evaluated. To this end, a controlled 3-month study was conducted in 6 replicated RAS containing a mixture of sexually mature and immature Atlantic salmon *Salmo salar* to determine whether ozone, as typically applied in RAS to improve water quality, is associated with a reduction in waterborne hormones. Post-smolt Atlantic salmon (1253 ± 15 g) were stocked into each RAS; 109 of 264 fish placed in each system were sexually mature males, and 5 were mature females. Water ozonation, controlled using an ORP set-point of 290–300 mV, was applied with the pure oxygen feed gas within the low-head oxygenators of 3 randomly selected RAS, while the remaining 3 RAS did not receive ozone. The RAS hydraulic retention time was 6.9 ± 0.3 days. Study fish were raised under these conditions for 12 weeks; during weeks 10 and 12, triplicate water samples were collected from the following locations in each RAS: i) culture tank, ii) makeup water, iii) pre-biofilter, iv) post-biofilter, and v) post-gas conditioning. Concentrations of 3 waterborne hormones – testosterone, 11-ketotestosterone (11-KT), and estradiol (17β-estradiol) – were quantified using enzyme immunoassays (EIA). Estradiol was significantly reduced by ozonation; testosterone and 11-KT were also reduced by ozonation, although these reductions were not observed across all sampling locations and events. Testosterone and 11-KT concentrations, however, were significantly reduced following water passage through the biofilters of both ozonated and non-ozonated RAS. The results of this study demonstrate the potential for ozone to be used in RAS as a means of preventing the accumulation of steroid hormones. Further research is required to assess whether reducing hormones in this manner impacts precocious sexual maturation in RAS-produced Atlantic salmon.

1. Introduction

Land-based, closed containment facilities utilizing recirculation aquaculture system (RAS) technologies are becoming more commonplace in the Atlantic salmon *Salmo salar* farming industry, not only to raise smolts and post-smolts prior to sea transfer but also, in a growing number of locations, to raise salmon to market size entirely on land (Summerfelt and Christianson, 2014). While the latter approach to salmon culture is still in its infancy, a major issue encountered thus far in land-based salmon growout is precocious sexual maturation, particularly in males (Davidson et al., 2016; Good and Davidson, 2016). Early maturation is detrimental to farm profitability due to the associated decrease in growth performance and feed conversion efficiency (McClure et al., 2007) and reduced product quality (Aksnes et al., 1986). Unfortunately, sexual maturation in Atlantic salmon is a complicated and highly flexible process (Taylor, 1991; Fjelldal et al., 2011), influenced by numerous factors including photoperiod (Taranger et al., 1998; Bromage et al., 2001), temperature (Taranger et al., 2003; Vikingstad et al., 2008), growth rate (Friedland and Haas, 1996; Duston and Saunders, 1999), and genetics (Wolters, 2010; Barson et al., 2015). The accumulation of steroid hormones in recirculating water has been suggested as a potential, additional instigator of sexual maturation in RAS facilities (Good and Davidson, 2016). Previous research has indicated that steroid hormones do indeed accumulate in RAS water (Good et al., 2014; Mota et al., 2014); however, their influence on salmon maturation has not been adequately characterized given the

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complexity of sexual development in salmon. Whether water ozonation at levels typically applied in RAS is sufficient to reduce accumulating hormones has likewise not been satisfactorily investigated.

All fish release conjugated (sulfonated or glucuronidated) or unconjugated ("free") steroid hormones into the aquatic environment through urine and feces (Vermeirssen and Scott, 1996) or through the gills (Ellis et al., 2005; Sorensen et al., 2005), respectively. Approaches to quantify hormones in water have been developed, such as radioimmunoassay (RIA) (e.g. Ellis et al., 2004) and enzyme immunoassay (EIA) (e.g. Kidd et al., 2010; Friesen et al., 2012), and have been validated in cultured species such as rainbow trout (*Oncorhynchus mykiss*) (Ellis et al., 2004). Various non-aquaculture studies have demonstrated that ozonation has the capacity to reduce or eliminate specific waterborne steroid hormones (e.g. Westerhoff et al., 2005; Snyder et al., 2008; Kawasaki et al., 2009); however, such studies have predominantly utilized relatively high ozone doses (e.g. 8 mg/L), and as such are difficult to compare to ozone levels typically observed in RAS operations (usually $<$ 1 mg/L). The possibility that ozone, as typically applied in RAS (i.e., at low-level, non-disinfecting dosages), can reduce waterborne hormones has thus far not been adequately investigated.

In the present study, we focused on three hormones, estradiol (i.e., 17β-estradiol), 11-ketotestosterone (11-KT), and testosterone, based on their major role(s) in the sexual maturation of fish, as well as on the availability of non-invasive techniques for their measurement in water samples. The major androgen produced by the testes of teleost fish is 11-KT, which is a derivative of testosterone (Taranger et al., 2010), and is associated with the onset of male sexual maturation in numerous fish species (e.g. Cavaco et al., 2001; Schulz and Miura, 2002; Campbell et al., 2003; Rodriguez et al., 2005). Circulating plasma levels of both 11-KT and testosterone are usually low in immature fish and rise markedly during spermatogenesis, declining shortly before the development of mature spermatozoa (Liley and Stacey, 1983; Fostier et al., 1987). Both of these androgens are also associated with reproductive behavior and the development of secondary sexual characteristics (Lofts, 1987), which in the case of male Atlantic salmon include bronze coloration and the development of a prominent kype (i.e., hooked jaw). In female Atlantic salmon, rising plasma estradiol levels are observed during commencement of secondary oocyte growth (Chadwick et al., 1987; King and Pankhurst, 2003). In general, estradiol is associated with female sexual maturation in many teleost species through, among other things, its role in promoting the synthesis and release of vitellogenin (the precursor of egg yolk, which is taken up by oocytes) (Skipper and Hamilton, 1977). Testosterone is a precursor for estradiol (Nagahama, 1987), and therefore this hormone plays a key role in both male and female Atlantic salmon development.

The following describes a 3-month study carried out to determine whether water ozonation, at levels typically applied in RAS, impacts the concentration of waterborne steroid hormones. A secondary objective was to determine the impact, if any, of biofiltration and/or gas conditioning on steroid hormone concentrations as recirculating water passes through these unit processes.

2. Materials & methods

2.1. Water recirculation aquaculture systems

The replicated (n = 6) experimental RAS used in this study have previously been described in detail (Davidson et al., 2011; Good et al., 2011; Davidson et al., 2013); the components of an individual RAS are shown in Fig. 1. To summarize, each system consisted of a fluidized-sand biofilter, CO_2 stripping column, low-head oxygenator (LHO), circular dual-drain culture tank (5.3 m^3), radial flow settler, microscreen drum filter (60 μm), heat exchanger, and a 1-HP centrifugal pump. The total RAS water volume was 9.5 m^3; water was recirculated at a rate of 380 L/min (100 gpm) through all processes, except only 60% was passed through the biofilter and 40% was passed through the heat exchanger. Makeup water originated from a freshwater spring source. All RAS were operated at an exchange rate of 99.7% on a flow basis, i.e. each unit of recirculating water volume consisted of 0.3% fresh makeup water. The RAS hydraulic retention time was 6.9 ± 0.3 days. A constant photoperiod (LD24:0; i.e., 24 h light, zero hours dark) was provided throughout the study, and fish were fed once an hour using automated feeders (T-drum 2000CE, Arvo-Tec, Finland) and a standard commercially available salmonid diet (Bio-Oregon, Westbrook, ME USA). Fish were fed to satiation based on standardized feeding charts, supplemented by observations of feeding activity and wasted feed. The mean feed loading was 1.5 ± 0.1 kg/day per m^3/day of makeup water flow.

2.2. Atlantic salmon

Fertilized, eyed Atlantic salmon eggs (Salmobreed AS, Bergen, Norway) were hatched on-site and raised in a flow-through system at 12°C under LD24:0 photoperiod until 40 g, at which point they received a 6-week LD12:12 artificial winter followed by a return to LD24:0 to induce smoltification. Post-smolt Atlantic salmon (102 ± 1 g) were then stocked into the replicated RAS, and raised for 8 months under high NO_3-N (99 ± 1 mg/L) versus low NO_3-N (10 ± 0 mg/L) conditions, described separately (Good et al., 2016). Following the Good et al. (2016) study, all fish were removed from the replicated RAS and combined into a holding tank to mix previously treated populations into a single source population for the present study. From this source population, salmon were selected for restocking each RAS (264 fish per RAS) with focus on distributing equal numbers of sexually mature fish; 109 mature males and 5 mature females were stocked into each RAS, such that the percentage of mature fish at study commencement was 43.2% in each culture tank. Sexually mature male and female Atlantic salmon were identified by characteristic morphological features (i.e., bronze coloration and prominent kype in males and ovipositor in females). Mean starting weight was 1253 ± 15 g, and mean starting density was 62 ± 1 kg/m^3. Water ozonation treatment (see below) began shortly afterward, and continued for 12 weeks; final mean salmon weight and density were 1853 ± 24 g and 91 ± 1 kg/m^3, respectively.

2.3. Ozonation

Three RAS were randomly selected to be ozonated using generators (Model G22, Pacific Ozone Technology, Benecia, CA, USA) that converted pure oxygen feed gas into ozone, which was then combined with the primary oxygen gas flow to the LHOs. To prevent ozone levels from reaching unsafe levels in the culture tank water, oxidation-reduction potential (ORP) was monitored in each tank via an ORP digital sensor (Model DRD1R5, Hach Company, Loveland, CO, USA) placed directly in front of the inlet flow. An ORP set-point of 290–300 mV was used, and SC100 Universal Controllers (Hach) provided proportional-integral-derivative control of generator output to maintain target ozone levels and prevent exposure of fish to toxic ozone residuals. An average of 1.79% ozone was contained in the feed gas produced by the ozone generators, and this was continuously monitored by Teledyne Instruments Ozone Monitor – Model 465H (Teledyne Instruments, City of Industry, CA, USA); data were recorded from the unit once daily.

2.4. Water sampling

For routine water quality assessments, water samples were collected weekly from culture tank side drains and tested on-site. Specific parameters, methodologies, and frequencies of testing are summarized in Table 1. To assess the effects of ozonation on waterborne hormones, 250 mL water samples were collected in triplicate in high density polyethylene bottles at the following locations in each RAS: i) makeup water, ii) pre-biofilter, iii) post-biofilter, iv) post-gas conditioning, and