Aquaculture 533 (2021) 736208

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Effects of ozone on post-smolt Atlantic salmon (*Salmo salar*) performance, health, and maturation in freshwater recirculation aquaculture systems

John Davidson [a,*], Steven Summerfelt [a,1], Åsa Maria O. Espmark [b], Vasco C. Mota [b], David Marancik [c], Ryan L. Earley [d], Anthony Snead [d], Christopher Good [a]

[a] The Conservation Fund Freshwater Institute, 1098 Turner Road, Shepherdstown, WV 25443, USA
[b] Nofima AS, Tromsø, NO 9291, Norway
[c] Department of Pathobiology, St. George's University, School of Veterinary Medicine, Grenada, West Indies
[d] Department of Biological Sciences, University of Alabama, 300 Hackberry Lane, Tuscaloosa, AL 35401, USA

ARTICLE INFO

Keywords:
Ozone
Recirculating aquaculture system
Atlantic salmon
Maturation
Waterborne hormones
Steroids

ABSTRACT

Steroid hormones accumulate in recirculation aquaculture systems (RAS) and may influence the reproductive physiology of farmed fish. Ozone reduces hormone concentrations in freshwater RAS used to rear Atlantic salmon, but its effect on reproductive development is unknown. Accordingly, an 8-month trial was carried out to evaluate the growth, health, and maturation of post-smolt Atlantic salmon (296 ± 4 g initial weight) reared in six replicated freshwater RAS (9.5 m^3 total volume) operated with or without ozone (N = 3/treatment). Residual ozone was controlled with an oxidation reduction potential (ORP) of 300–320 mV, and mean water temperature was maintained at 14.7 °C. Atlantic salmon growth was generally faster in ozonated RAS. Salmon from RAS with and without ozone weighed 2156 ± 101 and 1810 ± 15 g, respectively, by the end of the study. Caudal, anal, and pelvic fin damage was greater ($P < 0.05$) for salmon in ozonated RAS early in the trial but improved thereafter. No statistical differences in gill, skin, and skeletal muscle histopathology were observed between treatments at the end of the study. Waterborne estradiol, testosterone, and 11-ketotestosterone levels were periodically lower ($P < 0.05$) in ozonated RAS, but maturing salmon were more prevalent in these systems. At the end of the trial, percent maturation of salmon populations reared in RAS with and without ozone was 63 ± 7 and 48 ± 1%, respectively; however, maturity appeared to be related to fish size. Improved water quality was observed in ozonated RAS including reduced dissolved copper, iron, and zinc levels, total heterotrophic bacteria counts, and true color, and increased ultraviolet transmittance, which may have supported improved Atlantic salmon growth. Overall, ozone did not inhibit the onset or prevalence of Atlantic salmon maturation, but significant improvements in water quality and salmon growth performance resulted from its use.

1. Introduction

Many Atlantic salmon farms are now producing smolts and post-smolts using land-based recirculating aquaculture systems (RAS) (Bergheim et al., 2009; Dalsgaard et al., 2013), and a number of companies are producing or planning to produce market-size Atlantic salmon in RAS (Summerfelt and Christianson, 2014; Intrafish, 2018). Nevertheless, commercial development of a RAS industry for Atlantic salmon is still at an early stage, and precocious maturation has emerged as a challenge, particularly in mixed sex populations grown to market-size (Davidson et al. 2016; Good and Davidson, 2016). Atlantic salmon producers generally view early maturation as a significant problem due to coinciding physiological changes that include decreased growth and feed conversion efficiency (McClure et al., 2007), increased sensitivity to opportunistic infection (St-Hilaire et al., 1998; Taranger et al., 2010), and reduced flesh quality (Aksnes et al., 1986; Michie, 2001; Davidson et al. 2016; Davidson et al., 2017). These biological and product quality impacts generally equate to economic losses for Atlantic salmon farmers (McClure et al., 2007; Good and Davidson, 2016); therefore, early maturation should be reduced or eliminated in RAS-produced salmon to improve the economic viability of this aquaculture sector.

The onset and development of salmon maturation, however, is a

* Corresponding author.
E-mail address: jdavidson@conservationfund.org (J. Davidson).
[1] Present address: *Superior Fresh, W15506 Superior Fresh Drive, Hixton, WI 54635.*

https://doi.org/10.1016/j.aquaculture.2020.736208
Received 9 October 2020; Received in revised form 20 November 2020; Accepted 25 November 2020
Available online 2 December 2020

complex, multifactorial process that is influenced by a range of environmental (e.g., photoperiod, water temperature) and biological variables (e.g., feed intake, growth performance, condition factor, lipid reserves, and genetics) (McClure et al., 2007; Taranger et al., 2010; Good and Davidson, 2016). Therefore, causes for increased maturation in RAS are still under investigation. To add to the complexity, when RAS are operated with limited water exchange, dissolved nutrients and compounds accumulate in the culture water (Davidson et al., 2009; Martins et al., 2009), including some which can impact the endocrine system of fish, such as nitrate (Freitag et al., 2015; Good et al., 2017a; Kellock et al., 2018). Sex steroids are also produced by fish and can be excreted into water (Vermeirssen and Scott, 1996; Ellis et al., 2005; Sorensen et al., 2005). Recent trials have shown that steroid hormones including testosterone (T), 11-ketotestosternone (11-KT), estradiol (E2) (Good et al., 2014), and cortisol (Mota et al., 2014) can accumulate in RAS. Evidence of uptake and sensing of waterborne T by rainbow trout *Oncorhynchus mykiss* cultured in RAS has also been reported (Budworth and Senger, 1993), and Mota et al. (2014) suggested that sex steroid levels measured in RAS are within the olfactory sensitivity range of some fish species. Further, Leet et al. (2011) reported that exposure to exogenous hormones in natural environments can: i) disrupt biochemical and endocrine processes essential to reproduction, ii) alter gene expression related to sex determination and sexual differentiation, and iii) cause masculinization, femininization, intersex, and skewed sex ratio effects in fish populations. In addition, waterborne hormones are commonly administered via immersion to early life stage fish as a method to influence sexual differentiation and reversal (Piferrer and Donaldson, 1994; Hoga et al., 2018). Considering this body of research and the role that endogenous sex steroids play in fish maturation (e.g., Schulz and Miura, 2002; Taranger et al., 2010; Tokarz et al., 2015), it is reasonable to suspect that waterborne hormones could influence the endocrine function and onset of maturation in RAS-produced Atlantic salmon.

Within this framework, it is important to investigate water treatment technologies that could reduce hormone concentrations in RAS. For example, ozone, a commonly used water-oxidizing technology that imparts water quality improvements in RAS (Summerfelt and Hochheimer, 1997; Summerfelt, 2003; Davidson et al., 2011; Gonclaves and Gagnon, 2011; Powell and Scolding, 2018), reportedly reduces or eliminates specific waterborne hormones in non-aquaculture applications (Westerhoff et al., 2005; Broséus et al., 2009; Kawasaki et al., 2009). Moreover, Good et al. (2017b) found that ozone application to maintain 290–300 mV ORP reduced waterborne E2 and resulted in generally lower concentrations of T and 11-KT in a freshwater RAS stocked with a mix of immature and mature post-smolt Atlantic salmon (>1.2 kg). Similar research evaluating the potential for ozone to reduce or eliminate maturation in smaller, immature Atlantic salmon, putatively via reduction of waterborne steroid hormones, is therefore a worthwhile follow-up study to improve our understanding of salmon maturation in RAS.

To this end, a study was carried out to evaluate the effect of operating replicate RAS with and without low-dose ozone on the incidence of early maturation in post-smolt Atlantic salmon (<300 g initial weight), and to provide a comprehensive assessment of ozone's effect on salmon performance, health, and welfare. The authors hypothesized that the use of ozone would: i) reduce waterborne hormone concentrations, leading to reduced prevalence of early maturation, and ii) promote Atlantic salmon growth as a function of water quality improvements.

2. Materials and methods

2.1. Atlantic salmon

Mixed-sex Atlantic salmon were received as fertilized eyed eggs from Stofnfiskur (Hafnarfjörður, Iceland) and hatched onsite within a Heath-tray-style RAS incubation system. Following yolk sac absorption, juvenile salmon were transferred to a flow-through system with 24-h LED lighting where they were grown to 70–80 g. At this time, half of the fish were switched to 12-h:12-h light/dark (LD) to simulate an early winter and to induce smoltification per industry standard procedures, while the other half of the population remained on 24-h light (L). Photoperiod evaluation was included due to: i) the importance of this variable for maturation signalling, and ii) conflicting photoperiod × maturation results reported elsewhere (Fjelldal et al., 2011; Good et al., 2016; Hines et al., 2019). Following the 52-day artificial winter photoperiod, the adipose fin of salmon exposed to 24-h L was clipped for future identification, and fish were maintained for one additional month in a partial reuse system described by Summerfelt et al. (2004). The entire pre-study culture period was carried out using freshwater maintained at 12.5–14.5 °C. Thereafter, 500 salmon (250 fish from each photoperiod) were stocked within the six replicate RAS used for the trial (Fig. 1). To begin the study, mean Atlantic salmon weight among replicate RAS was 296 ± 4 g and initial biomass density was 28 kg/m^3. A 2-wk acclimation period was provided to allow fish to adjust to the new environment before adding ozone.

2.2. Recirculation aquaculture systems

Six replicate RAS operated with or without ozone ($N = 3$/treatment) were used for the 8-month study (Fig. 1) (Davidson et al., 2009). Each RAS (9.5 m^3 total volume) recirculated 340 L/min of freshwater through a 5.3 m^3 dual drain culture tank, radial flow settler, microscreen drum filter, fluidized sand biofilter, geothermal heat exchanger, gas conditioning column, and a low-head oxygenator (LHO) (Fig. 1). Three replicated RAS received ozone produced from a pure oxygen feed gas by a Model G22 generator (Pacific Ozone Technology, Benicia, CA, USA). Ozone gas (9–10% O_3 measured by Ozone Monitor M4654, Teledyne Instruments, San Diego, CA, USA) was added within the air space beneath the LHO water distribution plate (Fig. 1). To prevent ozone residuals from reaching unsafe levels, oxidation reduction potential (ORP) was monitored using a digital sensor (Model DRD1R5, Hach Company, Loveland, CO, USA) located near the tank inlet. SC100 Universal Controllers (Hach Company) provided proportional-integral-derivative control of ozone generator output to maintain target ORP levels at 300–320 mV.

RAS were operated with mean hydraulic retention times (HRT) of 14.9 ± 0.9 days (~7% of system water exchange/day) and feed loading rates of 3.6 ± 0.1 kg feed/m^3 of makeup water per day. RAS dilution rate was dictated by the discharged wastewater volume, which was sensed and replaced with new water via a float valve. Makeup water addition was measured in each RAS by a magnetic drive flowmeter (Model C700, Elster AMCO Water Inc., Ocala, FL, USA). Sodium bicarbonate ($NaHCO_3$; Church & Dwight Co. Inc., Ewing, NJ, USA) was periodically added to maintain alkalinity levels that support nitrification (Boyd et al., 2016). Lastly, a 12:12 LD photoperiod was provided throughout the trial, but approximately 5 lx was maintained during the "dark" period to facilitate 24-h feeding and semi-constant water quality conditions.

2.3. Feeding

Salmon were fed to apparent satiation using a computer operated system (TCFFI, Shepherdstown, WV, USA) programmed to deliver short feed bursts once per hour via automated feeders (T-drum 2000 CE, Arvotec, Huutokoski, Finland). Feeding rates were fine-tuned separately per RAS based on observations of feeding activity and wasted feed. Uneaten feed was collected four to five days per week from the cone bottom of radial flow settlers, rinsed to remove fecal material, and weighed in order to gain a general comparison of unconsumed feed amounts between treatments. A commercially available 44/29 (protein/fat - %) salmon diet (EWOS Dynamic Red ™, Cargill, Wayzata, MN, USA) was fed throughout the study.

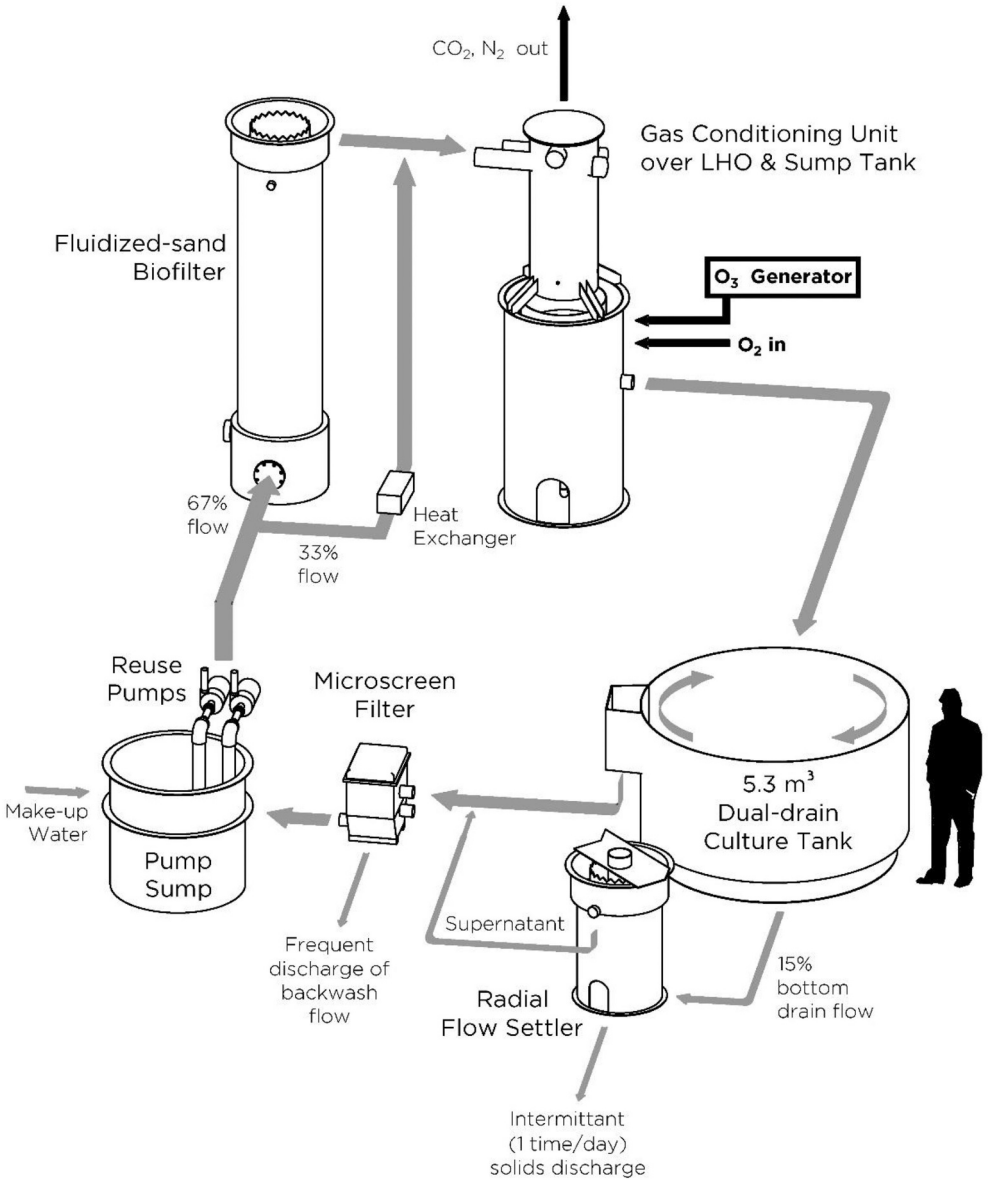

Fig. 1. Water flow and process design for an individual recirculation aquaculture system.

2.4. Fish sampling

Length and weight measurements of a random sample of 60 fish per RAS (~30 per photoperiod group) were collected to begin the study and thereafter at approximately 2-month intervals. Fish sample size was calculated using equations from Bhujel (2008) and Martin et al. (1987). Maturity status was also noted for all sampled fish where sexually mature salmon were identified by morphology characteristics, i.e., bronze coloration and prominent kype in males and ovipositor in females. External welfare indicators including eye cataracts, operculum, skin, snout, and fin damage were also scored for each fish ($n = 60$/RAS) according to guidelines established by Noble et al. (2018). Cataracts were scored with the naked eye using a 0–4 scale where absence was denoted 0 and severe cataracts covering >75% of the eye lens was scored as 4. All other welfare metrics were scored using a 0–3 scale where lack of damage was denoted 0 and severe damage/erosion was scored as 3. Welfare scores of fish sampled from each RAS were averaged and a grand mean was calculated for each treatment ($N = 3$). In accordance with onsite IACUC guidelines and maintenance of fish welfare, fish from each RAS were randomly culled midway through the trial to reduce the population by 50% and to maintain maximum fish density at <100 kg/m^3. Additionally, gonadosomatic index (GSI) percentage was assessed in a subsample of fish from each RAS ($n = 5$ - Month 2; $n = 30$ - Months 4, 6, 8) after fish began to demonstrate morphology consistent with early maturation. GSI (%) was calculated as follows: (gonad weight/ total body weight) * 100. Maturity was denoted for fish with GSI ≥ 1.0%.

Thermal growth coefficient (TGC), feed conversion ratio (FCR), and fish survival (%) were calculated bimonthly and/or cumulatively using the following formulae:

$$\mathrm{TGC} = \left(\text{End Weight}^{(1/3)} - \text{Start Weight}^{(1/3)}\right) / \left((\text{Days Between} * \text{Avg.Temp.}) \times 1000\right)$$

where weight is in grams, length is in mm, and temperature is in °C.

$$\mathrm{FCR} = \text{Cumulative Feed Delivered} / \text{Biomass Gain (BG)}$$

where BG = ((mean weight × number of fish $^{\text{after}}$) – (mean weight × number of fish $^{\text{before}}$)).

$$\text{Survival}\ (\%) = ((\text{Initial Number of Fish–Cumulative Mortalities\&Culls}) / \text{Initial Number of Fish})*100$$

2.5. Histopathology

Histopathology was carried out on five randomly selected fish per RAS at the completion of the study through assessments of gill tissue collected from the second arch, left side and a 0.5 × 0.5 cm section of skin tissue collected along the lateral line, ventral to the dorsal fin. All sampled fish were euthanized prior to tissue collection with 200 mg/L tricaine methanesulfonate. Representative samples of gill, skin, and underlying skeletal muscle were carefully removed using stainless steel scissors and forceps and preserved in 10% buffered formalin. Tissues were then processed routinely, sectioned at 4 μm, and stained with hematoxylin and eosin. Slides were examined blindly by a single pathologist using light microscopy and observed tissue alterations were semi-quantitatively scored on a 0-3-point scale based on cellular and extracellular changes and inflammatory infiltrates (0 representing no tissue change, and 3 representing severe changes observed). Specific pathological outcomes examined and scored included mononuclear cell infiltrates, eosinophilic granular cell infiltrates, goblet cell density, epithelial hyperplasia, lamellar adhesion and fusion (gill only), and cellular necrosis.

2.6. Water quality sampling and analyses

Water samples were collected from RAS tanks and makeup water and tested onsite using methods described by APHA (2012) and HACH Company (2003, 2015) (Table 1). Eleven select dissolved metals/elements were analyzed based on positive detection during previous studies in the same replicate RAS (Davidson et al., 2011, 2014). Metals analysis was carried out by REI Consultants Inc. (Beaver, WV, USA) on water samples collected once every two months.

2.7. Waterborne hormone analysis

Water for hormone analysis was collected from RAS tanks and makeup water after salmon from both treatments began to exhibit increasing morphologic signs of maturity. Samples were collected in 500 mL high density polyethylene bottles on study days 136, 164, 197, and 245, placed in freezer storage at -20 °C, and shipped in bulk to the University of Alabama after the study concluded. Waterborne hormones were extracted and assayed using enzyme-immunoassay (EIA) kits (Cayman Chemicals Inc., Ann Arbor, MI, USA) for T, 11-KT, E2 and cortisol in the same manner as described in Good et al. (2017b). To validate the EIA kits and determine appropriate dilution factors for each sample, 30 μL from each resuspended hormone sample of a particular type (i.e., tank or influent) was combined into a pool, which was then diluted from 1:1 (undiluted) to 1:32 (cortisol), 1:64 (11-KT 'influent') or 1:128 (T, 11-KT 'tank', E2) to generate serial dilution curves. All serial dilution curves were parallel to the standard curve, as assessed via the slope comparisons test (Zar, 1996): cortisol – tank: $t_9 = 0.021$, $p = 0.98$; cortisol – influent: $t_9 = 0.02$, $p = 0.99$; 11-KT – tank: $t_{12} = 0.016$, $p = 0.99$; 11-KT – influent: $t_{11} = 0.188$, $p = 0.85$; T – tank: $t_{12} = 0.022$, $p = 0.98$; T – influent: $t_{12} = 0.685$, $p = 0.51$; E2 – tank: $t_{10} = 0.121$, $p = 0.91$; E2 – influent: $t_{10} = 0.275$, $p = 0.79$. Samples were diluted as necessary to ensure that the concentrations would fall on the linear phase of the standard curve; these dilutions were: cortisol (tank and influent) – 1:4; E2 (tank and influent), T (influent), and 11-KT (influent) – 1:1 (i.e., no dilution); T (tank) and 11-KT (tank) – 1:10. Samples were run on two (cortisol, T, 11-KT) or three (E2) 96-well plates with pooled hormone extracts run in duplicate at the beginning and end of each plate to calculate intra- and inter-assay coefficients of variation, all of which were below 11% (intra-assay, cortisol – plate 1, plate 2: 4.4%, 4.3%; 11-KT: 4.2%, 4.1%; T: 1.4%, 9.9%; E2: 3.5%, 4.9%, 5.6%; inter-assay, cortisol: 3.5%; 11-KT: 5.8%; T: 10.6%, E2: 5.7%).

Table 1
Water quality parameters evaluated, methodologies, and frequency of testing.

Parameter	Method of Analysis	Frequency of Recording/ Testing
Dissolved Oxygen	Hach SC100 Controller & LDO® Probe	Daily
Oxidation Reduction Potential	Hach SC100 Controller & Differential ORP Sensor	Daily
Temperature	Hach SC100 Controller & Differential ORP Sensor	Daily
Specific Conductance	YSI 30 Salinity/Conductivity/ Temperature Meter	3–4 times weekly
Alkalinity	Hach Method 8203 - Sulfuric Acid Digital Titration pH endpoint Accumet #AB150	2–3 times weekly
pH	Standard Methods 4500-H^+ B – Electrode	2–3 times weekly
Biochemical Oxygen Demand	Standard Methods APHA 5210B - 5-day test (No prefiltration) YSI Model 58, YSI BOD probe #5905	Once weekly
Carbon Dioxide	Hach Method 8223 - Sodium Hydroxide Burette Titration pH endpoint Accumet #AB150	Once weekly
Dissolved Ozone	Hach Method 8311 (0.01–1.5 mg/L as O_3)	
Nitrate Nitrogen	Hach Method 8171 - Cadmium Reduction	Once weekly
Nitrite Nitrogen	Hach Method 8507 USEPA Diazotization	Once weekly
Total Ammonia Nitrogen	Hach Method 8038 USEPA Nessler	Once weekly
Total Heterotrophic Bacteria	Hach Method 8242 - Membrane Filtration, Fischer Isotemp Incubator #516D	Once weekly
Total Phosphorus	Hach Method 8190 – USEPA PhosVer3 with Acid Persulfate Digestion. DRB200 reactor and Hach Method 10,127 (Molybdovanadate w/ Acid Persulfate Digestion)	Once weekly
Total Suspended Solids	Standard Methods APHA 2540D - Dried at 103–105 ° C. Thelco Oven #6540, Mettler Toledo #AE240 and #PM30K	Once weekly
True Color	Hach Method 8025 - Platinum-Cobalt Standard	Once weekly
UV Transmittance	Hach Method 10,054 - Organic UV Absorbing (UV-254)	Once weekly
Dissolved Metals	Inductively Coupled Plasma Atomic Emission Spectrometry	Monthly - 4 events

-Spectrophotometers DR2700 and DR6000 (Hach Company, Loveland, CO, USA) were used for analysis of dissolved ozone, nitrate nitrogen, nitrite nitrogen, total ammonia nitrogen, and total phosphorus. Spectrophotometer DR4000 (Hach Company) was used for analysis of true color and UV transmittance.

2.8. Statistical analysis

Water quality data were analyzed using a restricted maximum likelihood mixed models test that assigned water quality criterion as dependent variables; treatment, time, and treatment × time as independent fixed factors; and RAS/tank as a random effect nested within treatment (Ling and Cotter, 2003; Thorarensen et al., 2015). Data transformation and/or removal of outliers was carried out as needed when analyzing water chemistry data. Fish performance, feeding, welfare, maturity metrics, dissolved metals, and waterborne hormone concentrations were analyzed using a two-sample Student's *t*-test (means comparison), or in the case of non-Gaussian distributed data, a Kruskal Wallis test. Two-factor ANOVA was utilized to evaluate side by side and interactive effects of primary treatment (ozone v. no ozone) and pre-study photoperiods. Ordered logit regression was carried out for scored histopathology data for each sampling point and tissue lesion type. A probability level of 0.05 was used to determine significance for all tests. Statistical analyses were carried out using SYSTAT 13 software

(2009) except for analysis of histopathology and hormones data, which were assessed using STATA v. 16.1 (StataCorp, College Station, TX, USA).

3. Results and discussion

3.1. Water quality

Important water quality criteria including alkalinity, dissolved oxygen, pH, and water temperature were controlled between treatments (Table 2). A range of other water quality variables were measured at significantly different concentrations between ozonated and non-ozonated RAS including ORP, total heterotrophic bacteria count (THBC), true color, and ultraviolet transmittance (UVT) (Table 2), as well as dissolved metals including copper, iron, and zinc (Table 3). Of these parameters, true color, THBC, copper, iron, and zinc levels were lower in ozonated RAS, while UVT and ORP were greater (Tables 2, 3), reflecting similar water quality improvements that have been observed onsite in ozonated RAS (Davidson et al., 2011; Good et al., 2017b). The implications of water quality differences to Atlantic salmon growth, health, and welfare are selectively discussed in the following sections.

3.2. Atlantic salmon growth and survival

First evidence of separation in Atlantic salmon growth curves was observed after two months as indicated by greater mean weights of sampled fish in ozonated (750 ± 9 g) versus non-ozonated RAS (637 ± 9 g) (Fig. 2). This trend continued throughout the study with statistical comparison indicating either higher mean weights in ozonated RAS or a borderline treatment effect (Fig. 2; Table 4). Resulting *P*-values at Months 2, 4, 6, and 8 were 0.001, 0.074, 0.011, and 0.073, respectively, where variance of means within treatment shifted the statistical outcome at Months 4 and 8. Metrics that considered fish weight such as fish biomass and density followed similar statistical trends (Table 4). Average TGC calculated across the study for salmon cultured in ozonated and non-ozonated RAS was 1.75 ± 0.04 and 1.57 ± 0.03, respectively ($P < 0.05$). However, bimonthly analysis indicated that TGC was greater for ozonated RAS during the first two months but similar between treatments thereafter (Table 4), suggesting that the brunt of the growth effect was dictated early in the study. By the end of the trial, salmon cultured in RAS with and without ozone weighed 2156 ± 101 and 1810 ± 15 g, respectively (Fig. 2; Table 4). Although growth was significantly impacted by treatment, survival was not. Cumulative Atlantic salmon survival in RAS with and without ozone was 98.7 ± 0.5 and 98.8 ± 0.2%, respectively.

In an attempt to discover a combination of variables that limit early maturation of post-smolt Atlantic salmon in RAS, fish exposed to two pre-study photoperiods were tracked throughout the study. It should be noted that salmon previously subjected to 12:12 LD entered the experiment at a significantly smaller mean weight (268 ± 4 g) compared to fish initially reared under continuous, 24-h L (330 ± 10 g). Likewise, Imsland et al. (2014) reported faster growth of juvenile Atlantic salmon subjected to continuous light versus a simulated natural photoperiod. During the present study, a significant growth effect related to pre-study photoperiod was observed at each sampling point except for the final event, indicating that salmon originally exposed to 12:12 LD exhibited compensatory growth (Fig. 3). Overall, however, the growth curves of salmon exposed to each pre-study photoperiod reflected the primary treatment effect, where fish growth was faster in ozonated RAS (Fig. 3). No interactive effects between ozone and pre-study photoperiod treatment were observed.

The reason for enhanced Atlantic salmon growth in systems with ozone is unclear. Davidson et al. (2011) reported a similar positive effect of low-dose ozone on rainbow trout growth when true color, heterotrophic bacteria counts, and dissolved copper were reduced and UVT was increased, among other improvements including reduced biochemical oxygen demand and total suspended solids. Therefore, it is reasonable to hypothesize that cumulative improvements to the culture environment instigated by ozone led to increased growth of post-smolt Atlantic salmon during the present study. In a review of literature on ozone application in aquaculture systems, Powell and Scolding (2018) speculated that the mechanisms for improved fish growth driven by ozone could be explained relative to reduced energetic costs of fish acclimating to water chemistry that might otherwise be suboptimal without ozone addition. Nevertheless, dramatic environmental improvements specifically related to water clarity should be considered. Of the water quality differences typically observed in onsite RAS when operating with and without ozone, true color was 13 times lower in ozonated RAS during the Davidson et al. (2011) trial and 22 times lower during the present study (Table 2, Fig. 4). In addition, UVT increased by approximately 27% as a result of ozonation during both trials. Clear water with reduced turbidity reportedly enhances the ability of salmonids to see and capture feed and can lead to increased growth (Sigler et al., 1984). A similar effect may apply to feed capture in experimental-scale tanks where feed remains suspended in the water for a short time.

Table 2
Water quality concentrations (mean ± standard error; mg/L unless otherwise noted) measured in RAS with and without ozone (N = 3) and makeup water.

Parameter	Ozone		No Ozone	Makeup Water
Carbonaceous Biochemical Oxygen Demand	1.6 ± 0.1		1.4 ± 0.1	0.4 ± 0.1
Carbon Dioxide	7.4 ± 0.4		6.5 ± 0.3	46 ± 2
Dissolved Oxygen	10.3 ± 0.12		10.2 ± 0.02	–
pH	7.62 ± 0.03		7.64 ± 0.04	7.30 ± 0.05
Nitrite Nitrogen	0.017 ± 0.009		0.022 ± 0.004	0.002 ± 0.000
Nitrate Nitrogen	105 ± 3		95 ± 3	2.5 ± 0.1
Oxidation Reduction Potential (mV)	307 ± 1	*	260 ± 5	–
Specific Conductance (μS)	1355 ± 20		1302 ± 15	–
Total Ammonia Nitrogen	0.194 ± 0.023		0.211 ± 0.010	0.018 ± 0.002
Temperature (°C)	14.7 ± 0.04		14.7 ± 0.05	–
Total Alkalinity	162 ± 8		178 ± 2	275 ± 4
Total Heterotrophic Bacteria (cfu/1 mL)	36 ± 7	*	135 ± 17	14 ± 3
Total Phosphorus (mg/L)	1.32 ± 0.14		0.88 ± 0.08	0.03 ± 0.01
Total Suspended Solids	2.1 ± 0.2		1.5 ± 0.1	0.5 ± 0.1
True Color (Pt-Co units)	2.1 ± 0.4	*	47 ± 2	1.1 ± 0.4
UV Transmittance (%)	87 ± 1	*	63 ± 1	98 ± 0.2

- Indicates data was not collected.
* Indicates significant difference between treatments ($P < 0.05$).

Table 3
Dissolved metals/trace element concentrations (mean ± standard error; N = 3) measured in RAS with and without ozone (N = 3) and makeup water.

Parameter (mg/L)	Ozone		No Ozone	Makeup Water
Calcium	107 ± 0.4		106 ± 0.5	110 ± 1.5
Copper	0.0072 ± 0.0004	*	0.0225 ± 0.0010	< det
Iron	0.012 ± 0.001	*	0.019 ± 0.002	< det
Magnesium	12.8 ± 0.01		12.8 ± 0.08	11.0 ± 0.29
Potassium	10.3 ± 0.3		10.1 ± 0.3	2.2 ± 0.1
Sodium	152 ± 7		145 ± 3	7.6 ± 0.2
Strontium	0.917 ± 0.004		0.913 ± 0.007	0.939 ± 0.022
Sulfur	15.3 ± 0.1		14.8 ± 0.1	6.2 ± 0.2
Zinc	0.052 ± 0.003	*	0.063 ± 0.001	0.062 ± 0.011

- Dissolved iron levels were generally above the minimum detection limit but below the practical quantitation limit.
* Indicates significant difference between treatments ($P < 0.05$).

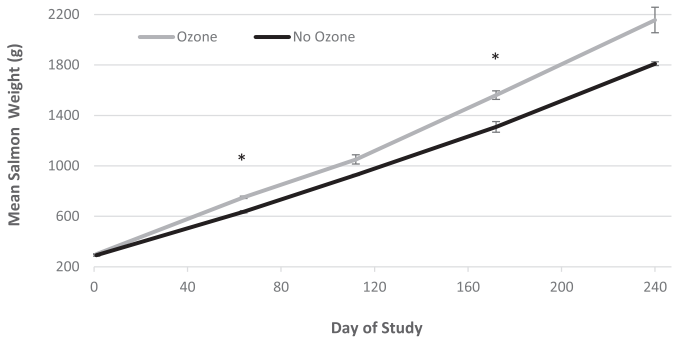

Fig. 2. Atlantic salmon weights (mean ± standard error; $N = 3$) in RAS with and without ozone over the study duration. * Indicates significant difference between treatments.

Table 4
Bimonthly growth performance, feeding, and fish production metrics (mean ± standard error; N = 3) for Atlantic salmon cultured in RAS with and without ozone.

Treatment	Response Variable	Bimonthly Fish Production, Feeding, and Performance Results			
		2	4	6	8
Ozone	Fish Weight (g)	750 ± 9 *	1051 ± 36	1561 ± 35 *	2156 ± 101
No Ozone		637 ± 9	928 ± 4	1309 ± 43	1810 ± 15
Ozone	TGC	2.6 ± 0.05 *	1.4 ± 0.21	1.6 ± 0.19	1.3 ± 0.08
No Ozone		2.1 ± 0.02	1.5 ± 0.05	1.3 ± 0.13	1.4 ± 0.17
Ozone	Fish Biomass (kg)	368 ± 5 *	511 ± 17	381 ± 10 *	454 ± 24
No Ozone		311 ± 4	448 ± 2	311 ± 4	381 ± 6
Ozone	Biomass Density	69 ± 1 *	96 ± 3	72 ± 2 *	86 ± 5
No Ozone	(kg/m^3)	59 ± 1	85 ± 0.3	61 ± 2	72 ± 1
Ozone	Feed Delivered (kg)	180 ± 5 *	162 ± 5 *	116 ± 14	128 ± 15
No Ozone		156 ± 3	128 ± 1	98 ± 4	96 ± 2
Ozone	Wasted Feed (kg)	1.32 ± 0.24 *	3.35 ± 0.43	3.85 ± 0.48	3.77 ± 0.08
No Ozone		2.23 ± 0.18	2.66 ± 0.06	4.42 ± 0.13	4.07 ± 0.31
Ozone	FCR	0.81 ± 0.02 *	1.16 ± 0.34	0.92 ± 0.01	1.04 ± 0.17
No Ozone		0.93 ± 0.02	0.91 ± 0.03	1.07 ± 0.23	0.91 ± 0.10

-TGC, feed delivered, wasted feed, and FCR calculated with representative data generated over 2-month intervals.
* Indicates significant difference between treatments ($P < 0.05$).

Post-study evaluation of feed sinking rates indicated that feed was suspended in the water column of the 1.2-m deep tanks for <10 s and flushed from the tank in approximately 30 s. Under these conditions, nominal inhibition of fish sight could impact feed capture.

Regardless of the exact environmental and/or physiological cause for increased Atlantic salmon growth in ozonated RAS, improved growth in the absence of significant maturation would likely facilitate economic benefits at a commercial farm due to reduced production time and associated expenditures related to energy, oxygen use, and labor. An economic analysis evaluating the capital and energy costs of operating ozone systems along with costs related to duration of the fish production cycle should be carried out to fully understand the tradeoffs.

3.3. Feed conversion

During the first two months of the study, salmon FCR was lower in ozonated RAS (0.81 ± 0.02) versus non-ozonated RAS (0.93 ± 0.02). Given that FCR calculations considered all feed inputs, this difference was likely driven by contrasting wasted feed amounts between treatments. This assertion is supported by periodic wet weight measurements of uneaten feed collected from radial flow settlers indicating nearly double the wasted feed in non-ozonated RAS during this period (Table 4). Per the previous discussion regarding feed capture response, it is interesting to note that the greatest true color measurements in non-ozonated RAS (Months 0–2) coincided with observations of increased wasted feed (Fig. 4). As the study progressed, fish production personnel effectively adjusted daily feed amounts according to wasted feed observations; therefore, differences in mean FCRs were not observed at other sampling intervals. As a result, cumulative FCR for salmon produced in RAS with and without ozone was similar between treatments over the study duration, i.e., 0.98 ± 0.05 and 0.95 ± 0.03, respectively.

3.4. Histopathology

Gill and skin tissue sections appeared in overall good health with only minor, subclinical histopathologic findings. No statistical associations were determined between observed lesions (presence and severity) and RAS ozonation treatment. The most common findings within gill tissue were mild eosinophilic granular cell and mononuclear cell infiltrates, increased goblet cell density, and rare epithelial hyperplasia and single cell necrosis; however, along with skin sections, cellular changes appeared uniform between all groups ($P > 0.05$). Previously, Good et al. (2011) reported increased gill epithelial hyperplasia and hypertrophy in rainbow trout exposed to ozonation (ORP set point = 250 mV) for four months in replicated RAS, compared to unexposed controls; however, these findings were not observed in the present study. Similar on-site research with Atlantic salmon (Good et al., 2017b) did not include histopathology evaluation; however, recent research carried out by Stiller et al. (2020) determined that approximately 40% of post-smolt Atlantic salmon (100 g mean weight) demonstrated gill epithelial lifting, hypertrophy, hyperplasia, and clubbing when exposed to ozone residuals resulting in 250 mV ORP for 10 days in flow-through brackish water. Stiller et al. (2020) also demonstrated that the prevalence of these lesion types, as well as gill lamellar fusion and necrosis, increased as ORP increased up to 500 mV. The absence of similar findings in the present study could be related to environment (i.e., freshwater RAS versus brackish flow-through), study fish (i.e., higher initial weight in the present study), or timing of tissue sampling (i.e., initial lesions associated with ozonated RAS could have resolved by the time of sampling).

3.5. External welfare indicators

No differences were observed between treatments for the following

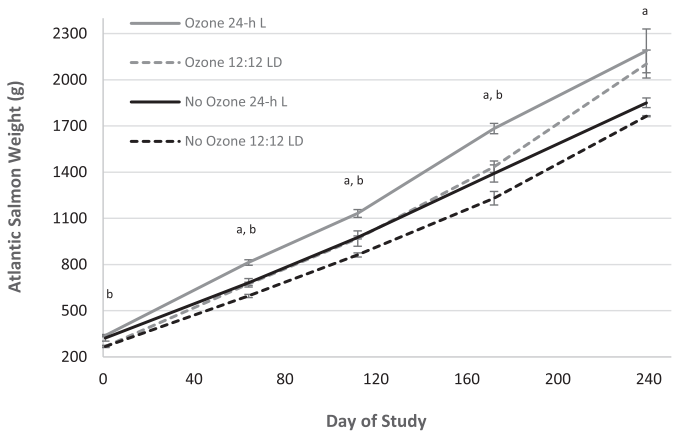

Fig. 3. Weights (mean ± standard error; N = 3) of Atlantic salmon exposed to two pre-study photoperiods (24-h light and 12:12 light/dark) from RAS with and without ozone over the study duration. [a] - Indicates significant effect of primary treatment (ozone v. no ozone). [b] - Indicates significant effect of pre-study photoperiod.

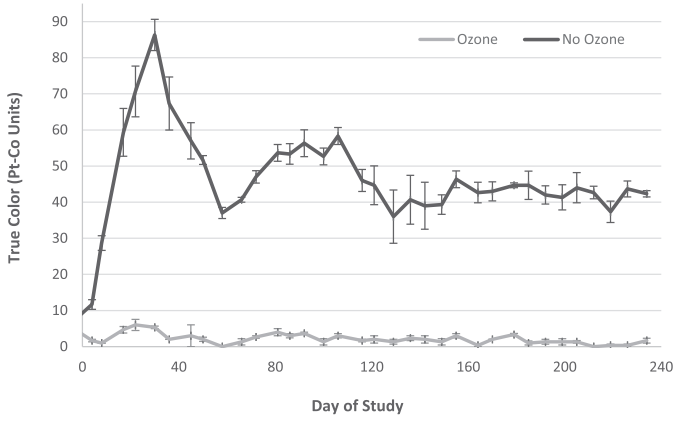

Fig. 4. True color (mean ± standard error; N = 3) in RAS with and without ozone over the study duration.

external welfare indicators: left and right eye cataracts, lesions, operculum damage, skin hemorrhages, and snout damage (Table 5). Mean welfare scores for these parameters were generally <1 indicating that most fish lacked these damage indicators (Noble et al., 2018). Scale loss, which can serve as a gateway for opportunistic infection, was greatest at Month 2, i.e. 1.5 ± 0.21 and 1.8 ± 0.20 for salmon from ozonated and non-ozonated RAS, respectively (Table 5). These slightly elevated scores may have been related to netting and relocating fish to begin the trial. Scale loss was significantly greater for salmon from non-ozonated RAS at Months 6 and 8, but the magnitude of differences was small and likely not of biological importance. Scale loss gradually improved for both treatments over the study duration (Table 5).

Fin damage defined by splitting of fin rays, tissue loss, and secondary issues such as opportunistic infection and hemorrhaging is common in farmed salmonids including Atlantic salmon (Turnbull et al., 1998; Ellis et al., 2002) and is therefore used as a welfare indicator (Stien et al., 2013; Noble et al., 2018). During the present study, fin damage scores were greatest for the caudal fin of salmon from both treatments which is consistent with observations from other studies. For example, Turnbull et al. (1998) found that farmed Atlantic salmon parr attacked the caudal and dorsal fins of conspecifics as a method of competitive aggression more frequently than other fins or areas of the body. Contrary to the findings of Turnbull et al. (1998), dorsal fin scores from the present study were low (Table 5); however, it is important to emphasize that scores were based on observations of active damage. Dorsal fins were damaged prior to the study but had healed, creating thickened nodular tissue that was less prone to further damage. Several important differences in fin scores were observed between treatments, however. For instance, salmon from ozonated RAS had greater damage of the caudal, anal, left and right pelvic fins at Month 2 (Table 5). Greater caudal fin damage was also observed for salmon from ozonated RAS at Month 4. With the exception of the caudal fin, however, fin scores for salmon cultured in ozonated RAS declined after Month 2 indicating a healing effect, while scores for fish from non-ozonated RAS gradually increased (Table 5). The only observation of greater fin damage noted for salmon from non-ozonated RAS was related to the left pectoral fin at Month 6.

During a previous study evaluating the effect of ozone on rainbow trout performance, health, and welfare, Good et al. (2011) did not observe significant dorsal or caudal fin damage; however, fin indices were only evaluated at the end of the study and an ORP setpoint of 250 mV was utilized (Good et al., 2011) versus the 300–320 mV range used during this trial. Although, the maximum fin scores noted during the present study only indicated minor damage, these slightly elevated scores still motivate practical considerations. For example, ozone was applied at the onset of the trial when RAS water contained relatively low levels of accumulating compounds. This approach was purposeful and related to the premise that constant reduction of waterborne hormones via ozonation (Good et al., 2017b) may limit early maturation. It stands to reason, however, that low-level ozone residuals present in the water early in the trial mildly affected salmon fin quality while other accumulating compounds were unavailable for ozone to oxidize. This theory is supported by dissolved ozone levels measured at Month 1 which averaged 0.02–0.03 mg/L, that then went undetected over the

Table 5
Fin damage and external welfare scores (mean ± standard error; N = 3) for Atlantic salmon from RAS with and without ozone.

Treatment	Welfare Variable	External Welfare Scores			
		2	4	6	8
Ozone	Dorsal Fin	0.1 ± 0.08	0.0 ± 0.03	0.0 ± 0.03	0.0 ± 0.03
No Ozone		0.1 ± 0.06	0.1 ± 0.03	0.0 ± 0.03	0.0 ± 0.03
Ozone	Caudal Fin	1.3 ± 0.07 *	1.5 ± 0.06 *	1.1 ± 0.23	1.6 ± 0.24
No Ozone		0.5 ± 0.00	0.8 ± 0.03	0.8 ± 0.02	1.1 ± 0.06
Ozone	Anal Fin	1.1 ± 0.06 *	0.9 ± 0.10	0.8 ± 0.09	0.9 ± 0.06
No Ozone		0.6 ± 0.03	0.8 ± 0.07	0.6 ± 0.05	0.8 ± 0.07
Ozone	Left Pelvic Fin	1.3 ± 0.06 *	1.2 ± 0.06	1.0 ± 0.14	1.0 ± 0.18
No Ozone		0.9 ± 0.07	1.2 ± 0.09	0.9 ± 0.06	1.1 ± 0.00
Ozone	Right Pelvic Fin	1.2 ± 0.09 *	0.9 ± 0.12	0.7 ± 0.11	0.9 ± 0.12
No Ozone		0.7 ± 0.03	1.1 ± 0.03	0.9 ± 0.13	1.1 ± 0.03
Ozone	Left Pectoral Fin	0.9 ± 0.10	0.7 ± 0.03	0.6 ± 0.07	0.7 ± 0.07
No Ozone		0.6 ± 0.07	0.9 ± 0.10	0.9 ± 0.07 *	1.0 ± 0.12
Ozone	Right Pectoral Fin	1.1 ± 0.12	0.7 ± 0.06	0.7 ± 0.12	1.0 ± 0.07
No Ozone		0.9 ± 0.07	1.0 ± 0.10	1.2 ± 0.12	1.2 ± 0.06
Ozone	Left Eye Cataract	0.1 ± 0.04	0.3 ± 0.06	0.3 ± 0.09	0.5 ± 0.17
No Ozone		0.1 ± 0.04	0.2 ± 0.01	0.2 ± 0.02	0.3 ± 0.07
Ozone	Right Eye Cataract	0.1 ± 0.03	0.4 ± 0.05	0.5 ± 0.12	0.8 ± 0.20
No Ozone		0.2 ± 0.05	0.4 ± 0.07	0.6 ± 0.11	0.5 ± 0.10
Ozone	Scale Loss	1.5 ± 0.21	0.8 ± 0.09	0.6 ± 0.09	0.5 ± 0.02
No Ozone		1.8 ± 0.20	0.9 ± 0.09	0.9 ± 0.05†	0.7 ± 0.06 *
Ozone	Snout Damage	0.3 ± 0.07	0.5 ± 0.14	0.3 ± 0.10	0.4 ± 0.07
No Ozone		0.2 ± 0.00	0.3 ± 0.04	0.3 ± 0.04	0.3 ± 0.04

Cataract scores (0–4 scale); All other welfare scores (0–3 scale).
* Indicates difference between treatments ($P < 0.05$). Notations beside significantly greater values.
† Indicates $P = 0.05$.

remainder of the study. These ozone levels are within the boundaries of the upper threshold (0.008–0.06 mg/L O_3) at which fish reportedly begin to experience somatic damage (Bullock et al., 1997). As such, a RAS facility might consider forgoing the use of ozone during the early months of system operation when tank water is relatively clear. In addition, although ORP was primarily maintained at 300–320 mV in ozonated RAS, ORP peaked beyond this range several times when solenoid valves responsible for controlling ozone delivery failed (Fig. 5). These short-term events cannot be ruled out as the cause for fin damage observed in salmon from the ozonated RAS.

3.6. Waterborne hormones

Testosterone, E2, and 11-KT concentrations were greater in RAS from both the ozone and no ozone treatments compared to the makeup water (Fig. 6), indicating that these sex steroids were produced and excreted by fish and subsequently accumulated in RAS. Cortisol levels in RAS tanks and influent makeup water were similar at each sampling point, indicating the likelihood of low-level cortisol contribution by the supply water. Albeit, other research has shown that factors such as reduced water usage, acute stressors, and water quality can also induce excretion and accumulation of cortisol in RAS water (Mota et al., 2017a; Mota et al., 2017b). Trends for waterborne T and 11-KT concentrations to be lower in ozonated RAS were evident (Fig. 6), with statistical differences noted at study days 197 & 245 (T) and 164 & 245 (11-KT). The general trend of increasing T and 11-KT in both ozonated and non-ozonated RAS points to increased fish production of these hormones as male maturation levels increased (Fig. 6); however, despite increasing levels of female maturation (Table 6) the same trend in waterborne E2 was not observed in non-ozonated RAS. Instead, generally consistent E2 concentrations were quantified across all sampling events (Fig. 6). As previously observed by Good et al. (2017b), E2 appears to be relatively sensitive to ozonation per the significantly lower levels observed in ozonated RAS at study days 136, 164, and 197. The final sampling at study day 245, which corresponded with elevated female maturation in both treatments (Table 6), demonstrated no significant difference in E2 concentrations, due to the relative increase in waterborne E2 in the ozone treatment group. Overall, these findings are consistent with previous trials carried out in the same replicate RAS. For example, Good et al. (2014) also reported mild accumulation of soluble T, 11-KT, and E2 in RAS, but while rearing initially larger (931 g) and more mature Atlantic salmon without ozone. Additionally, Good et al. (2017b) found that E2 was reduced by ozonation, while T and 11-KT levels were generally lower in ozonated RAS; albeit, not at every sampling point.

3.7. Atlantic salmon maturation

Reduction of waterborne hormone levels brought about by ozone did

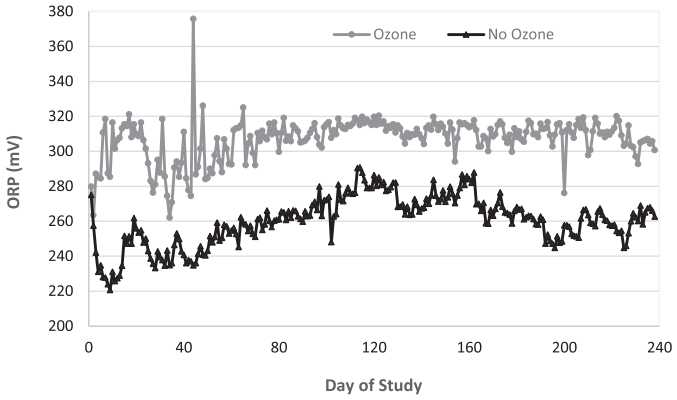

Fig. 5. Mean daily oxidation reduction potential (mV) in RAS with and without ozone (N = 3) over the study duration.

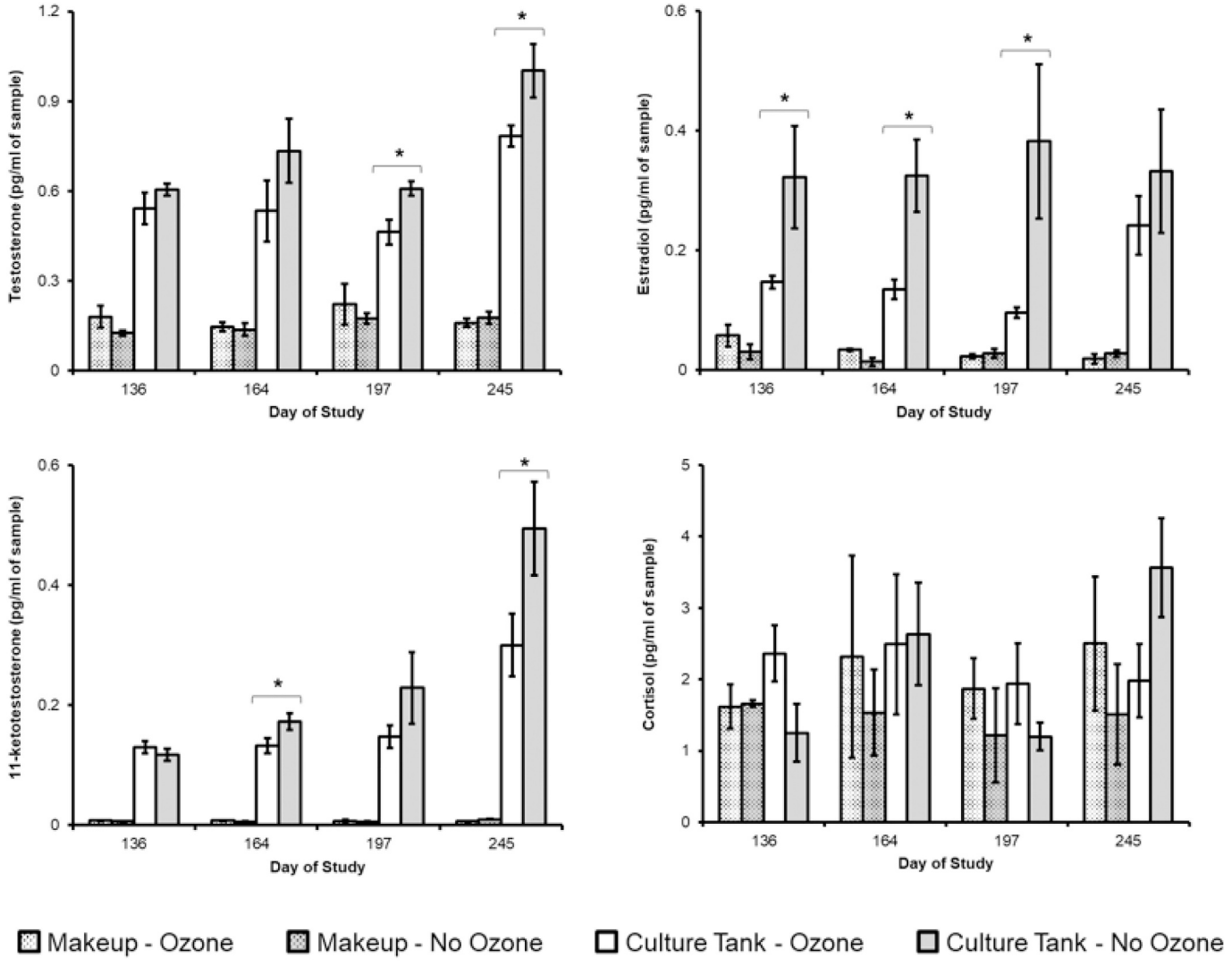

Fig. 6. Waterborne hormone levels (mean ± standard error; N = 3) in pg/mL of water sample, including testosterone, estradiol, 11-ketotestosterone, and cortisol in RAS with and without ozone at four sampling points spanning study days 136–245. Asterisks represent significant ($P < 0.05$) differences in hormones concentrations between ozonated and non-ozonated culture tank water samples.

Table 6
Atlantic salmon maturation percentages (mean ± standard error; N = 3) from bimonthly samples collected over the study duration.

Treatment	Variable	Number Sampled Fish/ RAS	Bimonthly Maturation Indices and Percentages				
			0	2	4	6	8
Ozone	Mean Population Weight (g)	60	300 ± 3	750 ± 9 *	1051 ± 36	1561 ± 35 *	2156 ± 101
No Ozone			292 ± 8	637 ± 9	928 ± 4	1309 ± 43	1810 ± 15
Ozone	Fish with External Maturation Indicators (%)	60	24.8 ± 4.5	17.3 ± 3.4	26.3 ± 3.8	45.0 ± 8.7	55.6 ± 6.9
No Ozone			21.9 ± 0.3	22.5 ± 5.5	31.3 ± 1.9	39.7 ± 5.5	41.1 ± 1.1
Ozone	Gonadosomatic Index (%)	30	–	1.6 ± 1.0 *	1.8 ± 0.3	2.6 ± 0.7	6.6 ± 0.1*
No Ozone			–	0.2 ± 0.01	1.5 ± 0.5	2.3 ± 0.1	4.1 ± 0.4
Ozone	Maturation (%) Gonadosamatic Index ≥1.0	30	–	13.0 ± 7.0 *	28.9 ± 2.8	41.9 ± 9.3	63.0 ± 7.0
No Ozone			–	0.0	18.9 ± 5.9	33.0 ± 2.0	48.0 ± 1.0
Ozone	Male Maturation (%) (GSI ≥ 1.0)	~14	–	–	57.9	63.4	69.0
No Ozone			–	–	36.4	54.9	52.9
Ozone	Female Maturation (%) (GSI ≥ 1.0)	~16	–	–	5.6	24.0	58.3
No Ozone			–	–	2.1	14.5	43.8
Ozone	Maturation (%) 12:12 Pre-study Photo (GSI ≥ 1.0)		–	–	30.6	38.8	62.7
No Ozone		~15	–	–	24.8	33.6	50.0
Ozone	Maturation (%) 24-h Pre-study Photo (GSI ≥ 1.0)		–	–	26.2	46.0	65.1
No Ozone		~15	–	–	17.1	32.8	45.8

- Indicates respective metrics were not evaluated at given sampling interval.
* Indicates difference between treatments ($P < 0.05$).

not inhibit maturation. Atlantic salmon cultured in RAS with and without ozone exhibited high rates of early maturity (Table 6), and mature male and female salmon were observed in both treatments at the end of the study (Table 6). However, salmon cultured in ozonated RAS exhibited higher gonadosomatic index at Months 2 and 8 (Table 6) compared to fish from non-ozonated RAS. When separating maturation data to evaluate effects of photoperiod across the two ozonation treatments, no significant effects of photoperiod were observed, but a statistical effect of ozone treatment was identified at the end of the trial for subjective and objective (related to GSI) maturity assessments (Fig. 7). Both of these data sets showed that salmon cultured in ozonated RAS demonstrated a higher incidence of early maturation at the end of the trial (Fig. 7). When considering cumulative maturity data, salmon in ozonated and non-ozonated RAS exhibited 63.0 ± 7.0% and 48.0 ± 1.0% maturity, respectively. Be that as it may, maturation differences observed between treatments appear to be related to fish growth. For example, when average maturation percentage was plotted with coinciding mean weight (Fig. 8), trendlines overlapped closely between ozone and no ozone treatments, suggesting that gonadal development was partly dictated or coincidental to fish size, and that slower growing salmon eventually would reach the same state of maturity. In hindsight, it would have been valuable to assess GSI at every sampling point to understand the exact timing of gonadal development. A small sample of five fish per RAS collected at Month 2 indicated that salmon in RAS operated with and without ozone had GSI of 1.6 ± 1.0% and 0.2 ± 0.01% (Table 6) suggesting that gonadal development began sooner in faster growing salmon cultured in ozonated RAS. Several studies have shown that increased Atlantic salmon growth rate is partly related to the onset of maturation, often overlapping with other variables (e.g., photoperiod and temperature) that direct reproductive development (e.g., Adams and Thorpe, 1989; Taranger et al., 2010; Fjelldal et al., 2011; Imsland et al., 2014).

In the context of understanding maturation onset, it is important to note that 20–25% of Atlantic salmon used for this study demonstrated morphology consistent with early maturation (e.g., bronze skin coloration and early kype formation) at a mean weight ≤ 300 g (Table 6). Anecdotally, this indicates that environmental cues experienced by fish before the study may have provided the directive for reproductive development. With this in mind, the environmental conditions of land-based systems used for early rearing may deserve more attention relative to maturation onset. The early rearing regime typically employed for Atlantic salmon cohorts at TCFFI consists of RAS incubation at 7–8 °C, flow-through fry culture at 12.5–14 °C, and intermediate production in a partial reuse system at 12.0–14.5 °C. Interestingly, Fjelldal et al. (2011) demonstrated that a combination of increasing water temperature and continuous light can trigger early maturation in male Atlantic salmon during and immediately after smoltification. Specifically, early male maturation was pronounced when parr were cultured at 16.0 °C with continuous, 24-h light compared to fish reared at 5 and 10 °C under various photoperiods (Fjelldal et al., 2011). In addition, Imsland et al. (2014) found that long-term rearing of Atlantic salmon under continuous light, but lower water temperature (8.3 vs. 12.7 °C) balanced growth while limiting early maturation. The pre- and in-study photoperiods that fish were exposed to during this trial did not inhibit maturation. It may also be important to note that the mean and maximum water temperatures for both RAS treatments were 14.7 °C and 16.2 °C, respectively.

4. Conclusions

Overall, ozone did not inhibit the prevalence of Atlantic salmon maturation in freshwater RAS despite notable reductions in waterborne

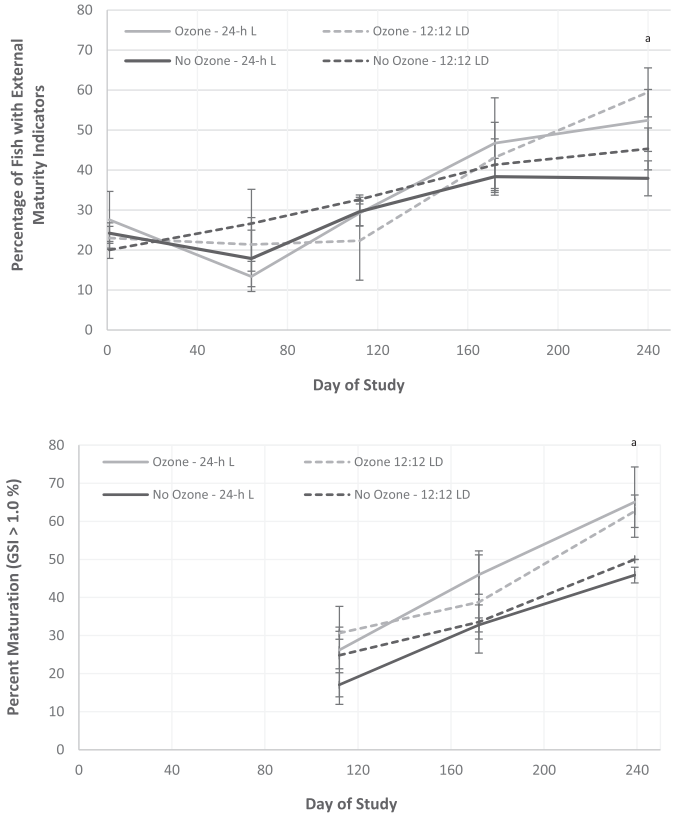

Fig. 7. Subjective maturity assessment (top) based on morphology indicators and objective maturity assessment (bottom) based on gonadosomatic index evaluation where salmon with GSI > 1.0% were considered mature. Percent maturation data presented as mean ± standard error; $N = 3$. Data provided for combinations of ozonation and pre-study photoperiod treatments at sampling points across the study duration. [a] - Indicates significant effect of primary treatment (ozone v. no ozone).

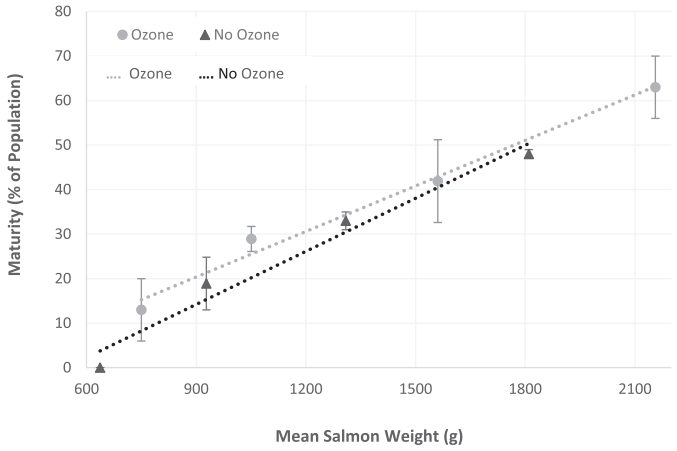

Fig. 8. Mean salmon weight plotted with coinciding percent maturity of Atlantic salmon populations reared in RAS with and without ozone ($N = 3$; mean ± standard error).

hormone levels. Additional research is therefore needed to determine an effective combination of environmental and/or biological conditions that reduce or eliminate early Atlantic salmon maturation in RAS. Given that a small percentage of fish exhibited morphology consistent with early maturation to begin the study, perhaps it would be interesting to evaluate the effect of ozone when rearing Atlantic salmon at a smaller size and earlier life stage, assuredly before the fish have received cues that signal a path towards maturation. As mentioned, more research is also needed to evaluate the potential effect of water temperature on early Atlantic salmon maturation, particularly given the warmer thermal conditions that are inherent of RAS. Lastly, notwithstanding the maturation findings, ozone had a positive effect on post-smolt Atlantic salmon growth that would likely reduce the duration of market-size salmon production in land-based RAS thereby leading to reduced production costs. If the maturation problem in RAS can be solved through establishment of an optimal set of environmental and biological conditions, then the use of ozone could be advantageous for RAS-based production of post-smolt Atlantic salmon.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest to report.

Acknowledgements

Special thanks to Natalie Redman, Megan Murray, Kevin Turner, and Destiny Evy for water chemistry analyses; Scott Tsukuda and Shanen Cogan for operations assistance; Lauren Cheeks, Courtney Harrison, and Brody Stelmach for fish husbandry and technical assistance; Kata Sharrer for engineering drawings; and Christine Lepine for data collection and organization. This study was part of the CtrlAQUA SFI, Centre for Closed-Containment Aquaculture and funded by the Research Council of Norway (Project No.: 237856/O30). Opinions, conclusions, and recommendations are the authors' and do not necessarily reflect views of the funding body. All experimental protocols complied with the Animal Welfare Act (9CFR) and were approved by TCFFI's Institutional Animal Care and Use Committee. The Conservation Fund and CtrlAQUA (NOFIMA) are equal opportunity employers and providers.

References

Adams, C.E., Thorpe, J.E., 1989. Photoperiod and temperature effects on early development and reproductive investment in Atlantic salmon (Salmo salar L.). Aquaculture 79, 403–409. https://doi.org/10.1016/0044-8486(89)90483-3.

Aksnes, A., Gjerde, B., Roald, S.O., 1986. Biological, chemical and organoleptic changes during maturation of farmed Atlantic salmon, Salmo salar. Aquaculture 53, 7–20. https://doi.org/10.1016/0044-8486(86)90295-4.

APHA (American Public Health Association), 2012. Standard Methods for the Examination of Water and Wastewater, 22nd ed. American Public Health Association, Washington, DC.

Bergheim, A., Drengstig, A., Ulgenes, Y., Fivelstad, S., 2009. Production of Atlantic salmon smolts in Europe – current characteristics and future trends. Aquac. Eng. 41, 46–52. https://doi.org/10.1016/j.aquaeng.2009.04.004.

Bhujel, R.C., 2008. Statistics for Aquaculture. Wiley-Blackwell, Ames, Iowa, USA.

Boyd, C.E., Tucker, C.S., Somridhivej, B., 2016. Alkalinity and hardness: critical but elusive concepts in aquaculture. J. World Aquacult. Soc. 47, 6–41. https://doi.org/10.1111/jwas.12241.

Broséus, R., Vincent, S., Aboulfadi, K., Daneshvar, A., Sauve, S., Barbeau, B., Prévost, M., 2009. Ozone oxidation of pharmaceuticals, endocrine disruptors and pesticides during drinking water treatment. Water Res. 43, 4707–4717. https://doi.org/10.1016/j.watres.2009.07.031.

Budworth, P.R., Senger, P.L., 1993. Fish-to-fish testosterone transfer in a recirculating water system. Prog. Fish-Cult. 55, 250–524.

Bullock, G.L., Summerfelt, S.T., Noble, A., Weber, A., Durant, M.D., Hankins, J.A., 1997. Ozonation of a recirculating rainbow trout culture system: I. effects on bacterial gill disease and heterotrophic bacteria. Aquaculture 158, 43–55. https://doi.org/10.1016/S0044-8486(97)00063-X.

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., Pedersen, P.B., 2013. Farming different species in RAS in Nordic countries: current status and future perspectives. Aquac. Eng. 53, 2–13. https://doi.org/10.1016/j.aquaeng.2012.11.008.

Davidson, J., Good, C., Welsh, C., Brazil, B., Summerfelt, S., 2009. Heavy metal and waste metabolite accumulation and their potential effect on rainbow trout performance in a replicated water reuse system operated at low or high flushing rates. Aquac. Eng. 41, 136–145. https://doi.org/10.1016/j.aquaeng.2009.04.001.

Davidson, J., Good, C., Welsh, C., Summerfelt, S., 2011. The effects of ozone and water exchange rates on water quality and rainbow trout Oncorhynchus mykiss performance in replicated water recirculating systems. Aquac. Eng. 44, 80–96. https://doi.org/10.1016/j.aquaeng.2011.04.001.

Davidson, J., Good, C., Welsh, C., Summerfelt, S., 2014. Comparing the effects of high vs. low nitrate on the health, performance, and welfare of juvenile rainbow trout Oncorhynchus mykiss within water recirculating aquaculture systems. Aquac. Eng. 59, 30–40. https://doi.org/10.1016/j.aquaeng.2014.01.003.

Davidson, J., May, T., Good, C., Waldrop, T., Kenney, B., Terjesen, B., Summerfelt, S., 2016. Production of market-size North American strain Atlantic salmon Salmo salar in a land-based recirculation aquaculture system using freshwater. Aquac. Eng. 74, 1–16. https://doi.org/10.1016/j.aquaeng.2017.08.003.

Davidson, J., Kenney, P.B., Barrows, F.T., Summerfelt, S., Good, C., 2017. Fillet quality and processing attributes of postsmolt Atlantic salmon, Salmo salar, fed a fishmeal-free diet and a fishmeal-based diet in recirculation aquaculture systems. J. World Aquacult. Soc. 49, 183–196. https://doi.org/10.1111/jwas.12452.

Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M., Gadd, D., 2002. The relationships between stocking density and welfare in farmed rainbow trout. J. Fish Biol. 61, 493–531. https://doi.org/10.1111/j.1095-8649.2002.tb00893.x.

Ellis, T., James, J., Scott, A., 2005. Branchial release of free cortisol and melatonin by rainbow trout. J. Fish Biol. 67, 535–540. https://doi.org/10.1111/j.0022-1112.2005.00740.x.

Fjelldal, P.G., Hansen, T., Huang, T., 2011. Continuous light and elevated temperature can trigger maturation both during and immediately after smoltification in male Atlantic salmon (Salmo salar). Aquaculture 321, 93–100. https://doi.org/10.1016/j.aquaculture.2011.08.017.

Freitag, A.R., Thayer, L.R., Leonetti, C., Stapleton, H.M., Hamlin, H.J., 2015. Effects of elevated nitrate on endocrine function of Atlantic salmon. Aquaculture 436, 8–12. https://doi.org/10.1016/j.aquaeng.2017.08.003.

Gonclaves, A.A., Gagnon, G., 2011. Ozone application in recirculating aquaculture system: an overview. Ozone Sci. Eng. 33, 345–367. https://doi.org/10.1080/01919512.2011.604595.

Good, C., Davidson, J., 2016. A review of factors influencing maturation of Atlantic salmon, Salmo salar, with focus on water recirculation aquaculture system environments. J. World Aquacult. Soc. 47, 605–632. https://doi.org/10.1111/jwas.12342.
Good, C., Davidson, J., Welsh, C., Snekvik, K., Summerfelt, S., 2011. The effects of ozonation on performance, health, and welfare of rainbow trout Oncorhynchus mykiss in low exchange water recirculation aquaculture systems. Aquac. Eng. 44, 97–102. https://doi.org/10.1016/j.aquaeng.2011.04.003.
Good, C., Davidson, J., Earley, R.L., Lee, E., Summerfelt, S., 2014. The impact of water exchange rate and treatment processes on waterborne hormones in recirculation aquaculture systems containing sexually maturing Atlantic salmon Salmo salar. J. Aquac. Res. Dev. 5, 1–7. https://doi.org/10.4172/2155-9546.1000260.
Good, C., Weber, G.M., May, T., Davidson, J., Summerfelt, S., 2016. Reduced photoperiod (18 hours light vs. 24 hours light) during first-year rearing associated with increased early male maturation in Atlantic Salmon Salmo salar cultured in a freshwater recirculation aquaculture system. Aquac. Res. 47, 3023–3027. https://doi.org/10.1111/are.12741.
Good, C., Davidson, J., Iwanowicz, L., Meyer, M., Dietze, J., Kolpin, D.W., Marancik, D., Birkett, J., Williams, C., Summerfelt, S., 2017a. Investigating the influence of nitrate nitrogen on post-smolt Atlantic salmon Salmo salar reproductive physiology in freshwater recirculation aquaculture systems. Aquac. Eng. 78 (Part A), 2–8. https://doi.org/10.1016/j.aquaeng.2016.09.003.
Good, C., Davidson, J., Earley, R.L., Styga, J., Summerfelt, S., 2017b. The effects of ozonation on select waterborne steroid hormones in recirculation aquaculture systems containing sexually mature Atlantic salmon Salmo salar. Aquac. Eng. 79, 9–16. https://doi.org/10.1016/j.aquaeng.2017.08.004.
HACH Company, 2003. DR/4000 Spectrophotometer Procedures Manual. Loveland, CO., Hach Company.
HACH Company, 2015. HACH Water Analysis Handbook, 8th ed., USA.
Hines, C.W., Fang, Y., Chan, V.K.S., Stiller, K.T., Brauner, C.J., Richards, J.G., 2019. The effect of salinity and photoperiod on thermal tolerance of Atlantic and coho salmon reared from smolt to adult in recirculating aquaculture systems. Comp. Biochem. Physiol. Part A 230, 1–6. https://doi.org/10.1016/j.cbpa.2018.12.008.
Hoga, C.A., Almeida, F.L., Reyes, G.R., 2018. A review on the use of hormones in fish farming: analytical methods to determine their residues. CyTA-J. Food 16, 679–691. https://doi.org/10.1080/19476337.2018.1475423.
Imsland, A.J., Handeland, S.O., Stefansson, S.O., 2014. Photoperiod and temperature effects on growth and maturation of pre- and post-smolt Atlantic salmon. Aquac. Int. 22, 1331–1345. https://doi.org/10.1007/s10499-014-9750-1.
Intrafish, 2018. Land-Based Salmon Farming: Aquaculture's New Reality. E. (Ed., Fischer.
Kawasaki, N., Ogata, F., Yamaguchi, I., Tominaga, H., 2009. Degradation characteristics of 17β-estradiol by ozone treatment with activated carbon. J. Oleo Sci. 58, 261–266. https://doi.org/10.5650/jos.58.261.
Kellock, K.A., Moore, A.P., Bringolf, R.B., 2018. Chronic nitrate exposure alters reproductive physiology in fathead minnows. Environ. Pollut. 232, 322–328. https://doi.org/10.1016/j.envpol.2017.08.004.
Leet, J.K., Gall, H.E., Sepúlveda, M.S., 2011. A review of studies on androgen and estrogen exposure in fish early life stages: effects on gene and hormonal control of sexual differentiation. J. Appl. Toxicol. 31, 379–398. https://doi.org/10.1002/jat.1682.
Ling, E.N., Cotter, D., 2003. Statistical power in comparative aquaculture studies. Aquaculture 224, 159–168. https://doi.org/10.1016/S0044-8486(03)00225-4.
Martin, S.W., Meek, A.H., Willeberg, P., 1987. Veterinary Epidemiology, Principles and Methods. Iowa State University Press, Ames, p. 343.
Martins, C.I.M., Pistrin, M.G., Ende, S.S.W., Eding, E.H., Verreth, J.A.J., 2009. The accumulation of substances in recirculating aquaculture systems (RAS) affects embryonic and larval development in common carp Cyprinus carpio. Aquaculture 291, 65–73. https://doi.org/10.1016/j.aquaculture.2009.03.001.
McClure, C.A., Hammell, K.L., Moore, M., Dohoo, I.R., Burnley, H., 2007. Risk factors for early sexual maturation in Atlantic salmon in seawater farm in New Brunswick and Nova Scotia, Canada. Aquaculture 272, 370–379. https://doi.org/10.1016/j.aquaculture.2007.08.039.
Michie, I., 2001. Causes of downgrading in the salmon farming industry. In: Kestin, S.C., Warriss, P.D. (Eds.), Farmed Fish Quality, 4. Fishing News Books, Oxford, UK, pp. 129–136.
Mota, V.C., Martins, C.I.M., Eding, E.H., Canário, A.V.M., Verreth, J.A.J., 2014. Steroids accumulate in the rearing water of commercial recirculating aquaculture systems. Aquac. Eng. 62, 9–16. https://doi.org/10.1016/j.aquaeng.2014.07.004.
Mota, V.C., Martins, C.I.M., Eding, E.H., Canário, A.V.M., Verreth, J.A., 2017a. Cortisol and testosterone accumulation in a low pH recirculating aquaculture system for rainbow trout (Oncorhynchus mykiss). Aquac. Res. 48, 3579–3588. https://doi.org/10.1111/are.13184.
Mota, V.C., Martins, C.I.M., Eding, E.H., Canário, A.V.M., Verreth, J.A., 2017b. Water cortisol and testosterone in Nile tilapia (Oreochromis niloticus) recirculating aquaculture systems. Aquaculture 468 (Part 1), 255–261. https://doi.org/10.1016/j.aquaculture.2016.10.023.
Noble, C., Gismervik, K., Iversen, M.H., Kolarevic, J., Nilsson, J., Stien, L.H., Turnbull, J. F. (Eds.), 2018. Welfare Indicators for Farmed Atlantic Salmon: Tools for Assessing Fish Welfare (351pp. ISBN 978-82-8296-556-9 https://nofima.no/en/fishwell/).
Piferrer, F., Donaldson, E., 1994. Uptake and clearance of exogenous estradiol-17β and testosterone during the early development of coho salmon (Oncorhynchus kisutch), including eggs, alevins and fry. Fish Physiol. Biochem. 13, 219–232. https://doi.org/10.1007/BF00004360.
Powell, A., Scolding, J.W.S., 2018. Direct application of ozone in aquaculture systems. Rev. Aquac. 10 https://doi.org/10.1111/raq.12169 (434–428).
Schulz, R.W., Miura, T., 2002. Spermatogenesis and its endocrine regulation. Fish Physiol. Biochem. 26, 43–56. https://doi.org/10.1023/A:1023303427191.
Sigler, J.W., Bjornn, T.C., Everest, F.H., 1984. Effects of chronic turbidity on density and growth of steelheads and coho salmon. Trans. Am. Fish. Soc. 113, 142–150.
Sorensen, P.W., Pinillos, M., Scott, A.P., 2005. Sexually mature male goldfish release large quantities of androstenedione into the water where it functions as a pheromone. Gen. Comp. Endocrinol. 140, 164–175. https://doi.org/10.1016/j.ygcen.2004.11.006.
St-Hilaire, S., Ribble, C., Whitaker, D.J., Kent, M., 1998. Prevalence of Kudoa thyrsites in sexually mature and immature pen-reared Atlantic salmon (Salmo salar) in British Columbia, Canada. Aquaculture 162, 69–77. https://doi.org/10.1016/S0044-8486(98)00208-7.
Stien, L.H., Bracke, M., Folkedal, O., Nilsson, J., Oppedal, F., Torgersen, T., Kittilsen, S., Midtlyng, P.J., Vindas, M.A., Øverli, Ø., Kristiansen, T.S., 2013. Salmon welfare index model (SWIM 1.0): a semantic model for overall welfare assessment of caged Atlantic salmon: review of the selected welfare indicators and model presentation. Rev. Aquac. 5, 33–57. https://doi.org/10.1111/j.1753-5131.2012.01083.x.
Stiller, K.T., Kolarevic, J., Lazado, C.C., Gerwins, J., Good, C., Summerfelt, S.T., Mota, V. C., Espmark, Å.M.O., 2020. The effects of ozone on Atlantic salmon post-smolt in brackish water – establishing welfare indicators and thresholds. Int. J. Mol. Sci. 21, 5109. https://doi.org/10.3390/ijms21145109.
Summerfelt, S.T., 2003. Ozonation and UV irradiation – an introduction and examples of current applications. Aquac. Eng. 28, 21–36. https://doi.org/10.1016/S0144-8609(02)00069-9.
Summerfelt, S., Christianson, L., 2014. Fish farming in land-based closed containment systems. World Aquac. Mag. 2014, 18–21. March. https://www.researchgate.net/publication/309075160_Fish_farming_in_land-based_closed_containment_systems.
Summerfelt, S.T., Hochheimer, J., 1997. Review of ozone processes and applications as an oxidizing agent in aquaculture. Prog. Fish-Cult. 59, 94–105. https://doi.org/10.1577/1548-8640(1997)059%3C0094:ROOPAA%3E2.3.CO;2.
Summerfelt, S.T., Davidson, J.W., Waldrop, T.B., Tsukuda, S.M., Bebak-Williams, J., 2004. A partial reuse system for Coldwater aquaculture. Aquac. Eng. 31, 157–181. https://doi.org/10.1016/j.aquaeng.2004.03.005.
Taranger, G.L., Carrillo, M., Schulz, R.W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F. A., Dufour, S., Karlsen, O., Norberg, B., Andersson, E., Hansen, T., 2010. Control of puberty in farmed fish. Gen. Comp. Endocrinol. 165, 483–515. https://doi.org/10.1016/j.ygcen.2009.05.004.
Thorarensen, H., Kubiriza, G.K., Imsland, A.K., 2015. Experimental design and statistical analyses of fish growth studies. Aquaculture 448, 483–490. https://doi.org/10.1016/j.aquaculture.2015.05.018.
Tokarz, J., Möller, G., de Angelis, M.H., Adamski, J., 2015. Steroids in teleost fishes: a functional point of view. Steroids 103, 123–144. https://doi.org/10.1016/j.steroids.2015.06.011.
Turnbull, J.F., Adams, C.E., Richards, R.H., Robertson, D.A., 1998. Attack site and resultant damage during aggressive encounters in Atlantic salmon (Salmo salar L.) parr. Aquaculture 159, 345–353. https://doi.org/10.1016/S0044-8486(97)00233-0.
Vermeirssen, E.L.M., Scott, A.P., 1996. Excretion of free and conjugated steroids in rainbow trout (Oncorhynchus mykiss): evidence for branchial excretion of the maturation-inducing steroid, 17,20β-dihydroxy-4-pregnen-3-one. Gen. Comp. Endocrinol. 101, 180–194. https://doi.org/10.1006/gcen.1996.0020.
Westerhoff, P., Yoon, Y., Snyder, S., Wert, E., 2005. Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. Environ. Sci. Technol. 39, 6649–6663. https://doi.org/10.1021/es0484799.
Zar, J.H., 1996. Biostatistical Analysis, 3rd ed. Prentice Hall, New Jersey. (662 pp.).