Stocking density limits for post-smolt Atlantic salmon (*Salmo salar* L.) emphasis on production performance and welfare

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Abstract

For the development of commercial scale semi-closed sea systems for farming post-smolt Atlantic salmon (*Salmo salar*), further knowledge is required on the interaction between fish density, farming conditions and fish welfare. In this experiment post-smolts (115.0 g ±13.6) were stocked at 5 different densities (25, 50, 75, 100 and 125 kg m^{-3}), and kept at these densities for 8 weeks. All treatments received an equal specific flow rate of 0.6 L kg $fish^{-1}$ min^{-1} of flow-through seawater (fully oxygenated, salinity 34 ‰ and temp. 9.3°C) and water oxygen (O_2), pH, carbon dioxide (CO_2) and total ammonia nitrogen (TAN) levels were monitored in the outlet and kept within recommended limits. Over the 8 week period, specific growth rate (SGR %) was significantly reduced in stocking densities of 50 kg m^{-3} and above. Increasing density from 100 kg m^{-3} to 125 kg m^{-3} lead to a 42 % decrease in SGR. Between 50 kg m^{-3} and 125 kg m^{-3} there was a correlation between reduced feed intake and increased stocking density and there was a linear increase in feed conversion ratio (FCR) with stocking density (25 kg m^{-3} to 125 kg m^{-3}). At the end of the 8 week period primary and secondary stress responses such as elevated plasma levels of cortisol, sodium, pCO_2 and decreased plasma pH were observed in the highest density treatment compared to other treatments. In combination with the reduced SGR in the highest density treatments these results indicate an allostatic overload *i.e.* the environment has exceeded the adaptive ability of the fish with chronic adverse effects on fish welfare. Stocking densities of 100 kg m^{-3} or more also increased pelvic fin damage and the prevalence of cataracts was higher in the 125 kg m^{-3} treatment. In conclusion, our results suggest that at this temperature and fish size it is feasible to rear Atlantic salmon post-smolts in densities up to 75 kg m^{-3} without compromising performance and welfare.

1. Introduction

Today the majority of post-smolt rearing in Norway takes place in open sea cages. However, new and alternative technologies are emerging, making it possible to move part of the post-smolt phase on land in closed recirculating aquaculture systems (RAS) or to large semi-closed containment systems (S-CCS) in sea (Rosten, et al., 2011; Thorarensen, et al., 2011). The overall production cost in S-CCS is likely to be higher than in open sea cages (Colt, et al., 2008) as a consequence of higher initial investments and possible need for oxygenation and water pumping. In this context, increased stocking density has been highlighted as an important factor that can contribute towards reducing overall production costs, provided that fish welfare and performance are not compromised. Several studies have been done on the subject of stocking density and its effects on fish (Ellis, et al., 2002; Hosfeld, et al., 2009; Kjartansson, et al., 1988). The majority of studies suggest that increased stocking density has a negative effect on fish welfare (Fagerlund, et al., 1981; Holm, et al., 1990; Schreck, et al., 1985; Trzebiatowski, et al., 1981). Reductions in growth and increased feed conversion ratio (FCR) as well as increased incidences of fin erosion are amongst the most commonly reported effects, although there is some disagreement as to the basic cause of these effects (Ellis, et al., 2002).

The current Norwegian regulation for the production of Atlantic salmon in open sea cages set an upper limit of 25 kg m^{-3} (Anon, 2004). However, with a high water exchange rate ensuring that vital water quality parameters, i.e. O_2, CO_2 and total ammonia nitrogen (TAN), are within acceptable limits it has been shown that it is possible to operate with stocking densities exceeding the current regulations (Hosfeld, et al., 2009). Hosfeld et al. (2009) found no negative effects on gill Na^+, K^+,-ATPase (NKA), plasma ion levels, plasma glucose, growth and condition in Atlantic salmon post-smolts after exposing them as pre-

smolts to densities up to 86 kg m^{-3} for 100 days in fresh water. These results are in line with Kjartansson et al. (1988), who detected no negative effects on stress responses and growth in large Atlantic salmon reared at densities from 30 to 125 kg m^{-3} in land-based systems. Although the findings of Kjartansson et al. (1988) and Hosfeld et al. (2009) suggest that smolts in freshwater and adult salmon in sea water can be farmed at relatively high densities, corresponding results on post-smolts are lacking. Hence, the introduction of new technology demands development of new production protocols, including new knowledge on the effect of increased stocking density on the post-smolt stage.

Welfare is a complex and currently debated topic and a stress response does not necessarily entail poor welfare but a physiological adaption to a changing environment. In fact, it has been suggested that the relationship between stress and welfare is not inversely related (i.e. increased stress leads to decreased welfare) but rather to follow an allostasis concept where too little or too much stress impairs welfare (McEwen, et al., 2003). In teleosts, elevated plasma cortisol levels commonly occur shortly after exposure to a stressor and are considered a primary response. Circulating cortisol is further involved in activating secondary responses like increased blood glucose, osmoregulatory and haematological changes which in turn allow the fish to react and compensate for the stressful stimuli (Barton, et al., 1991; Wendelaar Bonga, 1997; Wright, et al., 1989). However, long-term or repeated stress can lead to an allostatic overload of these adaptive mechanisms with chronic effects on the organism (Korte, et al., 2007; Schreck, 2010; Sterling, 2012). Stocking density, type of enclosure, water quality and handling may not only induce stress responses, but are also suggested as causes of fin and bodily damage in farmed fish, representing a clear welfare issue that must be addressed (Broom, 1991; Ellis, et al., 2002).

Earlier production cost models suggests that a yearly production of 80 kg m^{-3} in a S-CCS currently is still more expensive than today`s open sea cage production (Henriksen et al.,

2013). However, the development of new technology and by using S-CCS for strategic parts of the life cycle, like the post-smolt stage, will likely reduce the cost. Calculations by Iversen et al. (2013) also show that an increase of stocking density from the regulated limit (25 kg m^{-3}) to 80 kg m^{-3} will significantly reduce the coastal area used. Hence, there are several drivers for increasing stocking density and it is therefore highly relevant to establish safe stocking limits for post-smolts in S-CCS. Therefore in the present study, five stocking densities ranging from 25 kg m^{-3} to 125 kg m^{-3} were maintained throughout an 8 week period. This density range is also within the limits of what has previously been proven viable for other Atlantic salmon life stages (Hosfeld, et al., 2009; Kjartansson, et al., 1988). Welfare implications of stocking density were assessed by examining the overall stress response considering primary (cortisol), secondary (physiological) and tertiary (growth) responses as well as external morphological indicators.

2. Materials and methods

2.1. Fish stock and rearing conditions

The fish used in this study were out of season smolts produced by Lerøy Vest, Flateråker, in Western Norway. First feeding started in early February 2012 under constant light and in heated water (12-14°C). Between early May and early August the fish were maintained indoors in a green 7m rearing tank (volume: 70 m^{-3}) at constant light and water temperature (12°C). All fish were fed *ad lib* a commercial dry diet (EWOS, Bergen, Norway). A photoperiod regime known to stimulate parr-smolt transition was initiated in the beginning of August (Handeland, et al., 2001). This treatment included a decrease in day-length from LD24:0 to LD12:12 for 5 weeks followed by another 4 weeks on LD24:0. On October 8th, all fish showed normal morphological and physiological signs of smolting, including silvery scales, dark fin margins, low condition and high gill NKA activity (McCormick, 1993).

2.2 Experimental design

All experimental procedures were approved by the Norwegian Animal Research Authority (reference no. 4692). The study was carried out at the Industrial Laboratory (ILAB), Bergen Norway, between the 10th of October and 20th of December 2012. On October 10th 3750 smolts (weight = 115.0 g ±13.6, length = 22.2 cm ±1.4) were transported from the hatchery (Flateråker) to ILAB and distributed randomly among ten 1 m^2 square fiberglass tanks (500 L) with stocking density as the experimental factor, 25, 50, 75, 100 and 125 kg fish m^{-3}. Each treatment was conducted in duplicate tanks. In the period from the 16th to the 18th of October, the fresh water (treated with SiO_2) in each tank was gradually replaced with deep seawater (-105 m); i.e. from 0 to 17 ‰ on 16th of October, from 17 to 25 ‰ on 17th of October and from 25 ‰ to full strength seawater (34 ‰) on the 18th of October. Following

exposure to seawater, the fish were reared under a simulated natural light regime (60°25`N). The experimental period started on the 24th of October lasting till the 20th of December. In all groups, specific water flow was kept at 0.6 L kg fish^{-1} min^{-1} and temperature at 9.3°C (± 0.3). Water velocity in each tank was kept stable and equal by adjusting the angle on the inlet water pipe. Both temperature and oxygen saturation were measured once daily at 10:00-12:00 AM (YSI 550A, Yellow springs, OH, USA) in the outlet water of every tank, and pH (Seven Easy pH meter, Mettler-Toledo AG, Schwerzenbach, Germany) was measured every week (Table 1). The oxygen level in the outlet water was kept higher than 80 % saturation by oxygenating the water in the header tanks. Every second week water samples were collected from the outlets of each tank in sealable airtight glass bottles in order to monitor CO_2 (Fivelstad, et al., 2003) and in acid-washed tubes for TAN measurements. The carbon dioxide concentrations were calculated based on the percentage of carbon dioxide in the total carbonate concentration (Gebauer, et al., 1992). Before TAN was analysed pH was reduced below 2 in each sample using sulphuric acid (H_2SO_4), TAN concentrations were analyzed according to 'Norwegian Standard 2005, NS-EN ISO 11732' using a Seal autoanalyser (Omni process AB, Solna, Sweden). The measured water quality parameters in all treatments were within the recommendations for post-smolts in sea water systems (Thorarensen, et al., 2011). All tanks were checked twice daily and dead fish were removed immediately and weighed; however, the mortality throughout the experiment was negligible and not related to experimental treatment (Table 2). The fish in all treatments were fed a commercial freshwater dry diet (Optiline 3 mm, Skretting, Norway) in 10 % excess to table (Skretting) with an automatic feeder daily between 09:00-10:00 and 15:00-16.00 throughout the study. A freshwater feed was used to reduce the sinking rate of the pellets, hence increasing the time it was available to the fish, thus minimizing any density dependent effects on feed availability.

2.3 Performance analysis

To assess stocking density dependent effects on growth and condition, a sub-group of 30 randomly selected fish from each treatment were individually tagged (11 October, PIT tags, Trovan Ltd.), weight and length were measured during tagging and at the end of the experiment after 8 weeks. The growth was calculated as specific growth rate (SGR), were W_1 and W_2 are weights at days T_1 (start of experiment) and T_2 (after 8 weeks), according to the equation:

$$SGR= (\ln W_2-\ln W_1)*100/(T_2-T_1).$$

Fultons condition factor (CF), were W is weight and L is length, was calculated based on the formula:

$$CF=100W*L^{-3}$$

Bulk weight measurements of the total biomass in each tank were recorded at the start of the experiment, middle (4 weeks) and at the end (8 weeks). At week 4 the actual biomass gain was recorded and removed to maintain the original treatment density. To minimize disturbance in tanks during the experiment, the biomass gain at week 2 and 6 was estimated from the mean weight of the sampled fish (n=12) and removed. The density range in each treatment is given in table 2, however treatments are termed after their original and adjusted stocking density i.e. 25, 50, 75, 100 and 125 kg m^{-3}. Bulk weights were also used to assess feed intake and FCR. Fish were fasted 24 hours prior to tagging and bulk biomass measurements and anaesthetized with MS-222 (200 mg/kg, Sigma-Aldrich, St Louis, MO, USA). From week 4 to 8 the feed intake was monitored by daily collection of waste feed in each tank. Uneaten pellets were flushed out within 15 minutes, and filtered from the outlet water using an automatic collection system. The waste feed was stored in -20°C until the end of the experiment, and was then dried (24 hours, 70ºC) and weighed. Due to issues with the collection system in the start of the experiment feed intake could only be recorded between

week 4-8. Relative feed intake (RFI, % of body weight per day) was calculated using the formula:

$$RFI\% = 100*[C / ((B_1 + B_2)/2)]/ (T_2 – T_1)$$

Where C is feed consumption (dry weight; g) and B_1 and B_2 the actual biomass (g) at day T_1 and T_2 (Aas, et al., 2006). Feed conversion ratio (FCR) from week 4 to 8 was calculated for each tank as:

FCR= (kg feed consumed) / (kg final biomass - initial biomass + removed biomass + dead fish)

2.4 Blood and gill tissue sampling protocol

Blood and gill tissue were collected from each density treatment after 2, 4, 6 and 8 weeks, all fish were fasted 24 h prior to sampling. Twelve fish from each treatment were quickly netted and anesthetized in 200mg/l MS-222. Individual fish were weighed and their length measured. Subsequently, blood was then sampled with a heparinised syringe from the caudal blood vessels. One drop of blood was analysed immediately using an ISTAT analyser (Abbot Norge AS, Norway). The remaining blood was centrifuged (10 min at 4°C and 4000 rpm) and plasma was stored at -80°C for further analysis. Gill tissue, sampled from the second gill arch, was immediately immersed in ice-cold SEI, then frozen at -80^0C. Gill NKA was analyzed according to the procedure of McCormick (1993).

2.5 Blood chemistry

Analytical cassettes (EC8+) were used with the ISTAT analyser to measure blood levels of haematocrit (Hct), haemoglobin (Hb), glucose, sodium (Na^+), bicarbonate (HCO_3^-), blood pH and partial pressure of carbon dioxide (pCO_2). Both blood pCO_2 (Boutilier et al.,

1984) and pH (Heisler, 1984) values were adjusted according to the temperature difference between 37°C and the temperature of the fish. Values for HCO_3^- were calculated according to the Henderson Hasselbach equation (Boutilier et al., 1984) where the solubility of CO_2 and the apparent Pk were adjusted according to temperature. When used for diagnostics in fish some deviations between the ISTAT and conventional laboratory values have been found (Cooke, et al., 2008; DiMaggio, et al., 2010; Harrenstien, et al., 2005; Harter, et al., 2014) however it has been declared a useful tool for onsite analysis by Harrenstien, et al. (2005) and Cooke, et al. (2008), especially when the main objective is not to obtain absolute values but to compare relative differences between treatments. Therefore identical handling and sampling of fish was prioritized, to allow for comparison between treatments (Dimberg, 1988; Railo, et al., 1985).

Plasma cortisol levels were measured with a validated direct enzyme immunoassay (EIA) as outlined by Carey, et al. (1998). Briefly, 96-well microtiter plates were coated with rabbit anti-cortisol, polyclonal antibody (Cat# 20-CR50, Fitzgerald Ind. Int'l, North Acton, MA, USA; diluted 1:30000) for 3 hours at 37°C. To each well 2.5µl cortisol standard (Cat # 400364, Cayman Chemical Company, Ann Arbor, MI, USA) or sample along with 100 µl of cortisol–horseradish peroxidase conjugate (Cat. # 65-IC08, Fitzgerald Ind. Int'l; diluted 1:6000) was added, before overnight incubation. Color development using 200 µl/well 3,3′,5,5′-tetramethylbenzidine (TMB, Cat # 53-00-02, KPL inc., Gaithersburg, MA, USA) was monitored every 10 min at 650 nm by a temperature-controlled plate reader (Sunrise Basic™, software: Magellan™ V6.5, Tecan Group Ltd, Männedorf, Switzerland). When desired optic density was obtained (70 to 110 min) the reaction was terminated with 0.5 M HCl and absorbance was measured at 450 nm. Maximum binding (B_0= 150µl EIA +100 µl cortisol–horseradish peroxidase conjugate) and non-specific binding (NSB=150 µl EIA-

100µl cortisol–horseradish peroxidase conjugate) were determined. All standards were run in triplicate and samples in duplicate.

2.6 External welfare indicator analysis

At the final sampling point after 8 weeks of stocking density treatment, an external welfare analysis was performed on 10 fish from each tank (Hoyle, et al., 2007). Each fish was examined for the presence of fin erosions (pectoral, caudal, pelvic, dorsal and anal fins), cataracts, skin lesions and operculum shortening as described in (Kolarevic, et al., 2013). Briefly, each fish was scored an integer for each indicator, from 0 (no lesions) to 5 (severe lesions), except for operculum, cataract, and skin lesions score (0-2 score range). All fish were examined by the same operator, whom had no previous knowledge of the experimental treatments that the fish had been exposed to.

2.7 Statistics

All data sets were tested for normality using Kolmogorov-Smirnov test. The Hartley F-max test was used to test for homogeneity of variances. A two-way factorial ANOVA was used to study the effect of stocking density and treatment time on physiological parameters. Significant ANOVA's, $P < 0.05$, were followed by a Student-Newman-Keuls multiple comparison test. Due to unintentional disturbance in one of the replicate 25 kg m^{-3} treatment tanks during sampling at week 8 it was decided to remove cortisol data from that tank from the statistical analysis, other physiological parameters were tested (Student t-test) and no tank effects among replicate groups were found. A one-way ANOVA followed by a Student-Newman-Keuls multiple comparison test was used to compare growth rate (SGR) of tagged individuals between stocking density treatments and welfare score data after 8 weeks. Prior to statistical evaluation, the welfare score data was recalculated to proportions of the maximal

attainable score (of 2 or 5), and arcsine transformed. The relationship between stocking density SGR, FI and FCR was demonstrated by multiple regression analysis, using 95% as the critical level for significance. Statistical analyses were performed using STATISTICA (version 12) and all data are given as means ± SEM.

3. Results

3.1 Feed intake, feed efficiency and growth

There was no difference in mean weight among treatments at the start of the experiment, after 8 weeks the mean weight was significantly lower in the 100 and 125 kg m^{-3} treatments compared to lower stocking densities (Table 2, $P < 0.05$). A negative linear relationship between specific growth rate (SGR) and increased stocking density was observed between 25 kg m^{-3} and 100 kg m^{-3} (adjusted R^2: 0.92, $P < 0.001$), and between 100 kg m^{-3} and 125 kg m^{-3} (adjusted R^2:0.83, $P < 0.05$). Each incremental increase in stocking density from 25 kg m^{-3} to 75 kg m^{-3} had a negative effect on SGR with a reduction of 9% between 25 kg m^{-3} and 50 kg m^{-3} and 15% between 50 kg m^{-3} and 75 kg m^{-3} (ANOVA, $P < 0.05$). No significant difference in SGR was detected between the 75 kg m^{-3} and 100 kg m^{-3} treatments, however there was a 42% reduction in SGR between the 100 kg m^{-3} and 125 kg m^{-3} treatment ($P < 0.001$). Condition factor was reduced in the intermediate (50, 75 and 100 kg m^{-3}) treatments compared to the lowest stocking density (25 kg m^{-3}, $P < 0.05$), in the highest stocking density fish had a lower condition factor than all other treatments ($P < 0.05$). There was a positive linear relationship between increased stocking density and feed conversion ratio (FCR 25-125; adjusted R^2:0.57, $P < 0.05$) indicating that increasing stocking density has a negative effect on feed utilization. The relative feed intake (RFI) was lower in both the lowest (25 kg m^{-3}) and the highest (125 kg m^{-3}) stocking densities, however a significant correlation was only detected when comparing RFI 50-125 (adjusted R^2: 0.65, $P < 0.05$) and not RFI 25-100 (adjusted R^2:0.56, $P = 0.051$).

3.2 Gill NKA activity and plasma sodium, Na^+

Gill ATPase activity levels were similar between all treatment groups, ranging from 13.8 – 15.2 µmol ADP mg protein^{-1}h^{-1} throughout the study (Fig. 1A). Na^+ was affected by time and stocking density, with an increase in Na^+ in all treatments the first two weeks of the experiment ($P < 0.05$, Fig. 1B). At the last sample point (week 8) plasma Na^+ was significantly higher in the 125 kg m^{-3} group, contrary to other groups in which values remained stable ($P < 0.05$, Fig. 1B).

3.3 Plasma cortisol and blood glucose

Plasma cortisol levels were significantly affected by time and treatment ($P < 0.05$). After 4 weeks post-smolts kept at the intermediate stocking density (75 kg m^{-3}) had significantly elevated cortisol levels compared to other treatments ($P < 0.05$), levels were decreased at the 6 week sample point but still higher than all other treatments ($P < 0.05$, Fig. 2A). By the end of the experiment cortisol levels returned to basal levels in the 75 kg m^{-3} treatment. Fish in the 50 kg m^{-3} treatment had significantly higher cortisol levels than fish in the 100 kg m^{-3} treatment at week 4 ($P < 0.05$). After 8 weeks post-smolts kept in the highest stocking density (125 kg m^{-3}) had significantly elevated plasma cortisol ($P < 0.05$) compared to all other treatments. At this time point the mean cortisol concentration in the lowest stocking density (25 kg m^{-3}) was 4.6 ± 3.6 ng mL^{-1} and 33.6 ± 10.4 ng mL^{-1} in the 125 kg m^{-3} treatment ($P < 0.05$, Fig. 2A). Plasma glucose was affected by stocking density and time ($P < 0.05$, Fig. 2B). The 125 kg m^{-3} treatment was significantly reduced compared to 25 kg m^{-3} treatment after 2 and 4 weeks and was lower than 50 kg m^{-3} at 6 weeks ($P < 0.05$). At week 8 there was no significant difference in plasma glucose levels between treatments, however plasma glucose levels were significantly higher week 8 compared to week 6 in the highest stocking density ($P < 0.005$).

3.4 Blood pCO_2, pH and bicarbonate (HCO_3^-)

The pCO_2 in the blood increased with time in all treatments ($P < 0.05$, Fig. 3A). The general trend was an increase in plasma pCO_2 the first 4 weeks, followed by a period of stabilization between week 4 and 6. After 8 weeks, pCO_2 levels had increased by 2.6-fold in the 125 kg m^{-3} treatment, and were significantly higher compared to fish in the other treatments ($P < 0.05$, Fig. 3A).

There were no observed differences in blood pH between the five treatments the first 6 weeks of the experiment. At the end of the experiment the blood pH was significantly reduced in fish in the 100 kg m^{-3} treatment compared to fish in the 25 kg m^{-3} treatment ($P < 0.05$). Fish in highest stocking density (125 kg m^{-3}) had a lower blood pH than all other treatments ($P < 0.05$, Fig. 3B). No significant differences in blood HCO_3^-, HCT and Hb were evident between treatments at the end of the experiment (results not shown).

3.4 External welfare indicators

Fin damage such as erosion, splitting and malformations and fin ray damage were the most commonly observed signs of poor external welfare. Pectoral fin condition was adversely affected in densities of 100 kg m^{-3} and above ($P < 0.05$, Fig 4A). No significant external welfare effects were observed on other fins (pelvic, dorsal, anal and caudal). A higher prevalence of cataracts was observed in the highest density (125 kg m^{-3}) compared to treatment densities of 25 kg m^{-3}-75 kg m^{-3} ($P < 0.05$, Fig. 4B). Stocking density was not observed to affect skin or operculum condition.

4. Discussion

In the present study reduced body weight, negative effects on external welfare and changes in physiology were only observed in the two highest stocking densities, 100 and 125 kg m^{-3}, suggesting that the reduced growth observed in these treatments is directly related to stocking density. Increased competition between fish in the cohort and swimming speed at feeding times has been observed at high fish densities (Kebus, et al., 1992) and it has earlier been concluded that depressed growth can potentially be related to a reduction in access to food through competition or reduced visibility (Holm, et al., 1990; Refstie, 1977; Refstie, et al., 1976). In this study there was a linear increase in FCR with increased stocking density supporting that fish are spending more energy finding feed as density increased. As no effects on physiology and external welfare were observed in the 50 and 75 kg m^{-3} treatment, the reduced growth in these treatments might be related to the restricted tank depth i.e. the time feed is available in the tank. In a commercial setting the effect on growth may not have been as apparent as the tank depth is greater giving fish more time to find feed even if visibility is reduced due to increased stocking density. In support of this Hosfeld, et al. (2009) found no effects on growth in smolts stocked in densities up to 86 kg m^{-3} in freshwater land based systems for 100 days. The 42% reduction in growth between the 100 kg m^{-3} and 125 kg m^{-3} treatment together with the negative effects on feed utilization, feed intake, physiology and welfare suggest a direct relation between high stocking densities and tertiary (chronic) stress responses. In contrast, Kjartansson, et al. (1988) found no negative effects on growth in adult Atlantic salmon (~1.75 kg) reared in land based facilities in densities up to 100-125 kg m^{-3}. Negative effects have been reported at considerably lower densities for Atlantic salmon in open sea cages, in which most post-smolts in the size interval 0.1-1 kg are produced today (Oppedal, et al., 2011; Turnbull, et al., 2005). According to Oppedal, et al. (2011) densities

above 26.5 kg m^{-3} decreased growth rate, feed intake and feed utilization in adult salmon (~1 kg) in sea cages. However, large fluctuations in environmental factors such as temperature and O_2 within the sea cage can drive crowding. Therefore, Atlantic salmon are in fact commonly experiencing a much higher actual fish density than indicated by the stocked density (Oppedal, et al., 2011). In semi-closed sea systems oxygen can be added and water can be pumped in from below fluctuating surface layers (Rosten, 2011). Hence, it is reasonable to expect that rearing conditions are more similar to land-based tanks, where it is possible to produce a stable and homogenous tank environment (Davidson, et al., 2004). Fish may therefore distribute more evenly than in cages, allowing for operations at higher stocking densities. However, results from this study highlight the need for effective feeding solutions and monitoring when operating with high stocking densities in commercial scale closed containment systems.

To maintain optimal water quality in all treatments a biomass specific water flow of 0.6 L kg $fish^{-1}$ min^{-1} was used and this causes water retention time to decrease with increased density. In a large scale system this could lead to a high water velocity in the tank and drag near the outlet that could have negative effects on production performance (Solstorm, et al., 2015). In this experiment the inlet pipe was adjusted to create an equal water velocity in all tanks and with only 500 L of water the drag force from the tank outlet is expected to be negligible. Hence, it is unlikely that the effects observed in the higher density treatments are related to hydraulic retention time.

The reduced growth observed in the intermediate stocking densities, 50 and 75 kg m^{-3}, may be explained by the reduced availability of feed with increasing density, caused by the tank properties in this experiment. However, complex social interactions that increase with density may also be contributing. It has earlier been found that the frequency of aggressive acts and the complexity of the interactions increase with density in salmonids (Cole, et al.,

1980; Keeley, 2000; Li, et al., 1977). Measures were taken to sample each treatment exactly the same, however the handling every second week may have been perceived more stressful as stocking density increased, despite the intensity of stress being the same (Pottinger, et al., 1992). Established social hierarchies may have been disrupted leading to increased aggression after sampling, this may have been stronger at the highest densities. The elevated cortisol levels in the intermediate fish density treatment (75 kg m^{-3}), with a peak response at 4 weeks may be a reflection of such complex interactions. Elevated cortisol levels due to social interactions has earlier been reported in teleosts (Fox, et al., 1997; Gilmour, et al., 2005). By the end of the experiment plasma cortisol levels return to basal values (Barton, et al., 1991) in the 75 kg m^{-3} treatment, in combination with the lack of sustained secondary responses. This suggests that the increase in cortisol was an adaptive allostatic response to maintain internal stability. The significant cortisol increase in the highest density treatment (125 kg m^{-3}) after 8 weeks may indicate an acute response, to an accumulating allostatic load in which fish were able to compensate for earlier in the experiment. Besides an increase in cortisol secondary responses like increased blood glucose, Na^+, pCO_2 and decreased blood pH were also observed after 8 weeks in the highest stocking density. Increased blood CO_2 is also caused by increased activity (Stevens, et al., 1967; Wood, et al., 1977) further suggesting that competition/aggression in relation to high density may be taking place. Overall, the present results indicate an allostatic overload and a situation in which fish are no longer able to cope with increased stress in the highest stocking density.

The ion transporting enzyme Na^+, K^+,-ATPase (NKA) present in the basolateral membrane of the branchial epithelium is associated with the excretion of ions in a hyperosmotic environment (Marshall, et al., 1998). In the present study, the sharp increase in gill NKA activity followed by stabilization at a higher level in all treatments is consistent with the seawater acclimation process known to occur in salmonids shortly after transfer to

seawater (Berge, et al., 1995; Handeland, et al., 1998; Madsen, et al., 1989). The lack of difference in NKA activity between treatments in the first period of the experiment suggests that stocking density does not affect this seawater acclimation process. The drastic increase in Na^+ plasma levels in the 125 kg m^{-3} treatment at the end of the experiment, despite no differences in gill NKA activity, suggest that fish are unable to adjust gill NKA activity to regulate Na^+ levels. The ion-regulatory functions of NKA are energy dependent (Marshall, et al., 1998; Sinha, et al., 2015), and the reduced feed intake and glucose levels suggest that the energy reserves needed to elicit such a response may be prioritized in other physiological processes amongst fish in the highest stocking density. Stress can also impact the ion-regulating function of the epidermal tissue in gills, skin and intestine through an increase of paracellular permeability which could explain the influx of Na^+ (Segner, et al., 2012). Increased blood glucose in response to an acute stressor is also a typical secondary response reported in fish, with the function of dissipating energy in order to react to a threat (Barton, et al., 1991). In this study plasma glucose was reduced in the highest stocking density during the first 6 weeks, this may be related to a reduced feed intake in this treatment, however although on the low side all treatments are within the normal range reported for salmonids (Arnesen, et al., 1993; Miller Iii, et al., 1983). Though an overall lower blood glucose in the highest stocking density, there was a significant increase from week 6 to week 8 indicating that energy reserves are being mobilized in order to cope with a stressful stimuli. Generally, the responses observed in this study imply that there is a time period in which fish can cope with high stocking densities, but if this window is surpassed wide-spread physiological changes result.

Cataracts, fin, skin and operculur damage represent injuries to live tissue and are often found in farmed salmonids (Ellis, et al., 2008; Kolarevic, et al., 2014; Turnbull, et al., 2005). Damaged epithelia on the skin and fin bases can lead to osmotic disturbances and represent

invasion routes for pathogens and therefore increase the risk for disease (Stien, et al., 2013). Hence, these are important indicators of welfare and being externally visible they are relatively easy to study. In this study, stocking densities of 100 kg m^{-3} or above induced pectorial fin damage. The increased plasma Na^+ levels observed after eight weeks in the highest stocking density (125 kg m^{-3}) may be a consequence of damaged skin epithelia around the fin bases causing a reduced barrier function and influx of ions. Fin damage as a result of increased stocking density has earlier been reported for several species (e.g. Ellis, et al., 2008; North, et al., 2006), the main causes being aggressive behaviour, like biting and chasing, and mechanical abrasion (Turnbull, et al., 1998). Cataracts, opaqueness of the eye lens, may result in impaired vision and even blindness in farmed fish, further causing reduced feed intake and growth. In the present study cataract prevalence was increased in the highest stocking density. A similar observation was also found in adult Atlantic salmon by Oppedal, et al. (2011) where the number of cataracts increased when the fish were crowded for extended periods in sea cages, it has earlier been reported that high stocking density can increase cataract rates in tilapia (Cruz, et al., 1989) and cod (Björnsson, 2004) as a consequence of mechanical abrasion of the cornea (Ubels, et al., 1987). Overall, the effects on physiology and growth in the highest stocking density in combination with the visual signs of social interactions, damaged fins and cataracts, suggest that reduced welfare in this study may be related to aggression.

The present study was conducted at a temperature regime corresponding to the mean water temperature in the geographical area in Norway where it is currently of most interest to develop semi-closed sea systems. Effects of stocking density may be more adverse at higher temperatures due to the interacting effects of increased excretion (CO_2 and NH_3) reducing the water quality, thus further studies are needed to understand optimal post-smolt densities at different temperatures. Density effects will also depend on post-smolt size since smaller fish

have a higher mass excretion rate (Terjesen, 2008). Hence, density guidelines in this paper should be applied with consideration to the prevailing environmental and biological factors.

5. Conclusions

In conclusion, this study suggests that densities of 100 kg m^{-3} and above have a direct negative effect on growth. Increased FCR, plasma cortisol and secondary physiological stress responses were observed in the highest stocking density (125 kg m^{-3}) after 8 weeks. Furthermore, stocking densities of 100 kg m^{-3} and above had a negative effect on external welfare parameters such as fin condition and prevalence of cateracts. Our data suggests that it is feasible to rear Atlantic salmon post-smolts in densities up to 75 kg m^{-3} in semi-closed sea systems without compromising performance and welfare. Further studies in large scale systems should take these findings as a reference to verify density limits for commercial rearing of post-smolt salmon.

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Figure legends

Fig. 1. Gill NKA (A) and Blood sodium (Na^+) (B) in post-smolt Atlantic salmon after 0, 2, 4, 6 and 8 weeks of exposure to stocking densities of 25, 50, 75, 100 and 125 kg m^{-3}. All values are given as mean ± SEM (n=12). Different letters denote significant differences ($P < 0.05$) between density treatments at the below time points. NS= not significant.

Fig. 2. Plasma cortisol levels (A) and blood glucose (B) after 0, 2, 4, 6 and 8 weeks of exposure to five different density treatments (25, 50, 75, 100 and 125 kg m^{-3}) for 8 weeks. All values are given as mean ± SEM (n=6-12). Different letters denote significant difference ($P < 0.05$) between treatments at the below time points. NS= not significant.

Fig. 3. Blood partial pressure of CO_2 (A), blood pH (B) after 0, 2, 4, 6 and 8 weeks of exposure to stocking densities of 25, 50, 75, 100 and 125 kg m^{-3}. All values are given as mean ± SEM (n=12). Different letters denote significant difference ($P < 0.05$) between treatments at the below time points. NS= not significant.

Fig. 4. Pectoral and pelvic fin condition (A) and cataract prevalence (B) in post-smolt Atlantic salmon after 8 weeks of exposure to stocking densities of 25, 50, 75, 100 and 125 kg m^{-3}. Each data point is the tank mean ± SEM (n=2) and 10 fish per tank were scored. Scores are 0-2 for cataract and 0-5 for fins, higher value indicates severer damage. Different letters denote a significant difference between treatments ($P < 0.05$) per indicator. NS= not significant.

Table 1. Water quality at 5 different stocking densities in full strength sea water (34‰) displayed as averages (± standard error) over the 8 week experimental period (n=2 tanks). O_2 (Oxygen), TAN (total ammonia nitrogen) and CO_2 (Carbon dioxide) level were measured in outlet of each tank and are displayed as % saturation for O_2, CO_2 and TAN are in mg L^{-1}.

Parameter	**25 kg m^{-3}**	**50 kg m^{-3}**	**75 kg m^{-3}**	**100 kg m^{-3}**	**125 kg m^{-3}**
Temperature (°C)	9.2±0.01	9.2±0.01	9.2±0.01	9.2±0.01	9.2±0.01
O_2 (%)	91.8±0.9	86.4±1.1	90.1±0.8	87.6±1.1	86.7±0.1
pH	7.58±0.05	7.48±0.04	7.53±0.05	7.47±0.06	7.48±0.06
CO_2 (mg L^{-1})	3.6±0.4	4.5±0.4	4.3±0.5	4.7±0.6	4.6±0.6
TAN (mg L^{-1})	0.38±0.07	0.42±0.06	0.34±0.05	0.39±0.05	0.41±0.06

Table 2. Post-smolt Atlantic salmon performance at different stocking densities.

Parameter	**25 kg m^{-3}**	**50 kg m^{-3}**	**75 kg m^{-3}**	**100 kg m^{-3}**	**125 kg m^{-3}**
Density range (kg m^{-3})	25-35	50-62	75-94	100-123	125-142
Mortality (count)	1	2	2	1	5
Initial weight start (g)	111.1±1.8	118.1±2.6	119.0±3.0	114.4±2.0	111.2±2.5
Final weight (week 8)	217.4±5.8^{a}	217.9±7.7^{a}	202.4±8.5^{a}	181.1±5.3^{b}	147.4±5.5^{c}
SGR, 0-8 weeks	0.94±0.02^{a}	0.85±0.03^{b}	0.72±0.04^{c}	0.65±0.03^{c}	0.38±0.03^{d}
Condition factor	1.13±0.06^{a}	1.08±0.01^{b}	1.06±0.01^{b}	1.05±0.01^{b}	1.01±0.01^{c}
FCR, 4-8 weeks	0.87±0.06	1.12±0.00	1.06±0.00	1.01±0.02	1.63±0.01
RFI, 4-8 weeks	0.44±0.02	0.84±0.01	0.80±0.02	0.75±0.02	0.58±0.08

Mean weights, condition factor and specific growth rate (SGR; % bw day^{-1}) are based on individual fish (n=30) significant differences between treatment densities are denoted with different letters ($P < 0.05$). Feed conversion ratio (FCR) and relative feed intake (RFI; % bw day^{-1}) are measured on a tank level (n=2) values are given as means ± SEM.