Environmental and biological requirements of post-smolt Atlantic salmon (*Salmo salar* L.) in closed-containment aquaculture systems

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Scientific environment

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Abstract

Production of Atlantic salmon (*Salmo salar* L.), exceeds 2 million tonnes globally, and accounts for 90% of the salmon on the market. Presently, the predominant production of post-smolt Atlantic salmon occurs in open sea cages. Environmental concerns, during the production phase at sea are limiting further growth of the industry. In closed-containment aquaculture systems (CCS), the cultured fish are separated from the natural environment by a physical barrier; these can be land-based systems or closed units in the sea. Using CCS to shorten the time fish are reared in open sea cages has been highlighted as key to solving important challenges the industry is facing today. However, there is a lack of knowledge on the biological and environmental requirements of post-smolt Atlantic salmon in CCS. This thesis aims to provide insight on the effects of some key husbandry conditions on post-smolt performance and welfare in both sea- and land-based CCS.

Commercial feasibility of farming post-smolt Atlantic salmon in CCS in the sea relies on maximizing fish density. To assess stocking density limits, five different densities (25, 50, 75, 100 and 125 kg m^{-3}) were maintained in flow-through seawater systems for eight weeks. Increased stocking density had a negative effect on growth and feed utilization, and increasing density from 100 kg m^{-3} to 125 kg m^{-3} lead to a 42 % decrease in growth rate. After eight weeks, primary (elevated plasma cortisol) and secondary (hydro-mineral and acid-base) stress responses were observed in the highest density treatment compared to other treatments. Densities of 100 kg m^{-3} or more also increased pectoral fin damage and cataracts. Fish stocked at the medium (75 kg m^{-3}) density displayed more robust telencephalic activation of both stress and neural plasticity responses, compared to fish in the lowest (25 kg m^{-3}) and highest (125 kg m^{-3}) densities. Overall, the results suggest that stocking density can be maximized up 75 kg m^{-3} without compromising performance and welfare in Atlantic salmon post-smolts in CCS in sea. Given the peak expression of genes that are important for cognition and memory, densities around 75 kg m^{-3} may in fact be optimal for welfare.

Determining the mass-specific water flow (SWF) required by post-smolts will largely influence the design and dimensioning of closed-containment systems in the sea. Prominent physiological

regulatory responses to increased water carbon dioxide were observed in post-smolts kept in the lowest SWF (0.2 L kg fish^{-1} min^{-1}) in sea water flow-through systems. At these conditions the fish were able to compensate and maintain growth within the entire eight week period studied. However, the responses observed have an energetic cost revealed by increased oxygen consumption. Overall, it can be recommended that without any in-tank water treatment, specific water flow should be maintained above 0.3 L kg fish^{-1} min^{-1} as physiological regulatory responses are energy costly and reduced SWF can have a negative effect on other factors, not studied in this thesis, such as skin quality. Furthermore, our results suggest that fish density affects the ability of fish to react to additional challenges, more than in the case of specific water flow. This thesis also identifies several markers that when combined with an acute challenge test consistently reveals effects of pre-existing environmental conditions, and can be used to predict the fish´s resilience and potential for adaptation to changes in their environment.

The optimal strategy for rearing large post-smolts in land-based recirculating aquaculture systems (RAS), with respect to salinity, water velocity and timing of seawater transfer is not known. In a long-term study, from 70 g up to 800 g, post-smolts were reared in three separate RAS at different salinities (12, 22 and 32‰) and subjected to moderate (~1 bl s^{-1}) or low (~0.3 bl s^{-1}) water velocity. Results suggest that salinity isotonic to the fish (12‰) and moderate exercise training has a positive effect on post-smolt growth, feed efficiency, welfare and survival in RAS. At 250 and 800 g all treatments handled sea water transfer, at 450g handling and transfer caused high mortality in several treatments. Using water with salinity around 12‰ may therefore be an advantageous production strategy for large post-smolts in RAS provided that the post-smolts can handle the subsequent transfer to open sea cages, and this needs further investigation.

In conclusion, the knowledge gained on key husbandry conditions described in this thesis will contribute towards optimizing post-smolt Atlantic salmon production in closed-containment aquaculture systems on land and in the sea.

Sammendrag

Produksjonen av laks (*Salmo salar* L.), er over to millioner tonn på verdensbasis, og utgjør 90 % av all laks på verdensmarkedet. I dagens situasjon skjer den overveiende delen av produksjonen av post-smolt laks i åpne merder. Miljøutfordringene gjennom produksjonsperioden i sjøen begrenser i dag den videre veksten til næringen. I lukkede oppdrettssystemer (closed-containment aquaculture systems, CCS) er oppdrettsfisken skjermet fra det naturlige miljøet av en fysisk barriere, disse anleggene kan være landbaserte eller lukkede anlegg i sjø. Bruken av CCS for å redusere tiden fisken er i åpne merder kan bidra til å løse utfordringene knyttet til videre vekst i næringen. Det er imidlertid mangel på kunnskap om de biologiske og miljømessige forutsetningene for oppdrett av post-smolt laks i CCS. Målet med denne avhandlingen er å framskaffe ny innsikt i betydningen av viktige faktorer i oppdrett for prestasjonene og velferden til post-smolt laks, både i sjø- og landbasert CCS.

I en kommersiell sammenheng vil oppdrett av post-smolt laks i lukkede systemer i sjøen være avhengig av høy fisketetthet. For å avdekke hvilke grenser som gjelder ble fem tettheter (25, 50, 75, 100 og 125 kg m^{-3}) undersøkt i et forsøk med gjennomstrømmende sjøvann i en periode på åtte uker. Økende tetthet hadde negativ effekt på vekst og fôrutnyttelse, og en økning i tetthet fra 100 kg m^{-3} til 125 kg m^{-3} forårsaket en 42 % nedgang i vekstrate. Etter åtte uker var de primære (forhøyet plasma cortisol) og sekundære (vann-ione- og syre-basebalanse) stressresponsene aktivert i den høyeste tettheten sammenliknet med de andre behandlingene. Tettheter på 100 kg m^{-3} og høyere økte også forekomsten av skader på brystfinnene og katarakt. Fisk ved midlere tetthet (75 kg m^{-3}) viste en mer robust aktivering i telencephalon av stressresponser og responser knyttet til nerveplastisitet, sammenliknet med fisken i den laveste (25 kg m^{-3}) og høyeste (125 kg m^{-3}) tettheten. Sett samlet viser resultatene at fisketettheten kan økes til 75 kg m^{-3} uten at dette går ut over prestasjoner og velferd hos post-smolt laks i CCS i sjøen. Siden det høyeste uttrykket av viktige gener for kognitive egenskaper og hukommelse ble observert ved tettheter rundt 75 kg m^{-3} kan slike tettheter faktisk være optimale for velferden til fisken.

Å fastslå det spesifikke vannforbruket til post-smolt vil i stor grad bestemme design og dimensjonering av lukkede systemer i sjøen. Viktige fysiologiske regulatoriske responser på forhøyet karbondioksidinnhold i vannet ble observert i post-smolt som ble holdt ved det laveste

spesifikke vannforbruket (0.2 L kg fisk^{-1} min^{-1}) i et gjennomstrømningssystem med sjøvann. Under disse forholdene var fisken i stand til å kompensere og opprettholde veksten i løpet av den åtteukersperioden forsøket varte. De fysiologiske responsene vi observerte har imidlertid en energetisk kostnad, noe som ble avdekket gjennom et økt oksygenforbruk. Samlet sett vil anbefalingen være at i fravær av vannbehandling i karene bør spesifikt vannforbruk ligge over 0.3 L kg fisk^{-1} min^{-1}, dette siden fysiologiske regulatoriske responser er energikrevende og at redusert spesifikt vannforbruk kan ha andre negative effekter, som ikke er studert i denne avhandlingen, som f.eks. redusert skinnkvalitet. Videre viser våre resultater at fisketetthet i større grad påvirker fiskens evne til å respondere på nye utfordringer enn en reduksjon i spesifikt vannforbruk. I avhandlingen har vi også identifisert flere markører som, når de kombineres med en akutt utfordring, gjennomgående avdekker forutgående miljøforhold, og dermed kan brukes til å forutsi fiskens tilpasningsevne og muligheter for å forholde seg til endringer i oppdrettsmiljøet.

Den optimale strategien for oppdrett av post-smolt i landbaserte resirkuleringsanlegg (RAS) med tanke på saltholdighet, strømhastighet og tidspunkt for overføring til sjøvann er ikke kjent. I et langtidsstudie med fiskestørrelser fra 70 til 800 gram holdt vi post-smolt i tre forskjellige RAS ved ulike saltholdigheter (12, 22 og 32‰) og utsatte dem for moderat (~1 kroppslengde s^{-1}) eller lav (~0.3 kroppslengde s^{-1}) strømhastighet. Resultatene viser at en saltholdighet omkring fiskens egen (12‰) og moderat trening har positiv effekt på vekst, fôrutnyttelse, velferd og overlevelse hos post-smolt. Ved 250 og 800 g håndterte fisk fra alle behandlingene overgangen til sjøvann, mens ved 450 g forårsaket håndtering og overføring høy dødelighet i flere av gruppene. Det å bruke vann med en saltholdighet omkring 12‰ kan derfor representere en god produksjonsstrategi for stor post-smolt i RAS, under forutsetning av at fisken siden kan håndtere overføringen til åpne merder i sjøen. Videre forskning på dette området er nødvendig.

For å konkludere vil jeg si at den kunnskapen som er oppnådd i denne avhandlingen når det gjelder oppdrettsbetingelser vil bidra til en optimalisering av produksjonen av post-smolt laks i lukkede systemer på land og i sjøen.

Contents

List of publications

PAPER I

Calabrese, S., Nilsen, T.O., Kolarevic, J., Ebbesson, L.O.E., Pedrosa, C., Fivelstad, S., Hosfeld, C., Stefansson, S.O., Terjesen, B.F., Takle, H., Martins, C.I.M., Sveier, H., Mathisen, F., Imsland, A.K., Handeland, S.O., 2017. Stocking density limits for post-smolt Atlantic salmon (Salmo salar L.) with emphasis on production performance and welfare. Aquaculture. 468, Part 1, 363-370.

PAPER II

Calabrese, S., Nilsen, T.O., Kolarevic, J., Ebbesson, L.O.E., Fivelstad, S., Hosfeld, C., Pedrosa, C., Imsland, A.K., Terjesen, B.F., Stefansson, S.O., Takle, H., Sveier, H., Mathisen, F., Handeland, S.O., 2017. Water flow requirements of post-smolt Atlantic salmon (*Salmo salar* L.) reared in intensive seawater flow-through systems. *(Submitted manuscript Aquaculture)*

PAPER III

Calabrese, S., Nilsen, T.O, Handeland, S.O., Gorissen, M., Terjesen, B.F., Ebbesson L.O.E. Neural responsiveness to acute challenge tests: identifying environmental limits and future resilience in fish. *(Submitted manuscript Journal of Experimental Biology)*.

PAPER IV

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Nomenclature and abbreviations

ACT	acute challenge test
ACTH	adrenocorticotropic hormone
α-MSH	alpha-Melanocyte-stimulating hormone
BDNF	Brian-derived neurotrophic factor
CCS	closed-containment aquaculture systems
CF	condition factor
Cl^-	chloride
CO_2	carbon dioxide
CRF	corticotropin-releasing factor
CRF-BP	corticotropin-releasing factor binding protein
Dl	dorsolateral telencephalon
FCR	feed utilization/feed conversion ratio
FW	freshwater
H^+	hydrogen ions
Hb	haemoglobin
HCO^{3-}	bicarbonate
Hct	haematocrit
HPI	hypothalamic-pituitary- interrenal axis
MO_2	oxygen consumption
mRNA	messenger ribonucleic acid
N_2	nitrogen gas
Na^+	sodium
NeuroD	Neurogenic differentiation factor
NH_3	ammonia
NKA	Na+, K+ ATPase
NO^{2-}	nitrite
NO^{3-}	nitrate
pCO_2	partial pressure of carbon dioxide
RAS	recirculating aquaculture systems
RGI	relative feed intake
S-CCS	semi-closed containment systems
SGR	specific growth rate
SWF	specific water flow
TAN	total ammonia nitrogen
TSS	total suspended solids

1. INTRODUCTION

Aquaculture is the fastest growing food production industry globally, and in 2014 human consumption of farmed species surpassed that of wild caught fish for the first time (FAO 2016). Current, worldwide production of Atlantic salmon *Salmo salar* L. exceeds 2 million tones and it accounts for 90 % of the salmon on the market (FAO 2016). In Norwegian aquaculture alone a five-fold increase in volume and an eight-fold increase in value by the year 2050 is predicted (Olafsen et al. 2012). Farmed Atlantic salmon are hatched and raised in land-based fresh water facilities until they have undergone smoltification, a seawater preparatory transformation (Hoar 1988, Stefansson et al. 2008, Björnsson et al. 2011, McCormick 2013). Fish are then transferred to open sea cages and in terms of biomass this is where the predominant production of Atlantic salmon occurs (Oppedal et al. 2011). Once in seawater, these post-smolts are faced with many environmental and physiological challenges. Sea lice, diseases, escapes and fish mortality during the production phase at sea are considered the main hindrances to prospective growth in the industry (Gullestad et al. 2011). Shortening the time fish spend in open sea cages has been highlighted as a key factor in abating current challenges. This could be achieved by prolonging the time fish stay in closed-containment systems (CCS). CCS are defined as aquaculture rearing systems, in which the cultured fish is separated from the natural environment by a physical impermeable barrier (Ayer and Tyedmers 2009). The development of new rearing technologies in which the environment can be controlled, would also allow for optimization of rearing conditions. However, there is a lack of fundamental knowledge on the biological requirements of post-smolt Atlantic salmon in CCS. Hence, new knowledge on production of post-smolts in CCS could lead a paradigm shift in salmon aquaculture, as we know it today. This thesis will focus on establishing some of the necessary biological requirements of key husbandry conditions such as, stocking density, specific water flow (SWF), salinity and water velocity in both sea- and land-based CCS.

2. BACKGROUND

2.1 Atlantic salmon- life history

To optimise rearing conditions for Atlantic salmon, it is important to take into consideration the complexity of the life cycle of this species. The sensitivity to environmental challenges and preferred external conditions will highly depend on the life stage of salmon.

The Atlantic salmon, like many other salmonids, are anadromous fish meaning they spawn and hatch in freshwater (FW). Juvenile life stages are spent in FW before undergoing a sea water preparatory transformation, smoltification or parr-smolt transformation, which is onset by external cues like photoperiod and water temperature (Hoar 1988, Stefansson et al. 2008, Björnsson et al. 2011, McCormick 2013). Smoltification is defined as the morphological, physiological and behavioral changes that transform a darkly pigmented parr into a silvery smolt that is adapted to seawater (Hoar 1976, McCormick et al. 1987). Independent of their external environment salmonids maintain a relatively constant osmolality at approximately one-third (~10‰) of sea water (Brett and Groves 1979, McCormick et al. 1989). In FW, a hypo-osmotic environment, fish gain water and loss ions to the environment through diffusion and osmosis. To counteract these passive forces, dilute urine is produced and ions are actively taken up across the gills (Evans et al. 2005) and are absorbed from dietary sources (Baldisserotto and Olga Mimura 1994). The opposite occurs in sea water, a hyper-osmotic environment, fish loose water and gain ions. To counteract this water loss drinking rates are increased and both water and ions are absorbed through the intestinal epithelium and excess salt is mainly excreted by the gills and the kidney (Perrott et al. 1992, Sundell et al. 2003).

The major osmoregulatory changes that occur during smoltification are orchestrated by endocrine signals. Increases in the hormone cortisol are involved in the proliferation of the seawater ionocytes and increasing the ion-transporting enzyme Na^+, K^+ ATPase (NKA) in the basolateral membrane of the ionocytes (Specker 1982, Sakamoto et al. 2001, Björnsson et al. 2011). Once the salmon have entered and acclimated to sea water they are considered post-smolts. The initial acclimation to seawater occurs over a period of a few days and up to a couple of weeks depending on the environment i.e. temperature, salinity, in which gill NKA activity continues to increase until full osmoregulatory ability is reached (Handeland et al. 1998). Besides being the main site for gas exchange and active transport of ions in and out the fish, the branchial epithelium of the gill also has a primary role in the pH regulation of body fluids and excretion of nitrogenous waste (Claiborne et al. 2002). Hence the gill has a central role in the physiological adaption to internal and external changes.

Post-smolts

The post-smolt stage is the phase in the life cycle that has been found to be most sensitive and critical for survival in the sea both for wild and farmed Salmonids (Holtby et al. 1990). In farmed Atlantic salmon the transfer of fish from a land-based freshwater facility to open sea cages is a particularly stressful event in the production cycle (Roberts and Pearson 2005). The complex physiological and anatomical changes that occur during smoltification and the adaption to a new marine environment are energy demanding processes, rendering post-smolts more sensitive to stressors (Jarungsriapisit et al. 2016). Components of the immune system are also modulated or suppressed by the physiological changes accompanying smoltification (Maule et al. 1987, Melingen et al. 1995, Pettersen et al. 2003, Johansson et al. 2016), hence post-smolts are more susceptible to disease outbreaks the first period in sea water (Roberts and Pearson 2005). Successful smoltification at the time of transfer to sea water is also critical for acclimation, growth and feeding the initial time in the sea (Saunders and Henderson 1970, Boeuf 1993, Handeland et al. 1998, Boeuf and Payan 2001, Alne et al. 2011). Furthermore, the transfer in itself and the increase in environmental fluctuations in sea cages compared to land-based systems can cause stress and reduce growth and appetite post-transfer (Jørgensen and Jobling 1994, Handeland et al. 1998, Handeland et al. 2000). In general, growth in salmonids depends on fish size and is strongly influenced by temperature and photoperiod and other environmental factors (Brett and Groves 1979). Growth increases linearly with temperature up to a temperature optimum, and growth decreases with fish size. The temperature optimum for post-smolts ranges from 12.8° C for 70-150 g post-smolts to 14° C in 150-300 g post-smolts (Handeland et al. 1998).

2.2 Production environment

As for many farmed fish species a rapid intensification of Atlantic salmon smolt production has occurred to overcome bottlenecks, such as space and water limitations (Wedemeyer 1996, Terjesen et al. 2013b). Production intensity can be viewed in several ways, as increased water retention time in recirculating aquaculture systems (RAS), supplemental oxygen, or increased fish density i.e. the biomass of fish confined to a given water volume (kg m^{-3}) or as a function of specific water use, i.e., the rate of water exchange relative to the fish biomass (L kg $fish^{-1}$ min^{-1}) (Kristensen et al. 2012). In either scenery, the main limiting production factor is the amount of dissolved oxygen the water can supply the biomass (Willoughby 1968, Fivelstad et al. 2004). Oxygen saturations under 60 % induced stress responses, reduced appetite and decreased growth in post-smolt Atlantic salmon in sea cages (Remen et al. 2012) and even a slight increases from 70-75 % to 80-85 % have been shown to increase growth rate (Bergheim et al. 2006). In land-based Atlantic salmon smolt production the development of technology to add pure oxygen to the inlet and/or tanks dramatically increased production capacity in the late 80s and early 90s, making it possible to reduce the water flow per fish (Sanni and Forsberg 1996). If oxygen is added to a satisfactory level the buildup of metabolites, carbon dioxide (CO_2) and ammonia (NH_3), excreted from the fish will be the next factor limiting the further intensification of production (Fivelstad and Binde 1994). The combination of added oxygen and reduced water flow in intensive flow-through smolt production has a complex effect on water quality with increased levels of metabolites and decreased pH (Thorarensen and Farrell 2011). Hence, intensive production requires a more in depth knowledge on the physiology of the fish. Especially for anadromous salmonids since adverse effects of intensification may first become noticeable after transfer to sea water (Wedemeyer 1996).

In most cases, whenever oxygen is added to the rearing water the next limiting factor to production intensification will be CO_2 because approximately ten times more CO_2 than NH_3 is excreted (Sanni and Forsberg 1996). Acute, increases in water CO_2 are rapidly reflected in the blood of the fish as a reduction in pH, which lowers the oxygen uptake in the blood and transport to tissues. To restore the blood pH, hydrogen ions (H^+) are excreted from the blood and bicarbonate (HCO_3^-) is taken up from the surrounding water via the HCO_3/Cl exchanger in the gill epithelium (Claiborne et al. 2002). For the blood to remain electro-neutral the influx rate of chloride (Cl^-) is reduced concurrently, which acts to lower the Cl^- concentration, in most cases this adaptive response allows blood pH to be restored within 2-7 days (Lloyd and White 1967, Heisler 1984, Claiborne et al. 2002). Elevations in water CO_2 require fish to spend more energy on acid-base regulation and cardio-respiratory responses and may therefore have a negative effect on growth and feed utilization (FCR) (Fivelstad 2013). It has been shown that increased CO_2 has a dampening effect on cortisol secretion and can therefore disrupt smoltification (Pickering and Pottinger 1987). This is consistent with studies showing that hypercapnia reduces Na^+, K^+-ATPase activity (Fivelstad et al. 1999b, Fivelstad et al. 2003, Hosfeld et al. 2008). As current production of post-smolts predominantly occurs in open sea cages, where the ability to control environmental factors is restricted, knowledge regarding the effects of reduced SWF and high CO_2, on post-smolts in sea water is limited.

2.3 Fish welfare, allostasis and the brain

Defining fish welfare

To optimise welfare in any type of aquaculture facility it is first necessary to define fish welfare, and how it can be measured. Animal welfare is a complex concept and there is no absolute definition since the term is used in so many different ways. Most definitions either take into account the ability of an animal to function well, feel well or lead a natural life (reviewed by Lawrence 2008). Function-based definitions focus on the ability of an animal to cope or adapt to its environment, without being forced beyond its physical capacity. The nature based definitions include the ability of the animal to lead a natural life in an environment it is adapted to and express natural behaviors. Feeling-based definitions center around terms of emotional states. In this sense, good welfare requires that the animal feels well, is free from negative experiences such as pain and suffering and has access to positive experiences, as reviewed by Huntingford et al. (2006). The feeling based definition requires that fish are able to have conscious subjective experiences like suffering, and this is a subject of on-going debate (Sneddon et al. 2003a, b, Chandroo et al. 2004, Braithwaite and Ebbesson 2014, Rose et al. 2014). It has been argued that fish cannot experience pain and fear, since they lack the cerebral cortical structures that are responsible for these experiences in mammals (Rose 2002, Rose et al. 2014). However, it has been shown that sensory perception of harmful stimuli, nociception, exists in several fish species (Sneddon et al. 2003a, b, Braithwaite and Ebbesson 2014). Furthermore, there is clear evidence that fish have complex mental processes, such as memory and learning that shape the behavior of fish reviewed by Ebbesson and Braithwaite (2012). The embryonic development of the brain in mammals is very different from fish; instead of inversion pulling the two brain halves together like in mammals the fish brain develops through a process of eversion (Broglio et al. 2010). This results in functional homologues being in different regions of the brain in mammals and fish (Rodríguez et al. 2002, Rodríguez et al. 2005, Salas et al. 2006). Thus, it cannot be concluded that fish lack centers for processing complex emotional memory like fear based on the lack of amygdala and a cerebral cortex. In fact, the telencephalon of fish has been found to be the functional homologue of the mammalian hippocampus and amygdala (Mueller and Wullimann 2009, Mueller et al. 2011, Mueller 2012).

In the end these three different approaches to welfare are intertwined, since an animal being in an environment that it is adapted to normally functions well and feels good. Recognizing that both physical and psychological aspects are important for the well-being of fish, in this thesis I address the physical ability of post-smolts to adapt to their environment and also identify markers for the mental capacity of fish to make cognitive responses to environmental challenges.

The stress response

Stress is a fundamental biological process preserved among vertebrates, and is central in most discussions related to the welfare of farmed animals, including fish (Conte 2004). Stress has been defined as a condition in which the internal equilibrium, homeostasis, of the fish is threatened by an internal or external stimuli defined as a stressor (Wendelaar Bonga, 1997). A common misconception is that stress, in itself, is detrimental. The stress response is essential to fish and all beings and elicits a number physiological and behavioral changes considered as adaptive in order to cope with real or perceived stressors (Schreck 2010).

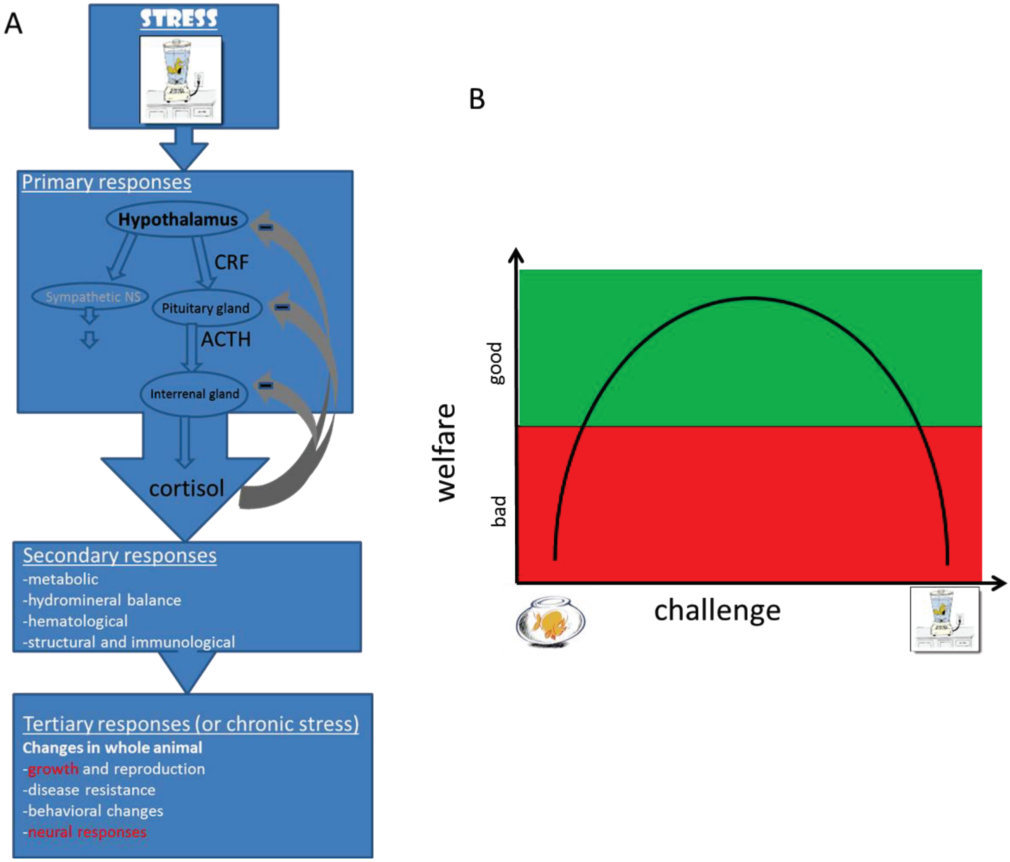

Figure 1: (**A**) The generalized stress response adapted from Wendelaar Bonga (1997); (**B**) The allostasis concept adapted from Korte et al. (2007).

The fish respond to environmental challenges with a series of neuroendocrine adjustments, the generalized stress response, that allow them to cope with internal and external challenges (Wendelaar Bonga 1997). The stress response, can be divided into primary, secondary and tertiary responses (see figure 1A). The primary response, resulting in the secretion of stress hormones, is mediated by the hypothalamic-pituitary- interrenal (HPI) axis. Sensory information of a possible threat is recognized by the central nervous system and travels to hypothalamus. This quickly stimulates a release of catecholamines from the head kidney chromaffin cells and corticotropin-releasing factor (CRF) from the hypothalamus. CRF in turn stimulates secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland (*pars distalis*), which results in the synthesis and release of cortisol (the main gluco- and mineralocorticoid hormone in teleosts) from interrenal cells of the head kidney (Wendelaar Bonga 1997). Thus, besides being an important developmental hormone, cortisol is also the predominant hormone involved in the response to stress. Once in circulation, cortisol initiates secondary responses in target tissue containing glucocorticoid receptors (Mommsen et al. 1999). Secondary responses include changes in metabolism, hydro-mineral balance, acid-base status, immunological and cellular responses in order to mobilize energy and defense systems to cope with the threat (see figure 1A; Barton 2002). However, long-term, or repeated activation of stress responses, leads to tertiary (chronic) responses. In this case, changes on a whole-animal level may be observed such as reduced growth and condition, suppression of the immune system, alterations in behavior and ultimately in survival (see figure 1A; Pickering and Pottinger 1989, Barton 2002).

Stress and allostasis

Welfare is closely linked to the stress concept, in the sense that poor welfare occurs when an organism cannot match its physiological response to that required by the environment (Korte et al. 2007). However, the relationship between stress and welfare is not linear (i.e. increased stress leads to decreased welfare), but rather follows an allostasis (maintaining stability through change) concept where too little or too much environmental input impairs welfare (Figure 1B; McEwen and Wingfield 2003, Korte et al. 2007, Koolhaas et al. 2011). Every challenge, internal or external, that elicits a cortisol response contributes to an allostatic load which can be described as the energy needed to adjust the physiological systems to adapt to the "new" environment and maintain homeostasis. Thus, as the allostatic load increases more energy is needed to maintain homeostasis (McEwen and Wingfield 2003). If the available energy is close to or equal with the energy required for allostasis, energy must be allocated away from "less critical" biological functions such as growth, reproduction and the immune system (McEwen 2002). In this situation the allostatic load becomes an allostatic overload, and there will be limited energy to cope with additional stressors (Korte et al. 2007, Koolhaas et al. 2011). At this stage, tertiary (chronic) stress responses are to be expected, welfare is threatened and the risk for pathologies is increased (Korte et al. 2007). However, it is important to continually emphasize that stress in itself is not negative. In the context of allostasis, a resilient animal will have the means to respond to environmental changes and allostatic responses will be efficiently terminated once stability is achieved (Karatsoreos and McEwen 2011).

Stress and the fish brain

Allostasis, allostatic load and overload not only apply to physiological functions in the body, but also in the brain where neural activity in response to experiences drives adaptive plasticity (Karatsoreos and McEwen 2011). In this context, the brain can be viewed as a central circuit board, by simultaneously controlling and enforcing mechanisms through incorporating influential factors such as memory and experiences in the adaption to environmental demands (Schreck 2010). Cognition is the interaction between perception, learning and memory and involves multiple complex neural processes (Schacter et al. 2012). Current literature on fish cognition indicates that many fish species, including salmon, are capable of learning and integrating multiple pieces of information that require more complex processes than associative learning (see Ebbesson and Braithwaite 2012, Grassie et al. 2013; Salvenes et al. 2013). Neural plasticity allows for the development and function of cognitive processes (Knudsen 2004, Ebbesson and Braithwaite 2012), and thus has a large role in adaptation to changing and challenging environments. Recent studies have shown that components of the stress and neural plasticity systems, respond differentially to a challenge, depending on the basal state of the fish (Ebbesson and Braithwaite 2012, Grassie et al. 2013, Madaro et al. 2015). To demonstrate, Grassie et al. (2013) discovered that exposure of Atlantic salmon to aluminium in acidified waters reduced neural plasticity and affected performance in a maze task. Thus the capacity for learning and memory underpins the ability to behave flexibly, and if cognition becomes impaired then the animal will find it hard to behave in appropriate ways (Ebbesson and Braithwaite 2012). The brain area most important for these complex neural processes in fish is the dorsolateral telencephalon (Dl) (Rodríguez et al. 2002, Wullimann and Mueller 2004, Broglio et al. 2010, Durán et al. 2010, Aoki et al. 2013), and has been recognized as the functional homologue of the mammalian hippocampus (Mueller and Wullimann 2009, Mueller et al. 2011, Mueller 2012). Neurogenic differentiation factor (NeuroD), a member of a family of proneural genes, is involved in the initiation and regulation of neural differentiation (Kiefer 2005). Recent studies have shown that expression levels of *neurod1* mRNA is a reliable measure of neurogenesis in fish, and a useful indicator of the neural plastic changes associated with memory and learning (Grassie et al. 2013, Salvanes et al. 2013). Brian-derived neurotrophic factor (BDNF) is the most abundantly expressed member of the nerve growth factor family, neurotrophins, and has an important role in neural plasticity through sculpting and refinement of synapses and through promoting neurogenesis and cell survival (Suri and Vaidya 2013). It has recently been shown that environmental challenges alters *bdnf* expression in the telencephalon of Atlantic salmon (Vindas et al. 2017).

Overall, in can be concluded that proper animal welfare is characterized by the animal's ability to functionally respond to challenges, both physiologically and cognitively (McEwen and Wingfield 2003).

2.3.1 Measuring welfare

Welfare, as described in the previous section, is complex and unfortunately using any single indicator when assessing welfare in an aquaculture environment gives little information on the overall well being experienced by fish. By evaluating indicators on every level of the stress response (primary, secondary and tertiary) the allostatic load a given situation inflicts upon the organism can be understood (see figure 1A). Underlining the importance of allostasis (stability through ability to change; Korte, et al. 2007) focus should be on measuring the ability to change. Hence, measuring the capacity to respond physiologically and cognitively to challenges can help identify environments that promote good fish welfare (Salvanes et al. 2013, Braithwaite and Ebbesson 2014). Measuring the ability to respond can also help identify sub-optimal environments, since physiological homeostasis and growth may be maintained in fish experiencing chronic mild stress, however these fish will have a reduced capacity to cope with additional challenges (Grassie et al. 2013, Madaro et al. 2015, Madaro et al. 2016, Vindas et al. 2017).

2.4 Tradition and trends in Atlantic salmon farming

Globally aquaculture is the fastest growing food production industry and, in none-Asia, Atlantic salmon is one of the most successfully farmed species. Commercial salmon farming in the sea started in the late 1960`s in Norway and in 1971 the production was a mere 531 tonnes. In Norway alone, 1.3 million tonnes of Atlantic salmon was produced in 2015 (FKD 2016).

Compared to terrestrial animal production (poultry, swine and cattle) salmon farming has a low carbon footprint per kg edible meat (ISFA 2015). Thus, according to FAO a substantial contribution in meeting the 70% increase, in global demand for food by the population in 2050, should come from Aquaculture (FAO 2011).

The predominant production of Atlantic salmon today occurs in large open sea cages in temperate coastal areas. Consequently, the direct contact of the cultured fish and surrounding ecosystems can have adverse environmental impacts. While salmon farming since its infancy has grown rapidly, the increase in production has slowed in recent years (FKD 2016). The ectoparasite sea lice (*Lepeophtheirus salmonis*) (Oppedal et al. 2011, Stien et al. 2012, Torrissen et al. 2013, Øverli et al. 2014) and escapees of farmed fish that can potentially have negative effects on wild salmon populations (Naylor et al. 2005) are current challenges to sustainability and growth in the industry. Operations related to treating against sea lice also cause increased stress, reduced growth and increased mortality in farmed salmon (Oppedal et al. 2011, Stien et al. 2012, Øverli et al. 2014). Chemical treatments are also a cause of environmental concern and sea lice are becoming increasingly resistant towards common treatments (Torrissen et al., 2013). Authorities in salmon producing countries are currently limiting production growth until sea lice issues are resolved (Torrissen et al. 2013). In Norway, it has been suggested that farming capacity should be regulated based on a sea lice outbreak risk model (Karlsen et al. 2016). Furthermore, the losses of fish are the highest during the production phase in open sea cages, and most losses occur shortly after seawater transfer (Bleie and Skrudland 2014). Approximately 16 % of the fish transferred to open sea cages do not make it to market size in Norway (Bleie and Skrudland 2014, FKD 2016). This is not only an economic burden for the industry, but a fish welfare and ethical issue affecting consumer perception of salmon farming. There are knowledge gaps regarding the cause of these losses. However, the quality of smolts that are stocked in sea cages, disease and environmental and physical factors at the sea sites are suggested as main causes (Gullestad et al. 2011).

It has been shown that smolt size matters for both hatchery reared smolts and wild salmonids with larger smolts having a higher survival rate in the ocean when conditions are sub-optimal (Holtby et al. 1990, Kallio-nyberg et al. 2004). It has been recognized that in an attempt to reduce losses, larger and more resilient smolts should be produced (FKD 2011), and there has been a steady trend in increasing the size of fish at transfer (Bergheim et al. 2009). Recently in Norway, there has been renewed interest in using CCS not only for smolts but also for post-smolt production and potentially till harvest with the primary purpose of limiting the time fish spend in open sea cages (Rosten et al. 2013, Terjesen et al. 2013a). In 2011, Norwegian legislation changed the allowable transfer size from closed systems from 250 g to 1 kg (FKD 2011). The industry is now looking into two main strategies for large post-smolt production up to 1 kg in CCS: 1) in land-based RAS and 2) in closed floating systems placed directly in the sea (Rosten et al. 2013, Terjesen et al. 2013b, Rud et al. 2016). These approaches will be explained in detail below.

2.5 Closed-containment aquaculture systems

Closed-containment aquaculture refers to a wide range of technologies that seek to isolate the culture environment from the natural environment, aiming to reduce or eliminate interactions between the two. Current CCS range from simple ditch systems to huge constructed tanks or raceways either on land or floating in the sea (Beveridge and Little 2002, Summerfelt et al. 2016). However, within the concept of CCS the treatment of both the incoming and effluent water is not distinguished. CCS includes single-pass flow-through systems in which the water only passes through the fish rearing units once, with a varying degree of treatment to the in- and effluent water (Rosenthal 1986, Bergheim et al. 2009). The latest technology to be employed for post-smolts is RAS in which water is (partially) reused after undergoing treatment (Rosenthal 1986, Martins et al. 2010, Terjesen et al. 2013b). While there is no strict classification, when approximately 60-70 % of the water is recirculated and the facility contains a bioreactor for nitrification, the system is classified as RAS, compared to partial reuse or flow-through systems (Timmons et al. 2001). Modern RAS usually recirculate more than 95 % of their water; however, a better designation for RAS is the % daily water exchange or make-up water related to feed loading (Martins et al., 2010).

2.5.1 Sea-based closed systems

Over the years multiple tests with floating closed structures in the sea have taken place (Skaar and Bodvin 1993); however these systems are still on the conceptual or pre-developmental stage and have not yet proven technically or commercially feasible for wide-spread use. Several test were undertaken in the late 80`s and 90`s, and conclusions were that the necessary technology and knowledge was not in place to render these profitable (Rosten et al. 2013). In recent R&D projects both rigid and flexible floating structures have been tested on a semi-commercial scale. One feature currently tested prototypes have in common is that the seawater is pumped in from a desirable depth (-15 to -30m) to avoid surface layers in which sea lice are the most abundant and optimize temperature (Figure 2; Rosten et al. 2011, Nilsen et al. 2017). All systems are required to have installations for supplemental oxygen.

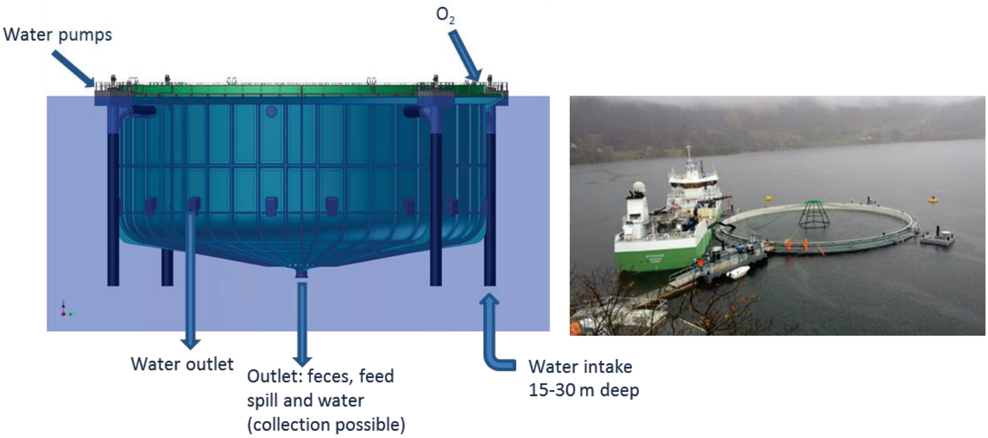

Figure 2. Sea-based closed-containment (CCS) prototype Neptun III, currently being tested by Marine Harvest. There is a variety of sea-based CCS concepts being tested that differ dramatically in shape and construction material however this illustration demonstrates the general principle (photo: Aquafarm equipment AS/Marine Harvest).

Functionally, these systems can be classified as flow-through because water is not reused but exits via outlets in the structure along with waste feed and feces. Partial or full sludge collection is possible in several of the systems currently being tested (Presthaug personal communication 2015). Since, the prototypes tested so far have minimal filtration of intake water and may not collect waste it has become common to define them as semi-closed containment systems (S-CCS; **paper I** and **II**; Rud et al. 2016). However, as previously mentioned the only current definition of a closed system is that it contains a physical barrier separating the fish from the external environment. Hence, for the purpose of this thesis they will simply be called closed sea systems.

Large scale commercial implementation of closed-sea systems will depend on that the productivity is increased compared to open sea cages. This is in part, due to the higher construction costs and potentially increased operational costs (Iversen et al. 2013). However, perhaps an even larger driver for intensification is space restriction both on land and in coastal areas (Henriksen et al. 2013). Compared to sea cages, the CCS currently available have a much lower volume per unit available for rearing fish. Hence, either larger units are needed or a more cost-effective solution would be increasing the fish density per unit compared to open sea-cages (Henriksen et al. 2013).

2.5.2 Land-based RAS

In RAS, to reduce the accumulation of ammonia excreted by the fish and in the decomposing feed and feces, bioreactors are used to remove the total ammonia nitrogen (TAN) by nitrification (Figure 3). Bioreactors provide carriers for biofilms containing nitrifying bacteria that convert TAN into nitrite (NO_2^-) and subsequently to nitrate (NO_3^-) (Timmons et al. 2001). Residual nitrate can be managed through filters with denitrifying bacteria, reducing nitrite and nitrate to nitrogen gas (N_2) (Colt 2006). In systems without denitrification, residual inorganic nitrogen compounds are controlled through daily water exchange (Terjesen et al. 2013b).

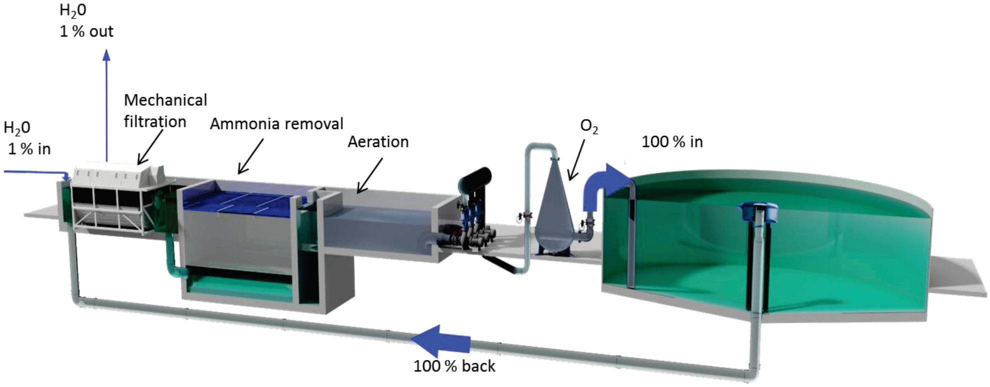

Figure 3. General principles of recirculating aquaculture (RAS). (Illustration: O.G. Kverneland, Akva group).

Until recently there were few commercial scale RAS in Norway, this is mainly due to the historically ample freshwater supply. However, it has been shown that any increases in land-based salmon production will be limited by water supply (Kittelsen et al. 2006). There are also studies on Atlantic salmon suggesting that RAS performs as well or better than classic flow-through systems in regards to growth, sea water survival and welfare (Terjesen et al. 2012, Kolarevic et al. 2014). This has sparked new interest for the development in RAS in Norway (Drengstig et al. 2011). In the Faroe Islands a complete shift to smolt production in RAS instead of flow-through systems took place after 2000, as well as clear strategy towards large post-smolts for stocking at sea (Bergheim et al. 2009). A shift in the industry is also being observed in Norway; traditional flow-through systems are being converted to RAS and all new facilities are RAS.

2.5.3 Husbundry conditions in CCS

Stocking density in sea based systems

The physiological effects of high stocking density on fish will depend on water exchange rate (i.e. water quality), food accessibility, and the degree and nature of social interactions (Ellis et al. 2002). The majority of studies suggest that increased stocking density has a negative effect on fish welfare (Fagerlund et al. 1981, Trzebiatowski et al. 1981, Schreck et al. 1985, Holm et al. 1990, Turnbull et al. 2005). However, there is some disagreement as to the basic cause of these effects (Ellis et al. 2002). Increased fish density will lead to a deterioration of water quality if not controlled for, and this may explain some of the variation in recommended density limits within the same species (Hosfeld et al. 2009, Ellis et al. 2012). In a study by Hosfeld et al. (2009) no negative effects on post-smolt Atlantic salmon were observed when parr in FW flow-through tanks on land were subjected to stocking densities up to 86 kg m^{-3}. Studies on a commercial scale in flow-through systems show minimal differences in post-smolt growth in densities up to 40 kg m^{-3} (Handeland et al. 2008). Furthermore, Kjartansson et al. (1988) suggested that adult Atlantic salmon can be stocked up to 100-125 kg m^{-3} in land-based sea water flow-through tanks. In contrast, other studies performed in open sea cages suggest that stocking above the regulated limit in Norway, 25 kg m^{-3} (Anon 2004), is negative for fish welfare and performance (Turnbull et al. 2005, Oppedal et al. 2011). Hence, stocking density limits for the post-smolt stage needs to be further examined to develop optimal economical and biological conditions in CCS.

Specific water flow (SWF) in sea based systems

To optimize rearing temperature and avoid surface layers where sea lice are the most abundant (Rosten et al. 2011, Nilsen et al. 2017), deep water is commonly pumped into CCS in the sea. The total water flow requirement can broadly be broken down into the flow necessary for maintaining sufficient water quality for the fish, and the flow required for ensuring adequate water velocity and for self-cleaning of the tanks. Even small reductions in the volume of water that needs to be pumped for these two purposes may have a significant effect on reducing costs (Holan et al. In prep.). Therefore, it is highly relevant to establish the specific water flow requirements of post-smolt Atlantic salmon in flow-through sea systems. As explained in detail previously, decreasing the water flow can deteriorate the water quality, thus it is important to establish safe levels for reduced SWF instead of relying on individual water quality parameters. The majority of knowledge on water quality requirements for salmon in flow-through systems is based on freshwater studies on earlier life stages from eggs/fry to smolt (Fivelstad and Binde 1994, Fivelstad et al. 1999a, Stefansson et al. 2007). Thus, it is of great relevance to establish safe limits and guidelines regarding SWF rates for post-smolts in sea water.

Salinity & exercise training

The optimal strategy for rearing large post-smolts in RAS, with respect to water salinity, temperature, exercise training (water velocity) and timing of seawater transfer is not known. There are several challenges related to production of large Atlantic salmon post-smolts in land-based RAS. Seawater RAS may have higher operating costs compared to FW or brackish water RAS, due to the lower efficiency of CO_2 (Moran 2010) and NH_3 removal in seawater (Nijhof and Bovendeur 1990, Chen et al. 2006). This will increase the scale of the bioreactors and/or increase the need for pumping water in saltwater RAS compared to freshwater RAS. A solution may be to produce salmon in brackish water since many studies show an improved growth rate in teleost fish at salinities between 8-20‰ (reviewed by Boeuf and Payan 2001). The improved growth rate might be correlated with a lower standard metabolic rate due to reduced energy expenditure on osmoregulation, however feed intake and feed conversion are also affected by salinity (Imsland et al. 2001, Árnason et al. 2013, Dietz et al. 2013, Zhao et al. 2013). Thus, producing post-smolts in RAS at a lower salinity could be a cost-efficient solution, if fish performance and welfare are not compromised and fish tolerate a later transfer to sea cages.

Exercise training increases the aerobic capacity of the fish through the combined effects on cardiac capacity and muscle morphology (Davison 1997, Castro et al. 2013). Training at speeds of 1.5 body lengths/second (bl s^{-1}) improves growth and feed conversion efficiencies in many salmonid species (reviewed by Davison 1997). Aerobic exercise through increased water velocity has been shown to improve growth rate and feed conversion of earlier life stages of Atlantic salmon (Castro et al. 2011) and increases the efficiency of energy and protein utilization for growth in 100 g Atlantic salmon post-smolts (Grisdale-Helland et al. 2013). Exercise training also reduces agonistic behavior, causing reduced stress levels, hence is beneficial for fish welfare (Christiansen et al. 1992, Adams et al. 1995, Castro et al. 2011). On the other hand it is not known if high water velocity can have detrimental effects, for example on skin health in posts-molts. Currently, land-based tanks are being built larger and larger, with volumes of thousands of cubic meters (Summerfelt et al. 2016). The hydrodynamic properties of these systems are, however, unknown and can conceivably result in high water velocity in the periphery of the tank. Thus, both in view of possible advantageous effects of training as well as for engineering purposes, the effects of water velocity in land-based RAS tanks needs to be established for post-smolts.

3. SCIENTIFIC AIM

Using CCS on land or in the sea to shorten the time Atlantic salmon are reared in open sea cages has been highlighted as key to solving important challenges the industry is facing today. Therefore, the overall aim of this thesis has been to increase the knowledge on the biological and environmental requirements of post-smolt Atlantic salmon in CCS. The focus of this thesis has been on key husbandry conditions that will need to be established before large-scale commercial production can commence (Figure 4).

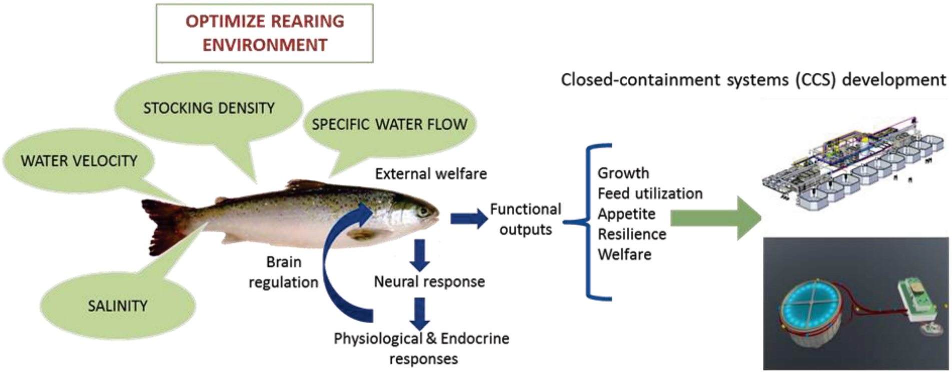

Figure 4. Thesis approach.

The four main objectives are as follows:

1. Commercial feasibility of farming post-smolt Atlantic salmon in CCS in the sea relies on maximizing fish density. However, profitability will depend on that the resulting rearing conditions do not have negative impact on fish physiology, performance and overall welfare. **Therefore threshold limits for stocking density need to be established.**
2. Furthermore, in closed-sea based systems water needs to be pumped in, in intensive large-scale commercial systems operating with high densities, even small reductions in the water volume that needs to be pumped may have a significant

effect on costs. **Therefore, it is highly relevant to establish the specific water flow requirements of post-smolt Atlantic salmon.**

3. Whereas physiological homeostasis can be maintained under chronic mild stress, an additional challenge might result in an allostatic overload impairing physiological and/or cognitive function and ability to cope with environmental changes. **It is therefore important to assess if increasing production intensity will affect the fish`s capacity to respond to new challenges and compromise welfare in CCS.**
4. The optimal strategy for rearing large post-smolts in RAS, with respect to water salinity and water velocity are not known. **Hence, the effects and interaction of salinity and exercise on growth, feed utilization, seawater tolerance, and welfare of large post-smolt Atlantic salmon reared in RAS needs to be examined.**

4. METHODOLOGICAL CONSIDERATIONS

With the aim to provide information on how key rearing conditions in CCS can be optimised out of a growth and welfare perspective, two main experiments were performed. A combined stocking density and SWF study was performed in flow-through sea water systems, aiming to simulate predicted conditions in sea-based closed system in southern parts of Norway. In the second experiment on salinity and exercise training, the RAS conditions described in **paper IV** were constructed to simulate RAS currently in commercial use (Terjesen et al. 2013b). The volume of the tanks (1m^3 and 3.2 m^3) used in these experiment compared to commercial prototypes (~100-21000m^3) are expected to have some effects on growth, feeding and energy expenditure (Espmark et al. 2016). Large scale CCS studies are needed to verify results in this thesis.

4.1 Experimental conditions

Experimental design stocking density and specific water flow limits

In both the stocking density and SWF trial, sea water acclimated post-smolts raised under identical conditions, from the same brood stock and facility were used. All sampling and analytical procedures were done the same way for both trials. Besides the experimental factor the only difference between the trials was that they were performed in separate rooms (see figure 5). Increases in biomass were removed as explained in **paper I** and **II** to maintain the original stocking density throughout the eight week trial. Effects of intensification 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 and SWF at different temperatures. Effects of intensification will also depend on post-smolt size since smaller fish have a higher mass excretion rate (Terjesen 2008). Hence, guidelines in this thesis should be applied with consideration to the prevailing environmental and biological factors.

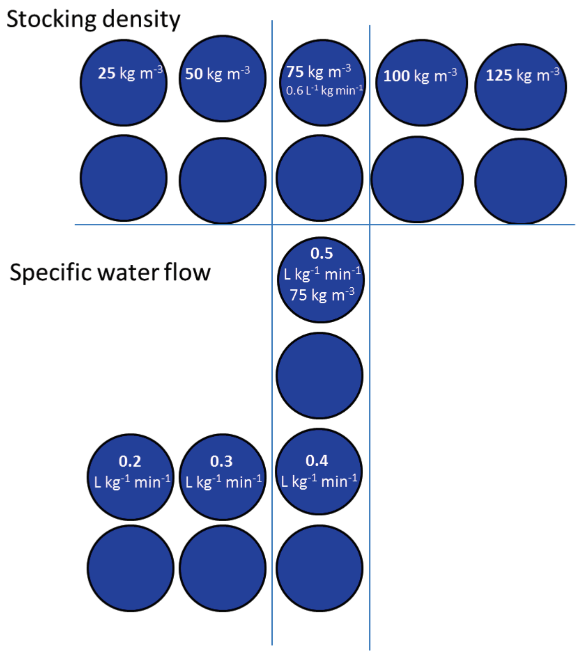

Figure 5. Experimental design of **paper I-III**.

In the stocking density trial (**paper I**), five densities levels: 25, 50, 75, 100 and 125 kg m^{-3} were tested, ranging from the regulated limit for open sea cage farming in Norway (Anon 2004) to the maximum that has been reported in other tests with salmonids (Kjartansson et al. 1988, Vijayan and Leatherland 1988, Jørgensen et al. 1993, North et al. 2006). In the stocking density trial, SWF was kept at 0.6 L kg $fish^{-1}$ min^{-1}. By maintaining a high SWF and oxygen saturation above 80 % in the outlet, the measured water quality parameters (see **paper I**) were within suggested recommended limits for post-smolts (Thorarensen and Farrell 2011) in all treatments. Hence, the causative effects of fish density in this trial are likely not related to water quality. A FW feed was used to reduce the sinking rate of the pellets increasing the time it was available to the fish, thus minimizing any density dependent effects on feed availability. It should be taken into consideration that in **paper I** density treatments were maintained for eight weeks at the same load, while in production density increases gradually and just stays high for a relatively short time.

In the SWF trial (**paper II**) four different levels of SWF were tested, 0.2, 0.3, 0.4 and 0.5 L kg fish^{-1} min^{-1}. This is within the range of SWFs previously investigated in other life stages (Fivelstad and Binde, 1994; Fivelstad, et al., 2004; Fivelstad, et al., 1999b) and slightly lower to identify a possible threshold level. The stocking density in this trial was kept at 75 kg m^{-3}. **Paper I** and **III** validates this density as feasible out of a welfare perspective; hence SWF limits suggested in **paper II** are relevant when fish are stocked at appropriate densities.

Experimental design acute challenge tests (ACT) paper III

To understand how any underlying suboptimal conditions, such as too high stocking density or too low SWF, affect stress responsiveness and neural plasticity, an acute challenge test (ACT) was performed after the eight-weeks treatment in different degrees of intensification. This was performed on a sub-sample of fish from the stocking density trial (25, 75 and 125 kg m^{-3}) and the SWF trial (0.2 and 0.4 L kg fish^{-1} min^{-1}) at the end of the experiments. Since fish from the 75 kg m^{-3} had an equal density as in the SWF trial this treatment was also used as a high SWF treatment (0.6 L kg fish^{-1} min^{-1}) in **paper III**. The ACT entailed a 15 min confinement described in detail in **paper III**.

Experimental design salinity and training in RAS

The aim of **paper IV** was to gain knowledge on the optimal strategy for rearing large post-smolts in RAS, with respect to salinity, water velocity and timing of seawater transfer. This trial was conducted with Atlantic salmon post-smolts at the Nofima Centre for Recirculation in Aquaculture (NCRA) at Sunndalsøra (Terjesen et al., 2013). The fish, RAS conditions, and experimental treatments are explained in detail in **paper IV**. In brief, salmon smolts (Bolaks strain; 70 g) were stocked in three separate RAS with salinities of 12, 22 and 32‰ and subjected to high (1.0 ± 0.13 bl s^{-1}) or low (0.27 ± 0.05 bl s^{-1}) water velocity. At an average weight of 250 and 450 g, sub-samples of fish from all treatments were transferred to full-strength seawater RAS (32‰), while the remaining fish were kept in the original tanks. At 800 g, all fish were transferred to flow-through seawater tanks to simulate final transfer to open sea cages (see figure 6 for experimental design).

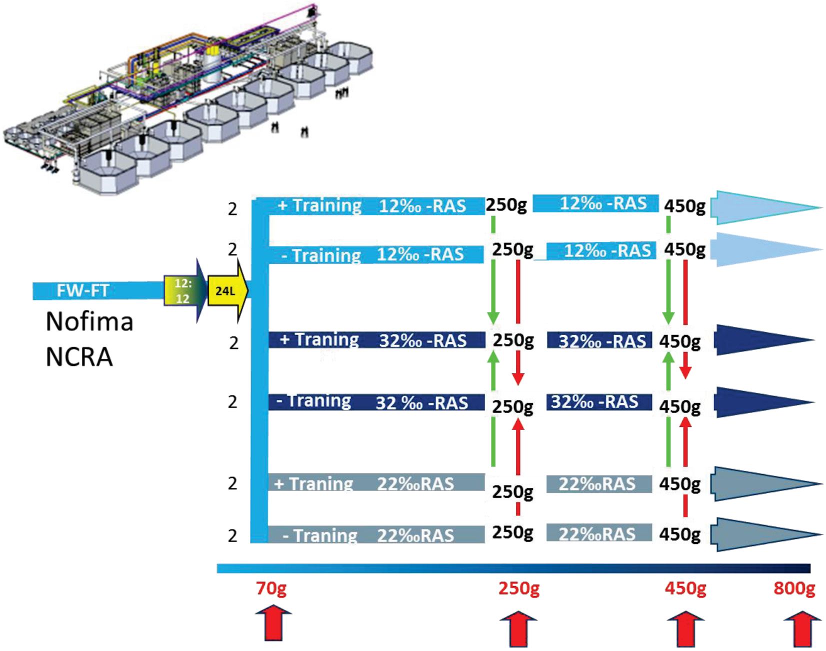

Figure 6. Experimental design of **paper IV.**

In this experiment each salinity was run in separate RAS. Replication on the tank level allows larger RAS to be used in studies on this scale (Terjesen et al. 2013b), reducing the possible effect of tank size on growth and physiology. Furthermore, RAS technology efficiency measured as bioreactor performance is also positively affected by scale (Kamstra et al. submitted). However, this means there is no replication on the system level and it could therefore be argued that different water qualities in the three RAS may be affecting the response variables studied. However, this is unlikely since water quality was carefully monitored and apart from the factor (salinity) no differences between treatments were observed (see **paper IV** for details) and all parameters were within levels regarded as safe for post-smolt Atlantic salmon (Colt 2006, Thorarensen and Farrell 2011).

4.2 Performance & welfare evaluation

Performance indicators

In fish, growth is a specific physiological function that is much more dependent on external factors, in comparison to other vertebrates like birds and mammals (Brett and Groves 1979, Boeuf and Payan 2001). Hence, the goal in any aquaculture environment is to optimize these external factors to improve growth rate. Growth is also closely linked to welfare, as reduced growth can be indicative of an allostatic overload (Pickering and Pottinger 1989, Wendelaar Bonga 1997, McEwen and Wingfield 2003). Feed conversion ratio (FCR; weight gain/feed consumed) is also an important performance indicator used is fish, with a lower FCR indicating a better feed utilization. The feed intake per tank was calculated from the difference between the amount of feed fed to each tank and the amount of uneaten feed collected, corrected for dry matter content in feed (Helland et al. 1996). Specific growth rate (SGR; **paper I, II** and **IV**) and condition factor (CF; **paper I, II** and **IV**) was followed in individually tagged fish. FCR (**paper I** and **IV**) and relative feed intake (RGI; **paper I** and **IV**) was estimated through bulk weight measurements.

Welfare assessment

Plasma cortisol was used as an indicator for **primary stress responses** in **paper I, III** and **IV** and is a widely used indicator of stress in fish (Barton and Iwama 1991, Pankhurst and Sharples 1992, Wendelaar Bonga 1997, Sopinka et al. 2016). However, plasma cortisol as a sole welfare indicator can be misleading, since short term increases are adaptive allowing fish to cope with an altered situation and is not a good predictor of functional output, such as learning and adaptation (Aerts et al. 2015, Sopinka et al. 2016). Furthermore, plasma cortisol rises rapidly in response to a stressor therefor the effects of sampling may bias results (Aerts et al. 2015). Hence, in this thesis plasma cortisol results are studied within the context of several other welfare indicators (secondary, tertiary and external welfare markers).

Secondary stress responses include systemic metabolic, hydro mineral and hematological adjustments (Barton and Iwama 1991), hence secondary responses can be measured as changes in blood chemistry. In this thesis (**paper I, II** and **IV**) an ISTAT analyser was used to measure blood levels of haematocrit (Hct), haemoglobin (Hb), glucose, sodium (Na^+), Cl^-, HCO_3^-, blood pH and partial pressure of carbon dioxide (*p*CO_2). When used for diagnostics in fish deviations between the ISTAT and conventional laboratory values have been found (Harrenstien et al. 2005, Cooke et al. 2008, DiMaggio et al. 2010, Harter et al. 2014). It has, however, been declared a useful tool for onsite analysis by Harrenstien et al. (2005) and Cooke et al. (2008), particularly 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 (Railo et al. 1985, Dimberg 1988). Measuring oxygen consumption (MO_2) can also be used as an indirect way to estimate metabolic rate and energy expenditure (Jobling 1981). However estimations can be difficult to obtain since MO_2 depends on many factors such as body mass, temperature, growth rate, feed intake, feed composition, swimming velocity and stress level (Thorarensen and Farrell 2011). To achieve a good basis for accurate MO_2 estimations in **paper II**, an automatic oxygen level regulation, monitoring and logging system was used.

Tertiary or chronic stress responses to different husbandry conditions were assessed by effects on growth (**paper I, II** and **IV**) and feed utilization (**paper I** and **IV**). Furthermore, fish experiencing chronic stress, will have reduced ability to respond and cope with additional challenges (Grassie et al. 2013, Madaro et al. 2015, Madaro et al. 2016, Vindas et al. 2017). Therefor in **paper III** fish previously exposed to different levels of intensification were subjected to an ACT. Markers for stress responsiveness and neural plasticity were assessed before and after the ACT and are given in table 1.

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. 2008). 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). Being externally visible, these markers are easily studied, and therefore pratical in larger scale studies to quickly compare welfare in different rearing environments. However, this is a subjective evalution and to reduce bias the same experienced examiner with no prior knowledge of the experimental treatments was used in all three studies (**paper I, II** and **IV**).

Table 1. Stress and neural plasticity markers studied in the telencephalon of post-smots in response to an acute challenge test (ACT; **paper III**).

Response	**Marker**	**Function**
Stress responsiveness	*corticotropin-releasing factor (crf)*	Stimulates pituitary corticotropic cells to release adrenocorticotropic hormone (ACTH) to bloodstream. Telencephalic CRF is also involved in modulating stress related behaviors (mammals).
	corticotropin-releasing factor binding protein (crfbp)	Modulates CRF and CRF related peptides activity by binding and reducing their bioavailability.
	11β-hydroxysteroid dehydrogenase 2 (11βhsd2)	Enzyme that coverts cortisol to (inactive) cortisone.
Neural plasticity	*neurogenic differentiation 1 (neurod1)*	Essential for numerous developmental processes like neurogenesis. Regulates and controls neural differentiation. Has been used as neural differentiation marker in fish.
	brain-derived neurotropic factor (bdnf)	Supports survival, encourages growth and differentiation of new neurons and synapses especially in areas in the brain (telencephalon) that are vital to learning and memory. Has been used as neural differentiation marker in fish.

Table references: Lee et al. 1995, Kiefer 2005, Johansen et al. 2012, Grassie et al. 2013, Salvanes et al. 2013, Suri and Vaidya 2013, Vindas et al. 2014, Gorissen et al. 2015, Madaro et al. 2015.

“Good” welfare indicators

Numerous markers exist for inferior or unacceptable welfare, however few indicators can identify advantageous and “good” fish welfare in aquaculture rearing situations. Therefore in **paper III**, we have targeted newly characterized neural response markers (see table 1; Grassie et al. 2013, Salvanes et al. 2013, Gorissen et al. 2015, Madaro et al. 2015, Vindas et al. 2017) that link environmental challenges to learning and memory potential, that are essential for coping and adapting to changing environments. Hence, the markers addressed in **paper III** indicate good welfare situations by identifying normal neural responses to acute challenges.

5. RESULTS AND DISCUSSION

Using CCS as a strategy to shorten production time in open sea cages requires new knowledge on the environmental and biological requirements of post-smolt Atlantic salmon in these systems. The results from **papers I-IV** provide insight on the effect of key husbandry conditions on post-smolt performance, physiology and welfare in both sea- and land-based CCS.

5.1 Closed-containment systems in sea

5.1.1 Stocking density

Commercial feasibility of farming post-smolt Atlantic salmon in CCS relies on maximizing fish density. Results from **paper I** show a 42 % decrease in post-smolt SGR when increasing density from 100 kg m^{-3} to 125 kg m^{-3}. A correlation between increased density (50-125 kg m^{-3}) and reduced feed intake and feed utilisation was also observed. At the end of the eight week period primary and secondary stress responses such as elevated plasma levels of cortisol, sodium, $p\mathrm{CO}_2$ 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. The rearing environment may not only induce stress responses, but can cause fin and bodily damage in farmed fish, representing a clear welfare issue that must be addressed (Broom, 1991; Ellis, et al. 2002). In **paper I**, stocking densities of 100 kg m^{-3} or more increased pectoral fin damage and the prevalence of cataracts was higher in the 125 kg m^{-3} treatment. In contrast to the results in **paper I** Kjartansson et al. (1988) found no negative effects of stocking density on measured parameters in adult Atlantic salmon reared in densities of 100-120 kg m^{-3} in land-based flow-through sea water systems. This suggests that the density limits vary between different life stages, even within the lifecycle phase at sea. Since post-smolts are using energy to adapt to a "new" marine environment (Roberts and Pearson 2005, Jarungsriapisit et al. 2016) they may be more sensitive to external factors, like proximity to conspecifics, than adults. In addition, in **paper I** several welfare indicators, such as external welfare markers were employed and these were not studied by Kjartansson et al. (1988) which might have resulted in different conclusions.

Generally, the responses observed in **paper I** imply that there is a time period in which fish can cope with high stocking densities, but if this time-window is surpassed then wide-spread physiological changes result. This is in line with previous studies on salmonids (Fagerlund et al. 1981, Trzebiatowski et al. 1981, Turnbull et al. 2005, North et al. 2006, Yarahmadi et al. 2016) and other species (Montero et al. 1999, Lupatsch et al. 2010) confirming that once a certain species specific stocking density threshold is passed negative effects on fish welfare are to be expected.

The primary, secondary and tertiary stress responses observed in post-smolts that had been exposed to the highest stocking density (125 kg m^{-3}) in **paper I**, indicate an allostatic overload and chronic stress situation. It can therefore be expected that these fish have a limited physiological and cognitive capacity to deal with additional challenges. This was confirmed in **paper III** as a reduced stress (telencephalic *crf* and *crf-binding protein* (*crfbp*)) and neuroplasticity (telencephalic *neurod1* and *bdnf*) response to a challenge in the highest density compared to the medium stocking density (75 kg m^{-3}). To our knowledge, this is the first study addressing the effect of stocking density on stress related neural responses in salmon. Also for the first time, **paper III** identifies molecular markers of conditions that promote advantageous and "good" fish welfare in an intensive aquaculture rearing situation, in contrast to the numerous markers that exist for inferior or unacceptable welfare.

Unexpectedly, in **paper III** a lack of response to a challenge test was also observed in the lowest stocking density (25 kg m^{-3}) compared to the medium density (75 kg m^{-3}). In **paper I**, the measured parameters do not suggest any adverse effects in the low (25 kg m^{-3}) stocking density treatment. However, there are several studies suggesting that also low fish densities can have adverse effects on welfare in Salmonids (North et al. 2006, Adams et al. 2007). In rainbow trout (*Oncorhynchus mykiss)*, high densities caused fin erosion and increased size variation; however, increased cortisol levels were only registered in the low density group (North et al. 2006). Authors suggested that low density lead to dominance hierarchies and therefore concluded that both low and high density affected welfare in rainbow trout. Upon smoltification, Atlantic salmon develop from a bottom-dwelling highly territorial parr to a pelagic schooling post-smolts

(McCormick et al. 1998) and it has been observed that in post-smolts aggressive behaviour decreases with increased density (Kjartansson et al. 1988). In mice social stress reduces neurogenesis in the adult hippocampus (Mitra et al. 2006). There is evidence that social stress also affects neural plasticity in fish, as Sørensen et al. (2007) discovered that brain cell proliferation is reduced in subordinate fish.

The functional consequences of stress are dependent on the intensity and the duration of the stressor; however, chronic stress is known to impair cognitive processes such as memory and learning (Conrad 2010). While physiological homeostasis and growth can be maintained under sub-optimal conditions, fish could be experiencing chronic mild stress in the low density treatment, which affected their ability to respond and cope with additional challenges (**paper I** and **III**). However, the results from **paper I** suggest that the intensity of the stressor and the adverse effects on welfare are not as profound in the lowest density treatment as in the highest density treatment. The cataracts and fin erosion observed in **paper I** suggest that fish in the highest density treatment are experiencing distress due to lack of space, continual or during feeding, which caused mechanical abrasion. Whereas, in the lowest density the nature of the social interactions is different, and reduced welfare is likely caused by aggressive attacks and establishment of social hierarchies (North et al. 2006). However, the lack of neural response to a challenge in both treatments suggests a reduced capacity to adapt to environmental changes, like the transfer to open sea cages (**paper III**). Overall, the results of **paper III** suggests that fish stocked at the medium density (75 kg m^{-3}) display a more robust telencephalic activation of both stress and neural plasticity responses, compared to low and high density treatments, i.e. the study was able to identify a set of conditions that promoted good welfare. Post-smolts reared in the medium density are thus better suited to respond physiologically and cognitively to environmental challenges. Furthermore, **paper III** identifies several markers that when combined with an acute challenge test consistently reveals pre-existing environmental conditions (favourable or chronic stress) in fish and predicts the fish´s potential for resilience and adaptation to changes in their environment.

5.1.2 Specific water flow

In **paper II** the aim was to document potential effects of reduced SWF on post-smolt performance and welfare in flow-through sea water systems at the stocking density, 75 kg m^{-3}. As SWF is reduced, less new water dilutes the metabolic waste from the fish. The effect of this increased intensity was clear on the water quality in **paper II**. Both TAN and CO_2 increased as conditions intensified, and with a rise in CO_2 level, the pH declined accordingly. The mean CO_2 concentration in the water was three times higher in the lowest (~15 mg L^{-1}) SWF treatment compared to the highest SWF (~5 mg L^{-1}; **paper II)**. The increase in water CO_2 was reflected in the blood as increased $p\mathrm{CO}_2$, pH, HCO_3^- and decreased Cl^- in the lowest water flow treatment (0.2 L kg $fish^{-1}$ min^{-1}) over the eight week experimental period. These observed blood chemistry alterations indicate a typical regulatory response in fish to increased water CO_2 (Eddy et al. 1977, Claiborne et al. 2002). Long-term reductions in plasma Cl^- in response to increased water CO_2 has earlier been observed in post-smolts exposed to 26 mg L^{-1} of CO_2 for 43 days (Fivelstad et al. 1998) and in parr exposed to 17-18 mg L^{-1} CO_2 for 42 days in FW (Hosfeld et al. 2008). Furthermore, increases in blood pH in response to hypercapnia have been observed in both rainbow trout in FW (Dimberg and Höglund 1987) and in post-smolt Atlantic salmon in seawater (Fivelstad et al. 1998). In **paper II**, fish in the two lowest SWF levels had a higher oxygen consumption (MO_2), suggesting that the observed physiological responses are energy costly. However, the typical diurnal variation in MO_2 following feeding (Jobling 1981) was reduced in the lowest SWF (0.2 L $kg^{-1}min^{-1}$) compared to other treatments. This may suggest a reduced feed intake, or that behavioural adaptions like reduced swimming activity may be important in reallocating energy during unfavorable water quality conditions (Pichavant et al. 2001, Santos et al. 2013).

Despite prominent physiological responses and increased MO_2 in response to reduced water flow no negative effects on growth performance were observed in **paper II**. This is consistent with Forsberg and Bergheim (1996), who did not detect any effects on growth and FCR in post-smolts in with seawater flow-through systems with a SWF of 0.2 L kg $fish^{-1}$ min^{-1} for 145 days. In contrast to **paper II** and Forsberg and Bergheim (1996), Fivelstad and Binde (1994) discovered that smolts in soft fresh water, had reduced growth after 65 days exposure to SWF of 0.27-0.33 L kg $fish^{-1}$ min^{-1}. The increased sensitivity in FW is likely related to the reduced buffering capacity of CO_2 or could also be life stage dependent (Fivelstad, et al., 1998). Furthermore, no negative effects of reduced SWF on osmoregulation and external macroscopic welfare were observed, suggesting that within the time period of this experiment post-smolts are able to compensate for reductions in water flow down to 0.2 L kg fish^{-1} min^{-1}. However, in a skin health analysis that was performed as part of the present experiment (not included in thesis) it was revealed that SWF of 0.3 L kg fish^{-1} min^{-1} and lower activated transcription of genes associated with immune responses and mucus production in the skin (Sveen et al. 2016).

The distinct enhancement of physiological responses in the lowest SWF throughout the study in comparison with higher SWF treatments indicates a greater allostatic load (**paper II**). However, on its own it is not clear whether this allostatic load, caused by altered stress physiology, is causing distress (Korte et al. 2007) in post-smolts. A reduced physiological and cognitive ability to respond to an additional challenge would imply that fish are experiencing an allostatic overload and a situation of distress (Grassie et al. 2013, Madaro et al. 2015, Vindas et al. 2017). In **paper III**, a reduced telencephalic upregulation of *crf* mRNA was observed in post-smolts in the low (0.2 L kg^{-1}min^{-1}) compared to the highest SWF treatment (0.6 L kg^{-1}min^{-1}) treatment. In mammals, telencephalic CRF is involved in the regulation of stress-related behavioural responses (Koob and Heinrichs 1999) and affects memory and learning (Radulovic et al. 1999). Furthermore, it has been observed that in chronically stressed Atlantic salmon an additional challenge reduces forebrain *crf* response (Madaro et al. 2015). This might suggest that the reduced telencephalic crf response in fish in the low SWF affects the ability of post-smolts to elicit important behavioural responses to cope with stress. However, for other stress responsiveness markers (plasma cortisol and alpha-Melanocyte-stimulating hormone (α-MSH); telencephalic *crfbp* and *11βhsd2)* and neuroplasticity markers (telencephalic *neurod1* and *bdnf*) a reduced response was not observed. The activation of stress and immune related responses in the skin and the tendency towards reduced stress and neuroplasticity markers in the brain **(paper III)** in the low SWF indicate that wide-spread effects on an organismal level may have become apparent in a longer-term study. Overall results suggest that without any in-tank water treatment specific water flow should be maintained above 0.3 L kg^{-1}min^{-1} (**paper I; III;** Sveen et al. 2016).

5.2 Salinity and exercise in land-based RAS

An alternative strategy in shortening production time in open sea cages is to prolong the time fish stay in land-based systems. In this long-term study on post-smolt production from 70 g- ~1 kg the water quality in the three RAS were within the range regarded as safe levels of CO_2, TAN, NO_2-N, total suspended solids (TSS) and turbidity for post-smolts in seawater (Thorarensen and Farrell 2011). Concentrations of CO_2 and TAN were low in both trials, regardless of the water salinity, with CO_2 concentrations below 5 mg L^{-1} and TAN concentrations below 0.7 mg L^{-1} respectively.

A higher growth rate at the lowest salinity (12‰) in RAS was established during the first months of the experiment when the fish increased in weight from 70 to around 250 g (**paper IV**). Fish also had a higher condition factor in 12‰ in this period. Over the whole trail a reduced salinity had a positive effect on growth rate and FCR. This is in agreement with results in several other teleost species, where salinities between 8-20% have a positive effect on growth and feed utilization compared to full strength seawater (Gutt 1985, Lambert et al. 1994, Imsland et al. 2001, Dietz et al. 2013). Lower metabolic costs associated with osmoregulation at an isotonic salinity may explain the higher growth potential found in several marine teleost species at salinities close to 10‰ (Boeuf and Payan 2001). In **paper IV**, trained fish had a higher CF and increased growth rate in all salinity treatments. These results support findings in earlier Atlantic salmon life stages where aerobic exercise improved growth and FCR (Castro, et al., 2011) and in other salmonid species (reviewed by Davison, 1997).

Sea water challenge tests and long-term transfer to full-strength sea water was performed at 250, 450 and 800 g. At 250 g and 450 g, a higher gill NKA activity was found in post-smolts reared in 32‰ compared to in 12‰. This is consistent with an expected increase in ion secretory capacity when external salinity is high (Specker 1982, McCormick and Saunders 1987, Handeland et al. 1998, Sakamoto et al. 2001). At 800g, there was no effect of salinity on NKA activity and enzyme activity. However, the 12‰ and 32‰ treatments at both fish sizes had similar plasma chloride levels below 150 mmol l^{-1} indicating that the differences in NKA did not affect ability to regulate ion homeostasis (Arnesen et al. 2003). Atlantic salmon (~3 kg) in flow-through systems display relatively low levels of gill NKA activity following transfer to both fresh- and sea water, while still being able to regulate ion homeostasis (Bystriansky and Schulte 2011). Thus, it appears as larger post-smolts can regulate body fluids with less gill NKA activity compared to small post-smolts (**paper I**; **paper II**; Nilsen et al. 2007, Stefansson et al. 2012). In general, the NKA activity (all treatments and fish sizes) was lower in the RAS experiment (**paper IV**) compared to the studies in sea water flow-through (**paper I** and **II**) suggesting that the activity of the enzyme is affected by the RAS environment. Indeed, a different expression pattern of genes in the gill involved with smoltification an ion-regulation have been observed in smolts reared in RAS compared to flow-through systems (Kolarevic et al. 2014), indicating that technology and resulting water quality may influence NKA activity. Training did not affect NKA activity **(paper IV)**.

At 450g high plasma Cl^- levels after seawater challenge tests were observed in all treatment groups, despite elevated gill NKA enzyme activity, indicating osmo-regulatory disturbances (Arnesen et al. 2003, Nilsen et al. 2010). Overall, the highest mortality when transferring fish to full strength sea water was observed at 450 g. After transfer, the mortality was increased in the 12‰ treatment compared to the 32‰. While indicators of stress were observed in all treatments at this time, fish in the 12‰ also had to adapt to a higher osmotic gradient compared to fish in 22‰ and 32‰, limited allostatic resources to deal with an additional stressor may explain the higher mortality. In this period there was also a negative effect of training on survival after transfer, the highest mortality among trained fish was observed in the 32‰. All trained treatments were terminated, due to the high mortality. The reason for the increased sensitivity to handling stress and subsequent mortality in 450 g post-smolt when transferred to new tanks compared to at 250 g and 800 g fish is not known. Elevated cortisol levels in the 450 g trained fish reared in 32‰ in conjunction with reduced skin quality, increased fin erosion, and prevalence of early cataracts implies a reduced welfare in this treatment and explains the poor survival seen in this period, particularly when the fish were handled. At 450 g, increased water velocity had a negative effect on skin condition especially in the 32‰ treatment. Hence, during periods of observed stress reductions in water velocity may be beneficial. It has been showed that in seawater raceways, water velocities of 1.5 BL s^{-1} have a negative effect on post-smolt performance and welfare (Solstorm et al. 2015, Solstorm et al. 2016).

The cumulative survival of post-smolts reared at a constant salinity until 800 g was above 97 % for salmon exposed to 12 and 22‰ whereas survival at 32‰ was only 67 and 77 % in trained and non-trained post-smolts, respectively. There is a lack of long-term studies at this scale to compare to, however the overall survival in the 12‰ treatment (99 %; **paper IV**), was high compared to an industry-wide survey in Norway were survival of small smolts stocked at sea until harvest was 84 % (Bleie and Skrudland 2014). At 800 g, all treatments that had been reared at constant salinity (12, 22 and 32‰) in RAS since 70 g were switched to flow-through sea water without handling, and after four weeks there was no difference in survival between treatments (pooled survival 95%). Thus, all post-smolts in all salinity treatments handled sea water transfer. This suggests that if you can minimize handling during production in closed systems, there is potential to reduce losses compared post-smolt production in open sea cages (Gullestad et al. 2011, Bleie and Skrudland 2014, FKD 2016). With the large tanks now being used in new RAS facilities for smolt/post-smolt production it is fully possible to produce fish up to 1 kg with minimal handling and tank transfer (Summerfelt et al. 2016)

Overall, results in **paper IV** indicate that a lower salinity increased survival, growth and feed utilization. Training had a positive effect on growth in all salinities. Early cataracts, reduced skin condition, growth and survival imply that rearing post-smolts in sea water RAS has a negative effect on welfare.

6. CONCLUSIONS

Compared to open sea-cages there is superior ability to control the rearing environment in CCS. However, the biological and environmental preferences of post-smolt Atlantic salmon in CCS are not sufficiently known. The present study provides insights on the effect of key husbandry conditions on post-smolt performance, physiology and welfare in both sea- and land-based CCS.

Commercial feasibility of farming post-smolt Atlantic salmon in closed sea systems relies on maximizing fish density. Stocking post-smolts in high density (125 kg m^{-3}) for eight weeks induced primary, secondary, tertiary stress responses and densities above 100 kg m^{-3} had a negative effect on external welfare. Furthermore, fish in the highest density treatment (125 kg m^{-3}) had reduced ability to cope with enviromental changes. Overall, the results suggest that stocking density can be maximized up 75 kg m^{-3} without compromising performance and welfare in Atlantic salmon post-smolts in CCS. In fact, for flow-through systems in sea, this fish density may be optimal for welfare, given the peak expression of genes in the telencephalon that are important for cognition and memory. The reduced ability of fish in the lowest density (25 kg m^{-3}) to elicit important responses in order to cope with enviromental challenges should be further elucidated.

Determining the SWF required by post-smolts will largely influence the design and dimensioning of CCS in the sea. Prominent physiological regulatory responses to increased water CO_2 were observed in post-smolts kept in the lowest SWF (0.2 L kg $fish^{-1}$ min^{-1}). However, no differences in growth were observed during the eight week period tested. The energetic cost of physiological responses was revealed by increased MO_2. Some effects of reduced SWF on neural responsiveness to environmental challenges were identified, however these need further investigation. However, it can be recommended that without any in-tank water treatment, SWF should be maintained above 0.3 L kg $fish^{-1}$ min^{-1} to avoid negative effects on skin quality and activating energy costly physiological regulatory responses. Future studies should concentrate on longer-term effects of reduced SWF/increased CO_2. Also, CO_2 limits for post-smolts in RAS should be established. Furthermore, effects of intensification should be confirmed at different temperatures and post-smolt sizes and in RAS once an optimal rearing salinity for post-smolts is established.

Whereas physiological homeostasis can be maintained under chronic mild stress, an additional challenge might result in an allostatic overload impairing physiological and/or cognitive function and ability to cope with environmental changes. **Paper III** indicates that fish density, more than SWF, affects the ability of fish to react to challenges. The reduced ability to respond to a challenge in the low and high density may be caused by social stress, however the link between social interactions and neurological and physiological plasticity needs to be further investigated. Furthermore, several markers were identified that when combined with an acute challenge test consistently reveals pre-existing environmental conditions (favourable or chronic stress) in fish and predicts the fish´s potential for resilience and adaptation to changes in their environment. These markers can be used to further optimize welfare in CCS in regards to other biological and environmental conditions not tested here.

An alternative strategy in shortening production time in open sea cages is to prolong the time fish stay in land-based systems. However the optimal strategy for rearing large post-smolts in RAS, with respect to salinity, water velocity and timing of seawater transfer is not known. Results suggest that salinity isotonic to the fish (12‰) and moderate training have a positive effect on post-smolt performance, welfare and survival in RAS. Using water with salinity around 12‰ may therefore be a production strategy for large post-smolts in RAS provided that post-smolts can handle the subsequent transfer to open sea cages. In future studies, timing of seawater transfer and handling during transfer needs to be further optimized. Furthermore, effects of the RAS environment on ability to cope with additional environmental challenges needs to be investigated.

Based on the present thesis rearing recommendations for post-smolt Atlantic salmon in CCS can be summarized as follows:

- It is feasible to rear post-smolts in densities up to 75 kg m^{-3} without compromising performance and welfare in closed sea systems;
- SWF should be maintained above 0.3 L kg $fish^{-1}$ min^{-1} in closed sea systems, without in-tank water treatment;

- Post-smolts reared in medium (75 kg m^{-3}) density are better suited to respond physiologically and cognitively to environmental challenges compared to fish in low and high densities. Fish density affects the ability of fish to react, more than SWF;
- In land-based RAS salinities isotonic (~12‰) to the fish are better for post-smolt growth and welfare compared to seawater. Exercise training is positive for post-smolt performance.

7. FUTURE PERSPECTIVES

Benefits and challenges

The reduced time fish are exposed and can spread sea lice may be one of the main drivers in Norway, for using CCS to produce post-smolt Atlantic salmon (Terjesen and Handeland 2014). However, there are several other potential benefits motivating the increased use of CCS for salmon (Gullestad et al. 2011, Rosten et al. 2013, Terjesen et al. 2013b, Terjesen and Handeland 2014):

- Improved control over the growing conditions, including temperature, water chemistry and avoiding large fluctuations in these;
- Risk reduction: disease, escapees and environmental impacts;
- Reduced operational costs related to sea lice and disease control;
- Better feed utilization due to control over the rearing conditions;
- Control over effluent, increased reclamation and exploitation of sludge resources;
- Reduced overall production time;
- Increased flexibility: more options for location, optimising transfer time and better management of open-cage sites;
- **Has the potential to significantly reduce production losses.**

Nevertheless, additional knowledge on rearing technologies and the biology of Atlantic salmon in CCS is needed to meet the predicted five-fold increase in production volume and an eight-fold increase in value by the year 2050 (Gullestad et al. 2011). This thesis indicates that it is feasible out of a fish welfare perspective to increase stocking density in flow-through sea water systems from the regulated limit for open sea cages up to ~75 kg m^{-3}. Future studies, in CCS should take these findings as a reference to verify density limits for commercial rearing of post-smolt salmon. However, there are large differences in water quality, technology and degree of environmental control between RAS and flow-through closed sea systems. Moreover, taking into consideration that the salinity preference of post-smolts is lower than full strength seawater in RAS **(paper IV)** optimal stocking densities for post-smolts in RAS still need to be identified. In general, the results gained in this thesis need to be verified in large-scale CCS. Additionally, the increased complexity of technology in CCS compared to open sea cages requires new knowledge and increased dependency on monitoring (Dalsgaard et al. 2013). Also, relatively little is known about microbiota, pathogens and effective control over disease outbreaks in these rearing systems (Rud et al. , Noble and Summerfelt 1996). Hence, further studies on technological solutions for environmental monitoring and biosecurity in CCS are needed.

Disadvantages of land- based RAS in comparison to closed sea systems include the land area needed for the facilities, and the increased energy use required for pumping water (Rosten et al. 2011). RAS technology has the advantage over closed systems at sea, in that it is already commercially available and been in use for some time for smolt production, although several challenges remain here as well, regarding biosecurity and environmental requirements (Terjesen et al. 2013b). An additional advantage of RAS is a year around stable temperature; since closed sea systems often take in deep water, temperatures will be lower in spring and summer compared both to RAS and also open sea cages (Holan et al. In prep.). In northern Norway where sea temperatures are colder, land-based RAS may be a strategic approach, as heat is produced as a byproduct of normal RAS operations and can be used to heat the water entering the tanks to optimal temperatures year around. A higher mean temperature in RAS may be positive for growth, however sexual maturation may become an issue especially when using higher temperatures and a 24 hour photoperiod (Davidson and Good 2015). Hence, understanding factors governing onset of sexual maturation in RAS is important when developing production strategies for large post-smolts.

Post-smolt production in CCS a strategic approach

Although, there are currently companies producing Atlantic salmon to market size in CCS (Iversen et al. 2013), these are often located near important markets and make up a very small fraction of the global production. The feasibility for countries producing large quantities of Atlantic salmon like Norway to shift the complete production to closed systems is debated, and may not even be the best option out of a sustainability perspective (Ayer and Tyedmers 2009).

However, CCS for strategic parts of the salmon life cycle has been suggested as both commercially feasible and a strategy to better manage and utilize open sea production (Rosten et al. 2013).

Concluding remarks

Consumer demand and the increased awareness of stakeholders to environmental and animal welfare issues of aquaculture will likely be a driver for increased use of CCS in Norway and elsewhere. The fundamental knowledge gained on the performance and welfare of post-smolts in different husbandry conditions in this thesis, will contribute towards a production protocol for post-smolt Atlantic salmon in commercial CCS. Furthermore, the markers of welfare addressed are tools that can be adapted in future studies optimising husbandry conditions. Overall, this work can assist in establishing CCS as a means to reduce the time Atlantic salmon spend in open sea cages, and therefore also reduce the environmental footprint of salmon farming and the risk for the salmon farmer.

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Paper I

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Stocking density limits for post-smolt Atlantic salmon (*Salmo salar* L.) with 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.

Statement of relevance: The data presented here are highly relevant for Aquaculture as further knowledge is required on the interaction between fish density, and fish welfare for the development of commercial scale semi-closed sea systems for post-smolt Atlantic salmon farming.

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 and Farrell, 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

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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 among 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 and Wingfield, 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 and Iwama, 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 7 m 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 and Stefansson, 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 analyzed 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 and Farrell, 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.

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

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:

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

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 h prior to tagging and bulk biomass measurements and anesthetized 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 min, 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 h, 70 °C) 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 200 mg/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 analyzed 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 °C. 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

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.

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 and McCormick (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 1:30,000) for 3 h at 37 °C. To each well 2.5 μL 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 ($B_0 = 150$ μl EIA + 100 μl cortisol–horseradish peroxidase conjugate) and non-specific binding (NSB = 150 μl EIA − 100 μL 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

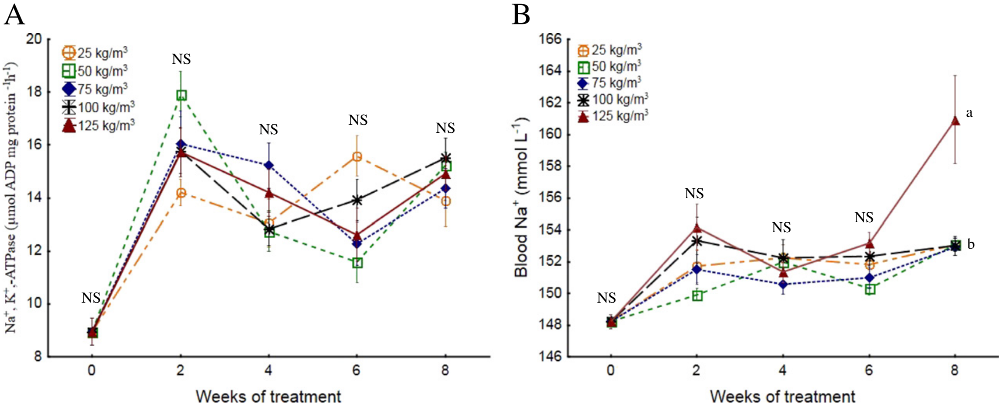

Fig. 1. Gill NKA (A) and Blood sodium (Na^+) (B) in post-smolt Atlantic salmon after 0, 2, 4, 6 and 8 weeks of exposure to stocking densities of 25, 50, 75, 100 and 125 kg m^{-3}. All values are given as mean ± SEM ($n = 12$). Different letters denote significant differences ($P < 0.05$) between density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

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).

3.5. 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 and Kittelsen, 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

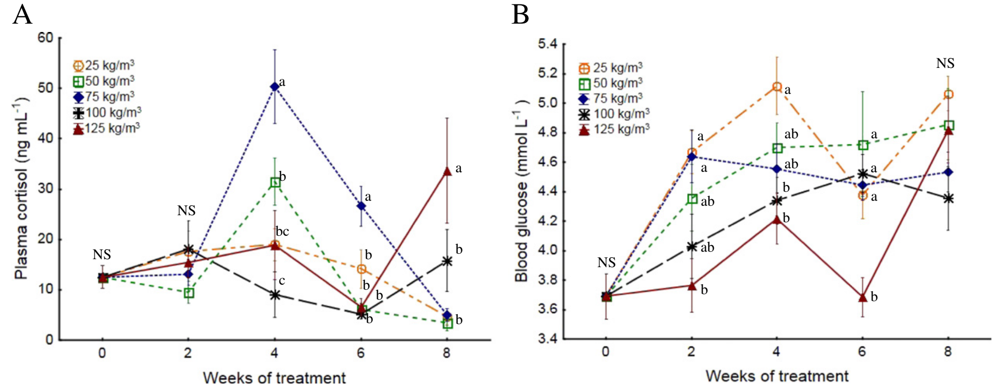

Fig. 2. Plasma cortisol levels (A) and blood glucose (B) after 0, 2, 4, 6 and 8 weeks of exposure to five different density treatments (25, 50, 75, 100 and 125 kg m^{-3}) for 8 weeks. All values are given as mean ± SEM ($n = 6$–12). Different letters denote significant difference ($P < 0.05$) between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

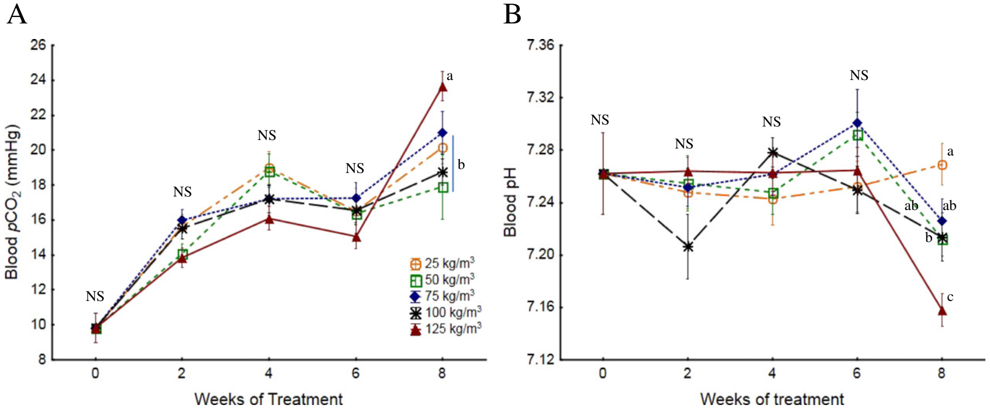

Fig. 3. Blood partial pressure of CO_2 (A), blood pH (B) after 0, 2, 4, 6 and 8 weeks of exposure to stocking densities of 25, 50, 75, 100 and 125 kg m^{-3}. All values are given as mean ± SEM ($n = 12$). Different letters denote significant difference ($P < 0.05$) between treatments at the below time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

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 et al., 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 and Summerfelt, 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 and Noakes, 1980; Keeley, 2000; Li and Brocksen, 1977). Measures were taken to sample each treatment exactly the same, however the handling every second week may have been

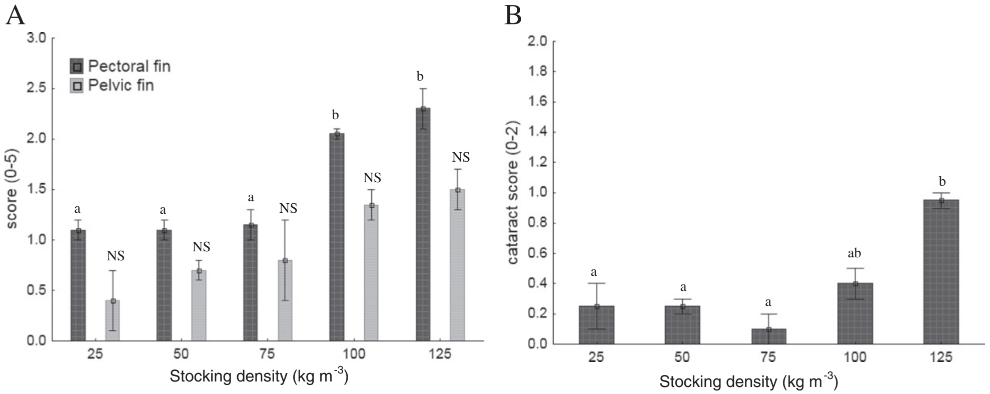

Fig. 4. Pectoral and pelvic fin condition (A) and cataract prevalence (B) in post-smolt Atlantic salmon after 8 weeks of exposure to stocking densities of 25, 50, 75, 100 and 125 kg m^{-3}. Each data point is the tank mean ± SEM ($n = 2$) and 10 fish per tank were scored. Scores are 0–2 for cataract 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.

perceived more stressful as stocking density increased, despite the intensity of stress being the same (Pottinger and Pickering, 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. An elevated cortisol level 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 and Iwama, 1991) in the 75 kg m^{-3} treatment, and the lack of sustained secondary responses 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 and Randall, 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 and Bryson, 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 and Naamansen, 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 and Bryson, 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 among 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 and Iwama, 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 and Krogdahl, 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 and Ridha, 1989) and cod (Björnsson, 2004) as a consequence of mechanical abrasion of the cornea (Ubels and Edelhauser, 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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