Documentation of growth and insulin-like growth factor I in Atlantic salmon (*Salmo salar*) post-smolts reared in large scale semi-closed and open systems

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ABSTRACT

Understanding the variation of growth performance in different large scale production scenarios is of great importance to the aquaculture industry to develop efficient rearing procedures for cultured fish. This study investigated the growth performance of Atlantic salmon (*Salmo salar*) post-smolts reared in the semi-closed system, Preline, compared to post-smolts reared in a traditional open cage. The study consisted of three different parts: (1) Freshwater period, (2) post-smolt period (Preline vs control systems), and (3) adult period in open cages. Smolts with the same biological and genetic background were divided into two groups, and reared in separate systems during the post-smolt period. Temperature and salinity differed between systems since the Preline system accessed water from a depth of 35 m. Control fish showed a higher growth rate compared to Preline fish during the post-smolt period. After two and four months, they also showed a significantly higher weight, length and condition factor compared to Preline fish, which was associated with higher temperature. The insulin-like growth factor I (IGF-I) plasma concentration was higher in control fish after one and two months, which was also associated with higher temperatures. However, no differences in plasma IGF-I concentration were found between systems after four months. Preline fish showed a higher growth rate compared to control fish when transferred to an open cage during the adult period, suggesting that they may have experienced a training affect during the post-smolt period from the constant water flow in the Preline semi-closed system. A higher expression of IGF-I binding proteins (IGFIBP) mRNA expression in muscle was seen in control fish at the end of the post-smolt period, which may have inhibited growth during the adult period.

1. INTRODUCTION

1.1 Norwegian salmon aquaculture

Currently, typical marine aquaculture operations, such as Atlantic salmon production, occur in sea cages that are open to the environment. Traditional open cages are widely successful in Norwegian aquaculture because they are cost-effective and they efficiently utilize Norway's natural advantage of clean water, and natural occurring streams. The salmon aquaculture industry extends along most of the Norwegian coast and in fjords, with 990 active salmon farming sites in 2015 (Directorate of Fisheries, 2016). This has facilitated the unprecedented growth over the last decades. However, despite beneficial conditions along the near coast and fjords of Norway, the annual increase in production volume in today's industry has decreased notably during the last years due to challenges with salmon lice, diseases, organic pollution and escapees. Hence, future sustainable growth of the aquaculture industry is not reasonable without finding mitigating measures to these challenges.

In Norway, smolts are around 100 – 150 g when transferred to open sea cages during spring, and around 60 – 80 g during autumn (Directorate of fisheries, 2015). It has been documented that approximately 16 % of the fish transferred to seawater do not survive, and a high percentage of this mortality occurs during the early seawater phase, where reasons include poor smolt-quality, diseases/disease treatment, and escapees (Gullestad et al., 2011, Bleie and Skrudland, 2014). In such situations, development and implementation of alternative production strategies may play a vital role to secure future sustainable growth of the salmon aquaculture industry. One suggestion has been to produce larger and thus more robust smolts in closed containment systems prior to transfer to open sea cages, a trend that has already been implemented by the industry (Bergheim et al., 2009). The legislation has also been adopted, so the industry can now produce salmon on land up to 1 kg before transfer to open sea cages.

1.2 Semi-closed containment systems

Currently, there is considerable interest for testing and developing post-smolt production in closed or semi-closed systems in the ocean to provide a more stable environment where fish farmers can employ greater control over the rearing conditions. The term "Closed containment system" is used to describe a range of technologies that have an impermeable barrier to restrict interactions between farmed fish and the surrounding environment (Iversen et al., 2013). Extending the time that fish spend in controlled environments may optimize welfare parameters and reduce susceptibility to disease and thus permit sustainable growth. Additionally, the containment of fish may also contribute to reducing other major issues with open cage systems, such as genetic influence from escapees on wild populations, as well as spreading disease and parasite infestation, which is one of the major environmental challenges of the salmon farming industry in Norway. Such systems can also filter outgoing water, or collect waste, which can eliminate or significantly reduce pollution from feed, feces and chemical waste to the surrounding environment. However, if salmon are to grow in semi-closed systems for the full duration of production, the high costs of construction and operation of semi-closed systems may reduce overall profit, which makes it hard to compete with conventional open cage systems. An option is to use semi-closed systems as a supplement rather than a replacement to current production technology during the early sea phase, where post-smolts can grow to sizes around 0.5 to 1 kg and become more robust before being exposed to the open ocean. However, higher investment implies production at higher fish densities, which may influence welfare parameters and growth negatively. The recommended fish density for salmon in open sea cages is between 15-25 kg/m^3 (Turnbull et al., 2005). Results from previous studies have indicated that higher fish densities may reduce fish welfare (based on body and fin condition and plasma concentrations of glucose and cortisol) (Turnbull et al., 2005). However, it has recently been demonstrated that Atlantic salmon post-smolts under controlled laboratory conditions can grow and perform well up to densities of 75 kg/m^3 without compromising animal welfare, given that the water flow is above 0.6 L kg fish min^{-1} and water quality parameters are kept within recommended values (Calabrese, 2016, Calabrese et al., 2017). This indicates that it may be possible to increase the density limit in closed systems. For the salmon aquaculture industry, the growth performance of fish is an important influencing factor regarding economic benefit, along with avoidance of disease and escapees. As closed containment systems are a relatively recent development, it is important

that these systems are evaluated in regards to post-smolt salmon quality, welfare, individual growth potential, development, survival, and water quality parameters. Additionally, it is important to research whether salmon post-smolts can perform equally well, or even better, in these closed systems compared to open cages. In addition to small controlled laboratory scale experiments, this knowledge must also be validated with large-scale production experiments, such as the current study.

1.3 Measuring somatic growth in fish

Growth in fish is highly dependent of size and influenced by environmental factors such as water temperature, photoperiod, salinity, water quality, and the quality and abundance of food (Thorarensen and Farrell, 2011). Somatic growth is probably the ultimate measure and indicator of animal performance and welfare as it relates to the fish's ability to efficiently utilize available feed and regulate their metabolism to maximize growth (Beckman et al., 2001). Growth prediction is important for the aquaculture industry to achieve good estimates of feed allocation, and thus maximize profit and reduce waste. There is probably no single growth measure that stands out as the universal indicator of fish growth and one should preferentially use several indicators to get the best estimate and/or prediction of growth. The most common growth indexes used in today's industry are specific growth rate (SGR) (Brett, 1979, Austreng et al., 1987, Jobling, 1995, Jobling, 2003) and the thermal growth coefficient (TGC) (Iwama and Tautz, 1981, Alanärä et al., 2001). Both SGR and TGC provide solid measures of growth in production, and are used to calculate the amount of feed needed. However, they also comprise some inherent limitations since they overlook fundamental drivers of fish growth, and often lack biological interpretation (Thorarensen and Farrell, 2011). For instance, SGR is based on the incorrect assumption that fish growth is continually exponential, and does not consider that the growth rate of fish is highly dependent on body weight and water temperature (Dumas et al., 2010). Therefore, it is difficult to use SGR as a growth measure when comparing fish of different sizes or reared at broadly differing temperatures, and predictions are usually only appropriate for very short intervals (Dumas et al., 2010).

TGC allows for assessment of growth rate independent of temperature, making it a more flexible tool for estimating growth in commercial production (Thorarensen and Farrell, 2011). However, the assumptions of TGC can, as with SGR, be violated in different production

scenarios and should be used with caution. TGC assumes that growth increases steadily with increasing temperature. However, this assumption is invalid at the higher end of the thermal range since TGC declines (Jobling, 2003). Therefore, errors can be made when calculating TGC in situations where the temperature is above the optimum for growth. Thus, new indicators to support growth assessments is beneficial. In addition to using SGR and TGC, it has been suggested that hormones essential in modulating growth may provide promising candidates in the search for growth indicators in salmonids (Beckman, 2011). Understanding the hormones that control growth may lead to further optimization of growth conditions. However, the measure of hormone abundance does not always represent its biological effects due to many interacting factors (Beckman, 2011). Thus, it has proven challenging to identify and validate new reliable and robust indicators that not only report somatic growth in real time, but can also predict future growth trajectories in commercial production scenarios. If such growth indicators are to be established and used in real production scenarios, they must be tested at large scale to see if the kinetics of production, release and clearance of hormones react in the same way as they do in small scale laboratory studies. Research is particularly needed for closed containment systems, since the environment in these systems may differ from normal rearing conditions. The semi-closed system used in the current study induces a constant water flow through the system, increasing aerobic training for fish. This has been associated with increased growth (Jørgensen and Jobling, 1993, Castro et al., 2011) and muscle development (Castro et al., 2013), among other advantages.

1.4 Insulin-like growth factor (IGF-I)

Somatic growth in fish is under endocrine control, where environmental factors and nutrition act as external and internal stimuli, and are processed and transferred to the endocrine organs. These organs include the hypothalamus, and the pituary gland where hormones are synthesized and secreted (Moriyama et al., 2000). Insulin-like growth factor-I (IGF-I) is a key component of the complex system that regulates growth in fish (Reinecke, 2010). With fish, it is normal to talk of a "growth hormone (GH)-IGF-I axis" in which several components are involved. GH is released from the anterior pituary, and via it's receptor it is the primary stimulus for synthesis and release of IGF-I in the liver (Daughaday and Rotwein, 1989, Le Roith et al., 2001, Ohlsson et al., 2009). The liver is the principle source of circulating IGF-I in plasma, and the effect of IGF-I is primarily mediated by IGF-I cell surface receptors

(IGF1ra) (Mendez et al., 2001). IGF-I signaling in target tissues, such as skeletal muscle, has been shown to stimulate cell proliferation, differentiation and protein synthesis (Stickland, 1983, Le Roith et al., 2001, Fuentes et al., 2013). The half-life of circulating IGF-I in plasma is about 10 min. However most circulating IGFs are bound to high affinity IGF binding proteins (IGFBPs), which modulate the bioavailability of IGFs in the extracellular environment (Wood et al., 2005). These binding proteins prolong the half-lives of IGFs in plasma up to about 12 h in humans and fish (Duan, 1997, Shimizu et al., 2009).Therefore, plasma IGF-I levels are relatively stable in comparison to many other hormones that are essential for stimulating growth, such as GH, insulin, and thyroid hormones (Beckman, 2011). For instance, studies have shown that IGF-I is less responsive to short-term nutritional changes after fasting, and relates more to long-term integrated trends in nutritional status (Shimizu et al., 2009). From several teleost studies, evidence suggests that IGF-I levels in plasma are positively correlated with individual growth rates (Beckman et al., 1998, Beckman et al., 2004a, Beckman et al., 2004b, Kawaguchi et al., 2013, Kaneko et al., 2015). Hence, several studies and reviews have suggested that IGF-I may serve as a useful growth index (Dyer et al., 2004, Picha et al., 2008). A series of small scale studies have used salmon to assess the response of IGF-I to different environments and its reliability as a growth index (Beckman et al., 1998, Beckman et al., 2004b). However, it is unclear how environmental variation may interfere with IGF-I. Hence, controlled small scale laboratory experiments may provide valuable information about biological potential and requirements of post-smolts, but there is a lack of documentation when it comes to intensive production of post-smolt salmon in semi-closed containment systems in large scale production.

1.5 IGF-I in muscle

Apart from IGF-I and IGFIBP production in liver, other non-hepatic tissues produce IGF-I locally, including skeletal muscle (Mendez et al., 2001). Local IGF-I has generally only autocrine or paracrine effects, and the actions of IGF-I in muscle tissue are also mediated by binding to IGF-Ira in muscle (Párrizas et al., 1995, Le Roith et al., 2001, Mendez et al., 2001). Signaling promotes muscle cell proliferation, differentiation, protein synthesis and muscle hypertrophy (Stickland, 1983, Fuentes et al., 2013), which results in muscle growth (Beckman et al., 2004b). Evidence supports that both muscle-derived and liver-derived IGF-I regulate growth in muscle, although it is not known which of them is the main driver of this (Fuentes

et al., 2013). As with plasma IGF-I, muscle IGFBPs regulate IGF-I signaling by binding to IGF-I, thus taking the place of receptors (Duan, 1997).

1.6 Aims

The aim of the present study was to investigate whether post-smolt grow and perform equally well in the semi-closed system, Preline, as in an open control cage, regarding survival, weight gain, and growth rate. Additionally, IGF-I levels in plasma and IGF1, IGFBP and IGFra mRNA levels in muscle will be assessed to see if they differ between differing environments (temperature, salinity and water flow) in large scale production.

Hypotheses:

H_0: The growth of fish reared in the Preline semi-closed system is the same as fish reared in the control open cage and has no significant effect on the parameters below:

- H_{A1}: Weight gain and length, and hence growth rate (SGR and TGC) of fish reared in the Preline semi-closed system is significantly different to fish reared in the control cage

- H_{A2}: IGF-I plasma levels of fish reared in the Preline semi-closed system is significantly different to fish reared in the control cage

- H_{A3}: Muscle IGF-I mRNA levels of fish reared in the Preline semi-closed system is significantly different to fish reared in the control cage

- H_{A4}: Muscle IGFIBP mRNA levels of fish reared in the Preline semi-closed system is significantly different to fish reared in the control cage

- H_{A5}: Muscle IGF-Ira mRNA levels of fish reared in the Preline semi-closed system is significantly different to fish reared in the control cage

2. MATERIAL AND METHODS

2.1 Fish material

Atlantic salmon (*Salmo Salar*) smolts (spring 1+) from Sjøtroll Havbruk AS facilities located at Kjærelva, Fitjar, were used in the present study. Smolts were from the Salmobreed QTL duo strain. Eggs were incubated at a water temperature of 5,8 $^{\circ}$C, and hatched after 513 day-degrees (d$^{\circ}$C). The fish began first feeding 387 day-degrees after hatching (early May 2015), which occurred in a constant light environment (LD24:0) in approximately 14 $^{\circ}$C heated water. During the freshwater period, fish were kept indoors in green 7 m rearing tanks (70 m^3) at constant light and ambient water temperatures (Table 2.1) up until January 11th 2016. Thereafter, a photoperiod regime was initiated to stimulate smoltification, which was done according to standard protocols for 1+ smolts. This included decreasing day length from LD24:0 to LD12:12 for approximately 8 weeks (from January 11th to March 7th), and then increasing day length to LD24:0 for approximately 8 more weeks (from March 8th to April 30th). By the end of April, fish showed morphological signs that indicate a normal smoltification, including a lowered condition factor, dark fin margins, silvery scales and high NKA-activity (Stefansson et al., 2008). Fish were fed commercial freshwater/seawater dry diets (Ewos, Norway) from automatic feeders (Akvagroup) according to temperature and fish size.

Table 2.1: Average water temperature during the freshwater period.

Month	Minimum, $^{\circ}$C	Average, $^{\circ}$C	Maximum, $^{\circ}$C
06-2015	15,3	17,2	18,6
07-2015	17,0	20,3	25
08-2015	15,5	17,8	21,8
09-2015	13,8	15,3	16,4
10-2015	10,8	13,0	14,1
11-2015	7,1	8,8	10,9
12-2015	3,9	5,5	6,9
01-2016	2,4	3,6	4,5
02-2016	1,8	2,6	3
03-2016	3,0	3,8	4,7
04-2016	4,3	5,8	7,6
05-2016	6,8	8,3	9,5

2.2 Experimental protocol

The project consists of three different parts; (1) Freshwater period, (2) Post-smolt period in two separate rearing systems; Preline semi-closed system, and control open cage system, and (3) adult period in open cage systems (Fig. 2.1).
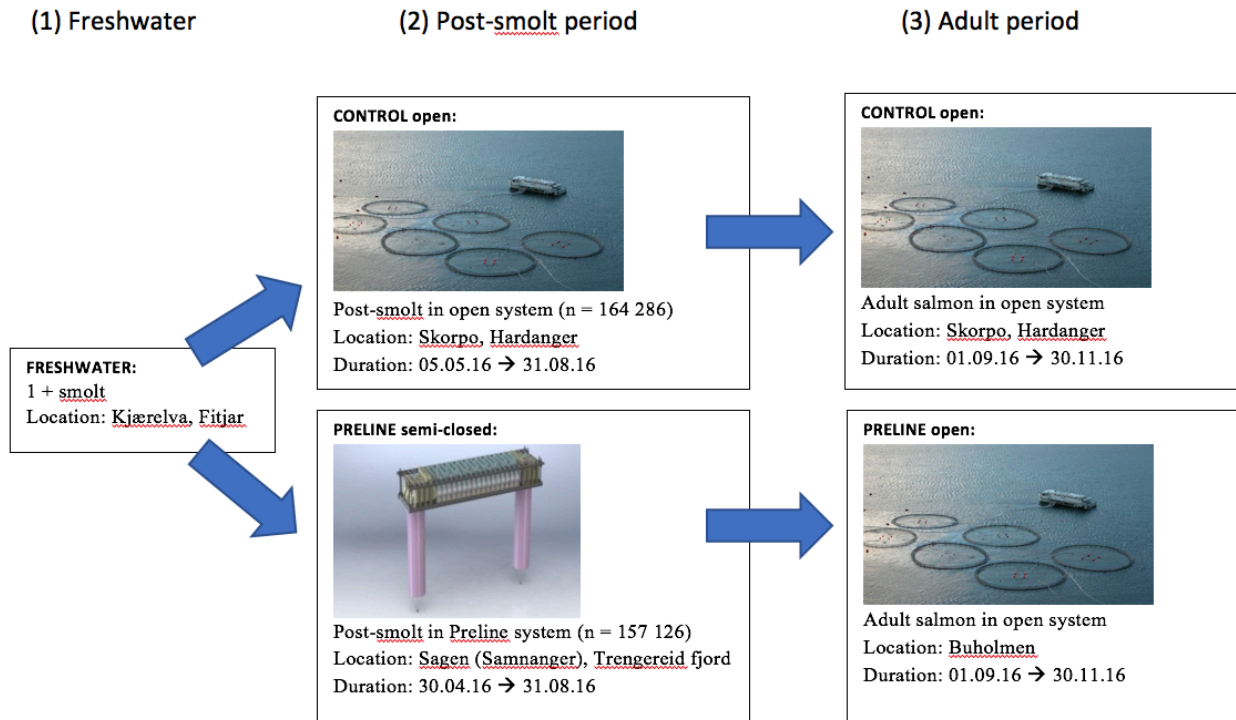
Figure 2.1: Overview of experimental design. One sampling conducted during the freshwater period, and three samplings conducted during the post-smolt period in each system. Data obtained from the adult period was provided by the farming company, Lerøy Vest, AS.

Seven separate sampling days were conducted in total. One sampling was conducted during the freshwater period on April 15th 2016 at Kjærelva, Fitjar, before a total of 157 126 and 164 286 smolts were transferred to the Preline semi-closed system and control open cage system on April 30th 2016 and May 5th 2016, respectively. All fish were transferred by the well boat "Mowistar". After transfer to seawater, three samplings were conducted during the post-smolt period in each system, which was approximately after one month (01.06.16 Control, 02.06.16 Preline), two months (29.06.16 Preline, 30.06.16 Control), and four months (29.08.16 Preline, 30.08.16 Control). Fish were in the Preline system from April 30th 2016 to August 31st 2016, and were thereafter transferred to an open cage system at Buholmen, Hordaland, Norway, for

the adult period. Fish in the control open cage system remained in the same system during the post-smolt period and adult period.

2.3 Experimental facilities

The Preline semi-closed system (Fig. 2.2) is located at Sagen in Samnanger, Hordaland in the Trengereid fjord in Norway (60°20'52.84"N, 5°38'30.64"E). This location has a depth of 100 m and is well protected from wind and waves with good water circulation. The Preline platform is 50 x 12 x 8 m, and holds approximately 2000 m^3 water volume, (max water flow 400 m^3/min, water exchange rate 5-6 min, and water current 10-20 cm/s). It is equipped with a 35 m deep water intake and outtake pipe system, which contributes to a constant water flow through the system. The control open cage system is located at Skorpo, Hardanger in Norway (59°57'5.79"N, 5°49'35.82"E) (Fig. 2.3). This location has a depth of approximately 250 m, and the open cage has a depth of 60 m. The cage is a traditional open 160m conical circular cage (Norwegian: Spissnot), which consists of an Akvaline ring. The open cage located at Buholmen was the same type of system as the control open cage system.
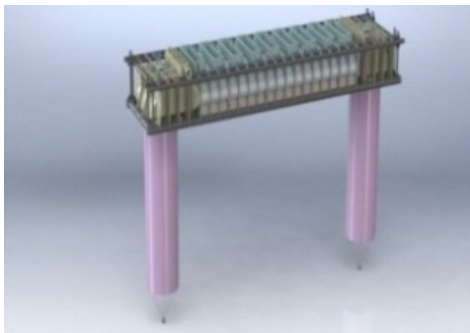
Figure 2.2: A diagram of the Preline semi-closed raceway system located at Sagen in Samnanger, Hordaland in the Trengerreid fjord in Norway (Sveier et al. 2015).
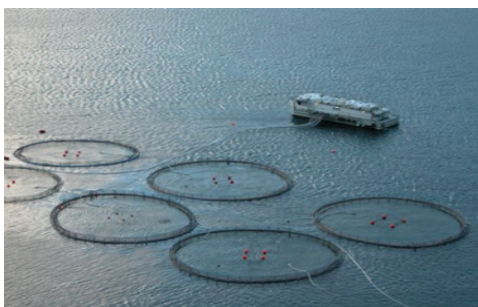
Figure 2.3: The control open cage system site located at Skorpo, Hardanger in Norway (Sveier et al. 2015).

2.4 Sampling protocol

All husbandry practices at the farms were conducted in accordance with standard protocol for Lerøy Vest, AS. Production data was measured by automatic systems and included temperature (°C), salinity (parts per thousand, ppt), and oxygen concentrations (%) (OxyGuard Commander, Sterner), and feeding (Fishtalk, Akvagroup, Bryne). These parameters were registered daily during the post-smolt period in the control open cage and the Preline semi-closed system from May 5th 2016 to August 31st 2016. They were also measured during the adult period in the control open cage and the Preline open cage at Buholmen from September 1st 2016 to November 30th 2016. During the same period, estimated weight data was calculated based on feed output (Fishtalk, FCE = 1.1) and registered daily. The water quality parameters were measured at three depths, 3m, 8m and 15m, in the control open cage system and the Preline open cage system at Buholmen. Accumulated mortality (%) was also registered by Lerøy in all systems from April 30th to August 31st in the Preline system, and from May 5th to August 31st in the control system, where fish were checked twice per day and dead fish were removed.

2.4.1 Fish material

For each sampling, a total of 30 fish were randomly selected, resulting in a total of 210 samples. A large net was used (Norwegian: Stornot) to catch the fish, which was lowered into systems to approximately 5 m while fish were fed, and lifted again shortly after. Fish were humanely euthanized with $NaCO_3$-buffered tricaine methanesulphonate (MS222, Sigma-Aldrich, St Louis, MO, USA) anesthetic, resulting in a total of 210 samples. For each sampling, blood samples were taken with 2 ml heparinized syringes, and put into 1,5 ml tubes for centrifugation. After centrifugation at 3000 rpm for 5 min at 4°C, 400 µl aliquots were transferred into 0,5 ml tubes, frozen on dry ice, and stored at -80 °C until use. Individual fish were weighed to the nearest 0,01 g, and measured to the nearest 0,1 cm to determine the average size and condition of the population. Muscle samples were taken above the lateral line behind the dorsal fin, where a small piece was stored in RNA later for molecular analysis.

2.4.2 Specific growth rate (SGR)

Specific growth rate (SGR) was calculated for the post-smolt period using the weight data from sampling points in early June (02.06.16 Preline, 01.06.16 Control) and late August (29.08.16 Preline, 30.08.16 Control), with the following equation:

$$SGR = (\ln W_2 - \ln W_1) / \Delta T$$

where W_2 and W_1 are weights measured at days T_2 and T_1, and ΔT is the number of days between T_1 and T_2.

2.4.3 Thermal growth coefficient (TGC)

All three systems used in the experiment (the Preline semi-closed system, the control open cage system, and the open cage at Buholmen) were in different locations, which varied in surrounding seawater temperature. Therefore, a weight model incorporating growth rate/day dependent on the daily temperature was used, Thermal Growth Coefficient (TGC), with the following equation:

$$TGC = (\text{Final weight}^{1/3} - \text{Start weight}^{1/3}) \times 1000 / \text{sum of daily temperature}$$

TGC was calculated for the post-smolt period using the weight data from sampling points in early June (02.06.16 Preline, 01.06.16 Control) and late August (29.08.16 Preline, 30.08.16 Control), and the measured temperature data from the farming company within the same dates. To see if the estimated weight data from the farming company was similar to the sampling data, TGC was calculated again during the post-smolt period using both estimated weight data and temperature measurements from the farming company. Since there were no sampling points during the adult period, TGC was calculated using estimated weight data from Lerøy, and the measured temperature data from August 31st 2016 to November 30th 2016.

A relative percentage increase in weight was also calculated for the control open cage and Preline open cage during the adult period using estimated weight.

2.4.4 Condition factor (CF)

The condition factor (CF) was measured for each individual fish using the following equation:

$$CF = (\text{weight}/(\text{length})^3) \times 100$$

2.4.5 Feed conversion ratio (FCR)

The feed conversion ratio (FCR) was calculated for the post-smolt and adult period with the following equation:

$$FCR_{\text{for each period}} = (\text{Biomass gain/feed consumption}) \times 100$$

2.5 Analytical techniques

The following techniques were used to analyze the parameters presented in this thesis.

2.5.1 Time-resolved fluoroimmunoassay (TR-FIA) for plasma IGF-I concentration

Plasma IGF-I levels were quantified by TR-FIA (competitive time-resolved fluoroimmunoassay) using the method described in Small & Peterson (2005), with some modifications explained further. The assay was conducted in DELFIA® pre-coated goat anti-rabbit IgG Microtitration strips (96-well format). The following reagents and solutions were used for the TR-FIA: Acid-ethanol mix (A/E mix), Tri-base, Blank Solution, Washing Buffer, Assay Buffer and Enhancement solution (Appendix 6.1). Recombinant salmon/trout IGF-I was used as a standard, masu salmon (1+) (Mori strain) pooled serum was used as Interassay Pool (IP) sample, anti-barramundi IGF-I (rabbit) as primary antiserum, and europium was used for labelling. Eight plates were used in total, one test plate and seven plates to analyze all unknown plasma samples. Prior to the assay, serum IGF-I was acid-ethanol extracted to dissociate the binding protein from the hormone peptide as described in Shimizu et al. (2000). Plasma samples were extracted with 1:4 ratio A/E mix and neutralized with 1:2 ratio tri-base. Each immunoassay was run over a period of three days.

Test plate: A test plate was run to find the volume (dilution factor) of extraction we needed to apply to each sample well so they would be within the range of the standard curve. The test plate consisted of a standard curve, IP samples (high-, mid- and low IP), a dilution of pooled samples, and three different amounts of unknown samples (10ul, 20ul, and 35ul) (Fig. 2.4). All were applied in duplicates.

Day 1: Prior to application, all wells were rinsed once with 200ul of washing buffer to create a wet environment. To generate the standard curve, 8ul of the standard stock was diluted in 492 ul assay buffer, and further serial diluted 1:1 eight times ranging from 0.0625 ng/ml (0.00625 ng/well) to 16 ng/ml (1.6 ng/well). For each dilution, 100ul was added into wells + 35ul blank solution. The standard zero consisted of 100ul Assay Buffer + 35ul blank solution to determine non-specific binding. To compare plates, an IP sample was used. For extraction of IP sample, 10ul of IP plasma was mixed with 40ul of A/E mix (1:4 ratio), and incubated at room temperature for 30 min. The IP sample was then neutralized with 20ul of Tri-base (1:2 ratio), and centrifuged at 10 000rpm for 10 min at 4°C. Thereafter, 50ul of the IP sample was serial diluted with 150ul blank solution into "high", "mid" and "low" IP, and 35 ul of each dilution was applied to correct wells + 100ul assay buffer. A dilution of pooled samples was prepared, using the first sample from each sampling group (seven samples in total). 10 ul from each sample was mixed, and then 30 ul of the pool was mixed with 120 ul of A/E mix, incubated for 30 min, neutralized with 60 ul of Tri-base, and centrifuged at 10 000rpm for 10 min at 4°C. Next, 100ul of the pool was serial diluted in 100ul blank solution ranging from x2 to x64, and 35ul of each dilution was applied to correct wells + 100 ul of assay buffer. The extraction of individual samples was done in the same way as the IP sample, however 25ul of plasma was used instead of 10ul (25ul plasma mixed with 100ul A/E mix and neutralized with 50ul tri-base). Individual samples were applied to wells in three separate amounts; 10ul, 20ul and 35ul + 100ul of assay buffer. The anti-body solution was prepared by mixing 5ul of anti-barramundi antiserum with 2ml of assay buffer. 20ul of the anti-body solution was added to all wells except blanks. The plate was shaken overnight at 600rpm at 4°C.

Day 2: the plate was first centrifuged at 3000 rpm for 1 min to remove droplets before removing cover, and then Eu-IGF-1 label was prepared by mixing 2ul of Eu-stock with 2ml of assay buffer. 20 ul of Eu-label was added to all wells except blanks, and the plate was shaken overnight overnight at 600rpm at 4°C.

Day 3: The plate was centrifuged to remove droplets, and well contents were dumped. All wells were rinsed with 200ul washing buffer six times. Enhancement solution was placed in room temp one hour before application into wells. 200ul enhancement solution was added to all wells, including blanks. Plate was then shaken in room temperature at 600rpm for 10 min, and IGF-1 concentrations were measured (Perker Elmer plate reader).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	"	0.065	"	0.125	"	0.25	"	0.5	"	1.0	"
B	2.0	"	4.0	"	8.0	"	16.0	"	Low IP	"	Mid IP	"
C	High IP	"	Dilution x64	"	Dilution x32	"	Dilution x16	"	Dilution x8	"	Dilution x4	"
D	Dilution x2	"	Dilution x1	"	Sample 1 (10ul)	"	Sample 1 (20ul)	"	Sample 1 (35ul)	"	Sample 31 (10ul)	"
E	Sample 31 (20ul)	"	Sample 31 (35ul)	"	Sample 61 (10ul)	"	Sample 61 (20ul)	"	Sample 61 (35ul)	"	Sample 91 (10ul)	"
F	Sample 91 (20ul)	"	Sample 91 (35ul)	"	Sample 121 (10ul)	"	Sample 121 (20ul)	"	Sample 121 (35ul)	"	Sample 151 (10ul)	"
G	Sample 151 (20ul)	"	Sample 151 (35ul)	"	Sample 181 (10ul)	"	Sample 181 (20ul)	"	Sample 181 (35ul)	"	BG (Blank)	"
H												

Figure 2.4: Display of the test plate set-up used for TR-FIA showing contents of each well, including the standard curv (wells A1 – B8), IPs (wells B9 – C2), dilution of pooled samples (wells C3 – D4, the first sample from each sampling group in three different amounts (wells D5 – G10), and blank control (wells G11 – G12). All done in duplicates.

Sample plates: Sample plate set-up consisted of a standard curve, IP samples and 30 individual samples (Fig. 2.5). The standard curve and IP samples were prepared in the same way as the test plate, and sample plates were also rinsed with washing buffer prior to application into wells. All samples were applied in duplicates.

Day 1: For extraction, 10ul of plasma was mixed with 40ul of A/E mix (1:4 ratio), and incubated at room temperature for 30 min. Samples were neutralized with 20ul of Tri-base, and centrifuged at 10 000rpm for 10 min at 4°C. Since IGF-1 concentrations were very high in test samples, an extra dilution step was added in addition to normal protocol, where 10ul from each centrifuged sample was diluted with 90ul of Blank Solution. 35ul from each sample was added to wells + 100ul assay buffer. The anti-body solution was added to all wells except the last two that were left as blanks. The plate was shaken overnight at 600rpm at 4°C. The second and third day followed the same procedure as test plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	"	0.065	"	0.125	"	0.25	"	0.5	"	1.0	"
B	2.0	"	4.0	"	8.0	"	16.0	"	Low IP	"	Mid IP	"
C	High IP	"	Sample	"	Sample	"	Sample	"	Sample	"	Sample	"
D	Sample	"	Sample	"	Sample	"	Sample	"	Sample	"	Sample	"
E	Sample	"	Sample	"	Sample	"	Sample	"	Sample	"	Sample	"
F	Sample	"	Sample	"	Sample	"	Sample	"	Sample	"	Sample	"
G	Sample	"	Sample	"	Sample	"	Sample	"	Sample	"	Sample	"
H	Sample	"	BG (Blank)	"								

Figure 2.5: Display of the plate set-up used for TR-FIA showing contents of each well, including the standard curv (wells A1 – B8), IPs (wells B9 – C2), individual samples (wells C3 – H2) and blank control (wells H3 – H4). All done in duplicates.

2.5.2 Isolation of total RNA from muscle samples

Total RNA was purified manually using FastPrep vials and TRI-reagent (Sigma, St. Louis, MO, USA) according to (Chomczynski, 1993) and as described in the manufacturer's protocol. Ten samples from each group were purified, resulting in a total of 70 samples. Before purification, 60 mg of muscle tissue was cut off, squeezed slightly to remove access RNA later and put into tubes containing 1 mL TRI-reagent and 6-7 mg zirconium oxide beads (1,4 µm, Bertin technologies, Versailles, France). Then samples were kept on ice for at least 5 min before being homogenized at room temperature for 15 seconds at 5000 rpm in a Precellys 24 (Bertin technologies, Versailles, France), followed by at least 5 minutes incubation at room temperature. The TRIZOL Reagent protocol for RNA-purification was then as follows: two hundred ul of chloroform (molecular grade, Sigma-Aldrich, Norway) was added to each vial and vortexed for 1 min. Samples were then centrifuged for 15 min at 4°C at 16000 rcf in an Eppendorf 5415 R centrifuge. After centrifugation, approximately 500 ul supernatant was

transferred to new vials and 500 ul isopropanol (Sigma Aldrich, Norway) was added. Vials were then inverted five times and left at RT for 10 min before being centrifuged for 10 min at 4°C at 16000 rcf. The supernatant was decanted and the remaining pellet was washed with 1 ml 80 % ice cold EtOH (Arcus, Norway). Vials were then centrifuged for 5 min at 4°C at 7500 rcf. and the ethanol was decanted out, followed by a brief flash-spin, after which the last drop of ethanol was carefully aspirated. Pellets were air dried for 5-10 min, reconstituted in 50 ul nucleasefree water and put on ice. Total RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, NC, USA) using 1.5 µl total RNA.

The RNA to protein ratios (260/280nm) ≥1.8 and the 260/230 nm ratio ≥2.0 indicated that RNA was sufficiently pure of contaminants for further downstream analysis (Bustin et al., 2013). The RNA integrity in a select number (12) of samples was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with the RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA) following the Agilent RNA Nano protocol according to the manufacturer's recommendations. The Agilent 2100 software classifies the RNA Integrity Number (RIN) of eukaryotic total RNA on a number system ranging from 1, being the most degraded, to 10 being the most intact (Mueller, 2004). All tested samples had RIN values above 9 and the assumption was made that this was representative for all samples. Remaining total-RNA was then put in -80 degree freezer for future use.

2.5.3 Reverse Transcriptase Synthesis (cDNA synthesis) of muscle samples

cDNA was synthesized using 2 µg total RNA in conjunction with the SuperScript[TM] III Reverse Transcriptase (RT) First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. One µl of oligo (dT) (50µM), 1µl of 10 mM dNTP Mix (10mM each dATP, dGTP, dCTP and dTTP at neutral pH) were added to 11 µl of total RNA for each sample before incubation at 65 [0]C for 5 min in the Thermal Cycler C 1000 Touch (Bio-Rad Laboratories, Inc. USA) to limit the formation of secondary structures, followed by incubation on ice at least 1 minute. Then a Master Mix containing 4 µl 5X First strand Buffer, 1µl 0.1 M DTT, 1 µl RNaseOUT™ Recombiant RNase Inhibitor and 1µl SuperScript™ III RT was added to each well using the Hamilton pipetting robot (leverandør og land) and then run on the Thermal Cycler for 50°C for 60 min, followed by

70°C for 15 min to inactivate the reaction. The cDNA samples were stored at $-20\ ^0$C until analyzed by Real-time PCR.

2.5.4 Polymerase chain reaction (PCR)

Quantitative PCR (q-PCR) was performed to quantify the muscle mRNA abundance of IGF-1, IGF-1bp1a and IGF1ra, using the C1000 Touch Thermal Cycler, CFX96 Real-Time System (Bio-Rad Laboratories, CA, USA) in conjunction with the software CFX Manager (version 3.1, Bio-Rad). The q-PCR reactions were performed in a total volume of 10µl containing 4,38 µl Gene Expression Master Mix (Bio-Rad Laboratories, CA, USA), 0,3 µl of forward and reverse primer (200 nm final concentration), 0,02 µl of nuclease free water and 3 µl of cDNA diluted 1:20. The q-PCR reactions were performed in 96-well plates (Bio-Rad) and the following thermal cycling protocol was used: 3 min at 95 ^{0}C, 40 rycles of 15 sec at 95^{0}C followed by 1 min at 60^{0}C. Standard melt curve profiles verified no primer dimer formation. Information about primer sequences for each qPCR assay is given in the table 2.2. All samples were run in duplicates and to confirm absence of DNA contamination, "non template control (NTC)" (Bio-Rad Laboratories, CA, USA) was included on all plates. Each plate had duplicate sample of pooled cDNA that served as a correction of differences between plates.

Table 2.2: Primer sequences for each qPCR assay (muscle samples)

Gene		Primer sequences (5' -> 3')	Accession no.	Reference
Ef1a	F	CCCCTCCAGGACGTTTACAAA	AF321836	(Olsvik et al., 2005)
	R	CACACGGCCCACAGGTACA		
IGF-1	F	ATGTCTAGCGCTCATTTCTT	EF432852	(Bower et al., 2008)
	R	GAATTCTTACATTCGGTAGTTCCTT		
IGF-1bpa	F	GGTCCCTGTCATGTGGAGTT	KC122927.1	(Hevrøy et al., 2015)
	R	TTCCAGAAGGACACACACCA		
IGF-1ra	F	TGCACAACTCCATCTTCACC	EU861008.1	(Hevrøy et al., 2013)
	R	GGGGCTCTCCTTCTGTCCTA		

All q-PCR assays were validated by running two-fold dilution series made from a representative pool of cDNA made from 12 random samples. Based on the dilution series all qPCR assays were run using a cDNA dilution 1:20. For efficiency calculations of the all assays, the threshold cycle (cq) values from dilution series was plotted against log

transformed concentrations and the slope of the curve then used according to the formula the following formula (Pfaffl, 2004):

$$E = 10^{(-1/slope)}$$

Normalization of the genes expression based on target versus reference gene was calculated according to (Pfaffl, 2004):

$$Ratio = E\,(Ct_{mean})ref \quad / \quad E(Ct_{mean})target$$

Ct – threshold cycle for each individual samples

E ref – qPCR efficiency of the reference gene

E target – qPCR efficiency of the target gene

Ct mean – average Ct value of duplicate reaction of individual sample

2.5.5 Screening for salmonid alphavirus (SAV) and piscine orthoreovirus (PRV)

Based on this histopathological investigation, a total of 90 heart samples from both sites, collected in the freshwater (n=30) and last sampling of the post-smolt phase (n = 60) (all stored in RNA later), were screened for Piscine orthoreovirus (PRV) and salmonid alphavirus (SAV), using real time RT-PCR analysis (Nylund et al., 2011). RNA from heart was extracted as described below. All RNA samples were stored at -20°C until further use.

The AgPt-IDTM one step RT-PCR kit (Applied assistant) was used to test the extracted RNA from heart tissues for presence of SAV and PRV. The following RT-PCR assays were used: the PRV-M2 assay targeting the M2 segment capsid protein of PRV (Nylund et al., 2015), and the nsP1assay, targeting the nsP1 gene of SAV (Andersen et al., 2007). The RT-PCR is described in Gunnarsson et al. (2017). The housekeeping gene elongation factor 1 alpha (EF1AA assay) was used as an internal control (Olsvik et al., 2005). Standard curves were generated using 10-fold serial dilutions of RNA in three parallels. Regression analysis, standard curve slopes s (cycle threshold, Ct, versus log quantity), amplification efficiency E (E = [101/(–slope)] − 1), and the coefficient of determination, R2, were calculated for all assays. Each run of the RT PCR consisted of 45 cycles and the samples were considered positive when the fluorescence signal increased

above a set threshold of 0.1. Negative controls, RNA extraction controls (lacking target RNA) and no template control, were included in all runs at a rate of 1 control per 10 samples to avoid false positives.

2.6 Statistical analysis:

All data was analysed using the statistical program, Statistica 13.2. Prior to statistical analysis, length, weight, and condition factor were tested for normality using the Kolmogornov-Smirnov test, and tested for homogeneity of variance using the Hartley F-max test. IGF-I plasma concentration and muscle mRNA levels of IGF-I, IGFIra, and IGFBP were tested for homogeneity of variance using the Levene's test, and were fulfilled for all groups except for IGF-I plasma concentration after four months in seawater. Therefore, for this group, homogeneity of variance was tested using the Hartley F-max test. A one-way ANOVA was used to test for significant differences in weight, length, condition factor, IGF-I plasma concentrations, and mRNA levels of IGF-I binding protein, IGF-I receptor and IGF-I in muscle, between the Preline semi-closed system and control open cage for each sampling. Significant one-way ANOVAs for IGF-I plasma concentration were followed by a Tukey HSD post hoc test for each sampling point to determine differences among experimental groups. To determine significant differences between sampling points within each system, a Kruskal-Wallis ANOVA was used for IGF-I plasma concentration, and mRNA levels of IGF-I, IGFIra, and IGFBP in muscle. Significant ANOVA's were followed by a Newman-Keuls test to see were differences were within each system. A simple linear regression was conducted to test the correlation between plasma IGF-I levels and fish size (weight, length and condition factor) after four months in seawater for both control fish and Preline fish. A one-way ANOVA was conducted to see if there were any differences in plasma IGF-I levels within the control group between fish that tested positive for SAV infection and fish that did not for the last sampling (four months in seawater). All statistical analyses are shown Appendix 6.5.

3. RESULTS

3.1 Mortality

The accumulated mortality rate of fish in the Preline semi-closed system was measured at 0.54 % on the first day of transfer (April 30th), compared to an accumulated mortality of 0 % in the control open cage system, where fish were transferred 5 days later (May 5th) (Fig. 3.1). During the time the Preline fish were in the semi-closed system, accumulated mortality increased to 1.34 %, while it increased to 0.98 % in the control cage. Approximately 20 days into the adult period, the accumulated mortality in the control open cage system exceeded the mortality in the Preline open cage system at Buholmen. On November 30th, mortality had increased to 3.52 % in the control open cage system, while it increased to 2.48 % in the Preline system.
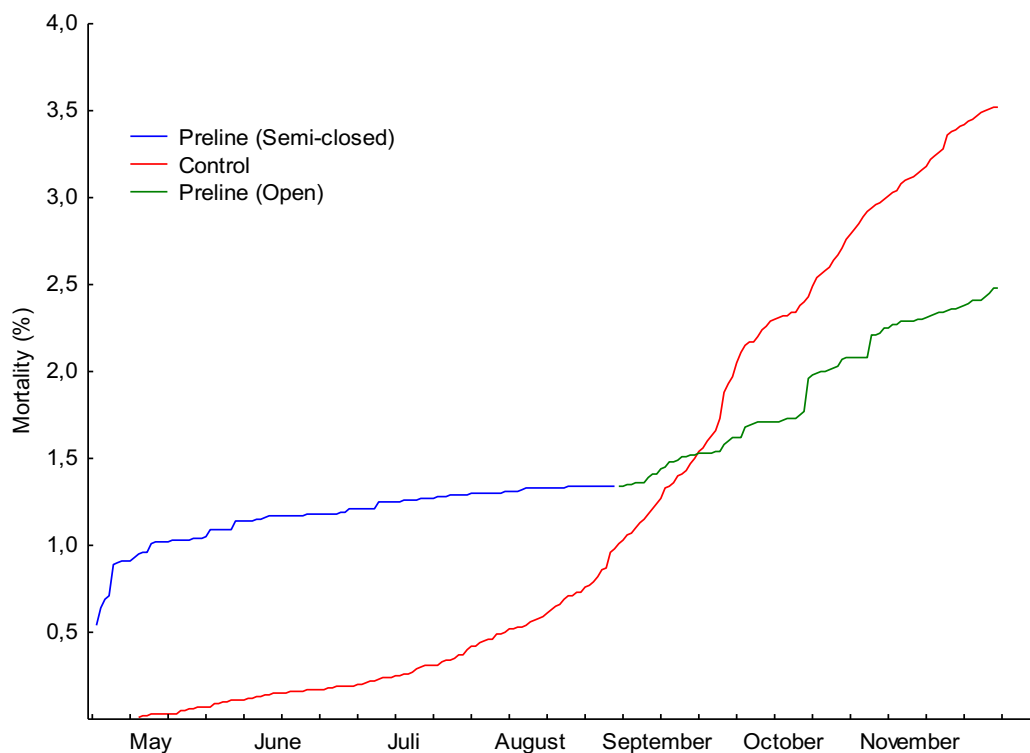
Figure 3.1: Accumulated mortality in the Preline semi-closed system (blue), control open cage system (red) and open cage system at Buholmen (green). Mortality in the Preline semi-closed system was registered from April 30th to August 31st, and in the Buholmen open cage system from September 1st to November 30th. The mortality in the control open cage system was registered daily from May 5th to November 30th.

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3.2 Temperature:

Although there was an increase in temperature in both systems during the post-smolt period (May 5th to August 31st), the average temperature was lower in the Preline semi-closed system, averaging at 9.6°C, compared to the control open cage system averaging at 12.9°C (Fig. 3.2). The temperature in the Preline system was relatively stable during May and June, averaging at 8 and 8.4°C. Temperatures had more fluctuations in July and August, but averaged at 10.2 and 11.4°C. The average monthly temperature in the control system was 9.4°C in May, and steadily increased the following months, showing an average temperature of 12.6°C in June, 14.3°C in July, and 15°C in August. After fish from the Preline system were transferred to the open cage at Buholmen on August 31st (adult period), the average monthly temperature was 16.3°C in September, and decreased to 10.1 °C in November. During the same time, the average monthly temperature decreased from 15.6°C to 9.6°C in the control open cage system.
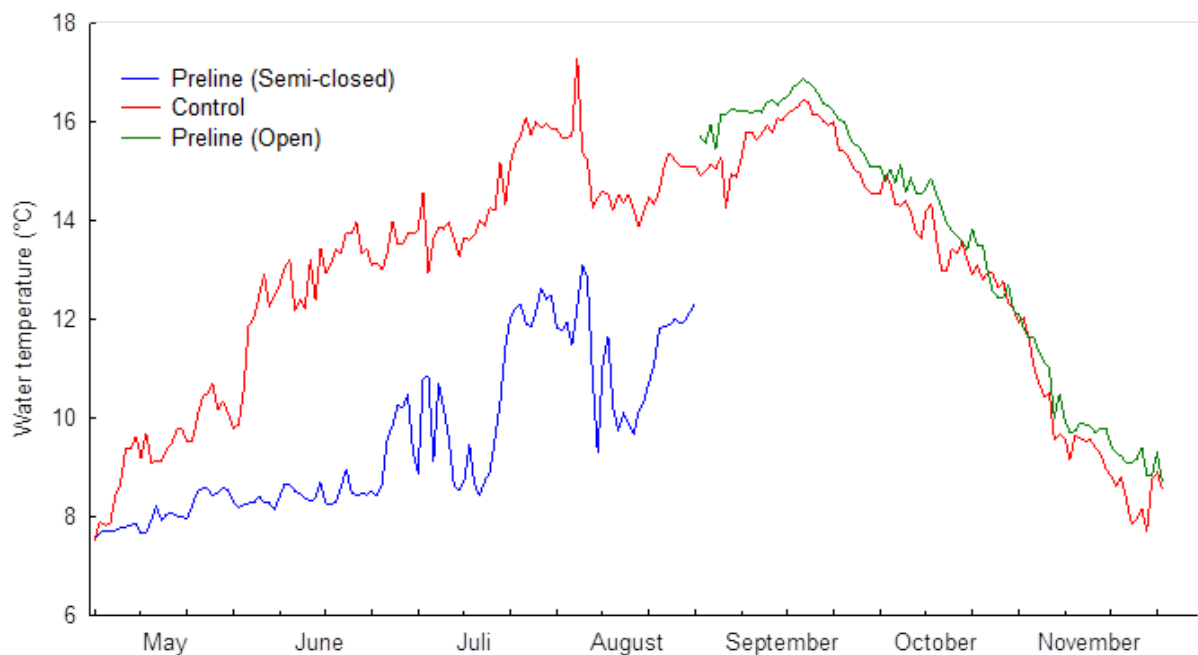
Figure 3.2: Average temperature (°C) in the Preline semi-closed system (blue), control open cage system (red) and open cage system at Buholmen (green). Temperature in the Preline system was registered from May 5th to August 31st, and water was taken from a depth of 30 m. The temperature in the control system and open cage system at Buholmen is an average of depths 3m, 8m, and 15m, registered daily from May 5th to November 30th in the control system and from September 1st to November 30th in the Buholmen system.

3.3 Salinity

There was a higher registered salinity in the Preline semi-closed system compared to the open cage system for the post-smolt period from May 5th to August 31st (Fig.3.3). During this period, the salinity varied between 26.2 and 34.7 ppt in the Preline system, averaging at 31.7 ppt, and between 21.6 and 27.5 ppt in the control system, averaging at 24.2 ppt. During the adult period (September 1st to November 30th), the salinity was still higher for Preline fish, averaging at 28.8 ppt in the open cage at Buholmen, compared to the control system, averaging at 24.6 ppt.
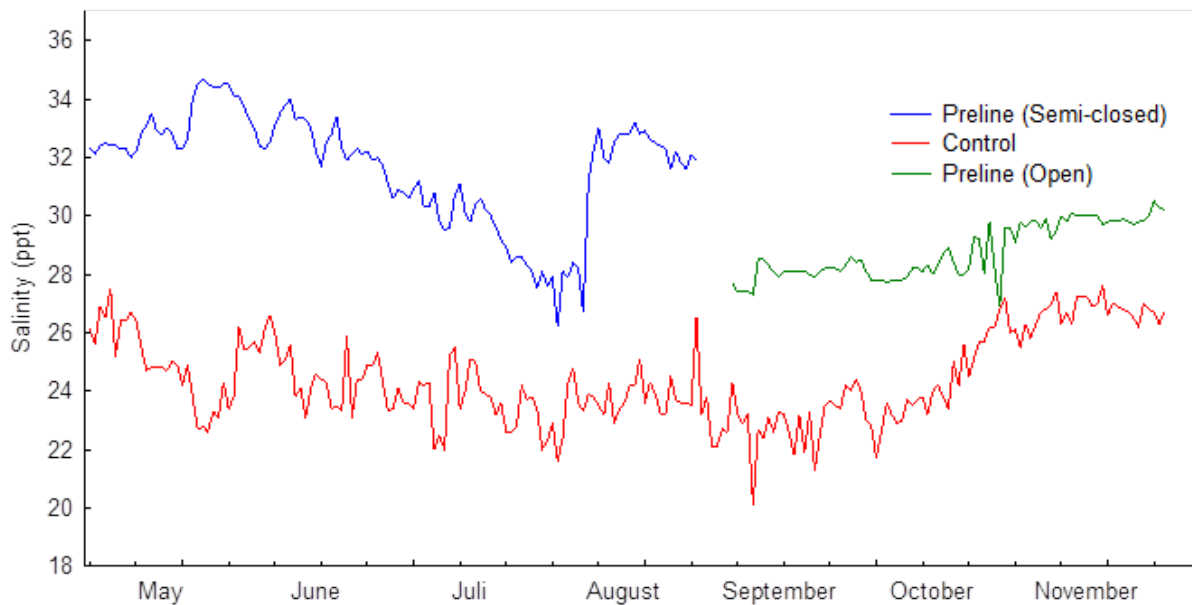
Figure 3.3: Average salinity (ppt) in the Preline semi-closed system (blue), control open cage system (red) and open cage system at Buholmen (green). Salinity in the Preline system was registered from May 5th to August 31st, and water was taken from a depth of 30 m. The salinity in the control system and open cage system at Buholmen is an average of depths 3m, 8m, and 15m, registered daily from May 5th to November 30th in the control system and from September 1st to November 30th in the Buholmen system.

3.4 Oxygen

During the post-smolt period in the Preline semi-closed system, oxygen concentrations measured from the outlet water were between 72.2 % and 131.6 % from May 5th to August 31st, averaging at 102.74 % (Fig. 3.4). During the same period, oxygen concentrations in the control open cage system, were measured between 83.8 % and 99.4 %, averaging at 97.21 %. After fish from the Preline system were transferred to the open cage at Buholmen on August 31st, oxygen concentrations were between 83.8 % and 99.4 % (from September 1st to November 30th), averaging at 92.96 %, while they were between 78 % and 96 % for the control open cage system, averaging at 86.63 %.
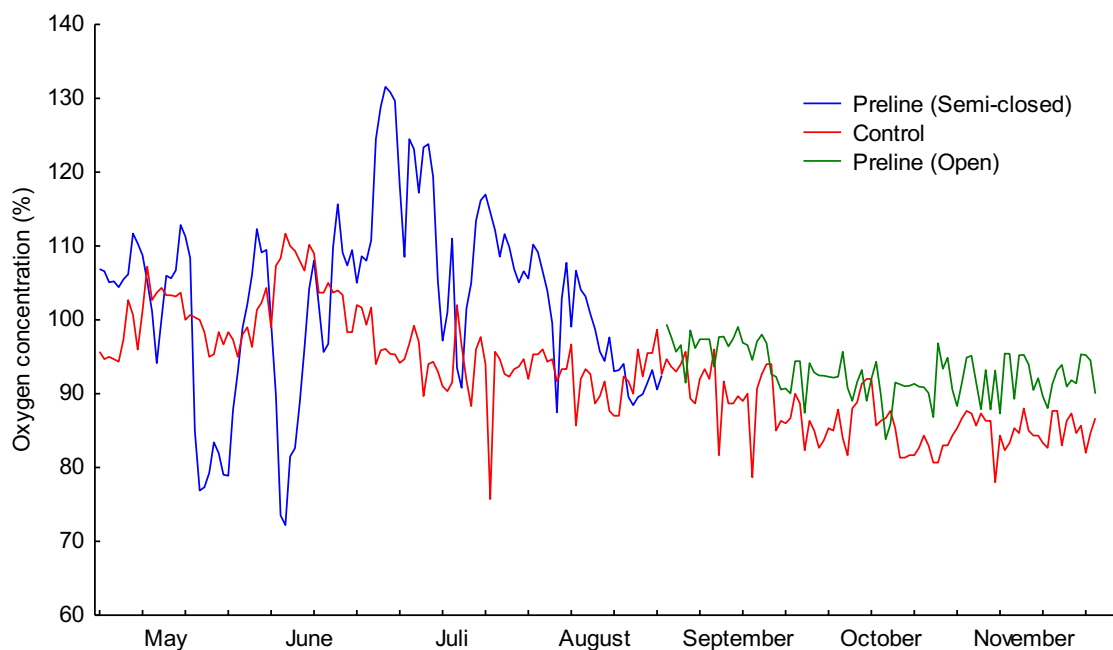
Figure 3.4: Average oxygen concentration (%) in the Preline semi-closed system (blue), control open cage system (red) and open cage system at Buholmen (green). Oxygen concentration in the Preline system was registered from the outlet water from May 5th to August 31st (water was taken from a depth of 35 m). The oxygen concentration in the control system and open cage system at Buholmen is the average of depths 3m, 8m, and 15m, and was registered daily from May 5th to November 30th in the control system and from September 1st to November 30th in the Buholmen system.
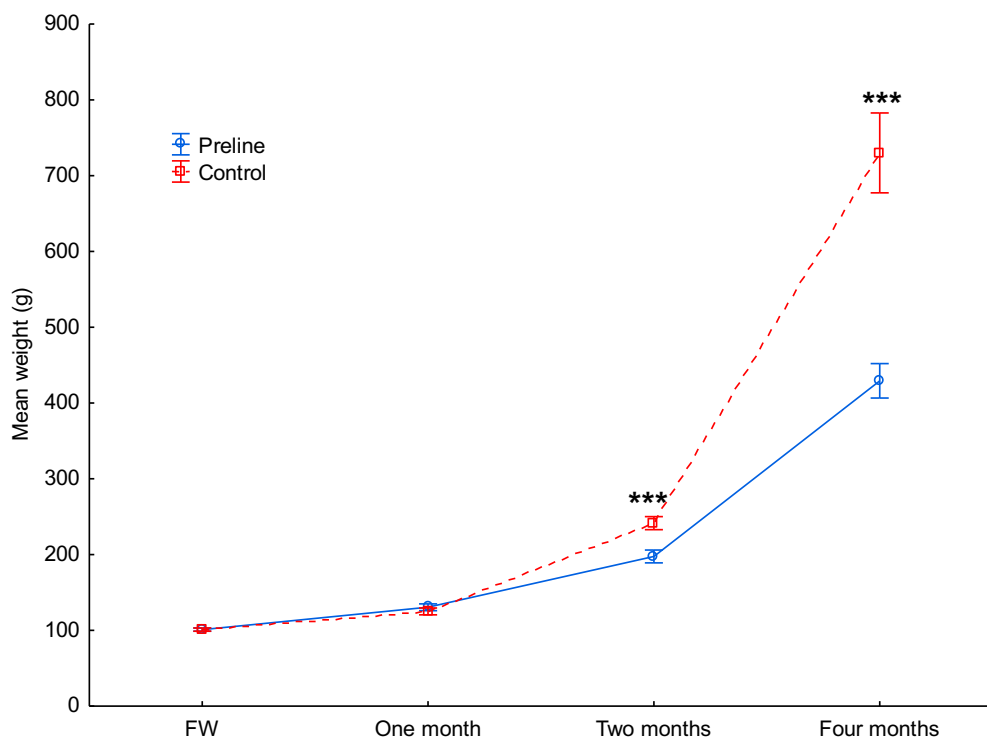
3.5 Mean weight

From the sampling conducted during the freshwater period, the weight of smolts (mean ± SEM) averaged at 101.00 ± 4.2 g on April 15th (Fig. 3.5). After approximately two months in separate rearing systems (sampling June 29th and 30th for Preline and Control, respectively), fish in the control open cage system had a significantly higher weight compared to fish in the Preline semi-closed system, with a difference of 43.97 g (one-way ANOVA, $p < 0.001$). Also after four months (sampling August 29th and 30th for Preline and Control, respectively), control fish had a significantly higher weight, with a difference of 300.73 g (one-way ANOVA, $p < 0.001$). The mean weight significantly increased within the Preline semi-closed system from $130.51 \pm 10,9$ g after one month to 429.27 ± 15.4 g after four months (one-way ANOVA, $p < 0.001$). The mean weight significantly increased within the control open cage system from 125 ± 4.4 g after one months to 730.00 ± 57.2 g after four months (ANOVA).

Figure 3.5: Mean weight (± SEM) of fish in freshwater (April 15th), and thereafter in the Preline semi-closed system (blue) and control open cage system (red) after approximately one month (June 1st/2nd for Control and Preline, respectively), two months (June 29[tt]/30th for Preline and Control, respectively), and four months (August 29th/30th for Preline and Control, respectively) of transfer to seawater. (n=30 per group per sampling). Asterisk indicates the level of significance: $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$.

3.6 Estimated mean weight

Similar results were shown in weight from the estimated weight data calculated by Lerøy Vest, AS (Fishtalk, FCE=1.1). After two months in separate rearing systems, fish in the control open cage system had a higher weight compared to fish in the Preline semi-closed system, with a difference of 31.6 g (Fig. 3.6). Control fish had higher weight also after four months, with a difference of 289.9 g. After the Preline fish were transferred to the open cage at Buholmen on August 31st, estimated weight data showed an increase in weight from 454.60 g on September 1st to 1474.70 g on November 30th. During the same period, the fish in the control open cage showed an increase in weight from 763.30 g to 1666 g. During this period, the relative increase in weight for Preline was 224.4 %, while it was 118.3 % for the control open cage system.
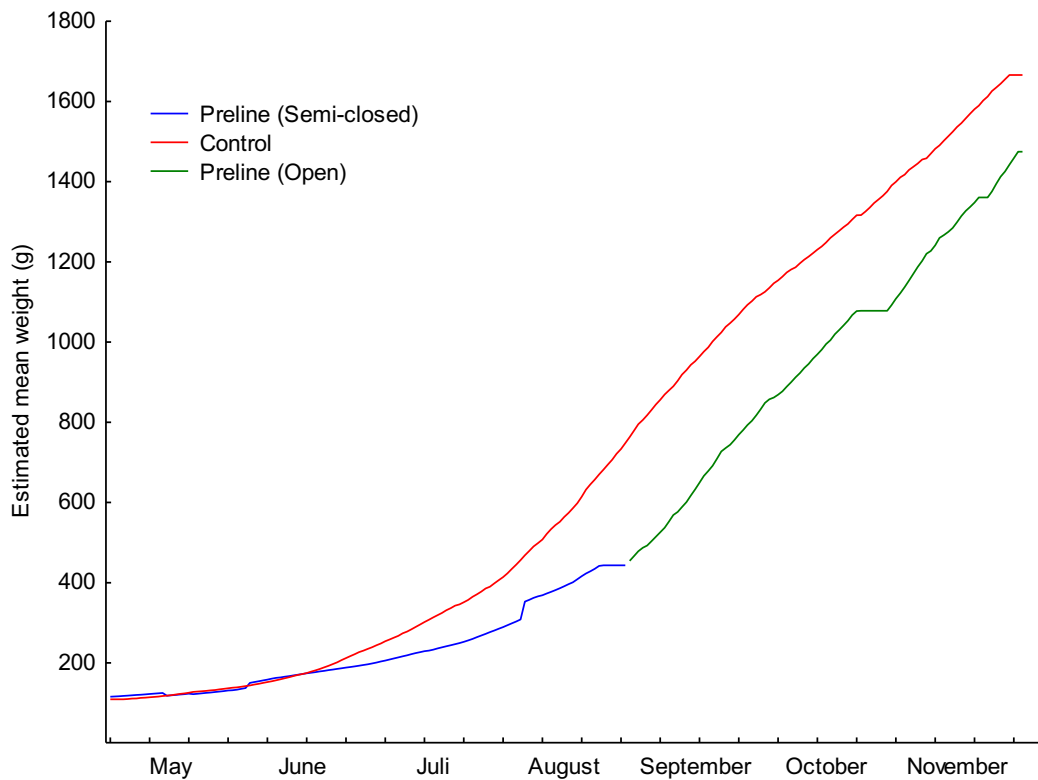
Figure 3.6: Estimated weight data from Lerøy (Fishtalk calculations, FCE=1.1), registered daily during the post-smolt period (from May 5th to August 31st), in the Preline semi-closed system, and control open cage system, and during the adult period in the Preline open cage system at Buholmen, and control open cage system (from September 1st to November 30th).

3.7 Mean fork length

From the sampling conducted during the freshwater period, the fork length of smolts (mean ± SE) averaged at 20.6 ± 0.2 cm on April 15th. After approximately two months (sampling June 29th and 30th for Preline and Control, respectively) in separate rearing systems, there was a significant difference in mean fork length between the Preline semi-closed system and open cage system (one-way ANOVA, $p < 0.001$), with a 1.8 cm higher mean fork length in the open cage system (Fig. 3.7). Also after approximately four months (sampling August 29th and 30th for Preline and Control, respectively), there was a significant difference in mean fork length between rearing systems (one-way ANOVA, $p < 0.001$), with a 5.3 cm higher mean fork length in the open cage system. Fish in the Preline system increased significantly in length from one month (23.2 ± 0.5 cm) to four months (33.4 ± 0.3 cm) (one-way ANOVA, $p < 0.001$). The control fish also increased significantly in length from one month (22.9 ± 0.2) to four months (38.7 ± 0.9 cm) (one-way ANOVA, $p < 0.001$).
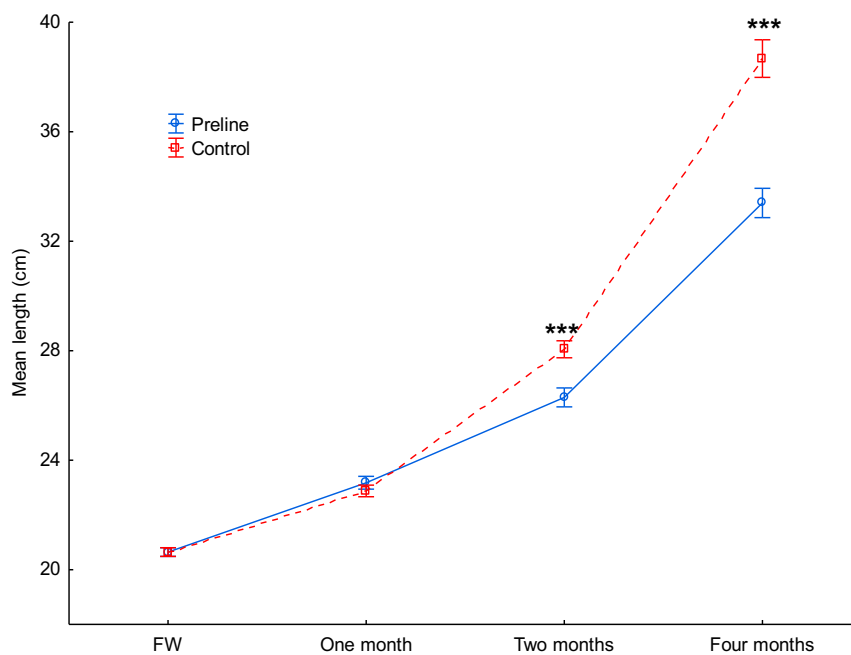
Figure 3.7: Mean fork length (± SEM) of fish in freshwater (April 15th), and thereafter in the Preline semi-closed system (blue) and control open cage system (red) after approximately one month (June 1st/2nd for Control and Preline, respectively), two months (June 29[tt]/30th for Preline and Control, respectively), and four months (August 29th/30th for Preline and Control, respectively) of transfer to seawater. (n=30 per group per sampling). Asterisk indicates the level of significance: $p < 0.05$=*, $p < 0.01$=**, $p < 0.001$=***.

3.8 Condition factor (CF)

From the sampling conducted during the freshwater period, the condition factor of smolts (mean ± SE) averaged at 1.15 ± 0.01 cm on April 15th (Fig.3.8). After one month in separate rearing systems, the condition factor decreased for both systems, showing 1.04 ± 0.01 for the Preline semi-closed system and 1.03 ± 0.01 for the control open cage system (sampling June 1st and 2nd for Control and Preline, respectively). After four months in separate rearing systems, fish in the control system had a significantly higher condition factor compared to fish in the Preline ed system, with values of 1.20 ± 0.02 and 1.12 ± 0.02, respectively (sampling August 29th and 30th for Preline and control, respectively) (one-way ANOVA, $p < 0.05$).
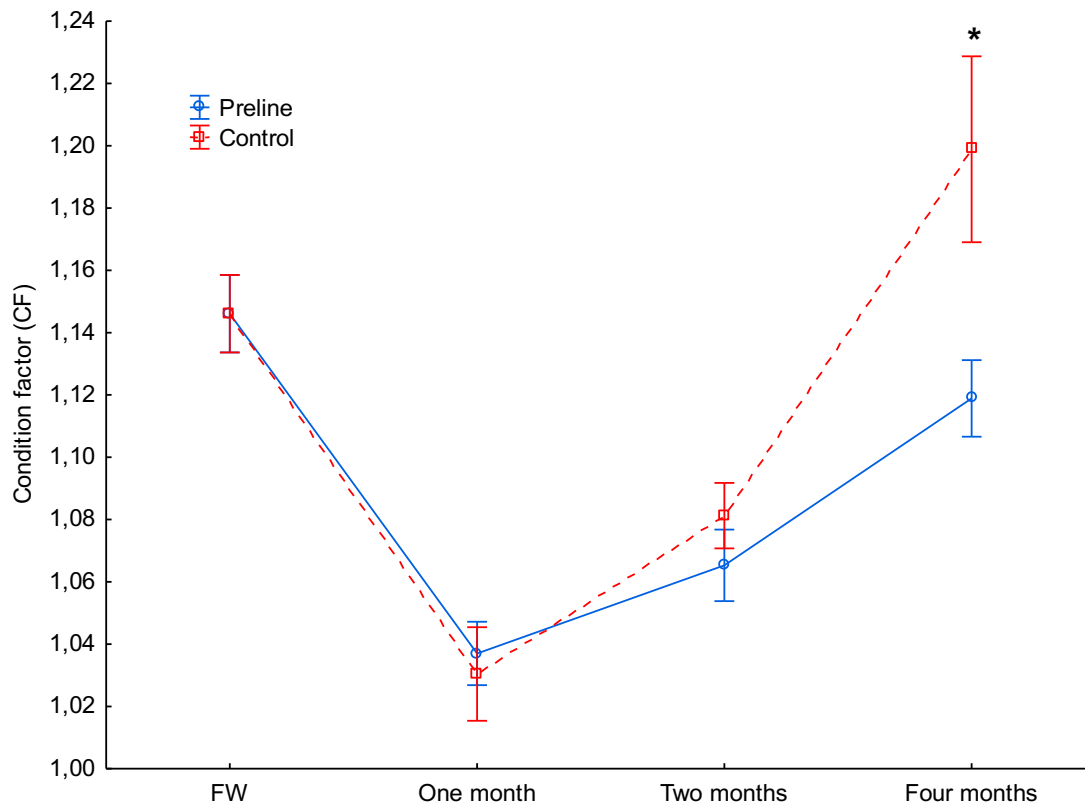
Figure 3.8: Mean condition factor (± SEM) of fish in freshwater (April 15th), and thereafter in the Preline semi-closed system (blue) and control open cage system (red) after approximately one month (June 1st/2nd for Control and Preline, respectively), two months (June 29[tt]/30th for Preline and Control, respectively), and four months (August 29th/30th for Preline and Control, respectively) of transfer to seawater. (n=30 per group per sampling). Asterisk indicates the level of significance: $p < 0.05$=*, $p < 0.01$=**, $p < 0.001$=***.

3.9 Specific Growth Rate (SGR)

Calculated SGR using weight data from sampling:

The SGR was higher for fish in the control open cage system compared to fish in the Preline semi-closed system during the post-smolt period, which was calculated to 1.939 and 1.338, respectively (Table 3.1). (Sampling points were 02.06.16 for Preline, and 01.06.16 for control) and 29.08.16 for Preline and 30.08.16 for control).

Calculated SGR using estimated weight data from Lerøy Vest, AS (Fishtalk, FCE=1.1)

When using estimated weight data, the same was shown where the SGR was higher for fish in the control open cage system compared to fish in the Preline semi-closed system during the post-smolt period, which was calculated to 1.846 and 1.362, respectively. During the adult period, estimated weight data showed that after the Preline fish were transferred to the open cage at Buholmen, the SGR value decreased to 1.298. However, this was higher compared to fish in the control open cage system during the adult period, where the SGR value was 0.870, which had decreased within the control cage after the post-smolt period.

Table 3.1: Calculated Specific Growth Rate (SGR) for the Preline semi-closed system and the closed open cage system during the post-smolt period using both weight data from sampling, and estimated weight data from Lerøy. Additionally, calculated SGR for the Preline fish at Buholmen and control open cage fish during the adult period using estimated weight data from Lerøy.

Group	SGR (weight from sampling)	SGR (estimated weight)	Average temperature (°C)	Sum temperature (°C)
Preline (post-smolt)	1,338	1,362	10,0	889
Control (post-smolt)	1,939	1,846	14,0	1272
Preline (adult)		1,298	13,5	1246
Control (adult)		0,870	13,0	1198

3.10 Thermal growth coefficient (TGC)

Calculated TGC using weight data from sampling:

The TGC was higher for fish in the control open cage system compared to fish in the Preline semi-closed system during the post-smolt period, which was calculated to 3.149 and 2.778, respectively (Table 3.2). (Sampling points were 02.06.16 for Preline, and 01.06.16 for control) and 29.08.16 for Preline and 30.08.16 for control).

Calculated TGC using estimated weight data from Lerøy Vest, AS (Fishtalk, FCE=1.1)

When using estimated weight data, the same was shown where the TGC was higher for fish in the control open cage system compared to fish in the Preline semi-closed system during the post-smolt period, which was calculated to 3.041 and 2.850, respectively. During the adult period, estimated weight data showed that after the Preline fish were transferred to the open cage at Buholmen, the TGC value increased to 3.001. This was higher compared to fish in the control open cage system during the adult period, where the TGC value decreased to 2.318.

Table 3.2: Calculated Thermal Growth Coefficient (TGC) for the Preline semi-closed system and the closed open cage system during the post-smolt period using both weight data from sampling, and estimated weight data from Lerøy. Additionally, calculated TGC for the Preline fish at Buholmen and control open cage fish during the adult period using estimated weight data from Lerøy.

Group	TGC (weight from sampling)	TGC (estimated weight)
Preline (post-smolt)	2.778	2.850
Control (post-smolt)	3.149	3.041
Preline (adult)		3.001
Control (adult)		2.318

3.11 Feed conversion ratio (FCR)

The feed conversion ratio calculated by Lerøy (Fishtalk, FCE=1.1) was higher in the control open cage system compared to the Preline semi-closed system for both the post-smolt period and the adult period (Table 3.3). The FCR was 1.04 in the Preline semi-closed system, which decreased to 1.03 after Preline fish were transferred to the open cage at Buholmen. Fish in the control open cage had a FCR of 1.08 during the entire post-smolt and adult period.

Table 3.3: The feed conversion ratio (FCR) for the Preline semi-closed system and the closed open cage system during the post-smolt period, and additionally FCR for the Preline fish at Buholmen and control open cage fish during the adult period.

Group	FCR
Preline (post-smolt)	1.04
Control (post-smolt)	1.08
Preline (adult)	1.03
Control (adult)	1.08

3.12 Plasma IGF-1 concentration

From the sampling conducted during the freshwater period, the plasma IGF-I concentration of smolts averaged at 72.3 ± 7.5 ng/ml on April 15th. After one month in separate rearing systems, fish in the control open cage system had a significantly higher plasma IGF-I concentration, showing 127.5 ng/ml, while fish in the Preline semi-closed system had a concentration of 95.3 ± 7.2 ng/ml (one-way ANOVA, $p < 0.01$) (sampling June 1st and 2nd for Control and Preline, respectively) (Fig. 3.9). Similarly, after two months, control fish had a higher concentration with a value of 204.8 ± 12.7 compared to fish in the Preline system with a value of 129.5 ± 8.0 (sampling June 29th and 30th for Preline and Control, respectively) (one-way ANOVA, $p < 0.001$). Within the control open cage system, there was an increasing trend in plasma IGF-I concentration, showing a significant difference between the freshwater sampling, and one and two months after transfer to seawater (Kruskal Wallis ANOVA, $p < 0.05$). Within the Preline semi-closed system, there was no significant difference between the freshwater period and one month in the system. However, there was a significant difference between one month and two months (Kruskal Wallis ANOVA, $p < 0.05$). No significant correlations were found plasma IGF-I levels and fish size (weight, length and condition factor) after four months in seawater for both control fish and Preline fish.
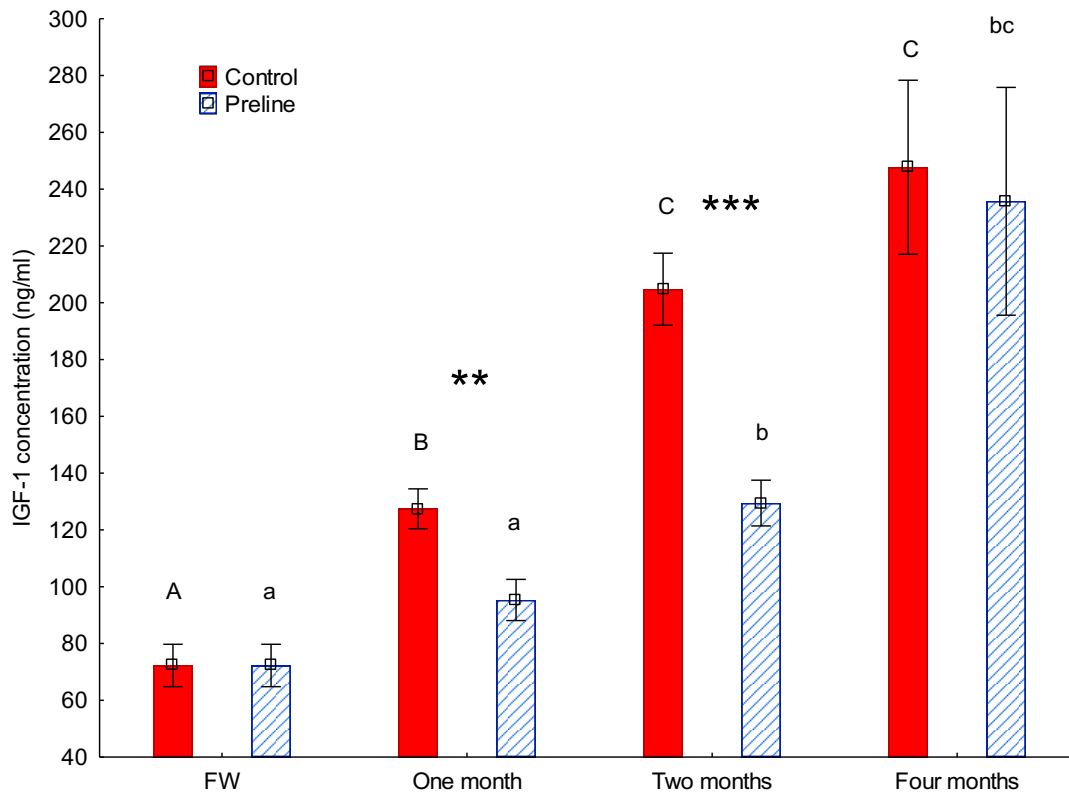
Figure 3.9: Mean plasma IGF-I concentration (± SEM) of fish in freshwater on April 15th (Preline and Control bar are based on same data), and thereafter in the Preline semi-closed system (blue) and control open cage system (red) after approximately one month (June 1st/2nd for Control and Preline, respectively), two months (June 29[tt]/30th for Preline and Control, respectively), and four months (August 29th/30th for Preline and Control, respectively) of transfer to seawater (n= 30 per group per sampling). Capital letters indicate significant differences within the control cage, and small letters indicate significant differences within the Preline system. Asterisk indicates the level of significance: $p < 0.05$=*, $p < 0.01$=**, $p < 0.001$=***.

3.13 IGF-I mRNA level muscle

During the post-smolt period, IGF-I mRNA levels in muscle ranged from 0.089 to 4.348 in the control system fish and from 0.195 to 2.718 in the Preline system fish, however, no significant differences were found between systems (Fig. 3.10).
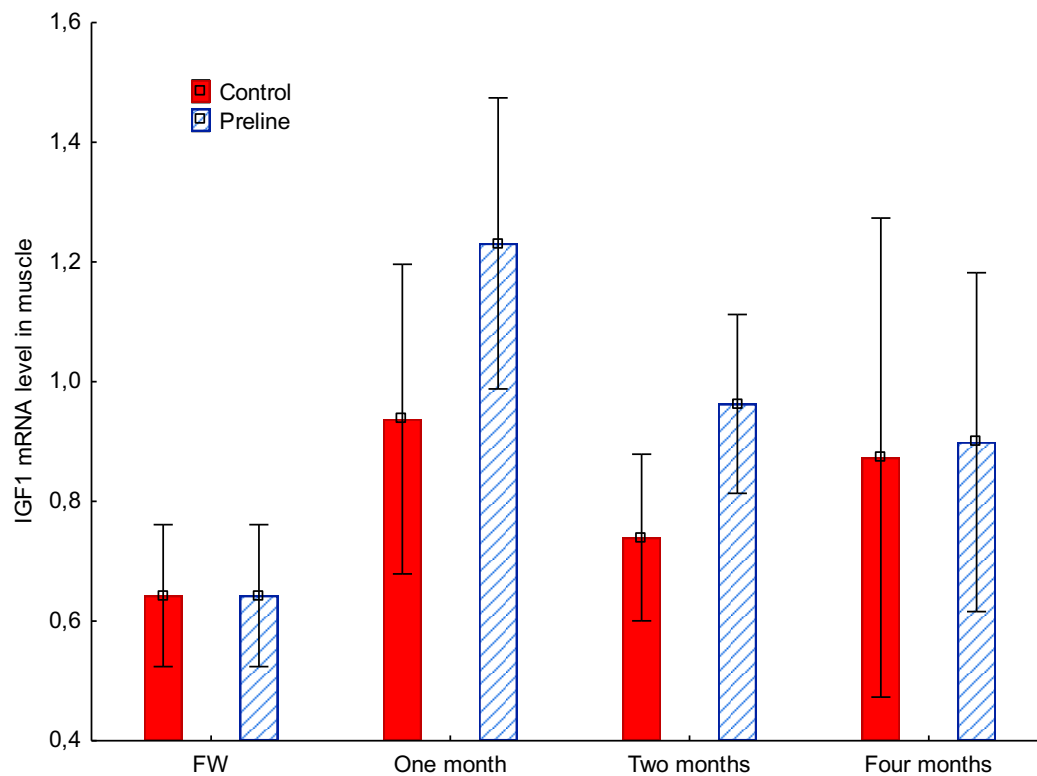
Figure 3.10: Mean IGF1 mRNA level in muscle ($\pm$ SEM) of fish in freshwater (April 15th), and thereafter in the Preline semi-closed system (blue) and control open cage system (red) after approximately one month (June 1st/2nd for Control and Preline, respectively), two months (June 29[tt]/30th for Preline and Control, respectively), and four months (August 29th/30th for Preline and Control, respectively) of transfer to seawater (n= 30 per group per sampling). Capital letters indicate significant differences within the control cage, and small letters indicate significant differences within the Preline system. Asterisk indicates the level of significance: $p < 0.05$=*, $p < 0.01$=**, $p < 0.001$=***.

3.14 IGF-I receptor mRNA levels in muscle

During the post-smolt period, IGF-I receptor mRNA levels in muscle ranged from 0.046 to 0.372 in the Preline system fish, and from 0.040 to 0.361 in the control system fish, however, no significant differences were found between systems (Fig. 3.11).
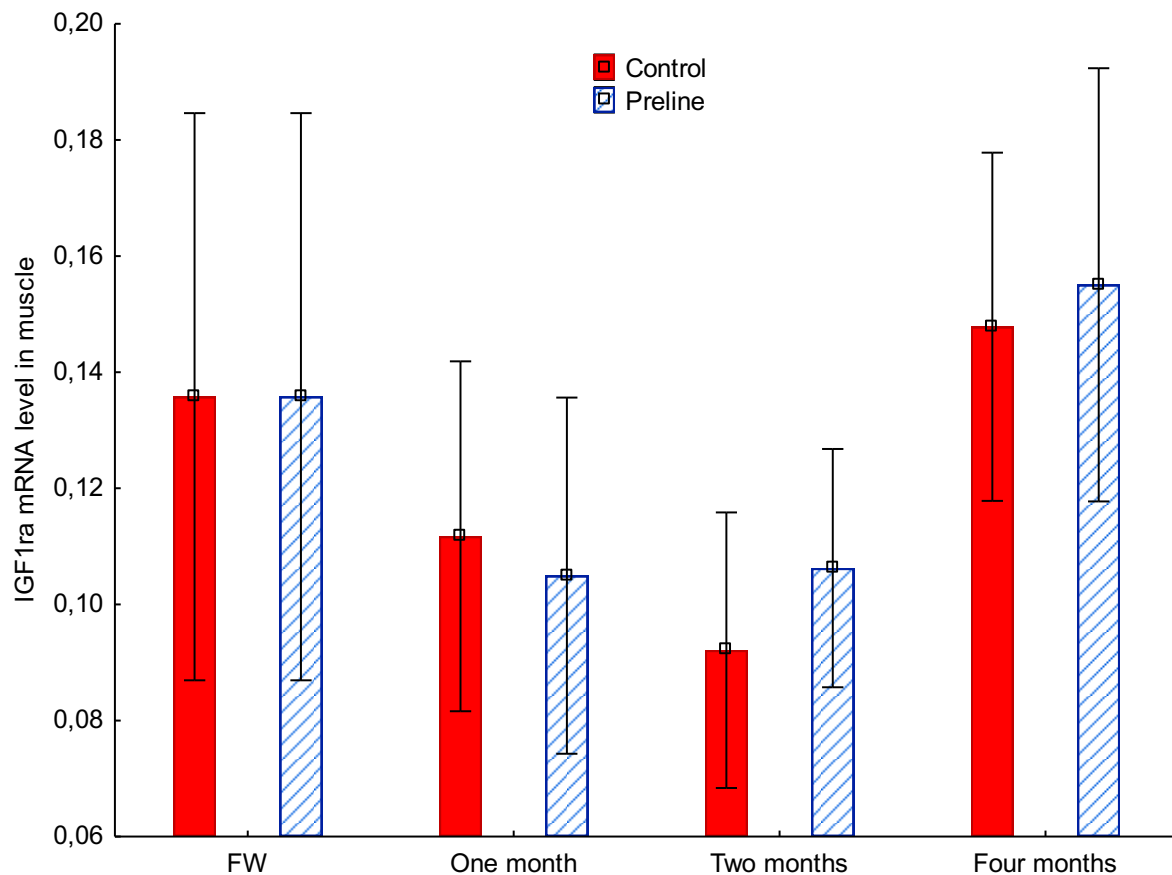
Figure 3.11: Mean IGF1ra mRNA level in muscle (± SEM) of fish in freshwater (April 15th), and thereafter in the Preline semi-closed system (blue) and control open cage system (red) after approximately one month (June 1st/2nd for Control and Preline, respectively), two months (June 29[tt]/30th for Preline and Control, respectively), and four months (August 29th/30th for Preline and Control, respectively) of transfer to seawater (n= 30 per group per sampling). Capital letters indicate significant differences within the control cage, and small letters indicate significant differences within the Preline system. Asterisk indicates the level of significance: $p < 0.05$=*, $p < 0.01$=**, $p < 0.001$=***.

3.15 IGF-I binding protein mRNA levels in muscle

During the post-smolt period, no significant differences in IGF-I binding protein mRNA levels in muscle were found between the Preline semi-closed system and the control open cage system for each sampling (Fig. 3.12). Within the control system, there were no significant differences from one to two months in the system, but there was a significant increase from two to four months increasing from $0.049 \pm 0,016$ to 0.390 ± 0.157 (Newman-Keuls test, $p < 0.01$).
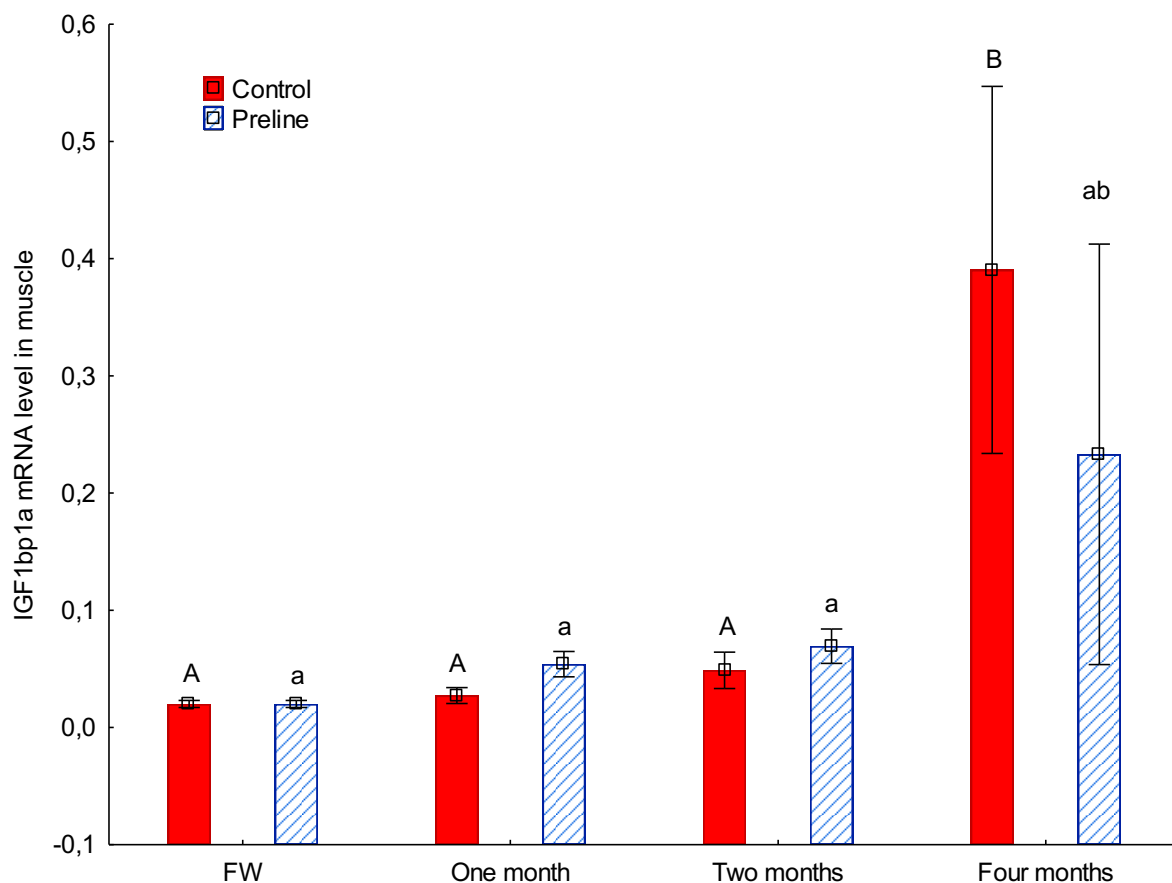
Figure 3.12: Mean IGF1bp1a mRNA level in muscle ($\pm$ SEM) of fish in freshwater (April 15th), and thereafter in the Preline semi-closed system (blue) and control open cage system (red) after approximately one month (June 1st/2nd for Control and Preline, respectively), two months (June 29[tt]/30th for Preline and Control, respectively), and four months (August 29th/30th for Preline and Control, respectively) of transfer to seawater (n= 30 per group per sampling). Capital letters indicate significant differences within the control cage, and small letters indicate significant differences within the Preline system. Asterisk indicates the level of significance: $p < 0.05$=*, $p < 0.01$=**, $p < 0.001$=***.

3.16 Screening for salmonid alphavirus (SAV) and piscine orthoreovirus (PRV)

From the pathological screening, it was seen that 5 fish from the control open cage system showed positive results for salmonid alphavirus (SAV) from the sampling conducted on August 30th (after four months in separate rearing systems). There were no significant differences in plasma IGF-I levels between SAV-positive fish and SAV-negative fish.

4. DISCUSSION

4.1 Discussion of results

4.1.1 Survival

The accumulated mortality in the Preline system began at 0.54 % on the first day of transfer to seawater, and exhibited a sharp increase up to approximately 1 % after the first two weeks, while the control open cage showed 0 % mortality the first week. In current study, the same batch of high quality smolts were used, and both groups were transferred from the smolt facility within 5 days, suggesting that the initial higher mortality in the Preline semi-closed system was not due to suboptimal smolt quality. A possible explanation could be that the average salinity in the Preline system was higher than the control system, resulting in a larger salinity gradient for transferred smolts. Higher mortalities in the beginning could also be linked to the high flow rate that fish were exposed to in the Preline semi-closed system. Studies examining exercise in fish, where fish have been exposed to high flow rates, have shown increased mortality rates during the first period of exposure (Davison and Goldspink, 1977, Totland et al., 1987). Nevertheless, the rate of increasing mortality decelerated in the Preline semi-closed system, and ended at a total accumulated mortality of 1.34 %, while the control open cage system showed a steady increase in mortality throughout the post-smolt period from 0 to 0.98 %. Since mortality was relatively stable after the first few weeks in the Preline system, the increased mortality in the beginning was most likely caused by injuries and/or stress from transportation.

Approximately 20 days after the Preline fish were transferred to the open cage at Buholmen, the accumulated mortality in the control cage exceeded the Preline open cage. In November, three months post-transfer from the post-smolt period, accumulated mortalities reached 2.48 % and 3.52% in the control cage and Preline open cage, respectively. This suggests that the Preline fish might have been more robust, possibly due to the training effect in the semi-closed system, which is discussed further in section 4.1.3. However, from the disease screening it was seen that about 16.7 % of fish in the control open cage from the last sampling during the post-smolt period (after four months), tested positive for salmonid alphavirus (SAV) infection. This virus causes pancreas disease, and symptoms include necrosis of exocrine pancreas, reduced appetite, growth and increased mortality (Nylund et al., 2015).

This may have contributed to increased mortality rates in the control cage during the adult period.

4.1.2 Growth

Temperature:

The regulation of growth and metabolism of Atlantic salmon are, like many teleost's, influenced by environmental factors, including temperature, salinity, photoperiod and oxygen (Brett, 1979, Boeuf and Payan, 2001). One clear finding in the present study was a significantly higher biomass development in the control open cage compared to post-smolts in the Preline semi-closed system. There were significant differences after two and four months of transfer to separate systems, where fish in the control system were about 300 g heavier than their cohorts in the Preline system after four months. Due to the ectothermic nature of salmon, environmental temperature is considered a major factor effecting growth. It has direct effects on metabolism and increases the efficiency of food energy transformation to net biomass development (Brett, 1979). Already in the starting month of the post-smolt period (May) the average temperature was higher in the control system, with a difference of 1.4°C. During the three continuing months (June, July and August), the average monthly temperature was always about 3-4°C higher in the control open cage system. This was expected since the Preline semi-closed system pumped water from a depth of 35 m. Studies have suggested that salmon prefer the highest available temperature up to 14°C (Oppedal et al., 2001), and avoid temperatures above 18°C (Johansson et al., 2006, Johansson et al., 2009). Different optimal temperatures for growth in post-smolts might also be size dependent. Handeland et al. (2008) found that the optimal temperature for growth of post-smolts ranging from 70-150 g was 12.8°C, and increased with about 1.2°C in the size range 70-300 g. Control fish have the advantage of swimming to higher and lower depths throughout the open cage, giving them access to temperatures that are equal to or close to optimal levels for their specific size range. Since this gives them a higher advantage for growth compared to the Preline fish, results indicate that the higher biomass development in control fish was associated with higher temperatures.

Oxygen

Temperature should be considered when determining the oxygen demand of fish, since it is the main controlling factor of metabolism (Fry, 1971). With differing temperatures in the two systems, oxygen concentration could have influenced differences in growth. Previous studies suggest that the oxygen concentration should be a minimum of 85 % saturation and a maximum of 120 % saturation to maintain maximum growth of Atlantic salmon in temperatures ranging from 5-15°C (reviewed by Thorarensen and Farrell, 2011). In the current study, oxygen concentrations in the control cage were well within the recommended ranges, averaging at 97.21 %, with a one day exception showing a minimum of 83.8 %. Oxygen concentrations in the Preline semi-closed system ranged between 72.2 % and 131.6 %, which could potentially restrict the growth potential of fish. However, concentrations fluctuated a lot, and were at the minimum and maximum values only for a limited amount of time. The average concentration was 102.74 %, indicating that values were within the recommended ranges for the vast majority of the time. Thus, oxygen concentrations probably didn't have a large effect on growth within both systems.

Salinity

Once salmon smolts have entered the marine environment they need to acclimatize to higher salinity, which is often associated with a lag phase in growth before being followed by an increase in growth rate (Stefansson et al., 2008). This may reflect why post-smolts in both systems did not differ so much in weight and length after one month, despite a clear difference in environmental rearing factors such as temperature and salinity. However, differences in weight after two and four months may have been affected by the higher salinity in the Preline system compared to the control. Previous studies on other marine teleosts have found that lower salinities, around 15-25ppt, might improve growth due to better feed utilization (Imsland et al., 2008). In contrast, Duston (1994) showed that the growth rate of Atlantic salmon smolts was independent of salinity for values ranging from 20ppt to 31ppt. In the current study, salinity ranged within the same values as the latter study, averaging at 24.2 and 31.7 ppt in the control and Preline system, respectively. Since Duston (1994) also studied Atlantic salmon smolts, it is likely that salinity did not have a large effect on the growth of fish in both systems in the current study.

4.1.3 Growth rate

Specific growth rate (SGR)

Growth rate was measured using both the Specific Growth Rate (SGR) and the Thermal Growth Coefficient (TGC). During the post-smolt period, the SGR was higher in the control open cage system (SGR = 1.939) compared to the Preline semi-closed system (SGR = 1.338). After the Preline fish were transferred to the open cage at Buholmen, they showed a higher growth rate (SGR = 1.298) compared to the control open cage system during the adult period (SGR = 0.870). As mentioned, SGR does not consider fish size and temperature in regards to fish growth. Therefore, it is difficult to use SGR to compare the growth rate of fish that vary in size and are reared at differing temperatures (Dumas et al., 2010), such as the post-smolt period in the current study.

Thermal growth coefficient (TGC)

TGC seems to be a good predictor of expected mean growth when comparing growth between fish reared at differing temperatures with differing sizes since it regards temperature as an independent factor. The current study showed that when correcting for temperature, the control open cage system still had a higher growth rate (TGC = 3.149) compared to fish in the Preline system (TGC = 2.778). Although growth rates vary among production sites, the mean TGC from several recent growth studies of Atlantic salmon post-smolts was 2.71 (reviewed by Thorarsen and Farell, 2011). Additionally, a previous study on the Preline semi-closed system showed the same growth rate for the semi-closed system and open cage during the post-smolt period when correcting for temperature (TGC =2.141) (Sveier et al., 2015). Thus, the Preline fish grew better in the semi-closed system in the current study compared to the previous generation despite similar temperature profiles within the system in both studies. They also grew at a similar rate compared to the mean of other open cage facilities. However, repeated future studies on growth rate in the Preline system are needed to determine accurate predictions.

During the adult period, fish exhibited an increased growth rate in the Preline open cage system at Buholmen (TGC = 3.001) compared to control fish (TGC = 2.318). Although fish were larger in the control system compared to the Preline open cage at the end of November (1666 g and 1475 g, respectively), the relative increase in weight was greater for the Preline

fish (224,4%) compared to the control fish (118,3%). This was unexpected since temperatures were very similar between the two open cage systems during the adult period, averaging at 13.5°C and 13°C for the Preline open cage and control cage, respectively. A possible explanation for higher growth rates in Preline fish might be associated to compensatory growth. Several studies have found that Atlantic salmon show strong compensatory growth after first being exposed to low temperatures (2-6°C), and then to ambient temperatures (11-14°C), in which they grew faster than control fish exposed to constant ambient temperatures (Mortensen and Damsgård, 1993, Nicieza and Metcalfe, 1997, Maclean and Metcalfe, 2001). However, these studies showed compensatory growth after being exposed to abnormally low temperatures, in which Preline fish were not. Another explanation for differences in growth rate might be linked to the SAV infection in the control group from the last sampling during the post-smolt period, which may have effected growth in the control group negatively during the adult period. A reason for increased growth rate in Preline fish after transfer to the open cage at Buholmen, might be associated to robustness due to a training effect in the semi-closed system (Jørgensen and Jobling, 1993, Castro et al., 2011). A study parallel to the current looked at the effect of training in the Preline system on muscle fibre size and distribution, and found a higher frequency of small muscle fibres in the exercised group after four months in the Preline system. This was associated with high muscle fibre recruitment in trained fish, which might have influenced growth during the adult period (Øyvind Moe, 2017, personal communication).

Feed conversion ratio (FCR)
The feed conversion ratio calculated by Lerøy (Fishtalk, FCE=1.1) was higher in the control open cage system (1.08) compared to the Preline semi-closed system (1.04) during the post-smolt period. This may have been due to more precise feeding in the Preline semi-closed system, and less drifting of feed due to confinement. Additionally, studies have shown that a training may result in lower FCR (Leon, 1986, East and Magnan, 1987, Christiansen et al., 1992).

4.1.4 Plasma IGF-I concentrations

The current study showed relatively high plasma IGF-I levels in freshwater smolts, followed by a further increase in IGF-I levels after transfer to seawater. These results are in accordance with several studies reporting increasing plasma IGF-I levels in smolts and post-smolts upon entering the marine environment (reviewed by Beckman, 2011, McCormick, 2013). Fish reared in the control cage had significantly higher plasma IGF-I levels compared to fish in the Preline system after one and two months in separate rearing systems. These differences were also reflected by higher growth (weight and length) in the control cage during the post-smolt period. The differences in plasma IGF-I levels are likely due to the differences in rearing temperature and/or salinity between systems, since environmental factors are known to have clear effects on both plasma IGF-I levels and growth (Brett, 1979, Beckman, 2011). For instance, plasma IGF-I levels decreased in coho salmon that were fed ad libitum and transferred from 10°C to 2.5°C, while they increased when fish were transferred from 2.5°C – 10 °C (Larsen et al., 2001). In rainbow trout, plasma IGF-I levels were two-fold higher for fish reared at 16°C compared to fish reared at 8°C after 6 weeks (Gabillard et al., 2003). Several other studies in teleosts reported similar responses in plasma IGF-I levels when exposed to changes in different temperatures (Silverstein et al., 2000, Davis and Peterson, 2006, Imsland et al., 2007, Luckenbach et al., 2007). These studies displayed a large temperature transition, while Beckman et al. (1998) showed similar results when generating a seasonal increase in temperature for chinook salmon. Collectively, previous literature is in concordance with results found in the current study after one and two months in separate rearing systems, where the higher seasonal temperature in the control open cage is associated with higher IGF-I levels in plasma. Higher IGF-I levels in plasma corresponds to a higher growth rate in the control cage. This correspondence is seen for other teleost species, such as gilthead seabream, *Sparus aurata* (Mingarro et al., 2002), Turbot, *Scophthalmus maximus* (Imsland et al., 2007), chinook salmon, *Oncorhynchus tshawytscha* (Beckman et al., 1998), and coho salmon, *Oncorhynchus kisutch* (Pierce et al., 2001). However, no significant correlations in plasma IGF-I levels and growth (weight and length) were found after four months in separate rearing systems for the control and Preline fish in the current study, which may reflect a difference between large scale and small scale studies.

Additionally, circulating IGF-I levels Preline fish increased to similar levels as those observed in control fish after four months in separate rearing systems, despite higher rearing temperatures and significantly higher length and weight in the control open cage. Due to the training effect in the Preline system, it is possible that elevated plasma IGF-I levels in the Preline system reflect the initiation of enhanced growth at the time of transfer to Buholmen. The SAV infection in the control group from the last sampling was also considered when interpreting these results. Plasma IGF-levels in SAV positive fish were tested against SAV negative fish, and no differences were found. Thus, it was not likely that SAV played a role in these differences. Beckman (2001) argued that IGF-I and growth rate must change in a similar manner for IGF-I to be a reliable measure of growth (concordant relation). This is seen in the current study after one and two months in separate rearing systems, although it is not seen after four months (discordant relation). One should therefore be cautious when interpreting and comparing the significance of plasma IGF-I levels and growth results between large scale and small scale studies. Additional large scale studies are required to further improve our understanding of the underlying mechanisms responsible for these variations.

4.1.5 IGF-I and muscle

Local IGF-I mRNA levels and IGF-I receptor levels in muscle did not differ between the two rearing systems during the post-smolt period. Since muscle growth is mediated by the interaction of these two (Beckman et al., 2004b), it was expected to find higher levels in the control fish since they had a higher weight gain. No differences between systems indicates that temperature is not an influencing factor on both IGF-I mRNA and IGF-I receptor levels. Previous studies have also suggested that temperature does not regulate IGF-I mRNA in muscle in the long term (i.e 6 weeks) (Gabillard et al., 2003). Hence, the growth promoting effects from temperature were probably mainly driven by liver-derived IGF-I (circulating IGF-I in plasma), rather than local muscle-derived IGF-I. However, local muscle IGF-I mRNA expression should be interpreted with caution, as interactions with IGFBPs may inhibit biological action (Beckman, 2011). In fact, the affinity of IGFBPs may equal or surpass that of receptors (Duan, 1997). The current study showed an increase in muscle IGFBP mRNA in the control fish after four weeks, which may be an indication of inhibition of IGF-I action. The training effect of the Preline system should be taken into consideration, since the effect causes an increased rate of muscle fibre recruitment (Øyvind Grøner Moe,

2017, personal communication). Hence, since IGF-I signaling causes muscle hypertrophy, the lower levels of IGFIBP in the Preline system indicates less inhibition of biological actions, and thus increases hypertrophy. If such a difference in IGFBP mRNA levels persist through the adult period, this might be one mechanism explaining higher growth rates in the Preline open cage at Buholmen during the three months in the adult period.

4.2 Conclusions

This thesis studied the growth performance of post-smolts in the Preline semi-closed system compared to the growth of post-smolts in an open control cage. During the post-smolt period, the Preline fish had a lower weight gain and length compared to the control fish, mainly due to lower temperatures in the Preline system. The SGR showed that control fish had a higher growth rate compared to Preline fish throughout the post-smolt period, and the TGC showed that this was independent of higher temperatures in the control cage. Thus, H_{A1} can be confirmed since the growth (weight and length) and growth rate (SGR and TGC) of fish in the Preline system and control cage differed. Plasma IGF-I concentration was higher in control fish for the first two months in separate rearing systems, which corresponds to higher growth rate and higher temperatures in the control fish. However, plasma IGF-I levels did not differ after four months despite higher rearing temperatures and significantly higher length and weight in the control open cage. A possible explanation for the lack of difference is the effect of aerobic exercise in the Preline system, which is associated with a higher recruitment of muscle fibres. IGF-I signaling is known to mediate hypertrophy (enlargement of cells), which may have influenced the higher growth rate in Preline fish after transfer to the open cage at Buholmen. H_{A2} is confirmed for the two first months in separate rearing systems since plasma IGF-I levels of Preline fish differed from the control fish, however, it is rejected after four months since plasma levels were no longer significantly different. The present study found a steady state of muscle IGF-I and IGF-I receptor mRNA levels in fish from both systems, suggesting that the higher growth rate from temperature in the control cage was mainly driven by plasma IGF-I levels, and not local muscle IGF-I. After four months in separate rearing systems, the control fish also had higher levels of IGFIBP, which may have inhibited IGF-I signaling and growth during the adult period. H_{A3} and H_{A4} is therefore rejected since IGF-I mRNA levels and IGF-I receptor mRNA levels in muscle did not differ between systems. H_{A5}

is rejected for the first two months, but confirmed after four months since IGFIBP mRNA levels differed in separate rearing systems.

4.3 Future perspectives

There are huge advantages that come with the possibility of having a more controlled rearing environment in semi-closed systems, and it appears the Preline semi-closed system has the potential to produce robust fish that grow well when exposed to the open ocean. Since IGF-I is a promising candidate as a growth index for fish, it is important that its behavior is measured in realistic production scenarios. It can be difficult to obtain concluding evidence in large scale production studies due to many influencing factors, and such studies should be repeated over several years to determine and strengthen trends. It would be interesting to expand the current study in relation to IGF-I, and measure its association to growth in fish after being reared in the semi-closed system (the adult period). Additionally, these measurements should also be conducted for 0+ smolts, since temperatures within the Preline system will differ for autumn/winter rearing compered to spring/summer rearing. Measurements of IGF-I in differing environments during different seasonal temperatures can help aquaculture producers understand the variation in growth, and thus design efficient rearing procedures for cultured fish.

4.4 Discussion of methods

The present study represents the third time post-smolts have been reared in the Preline semi-closed system, and is therefore an ongoing project. The goal is to rear post-smolts up to at least 0,5-1kg, making them larger and more robust for the open cage seawater phase. In a biological context, the post-smolt phase is often referred to as the first 1-3 months following transfer of smolts to the marine environment (Stefansson et al., 2008, Stefansson et al., 2012). From an industry point of view the post-smolt term is often used in a much wider context, typically referring to fish sizes ranging between 100 grams and up to 1 kg over a period of up to six months. Hence, the post-smolt term is used rather obscurely when referring to new production strategies in the industry. In the current study, the four-month period during which the Preline fish spent in the semi-closed system was defined as the post-smolt period, while the adult period refers to the period after fish were transferred to the open cage at Buholmen, in which we have growth data for three months. Fish were transferred to the control open cage system 5 days later than the Preline semi-closed system. It would have been ideal if smolts were transferred to separate rearing systems on the same day. However, this was difficult to achieve with such a large-scale experiment due to the capacity of well boats.

4.4.1 Water parameters

Temperature, salinity and oxygen were registered daily during the post-smolt period in the Preline system from April 30th to August 31st. However, since the control fish were transferred to the open cage 5 days later than the Preline fish, these parameters were given from May 5th in the results section to compare the two systems during the same time. These parameters varied between the Preline and control systems, since Preline accesses water from a depth of 35 m. Control fish could access the entire depth range throughout the open cage, so knowing the exact temperature profile in this system is difficult. These parameters also differed during the adult period when both groups were in open cages, since cages were in two different locations. It was not possible to have cages in the same location, since this study was conducted at full scale production.

4.4.2 Growth rate

The estimated weight data based on feed output (Fishtalk calculations, FCE = 1.1) was similar to the weight measurements conducted during the post-smolt period. Therefore, they were expected to represent accurate weight measurements during the adult period. We did not use

individually tagged fish when calculating TGC, but a sample of 30 fish was thought to represent accurate measurements that were representative for the population. Additionally, TGC might produce systematic errors when being used for temperatures that exceed the optimum for growth (above 16°C) (Jobling, 2003). Temperatures were above optimum only for a limited amount of time in the current study. Therefore, it is likely that the calculated TGC values represented accurate growth rates.

4.4.3 Screening for salmonid alphavirus (SAV) and piscine orthoreovirus (PRV)

Since salmonid alphavirus (SAV) was detected at other facilities in the same fjord as the control open cage, it was suspected that the control cage was also exposed to the virus. Symptoms of SAV include necrosis and degeneration in heart and skeletal muscle, and from a parallel study to the current one, using the same sampling fish, heart and muscle were being investigated for symptoms (Øyvind Moe, 2017, personal communication). Piscine orthoreovirus (PRV) causes heart and skeletal muscle inflammation (HSMI), in which can cause severe inflammation and necrosis of cardiac and red skeletal muscle (Nylund et al., 2015). To detect if SAV and PRV were present, a real-time PCR analysis was run. Five fish tested positive for SAV in the last sampling during the post-smolt period (on August 30th), which was a prevalence of approximately 16.7 %. Since an infection with SAV may cause necrosis of exocrine pancreas, reduced appetite, growth and increased mortality (Nylund et al., 2015), it was discussed whether these fish should be excluded from the study. However, a one-way ANOVA was conducted to test if SAV infected fish differed in plasma IGF-I levels compared to non-infected fish. No significant differences were found, and the fish were therefore included in the study. The mRNA levels of IGF-I, IGF-I receptor and IGF-I binding protein in muscle were not tested for differences because the muscle samples from infected fish were not used in the study. All muscle samples used in the study showed to be a carrier of PRV, and it was therefore not possible to run any statistical tests since we only had one group. However, since PRV is relatively abundant in farmed salmon, and appears often in high levels without associated pathology (Nylund et al., 2015), the infection most likely did not influence the growth of fish.

4.4.4 Statistical analysis

Due to constraints associated with a large scale commercial production site and the fact that there is only one existing Preline system, it was not possible to have several replicates for

each treatment (rearing system). We chose to sample thirty fish for each sampling since this number is used in normal practice when establishing diseases for fish, and should be a good representation of the population. TGC and FCR were calculated from estimated weight data provided by the farming company, Lerøy Vest AS, and were not estimated for individual fish. It was not possible to conduct a statistical test on these values, since we only had one replicate for the post-smolt period and adult period for both systems.

4.4.5 Time-resolved fluoroimmunoassay (TR-FIA) for plasma IGF-I concentration

For the determination of plasma IGF-I levels, TR-FIA (competitive time-resolved fluoroimmunoassay) provided a time-efficient non-isotopic method to safely quantify plasma levels of IGF-I (Small and Peterson, 2005). Radioimmunoassay (RIA) is another common and frequent method used to analyze plasma IGF-I levels in salmonids (Moriyama et al., 1994). Both are immunological methods that based on the anti-IGF-I antibodies to recognize a certain epitope at the IGF-I peptide in plasma. The main difference between TR-FIA and RIA is that the latter uses radioactive isotopes as a tracer/reporter. TR-FIA was chosen for the current study since the methanol acid extractions allow the IGF-I binding protein to be removed, unmasking the epitope of the hormone and thereby providing a more accurate measurement of the total IGF-I levels in plasma samples. Sensitivity of the assay was 0.25 ng/ml) (Appendix 6.2). The inter-assay and intra-assay variation was less than 15% and considered sufficient in current study. Serial dilutions of plasma were parallel to the standard curve (Appendix 6.3).

4.4.6 Isolation of total RNA using the QIAsymphony robot

Initially, total RNA from muscle samples was first attempted purified using the QIAsymphony nucleic acid purification robot in conjunction with QIAsymphony RNA kit as described in the manufacturer's protocol. This is an automated high throughput analytical platform that can purify up to 200 samples per day. The QIA symphony procedure requires a similar homogenization of tissue samples as for the TRI reagent protocol described in the material and methods section. The main difference is that the QIAsymphony RNA kit requires 600-800 ul of RLT plus lysis buffer instead of 1 ml of TRI reagent. One limiting factor is the amount of tissue the in the QIAsymphony system. First, 20-25 mg of muscle tissue was applied in 600 ul buffer. This gave very low total RNA concentrations, so for the next batch 35-40 mg of tissue was used in 800 ul of buffer. Unfortunately, the amount of total RNA yield

ranged between 2,55 – 95,5 ng/ul in a total volume of 50 ul per sample, which was not sufficient to proceed with downstream analysis such as cDNA synthesis and qPCR. In an attempt to concentrate the RNA in low abundance samples, total RNA was precipitated from 10 samples by adding NaAc (1/10 of sample volume) and 100% ice cold ethanol, EtOH, (x3 sample volume) and then stored at -80°C freezer overnight. Samples were then centrifuged, washed in EtOH and pellet dried and reconstituted in 10-ul nuclease free water. However, the total RNA concentration in most of the samples were low and considered to be "out of range" for further cDNA synthesis and q-PCR. The QIAsymphony nucleic acid purification robotitc system is quite new in our laboratory, and further optimization of protocols using low amount of tissues and tissues that are rich in lipids and carbohydrates is needed, as this is known to influence yield.

Based on the low yield of total RNA from the QIAsymphony robot protocol it was decided to switch to the TriReagent protocol described in the Material and methods section. The TriReagent protocol is a manual protocol that has been in use for more than a decade in our lab and it has proven to give high yield and quality of total RNA from fish tissue (Tom Ole Nilsen, Uni, personal communication). As sufficient amounts of high quality total RNA was vital to be able to quantify the different components of the IGF-I system in muscle total RNA, the remaining 70 tissue samples not used in the QIAsymphony robot was purified manually with the TriReagent protocol. Based on measurements using the NanoDrop-1000 (Thermo Scientific, NC, USA), it was clear that the A260/280 ratios greater than 1.8 indicated sufficient RNA purity and low protein contamination. Moreover, high A260/230 ratio suggested a residual contamination of organic compounds, such as phenol and alcohol (Bustin, 2002) from the TriReagent RNA isolation protocol. Hence, we did not expect a negative effect on downstream applications such as cDNA synthesis and quantitative PCR (Pfaffl, 2004) as was indicated sufficient efficiency of q-PCR assays used in this study (Fig. 4.1).

### 4.4.7 Reverse Transcriptase Synthesis (cDNA synthesis) of muscle samples (test)

It is widely accepted that almost all RNA isolation methods results in residual amounts of genomic DNA (Pfaffl, 2004). Such unwanted genomic DNA (gDNA) can be removed by DNase treatments of our RNA samples as even the smallest DNA contamination may interfere with the desired "specific amplification" given the high sensitivity of the qPCR

method (Pfaffl, 2004). However, DNase treatment often leads to partial degradation of total RNA and, if possible, should be avoided. Based on no –RT signal in our assays we considered the signal measured in our samples to reflect mRNA abundance of each transcript in muscle tissues. Hence, we concluded that there was no signal from residual gDNA using the protocols described in the present study. As RNA cannot serve as a template for PCR, the formation of a DNA template is necessary and RNA is used as the template for the formation of single stranded (ss) complementary DNA (cDNA) through reverse transcriptase. The RT step is the source of the most variability in a kinetic RT-PCR experiment (Pfaffl, 2004), and it is crucial that all samples are treated in a standardized manner throughout the experiment. We added all components into one master mix, as described in the Material and Methods section, before aliquots of the master mix were added to the samples. Furthermore, all batch number for reagents, primers and laboratory supply were kept as similar as possible in order to minimize variation throughout the experiment. The complex nature of the RT-qPCR method and the numerous steps involved going from tissue sampling to the end expression results provide potential of pitfalls that may easily lead to methodological errors (Bustin and Nolan, 2004).

We used the Hamilton pipetting robot to dispense the different ingredients to ensure high accuracy and reproducibility (Tom Ole Nilsen, Uni, 2017, personal comment). The SuperScript III kit is widely used for cDNA synthesis applied in both regular PCR and RT-qPCR and have proven to give excellent results in our laboratory. In this study, a two-step RT-qPCR was chosen, which involves creating the cDNA in one separate RT reaction tube before adding a small amount of diluted aliquot of cDNA as template to the RT-qPCR reaction. This method enhances the flexibility by allowing storage and multiple testing with the same cDNA stock compared to a single-step procedure (Tom Ole Nilsen, 2017, personal comment). The choice of priming strategy (gene specific, random hexamer or oligo dT) has also proven to be important, as different priming methods have shown to provide different sensitivities and efficiencies (Raja et al., 2000). We chose to use the oligo (dT) primer, which by experience has proven to be a very reliable choice in our laboratory when quantifying gene expression in most assays (Tom Ole Nilsen, 2017, personal comment). Real time q-PCR is a modern and commonly used method to measure mRNA level according to its high sensitivity, reproducibility, and precision in detecting mRNA transcripts over a wide quantification range

(Bustin, 2002). Based on prior experience from screenings of suitable reference gene, the endogenous reference gene Ef1-a was evaluated and found sufficiently stable to use in this study (Olsvik et al., 2005). The working concentrations of cDNA to be used in the q-PCR analysis were determined by generating a pool of cDNA that contained an aliquot from representative samples. From the pool cDNA a two-fold dilution series was made and used as template to validate each of the gene expression assays used in the present study (Fig 4.1). Based on dilution series for each of the assays all genes were found to display a sufficient PCR efficiency and analyzed using a template cDNA diluted 1:20.
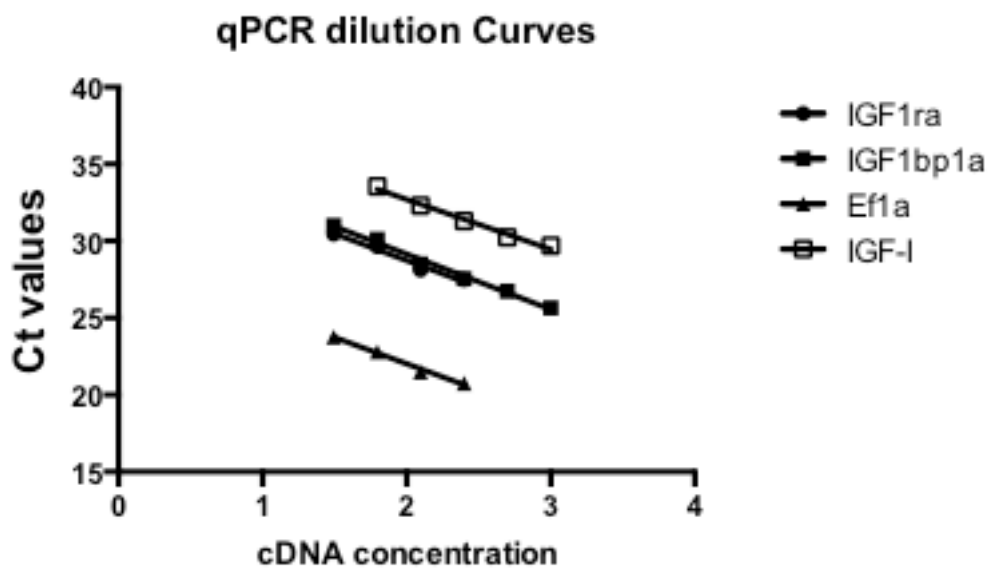
Figure 4.1: Real-time RT-PCR threshold cycle (Ct) values ± S.E.M. against cDNA dilution series from Atlantic salmon muscle for qPCR assay used in present study. Linear regression results IGF1ra: $y = -3.472x + 35.68$, $R^2 = 0.975$; IGF1bp1a: $y = -3.595x + 36.34$, $R^2 = 0.991$; IGF1: $y = -3.246x + 39.20$, $R^2 = 0.984$; Ef1a: $y = -3.425x + 28.87$, $R^2 = 0.989$.

Several probes and dyes may be used to detect DNA amplification in real time, and some bind to specific sections of the DNA, whereas others bind to DNA in general (Bustin, 2000). For this study, SYBRgreen was used, which binds to the double stranded DNA and the bound dye emits a detectible fluoresces (Bustin, 2000). The amount of fluorescence will increase proportionally with the quantity of amplified DNA and is recorded in real-time (Bustin, 2000). As SYBRgreen binds to all dsDNA present, specific primers and low DNA contamination are required to ensure a reliable PCR result (Bustin, 2000).

This dye binds to the double stranded DNA and emits a detectible fluoresces proportional to the quantity of amplified DNA, which then will be recorded in real-time when it reaches a predetermined threshold set by the operator (Bustin, 2000). However, as it binds to all dsDNA present, specific primers and low genomic DNA contamination are vital to ensure a reliable result (Bustin, 2000). This was addressed by the great care taken in each step prior to this, e.g. RNA integrity, purity tests and screenings as discussed above. In addition, NTC wells applied to each plate and these showed no signal and thus no contaminations in our qPCR reactions. It should be mentioned that several of the assays planned to quantify the IGF system in present study had all been validated and used in published studies. However, we were not able to secure a reproducible result for all the assays (Hevrøy et al., 2013, Hevrøy et al., 2015). Hence, we chose to only proceed with the assays reported in the results in present study as those were the only assays that gave a reliable quantification signal.

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6. APPENDIX

6.1 Reagents used for TR-FIA

A/E mix:

1. First prepare 2M HCL: 1.67ml of 37% HCL + 8.33ml of Distilled Water

2. **A/E mix** = 8.75ml (8750 ul) Ethanol 99.5% + 1.25ml (1250 ul) 2N HCL

Tri-base:

Blank Solution:

(Ratio 1:4:2)

For one plate: 750 ul Assay Buffer + 3000 ul A/E mix + 1500 ul Tri-Base

Washing Buffer:

Purchased from Perkin Elmer (concentrated stock). Diluted with distilled water (1:25) as needed (i.e. 20 ml original concentration + 480 ml dH_2O = 500 ml washing buffer). *Wash buffer is a 25-fold concentrate of Tris-HCl buffered (pH 7.8) salt solution with Tween 20. It contains Germall II[1] as preservative.*

Assay Buffer:

Purchased from Perkin Elmer. *Assay Buffer is a ready for use Tris-HCl buffered NaCl solution (pH 7.8), containing < 0.1% NaN_3, bovine serum albumin (BSA), bovine gamma globulins, Tween 40, diethylenetriaminepentaacetic acid (DTPA), and a inert red dye.*

Enhancement solution:

Purchased from Perkin Elmer. *Ready for use solution with Triton X-100[1], acetic acid and chelators.*

6.2 Sensitivity of TR-FIA
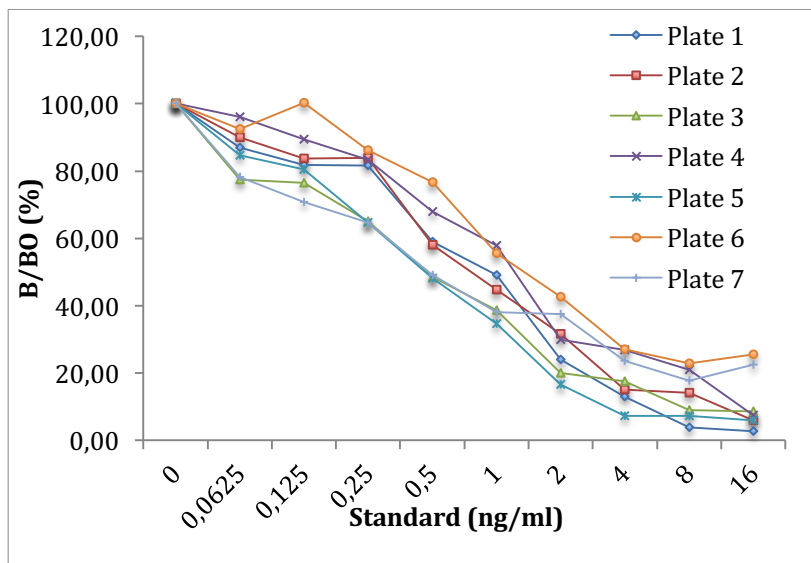
Figure 6.1: Standard curves for all sample TR-FIA plates

6.3 Plasma dilutions and standards curve for TR-FIA
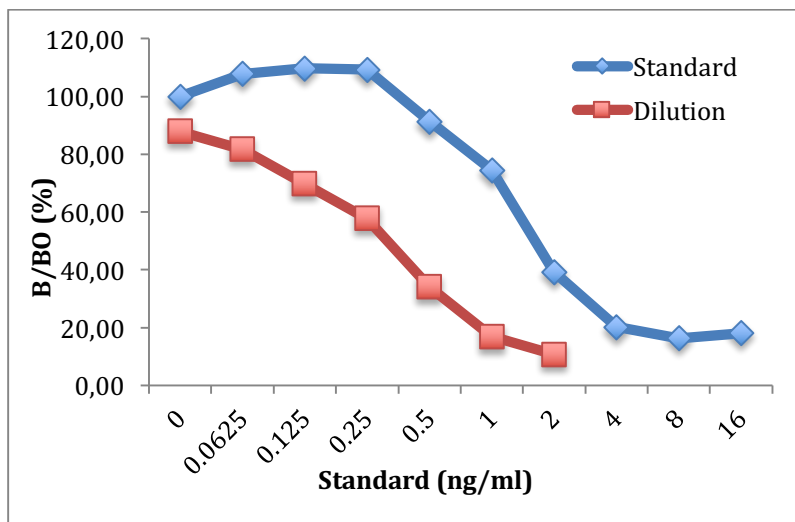
Figure 6.2: Serial dilutions of plasma for each plate and standards curve showing parallelism.

6.4 Temperature graphs

Depth: 3m
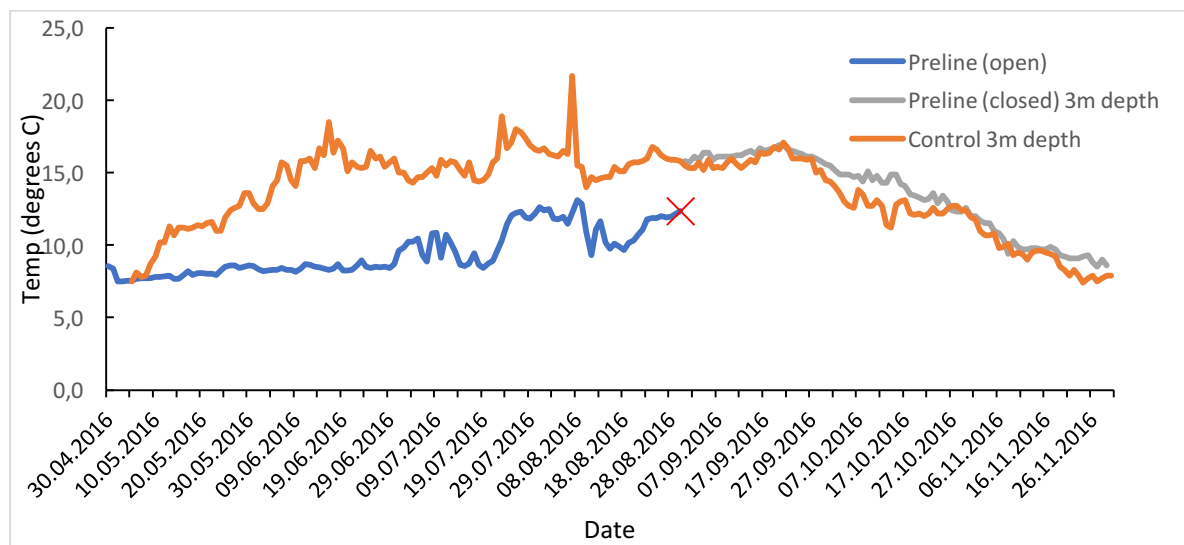
Figure 6.3: Temperature in the Preline system (blue), (water taken from 35 m depth), up until transfer to open net pen located at Buholmen (31.08.16), and thereafter the temperature at Buholmen at 3 m depth (grey). Orange line shows temperature at the Control facility at 3 m depth throughout the whole period.

Depth: 8m
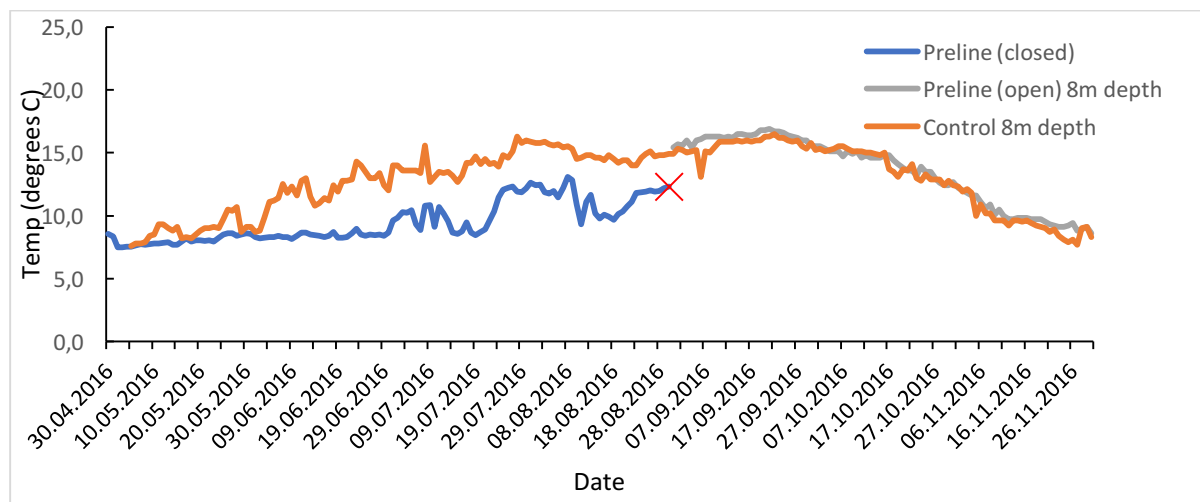
Figure 6.4: Temperature in the Preline system (blue), (water taken from 35 m depth), up until transfer to open net pen located at Buholmen (31.08.16), and thereafter the temperature at

Buholmen at 8 m depth (grey). Orange line shows temperature at the Control facility at 8 m depth throughout the whole period.

Depth: 15m

Figure 6.5: Temperature in the Preline system (blue), (water taken from 35 m depth), up until transfer to open net pen located at Buholmen (31.08.16), and thereafter the temperature at Buholmen at 15 m depth (grey). Orange line shows temperature at the Control facility at 15 m depth throughout the whole period.

6.5 Salinity graphs
Depth 3m
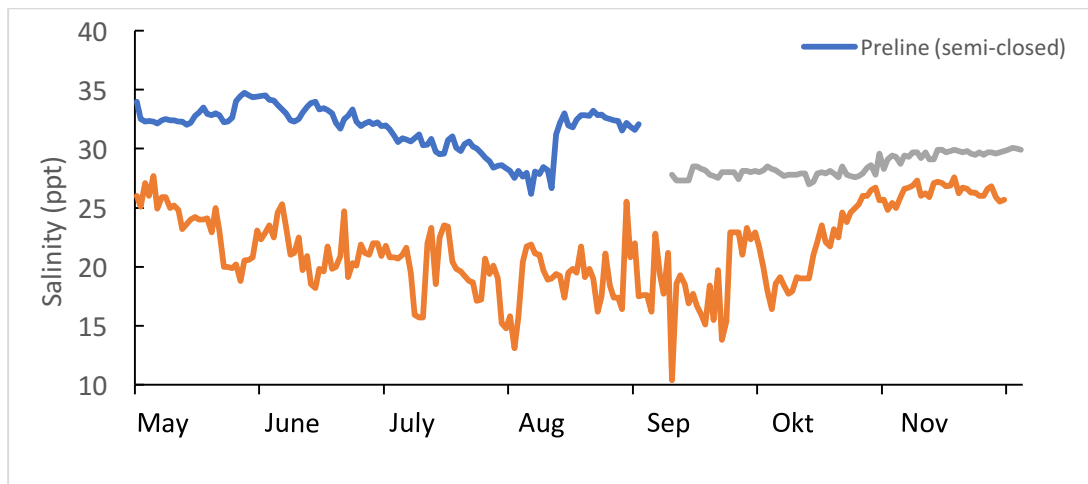
Figure 6.6: Salinity in the Preline system (blue), (water taken from 35 m depth), up until transfer to open net pen located at Buholmen (31.08.16), and thereafter the salinity at Buholmen at 3 m depth (grey). Orange line shows salinity at the Control facility at 3 m depth throughout the whole period.

Depth 8m
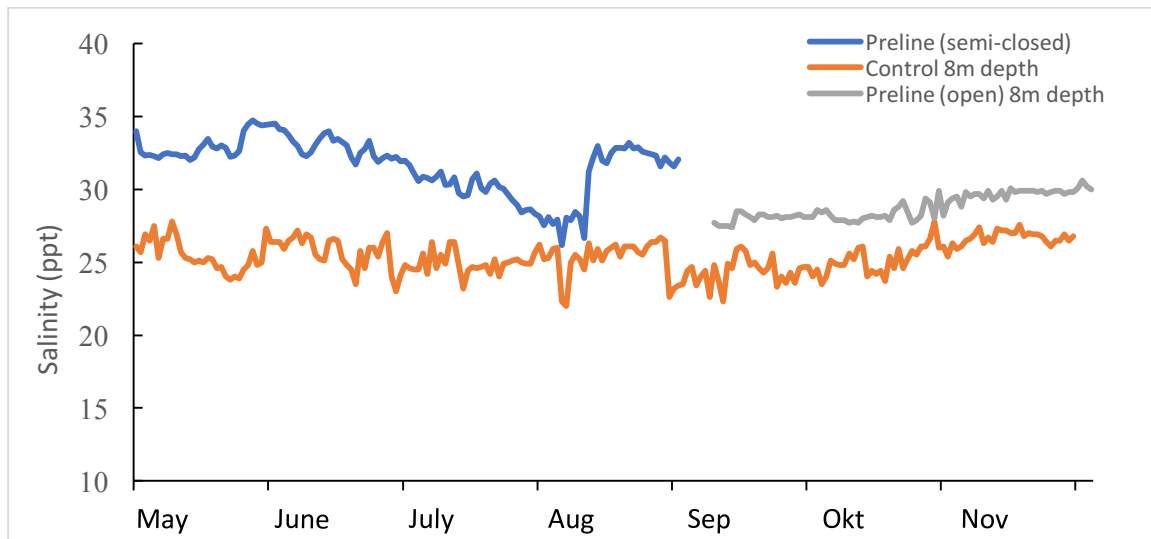
Figure 6.7: Salinity in the Preline system (blue), (water taken from 35 m depth), up until transfer to open net pen located at Buholmen (31.08.16), and thereafter the salinity at Buholmen at 8 m depth (grey). Orange line shows salinity at the Control facility at 8 m depth throughout the whole period.

Depth 15m
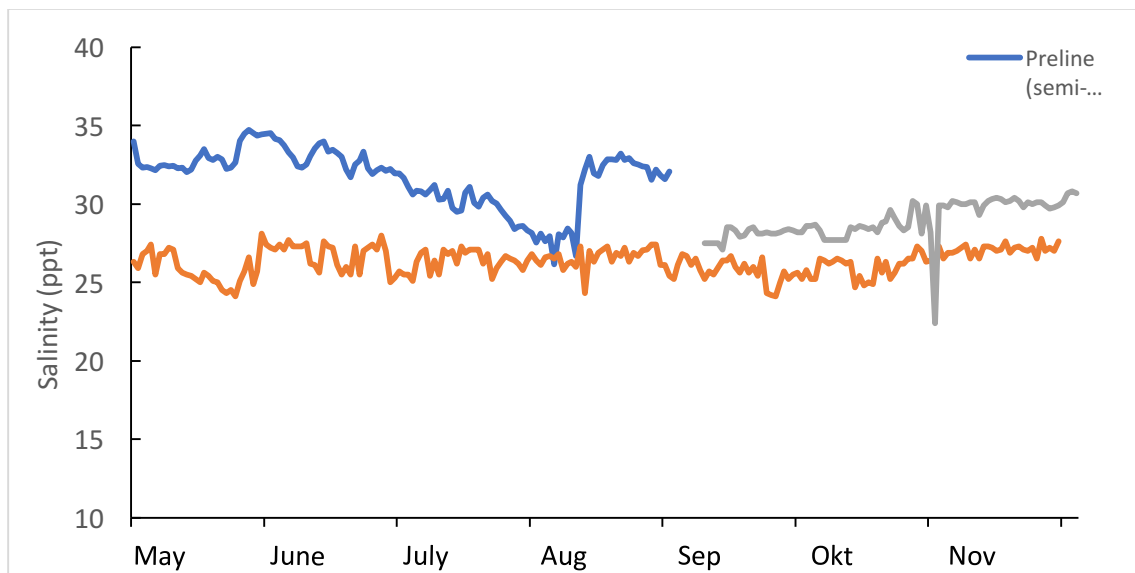
Figure 6.8: Salinity in the Preline system (blue), (water taken from 35 m depth), up until transfer to open net pen located at Buholmen (31.08.16), and thereafter the salinity at Buholmen at 15 m depth (grey). Orange line shows salinity at the Control facility at 15 m depth throughout the whole period.

6.6 Statistical analysis

One-way ANOVA tests for difference in weight, length and condition factor between systems for each sampling

**TABLE 6.1:** Test results from a one-way ANOVA on weight difference after one month in separate rearing systems (June 1/2nd), during the post-smolt period

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	979341,7	1	979341,7	1595,255	0,000000
Behandling-2	455,0	1	455,0	0,741	0,392857
Error	35606,7	58	613,9		

TABLE 6.2: Test results from a one-way ANOVA on weight difference after two months in separate rearing systems (June29/30th), during the post-smolt period

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	2891526	1	2891526	1321,029	0,000000
Behandling-3	29004	1	29004	13,251	0,000582
Error	126953	58	2189		

TABLE 6.3: Test results from a one-way ANOVA on weight difference after four months in separate rearing systems (August 29/30th), during the post-smolt period

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	20158604	1	20158604	407,5520	0,000000
Behandling-4	1356578	1	1356578	27,4263	0,000002
Error	2868834	58	49463		

TABLE 6.4: Test results from a one-way ANOVA on length difference after one month in separate rearing systems (June 1/2nd), during the post-smolt period

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	31813,64	1	31813,64	21202,43	0,000000
Behandling-2	1,35	1	1,35	0,90	0,346793
Error	87,03	58	1,50		

TABLE 6.5: Test results from a one-way ANOVA on length difference after two months in separate rearing systems (June29/30th), during the post-smolt period

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	44303,40	1	44303,40	13688,94	0,000000
Behandling-3	46,46	1	46,46	14,36	0,000362
Error	187,71	58	3,24		

TABLE 6.6: Test results from a one-way ANOVA on length difference after four months in separate rearing systems (August 29/30th), during the post-smolt period

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	77911,27	1	77911,27	6849,775	0,000000
Behandling-4	417,65	1	417,65	36,719	0,000000
Error	659,71	58	11,37		

TABLE 6.7: Test results from a one-way ANOVA on condition factor difference after one month in separate rearing systems (June 1/2nd), during the post-smolt period

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	64,11417	1	64,11417	13002,42	0,000000
Behandling-2	0,00065	1	0,00065	0,13	0,717474
Error	0,28599	58	0,00493		

TABLE 6.8: Test results from a one-way ANOVA on condition factor difference after two months in separate rearing systems (June29/30th), during the post-smolt period

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	69,11486	1	69,11486	19001,80	0,000000
Behandling-3	0,00382	1	0,00382	1,05	0,309430
Error	0,21096	58	0,00364		

TABLE 6.9: Test results from a one-way ANOVA on condition factor difference after four months in separate rearing systems (August 29/30th), during the post-smolt period

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	80,58043	1	80,58043	5154,859	0,000000
Behandling-4	0,09595	1	0,09595	6,138	0,016167
Error	0,90665	58	0,01563		

Control:

TABLE 6.10: Test results from a one-way ANOVA on length difference from one month (June $1/2^{nd}$) to four months (August $29/30^{th}$), during the post-smolt period for the control cage

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	56826,04	1	56826,04	7341,010	0,00
Behandling	3743,02	1	3743,02	483,538	0,00
Error	448,97	58	7,74		

TABLE 6.11: Test results from a one-way ANOVA on weight difference from one month (June $1/2^{nd}$) to four months (August $29/30^{th}$), during the post-smolt period for the control cage

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	10965512	1	10965512	260,9824	0,000000
Behandling	5490278	1	5490278	130,6702	0,000000
Error	2436944	58	42016		

TABLE 6.12: Test results from a one-way ANOVA on CF difference from one month (June $1/2^{nd}$) to four months (August $29/30^{th}$), during the post-smolt period for the control cage

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	74,54606	1	74,54606	4453,088	0,000000
Behandling	0,42563	1	0,42563	25,426	0,000005
Error	0,97094	58	0,01674		

Preline:

TABLE 6.13: Test results from a one-way ANOVA on length difference from one month (June $1/2^{nd}$) to four months (August $29/30^{th}$), during the post-smolt period for the Preline system

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	48008,13	1	48008,13	9351,291	0,00
Behandling	1566,73	1	1566,73	305,176	0,00
Error	297,76	58	5,13		

TABLE 6.14: Test results from a one-way ANOVA on weight difference from one month (June 1/2nd) to four months (August 29/30th), during the post-smolt period for the Preline system

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	4700350	1	4700350	583,1495	0,00
Behandling	1338839	1	1338839	166,1033	0,00
Error	467496	58	8060		

TABLE 6.15: Test results from a one-way ANOVA on CF difference from one month (June 1/2nd) to four months (August 29/30th), during the post-smolt period for the Preline system

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	69,71894	1	69,71894	18238,75	0,000000
Behandling	0,10057	1	0,10057	26,31	0,000004
Error	0,22171	58	0,00382		

Tests for homogeneity of variance for Plasma IGF-I concentration

Table 6.16: Test results from Levene's test for homogeneity of variance on plasma IGF-I concentration after one month (June 1/2nd)

	MS Effect	MS Error	F	p
IGF-1p	72,04776	649,8044	0,110876	0,740350

Table 6.17: Test results from Hartley F-max test for homogeneity of variance on plasma IGF-I concentration after two months (June 29th/30th)

	Hartley F-max	Cochran C	Bartlett Chi-Sqr.	df	p
IGF-1p	2,485240	0,713076	5,713391	1	0,016836

Table 6.18: Test results from Levene's test for homogeneity of variance on plasma IGF-I concentration after four months (August 29th/30th)

	MS Effect	MS Error	F	p
IGF-1p	4099,535	20329,51	0,201654	0,655153

Table 6.19: Test results from a one-way ANOVA on plasma IGF-I concentration difference after one month in separate rearing systems (June 1/2nd)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	744467,9	1	744467,9	488,6558	0,000000
Group	15482,7	1	15482,7	10,1626	0,002310
Error	88363,1	58	1523,5		

Table 6.20: Test results from a Tukey HSD test on plasma IGF-I concentration difference after one month in separate rearing systems (June 1/2nd)

Cell No.	Group	{1} 127,45	{2} 95,327
1	Control		0,002457
2	Preline	0,002457	

Table 6.21: Test results from a one-way ANOVA on plasma IGF-I concentration difference after two months in separate rearing systems (June 29th/30th)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	1675664	1	1675664	496,1627	0,000000
Group	85108	1	85108	25,2004	0,000005
Error	195880	58	3377		

Table 6.22: Test results from a Tukey HSD test on plasma IGF-I concentration difference after two months in separate rearing systems (June 29th/30th)

Cell No.	Group	{1} 129,45	{2} 204,78
1	Preline		0,000115
2	Control	0,000115	

Table 6.23: Test results from a one-way ANOVA on plasma IGF-I concentration difference after four months in separate rearing systems (August 29th/30th)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	3388831	1	3388831	91,78198	0,000000
Group	2085	1	2085	0,05647	0,813027
Error	2067667	56	36923		

Table 6.24: Test results from a Kruskal Wallis ANOVA on plasma IGF-I concentration difference from one month (June 1/2nd) to four months (August 29/30th), during the post-smolt period for the control cage

Depend.: C-IGF-1p	1 R:22,321	2 R:50,133	3 R:81,400	4 R:80,414
1		0,010836	0,000000	0,000000
2	0,010836		0,002141	0,003647
3	0,000000	0,002141		1,000000
4	0,000000	0,003647	1,000000	

Table 6.25 Test results from a Kruskal Wallis ANOVA on plasma IGF-I concentration difference from one month (June 1/2nd) to four months (August 29/30th), during the post-smolt period for the Preline semi-closed system

Depend.: P-IGF-1p	1 R:32,321	2 R:44,967	3 R:69,233	4 R:88,690
1		0,935812	0,000207	0,000000
2	0,935812		0,033546	0,000004
3	0,000207	0,033546		0,165705
4	0,000000	0,000004	0,165705	

Tests for homogeneity of variance for IGF1bp1a, IGF1ra and IGF1 mRNA levels in muscle

Table 6.26: Test results from Levene's test for homogeneity of variance on IGF1bp1a, IGF1ra and IGF1 mRNA levels in muscle after one month (June 1/2nd)

	MS Effect	MS Error	F	p
IGF1bp1a	0,000334	0,000397	0,841518	0,371091
IGF1ra	0,000011	0,003562	0,003076	0,956381
IGFI	0,000075	0,227461	0,000329	0,985722

Table 6.27: Test results from Levene's test for homogeneity of variance on IGF1bp1a, IGF1ra and IGF1 mRNA levels in muscle after two months (sampling June 29th and 30th)

	MS Effect	MS Error	F	p
IGF1bp1a	0,000000	0,001242	0,000236	0,987900
IGF1ra	0,000031	0,001536	0,020450	0,887877
IGFI	0,000052	0,067501	0,000764	0,978259

Table 6.28: Test results from Levene's test for homogeneity of variance on IGF1bp1a, IGF1ra and IGF1 mRNA levels in muscle after four months (sampling August 29th and 30th)

	MS Effect	MS Error	F	p
IGF1bp1a	10,33096	0,824808	12,52528	0,002521
IGF1ra	0,00231	0,003839	0,60096	0,448860
IGFI	0,04289	0,605926	0,07079	0,793389

ANOVA tests for IGF1BP mRNA level in muscle

Table 6.29: Test results from a One-way ANOVA on IGF1bp1a mRNA level in muscle difference after one month in separate rearing systems (June 1/2nd)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0,032992	1	0,032992	40,48945	0,000005
Group	0,003571	1	0,003571	4,38228	0,050743
Error	0,014667	18	0,000815		

Table 6.30: Test results from a One-way ANOVA on IGF1bp1a mRNA level in muscle difference after two months in separate rearing systems (June 29th/30th)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0,069743	1	0,069743	30,49162	0,000030
Group	0,002128	1	0,002128	0,93020	0,347592
Error	0,041171	18	0,002287		

Table 6.31: Test results from a One-way ANOVA on IGF1bp1a mRNA level in muscle difference after four months in separate rearing systems (August 29th/30th)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	12,08031	1	12,08031	5,509985	0,030546
Group	5,92205	1	5,92205	2,701124	0,117630
Error	39,46392	18	2,19244		

Table 6.32: Test results from a Kruskal-Wallis ANOVA on IGFIBP mRNA level in muscle difference from one month (June 1/2nd) to four months (August 29/30th), during the post-smolt period for the control system

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	5,02258	1	5,022576	4,941018	0,032595
Time	12,47196	3	4,157322	4,089814	0,013467
Error	36,59423	36	1,016506		

Table 6.33: Test results from a Newman-keuls test on IGFIBP mRNA level in muscle difference from one month (June 1/2nd) to four months (August 29/30th), during the post-smolt period for the control system

Cell No.	Time	{1} ,02007	{2} ,02725	{3} ,04874	{4} 1,3213
1	1		0,987490	0,997845	0,031882
2	2	0,987490		0,962369	0,018390
3	3	0,997845	0,962369		0,007841
4	4	0,031882	0,018390	0,007841	

Table 6.34: Test results from a Newman-keuls test on IGFIBP mRNA level in muscle difference from one month (June 1/2nd) to four months (August 29/30th), during the post-smolt period for the Preline system

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0,354284	1	0,354284	4,357159	0,043993
Time	0,270034	3	0,090011	1,107004	0,358943
Error	2,927185	36	0,081311		

ANOVA tests for IGF1ra mRNA level in muscle

Table 6.35: Test results from a One-way ANOVA on IGF1ra mRNA level in muscle difference after one month in separate rearing systems (June 1/2nd)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0,234734	1	0,234734	25,38984	0,000085
Group	0,000230	1	0,000230	0,02485	0,876488
Error	0,166413	18	0,009245		

Table 6.36: Test results from a One-way ANOVA on IGF1ra mRNA level in muscle difference after two months in separate rearing systems (June 29th/30th)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0,196760	1	0,196760	39,97588	0,000006
Group	0,000998	1	0,000998	0,20278	0,657864
Error	0,088596	18	0,004922		

Table 6.37: Test results from a One-way ANOVA on IGF1ra mRNA level in muscle difference after four months in separate rearing systems (August 29th/30th)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0,434539	1	0,434539	40,78048	0,000007
Group	0,000247	1	0,000247	0,02322	0,880677
Error	0,181144	17	0,010656		

Table 6.38: Test results from a Kruskal-Wallis ANOVA on IGFIra mRNA level in muscle difference from one month (June 1/2nd) to four months (August 29/30th), during the post-smolt period for the control system

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0,594019	1	0,594019	49,94987	0,000000
Time	0,018549	3	0,006183	0,51992	0,671281
Error	0,428123	36	0,011892		

Table 6.39 Test results from a Kruskal-Wallis ANOVA on IGFIra mRNA level in muscle difference from one month (June 1/2nd) to four months (August 29/30th), during the post-smolt period for the Preline system

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0,613049	1	0,613049	49,03340	0,000000
Time	0,016824	3	0,005608	0,44855	0,719889
Error	0,437594	35	0,012503		

ANOVA tests for IGF1ra mRNA level in muscle

Table 6.40: Test results from a One-way ANOVA on IGF1 mRNA level in muscle difference after one month in separate rearing systems (June 1/2nd)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	23,51268	1	23,51268	37,29198	0,000009
Group	0,43078	1	0,43078	0,68324	0,419296
Error	11,34904	18	0,63050		

Table 6.41: Test results from a One-way ANOVA on IGF1 mRNA level in muscle difference after two months in separate rearing systems (sampling June 29th and 30th)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	14,49223	1	14,49223	69,48815	0,000000
Group	0,24924	1	0,24924	1,19507	0,288725
Error	3,75402	18	0,20856		

Table 6.42: Test results from a One-way ANOVA on IGF1 mRNA level in muscle difference after four months in separate rearing systems (sampling August 29th and 30th)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	14,87649	1	14,87649	12,53141	0,002516
Group	0,00318	1	0,00318	0,00268	0,959353
Error	20,18132	17	1,18714		

Table 6.43: Test results from a Kruskal-Wallis ANOVA on IGFI mRNA level in musle difference from one month (June 1/2nd) to four months (August 29/30th), during the post-smolt period for the Control system

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	25,48426	1	25,48426	39,12624	0,000000
Time	0,52695	3	0,17565	0,26968	0,846824
Error	23,44803	36	0,65133		

Table 6.44: Test results from a Kruskal-Wallis ANOVA on IGFI mRNA level in muscle difference from one month (June 1/2nd) to four months (August 29/30th), during the post-smolt period for the Preline system

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	33,94101	1	33,94101	82,67079	0,000000
Time	1,75127	3	0,58376	1,42187	0,252818
Error	14,36947	35	0,41056		