AQUA EXCEL 2020

AQUAculture infrastructures for EXCELlence in European fish research towards 2020 — AQUAEXCEL2020

D6.4 Atlantic salmon and sea bass transfer protocols. Effects of internal transfer (cage to tank and between tanks) and sampling on salmon and sea bass, and the effect on acclimation time

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 652831. This publication reflects only the view of the author, and the European Commission cannot be held responsible for any use which may be made of the information contained therein

Executive Summary

Task 6.2.1 (Atlantic salmon)

Objectives:
Handling of fish is necessary, both in commercial farming operations and in research facilities, but will cause stress and discomfort for the fish. The time it takes for the fish to resume normal physiology and behaviour is called acclimation time, and it will depend on the severity of the stress experienced by the fish. The previous life history and handling procedures of experimental fish may affect the outcome and quality of experiments in terms of feed intake and growth as well as variability among experimental units. Handling operations in small sale research facilities are often not comparable to operations in large scale facilities, and acclimation time to new experimental units is also often a bottleneck. Transfer of fish between tanks is done by pumping in commercial facilities whereas in small-scale trials in research facilities netting of fish is normally used.

Rationale:
To test how these different handling procedures affect the stress response and acclimation time in Atlantic salmon (*Salmo salar*) smolts, an experiment with a 2x2 factorial design with handling procedure (pumping or netting) and sedation/no sedation as variables was performed. Atlantic salmon smolts were pumped or netted from 4 large tanks (3.3 m^3) with water salinity 12 ppt to 12 smaller tanks (0.5 m3) with water salinity of 32 ppt where they stayed for a period of 30 days. To measure both the acute stress responses and long term effects, samples of blood, skin and gills were taken before transfer and at regular intervals after transfer and analyzed for cortisol, blood glucose and lactate, serum ion concentrations, skin histology and gene expression. Fish welfare and growth rate were also assessed. Feed intake was measured on a daily basis both in the large and in the small tanks for the 30-day trial period, to see if handling method affected the time to resume normal feed intake.

Main Results:
The feed intake in the 3.3 m^3 was close to 2 % of bodyweight. The day after transfer, the feed intake was reduced to 0.9 %, but fish in all treatments were feeding. Fish that had been pumped took a few days longer to resume feed intake compared to fish that had been netted. However, after 5 days and for the rest of the trial there were no differences in feed intake between treatments. The fish grew well during the 30-day trial (final bodyweights 190-200 g, TGC of 2.5-2.7, SGR 1.8-1.9%) and mortality was low (less than 1%). There were no effects of treatment on growth and feed conversion ratio.
Cortisol levels were higher in netted fish, but there was no significant effect of sedation. There was however a tendency for an interaction between sedation and handling method so that the combination of netting and no sedation gave higher cortisol concentrations than the other treatments 1 h after handling. The highest glucose levels were measured at the sampling points 6 and 24 h after transfer (maximum of 4.4 mmol/l compared to basal levels of 2.7 mmol/l). Glucose was higher in fish that were netted compared to fish transferred by pumping 24 h after transfer. Lower blood glucose levels were found after sedation. In fish that were not sedated, blood lactate concentrations 1 hour after handling were higher in fish transferred by pumping than by netting. Sedation reduced blood lactate concentrations and there was no effect of handling method in sedated fish. In this experiment, salmon smolts were transferred from 12 ppt to 32 ppt. As expected, the serum concentrations of Cl, Na and

Mg was elevated after transfer compared to basal levels at 12 ppt in all treatments. However, within 48 h they stabilized within the normal range for seawater tolerant smolts in seawater. The first 6 h after handling, netting led to higher serum concentrations of all three ions, and sedation lowered serum ion concentrations confirming the stress-reducing effect of sedation in the acute stress phase. The enzyme sodium potassium ATP-ase (NKA) is involved in pumping of ions out of the chloride cells in the gills when the fish is in seawater. The basal activity was 19.4±2.3 µmol ADP/mg protein/h which indicate a seawater adapted smolt. The activity of NKA was reduced after netting and pumping the fish to the smaller tanks, but the activity was higher in netted fish where it also increased gradually until 10 d after handling. There was no effect of sedation on NKA activity. The effect of netting was still significant 10 days after transfer, whereas at 30 days after transfer there was no significant effects of transfer protocol on NKA activity.
There were several effects of treatment on the skin morphology 48 h after handling the fish. The number of mucus cells was higher in pumped fish than in netted fish, and netting tended to reduce the thickness of the epidermis. The size of mucus cells was not affected by handling method, but sedated fish had smaller mucus cells compared to fish that were not sedated. Gene expression in skin samples were analyzed before transfer from 12 ppt (basal values) and 48 h after transfer to new tanks in seawater. *Claudin 10* is a gene involved in formation of pores for cation transport through tight junctions, suggesting a role in osmoregulation in A. salmon. The expression of this gene after 48 h in seawater was increased compared to basal values. There was a tendency for a higher expression of *Claudin 10* in sedated fish. The expression of genes in the skin related to cellular stress responses (*HSP70, iNOS)* were not affected by treatment 48 h after handling. Expression of genes indicating mucus production (*MuC5ac, MuC2*) were higher in fish that were not sedated, and expression of *MuC5ac* was higher in fish that were pumped compared to netted fish. There were no effects of handling method or sedation on fin erosion and eye damage.

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Task 6.2.2 (Sea bass)

Objectives:
Transferring of fish for experimental purposes poses several challenges for the animals due to shift in the environmental conditions before- and post-transfer. Acclimation to the new conditions, which is a prerequisite for conducting fish experiments, may require substantial time which is species-specific and depends on the direction and intensity of environmental change. The objective of this study was to assess the effects, both in terms of intensity and duration, of change in environmental conditions between marine cages and tanks in experimental facilities for European sea bass *(Dicentrarchus labrax*). The aim was to determine the appropriate duration for acclimation time in order to improve research quality and facilitate experimental design.

Rationale:
The effect of change in environmental conditions was assessed by two trials performed at the research facilities of HCMR. In these trials, post-transfer fish were monitored over a

period of several weeks following their transfer from cages to tanks and the effects were evaluated by close monitoring of biochemical, haematological, hormonal, behavioural and husbandry variables. The fish were initially distributed in eight 500l tanks and every week one tank was sampled at random for the above parameters. Moreover, the size of the fish was taken into consideration in order to test the hypothesis that acclimation capacity is related to age. For this reason, the trials were performed on fish of two different sizes, small and large, referring to fish of approximately 150g and 300g.

Main Results:

Handling and transferring to new conditions causes effects on fish that may require weeks of acclimation in order to be reverted. Elevated values of post-transfer physiological variables such as cortisol and lactate were indicative of the initial stress response but physiological normality was attained fast, within the first week after transfer. However, reverting to physiological normality does not entail that adaptation to the new conditions is complete and results from the behavioural monitoring of small fish suggest that behavioural adaption requires an additional week. Furthermore, according to husbandry parameters, normality with respect to feed intake and FCR may require over a month to be achieved. Our findings suggest that acclimation is size-dependant, with smaller fish exhibiting signs of faster adaptation. Smaller fish required 3-4 weeks for adaptation while for large fish this took up to 5 weeks. This was further supported by the significantly lower mortality rates recorded for the smaller fish.

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Table of contents

I. Subtask 6.2.1: Handling impact during transfer or sampling on acclimation time

1.1 Introduction and objectives

In order to be able to improve and develop the aquaculture industry, research of high quality is necessary. Handling of fish is necessary, both in commercial farming operations and in research facilities, but will cause stress and discomfort for the fish. Transfer to seawater is often associated with increased mortality and Atlanitc salmon smolts are more sensitive to handling and confinement stress than salmon parr (Carey and McCormick 1998) and in addition the fish has to adjust to higher salinity and often changes in temperature. Stress may be defined as a condition where the dynamic equilibrium of an organism (homeostasis) is disturbed as a result of actions of internal or external stimuli called stressors. The physiological response to stressors is often divided into primary, secondary and tertiary responses (Wendelaar Bonga 1997). The primary response involves the neuroendocrine responses, with stimulation of the hypothalamic-pituitary-interrenal (HPI) axis and release of corticosteroid hormones such as cortisol. Secondary stress responses include changes in osmoregulation and metabolism and haematology (haematocrit, blood pH, CO_2 etc), immune function and cellular responses. Tertiary stress responses involve the effects on performance of the animal in terms of growth, behaviour and survival. The present experiment will address effects on all three levels. The time it takes the fish to resume normal physiology and behaviour is called acclimation time, and it will depend on the severity of the stress experienced by the fish.

The previous life history and handling procedures of experimental fish may affect the outcome and quality of experiments in terms of feed intake and growth as well as variability among experimental units. Handling operations in small scale research facilities may not be comparable to operations in large scale facilities, and acclimation time to new experimental units is also often a bottleneck. Transfer of fish between tanks is done by pumping in commercial facilities whereas netting of fish is normally used in small-scale trials in research facilities. The time it takes for the fish to resume feed intake and normal physiological functions can be affected by the handling procedures used for transferring the fish to new units. The experimental units in a trial is often smaller than the holding tanks used to keep the fish before trials, and fish often need some time to adjust to a smaller tank size. In a previous study, it was shown that different tank sizes matters for experimental fish performance (Espmark *et al.*, 2016). Suboptimal feed intake and growth reduce the relevance of a trial when comparing the results with industrial farming conditions. All handling is stressful for fish, but different handling protocol may induce different stress responses, and the use of sedation can potentially reduce the stress response (Iversen and Eliassen 2009). Sedation during transfer and transport of salmon smolts is becoming more common in commercial productions, but the effects in terms of stress are not well documented. The aim of the present experiment was therefore to examine the effect of different transfer protocols (pumping and netting) and sedation on acclimation time of Atlantic salmon smolts. The following research questions were addressed:

- What is the optimal transfer protocol for Atlantic salmon smolts in research facilities?
- How long does it take after handling stress before fish resume feed intake and normal physiology (acclimation time)?
- What is the effect of transfer on growth, physiology and welfare?
- How is the acute stress response affecting growth and feed utilisation?

1.2 Methods

1.2.1 Experimental design

The experimental design was a 2x2 factorial design where handling procedure (either pumping or netting) and sedation or no sedation during transfer of Atlantic salmon smolts (118 g, Aquagen strain) from 4 large tanks (3.3 m^3) with water salinity 12 ppt to 12 smaller tanks (0.5 m^3) with water salinity of 32 ppt (Figure 1). Fish were either sedated with iso-eugenol, 2.7 mg/l in the 3.3 m^3 tanks and then immediately pumped (Heathro Vaki Fish pump) or netted over in the 0.5 m^3 tanks or they were pumped or netted without sedation. Before pumping and netting, the water level in the 3.3 m^3 tanks were reduced to 1/3 of the original level, to facilitate transfer. The fish that were netted were transferred to the 0.5 m^3 tanks in the nets placed in buckets to minimize the time out of the water. The fish that were pumped were pumped directly from the 3.3 m^3 to the 0.5 m^3 tanks. Both the 3.3 m^3 and the 0.5 m^3 tanks were equipped with a system for collection of uneaten pellet, so that feed intake could be calculated on a daily basis based on the dry matter content of the collected pellet according to Helland et al (1996). Thus, the time before resumption of feed intake after pumping and netting and potential effects of sedation could be estimated. The fish were fed every 20 minutes with an automatic belt feeding system. In the 0.5 m^3 tanks the fish were kept in flow-through seawater (32 ‰) with mean temperature 12.6 °C, pH above 7.6, oxygen saturation above 85% and CO_2 below 5 mg/l. The fish were kept on 24 h light during the trial period in 0.5 m^3 tanks.

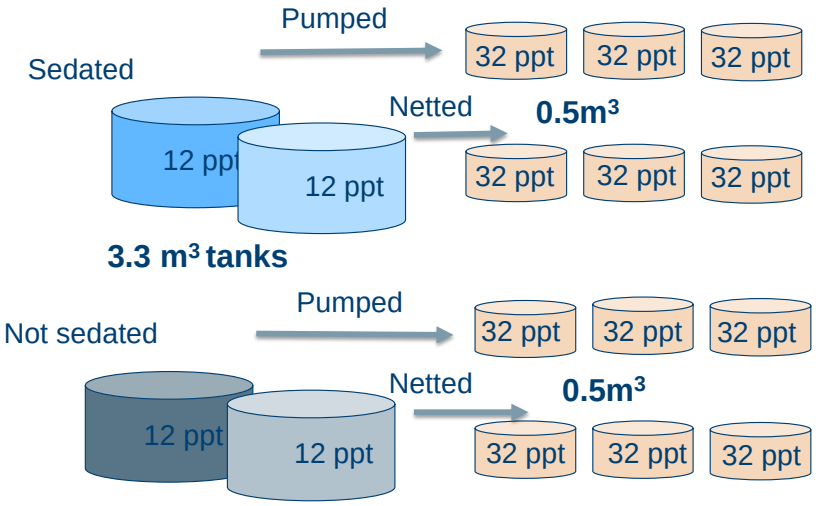

Figure 1: Experimental design

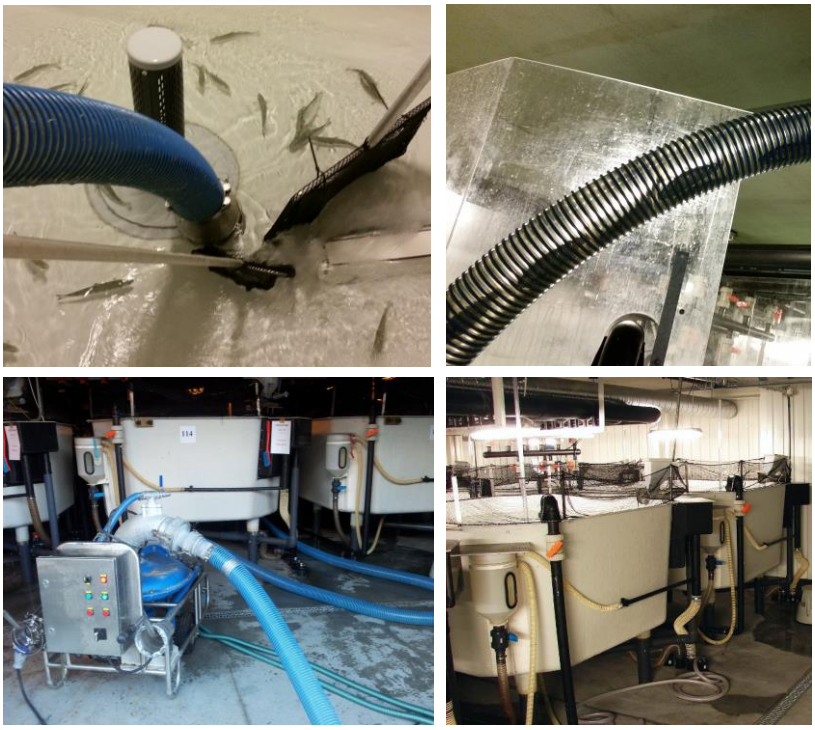

Figure 2: Equipment and setup for pumping of fish. Before pumping with a Heathro Vaki pump, the water level was lowered with 1/3 the original level, in order to facilitate pumping

1.2.2 Samples and registrations

Before pumping or netting the fish (time = 0h), 5 fish were sampled from each of the 4 3.3 m^3 tanks (blood, gills, skin) and external welfare indicators (skin lesions, operculum shortening and fin damage) were evaluated by a scoring systems developed by Kolarevic et al (2013). Each of the welfare indicators was given a score between 0 and 2, where 0 is good and 2 represents bad condition. Evaluation of fin condition (pectoral, pelvic, anal, dorsal and caudal fin) was done by a scoring system from 0 to 5 developed by Hoyle et al (2007), where 0 indicates no visible damage and 5 indicates total erosion of the fin (Figure 3). The same samples and registrations were also done on 5 fish per tank at times 1, 6, 24, and 48 hours and 10 and 30 days after transfer to smaller tanks. The response variables measured were serum cortisol, and serum concentrations of Cl, Na and Mg, whole blood glucose and lactate, gill sodium/potassium ATPase (NKA), skin histology and expression of genes related to stress and mucus secretion and cell repair in skin.

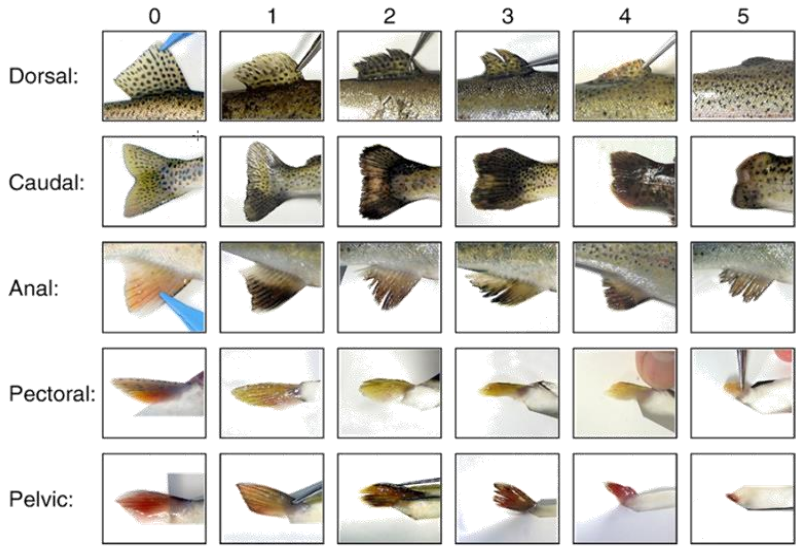

Figure 3: Scoring system used for evaluation of external welfare indicators (Hoyle *et al.* 2007). The fish above shows the external welfare indicators and fins measured

1.2.3 Analytical procedures

Blood glucose was measured in whole blood by Freestyle Lite glucose measuring system (Abbot). Lactate was measured in whole blood with Arkray LactatePro test meter equipped with LactatePro Test Strips (Shiga, Japan). Blood samples were the centrifuged and serum immediately frozen on -20 °C for later analysis of concentrations of cortisol, chloride, sodium and magnesium. Cortisol was analysed by ELISA (Demeditec Diagnostics). Cl, Na and Mg were analysed on a Horiba Pentra C400. Then a small piece of the second gill arc on the right side were sampled and analysed for NKA activity (µmol ADP mg protein h^{-1}) by Pharmaq Analytic according to the method described in McCormick (1993).
Skin samples (1 cm^2) were taken above the lateral line on the left side behind the dorsal fin. Half the sample was frozen on liquid nitrogen for analysis of gene expression with qPCR and the other half was fixated in formalin. To characterize skin surface morphology and density of mucus cells, staining with fluorescein labeled lectins was used on whole-mount skin samples. Histological image analysis were conducted with NIS-Elements software and bright field microscope (Nikon Eclipse Ci-L). The following steps were done with each sample: Visual microscopic overview of the sample, tuning focus to match with computer image, capturing image, burn scale bar, saving image, draw polygonal line to measure epidermis length, measuring width of the epidermis, manually measuring area of mucus cells, and counting number of mucus cells.
Samples for gene expression in skin were placed in RNAlater Stabilization Solution (ThermoFisher Scientific) and stored at -80°C until analysis. Total RNA from the skin samples (N = 4 per tank; 4 tanks for 0h samples and 12 tanks for 48h samples) was extracted using PureLink™ Pro 96 well purification kit (Thermo Fisher Scientific) with PureLinkTM DNase Set (ThermoFisher Scientific) according to the manufactures instructions. RNA concentrations were measured using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized on 500 ng RNA with SuperScript® VILOTM cDNA Synthesis Kit (ThermoFisher Scientific) according to the manufactures instructions. Primers sequences were designed using ePrimer3 from the EMBOSS online package and synthesized by Invitrogen (Table 1). Quantitative real-time PCR was performed on LightCycler 480 (Roche) using LightCycler® SYBR Green I Master (Roche). Reactions of 10 µL (5 µL 2xSYBR Green I Master, 0.5 µL F-primer (10 µM), 0.5 µL R-primer (10 µM) and 4 µL cDNA (1:10) were run in parallels. The conditions for the qPCR reaction were 95ºC for 5 min (pre-incubation), 95ºC for 15 s and 60ºC for 30 s (amplification, 45 cycles), 95ºC for 5 s

followed by continuous increase from 65ºC to 95ºC for one minute (melt curve). Cycle threshold values (Ct) were calculated using the second derivative method in the LightCycler 480 software (version 1.5.0.39). For evaluation of the results, the mean Ct of duplicates was used. Duplicate measurements that differed > 0.5 Ct values were removed and reanalysed

Table 1: Forward and reverse primers for real-time PCR

Gene name	Symbol	Accession number		Primer sequence
Inducible nitric oxide synthase	iNOS	AF088999.1	F	GCTAAACTGTGCCTTCAACTCCA
			R	CTCCATTCCCAAAGGTGCTAGTTA
Mucin-like 2	MUC2	JT815394.1	F	ACCACCCTGAACCATCAGTC
			R	CTCCTTCAACATCGCATCAA
Mucin-like 5ac	MUC5ac	JT819124.1	F	AGGCGTCCTTGTCCAAATAA
			R	CCTCTGGAAACTGGATGGTC
Claudin 10	Claudin10	BK006391	F	ATCAAGGTGGCCTGGTACTG
			R	GACCAGAGCACAGGGAAGTC
Cathepsin B	CathepsinB	NM_001140522.1	F	CCGGATACACACCTGGCTAC
			R	ACCCTCTACAGGCCCATTCT
Heat shock 70 kDa	HSP70	XR_001327470.1	F	TGACGTGTCCATCCTGACCAT
			R	CTGAAGAGGTCGGAACACATCTC
Elongation factor 1 alfa	Elf1a	AF321836.1	F	CACCACCGGCCATCTGATCTACAA
(Internal standard)			R	TCAGCAGCCTCCTTCTCGAACTTC

1.2.4 Calculations and statistical analysis

Specific growth rate (% day^{-1}) between two sampling points was calculated as:*SGR = (ln BW_2 – ln BW_1) x 100/d (BW= bodyweight, d = number of days)*

The thermal growth coefficient, TGC, was calculated as:
TGC =1000($BW_2^{1/3}$- $BW_1^{1/3}$) x (number of day degrees)$^{-1}$*

The feed intake per tank was calculated from the difference between the amount of feed fed to each tank and the amount of uneaten pellet collected. Individual daily and cumulative feed intake was calculated by dividing the feed intake per pen with the number of fish in the tank. The feed conversion ratio (FCR) was calculated as: feed eaten (kg)/weight gain (kg).

Statistical analysis on growth, feed intake and utilisation, welfare scores and blood parameters were performed in SAS Jmp. A two-way mixed model ANOVA with handling method (pumping or netting) and sedation/no sedation as fixed factors was performed for each sampling point. P -values < 0.05 were considered significant. Response variables given in percent were arcsin transformed before analysis by ANOVA.

1.3 Results

1.3.1 Acute stress responses

Serum cortisol peaked 1 hour after handling in all treatments, and there was significantly higher cortisol levels in netted fish ($p < 0.01$) but there was no significant effect of sedation. There was however a tendency for an interaction between sedation and handling method ($p = 0.08$) so that the combination of netting and no sedation gave higher cortisol concentrations than the other treatments (Figure 4). 6 h after handling the serum cortisol concentrations had decreased, but there were no treatment effects on serum cortisol 6 hours after handling. After 24 hours, serum cortisol was higher in fish that had been pumped ($p < 0.05$), and there was also a significantly higher concentration in sedated fish ($p < 0.005$), thus the highest cortisol concentration was found in fish that had been sedated before pumping. After 48 hours, there was no effect of handling method, but cortisol levels were higher in fish that were not sedated. After 10 and 30 days there were no longer any differences between treatments. The serum cortisol concentration was not significantly different at sampling points 48 h and 10 and 30 days, but it was slightly higher than the concentrations before the experiment started. The basal values were obtained from fish that had been starved for 48 h before exposed to handling by pumping and netting, whereas for the other sampling points, concentrations were measured in fish that were feeding, and this could have influenced the results.

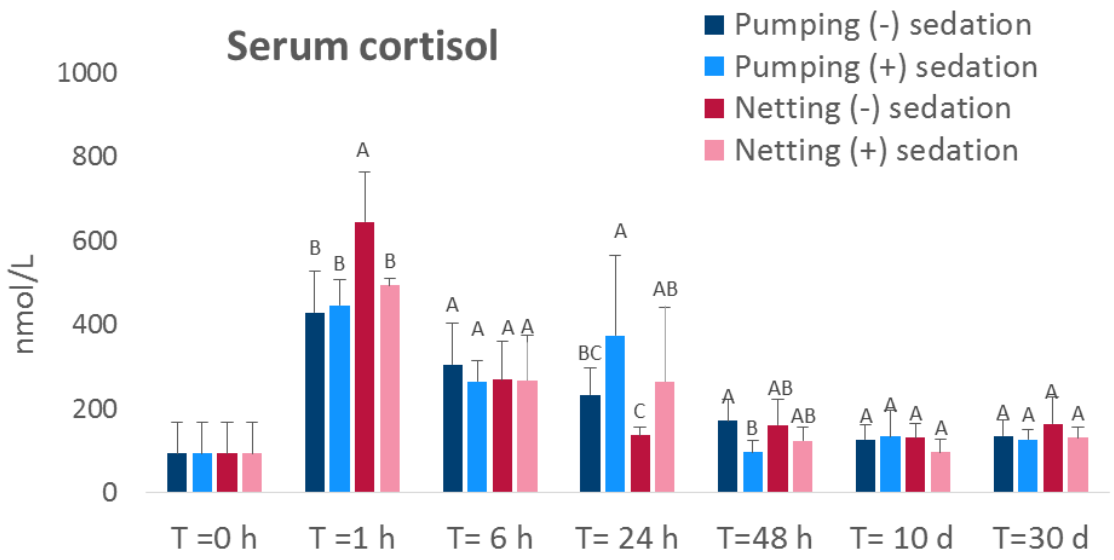

Figure 4: Serum cortisol concentration in the different treatments before handling (T = 0 h) and up 30 days after handling. Values are means per treatment ± SD (n = 3 tanks per treatment, 5 fish were sampled per tank). Different letters indicate significant differences between treatments within each sampling point.

The highest glucose levels were measured 6 and 24 h after transfer (maximum of 4.4 mmol/l compared to basal levels of 2.7 mmol/l). Glucose was higher in fish that were netted compared to fish transferred by pumping from 24 h to 10 days after transfer ($p < 0.05$) (Figure 5A). One hour after handling, blood glucose was higher in fish that were sedated with iso-eugenol ($p < 0.05$), whereas lowered glucose levels were found after sedation both at 6 and 24 h after handling compared to fish that were not sedated ($p < 0.05$). After 30 days,

there were no significant effects of handling or sedation on blood glucose. Blood lactate peaked 1 h after transfer (maximum of 15.3 mmol/l) but decreased rapidly, and at 24 h after transfer the lactate levels were close to the level before pumping and netting (4.5 mmol/l) where they remained for the rest of the trial (Figure 5B). In fish that were not sedated, blood lactate concentrations were higher in fish transferred by pumping than by netting 1 hour after handling. Sedation reduced blood lactate concentrations 1 h after handling ($p < 0.0001$) and there was no effect of handling method in sedated fish. After 6 hours, the lactate levels were still higher in fish that were pumped without sedation compared to the other treatments ($p < 0.01$).

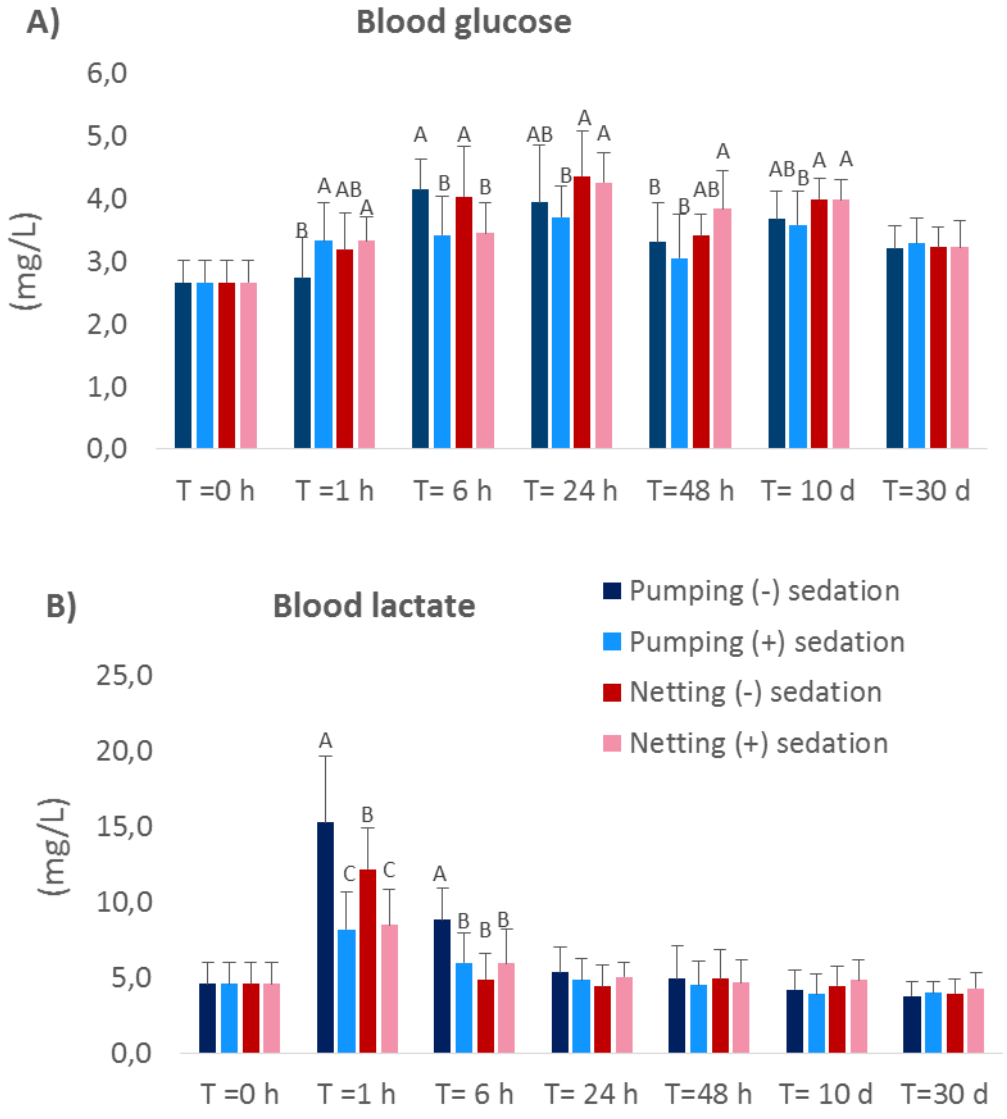

Figure 5 A) Blood glucose and B) blood lactate in the different treatments before handling (T = 0 h) and up 30 days after handling. Values are means per treatment ± SD (n = 3 tanks per treatment, 5 fish were sampled per tank). Different letters indicate significant differences between treatments within each sampling point.

1.3.2 Effects on osmoregulation

In this experiment, salmon smolts were transferred from 12 ppt to 32 ppt. As expected, the serum concentrations of Cl, Na and Mg was elevated after transfer compared to basal levels at 12 ppt in all treatments (Figure 6). However, after 48 h they stabilized within the normal range for seawater tolerant smolts in seawater (Cl<140, Na <170, Mg<1.4).

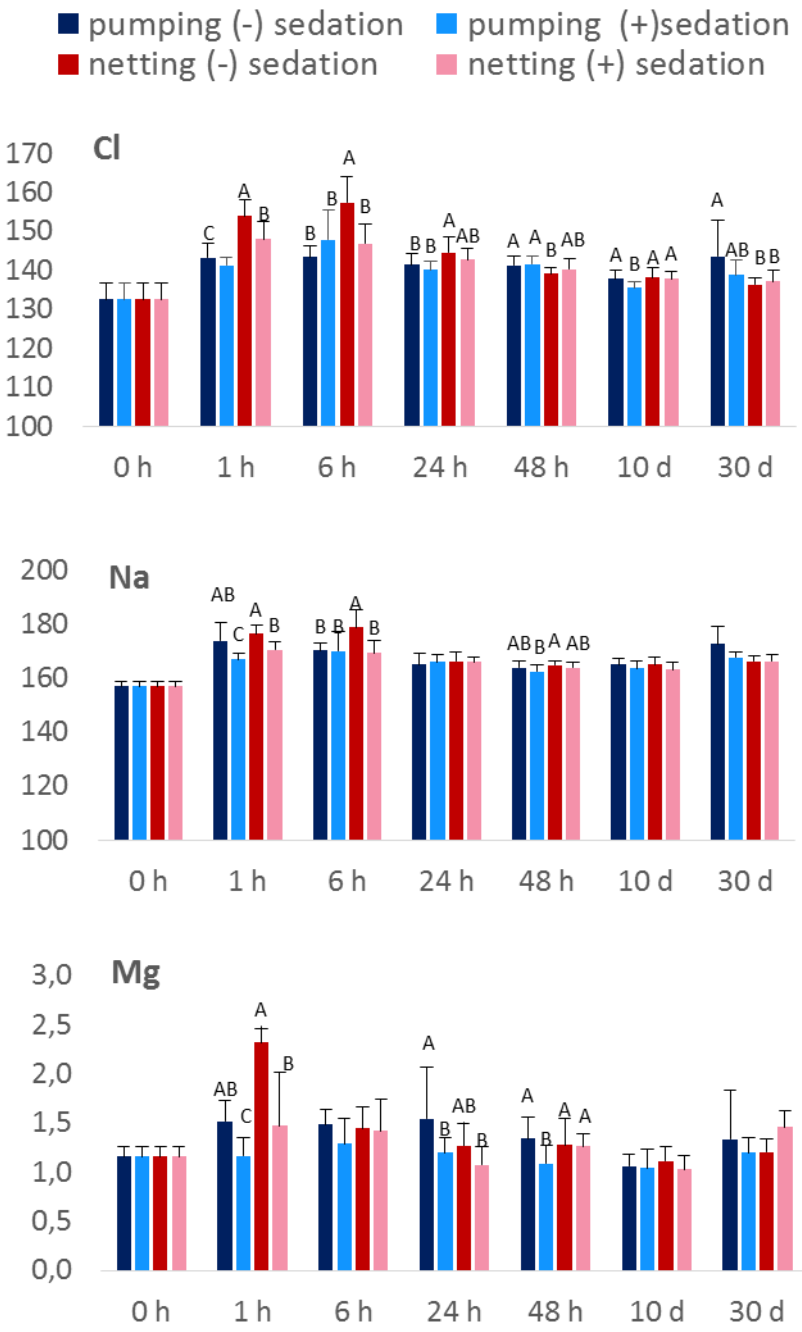

Figure 6 serum chloride (Cl) Sodium (Na) and magnesium (Mg) in the different treatments before handling (T = 0 h) and up 30 days after handling. Values are means per treatment ± SD (n = 3 tanks per treatment, 5 fish were sampled per tank). Different letters indicate significant differences between treatments within each sampling point.

However, serum ion concentrations were affected by transfer protocol. The first 6 h after handling, netting led to higher serum concentrations of all three ions, and sedation lowered

serum ion concentrations confirming the stress-reducing effect of sedation, as indicated by the effects on blood glucose and lactate. For Na, there were no significant treatment effects after 24 h, and a marginal lowering effect of sedation after 48 h ($p < 0.05$). Cl concentrations were higher in netted fish at 24 h after handling ($p < 0.01$), whereas after 48 h concentrations were higher in pumped fish ($p < 0.05$). There was no significant effect of sedation on serum Cl concentrations at 24 and 48 h and after 10 and 30 days. There was however an effect of netting after 10 and 30 days, giving slightly higher Cl concentrations ($p < 0.05$). The serum Mg concentration was higher in pumped fish and fish that were not sedated after 24 h. The effect of sedation was still evident after 48 h whereas the effect of handling was not. After 10 and 30 days there were no significant effects of treatment on serum Mg concentrations.

The enzyme sodium/potassium ATP-ase (NKA) is involved in pumping of ions out of the chloride cells in the gills when the fish is in seawater. The basal activity was 19.4±2.3 µmol ADP/mg protein/h, which indicate a seawater adapted smolt. The activity of NKA was reduced after netting and pumping the fish to the smaller tanks ($p < 0.001$). The activity was higher in netted fish where it also increased gradually until 10 d after handling (Figure 7), which could be related to the higher serum ion concentrations in these fish that may have triggered the expression of NKA. There was no significant effect of sedation on NKA activity. The effect of netting was still significant 10 days after transfer, whereas at 30 days after transfer there was no significant effects of transfer protocol.

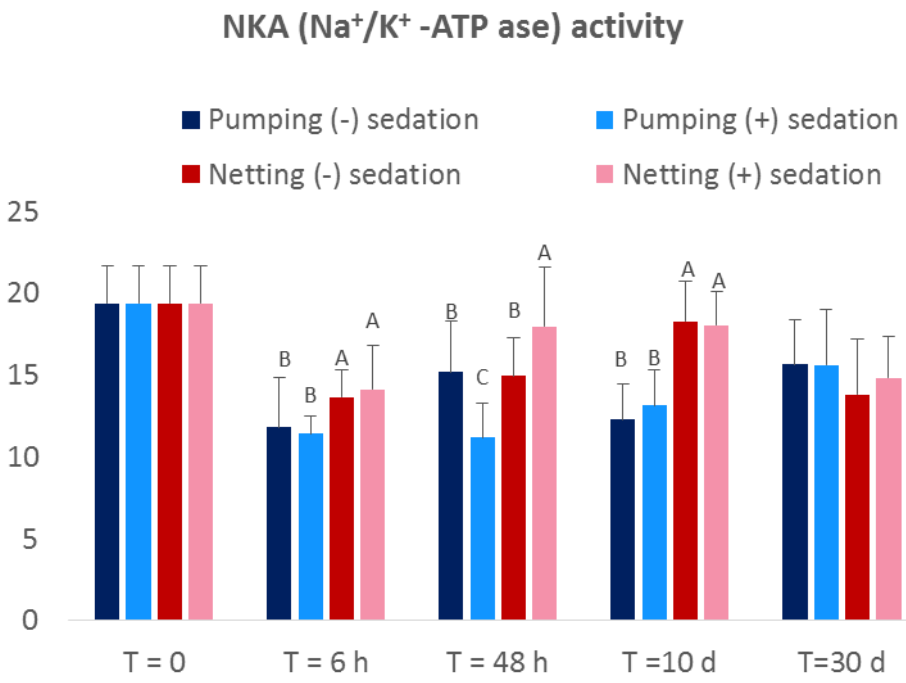

Figure 7. Activity of the enzyme sodium potassium ATP-ase (NKA) (µmol ADP/mg protein/h) in the different treatments before handling (T = 0 h) and up 30 days after handling. Values are means per treatment ± SD (n = 3 tanks per treatment, 5 fish were sampled per tank). Different letters indicate significant differences between treatments within each sampling point.

1.3.3 Skin morphology and gene expression

There were several effects of treatment on the skin morphology 48 h after handling the fish. The variables that were quantified were number of mucus cells per µm^2, and the size (area) of each mucus cell, and the width and surface area of epidermis (figure 8). The number of

mucus cells was higher in pumped fish than in netted fish ($p < 0.01$) (Figure 9). The area of each mucus cell was however not affected by handling method, but sedated fish had a smaller mucus cell area than fish that were not sedated ($p < 0.05$). The effects of treatment on epidermis surface area was similar to the effects on mucus cell area, sedation gave a lower epidermis surface area ($p< 0.05$). Epidermis width responded similar to the number of mucus cells, netting tended to reduce epidermis width ($p = 0.056$).

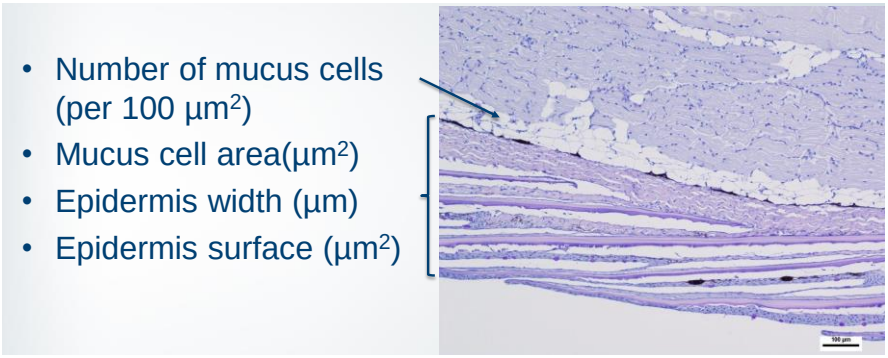

Figure 8: Microscopy image of skin sample showing location of mucus cells and epidermis

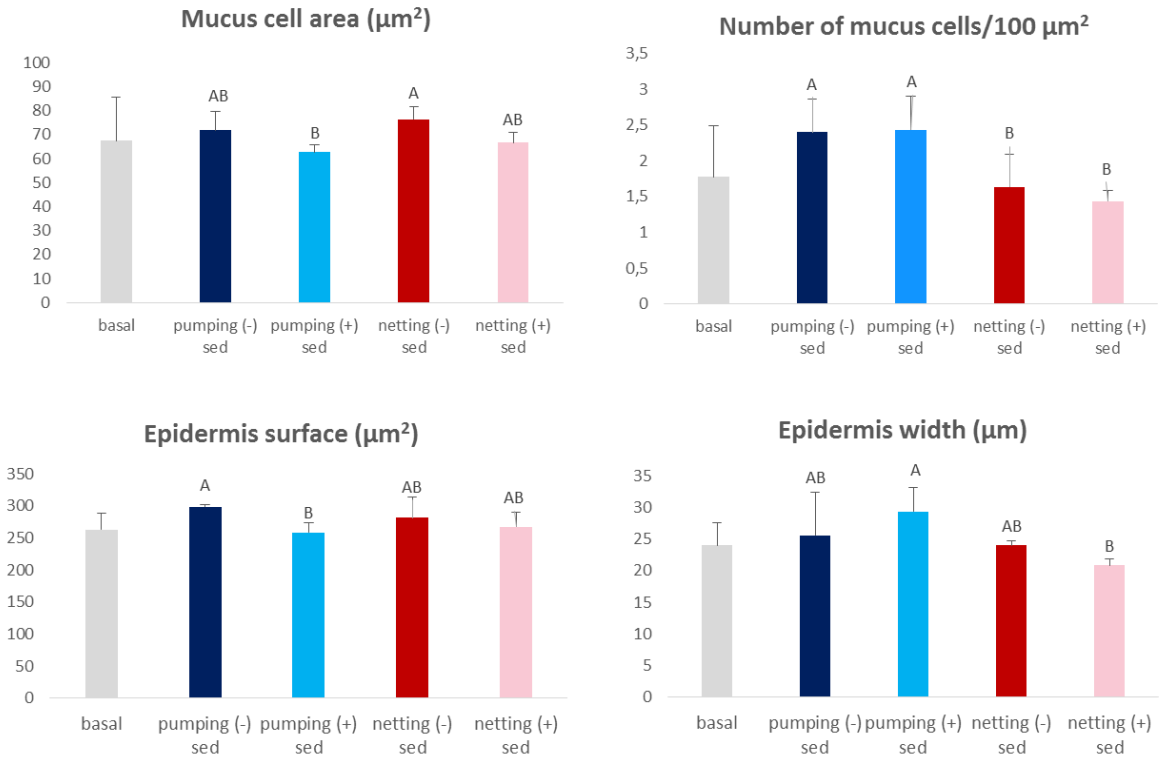

Figure 9: Mucus cell area, number of mucus cells, epidermis surface area and width in salmon before (basal) and 48 h after handling by pumping/netting with and without sedation. Values are means per treatment ± SD (n = 3 tanks per treatment, 5 fish were sampled per tank). Different letters indicate significant differences between treatments.

Gene expression in skin samples were analysed only at T=0 (basal values) and 48 h after transfer to tanks in seawater. *Claudin 10* is a gene involved in formation of pores for cation transport through tight junctions (Krause *et al.*, 2008) and a large number of caludins are found in fish skin, including *claudin 10* (Kolosov *et al.*, 2013). Differential expression of claudins in skin in seawater and freshwater suggest osmoregulatory functions of claudins in skin of teleosts (Kolosov *et al.*, 2013). 48 hours after pumping and netting, the expression *of*

claudin 10 in skin was increased compared to basal values ($p < 0.05$), but there was no significant effect of handling method or sedation due to large individual variation. There was however a tendency for a higher expression in sedated fish ($p=0.11$). *Claudin 10* is involved in osmoregulation, its transcription in the gill increases during seawater acclimation in Atlantic salmon (Tipsmark *et al.*, 2008). So, the increase in skin after handling stress and transfer to 32 ppt suggest a role in osmoregulation in skin, making the skin more permeable to ions. It is possible that it was the salinity increase, and not the handling stress that caused the increase in expression. The expression of genes in the skin related to cellular stress responses (*HSP70, iNOS)* were not significantly affected by treatment 48 h after handling (Figure 10). Expression of *Cathepsin B*, a gene indicating cellular turnover, was higher in fish that were not sedated compared to sedated fish ($p < 0.05$) but there was no effect of handling method. Expression of genes indicating mucus production (*MuC5ac, MuC2*) were also significantly higher in fish that were not sedated ($p < 0.05$). Expression of *MuC5ac* was also higher in fish that were pumped compared to netted ($p < 0.01$).

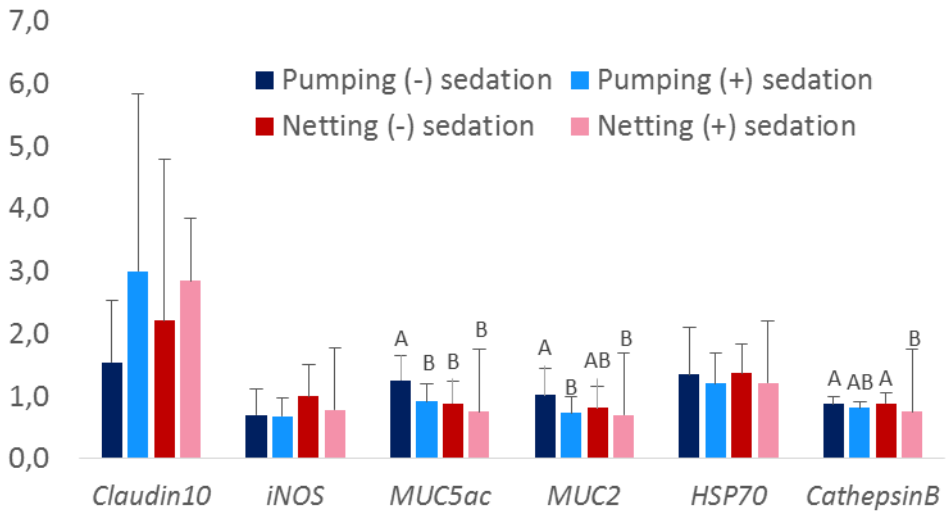

Figure 10: Expression of genes related to stress responses and mucus secretion in skin 48 h after transfer to seawater by pumping/netting with and without sedation. The values are expressed relative to basal values (basal value = 1). Values are means per treatment ± SD. (n=3 tanks per treatment, 4 fish per tank were analysed). Different letters indicate significant differences between treatments.

1.3.4 External welfare indicators

There were no significant effects of handling method or sedation on morphological welfare indicators measured during the experiment.

Fish used in the experiment had visible fin and eye damages before handling.

The frequency of individuals with dorsal and pectoral damage at 0h was 81% and 94% respectively. The prevalence of dorsal fin damage increased for all treatments 1h post handling and was reduced 24h post treatment for majority treatments. At the end of the experiment, the frequency of dorsal fin damage was lower in netted fish compared to pumped fish and in comparison to 0h (Figure 11). Pectoral fin damage prevalence remained high throughout the experiment. Caudal fin damage frequency was observed in 56% examined fish at the start of the experiment and was in particular high for treatments that were not sedated prior to handling.

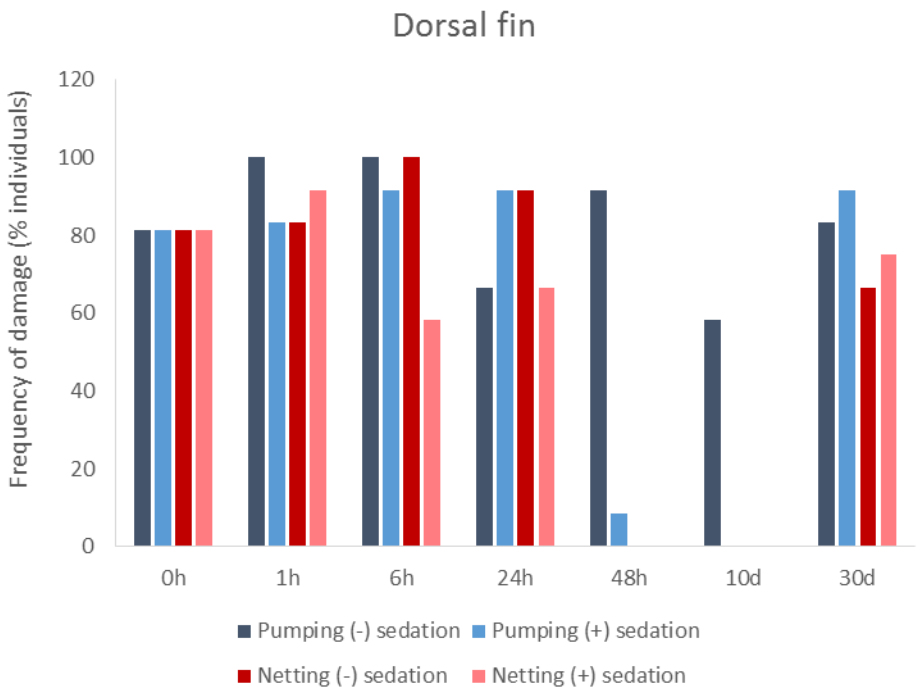

Figure 11. Frequency of dorsal fin damage in all treatments at 0, 1,6,24,48 h and 10 and 30 days post-treatments (n = 3 tanks per treatment, 5 fish were sampled per tank).

Severity of dorsal, caudal and pectoral fin increased 1h post-treatment for all groups (Figure 12). However, there were no significant differences between treatments at this point. Skin lesion score almost doubled for groups that were not sedated 1h post-treatment, while severity of the eye damage remained the same as prior start of the experiment. Fish that were netted and sedated showed tendency (p=0,06) for significantly lower pelvic fin score at the end of the experiment compared to other sampling time points. This might have been related to the fact that fish were moved from larger to smaller tanks.

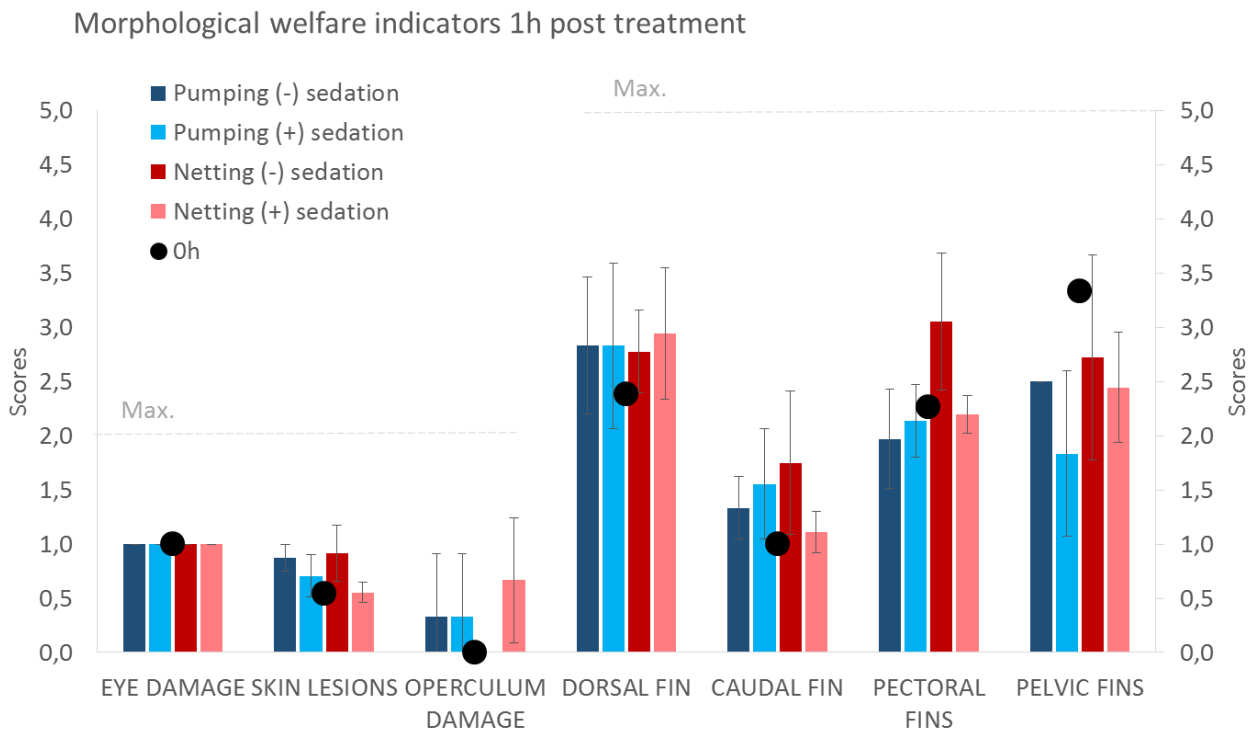

Figure 12. Scores for skin, opercular and eye damage (0-2) and fin damages (0-5) in the different treatments before handling (T = 0 h; presented as a black dot) and 1h after handling. Values are means per treatment ± SD (n = 3 tanks per treatment, 5 fish were sampled per tank).

1.3.5 Feed intake and growth

Feed intake was measured daily in all tanks, both in the 3.3 m^3 before pumping/netting and after transfer to the 0.5 m^3 tanks. The feed intake in the 3.3 m^3 was close to 2 % of bodyweight. The day after transfer, the feed intake was reduced to 0.9 %, but fish in all treatments were feeding. Fish that had been pumped took a few days longer to resume feed intake compared to fish that had been netted (Figure 13). However, after 5 days and for the rest of the trial there were no differences in feed intake between treatments. The fish grew well during the 30-day trial (final bodyweights 190-200 g, TGC of 2.5-2.7, SGR 1.8-1.9%) and mortality was low (less than 1%). There were no effects of treatment on growth and feed conversion ratio (Figure 14).

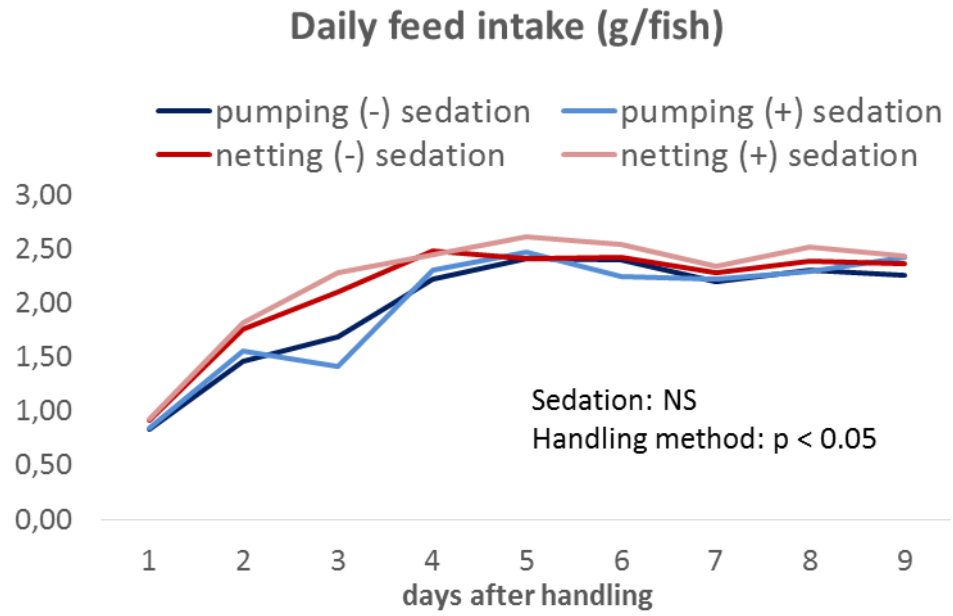

Figure 13. Daily feed intake (% of bodyweight) the first 9 days after transfer to smaller tanks. by pumping/netting with and without sedation. Values are means per treatment (n = 3 tanks).

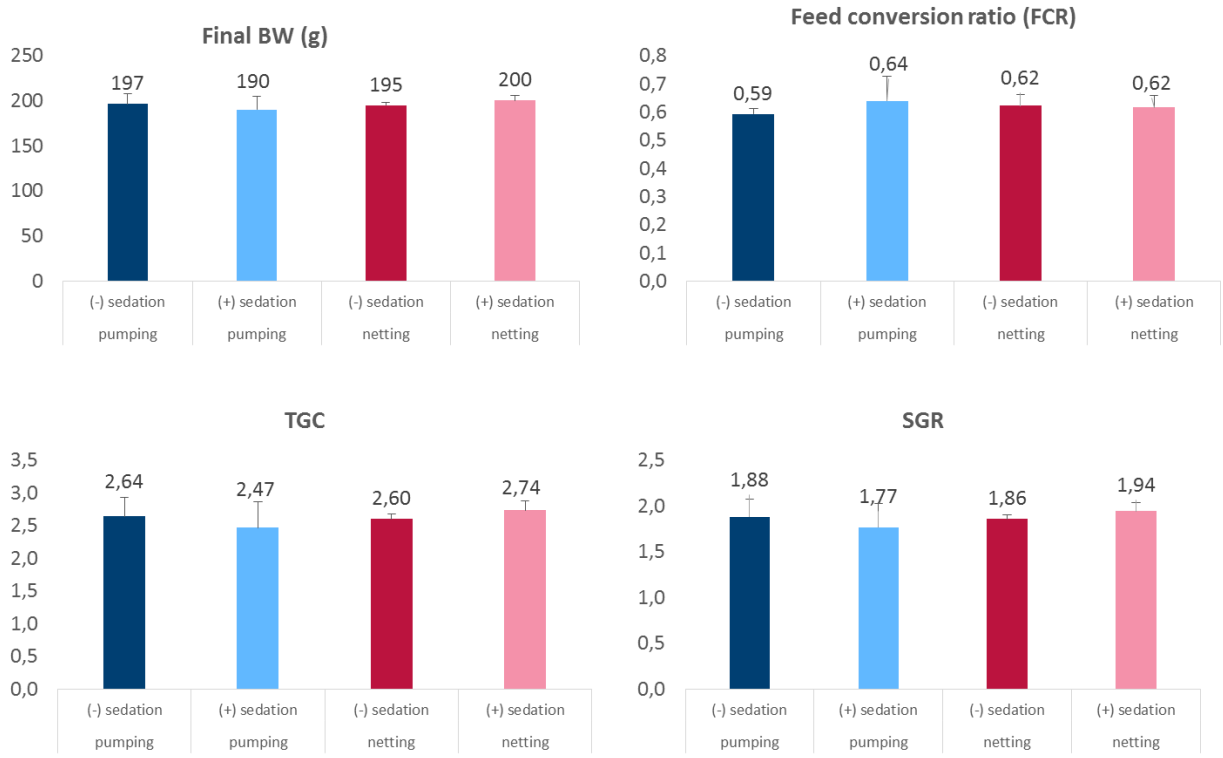

Figure 14. Final bodyweight (g), feed conversion ratio (FCR), and growth rate (TGC and SGR) in salmon transferred to smaller tanks in seawater bu pumping/netting with and without sedation. Values are means per treatment ± SD (n = 3 tanks per treatment).

Discussion

In this trial, Atlantic salmon smolts were transferred from 12 ppt to 32 ppt using four different protocols. Pumping of fish between tanks, wellboats and net pens is the common practice in

commercial salmon farming, whereas transfer of fish between tanks by netting is the most common practice in research facilities. If these protocols differ in how they affect stress response, acclimation time and welfare in salmon smolts this may be important to account for when designing experiments as well as when implementing results from research in commercial farming. Sedation is being used during routine operations in aquaculture such as vaccination, but the use of sedation during transport and transfer of fish is increasing. Anaesthetics based on iso-eugenol is being used in commercial fish-farming in Norway and has shown promising effects as a stress reducing sedative that may improve fish welfare and survival (Iversen and Eliassen 2009). Iso-eugenol has been shown to reduce cortisol secretion at concentrations above 40 mg/l (Iversen *et al.*, 2003) which is higher than what is used in the current study. However, sedation with iso-eugenol reduced the cortisol concentration in serum in netted fish 1 h after handling, whereas there was no effect of sedation in pumped fish. At 6 h after handling there were no treatment effects on serum cortisol, but after 24 h there were higher cortisol in fish that had been sedated. At this sampling point, the lowest cortisol level was found in fish that was netted without sedation. At 48 h, cortisol levels were again higher in fish handled without sedation. Sedation with iso-eugenol reduced blood glucose and lactate levels the first 24 h after handling, indicating a stress reducing effect of sedation. Lower concentrations of serum Cl, Na and Mg in sedated fish confirm the stress-reducing effect of sedation, particularly when handled by netting. Fish in seawater is hypo-osmotic to the surrounding water, and osmoregulate by drinking seawater and actively secreting ions over the gill epithelium and kidney. The enzyme sodium-potassium ATPase (NKA) is involved in pumping of ions out of the epithelial chloride cells to the surrounding water. In freshwater, NKA is involved in ion uptake (Evans *et al.*, 2005). Cortisol has been shown to be important for seawater tolerance and osmoregulation, and cortisol increase the activity of NKA (McCormick *et al.*, 2008). In the present study, the highest NKA activity was found in netted fish, which also had the highest cortisol concentrations after handling. The effect of netting on NKA activity was evident even 10 days after handling the fish. However, the serum concentrations of Cl, Na and Mg was also higher in netted fish the first 1-6 h after handling, particularly in fish netted without sedation, indicating a higher stress level in these fish. The largest effect of sedation was found on serum Mg concentrations 1 h after transfer. Iversen and Eliassen (2009) found elevated plasma Mg two weeks after seawater transfer in A. salmon smolts that had not been sedated during transport whereas in smolts that were sedated the plasma Mg concentrations were reduced to pre-stress levels within 12 h. In the study by Iversen and Eliassen (2009) there were no correlation between the primary stress response and the secondary stress indicators (plasma glucose and ion concentrations). In the present study, the highest ion concentrations were found in the treatment with the highest cortisol concentrations 1 h after handling (netting without sedation).

Blood or plasma glucose is a commonly used indicator of secondary stress responses in fish, and increased blood glucose levels can be observed within one hour after exposure to a stressor. Mobilisation of energy in the form of glucose is necessary to meet the increased energy demand to overcome the effects of the stressor. Both adrenalin and cortisol mobilise and stimulate gluconeogenesis. However, the correlation between the primary stress response indicator cortisol and blood glucose is not always evident, and both an increase and decreases in plasma glucose has been observed after cortisol administration (Wendelaar Bonga 1997, Mommsen *et al.*, 1999). The glucose levels in carnivorous fish like A. salmon may be highly variable and dependent on feeding rate and time since last meal, and may thus not be a reliable indicator of stress level. In the present study all the fish were not fed the last 48 h before handling, and the effects on blood glucose 1-6 h after handling should thus be linked to the stress associated with handling protocol since the fish at that point had not started feeding after transfer. At 6 h after transfer, blood glucose was lower in sedated fish while there was no difference between netted and pumped fish, even though netted fish had higher plasma cortisol concentrations 1 h after handling. After 24 and 48 h blood glucose was higher in netted fish, and this could be due to the higher cortisol level in netted fish after handling, but netted fish also had a higher feed intake than pumped fish until

4 days post handling, and this could have affected blood glucose as well. The reason for the higher feed intake in netted fish the first days after handling is currently not known. If it was an adaptive mechanism to cope with increased energy demand in the most stressed fish, it would be expected to find a higher feed intake in the fish that were not sedated since they had the highest cortisol and glucose levels, and lactate levels. However, there were no effect of sedation on the resumption of feed intake, and all treatment groups started to feed normally within 5 days after handling and transfer to seawater. Furthermore, for the 30-day trial period there were no significant effects of handling protocol on growth, feed intake and feed utilisation.

Fish skin is the barrier between the internal and external environment of the fish, and an intact skin is important for avoiding entrance of pathogens. The skin is covered by a layer of mucus, and mucus secretion of skin and gills increase in response to stress (Shepard, 1994, Varsos *et al.*, 2008). It could be anticipated that fish that were not sedated would struggle more both during netting and pumping, and could thus be more susceptible to skin damage compared to sedated fish. The skin damage scores increased as a result of both pumping and netting over in smaller tanks, from a basal value of 0.4 before handling, mean values between 0.4 and 1 was observed for the rest of the trial. At samplings 1 and 6 h after handling, there were lower skin damage scores in sedated fish compared to fish that were not sedated. There were higher skin damage scores in pumped fish 24 h after handling. The results on skin histology showed a higher number of mucus cells and a thicker epidermis in pumped fish 48 h after handling, which could indicate a more severe effect of pumping on the mucus layer compared to netting. There was however no effect of sedation on these parameters, but sedation had an effect on the size of mucus cells, larger mucus cells were found in non-sedated fish, which could indicate increased mucus production. Gene expression of selected genes relates to mucus secretion and stress in skin were done 48 h after handling of the fish. For the genes related to mucus secretion (*MUC5ac, MUC2*) there were increased expression in skin of fish that were not sedated, and for *MUC5ac* the expression was also higher in pumped fish compared to netted fish. Genes indicating cellular stress (*iNOS, HSP 70, Cathepsin B*) also showed slightly higher expression in fish that were not sedated, but no effects were found for handling method. The only gene that was expressed at higher levels compared to basal levels was *Claudin 10*. Claudins are a family of transmembrane proteins of tight junctions between cells, and they determine the permeability of epithelia and endothelia. Claudins can tighten the cleft between cells completely, or they can form paracellular ion channels (Krause *et al.*, 2008). *Claudin 1*0 has been shown to form paracellular cation channels increasing the permeability for cations and is considered to belong to the pore-forming claudins (Krause *et al.*, 2008). Studies on knock-out mice has shown that *Claudin 10* is essential for Na^+ transport, in *Claudin 10* deficient mice, transport of Na^+ was decreased while transport of Mg^{2+} and Ca^{2+} was increased. As a result, the *Claudin 10* deficient mice developed hypermagenesemia and nephrocalcinosis (Breiderhoff *et al.*, 2012). The increased expression of *Claudin 10* observed after transfer to seawater in the present study could indicate an increased permeability of the skin after seawater transfer, and be an adaptation to seawater. But the expression also seems to be influenced by sedation; all though the effect of sedation was not significant, sedated fish tended to have higher expression of *Claudin 10* than fish that were not sedated. Fish that were not sedated had higher concentrations of serum ions 1-6 h after transfer, particularly of Mg^{2+}. However, the serum Mg^{2+} returned to basal values within 48 h in the present study, in contrast to what was found by Iversen and Eliassen (2009) where Mg^{2+} concentration was still elevated 1 week after transfer in un-sedated fish while returning to basal within 12 h in sedated fish. The stress load was however more severe in the study by Iversen and Eliassen, with fish being loaded and unloaded from a truck, and transported for 2 h, and the non-sedated group experienced higher mortality than the sedated group, which was not the case in the present study. Thus, the stress reducing effect of sedation during handling and transport is dependent on the severity of stress experienced by the fish

Conclusions

There were no large differences between using pumping and netting for transfer of A. salmon smolts between indoor tanks in terms of the primary, secondary and tertiary stress response.

Sedation of the fish with iso-eugenol had some stress reducing effects: Sedation reduced serum cortisol, blood lactate and serum ions shortly after handling. However, there was a delayed increase in serum cortisol in sedated fish 24 h after handling.

Sedation also resulted in better skin health as assessed by histology, gene expression and visual assessment.

Feed intake was resumed to pre-handling levels within 5 days in all treatment groups, but it took a few days longer to feed normally for the pumped fish. During the 30-day trial, there were no significant effects of handling method or sedation on feed intake and growth

Acknowledgements

We would like to thank the technical staff at Nofima for setup and technical solutions during the trial, in particular Frode Nerland and Britt Kristin Megård Reiten. We also thank Gunnhild Seljehaug Johansson and Hanne Johnsen for analysing skin histology, Katrine Hånes Kirste for qPCR and Tonje Kristin Vikan for serum ion analysis. Finally, we would like to thank Bendik Fyhn Terjesen for contributing to the initiation of this work.

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II Subtask 6.2.2: Effects of environmental change before trials

2.1 Introduction and objectives

Farmed fish are transported several times during their life cycle with at least one transfer occurring between hatcheries and grow-out facilities (Rosten, 2010). In particular, laboratory fish research heavily relies on obtaining live specimens from hatcheries, nurseries, and commercial or experimental farms. An important consideration when conducting fish experiments in vivo is the use of animals that are in a stable physiological, biochemical, and behavioural state so that the effects of potential treatments can be clearly recognized. If this condition is not satisfied and animals are found in transitioning states, then the underlying mechanism interfere with the effects of the research treatment. Subsequently, this undermines the quality of the research and renders the significance of potential findings questionable.

Transferring of fish for experimental purposes poses several challenges for the animals. These relate both to the shifting environmental conditions and the transferring procedures themselves.

With respect to the former, although differences in environmental parameters between the various facilities can be minimized with careful transferring protocols, they are still bound to occur due to the extensive list of parameters involved. These differences may relate to common water physicochemical parameters such as temperature, acidity, pH, turbidity, hardness, dissolved oxygen (DO), concentration of heavy metals and organic nutrient load as well to the hydrodynamic conditions (Hjeltnes *et al.*, 2008). Moreover, rearing conditions relating to the rearing volume and type as well as the feeding schedule or feed composition are also likely to differ. It follows that the magnitude of the effects on the fish will depend upon the degree of change in these parameters as well as the species-specific tolerances and rearing requirements.

As far as the transferring procedures are concerned, they are often conducted in a way that minimizes disturbance to the fish. For instance, sedation is used when physical handling is required, and additional oxygenation is provided when necessary. Nevertheless, handling procedures during sampling remain highly stressful for the fish. The fish are aggregated in smaller confinements within the cages, are chased with nets or other devices, are exposed to air and low oxygen conditions for a duration the may last several minutes and lastly, they are subjected to various forms of physical handling that entails transfer between buckets, tanks, and containers. Such physical handling is known to cause injuries, removal of the skin's protective mucus layer and infections. In many cases, substantial mortalities are recorded for several days after sampling procedures, although some species appear relative high levels of tolerance to them (Ramsey *et al.*, 2009). Moreover, the magnitude of stress caused by handling is reflected by elevated hormonal concentrations in the blood. For instance, many commercial Mediterranean species such as European sea bass and meagre are known to evoke an acute stress response to physical disturbance as in the case of chasing for sampling purposes (Samaras *et al.,* 2016). Cortisol, which is the main stress hormone shows high levels right after sampling and in fact this appears to be affected by both the duration and the intensity of the handling (Fatira *et al.*, 2014). Therefore, many physiological variables are influenced by the handling operations and this hinders the detection and interpretation of results in an experimental setting. Although it is regarded that the initial response to acute stress has a relatively short lifetime (followed by a slower secondary and tertiary response),

its exact duration is not known for the majority of species and for the various handling procedures (Iwama *et al.*, 2006).

Due to the aforementioned challenges, fish invoke regulatory mechanisms in order to cope with the environmental changes. Although shift in behaviour is fast and is considered the first line of regulation animals employ, it is of limited use for farmed animals because confinement does not allow escape from their rearing conditions. Therefore, physiological regulation plays the most vital role on post-transfer fish. The new approach to understanding such regulatory mechanisms is through the physiological framework of allostasis (Korte *et al.*, 2007; Ramsey and Woods, 2014). Allostasis, which is described by *stability through change*, involves mechanisms that alter the physiological variables according to the levels of anticipated change. This equips the organisms with plasticity to cope with a wide range of environmental changes by adaptive changes to their metabolism, and the immune and cardiovascular systems. However, if changes exceed certain species-specific thresholds then the allostatic load becomes too high and the animals become prone to pathologies (Korte *et al.*, 2007).

Because not all allostatic mechanisms are activated at the same speed, a substantial acclimation time is required to achieve a new steady state. Over time, the fast regulatory mechanisms that were employed at the initial exposure of the fish to the new conditions give way to more permanent modifications which allow optimization of performance under the new conditions. During this acclimation period, biochemical, morphological and behavioural changes may occur. For instance, the lipid structure of cell membranes changes in response to acclimation to different temperatures which in turn affects the membrane fluidity and its permeability (El-Sheekh*et al.*, 2017; Mellery *et al.*, 2016). In addition, expression of specific proteins called Heat Shock Proteins (HSP), that have a protective role for cell functions, is known to be induced under stress conditions (Roberts *et al.*, 2010). In other cases, acclimation mechanisms may involve tissue reorganization and alteration of organ size as is the case of heart hypertrophy in response to high temperature (Keen *et al.*, 2016), which is believed to be caused by low oxygen conditions (Pörtner *et al.*, 2017). Finally, regulation of enzymatic activity in the gut is a long term mechanism for acclimation to new diets. Overall, although the process is documented for many species, the complexity and diverse nature of the underlying mechanisms involved in acclimation are not fully understood while the acclimation time required seems to be highly species-specific.

The objective of this study was to assess the effects of change in environmental conditions that fish are subjected to before initiation of experimental trials. Specifically, the study investigated the effects of transfer of European sea bass (*Dicentrarchus labrax*) from marine Mediterranean cages to tanks both in terms of intensity and duration. The aim was to determine the appropriate duration for acclimation time in order to improve research quality and facilitate experimental design. To achieve that, two trials were conducted at the research facilities of HCMR. In these trials, post-transfer fish were monitored over a period of several weeks following their transfer from cages to tanks and the effects were evaluated by close monitoring of biochemical, haematological, hormonal, behavioural and husbandry variables. Moreover, the size of the fish was taken into consideration in order to test the hypothesis that acclimation capacity is related to age. For this reason, the trials were performed on fish of two different sizes, small and large, referring to fish of approximately 150g and 300g.

2.2 Methods

2.2.1 Site and rearing

The trials were conducted between Oct 2016 and Jun2017 at the intensive hatchery facilities of HCMR in Heraklion, Crete. Juvenile E.sea bass was obtained from the HCMR pilot scale farm at Souda Bay, north-west Crete, where they had been reared under natural photoperiod and temperature in rectangular cages of 1000m^3 volume (Figure 1, left). The fish were captured using standard sampling procedures (Samaras *et al.*, 2017) and transferred to the experimental facilities via truck in plastic containers of 2 m^3 volume (Figure 1, right). The duration of transit did not exceed 4 hours while a preventive treatment protocol was implemented during transfer to minimize this risk of contaminating the research facilities with pathogens.

Figure 1. Rearing cages at the HCMR pilot scale farm (left) and 2 m^3 containers used for transferring fish (right).

2.2.2 Experimental setup

The effect of different environmental conditions before trials was investigated for two fish sizes, small and large. In both cases, blood samples were collected from eight fish at the farm site right before transfer, which were used as controls for the physiological variables. In addition, 10 individuals were sampled upon their arrival at the experimental facilities to determine the post-transfer values.

Small fish

The first trial considered small fish of mean weight 136±20g and was conducted between 26 Oct to 21 Dec 2017. After transfer to the HCMR facilities, they were randomly distributed in eight 500l tanks, thus forming eight experimental groups. In total, 112 fish were used, which resulted in an initial density of 3.6 kg/m^3. Subsequently, a group was chosen at random every week for growth and physiological parameter monitoring. Every group was only sampled once during the eight-week trial. Growth was monitored by weight measurements for estimation of the Specific Growth Rate (SGR) while the consumption of feed was recorded daily to allow for the calculation of total feed intake and Feed Conversion Ratio (FCR). SGR was calculated as the percentage of weight gain on a daily basis and FCR as the feed given over the weight gained.

Physiological monitoring was performed with blood collection from the caudal vain via heparinized syringes for the quantification of biochemical, haematological, and hormonal parameters. After determination of haematocrit (HTC), the blood samples were centrifuged at -4^{o}C and stored at -20^{o}C until further analysis.

Behaviour related to swimming activity was monitored continuously with cameras attached above each tank. The parameters considered were the swimming level, the swimming speed, and the schooling behaviour. These were evaluated semi-quantitatively by assigning behaviour to a number of categorical values. Swimming level was assessed at three levels, where the first corresponded to swimming at the bottom and the third at the top level of the tank. Similarly, swimming speed was divided in five categories with the first indicating low and the fifth high swimming speeds. Finally, schooling behaviour was assigned values between 1-10 which corresponded to increasing schooling behaviour from no schooling (level 1) to high schooling (level 10).

Large fish

The second trial considered larger individuals of mean weight 274±64 g. The trials lasted eight weeks, from 28 Apr to 20 Jun 2017, while rearing and sampling procedures were conducted as described for small fish. Growth performance and physiological parameter monitoring was also performed as above. In addition, oxygen levels in the tanks were monitored continuously via automatic recorders. In total, 112 individuals were used, which resulted in fish densities that did not exceed 8.2 kg/m^3.

2.2.3 Analytical procedures

The cortisol concentration in plasma was determined with a commercially available immunoassay kit (DRG-Diagnostics, Marburg, Germany) which has been previously evaluated for E. sea bass (Samaras *et al.*, 2016). Regarding the quantification of plasma glucose and lactate, calorimetric assays were used (glucose: Biosis, Greece; lactate: Spinreact, Spain). Finally, haematocrit levels were assessed via the use of capillary tubes after centrifugation in a haematocrit microcentrifuge for 5 minutes at 15,000 rpm.

2.2.4 Statistical analysis

For the statistical analysis the statistical package SigmaStat 3.1 was used. Comparison between means of the response variables was performed with t-test and the data were checked for meeting the analysis criteria prior to the analysis. Normality was assessed with the Kolmogorov-Smirnov test and the significance level was set at p=0.05.

2.3 Results

2.3.1 Small fish

The effect of transferring procedures on the growth performance of small E. seabass is shown in Figure 2. Feed consumption was low during the first week at 22 g/fish but reached values of 114 g/fish by the third week and remained high until the end of the trial. Low feed intake during the first week resulted in a low FCR (FCR=2.1) for that week. However, FCR exhibited threefold increase during weeks two and three. From week four onwards, FCR dropped significantly and remained between values 3.1-4. Regarding growth, SGR exhibited progressive decline over time from 1.5/day in the first week to 0.5/day at the end of the experiment. Finally, during the trial, five out of the 112 fish died which resulted in a total mortality of 4%.

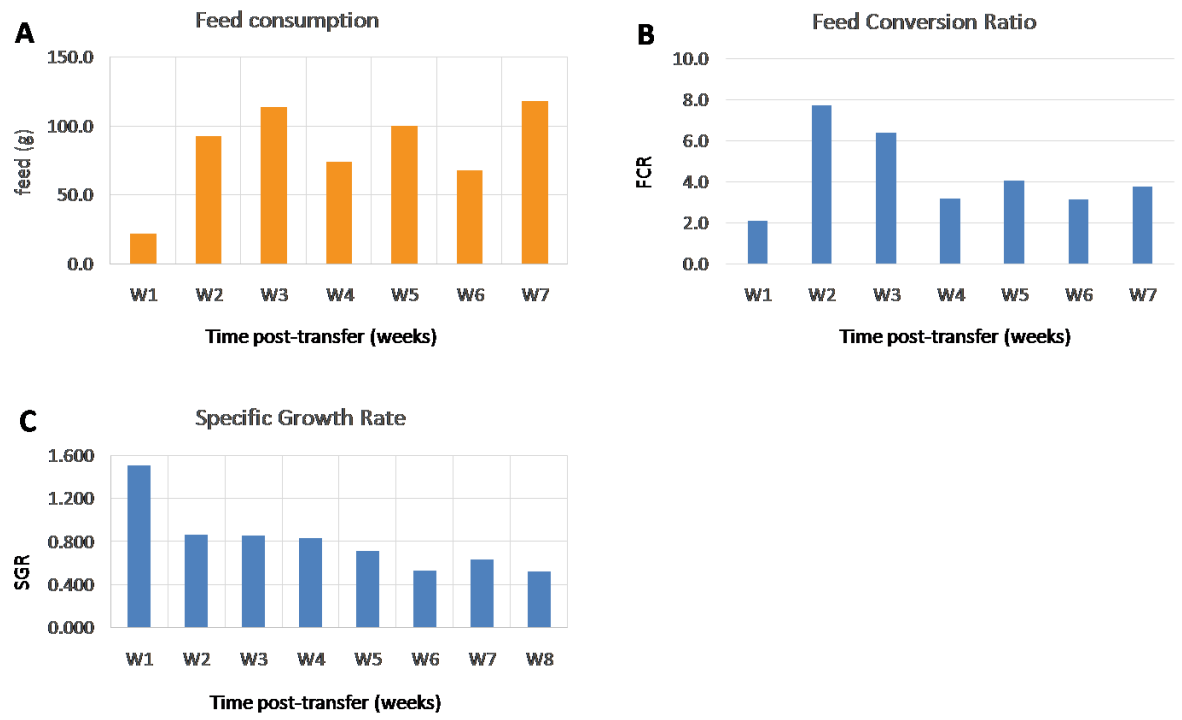

Figure 2. Individual feed consumption (A), FCR (B), and SGR (C) for the small E. sea bass sampled weekly after transfer from cages to tanks.

With respect to the physiological parameters, the concentrations of cortisol, HTC, lactate, and glucose in blood over the course of the trial are given in Figure 3. Differences were most prominent for cortisol and particularly between the control values obtained at the pilot scale farm and those right after transfer to the experimental facilities, with the latter exhibiting a two-fold increase (Figure 3, A). However, cortisol levels quickly returned to their normal levels with no significant deviations from the control value over the next eight weeks. Moreover, for weeks 2, 3, and 6, cortisol concentrations did not differ from the post-transfer values either.

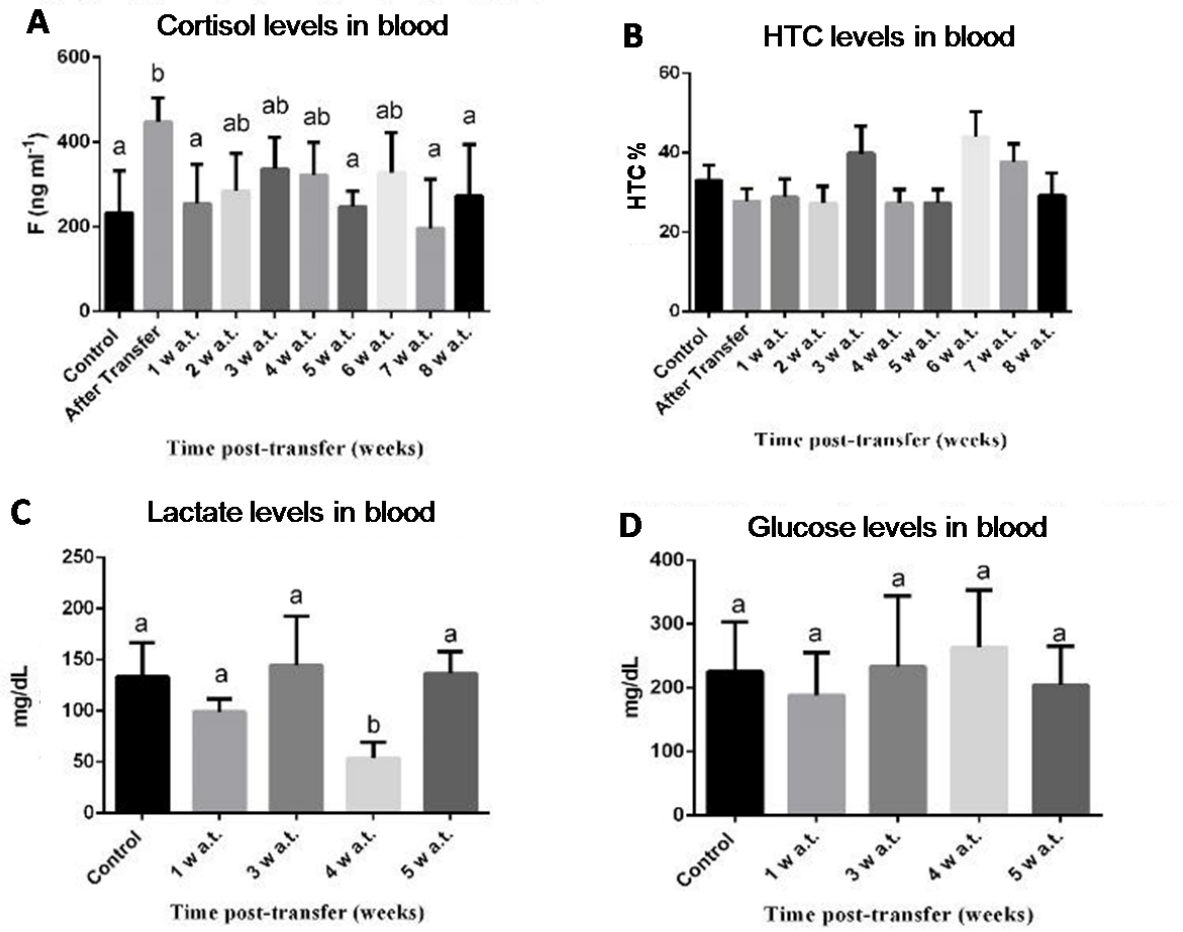

Figure 3. Concentration of cortisol (A), HCT% (B), concentration of lactate (C), and concentration of glucose (D) of the small E.sea bass sampled weekly after transfer from cages to tanks. Whiskers represent the standard deviation and different letters indicate statistically significant differences.

Regarding the other physiological parameters, no notable differences were observed over the course of the trial (Figure 3, B-D). Haematocrit values showed low variation over timewith slightly elevated levels at weeks three and six (42% and 46% respectively), while small differences were also observed for lactate and glucose. These differences were not statistically significant with the exception of lactate in the fourth week when concentration dropped by half compared to control levels.

The results from monitoring the behavioural variables are shown in Figures 4 and 5. No tank effect was detected and all groups exhibited the same overall pattern with respect to swimming level, swimming speed and schooling behaviour over time. The swimming level appeared unaffected by transfer procedures and fish consistently showed preference for the middle and top parts of the tank throughout the experimental period. However, the impact of handling and transfer on the swimming speed is evident during the first two weeks. Swimming speed was the lowest for the first two days post-transferand gradually increased until the end of the second week. After this point, fish exhibited medium levels of swimming speed which remained constant until the end of the trial. With respect to schooling behaviour, it followed a similar pattern with swimming speed. Fish tended to aggregate closely the first

few days and exhibited high schooling behaviour for the first two weeks whereas no apparent schooling pattern was observed from the third week onwards.

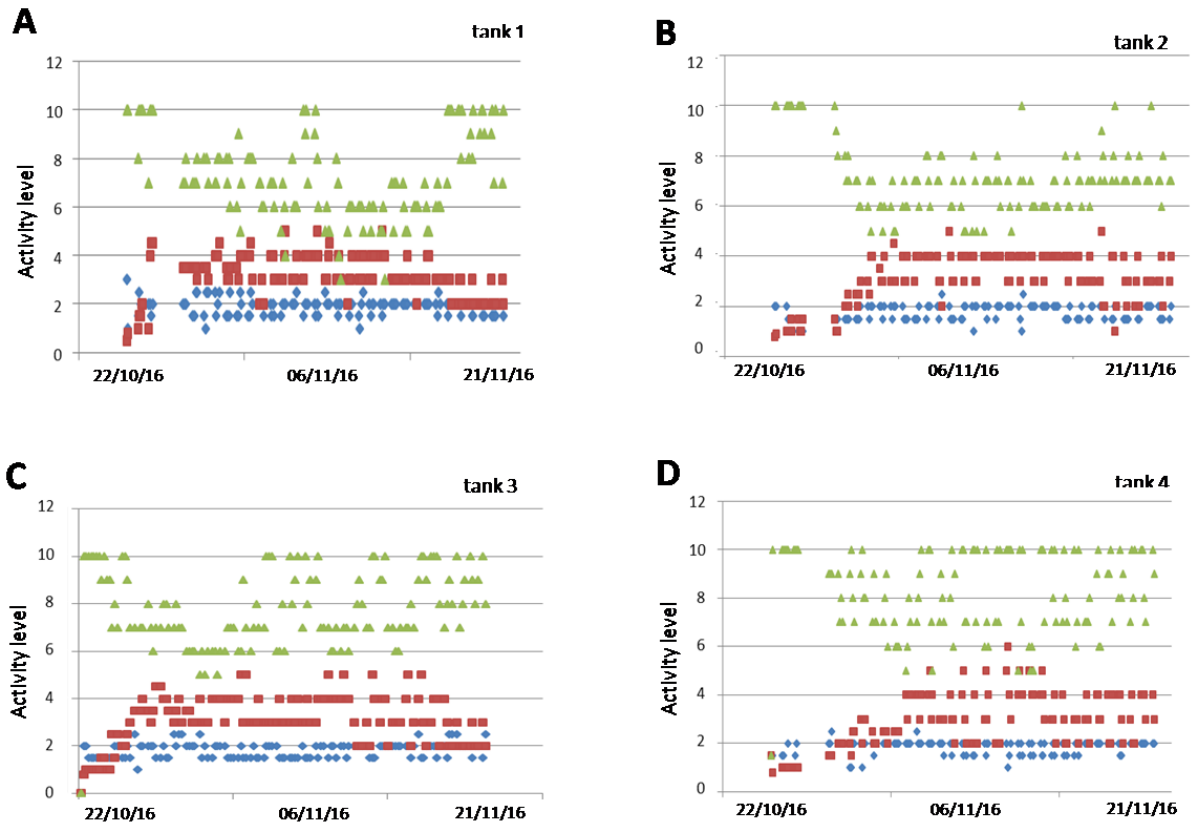

Figure 4. Monitoring of swimming behaviour of small E. seabass between 22/10/16 - 21/11/16 for tanks 1-4 (A-D). The three monitoring variables are represented with different colours, blue is for swimming level in the tank, red for swimming speed, and green for schooling behaviour.

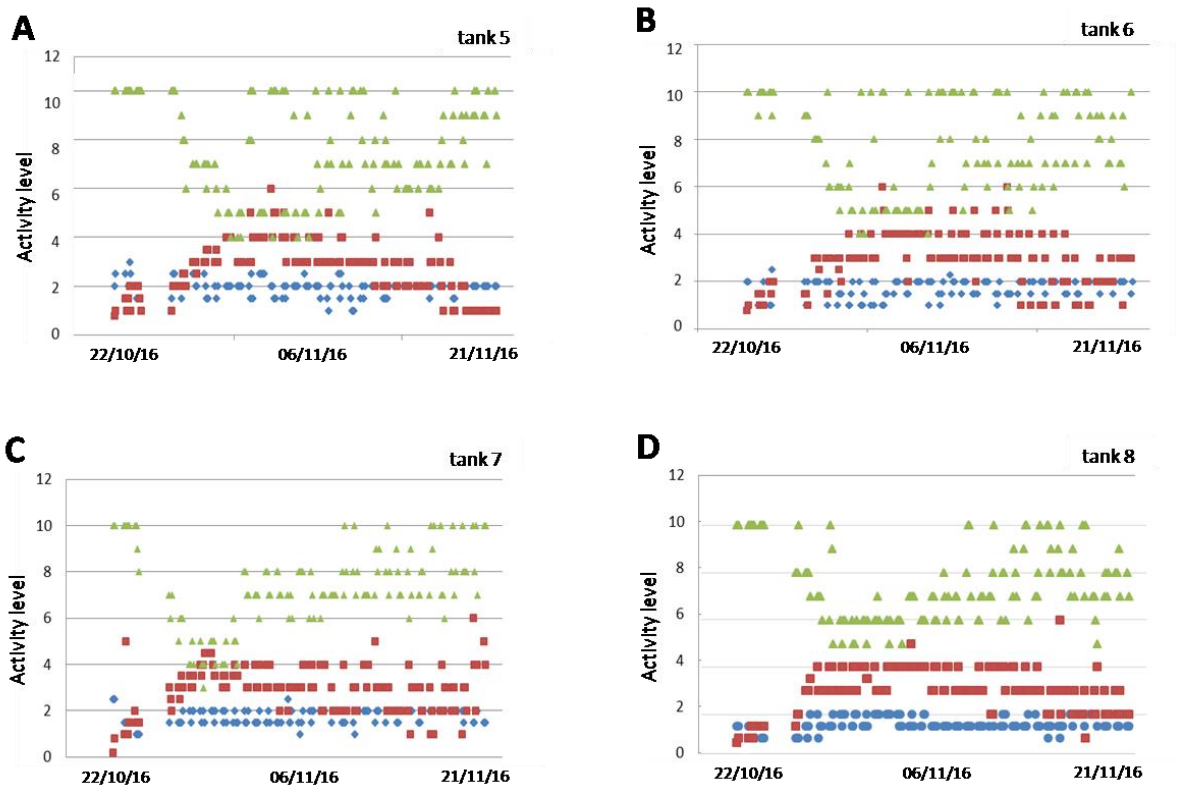

Figure 5. Monitoring of swimming behaviour of small E. seabass between 22/10/16 - 21/11/16 for tanks 5-8 (A-D). The three monitoring variables are represented with different colours, blue is for swimming level in the tank, red for swimming speed, and green for schooling behaviour.

2.3.2 Large fish

The growth performance of large E. sea bass is given in Figure 6.

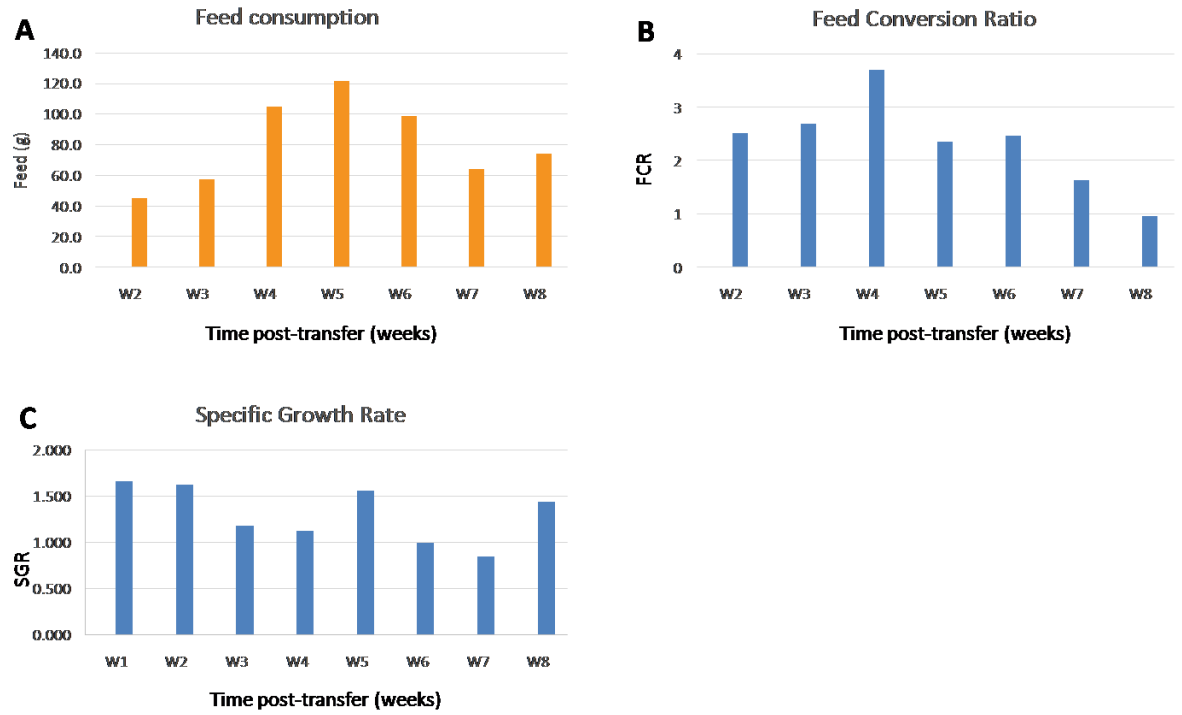

Figure 6. Feed consumption (A), FCR (B), and SGR (C) for the large E. sea bass sampled weekly after transfer from cages to tanks.

Feed consumption was very low for the first weeks of the trial. Especially during the first week fish showed near zero appetite, resulting in negligible feed consumption overall. However, it increased progressively during the next weeks raising from 45 g/fish/week in week one to a maximum of 122 g/fish/week in week five. For the remaining weeks individual feed consumption ranged between 64 and 99 g. Conversion of feed to biomass was low with high FCR values being estimated for the first three weeks. FCR did not drop significantly until week five with the lowest value recorded for the last week of the trial (FCR=0.97), thus, indicating that large fish require a longer acclimation time compared to small ones. Regarding SGR, starting from 1.6/day in the first week it declined over time with notably high values in weeks five and eight (1.6 and 1.4/day respectively).

The levels of the monitored physiological parameters for large E. seabass over the eight-week trial (cortisol, HTC, lactate, and glucose) are given in Figure 7. All parameters were significantly elevated right after transfer compared to the control values and returned to their typical range during the following weeks. In the case of cortisol, the initial post-transfer increase was followed by rapid decline during the first week which was restored over weeks two and three. Significantly lower values were also recorded in weeks four and seven. With respect to haematocrit, all temporal changes were contained within a narrow 5% range. Yet, such differences were significant for the first two weeks while from week three onwards no significant change was observed. Finally, lactate and glucose exhibited the same pattern over time. Post-transfer concentrations were notably raised (threefold and twofold

respectively) compared to the controls while they were quickly restored to typical levels by the first week and remained unaffected until the end of the trial.

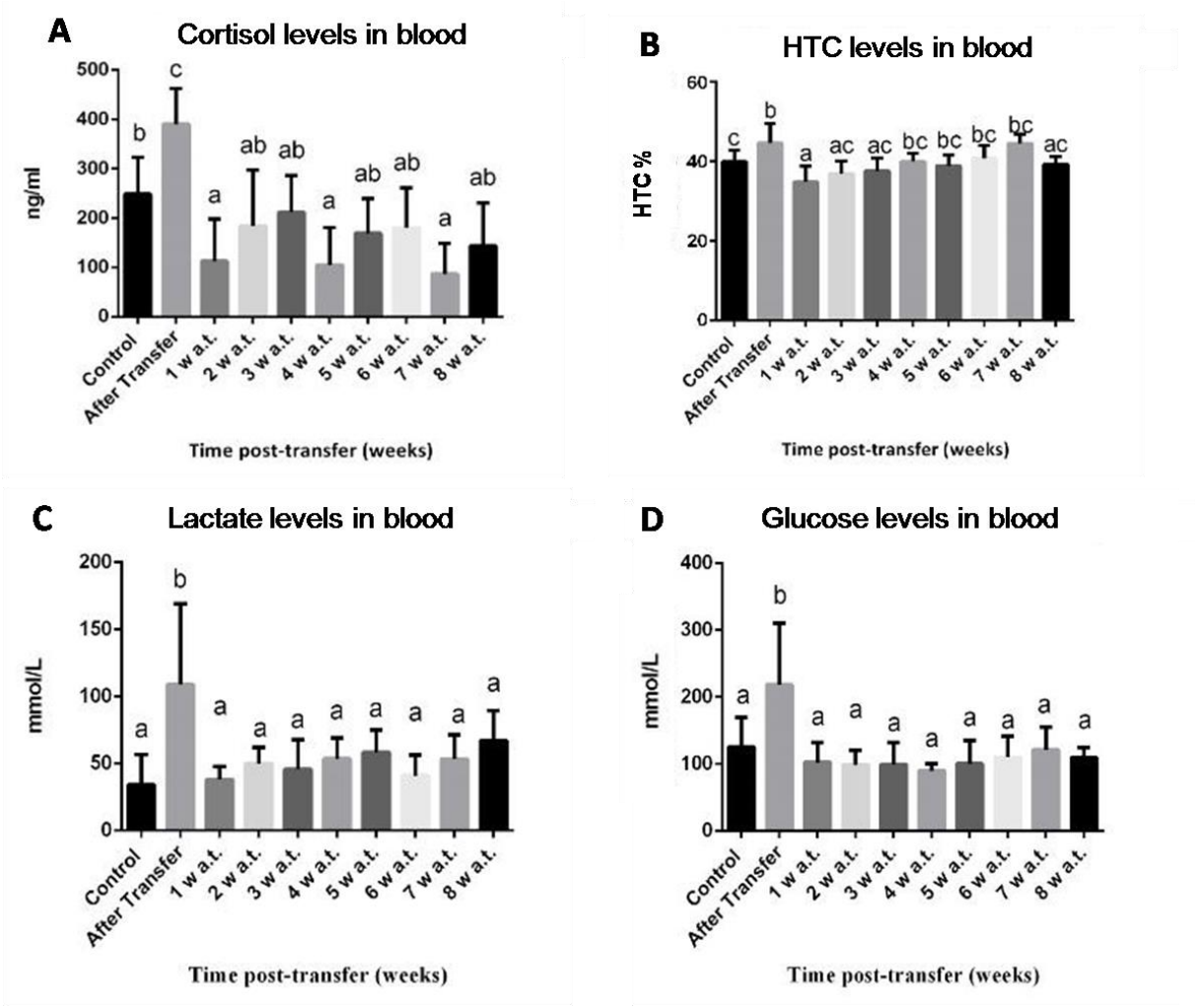

Figure 7. Concentration of cortisol (A), HCT% (B), concentration of lactate (C), and concentration of glucose (D) of the large E. seabass sampled weekly after transfer from cages to tanks. Whiskers represent the standard deviation and different letters indicate statistically significant differences.

Monitoring of the behavioural variables is shown in Figures 8 and 9. Overall, observations did not differ between tanks, nor with the results from the small E. sea bass. Both swimming level and schooling behaviour did not show a clear pattern over time while swimming speed seemed to be affected only in the beginning of the trial. Swimming speed was the lowest for the first day post-transfer and gradually increased during the next two weeks. After this point, fish exhibited medium levels of swimming speed until the end of the trial.

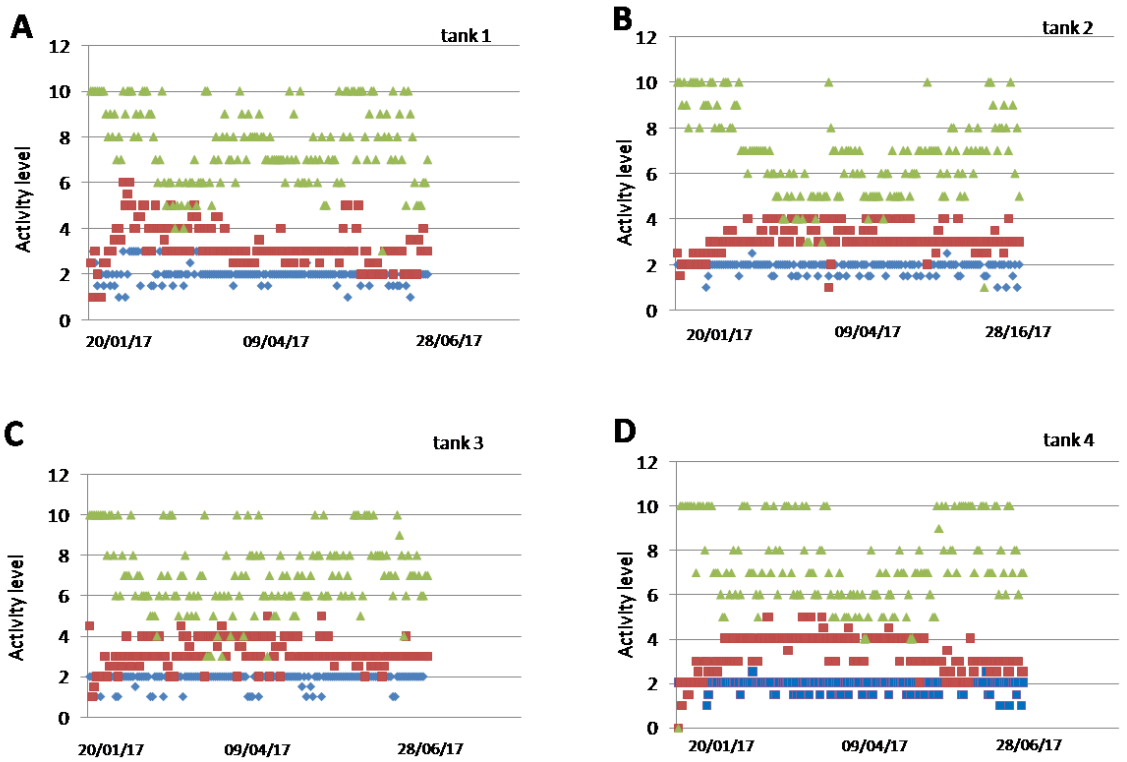

Figure 8. Monitoring of swimming behaviour of large E. sea bass between 20/01/17 - 28/28/17 for tanks 1-4 (A-D). The three monitoring variables are represented with different colours, blue is for swimming level in the tank, red for swimming speed, and green for schooling behaviour.

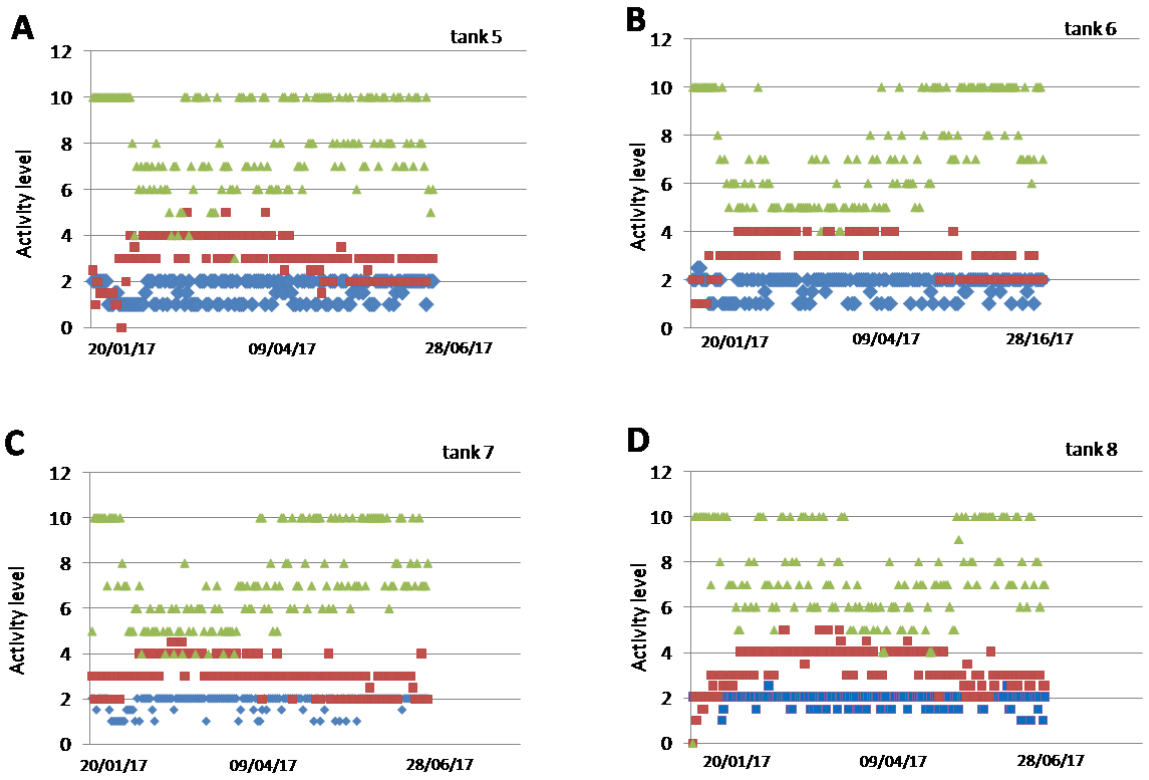

Figure 9. Monitoring of swimming behaviour of large E. sea bass between 20/01/17 - 28/28/17 for tanks 5-8 (A-D). The three monitoring variables are represented with different colours, blue is for swimming level in the tank, red for swimming speed, and green for schooling behaviour.

With respect to the oxygen consumption, this is depicted in Figures 10 and 11 for each experimental group over the eight weeks. This monitoring parameter appeared unaffected by the transfer procedures and remained constant over time for all groups at approximately 0.72 mg/kg/min.

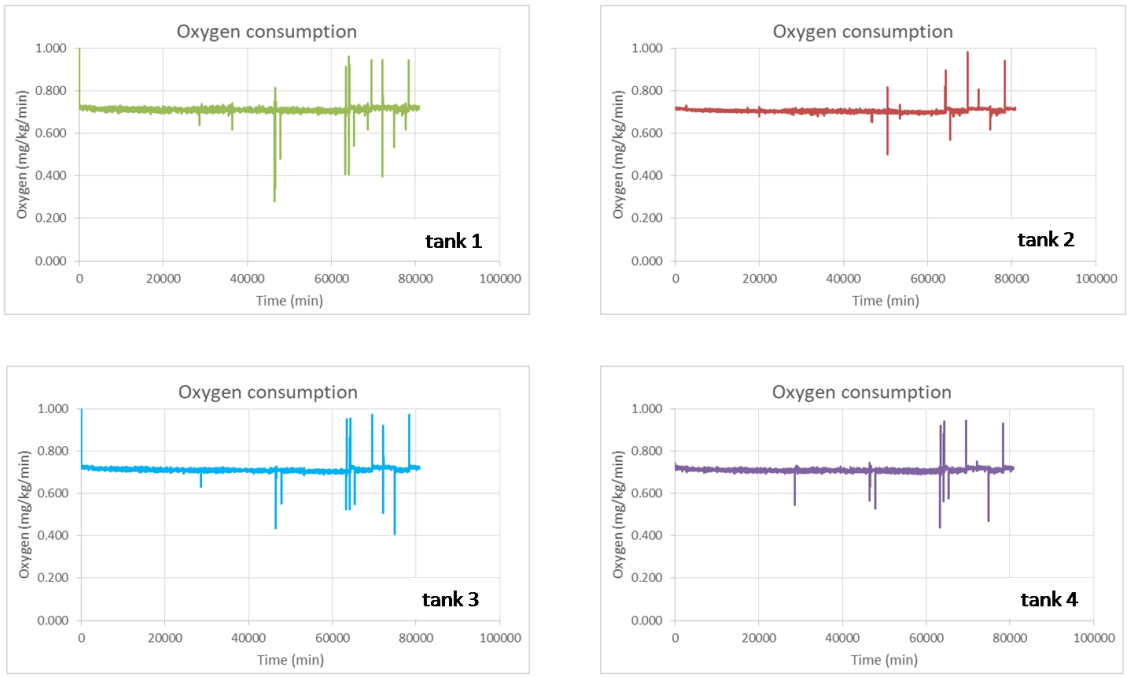

Figure 10. Oxygen consumption (mg/kg/min) during the experimental period for tanks 1-4.

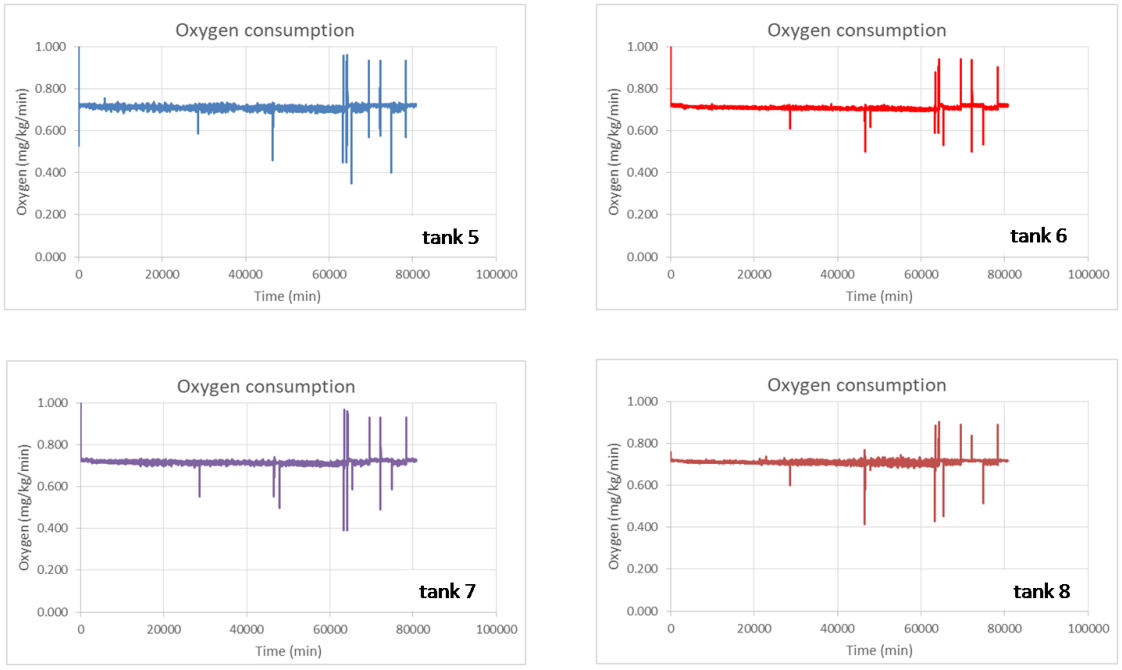

Figure 11. Oxygen consumption (mg/kg/min) during the experimental period for tanks 5-8.

Discussion

The effect of different environmental conditions before trials was assessed on small and large E. sea bass via monitoring of biochemical, haematological, hormonal, behavioural and husbandry variables over a period of eight weeks following their transfer from cages to tanks. Overall, the results suggest that fish can attain physiological and behavioural normality fast but require substantially longer acclimation time to fully adapt to the new conditions. Moreover, acclimation appears to be affected by size.

As corroborated by studies on multiple stressors, all fish exhibited elevated post-transfer values of their hormonal, biochemical, and haematological variables (Gesto *et al.*, 2016; Hoffmayer *et al.*, 2015; Silveira *et al.*, 2018). However, all variables reverted to their normal range by the end of the first week, which held irrespective of their size. In contrast, according to husbandry parameters acclimation lasted 3-4 weeks for the small fish and 4-5 for the large, with feed consumption being generally low and its conversion to biomass (FCR) exhibiting sub-optimal values during that time. Such requirements for long acclimation time are generally taken into account for experiments determining metabolic rates or evaluating temperature effects and toxicity to heavy metals (Chabot *et al.*, 2016; Hashemi *et al.*, 2008). Nevertheless, practical limitations as well as specific research goals may impose experimental settings with only a few days of acclimation time. Our findings, raise concerns on whether short acclimation periods suffice for obtaining scientifically robust results and suggest caution when acclimation requirements are considered during the stage of experimental design. Considerations should take into account species- and size-specific differences as well as the magnitude of change in the environmental parameters.

Regarding behaviour, our findings suggest that fish acclimate fast to the new conditions. In the beginning of the trial, post-transfer fish showed signs of low swimming activity and preference for the lower parts of the tanks. However, this effect disappeared after a few days for the majority of fish and, within the first two weeks, all fish resumed normal swimming behaviour irrespective of their size. This comes in line with the findings of Melvin *et al.* (2017) who reviewed the effects of acclimation time on swimming behaviour over a large number of published studies and concluded that in most cases fish exhibit extremely brief acclimation times.

Acclimation to different environmental variables requires different acclimation periods. Therefore, trials like the present are useful for providing insight into the general acclimation requirements of the species. For instance, it is known that acclimation to different levels of pH is relatively fast but acclimation to different temperatures requires longer acclimation time (Enzor *et al.*, 2013). Moreover, it appears that altering multiple environmental parameters simultaneously acts synergistically and results in even longer acclimation times. Shift of both pH and temperature conditions can result in acclimation time of up to 4 weeks for some species while others exhibit elevated metabolism (Resting Metabolic Rate, RMR) even after this period, which further demonstrates that the capacity for acclimation is highly species-specific (Enzor *et al.*, 2013). This is confirmed by our trials which suggested an acclimation time of minimum three weeks despite behavioural and physiological normality appearing to be achieved much faster; only 1-2 weeks post-transfer. Moreover, our findings are in line with the general recommendations for estimation of metabolic rates in fish which assumes fully acclimated individuals. Although the exact mechanisms involved in the acclimation process have not been disentangled, it is commonly accepted that fish must be acclimated for a period that lasts from several weeks to months prior to such experiments (Chabot *et al.*, 2016; Kir *et al.*, 2017).

Finally, our results suggest that smaller fish have higher capacity for adapting to the new conditions compared to their larger counterparts. Not only did they revert to normality1-2 weeks earlier than larger fish, with respect to husbandry parameters, but they also exhibited negligible mortalities. In contrast, the inability of larger individuals to adapt and achieve a stable status fast, resulted in marked mortalities in the first week of the experiment. In many species, small juveniles exhibit higher metabolism and grow faster than large ones, which is

believed to be strategy to shorten the period when fish are most vulnerable to predation (Gale *et al.*, 2013). This, coupled with the fact that small fish often have to adapt to new environments and nutritional sources as they grow (type or size of prey) may explain why our findings suggest a size-dependent acclimation period.

Conclusion

In conclusion, handling and transferring to new conditions causes effects on fish which may require weeks of acclimation in order to be reverted. The acute stress caused by such procedures is evident by the values of the control and post-transfer physiological variables assessed here, with cortisol and lactate providing the clearest patterns. However, the initial stress response is soon relaxed and physiological parameters are restored to their typical range within a week from transfer to the new conditions. The speed with which physiological normality is attained appears to be unaffected by the fish size. That being said, reverting to physiological normality does not entail that adaptation to the new conditions is complete. Results from the behavioural monitoring of small fish suggest that behavioural adaption requires and additional week. Furthermore, according to husbandry parameters, normality with respect to feed intake and FCR may require over a month to be achieved. This in fact depends upon the fish size, with smaller fish exhibiting signs of faster adaptation. Finding from this study suggest that smaller fish require 3-4 weeks for adaptation while for large fish this may take up to 5 weeks. Finally, the capabilities of smaller fish to adapt to new conditions easier and faster than large fish is clearly demonstrated by the significantly lower mortality rates recorded for the former.

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Glossary

AQUAEXCEL2020: AQUAculture Infrastructures for EXCELlence in European Fish Research towards 2020
acclimation: The process of gradual, reversible adjustment to environmental change.
allostasis: The process of achieving a state of internal equilibrium via physiological or behavioural change.
hypertrophy: Organ enlargement due to increase in size of its constituent cells.
HCMR: Hellenic Centre for Marine Research

Definitions

BW: Bodyweight
DO: Dissolved Oxygen
FCR: Feed Conversion Ratio
HSP: Heat Shock Proteins
HTC: Haematocrit
RMR: Resting Metabolic Rate
rpm: rounds per minute
SGR: Specific Growth Rate
TGC: Thermal Growth Coefficient

Document information

EU Project N°	652831	**Acronym**	AQUAEXCEL2020
Full Title	AQUAculture Infrastructures for EXCELlence in European Fish Research towards 2020		
Project website	www.aquaexcel.eu		

Deliverable	**N°**	D6.4	**Title**	Atlantic salmon and sea bass transfer protocols. Effects of internal transfer (cage to tank and between tanks) and sampling on salmon and sea bass, and the effect on acclimation time.
Work Package	**N°**	6	**Title**	Experimental fish management

Date of delivery	**Contractual**	31/01/2019 (Month 40)	**Actual**	08/03/2019 (Month 42)
Dissemination level	X	**PU Public, fully open, e.g. web**		
		CO Confidential, restricted under conditions set out in Model Grant Agreement		
		CI Classified, information as referred to in Commission Decision 2001/844/EC.		

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Version log			
Issue Date	**Revision N°**	**Author**	**Change**
08/02/2019	1	Asa Espmark	First review by WP leader
20/02/2019	2	Rolf Erik Olsen	Second review
08/03/2019	3	Marc Vandeputte	Validated by the coordinator

Annex 1: Check list

Deliverable Check list (to be checked by the “Deliverable leader”)

	Check list		Comments
BEFORE	I have checked the due date and have planned completion in due time		*Please inform Management Team of any foreseen delays*
	The title corresponds to the title in the DOW		*If not please inform the Management Team with justification*
	The dissemination level corresponds to that indicated in the DOW		
	The contributors (authors) correspond to those indicated in the DOW		
	The Table of Contents has been validated with the Activity Leader		*Please validate the Table of Content with your Activity Leader before drafting the deliverable*
	I am using the AQUAEXCEL2020 deliverable template (title page, styles etc)		*Available in “Useful Documents” on the collaborative workspace*
The draft is ready			
AFTER	I have written a good summary at the beginning of the Deliverable		*A 1-2 pages maximum summary is mandatory (not formal but really informative on the content of the Deliverable)*
	The deliverable has been reviewed by all contributors (authors)		*Make sure all contributors have reviewed and approved the final version of the deliverable. You should leave sufficient time for this validation.*
	I have done a spell check and had the English verified		
	I have sent the final version to the WP Leader, to the 2nd Reviewer and to the Project coordinator (cc to the project manager) for approval		*Send the final draft to your WPLeader, the 2nd Reviewer and the coordinator with cc to the project manager on the 1st day of the due month and leave 2 weeks for feedback. Inform the reviewers of the changes (if any) you have made to address their comments. Once validated by the 2 reviewers and the coordinator, send the final version to the Project Manager who will then submit it to the EC.*