

**The effect of allostatic load on hypothalamic–pituitary–Interrenal (HPI) axis before and after secondary vaccination in Atlantic salmon postsmolts (*Salmo salar* L.)**

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## **Abstract**

The experiment consisted of three experimental groups; 1) “vaccine and stress”, 2) “stress and vaccine” and 3) control. All groups have previously been vaccinated 6 months prior to the start of the experiment. At the start of the experiment the “vaccine and stress” group was vaccinated with Pentium Forte Plus for the second time (25.02.2008), and then given a daily stressor (crowding stressor, 267 kg m<sup>-3</sup> in 15 min) for a period of four weeks. The “stress and vaccine” group was given a similar daily stressor for four weeks, and then vaccinated for the second time. The control group was neither stressed nor vaccinated a second time. The results indicates that a fish in the “stress and vaccine” group enters an allostatic overload type 2 due to oversensitivity to ACTH, a reduced efficient negative feedback system, with elevated baseline levels of plasma cortisol, and reduced immune response with pronounced effects on the wellbeing of the animal. The “vaccine and stress” group entered an allostatic overload type 1 response, with oversensitivity to ACTH and transient reduced efficient negative feedback system, but at the end of the experiment the fish had recovered. This study shows that if plasma cortisol becomes elevated prior to vaccination it could instigate an allostatic overload type 2 with dire consequences on animal welfare. To reduce the risk of compromising the animal welfare during commercial vaccination of salmon one propose to grade the fish minimum a week prior to vaccination, or grade simultaneously with vaccination. This could reduce the overall allostatic load during handling and vaccination, and secure a healthy fish, with intact immune response and improved animal welfare.

## Introduction

Stress is defined as a condition in which the “dynamic equilibrium” of an organism, called homeostasis, is threatened or disturbed as a result of the actions of internal or external stimuli, commonly defined as stressors (Selye 1950, 1973; Wendelaar Bonga 2011; Wendelaar Bonga 1997; Varsamos et al. 2006). The physiological response to stressors has received great attention over the last decades and a great amount of data has attempted to complete, or even replace Selye’s “non-specific” stress concept. This stress concept is also known as the “General Adaptation Syndrome”, which describes an alarm, resistance and exhaustion stage (Varsamos et al. 2006).

In the later years the concept of allostasis has been introduced to complement the concept of stress, and more precisely try to describe the role primary mediators (e.g. glucocorticosteroids) has in response to a stressor (McEwen 1998; McEwen and Wingfield 2003; Goymann and Wingfield 2004; McEwen 2005; Wingfield 2005). In the study of McEwen and Wingfield (2003), they considered allostasis to be “*the ability to achieve stability through change.*” This is a process that supports homeostasis, i.e., those physiological parameters essential for life defined above, as environments and/or life history stages change. An allostatic state refers to altered and sustained activity levels of the primary mediators (e.g. glucocorticosteroids) as a response to a stressor (McEwen and Wingfield 2003). The cumulative result of an allostatic state is allostatic load. Within limits an organism can cope with, adapt to, or tolerate stressors to keep homeostasis. However, when the system is not dealing well with the disturbing factor(s) the increased allostatic load results in allostatic overload, which either could be adaptive (overload type 1) or malicious for the animal (overload type 2) (McEwen and Wingfield 2003; McEwen 2005; Wingfield 2005; Juster et al. 2010).

Stress is unavoidable in natural as well as in aquaculture environment. Exposure of fish to common stressors, such as handling, netting, transport, vaccination, and confinement, activates an allostatic response through the hypothalamo–pituitary interrenal axis (HPI). Resulting in increased blood levels of catecholamines and cortisol (Wendelaar Bonga 2011; Wendelaar Bonga 1997). Cortisol is often associated with the detrimental effects of stress, including decreased growth, reproductive dysfunction (Morgan et al. 1999; Schreck et al. 2001; Schreck 2010; Mommsen et al. 1999), increased incidence of disease (Barton 2002; Davis et al. 2002, 2003; Einarsdottir et al. 2000a; Einarsdottir et al. 2000b; Weyts et al. 1999),

reduced seawater tolerance (Iversen et al. 1998; Liebert and Schreck 2006; Sandodden et al. 2001) and survival (Iversen et al. 2005; Portz et al. 2006; Finstad et al. 2003; Iversen et al. 1998; Hasan and Bart 2007).

Salmon pancreas disease (PD) is an emerging disease in the Atlantic salmon farming industry in Europe. In Norway a significant increase of disease outbreaks, in the southern part of the country, have occurred since 2003 (Kristoffersen et al. 2009). A vaccine has been introduced as preventive measure to this disease, due both legal and practical issues the salmon parr needs to be vaccinated twice prior to transfer to sea; first with PD-vaccine and then 3 weeks later (240 days degrees) with the combination vaccine protecting against common diseases as Furunculosis, Vibriosis, Coldwater vibriosis, Winter sore, IPN and ISA (MSD animal health, Norway).

This procedure with a second vaccination (stressor) puts additional strain on the animal with possible negative effects on the HPI-axis and animal welfare. In this study we wish to study the possible effect of an allostatic overload induced by a secondary vaccination on hypothalamic–pituitary–interrenal (HPI) axis, primary, secondary and tertiary stress responses in Atlantic salmon postsmolts (*Salmo salar* L.) and its impact on animal welfare.

## **Materials and methods**

### Experimental animals and rearing conditions

One-year-old (1+) hatchery-reared Atlantic salmon postsmolts (Aqua Gen strain, Sunndalsøra, Norway) with a mean body weight  $178.2 \text{ g} \pm 29.4$  (S.D.) and a mean body length  $26.3 \text{ cm} \pm 1.5$  (S.D.) were used in the experiment. This experiment was performed at Faculty of Biosciences and Aquaculture facilities in northern Norway, Bodø. The salmon were reared at natural freshwater temperatures (1–18 °C) and held at natural light conditions (67°17'N, 14°23'E) until smoltification, and then transferred to seawater (33 ppt). Commercial dry feed, dispensed from automatic feeders, was provided in excess. 1.5 month prior to the start of the experiment (25.02.08), 450 fish was distributed in to three isolated tanks of  $1.0 \text{ m}^3$  supplied with seawater (33 ppt) with an average water temperature of  $7.3 \pm 0.3$  °C. The total number of fish in each tank was 150, and the initial biomass was  $26.7 \text{ kg m}^{-3}$ .

## Experimental protocol

The experimental animals were first time vaccinated with Pentium Forte Plus (Novartis Animal Vaccines Ltd., UK) 6 months prior to the onset of the experiment. After 450 degree days a positive antibody titre *Aeromonas salmonicida* subsp. *salmonicida* with a ELISA method (as described below), was confirmed in 36 randomly selected postsmolts (fig. 4, 0 days degrees), thus confirming a positive antibody titre response to the vaccine prior to start of the experiment.

The experiment consisted of three experimental groups; 1) “vaccine and stress”, 2) “stress and vaccine” and 3) control. The Control group was kept undisturbed and isolated during the length of the experiment (25.02-25.04.2008), while the “vaccine and stress” group was vaccinated for the second time with Pentium Forte Plus (Novartis Animal Vaccines Ltd., UK) at start of the experiment (25.02.2008) and then given a daily stressor (crowding stressor, as described below) for four weeks. Finally the “stress and vaccine” group was given a daily stressor (crowding stressor, as described below) for four weeks, and then vaccinated for the second time with Pentium Forte Plus (Novartis Animal Vaccines Ltd., UK) (25.03.2008).

## Daily stressor

The daily applied stressor in group “vaccine and stress” and “stress and vaccine” consisted of a crowding stressor produced by lowering the water level by submersible water pumps (Flygt, SX-pump, Xylem, NY, USA) to approximately 10 cm (267 kg m<sup>-3</sup>), and kept crowded for 15 minutes repeated daily for 4 weeks. To ensure that the fish did not adapt to the stressor, the stressor was applied at irregular interval (daily) between 10 am to 2 pm daily, and this was controlled by a self-made programmable logic controller (PLC).

## Blood sampling and analytic procedures

To determine the effect of the different treatments on primary (plasma cortisol) and secondary (plasma chloride and magnesium) stress responses, blood samples were obtained prior to start of the experiment (pre-stress), and 1, 2, 3, 4, 5 and 6 weeks after start of the experiment. The blood sample was taken Monday morning every week at 8 am to ensure that the fish had at least 18 hours rest after last applied stressor. The fish were rapidly transferred to a bucket containing a metomidate solution of 5 mg/L. This concentration which has shown to be sufficient in inducing rapid anaesthesia and preventing an increase in blood plasma cortisol (Iversen et al. 2003; Olsen et al. 1995). Blood samples from six postsmolts (per group) at each sampling time were obtained from the caudal vein complex using size 0.50-x16-mm

heparinised syringes. In sum the total sampling time per group was approximately 3.5 min. The blood was centrifuged at 5000 rpm for 5 min. and plasma was removed and stored in cryo tubes at -36 °C until analyses were performed.

Radioimmunoassay (RIA) techniques were used to measure blood plasma cortisol concentrations as described in Iversen et al. (1998). Previous tests at our laboratory (University in Nordland, Bodø, Norway) gave the following assay specifications: Sensitivity of 1.8 nmol L<sup>-1</sup> (nM) (samples with hormone levels below detection limit were assigned in the value of assay sensitivity); non-specific binding (NSB) of 2.1 to 3.7 % of total activity; intra-assay coefficient of variation less than 7.0 % and inter-assay coefficients of variation of 5.1 % at 50 nM. Measurements of 4, 17, 34 and 69 nM radio labelled cortisol added to plasma, showed a recovery of 90, 94, 96 and 95 %, respectively. A serial dilution of plasma from stressed Atlantic salmon resulted in a line parallel to the dose response curve.

Plasma was also analysed for chloride levels using a Sherwood Chloride Analyser 926 (Sherwood Scientific Inc. USA), respectively. Magnesium (Mg<sup>2+</sup>) was analysed by a fluidtest® Mg-XB (Biocon®, Germany) adapted for plate count reader. Samples below the detection limit were given a value corresponding to the tests sensitivity, which was 0.4 mmol L<sup>-1</sup> (mM).

#### Stimulation and suppression test of HPI-axis

Stimulation (ACTH) and dexamethasone (DEX) suppression test was conducted in accordance to previous study by Pottinger and Carrick (2001), with some minor modifications. Briefly, at pre-stress, 4 and 6 weeks after start of the experiment 12 fish per group (a total of 36 fish), were netted from their respective tanks, then anaesthetised (as described above), and injected intraperitoneally with 1 mg kg<sup>-1</sup> dexamethasone (Sigma-Aldrich) in ethanol: phosphate-buffered saline (PBS; 1:3; 1µg µL<sup>-1</sup>). Finally they were transferred to three holding tanks (0.5 m<sup>3</sup>). After 24 h the fish were netted, anaesthetised, and 6 fish from each group was either given an intraperitoneal injection of 0.5 mL kg<sup>-1</sup> adrenocorticotrophic hormone (ACTH, fragment 1-24; Sigma-Aldrich in PBS at 45 µg mL<sup>-1</sup>) or 0.5 mL kg<sup>-1</sup> PBS, to measure the function of the negative feedback system. The ACTH and PBS groups were kept separate by an artificial wall inserted in the three different holding tanks. Two hours after the ACTH/PBS administration the fish were netted, anaesthetised and blood sampled. The blood was centrifuged at 5000 rpm for 5 min. and plasma was removed and stored in cryo tubes at -36 °C until plasma cortisol analyses were performed.

### Antibody response

The antibody response in blood plasma was measured against *Aeromonas salmonicida* subsp. *salmonicida*, using an indirect enzyme linked immunosorbent assay (ELISA). Ab titres were determined using a modification of the method previously outlined by Adams et al. (1995) and with suggested modification by the monoclonal antibody (MAb) manufacturer (Aquatic Diagnostics, Stirling, Scotland UK). 96-well microtitre plates with pre-coated *Aeromonas salmonicida subspecies salmonicida* supplied by Aquatic Diagnostics (Stirling, Scotland UK) were used. ELISA readings at optical density (OD) 450 nm are later referred to as antibody values. As negative control, PBS was added instead of salmon plasma, while the plate-to-plate and day-to-day variations were monitored using pooled control serum (positive control).

### Fin erosion

Fin erosion at the dorsal fin was measured at the end of experiment (25.04.2008) using a method described by Hoyle et al. (2007) with minor modification utilising an ordinal scale of 0, 1, 2 and 3, corresponding to erosion (0% of fin eroded), mild erosion (1–24% of eroded), moderate (25–49% of fin eroded) and severe erosion (>50% of fin eroded), respectively (n=72).

### Mortality

Mortality was daily registered until end of the experiment (25.04.2008). For each dead salmon postsmolt size (g) and length (cm) was noted. Overall mortality was expressed as % dead of total number of fish (n=150) in each experimental group.

### Statistical analysis

Statistical tests were performed using the statistical program SPSS 18.0 for Windows. All datasets were tested for normality using a Kolmogorov-Smirnov test, and for homogeneity of variance using a Levene test. A one-way ANOVA test was thereafter performed at each sampling time in regard to various physiological parameters to test for differences between the test groups and within the groups (Sokal and Rohlf 1987). If the F-values were significant, a Bonferroni post hoc test was used to determine which groups differed. A Kruskal-Wallis ANOVA (non-parametric) and a Mann-Whitney U-test with a Bonferroni-adjusted significance level were used when requirements for parametric statistics were not met. Significant differences were established at 0.05 levels. Results are given as means  $\pm$  standard deviation (SD).

## Results

### Plasma cortisol

The changes in baseline levels of plasma cortisol during the experiment are shown in Fig. 1. The average plasma cortisol prior to handling (pre-stress) were 17,7 ( $\pm$  21,4) nM (control), 5,0 ( $\pm$  4,5) nM (vaccine and stress) and 4,6 ( $\pm$  7,6) nM (stress and vaccine). Plasma cortisol in the control group ranged from 2,0 ( $\pm$  7,6) nM (week 2) to 43,7 ( $\pm$  42,9) nM (week 4). It was not observed any significant changes from pre-stress levels throughout the experiment in the control group. Baseline levels of plasma cortisol in the “vaccine and stress” group ranged from 5,0 ( $\pm$  4,5) nM (pre-stress) to 212,4 ( $\pm$  65,0) nM (week 4), and this group had significant elevated plasma cortisol compared to pre-stress levels, control and “stress and vaccine” group at week 1 and 2 after start of the experiment. Plasma cortisol in the “stress and vaccine” group ranged from 4,6 ( $\pm$  7,6) nM (pre-stress) to 712,2 ( $\pm$  311,1) nM (week 5), and the plasma cortisol was significant elevated from pre-stress levels at week 3, 4 5 and 6, and significantly elevated from the control group and the “vaccine and stress” group at week 5 and 6 (fig. 1).

### Osmoregulation (secondary stressresponses)

The changes in plasma chloride (secondary stressresponse) are shown in fig. 2a. The average plasma chloride prior to handling (pre-stress) were 135,5 ( $\pm$  4,9) mM (control), 134,5 ( $\pm$  5,3) mM (vaccine and stress) and 133,7 ( $\pm$  5,8) mM (stress and vaccine). Plasma chloride in the control group ranged from 120,2 ( $\pm$  8,5) mM (week 2) to 135,5 ( $\pm$  4,9) mM (pre-stress). It was not observed any significant changes from pre-stress levels throughout the experiment in the control group. Plasma chloride in the “vaccine and stress” group ranged from 133,3 ( $\pm$  8,5) mM (week 4) to 148,8 ( $\pm$  15,9) mM (week 1), and there were no significant changes in plasma chloride compared to pre-stress levels during the experiment. Plasma chloride in “stress and vaccine” group ranged from to 133,6 ( $\pm$  5,8) mM (week 5) to 163,3 ( $\pm$  15,2) mM (week 6), and the plasma chloride was significant elevated from pre-stress levels at week 3, 4 5 and 6, and significantly elevated from the control and “vaccine and stress” group at week 4 and 6 (fig. 2a).

The changes in plasma magnesium (secondary stressresponse) are shown in fig. 2b. The average plasma magnesium prior to handling (pre-stress) were 1,3 ( $\pm$  0,2) mM (control), 1,1 ( $\pm$  0,2) mM (vaccine and stress) and 1,1 ( $\pm$  0,3) mM (stress and vaccine). Plasma



magnesium in the control group ranged from 0,6 ( $\pm$  0,2) mM (week 5) to 1,3 ( $\pm$  0,2) mM (week 1), and there were no significant changes in plasma magnesium compared to pre-stress levels during the experiment. In the “vaccine and stress” group plasma magnesium ranged from 0,6 ( $\pm$  0,1) mM (week 4) to 1,1 ( $\pm$  0,2) mM (pre-stress). It was not observed any significant changes from pre-stress levels throughout the experiment in the “vaccine and stress” group. Plasma magnesium in “stress and vaccine” group ranged from 0,8 ( $\pm$  0,3) mM (week 5) to 1,9 ( $\pm$  0,2) mM (week 6), and the plasma magnesium was significant elevated from pre-stress levels at week 5 and 6, and significantly elevated from the control group and the “vaccine and stress” group at week 5 and 6 (fig. 2b).

#### Stimulation and suppression test of HPA-axis

The mean plasma cortisol levels in the DEX-injected fish 2 h following ACTH inoculation increased significantly when compared to pre-stress. This occurred during week 4 and 6 in both the “vaccine and stress” (fig. 3b) and the “stress and vaccine” group (fig. 3c), however no such changes in ACTH sensitivity was observed in the control group (fig. 3a).

In the DEX-injected fish which received PBS, plasma cortisol increased significantly (compared to pre-stress) in week 4 and 6 for the “vaccine and stress” group (fig. 3b) and the “stress and vaccine” group (fig. 3c), respectively. No such change in the control group was observed (fig. 3a).

#### Antibody response (secondary stressresponse)

Fig. 4. shows the mean antibody titre prior to and 245 days degrees after secondary vaccination. At 0 days degrees the antibody titre were 0,18 ( $\pm$  0,06) OD (control), 0,18 ( $\pm$  0,04) OD (vaccine and stress) and 0,2 ( $\pm$  0,05) OD (stress and vaccine). The negative and positive control was at the same time was 0,09 ( $\pm$  0,01) and 0,18 ( $\pm$  0,03) OD, respectively. 245 days degrees after secondary vaccination there was a significant increase in antibody titre in “vaccine and stress” group, and reach at peak of 0,46 ( $\pm$  0,10) OD (fig. 4).

#### Fin erosion (tertiary stressresponse)

At the end of the experiment (25.04.2008) the overall fin erosion ranged from 1,1 ( $\pm$  0,4), 1,7 ( $\pm$  0,4) and 2,7 ( $\pm$  0,5) for the control, the “vaccine and stress” and the “stress and vaccine” groups, respectively. The “stress and vaccine” group experienced significantly higher grade of fin erosion at the end of experiment compared to the control and the “vaccine and stress” groups.

Mortality (tertiary stressresponse)

At the end of the experiment (25.04.2008) the overall mortality ranged from 0,1, 44 and 66% for the control, the “vaccine and stress” and the “stress and vaccine” groups, respectively.

## **Discussion**

Vaccination is the most important tools to prevent outbreaks of a number of bacterial and viral diseases in farmed Atlantic salmon (*Salmo salar*), and is largely responsible for reducing the use of antibiotics and continuing sustainable growth of Norwegian aquaculture in the 1990s (Drangsholt et al. 2011; Gudding et al. 1999). To maintain the health and welfare of farmed Atlantic salmon, individual fish are vaccinated prior to sea water entry, however the injection of these vaccines could lead to unavoidable stress and is often associated with short-term increases in plasma cortisol (Funk et al. 2004; Skinner et al. 2010). The allostatic load associated with handling and vaccination have increased in the later years in commercial salmon aquaculture industry due to pancreas disease (PD) and the need of a secondary vaccination and handling of smolt prior to seawater transfer.

Stressors effects on the immune system have mainly been attributed to elevated levels of cortisol, and there is a huge complexity in the interactions between the immune- and endocrine system, which is most likely governed by a bi-directional communication between HPI-Axis and immune system (Wendelaar Bonga 2011; Pérez-Casanova et al. 2010; Stolte et al. 2008; Fast et al. 2008; Engelsma et al. 2002). In the current experiment baseline level of plasmacortisol in the “vaccine and stress” group was elevated week 1 and 2 after second vaccination, which after it returned back to pre-stress levels (and similar to the control group). A similar but more aggravated increase in baseline levels of plasma cortisol after second vaccination was detected in the “stress and vaccine” group, but no recovery was obtained. A plausible explanation for this increase in baseline levels of plasma cortisol is due to an increased inflammatory response to oil-adjuvant vaccine, which activate different cytokines during inflammation. In mammals it has been shown that inflammation promotes the release of cytokines such as TNF, IL-1, IL-6 and IL-12, which may result in the activation of a stress response (Tort 2011; Calcagni and Elenkov 2006). Especially IL-6 appears to have a significant role as a mediator of the HPI activation. IL-6 receptors have been detected in mammalian brain tissues and neuroendocrine glands, thus stimulating CRH, prolactin and GH secretion. ACTH secretion seems also to be some control of IL-6 as high IL-6 levels have

shown correlated with higher ACTH and cortisol levels (Zarkovic et al. 2008). In fish, several studies have shown a similar bi-directional communication between HPI-Axis and cytokines (Tort 2011). Holland et al. (2002) showed that recombinant IL-1 injection raises cortisol levels, and injection of lipopolysaccharide (LPS) raised plasma cortisol in yellow perch (Haukenes and Barton 2004) and promoted StAR HK expression in gilt-head bream (Castillo et al. 2008). Similar an injection of LPS both in sea bream and trout have shown to promote increased cortisol secretion and increased cortisol receptor expression in most tissues, 6 to 72 h after the initial injection (Acerete et al. 2007).

However all increase in baseline plasma cortisol cannot be explained through a bi-directional communication between HPI-Axis and immune system in this experiment. The “stress and vaccine” group experienced two weeks prior to the second vaccination elevated baseline levels of plasma cortisol compared to pre-stress levels, and the secondary vaccination (week 4) seemed just to be the additional stressor that “pushed” the animals in to a chronic stress state or allostatic overload type 2 situation. Similar studies of base-line plasma cortisol have shown that an unstressed fish had a baseline levels as low as 13.8 nM, while chronic stressed fish had a baseline level above 27.5 nM (Maule et al. 1987; Pickering and Pottinger 1989; Van Zwol et al. 2012). This seemed to be supported by the fact that secondary and tertiary stress responses as plasma chloride, magnesium, fin rot and mortality, were significantly increased in the “stress and vaccine” group compared to the “vaccine and stress” and the control group. Earlier studies have shown that cortisol is often associated with the detrimental effects of stress including: decreased growth rates, reproductive dysfunction (Morgan et al. 1999; Schreck et al. 2001; Mommsen et al. 1999), increased incidence of disease (Barton 2002; Davis et al. 2002, 2003; Einarsdottir et al. 2000a; Einarsdottir et al. 2000b; Weyts et al. 1999), reduced seawater tolerance (Ventura et al. 2011; Iversen et al. 2009; Mommsen et al. 1999; Redding and Schreck 1983; Sandodden et al. 2001) and survival (Iversen et al. 2005; Portz et al. 2006; Finstad et al. 2003; Iversen et al. 1998; Hasan and Bart 2007). It has also been suggested that if the fish is not permitted enough time to recover completely after stress, a second, normally nonfatal, stressful occurrence could be fatal (Carmichael 1984), as observed in this experiment.

The actions of stress or cortisol on the fish immune system can be described to be regulatory and not necessarily inhibitory. The effects on immune system and the final outcome may depend on the severity and duration of the stressor as it does in mammals (Weyts et al. 1999). Recent studies have suggested that the observed differences in immune

responsiveness and disease susceptibility in relation to elevated cortisol levels, are specific to species, strain, antigen type, and possibly to the timing of the stressor (Engelsma et al. 2003; Funk et al. 2004; Skinner et al. 2010). In this experiment stress prior to vaccination negatively affected the production of antibody titre, while vaccination before applying a daily stressor did not. The “vaccine and stress” group responded with a 2.5 times increase in antibody titre, 245 degrees days after the second vaccination, which is common during a second immunisation (Einarsdottir et al. 2000a; Einarsdottir et al. 2000b). After the “boost” injection, the secondary immune response emerged faster and resulted in a greater antibody titre than the primary immune response. This is in accordance with earlier findings involving brown trout (*Salmo trutta* L.), Atlantic salmon (*Salmon salar*) and Arctic charr (*Salvelinus alpinus*), where the initial humoral immune response produced antibody titre to SRBC, and second dose of SRBC produced higher serum agglutinin (titre) compared the initial dose (Einarsdottir et al. 2000a; Einarsdottir et al. 2000b; Ingram 1985). A secondary immune response (“boost”) was not observed in the group “stress and vaccine”. It seems that applying the stressor before initiating the immune response could have dire consequences on the immunisation. Recent findings supports these observations, as studies shows that if plasma cortisol levels becomes elevated after the initiation of the innate and adaptive immune responses, the overall disease susceptibility and the production of pathogen-specific Abs remains unaffected (Espelid et al. 1996; Funk et al. 2004; Lovy et al. 2008; Skinner et al. 2010).

Quantification of chronic stress conditions has proven to be complicated in fish. Many researchers reporting transient elevation of cortisol levels during crowding stress (Barcellos et al. 1999; Ruane et al. 2002), while others report no effect (Da Rocha et al. 2004; Fanouraki et al. 2007; Vijayan and Leatherland 1990) or even a reduction (Leatherland and Cho 1985). However without a high number of individuals during a survey of baseline cortisol, a single parameter related to the HPI axis could be insufficient to establish whether the fish is chronically stressed or not. For instance might different genetic strains exhibit different resting levels (baseline) of cortisol due to the effects at any levels in the HPI axis (Fevolden et al. 2002; Pottinger and Carrick 1999; Tanck et al. 2002; Tanck et al. 2001). Each level of the axis (hypothalamus, anterior pituitary, interrenal cells) is subjected to opposite influences, trophic via their respective stimulating inputs (such as CRH to the pituitary or ACTH to the interrenal cells) and inhibitory via corticosteroid hormones (negative feedback) (Mormede et al. 2007). In mammals several changes in the HPI-axis has been documented during a chronic stress-state such as weight loss (catabolic effect of cortisol and catecholamines), proliferation

of the corticotrope cells in the anterior pituitary (trophic effect of CRH), inhibition of ACTH synthesis (by cortisol) and reduction of the feedback effect of GR agonists on ACTH release, increase of the size of the adrenal glands and of the response of the adrenals to ACTH (a trophic effect of ACTH) (Mormede et al. 2007). This resetting of the HPI axis at a “new” level of activity, that Selye (1973) described as the stage of resistance, is also known as allostasis (McEwen 1998; McEwen and Wingfield 2003; Goymann and Wingfield 2004; McEwen 2005; Wingfield 2005). Different approaches could be used to detect these changes, including stimulation tests (by CRH, CRH/vasopressin, ACTH, insulin-induced hypoglycaemia) that measure the relative sensitivity of the pituitary and/or the interrenal cells, and the use of a inhibition test utilising dexamethasone (a synthetic steroid with glucocorticoid activity) to demonstrate the reduced efficiency of the negative feedback by corticosteroids (Mormede et al. 2007). In this experiment both the “vaccine and stress” group and the “stress and vaccine” group become oversensitive to administration of a weight-adjusted dose of ACTH compared with the control group, and the oversensitivity increased with duration of the experiment. Few studies on ACTH sensitivity have been done on fish, however Pottinger and Carrick (2001) showed that two lines of rainbow trout selected for high (HR) and low (LR) responsiveness to a standard crowding test had different responsiveness to weight-adjusted dose of ACTH. The LR strain had significant lower production of plasma cortisol compared to HR strain. In domesticated mammals an injection of ACTH have shown an increased cortisol response in animals reared in poor conditions or subjected to repeated stressors. For instance, calves submitted to prolonged spatial and social restriction (Friend et al. 1985), repeated regrouping (Veissier et al. 2001), growing pigs in restricted space (Meunier-Salaun et al. 1987), tethered sows (Janssens et al. 1994).

The dexamethasone (DEX) suppression test has been developed in humans to detect HPI axis changes in melancholic patients (Wilens et al. 1984; Banki et al. 1986; Kumar et al. 1986). In humans DEX have shown to reduce the morning peak of cortisol, but more in healthy humans than in depressed patients, and the maximum post-DEX effects on plasma cortisol levels have shown to reflect the overall severity of depression during melancholia (Kumar et al. 1986). The response to DEX to chronic stress in this experiment seems to be similar to that of depressive humans, and the severity of the response to DEX seems to be most severe in the “stress and vaccine group”, followed by the “vaccine and stress” group and control group. Similar “collapse” as observed in “stress and vaccine”, with no changes in

plasma cortisol response after injection in DEX, has also been documented in pigs submitted to a prolonged reduction in their space allowance (Meunier-Salaun et al. 1987).

Too the best of our knowledge no such studies have been done on fish, where one combines baseline levels of plasma cortisol, sensitivity of the interrenal cells (ACTH), and efficiency of the negative feedback by corticosteroids (DEX) during prolonged stress. The results indicates that a fish who enters an allostatic overload type 2 (as the “stress and vaccine” group) is oversensitive to ACTH, have a reduced efficient negative feedback system, and elevated baseline levels of plasma cortisol with pronounced effects on wellbeing of the animal. The “vaccine and stress” group represent an allostatic overload type 1 response with oversensitivity to ACTH and transient reduced efficient negative feedback system, but at the end of the experiment the fish had recovered. However fish in the “vaccine and stress” group had elevated mortality compared to control and experienced reduced animal welfare. In a simplified and general way one can conclude the type 1 overload could be associated with more acute stressors with temporary changes in the sensitivity of the HPI-axis and type 2 overloads being associated increasingly with more chronic stressors with severe and malicious effect on the HPI-axis and the welfare of the being. However, as Schreck (2010) emphasised the distinction between acute and chronic stress is quite blurred. Thus, it is difficult to generalise about the effects of various stressors, since several stressors are often experienced simultaneously, sequentially, or in series during different life scenarios. Likewise the effects of the different stressors and severities are more an ongoing process, where the outcome of stress is depending on the mental and physical fitness of the individual organism.

The study shows that if plasma cortisol becomes elevated prior to vaccination it could instigate an allostatic overload type 2 with dire consequences on animal welfare. To reduce the risk to compromise the animal welfare during commercial vaccination of salmon one propose to grade the fish minimum a week prior to vaccination, or grade simultaneously with vaccination. This could reduce the overall allostatic load during handling and vaccination, and produce a healthy fish, with intact immune response and improved animal welfare.

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version of the manuscript. The experiment described has been approved by the local responsible laboratory animal science specialist under the surveillance of the Norwegian Animal Research Authority (NARA) and registered by the Authority.

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## Figure captions

Fig. 1. The effects of four weeks of daily applied stressor on baseline levels of plasma cortisol either before (“vaccine and stress”) or after (“stress and vaccine”) secondary vaccination. Note that control group was not subjected to either secondary vaccination or daily stressor. Values are expressed in mean  $\pm$  SD (n=6). Significant changes ( $p < 0.05$ ) from pre-stress are indicated with \*. Significant changes ( $p < 0.05$ ) between groups are indicated with #.

Fig. 2. Average plasma chloride (a) and magnesium (b) levels in Atlantic salmon postsmolts pre-stress, and during and after 4 weeks of daily applied crowding stress either before (pre-stress; “vaccine and stress” or after (week 4; “stress and vaccine”) secondary vaccination. Note that control group was not subjected to either secondary vaccination or daily stressor. Values are expressed in mean  $\pm$  SD (n=6). Significant changes ( $p < 0.05$ ) from pre-stress are indicated with \*. Significant changes ( $p < 0.05$ ) between groups are indicated with #.

Fig. 3. Average plasma cortisol levels in Atlantic salmon postsmolts 2 h following intraperitoneal injection with either phosphate buffered saline (PBS; 0.5 mL kg<sup>-1</sup>); or adrenocorticotrophic hormone (ACTH 45  $\mu$ g mL<sup>-1</sup>, 0.5 mL kg<sup>-1</sup>). All fish were injected 24 h previously with dexamethasone (DEX; 1 mg kg<sup>-1</sup> in ethanol : PBS; 1:3; 1  $\mu$ g L<sup>-1</sup>). Fig. 3a. Control group not given either secondary vaccination or daily stressor, fig. 3b. The “vaccine and stress” group given a secondary vaccination and then a daily stressor and fig. 3c. The “stress and vaccine” daily stressor for 4 weeks and the vaccinated for second time. Values are expressed in mean  $\pm$  SD (n=6). Significant changes ( $p < 0.05$ ) from pre-stress are indicated with \*. Significant changes ( $p < 0.05$ ) between groups are indicated with #.

Fig. 4. The effect of four weeks of daily applied stressor on antibody titre either after (“vaccine and stress”) or before (“stress and vaccine”) secondary vaccination. Note that control group was not subjected to either secondary vaccination or daily stressor. Values are expressed in mean  $\pm$  SD (n=12). Significant changes ( $p < 0.05$ ) from pre-stress are indicated with \*. Significant changes ( $p < 0.05$ ) between groups are indicated with #

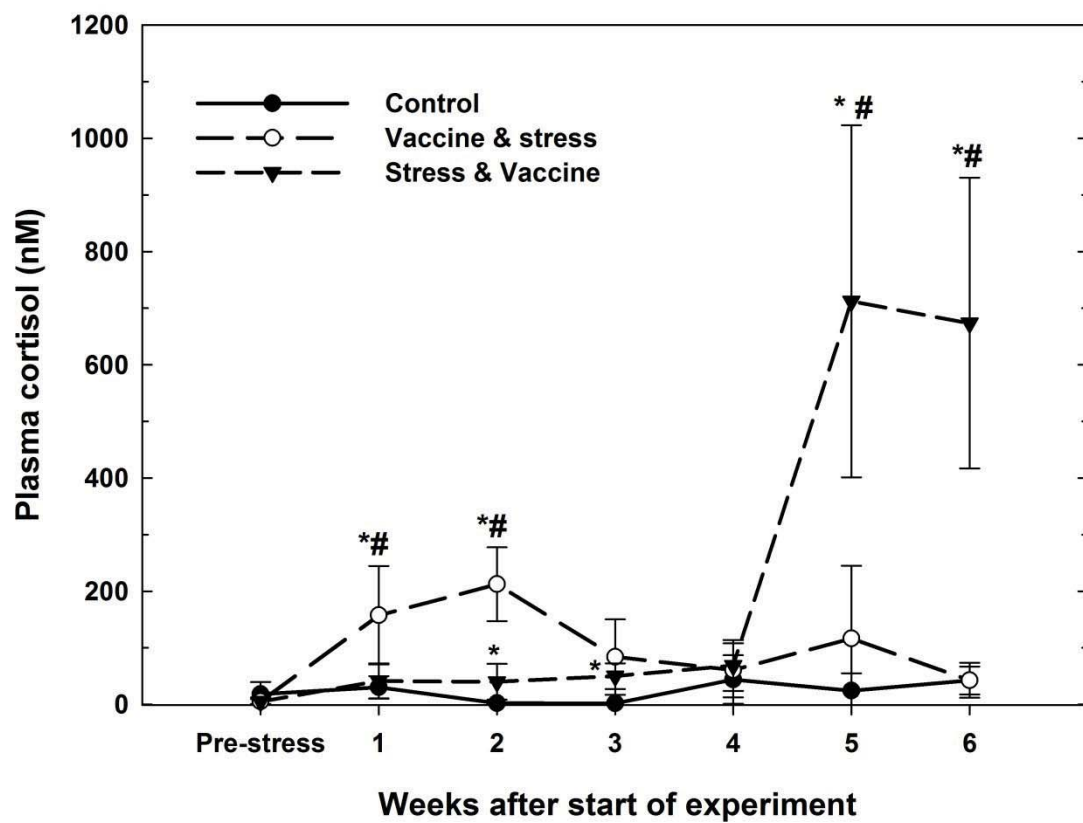


Fig. 1

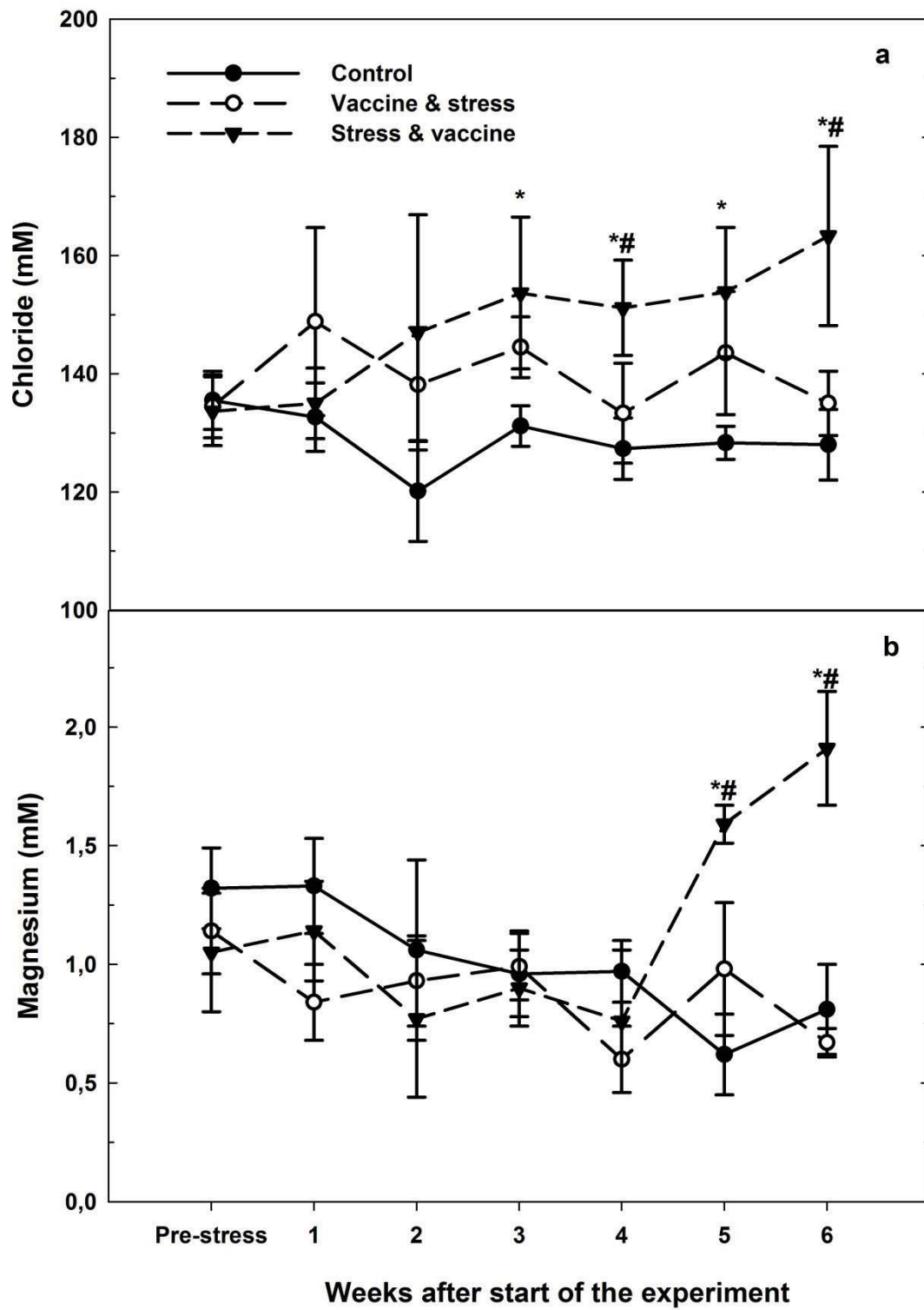


Fig. 2

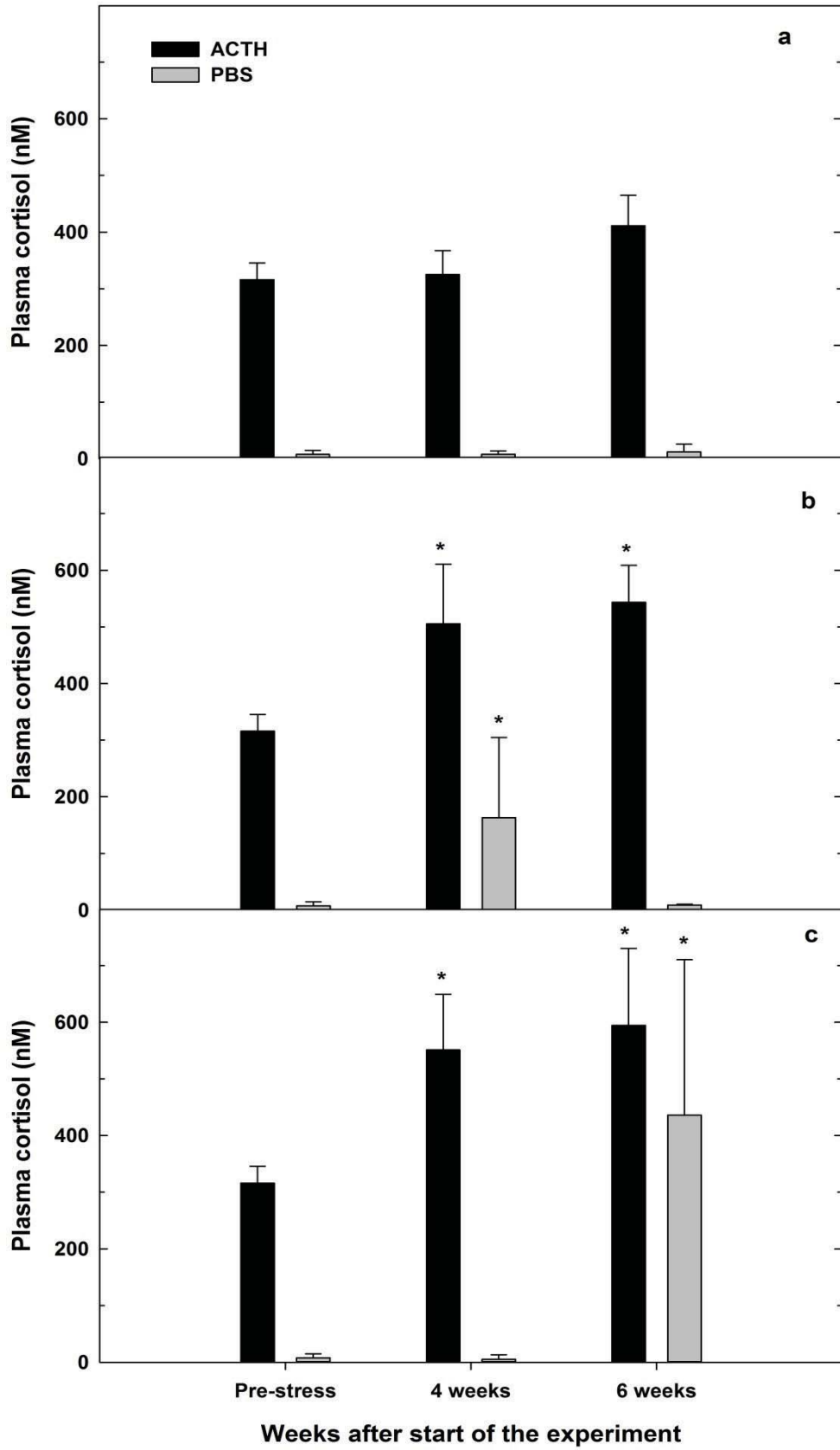


Fig. 3.

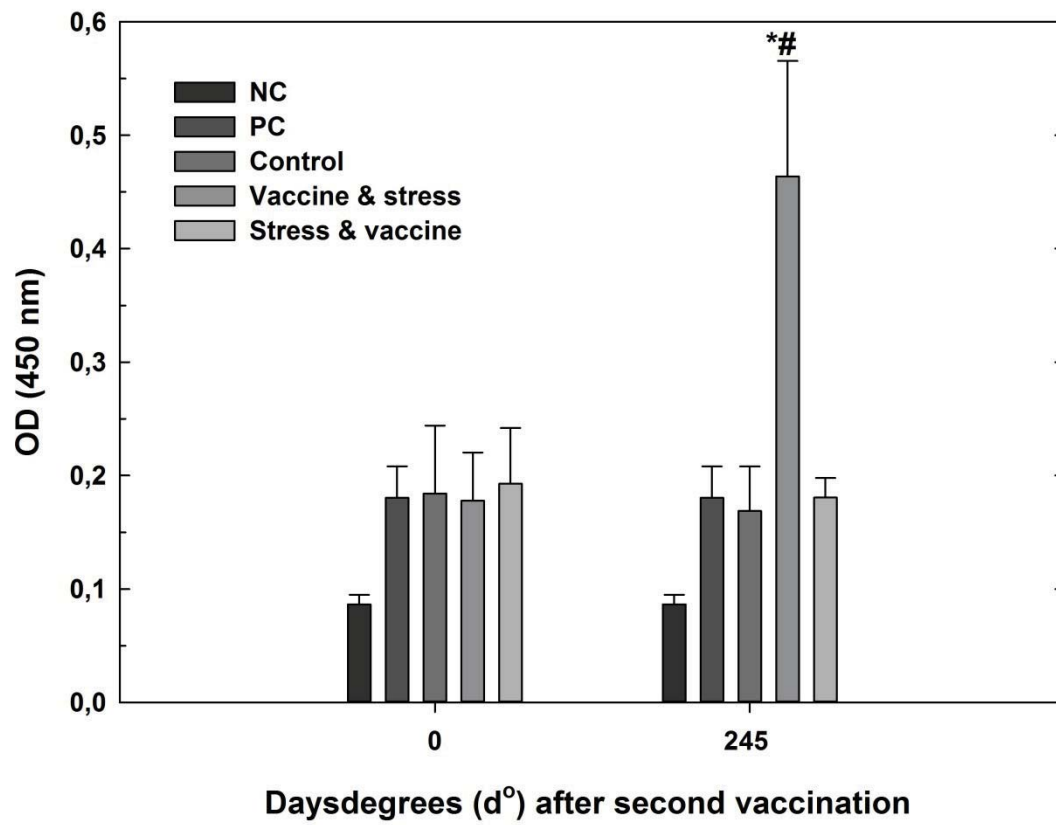


Fig. 4.