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Systemic and mucosal stress responses of Atlantic salmon (*Salmo salar*) to peracetic acid

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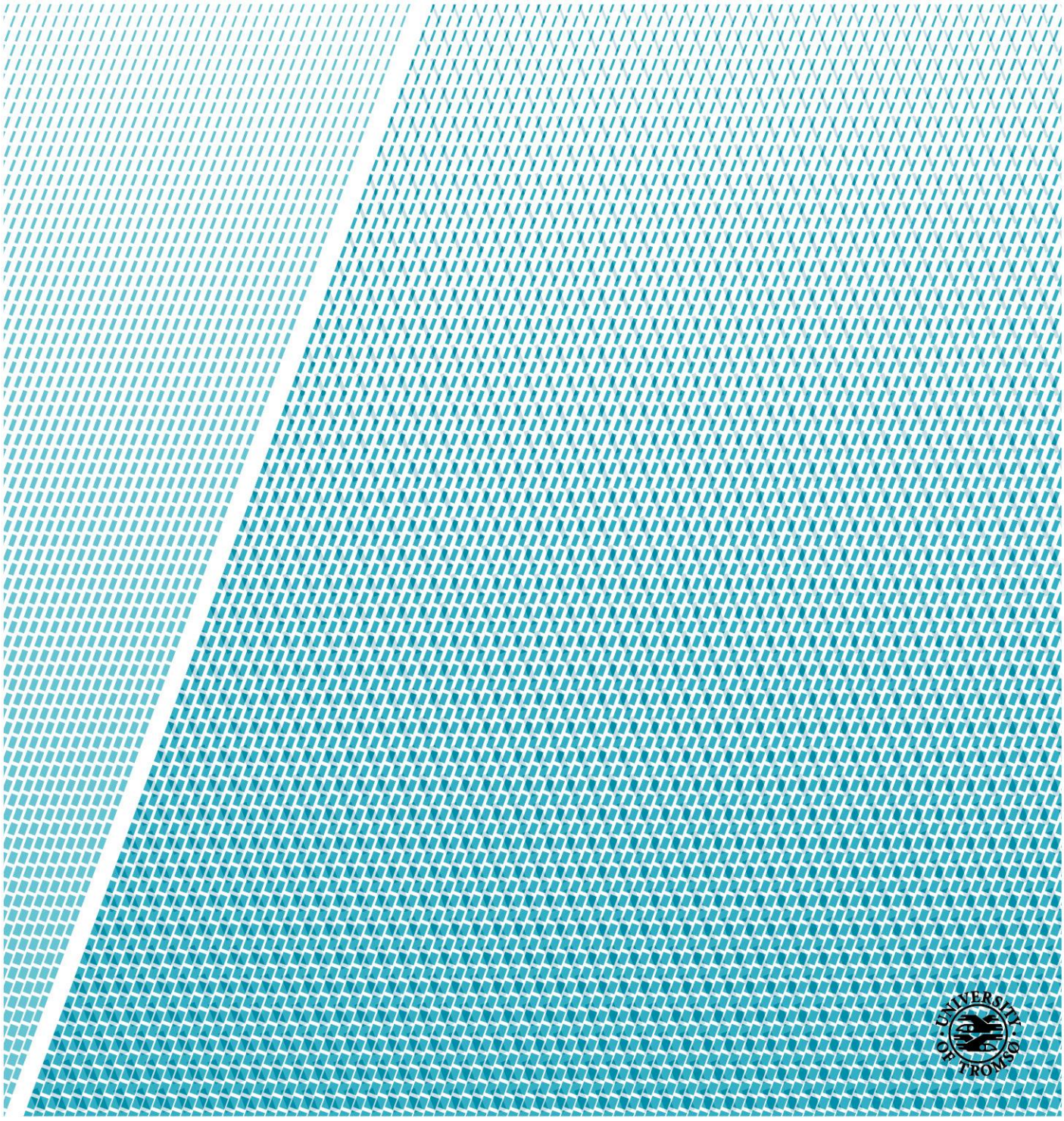

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Abstract

Infectious diseases are still a major problem in salmon aquaculture despite several attempts on developing prophylactic measures. Amoebic gill disease (AGD) is considered a growing threat in Norwegian salmon aquaculture. It has increased in prevalence and geographic distribution in the last years, resulting in considerable economic losses. Current treatments (i.e., freshwater and hydrogen peroxide bathing) are not optimal as they have both practical and welfare issues. It is, therefore, necessary to find new treatment alternatives. Peracetic acid (PAA) is under consideration as a potential treatment against AGD, but it is vital in the development of new chemotherapeutic measures that the consequences to the health and welfare of fish be identified. The overall aim of this thesis was to evaluate the systemic and mucosal stress responses of Atlantic salmon (*Salmo salar*) post smolts to peracetic acid. In Trial 1, salmon were first exposed to different nominal concentrations (0, 0,6 and 2,4 ppm) of PAA for 5 minutes, followed by a re-exposure to the same concentrations for 30 min 2 weeks later. Sampling was performed before exposure, 2 h, 48 h, and 2 w after exposures on both occasions. In Trial 2, salmon were subjected to crowding stress prior to PAA exposure at 4,8 ppm, double than the highest concentration tested in Trial 1. The fish were sampled before exposure, 1 h, 4 h, and 2 w after exposure. The treatments in both trials did not dramatically affect the overall external welfare status of fish. Both systemic and mucosal stress indicators were affected by the treatments at varying levels, and it was apparent that the fish were able to mount an appropriate response to the physiological demands of PAA exposure. In particular, the cortisol levels increased in the early hours after exposure, then followed by a rapid decrease and was back at baseline levels 2 weeks post-exposure. Prior exposure history to PAA did not markedly affect the stress responses of fish when re-exposed to PAA. Crowding stress before PAA treatment, however, did influence some of the stress indicators (lactate, glucose and antioxidant genes in the gills). Nonetheless, the changes were not substantial. In addition, a preliminary *in vitro* experiment revealed the amoebicidal activity of PAA against *Paramoeba perurans,* the causative agent of AGD. In conclusion, the results of this study showed that PAA at the tested concentrations did not dramatically compromise the health and welfare of salmon. The fish were able to mount a robust adaptive response to different PAA doses, exposure time and a potential confounding factor. The preliminary data on the amoebicidal activity of PAA further supports the potential of PAA as a potential treatment for AGD.

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List of abbreviations

AGD	Amoebic gill disease
cDNA	Complementary deoxyribonucleic acid
cu/znsod	*copper and zink superoxide dismutase*
DNA	Deoxyribonucleic acid
gp	*glutathione peroxidase*
gr	*glutathione reductase*
GSH	Sulfhydryl form glutathione
GSSG	Glutathione disulphide
H_2O_2	Hydrogen peroxide
mnsod	*manganese superoxide dismutase*
NH_3	Ammonia
NO_2-N	Nitrite nitrogen
NO_3-N	Nitrate nitrogen
OWI	Overall welfare index
OH•	Hydroxyl radical
O_2-	Superoxide radical
PAA	Peracetic acid
ppm	Parts per million
RAS	Recirculation aquaculture system
RNA	Ribonucleic acid
ROS	Reactive oxygen species
sod	*superoxide dismutase*
TAN	total ammonia nitrate
WI	welfare index
qPCR	quantitative polymerase chain reaction
$^1O_2$	singlet oxygen

1 Introduction

1.1 Norwegian Atlantic salmon production

Aquaculture has expanded remarkably over the past decades and is still growing. One of the most cultured species is Atlantic salmon (*Salmo salar*). and Norway is the leading global producer. In 2017 alone, the production of salmon was 1,2 million tons (Figure 1) (Statistics Norway, 2018). Traditional salmon farming produces smolt on land before the fish are transferred to open net pens in the sea for the rest of its production cycle; this can take up to 2 years. Some of the biological and societal challenges to this include organic waste release, fish escapes and diseases (Black, 2001). The pathogenic organisms in the surrounding environment are a constant threat to fish production. When environmental conditions favour the growth and proliferation of many of these opportunistic pathogens, disease outbreaks can take place. Diseases like heart and skeletal muscle inflammation (HSMI), ulcers, parasites and gill diseases are significant problems in the open net pens, causing devastation both biological and economic (Hjeltnes *et al.*, 2018).

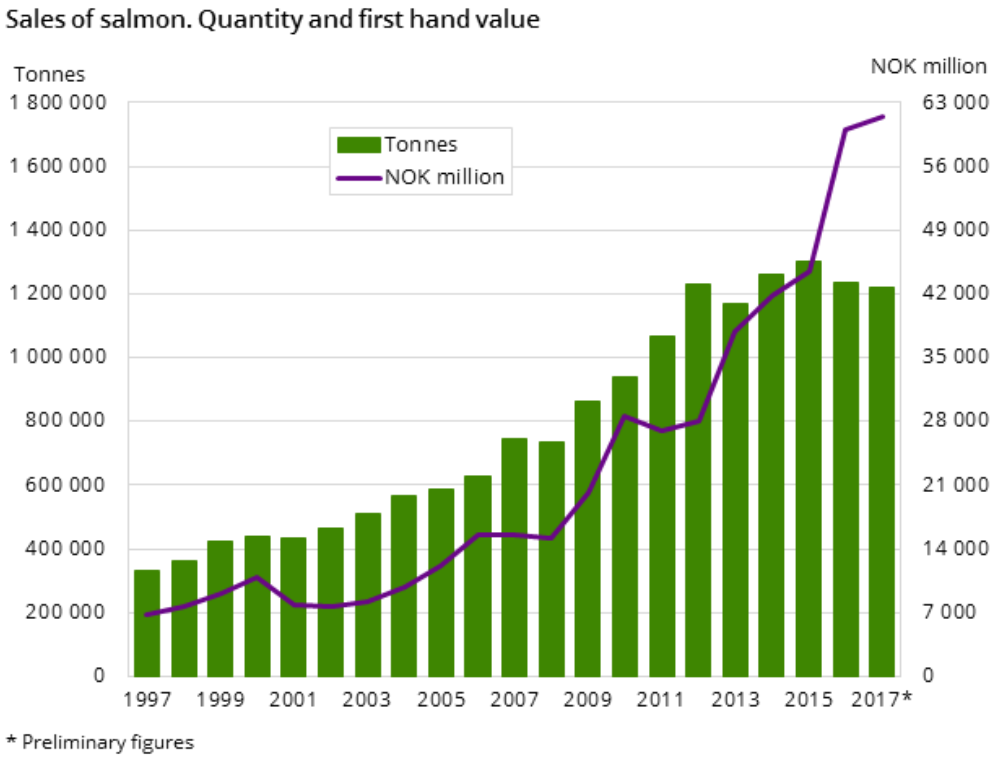

Figure 1: Quantity of Atlantic salmon sold in Norway and first hand value in the period 1997 – 2017. Source: Aquaculture, Statistics Norway.

Diseases and fish welfare in open net pens are perennial problems, hence, closed production systems have been in focus in the last years. Closed systems can be land-based recirculation aquaculture systems (RAS) where water is recycled in different degrees or sea-based in which floating tanks obtain water from a certain depth (Thorarensen and Farrell, 2011; Bregnballe, 2015). Closed sea-based systems are not yet fully commercialised, and many prototypes are still under development (Snøfugl, 2018). Nonetheless, the solutions they offer to combat the constant problems on sea lice, amoebic gill disease (AGD) and escapees, among others, are promising. The risk for pathogens is relatively low in RAS due to the minimal water use and fish are better protected from varying environmental conditions such as in open net pens. However, if a pathogen enters the system, it can spread to other connected tanks as well (Bregnballe, 2015). Disinfectants and chemical treatments are used in RAS to maintain optimum water quality and to improve biosecurity (Bregnballe, 2015). Many disinfectants have been used like ozone, hydrogen peroxide (H_2O_2) and formalin, with varying effectiveness and risks, both to the biofilter and the fish (Bregnballe, 2015). Peracetic acid (PAA) has been proven to be a suitable disinfectant in RAS and its importance have been acknowledged in a global scale (Liu *et al.*, 2016; Pedersen *et al.*, 2009; Liu *et al.*, 2017)

1.2 Peracetic acid in aquaculture

Peracetic acid is an organic peroxide and is both corrosive and flammable in its undiluted form (Liu, 2017). The commercially available product is an equilibrium mixture of acetic acid, H_2O_2 and water and degrades into acetic acid, water and oxygen at varying kinetic order depending on other factors, such as, organic matter, temperature, light, among many others (Wagner *et al.*, 2002; Pedersen *et al.*, 2009). Thus, it has been recognised to have a very low environmental impact (Straus *et al.*, 2017). H_2O_2 contributes to the disinfection power of PAA, being one of the components of the commercial product, but PAA is a more potent active component of the commercial product. Commercially available PAA solutions contain 5 – 20 % v/v of pure PAA. It is also active at low concentrations against a broad spectrum of microorganism and shown to be bactericidal, virucidal, fungicidal and sporicidal (Baldry, 1983; Kitis, 2004). This is mainly attributed to the fat solubility of PAA. Moreover, its fast-acting property is also credited to its degradation which is by chemical oxidation, whereas H_2O_2 is dependent on microbial breakdown, but both will spontaneously decompose to water and oxygen (Block, 1991).

PAA toxicity has been demonstrated on several aquatic pathogens, such as, infectious salmon anemia virus, *Ichthyophthirius multifiliis*, *Aphanomyces* spp., *Flavobacterium columnare*, *Saprolegnia* spp., *Aeromonas salmonicida* and *Yersinia ruckeri* (Smail *et al.*, 2004; Meinelt *et al.*, 2009; Straus and Meinelt, 2009; Sudová *et al.*, 2010; Jussila *et al.*, 2011; Marchand *et al.*, 2012; Picón-Camacho *et al.*, 2012; Straus *et al.*, 2012; Farmer *et al.*, 2013; Meinelt *et al.*, 2015). The effective concentration of PAA have been proven to be less than 2 ppm against various pathogens, and furthermore, concentrations around 1 ppm PAA are reported to have no effect on the biofilter (Pedersen *et al.*, 2013), thus a suitable disinfectant in RAS (Pedersen *et al.*, 2009; Liu *et al.*, 2016; Liu *et al.*, 2017; Liu, 2017). In contrast, H_2O_2 needs much higher concentrations for the same level of disinfection and can potentially harm the biofilter (Wagner *et al.*, 2002; Liu, 2017).

The acute toxicity of PAA to different commercially important fish species varies. Straus *et al.* (2017) found that the mean 24-h lethal concentration (LC_{50}) ranged from 2,8 – 9,3 ppm PAA and the 24-h no-observed-effect-concentration (NOEC) ranged from 1,9 – 5,8 ppm PAA for the 12 different species tested. Atlantic salmon was not included in the study, but rainbow trout (*Oncorhynchus mykiss*) had LC_{50} at 4,17 ppm PAA and NOEC at 2,8 ppm PAA. These values can be used as guidelines to determine the safe PAA concentrations for use in aquaculture.

1.3 Stress in fish

Stress is defined as a situation where homeostasis is threatened by some kind of stressor, and it is re-established by a series of stress response. The stress responses are considered adaptive mechanisms that allow fish to handle the stressors and maintain homeostasis (Barton, 2002). By measuring stress indicators, it is possible to determine how health, performance and welfare are affected by different treatments, husbandry practices, environmental changes, among others (Sopinka *et al.*, 2016). In aquaculture, several factors may act as a stressor including poor water quality, handling, confinement, hypoxia, transport or exposure to chemotherapeutants, to name a few.

The responses are defined as primary, secondary and tertiary (Figure 2). The primary level involves an increase in catecholamine release from the chromaffin tissue and cortisol (corticosteroids) release from the interrenal tissue of the head kidney; the latter is slightly delayed and thus easiest to measure (Barton, 2002). Plasma level of cortisol rises within a few minutes after exposure to the stressor and the return to baseline level can take one or more

hours and is thus a reliable stress indicator (Barton and Iwama, 1991; Noble *et al.*, 2018). The secondary level includes changes in metabolism, hydromineral balance and cardiovascular system (Barton, 2002). Both plasma lactate and plasma glucose can increase as a secondary response. The catecholamines induce the release of glucose from the liver through glycogenolysis to assure energy to the muscles, and the absence of oxygen results in anaerobic metabolism and thus, increase lactate (Sopinka *et al.*, 2016). Both happens over a slower timescale compared with cortisol and are likewise used to supplement the acute stress indicators in fish (Sopinka *et al.*, 2016; Barton and Iwama, 1991). If the stressor is chronic or aggregated, a tertiary response occurs which may last for weeks or months, affecting growth, condition, disease resistance, behaviour and survival of the fish (Barton, 2002).

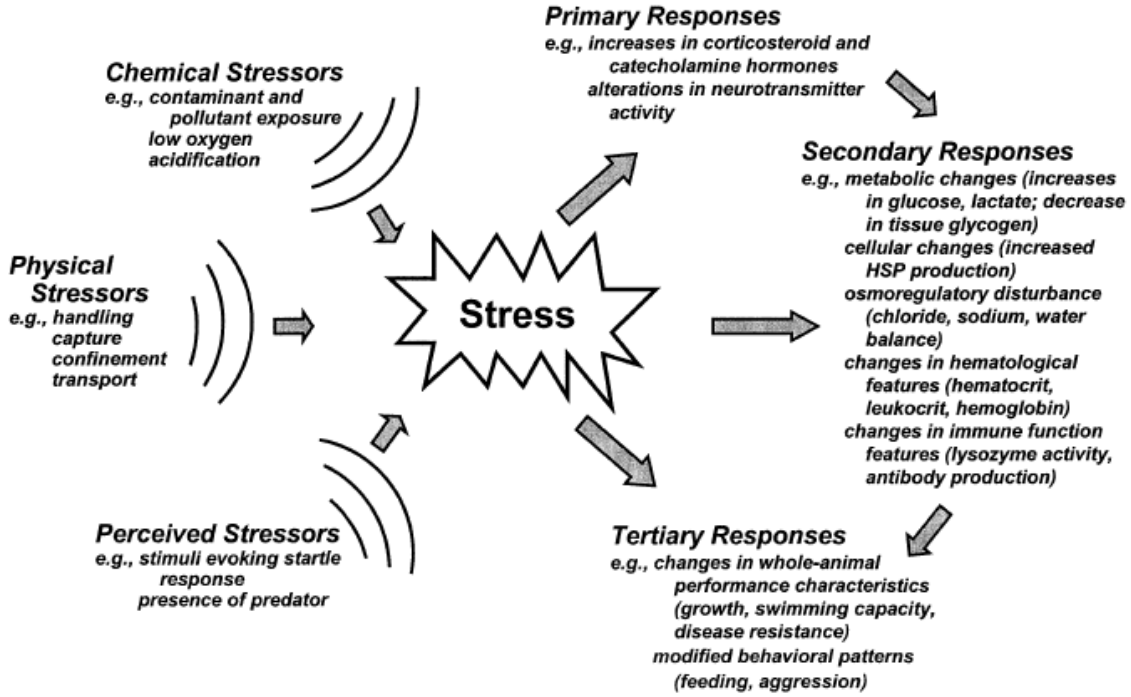

Figure 2: Different type of stressors which can initiate stress responses in the fish, divided into primary, secondary and tertiary responses (Barton, 2002). Reproduced under license number 4585471132787 from Oxford University Press.

Oxidative stress occurs when production and accumulation of reactive oxygen species (ROS) overwhelm the scavenging capacity of antioxidants which can then result in the damage of lipids, proteins and deoxyribonucleic acid (DNA) (Lesser, 2006). ROS such as superoxide radical (O_2^-), singlet oxygen ($^1O_2$), H_2O_2 and hydroxyl radical (HO•) (Lesser, 2006) can be produced as a result of metabolic rate, water temperature, changes in O_2 availability, or be introduced to the environment through for example chemicals containing H_2O_2 or PAA as ROS are formed when they dissociate (Tort *et al.*, 2005; Clotfelter *et al.*, 2013; Liu, 2017). The fish have a natural antioxidant defence system to maintain homeostasis (Sopinka *et al.*,

2016) and four antioxidant molecules known to be responsive during oxidative stress are *glutathione reductase* (*gr*), *copper* and *zink superoxide dismutase* (*cu/znsod*), *manganese superoxide dismutase* (*mnsod*) and *glutathione peroxidase* (*gp*) (Solberg *et al.*, 2012). The four antioxidant molecules, together with other antioxidants, scavenge and transforms ROS by detoxification to protect the cells from oxidative damage (Tort *et al.*, 2005). Superoxide dismutase occurs as different metalloproteins (Mn/Cu/Zn/Fe) which breaks down O_2^- into H_2O_2, which is then further broken down to water (H_2O) (Weydert and Cullen, 2009). For Glutathione peroxidase to function it requires secondary enzymes, like Glutathione reductase, and co-factors, like sulfhydryl form glutathione (GSH) (Weydert and Cullen, 2009). Glutathione reductase is dependent on the co-factor nicotinamide adenine dinucleotide phosphate (NADPH) to reduce glutathione disulphide (GSSG) to GSH (Weydert and Cullen, 2009). Thus O_2^- conversion is dependent on Superoxide dismutase, Glutathione peroxidase and Glutathione reductase.

A few studies have reported the stress responses after PAA exposure in fish, and interestingly, none on salmon. Plasma stress indicators have been studied in rainbow trout (Liu, 2017; Gesto *et al.*, 2018) and mirror carp (*Cyprinus carpio*) (Liu, 2017), as well as ROS changes (Liu, 2017) following PAA exposure. They reported that PAA could be used as a welfare-friendly disinfectant as the fish displayed a normal adaptive stress response. As PAA is being developed as an alternative treatment for salmon (FHF project nr 901472) it is important to explore the health-related impacts of PAA exposure to document the physiological changes that will help to establish safe limits recommended levels.

1.4 The mucosal barrier in fish

Fish are in constant contact with the aquatic environment where biological, physical and chemical threats are always present (Lazado and Caipang, 2014). The mucosal organs (i.e. skin, gills and gut) are sensitive and responsive to environmental changes (Peterson, 2015; Balasch and Tort, 2019) and thus are considered as the first crucial line of defence (Salinas, 2015). The mucosa-associated lymphoid tissue (MALT) is the collective term to refer the main organ of defence at the mucosa and is separated into gut-associated lymphoid tissue (GALT), nasal-associated lymphatic tissue (NALT), skin-associated lymphoid tissue (SALT) and gill-associated lymphoid tissue (GIALT) (Lazado and Caipang, 2014).

Gills are a multifunctional organ with a single epithelial layer, large respiratory surface area and are covered by a mucous layer (Peterson, 2015). They are responsible for respiration,

osmoregulation, pH regulation, control of hemodynamic and nitrogenous waste excretion, among others (Peterson, 2015; Koppang *et al.*, 2015). They form a semipermeable barrier between the fish and the environment (Koppang *et al.*, 2015). Skin, like gills, is a multipurpose tissue with a mucus layer and has a large surface area which surrounds the entire fish body (Peterson, 2015). It serves several functions such as, protection against physical damage and microorganism invasion, conservation of hydrodynamics, and are very important in maintaining physiological homeostasis, such as osmotic balance (Peterson, 2015). The mucous layer contributes to the immune defence as a physical barrier against pathogens (Peterson, 2015), as it forms a thin barrier that separates the fish from the environment (Ángeles Esteban and Cerezuela, 2015). Mucus also contains biologically active substances such as, mucin, antimicrobial peptides, lysozymes, and lectins (Ángeles Esteban and Cerezuela, 2015; Peterson, 2015; Cabillon and Lazado, 2019), and several enzymes (i.e. protease, oxidase, peroxidase) (Salinas and Parra, 2015). The microorganisms residing on the mucosal surfaces contribute to the defence mechanism, through the production of inhibitory compound, inhibition of pathogen adhesion, colonisation of beneficial microbes, among others (Cabillon and Lazado, 2019). The physical barrier of the mucosa captures and eliminates pathogens, and if the pathogens penetrate the barrier, the cellular and humoral components will act upon the foreign object and trigger a cascade of response. Some cells develop a memory that when they encounter the same foreign object, they already have the repertoire of responses ready (Cabillon and Lazado, 2019).

1.5 Amoebic gill disease

AGD is caused by the facultative parasitic amoeba *Paramoeba perurans* (Young *et al.*, 2007; Powell and Kristensen, 2014). The parasite has been reported to affect several species like rainbow trout, brown trout (*Salmo trutta*), chinook salmon (*Oncorhynchus tshawytscha*), lumpsucker (*Cyclopterus lumpus*) and Ballan wrasse (*Labrus bergylta*) (Kim *et al.*, 2017; Hjeltnes *et al.*, 2018), but among salmonids Atlantic salmon appears to be the most susceptible (Mitchell and Rodger, 2011). The first outbreak reported was in the mid-1980s in Tasmania, Australia, and has since then occurred in many countries, for example United States, Chile, Ireland, Spain, Scotland and Japan (Mitchell and Rodger, 2011; Powell and Kristensen, 2014). The first documented outbreak in Norway was in 2006, the second in 2012, and have since increased in prevalence, geographic distribution, and have caused

considerable losses (Hjeltnes *et al.*, 2018). The parasite was confirmed from Vest-Agder to Nordland in 2017, often in coherence with another disease (Hjeltnes *et al.*, 2018).

There is limited knowledge about the amoeba's habitat and lifecycle. *P.perurans* has a parasitic stage and a free-living stage with long pseudopodia, which is possibly the transmission stage that can spread over long distances and may survive for days or weeks (VKM, 2014). Spreading can also happen through moving hosts, infected equipment or infected fish (VKM, 2014). Atlantic salmon can be affected throughout the whole seawater phase but post-smolts are most susceptible (Mitchell and Rodger, 2011; VKM, 2014). Outbreaks are most common with temperatures between 12 – 20 °C and high salinity (35 ‰) (Mitchell and Rodger, 2011; Hjeltnes *et al.*, 2018), but has been reported at temperatures as low as 5 – 6 °C and salinity around 28 ‰ (VKM, 2014). The parasite is mainly on the gills and AGD causes white-grey spots with increased gill mucus on the gills, lamellar fusion, reduced swimming activity, reduced growth, respiratory distress and mortality (Mitchell and Rodger, 2011; VKM, 2014). Methods to prevent AGD outbreaks are not known but healthy, robust fish in a good environment reduces the risk and early detection is important (VKM, 2014).

The preferred treatment against AGD is freshwater (Powell and Kristensen, 2014). It is considered more environmentally friendly and does not appear to harm the salmon (Powell *et al.*, 2001). The process takes one working day to complete and involves moving the fish to the bathing site with technical equipment, thus it has high infrastructure cost and is labour expensive (Powell and Kristensen, 2014). Freshwater can also be a limited resource, does not eradicate the amoeba and re-infection is common (Clark *et al.*, 2003). Chlorine-based Chloramine-T and H_2O_2 have also been used as a treatment against AGD. However, Chloramine-T toxicity is enhanced in seawater (Powell and Harris, 2004) and H_2O_2 is proven to be less effective, requires a high concentration and exposes the fish to a higher risk of mortality (Adams *et al.*, 2012; Powell and Kristensen, 2014).

Despite several attempts on developing prophylactic methods for infectious diseases, they remain a major bottleneck in salmon aquaculture. Sea lice are the main parasitic infection problem, but amoebic gill disease has over the last couple of years become a more significant threat (Hjeltnes *et al.*, 2018). Knowledge about AGD has been one of Fiskeri- og Havbruksnæringens Forskningsfinansiering (FHF) priorities in the last years, and it is still a priority in 2019. More knowledge about the amoeba and how to treat it is essential for the aquaculture industry. Because of the welfare and practical issues with current treatments,

there is a need for a new treatment method against AGD. PAA is now under consideration as a potential treatment for AGD, as it has been proven to be effective against several aquatic pathogens at low concentrations and effective doses are within the tolerable limits of different fish species.

1.6 Objective

The potential of peracetic acid as a treatment against AGD is outlined by several advantages: 1) PAA degrades completely into harmless, neutral residuals and is thus environmentally friendly; 2) The toxicity to PAA varies in different fish species, but it is higher than what was proven to be effective against many pathogens; 3) PAA has been reported to induce a normal stress response in fish; 4) PAA is reported as a sustainable disinfectant in RAS because some organisms, like microalgae, is not susceptible to the treatment and concentration ≤ 1 ppm did not affect the biofilter, and most importantly; 5) it has anti-parasitic activity and is active at low concentrations (≤ 2 ppm) against a wide spectrum of microorganism. Moreover, PAA is also a more potent disinfectant than H_2O_2.

This thesis is a part of an on-going initiative that explores the potential of peracetic acid as a treatment for AGD. It is vital in the development of new chemotherapeutic measures that a strategy should not impose a significant health and welfare concerns to the fish. It is equally important to establish the amoebicidal effect of PAA and identify physiological and morphological alterations following treatment, to identify safe limits and ensure welfare. As of now, the stress responses in Atlantic salmon exposed to PAA are not known.

The overall aim of this thesis was to evaluate the systemic and mucosal stress responses of Atlantic salmon (*Salmo salar*) to peracetic acid. The objective was driven by the main hypothesis that PAA, an oxidising agent with anti-parasitic activity against *Paramoeba perurans* is a stressor that triggers strong systemic and mucosal stress responses and that repeated exposure will lead to compromised welfare of salmon.

Specific objectives

1) Document the changes in the external welfare indicators following exposure to PAA.
2) Determine the levels of cortisol, glucose and lactate in the plasma of salmon repeatedly exposed to PAA.
3) Identify the influence of crowding prior to PAA exposure on the stress responses of salmon.

4) Asses the regulation of antioxidant defences in the mucosal tissue following PAA exposure.
5) Evaluate the antiparasitic activity of PAA against *P.perurans*.

2 Materials and Methods

2.1 Ethics statement

All fish handling procedures employed in the study were in accordance with national and EU legislation (2010/63/EU) on animal experimentation.

2.2 Peracetic acid

Peracetic acid (Divosan Forte, PAA) was supplied by Lilleborg AS (Oslo, Norway). The disinfectant is a stabilised PAA solution (15 %) which is non-foaming and completely free-rinsing. The actual amount of PAA in the solution was verified by the Technical University of Denmark (DTU) Aqua laboratory (Hirtshals, Denmark) to be at approximately ~ 18 %. The solution was stored at 4 °C. During each exposure, the concentration of PAA in the water was experimentally verified in real-time to ensure that the fish were exposed to the target concentration from start to termination of exposure.

2.3 Exposure Experiments

2.3.1 Trial 1: Effects of different PAA concentrations and exposure durations

2.3.1.1 Fish and husbandry conditions in the holding facilities

Salmon post-smolts were purchased from Danish Salmon A/S (Hirtshals, Denmark). The fish were transported to the recirculation aquaculture facility of DTU Aqua, sorted and moved to six 1 m^2 holding tanks (water volume ≈ 600 L), with 60 fish in each tank. The RAS had a 40 µm drum filter, a submerged fixed bed biofilter and a trickling filter with a makeup water exchange at approximately 0,4 m^3/h equivalent to a retention time of 1,5 days. Internal recirculation allowed more than two times the tank exchange per hour.

The fish were acclimated for three weeks under stable laboratory conditions, with daily monitoring of water quality parameters that were kept within safe limits (Table 1). The tanks had no direct light above them and the photoperiod in the experimental hall was set at 16L:8D (06.00 – 22.00), similar to the natural photoperiod in April-May (57º35’N 09º57’E). The fish were fed (Biomar, EFICO Enviro, 4,5 mm) at a ratio of 1,0 – 1,5 % total biomass per day through a belt feeder. Feeding was gradually increased during the acclimation period and

feeding behaviour of the fish in terms of uneaten feed pellets was registered by daily inspection of the swirl separator.

Table 1: Rearing conditions prior to and during Trial 1.

Parameter	Value	Unit
TAN[1]	≤ 0,2	mg N/L
NO_2^-N	≤ 0,2	mg N/L
NO_3^-N	≤ 5	mg N/L
pH	7,5 - 7,7	
Temperature	15 ± 1	°C
Oxygen	80 - 90	% saturation
Oxygen	7,8 - 9,1	mg O_2/L
Salinity	33 - 34	ppt
Makeup water	~ 10	m^3/d
System retention time	~ 1,5	d
Daily feed (total)	0,6 - 1,1	kg/d

[1]NH_3 < 0,01 ppm

2.3.1.2 Collection of pre-exposure fish

A day before the first exposure two fish from each of the holding tanks were sampled for plasma as described in detail in Section 2.3.1.5

2.3.1.3 First PAA exposure

Feeding was temporarily ceased 24 h prior to PAA exposure. Fish (150,26 ± 5,56 g of weight and 22,82 ± 0,26 cm of length, mean ± SE) were netted from the holding tank, transferred to a transportation container and immediately thereafter into the exposure tank with a similar volume (Figure 3A-C). Each holding tank had its equivalent exposure tank, and water quality parameters were identical between these two tanks. The fish were allowed to settle for 10 min before the PAA solution was added to the tanks to achieve the following final concentrations: 0 (seawater), 0,6 and 2,4 ppm (Figure 3D). The concentrations were pre-selected based on an earlier report on the toxicity of PAA on rainbow trout (Straus *et al.*, 2017). Each treatment group had two replicate tanks. During the exposure period, the water flow was stopped, and the degradation of PAA in the water matrix was followed (Figure 3E). After 5 min, fish were immediately netted out of the tank and returned to their corresponding holding tank (Figure

3F). Post-exposure samplings were carried out thereafter as detailed in Section 2.3.1.5. Two days after the PAA exposure, feeding was resumed as described in Section 2.3.1.1. All husbandry conditions during post-exposure rearing were similar to pre-exposure conditions.

Figure 3: PAA exposure experiment. A) Fish were netted from holding tank and B) transferred to a transportation bucket. C) Fish were immediately transferred to the exposure tank, allowed to settle for 10 min before D) PAA was added. E) During the exposure period, the concentration of PAA was analysed in real-time. F) After the exposure period, the fish were netted out of the exposure tank, transferred to the transportation bucket and returned to their corresponding holding tank. Note: The holding and exposure tanks were in the same experimental hall.

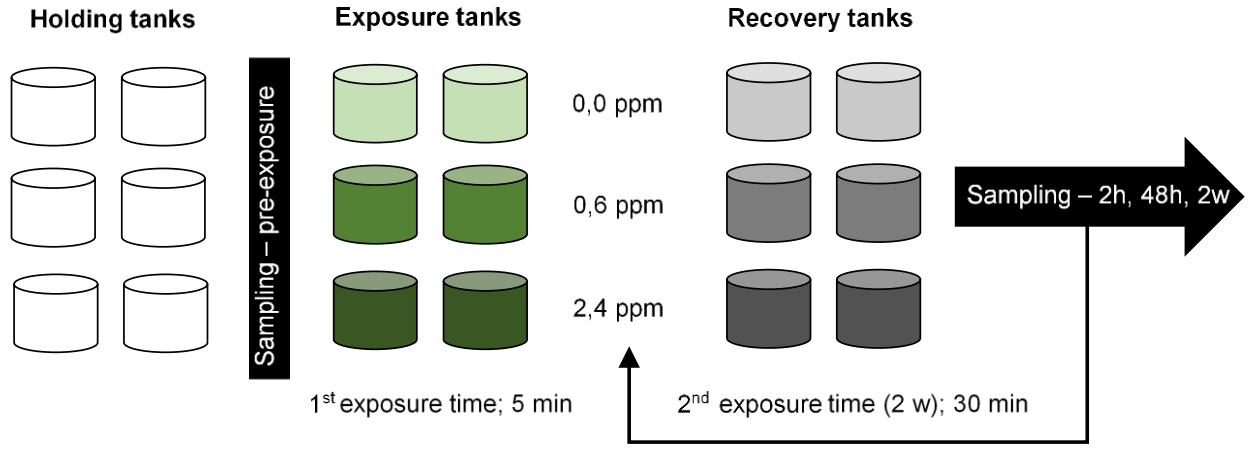

Figure 4: Overview of the initial and re-exposure experiments in Trial 1.

2.3.1.4 Re-exposure

Two weeks after the first exposure, the fish were re-exposed to the same concentration of PAA. The fish had an average weight of 150,26 ± 5,56 g and length of 24,54 ± 0,28 cm (mean ± SE). The protocol used in the re-exposure experiment was identical with the approach employed in the initial exposure (Section 2.3.1.3), with a slight modification on the duration of exposure. Instead of 5 min, fish were re-exposed to PAA at a similar concentration used in the first trial for 30 min (Figure 4). Fish were returned to their corresponding recovery tank and post-exposure samplings were carried out thereafter. Post-exposure husbandry strategies as described in Section 2.3.1.1 were followed.

2.3.1.5 Sample collection

Sample collection was performed 2 h, 48 h and 2 w after exposure for each experiment. Five fish were taken from each replicate tank and were humanely euthanised with an overdose of 20 % benzocaine solution. After the length and weight were measured, the external welfare status of the fish was assesed following the FISHWELL handbook (Noble *et al.*, 2018). A set of 11 external welfare indicators were evaluated and rated using 0 – 3 scale, where 0 means the “best” while 3 means the “worst”. To ensure objectivity and limit biases, only one person evaluated the fish throughout the experiment. Moreover, the evaluator was not informed about the treatment history of the fish. The condition factor was calculated according to the equation:

$$K = \frac{Weight\ (g)}{Length\ (cm)\text{^}3} * 100$$

Blood was withdrawn from the caudal artery using a heparinised vacutainer, centrifuged at 1000 x *g* for 10 mins at 4 °C and plasma was collected and kept at – 80 °C until analyses. The same sampling protocol was applied for fish that were collected before exposure (Section 2.3.1.2).

2.3.2 Trial 2: Effects of crowding stress before PAA exposure

2.3.2.1 Fish and husbandry conditions in the holding facilities

Salmon post-smolts were purchased from Danish Salmon A/S. The fish were transported to the aquaculture facility of DTU Aqua, sorted and moved to two 4 m^2 holding tanks (water volume ≈ 1500 L) in a seawater flow-through system, with 100 fish in each tank. The fish acclimated for two weeks under stable laboratory conditions, with daily monitoring of water quality parameters (Table 2). The photoperiod was set at 24L:0D and the dietary ration of 1 – 1,5 % total biomass (Biomar, EFICO Enviro, 4,5 mm) per day were provided through a belt feeder.

Table 2: Rearing conditions prior to and during trial 2.

Parameter	Value	Unit
TAN[1]	≤ 0,2	mg N/l
NO_2^-N	≤ 0,2	mg N/l
NO_3^-N	≤ 5	mg N/l
pH	7,6 – 7,8	
Temperature	11 ± 1	°C
Oxygen	85 - 98	% saturation
Oxygen	8,5 - 9,5	mg O_2/l
Salinity	33 - 35	ppt
Makeup water	~ 10	m^3/d
System retention time	~ 1,5	d
Daily feed (total)	0,6 - 1,1	kg/d

[1]NH_3 < 0,01 ppm

2.3.2.2 Crowding stress and PAA exposure

Fish were starved for 24 h prior to stress and PAA exposure. Before the experiment was carried out, 4 fish were collected from each holding tank to represent the "pre-exposure" fish. Samples were collected similarly as the samples for post-exposure, as described in detail below (Section 2.3.2.3).

Twenty five post-smolts (131,25 ± 2,30 g of weight and 23,99 ± 0,20 cm of length, mean ± SE) were transferred from the holding tank to a closed-system 500 L exposure tank, with a density of 15 kg/m^3 (Figure 5). The fish were allowed to settle for 15 min before a group was subjected to crowding stress for 1 h, by lowering the water volume to attain a density of 75 kg/m^3. Aeration was provided during crowding stress. Fifteen min after the water level returned to the initial level, one group of the stressed fish was exposed to 4,8 ppm PAA, double the highest concentration tested in Trial 1, while the other stressed group was exposed 0 ppm (seawater) for 30 mins. Likewise, non-stressed fish was exposed to a similar PAA protocol as the stressed groups. After the exposure period experimental fish were moved to new recovery tanks (water volume ≈ 600 L) connected to RAS. The RAS had a 40 µm drumfilter, a submerged fixed bed biofilter and trickling filter with a makeup water exchange at approximately 0,4 m^3/h equivalent to a retention time of 1,5 days. Internal recirculation allowed more than two times the tank exchange per hour. Water quality parameters were supervised daily and kept within safe limits (Table 2). The tanks had no direct light above them, and the photoperiod in the experimental hall was set at 14L:10D (06.00 – 20.00). The fish were fed (Biomar, EFICO Enviro, 4,5 mm) at a ratio of 1 – 1,5 % total biomass per day via a belt feeder. Feeding behaviour of the fish in terms of uneaten feed pellets was registered by daily inspection of the swirl separator. Each treatment group was represented with duplicate tanks.

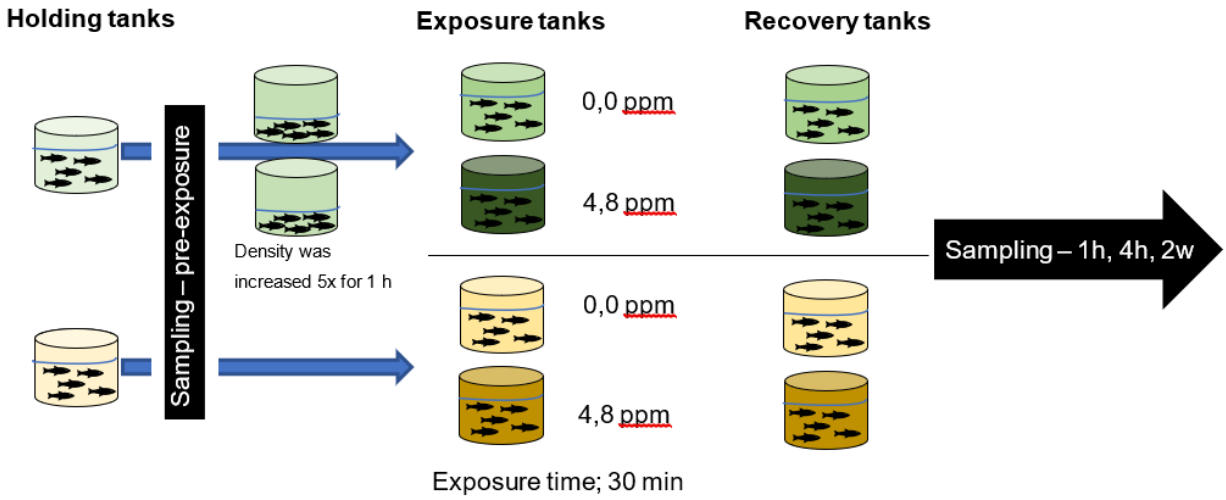

Figure 5: Overview of the experiment in Trial 2. Each treatment was represented with a duplicate tank (not shown in the diagram). Note: The holding and exposure tanks were in separated halls.

2.3.2.3 Sample collection

Sampling was performed 1 h, 4 h, and 2 w after PAA exposure. Sampling protocol as described in Section 2.3.1.5 was followed. In this trial, tissue samples for gene expression analyses were also collected. A portion of the dorsal skin and the second gill arch were dissected and transferred to RNAlater (Ambion, USA). Tissue samples in RNAlater were left at room temperature overnight and thereafter kept at – 80 °C before RNA extraction.

2.4 Stress indicators in the plasma

The plasma samples were aliquoted and kept at - 20 °C. Before analyses they were thawed on ice, vortexed (REAX 2000, Heidolph. Germany) and spun-down (C1301B, BioNordika, Norway). Three commercially available assay kits were used to analyse plasma stress indicators. A pre-test was done on selected samples from each treatment and sampling point to determine the most suitable dilutions of the plasma for the assay. Samples giving reading beyond the upper detection limit of the standard curve were further diluted. All samples and standards were run in duplicates.

2.4.1 Cortisol

Plasma cortisol was analysed using an Enzyme-Linked Immunosorbent Assay (ELISA) kit (402710, Neogen, USA) following the manufacturer's protocol, with modifications. Cortisol was not extracted from the plasma prior to performing the assay. Previous trials showed that

linearity and reproducibility of the assay were not affected when using unextracted samples (Gesto, pers.communication, (Gesto *et al.*, 2018)). Extraction buffer (ELISA kit) was diluted 5-fold with distilled water and the diluted extraction buffer was used to dilute the plasma samples. Standards were likewise prepared as described in the protocol. Fifty µL of each standard and 50 µL of each diluted sample were pipetted into their respective well. Fifty µL diluted enzyme mix was pipetted into the same well containing either the standards or samples. The plate was covered with aluminum foil and incubated at room temperature for 1 h. After the incubation period, the content of the plate was dumped out and the plate was tapped against a clean lint-free towel to remove any remaining liquid. Each well was washed three times with 300 µL 10x diluted wash buffer. K-blue substrate (150 µL) was added to each well and in two empty wells as blanks. The plate was covered and incubated at room temperature for 30 min. The plate was gently shaken before the absorbance was measured at 650 nm (Versamax tunable microplate reader, Molecular Devices, USA).

2.4.2 Lactate

Plasma lactate was analysed with Lactate Assay Kit (MAK064, Sigma-Aldrich, USA). The solutions were equilibrated to room temperature prior to use, except for the Enzyme Mix which was kept on ice. The plasma samples were diluted with Lactate Assay Buffer. Briefly, 220 µL Lactate Assay Buffer was added to Lactate Enzyme Mix and mixed by pipetting. A 1-fold six-point dilution series of standards was prepared by diluting the stock standard solution with the Assay Buffer. Fifty µL of each standard and 50 µL of the diluted sample was pipetted into their wells in a 96-well plate (CLS3599, Sigma). Master Reaction Mix was prepared by mixing 46 µL Lactate Assay Buffer, 2 µL Lactate Enzyme Mix and 2 µL Lactate Probe for each standard/sample wells. The plate was covered in aluminum foil, gently shaken and incubated at room temperature for 30. The absorbance was measured at 570 nm (Versamax tunable microplate reader).

2.4.3 Glucose

Plasma glucose was analysed with Glucose Assay Kit (ab65333, Abcam, USA). The solutions were equilibrated to room temperature prior to use, except for the Enzyme Mix which was kept on ice and the Glucose Probe which was warmed to 37 °C to melt crystallised DMSO in the solution. The plasma samples were diluted with Glucose Assay Buffer. Briefly, 220 µl Assay Buffer was added to Glucose Enzyme Mix and mixed by pipetting. A 1-fold six-point

glucose standard was prepared from the stock solution with the Glucose Assay Buffer as the diluent. Wells of the 96-well microplate (CLS3599) were added either 50 µL of each standard or 50 µL diluted plasma sample. Thereafter, 50 µL Master Reaction Mix containing 46 µL Glucose Assay Buffer, 2 µL Glucose Enzyme mix and 2 µL Glucose Probe was added to each of the wells. The plate was covered in aluminum foil, mixed by gentle agitation and incubated for 30 min at 37 °C. After the incubation period, the absorbance was measured at 570 nm (Versamax tunable microplate reader).

2.5 Gene expression analyses

2.5.1 RNA isolation

Total ribonucleic acid (RNA) was isolated using the MagMAX ™-96 Total RNA Isolation Kit (AM1830, Ambion) modified after the producer's protocol.

Six zirconium oxide beads (1,4 mm, KT03961, Bertin Technologies, France) and 200 µL Lysis/Binding Solution (AM1830, Ambion) were added to microcentrifuge tubes (16466-058, VWR, USA). Approximately 10 mg tissue from the samples in RNAlater (Ambion) were cut out and transferred to the tubes. The samples were homogenised using the Precellys 24 (Bertin Technologies) for 45 seconds with 3 x 15 seconds interval pulses. Proteinase K (AM2548, Ambion) (20 µL) was added before incubation at 37 °C (BTD, Grant, United Kingdom) for 90 min. The homogenised samples were frozen (- 80 °C).

For initial purification, Wash solution 1 and 2, RNA Rebinding Solution and Bead Mix were prepared according to the producer's protocol. The samples were centrifuged for 2 min at 2500 rpm (KUBOTA 3500, Japan) and 50 µL sample was mixed with 50 µL Lysis/Binding Solution. The Deep-Well plates (4388476, Applied Biosystems, USA), MagMAX Express 96 plate (4388474, Applied Biosystem), and MagMax Express Tip Comp (4388487, Applied Biosystems) were prepared following appendix B, for animal tissues, before the plates were placed in the MagMAX Express-96 processor (4456933, Applied Biosystems) and a cleaning protocol (internal Nofima protocol) was used. Re-binding solutions were added manually when the machine paused. When the program was finished, the Express 96 plate was placed on a cold magnetic stand for 10 min. The samples (35 µL) were transferred to a 96-well RNAplate (AB17500, Bioplastics, Netherlands) and frozen (- 80 °C).

The total RNA concentration and quality were determined by measuring its absorbance using the NanoDrop 8000 spectrophotometer (Thermo Scientific, USA). The ratio between RNA

and protein contaminants were measured and RNA was considered pure at A_{260}/A_{280} ratio between 1,8-2,1.

2.5.2 Genomic DNA treatment

A second purification was performed on the samples. TURBO DNA-free Second Digest Protocol[2] (Thermo Scientific) was followed to make the TURBO DNA-free Second Digest buffer (calculation in appendix ii). Then TURBO DNA-free Kit (AM1907, Ambion) was used for DNase treatment where 1 µl Second Digest buffer and 1 µL Turbo DNase was added to the samples, before incubation at 37 °C (BTD, Grant) for 20 min. Thereafter, 1 µL DNase Inactivation Reagent was added, before incubation for 3 min at room temperature and centrifugation at 12 000 rpm/2 min. The supernatant containing genomic DNA-treated RNA was transferred to tubes and frozen (– 80 °C).

2.5.3 cDNA synthesis

Total RNA was reverse transcribed to complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems) with minor modifications. The 25 µL reaction was set up containing 15 µL 200 ng total RNA, 2,5 µL 10X RT Buffer, 1 µL 25X dNTP, 2,5 µL 10X RT random primers, 1,25 µL Multiscript Reverse Transcriptase, 1,75 µL nuclease-free H_2O, and 1 µL Oligo d(T) (N8080128, Invitrogen, USA). The plate was sealed and centrifuged. The thermocycling parameters (2720 Thermal cycler, Applied Biosystem) were as follows: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min and 4 °C ∞. The stock cDNA was diluted 1:8 by nuclease-free H_2O and additional 1:40 for further use. The samples were stored at – 20 °C.

[2] https://www.thermofisher.com/no/en/home/references/ambion-tech-support/nuclease-enzymes/turbo-dna-free-second-digest-protocol.html

2.5.4 Real time qPCR analyses

2.5.4.1 Primers

Nuclease-free H_2O was added to the primers following the technical datasheet (Sigma) to make 100 µM stock solutions. The stock solutions were diluted 1:20 by nuclease-free H_2O to 5 µM solutions, the solutions were stored at -20 °C.

Table 3: Primer sequences. F: Forward. R: Reverse. Efficiency (E) were calculated according to equation $E = 10^{(-1/slope)}$

Gene/Primer	Abbreviations	Sequence (5'-3')	E (%E)	Ref
glutathione reductase F	*gr*	CCAGTGATGGCTTTTTGAACTT	1,950 (97,48)	(Solberg *et al.*, 2012)
glutathione reductase R		CCGGCCCCCACTATGAC		
cu/zn superoxide dismutase F	*cu/znsod*	CCACGTCCATGCCTTTGG	1,908 (95,40)	(Solberg *et al.*, 2012)
cu/zn superoxide dismutase R		TCAGCTGCTGACAGTCACGTT		
mn superoxide dismutase F	*mnsod*	GTTTCTCTCCAGCCTGCTCTAAG	1,871 (93,53)	(Solberg et al., 2012)
mn superoxide dismutase R		CCGCTCTCCTTGTCGAAGC		
glutathione peroxidase F	*gp*	GATTCGTTCCAAACTTCCTGCTA	1,876 (93,80)	(Solberg et al., 2012)
glutathione peroxidase R		GCTCCCAGAACAGCCTGTTG		
β-actin F	*β-actin*	CAGCCCTCCTTCCTCGGTAT	2	(Julin *et al.*, 2009)
β-actin R		CGTCACACTTCATGATGGAGTTG		
18s F	*18S*	TGTGCCGCTAGAGGTGAAATT	2	(Kileng *et al.*, 2007)
18s R		GCAAATGCTTTCGCTTTCG		
elongation factor 1 alpha F	*eF1α*	CGCCAACATGGGCTGG	2	(Julin *et al.*, 2009)
elongation factor 1 alpha R		TCACACCATTGGCGTTACCA		

2.5.4.2 Positive control

A pooled sample was made with 60 µL 1:40 cDNA from 26 different skin and gill samples were pipetted into a pooled sample, vortexed and spun down, to function as positive control. The pooled sample was aliquoted and stored at - 20 °C.

2.5.4.3 Realtime qPCR

Realtime quantitative polymerase chain reaction (qPCR) was conducted with cDNA diluted 1:40 in triplicates in 384-plates (4343470, Applied Biosystems) using the QuantStudio 5 Real-Time PCR system (Applied Biosystems). Each reaction contained 10 µL Power SYBR Green PCR Master Mix (4368702, Applied Biosystems), 1,2 µL of each Primer F/R (5µM), 0,6 µL nuclease-free H_2O and 7 µL of 1:40 cDNA. Positive and non-template control (NTC) were included in the setup. The following cycling parameters were used: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. To view the melting peak of each amplicon a third dissociation step of 95 °C for 15 sec, 60 °C for 1 min and 95 °C for 15 sec was used.

The Pflaffle method (2001) was used to calculate relative gene expression according to the equation:

$$Ratio = \frac{(Etarget)\text{^}(\Delta CPtarget\ (control - sample))}{(Eref)\text{^}(\Delta CP(control - sample))}$$

The sample with the lowest expression level was chosen as calibrator. The transcript level of the target gene was normalised relative to the geometric mean of 3 reference genes.

To test the primers and make standard curves a pooled cDNA (1:10) sample with gills and skin was made and then a two-fold eight-step serial dilution was performed by nuclease-free H_2O on the pooled cDNA, before realtime qPCR was done as described above. The C_t-values of each gene was used to make standard curves and calculate the slope. Primer efficiency (E)(Table 3) was calculated following Pfaffl (2001) equation:

$$E = 10\text{^}(-\frac{1}{slope})$$

Amplification efficiency is often given in % (%E = (E-1) * 100).

2.6 Amoeba-PAA *in vitro* studies

The amoeba (*P. perurans)* used in this study was isolated from an AGD outbreak in a commercial farm in Norway and a gift from the Norwegian Veterinary Institute (Sigurd Hytterød).

The amoebas were routinely grown in a specific growth media, Malt Yeast Broth (MYB) in special cell culture flasks (TC 25 cm^2 with filter) with seawater (35 ppt) and incubated at 15 °C. The amoebic cells were split and washed with filtered autoclaved seawater every second week.

2.6.1 Amoeba counting and seeding

Haemocytometer was used to quantify the amount of amoeba. A cell scraper was used to loosen the amoebas from the culture bottle before 10 µl was added to the counting chamber. The count number was then used to determine the number of amoeba per µL or mL, before adjusting µl amoeba culture with µl MYB to obtain a required number of amoeba/µL for the seeding. A 96-well plate was then seeded according to the *in vitro* plan described below and allowed to adhere overnight.

2.6.2 Tests for viability

Four commercially available assay kits were tested to determine the most suitable system to study amoeba viability after PAA exposure *in vitro*; 1) Neutral Red (TOX-4, Sigma); 2) Resazurin (TOX-8, Sigma); 3) MTT (CGD1, Sigma); and 4) WST-1 (CELLPRO-RO, Roche, Switzerland). To test the ideal seeding condition prior to PAA *in vitro* exposure, a preliminary trial was performed by seeding a well of the microplate with 100 amoebae/µL. Thereafter, one plate was placed in a 15 °C incubator for approximately 24 h, while the other plate was placed in the same incubator for 30 min, prior to PAA exposure and viability tests. The four kits are described briefly in the following sections.

2.6.2.1 *In vitro* toxicology assay kit Neutral Red Based

Ten µL of 0,33% Neutral Red Solution (TOX-4, Sigma) was added to the wells containing amoeba and mixed; then the plate was returned to the incubator for 3 h. The media was pipetted out, and the wells rinsed with 100 µL Neutral Red Assay Fixative. The fixative was pipetted out and 100 µL Neutral Red Assay Solubilization Solution added and the mixture was pipetted up and down. The plate was incubated for 10 min at room temperature before absorbance was measured at 650 nm (Versamax tunable microplate reader).

2.6.2.2 *In vitro* toxicology assay kit Resazurin Based

Ten µL Resazurin Solution (TOX-8, Sigma) was added to the wells containing amoeba and mixed, then the plate was returned to the incubator for 3 h. The absorbance was measured every hour during the incubation period at 650 nm (Versamax tunable microplate reader).

2.6.2.3 Cell growth determination kit, MTT based

Ten µL MTT Solution (CGD1, Sigma) was added to the wells containing amoeba and mixed before the plate was returned to the incubator for 3 h. The solution was pipetted out and 100 µL MTT Solvent added and mixed by pipetting up and down. The plate was incubated for 1 h and measured at 650 nm (Versamax tunable microplate reader).

2.6.2.4 Cell Proliferation Reagent WST-1

Ten µL Cell Proliferation Reagent WST-1 (CELLPRO-RO, Roche) was added to the wells containing amoeba and mixed. The plate was incubated for 4 h and measured at 450 nm, with 650 nm as reference wavelength (Versamax tunable microplate reader).

2.6.3 *In vitro* PAA exposure of amoeba

Two plates were seeded with amoeba as described above and allowed to settle for 24 h in the incubator. The preliminary trial revealed that the assays were more reproducible when the plate with amoeba was allowed to stay in the incubator for 24 h. The PAA solution was prepared by diluting Divosan Forte (15 %) with distilled water to achieve appropriate working concentrations. The seeded amoebas were exposed to PAA at 3 varying doses: 0 (distilled water), 0,6 and 2,4 ppm. Unexposed amoeba served as control. Two exposure duration was studied: 15 and 30 min. After the incubation period, the viability of amoebic cells was evaluated using the WST-1 assay described in Section 2.6.2.4. Preliminary testing of the four

kits revealed that WST-1 was most suited for studying the amoeba viability, hence was used in the study.

2.7 Statistical analyses

The data were processed in Sigmaplot for Windows version 14.0 (Systat Software). A Shapiro-Wilk test was used to evaluate the normal distribution and a Brown-Forsyth test to check for equal variance. The differences between groups were significant if $p < 0,5$.

For the first exposure in Trial 1 the effect of stress between baseline and treatment groups was analysed using one-way analyses of variance (ANOVA). A two-way ANOVA was used to test for differences in time, treatment and their interaction, time x treatment, for the first exposure and the re-exposure in Trial 1. For the re-exposure, data from 2 weeks after first exposure functioned as a baseline and was included in the two-way ANOVA.

A three-way ANOVA was used to test for time, treatments and stress and their interactions, time x treatment, time x stress, treatment x stress and time x treatment x stress, for Trial 2. Backward elimination was used to remove insignificant factors from the ANOVA to increase the fit to the model. A post-hoc test, Holm-Sidak, was applied in an ANOVA with significant interactions occurred.

For the amoeba *in vitro*, the effect of PAA concentration and exposure duration was analysed using a two-way ANOVA.

Kruskal-Wallis factor ANOVA and Dunn's post hoc were used if the requirement for parametric statistics were not met. The transformation was applied where necessary, to meet the assumptions of the two- or three-way ANOVA. If the transformation was unsuccessful, the residuals were plotted (appendix i) for examination. If passed, an ANOVA test was performed.

ANOVA with Holm-Sidak post hoc or Kruskal-Wallis with Dunn's post hoc with Bonferroni adjusted the significant level (0,007) was used on four genes which did not pass transformation.

Results are given as mean ± standard error (SE).

3 Results

3.1 Welfare and stress responses of salmon – Trial 1

3.1.1 Growth of fish

The mean weight (g), length (cm) and condition factor (K) of all treatment groups are listed in Table 4. All fish appeared to be in a good condition where size and condition factor were almost identical among groups.

Table 4: Growth measurements during Trial 1. Length (cm) and weight (g): mean ± SE. exp – exposure, K – condition factor. N = 8

Length	**2 h first exp**	**48 h first exp**	**2 w first exp**	**2 h re-exp**	**48 h re-exp**	**2 w re-exp**
Pre	23,03 ± 0,30					
0	23,19 ± 0,26	23,92 ± 0,44	24,95 ± 0,39	25,01 ± 0,35	25,63 ± 0,26	26,15 ± 0,40
0,6	22,98 ± 0,36	23,89 ± 0,38	24,26 ± 0,51	24,58 ± 0,30	24,96 ± 0,27	25,95 ± 0,42
2,4	22,30 ± 0,40	23,79 ± 0,28	25,11 ± 0,49	24,04 ± 0,44	24,89 ± 0,29	26,01 ± 0,41
Mean	*22,88 ± 0,20*	*23,86 ± 0,05*	*24,77 ± 0,26*	*24,54 ± 0,28*	*25,16 ± 0,24*	*26,04 ± 0,06*
Weight	**2 h first exp**	**48 h first exp**	**2 w first exp**	**2 h re-exp**	**48 h re-exp**	**2 w re-exp**
Pre	137,70 ± 4,96					
0	157,57 ± 6,87	150,10 ± 8,39	178,45 ± 8,97	168,73 ± 9,34	185,17 ± 5,38	199,95 ± 10,05
0,6	153,87 ± 8,91	147,47 ± 6,28	164,95 ± 10,50	163,82 ± 8,75	171,59 ± 6,24	199,51 ± 8,74
2,4	139,34 ± 8,43	143,61 ± 5,30	184,50 ± 14,65	152,82 ± 9,40	175,35 ± 6,20	211,86 ± 10,45
Mean	*147,12 ± 5,03*	*147,06 ± 1,88*	*175,97 ± 5,78*	*161,79 ± 4,70*	*177,37 ± 4,05*	*203,77 ± 4,04*
K	**2 h first exp**	**48 h first exp**	**2 w first**	**2 h re-exp**	**48 h re-exp**	**2 w re-exp**
Pre	1,1					
0	1,3	1,1	1,1	1,1	1,1	1,1
0,6	1,3	1,1	1,2	1,1	1,1	1,1
2,4	1,3	1,1	1,2	1,1	1,1	1,2

3.1.2 Welfare scores

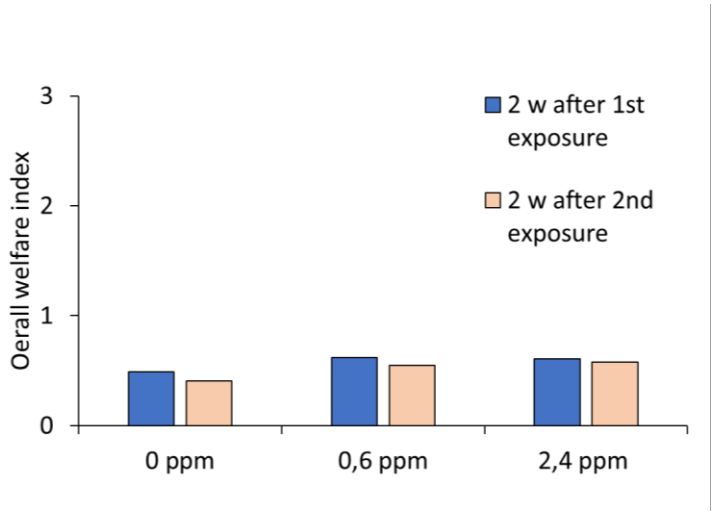

Figure 6: Overall welfare index in Trial 1, 2 w after both PAA exposures. The 0 represents the "best" and 3 represents the "worst".

Welfare scores for each parameter are in Appendix iii.

Overall welfare index (OWI) was expressed as the average of the frequency and score of all indicators evaluated. The OWIs for both exposures in Trial 1 are shown in Figure 6. There were minor differences between the first and re-exposure experiments, with, groups exposed to PAA appeared to exhibit relatively higher scores than the unexposed group. The OWIs were 0,49, 0,62 and 0,61 for the first exposure and 0,40, 0,44 and 0,58 for the re-exposure for *0*, *0,6* and *2,5 ppm* group, respectively. The welfare indices (WIs) including emaciation, eye damages, snout damages, vertebral deformities, and jaw deformities were not observed in any of the fish in either of the exposure experiments (Figure 7). Operculum and pelvic fin damages had scores lower than 1, caudal fins were scored to ca 1 and pectoral fins were scored between 1,1 to 1,5. The highest damage was seen in the dorsal fins (mostly active damages in the form of splitting) and skin (mostly scale loss), reaching 1,7 (first exposure, *2,4 ppm*) and 2 (re-exposure, *0,6 ppm*) for PAA exposed fish. There seemed to be higher scores in some indicators in fish exposed to PAA, such as in 4 of 11 WIs (skin, operculum, dorsal fin, pectoral fins) in the first exposure and 6 of 11 WIs (skin, operculum, dorsal fin, caudal fin, pectoral fins and pelvic fins) in the re-exposure. However, the difference in the scores between the control group and exposed groups was low and ranged between 0,3 – 0,75. Additionally, skin damage following re-exposure had higher scores for all groups compared with the documented cases during the first exposure.

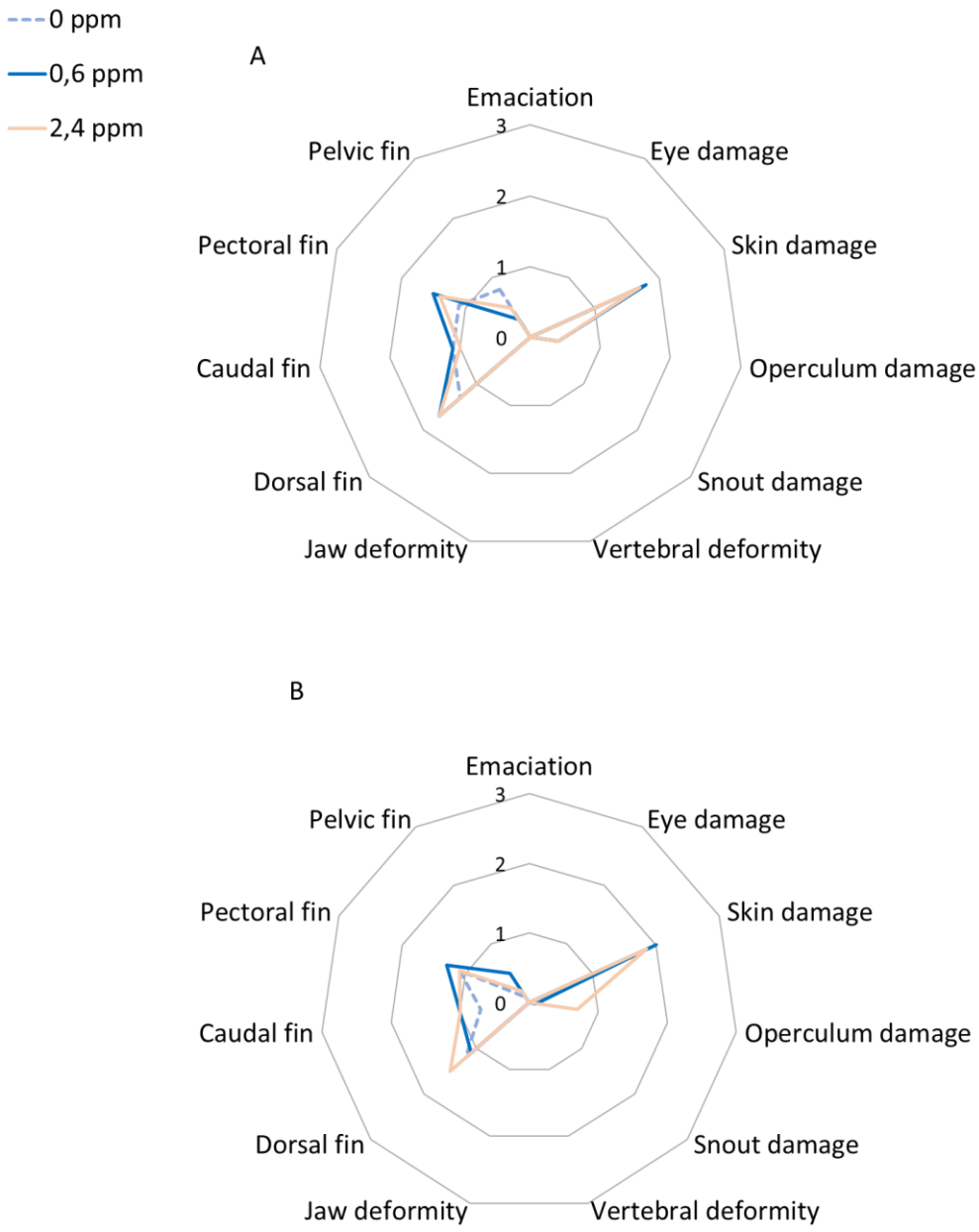

Figure 7: Welfare scores 2 w after A) first exposure and B) re-exposure in Trial 1. The 0 represents the "best" and 3 represents the "worst". N = 8

3.1.3 Stress responses in plasma – Trial 1, First Exposure

3.1.3.1 Cortisol

The baseline cortisol level (pre) was 6,38 ± 1,04 ng/mL. All groups showed a significant increase of at least 2-fold in the plasma cortisol levels (Figure 8A) 2 h after exposure compared with the pre-exposure level. The plasma cortisol levels decreased thereafter, especially in *0* and *2,4 ppm* groups where the levels were almost similar to the baseline value. However, the group exposed to *0,6 ppm* PAA had elevated levels compared with the other

treatment groups at 48 h. A similar tendency was also observed 2 w after exposure, but there was no significant difference with the other exposed groups or with the baseline value.

3.1.3.2 Glucose

Figure 8B shows the change in the plasma glucose following PAA exposure. The average baseline plasma glucose value was 4,20 ± 0,58 mmol/L. The levels slightly increased 2 h after exposure compared with the baseline value. However, the changes were not significant. The plasma glucose level in all groups decreased significantly 2 w after exposure, especially when compared with the values identified at 2 h after exposure. The significant decrease ranged from 31 – 44 % reduction compared with the values 2 h after exposure.

3.1.3.3 Lactate

The average plasma lactate level prior to exposure (pre) was 227,41 ± 39,07 ng/µL. There were fluctuations in the plasma lactate levels in the succeeding sampling points with levels ranging from 234 – 333 ng/µL. Nonetheless, no significant differences between groups and through time were found (Figure 8C).

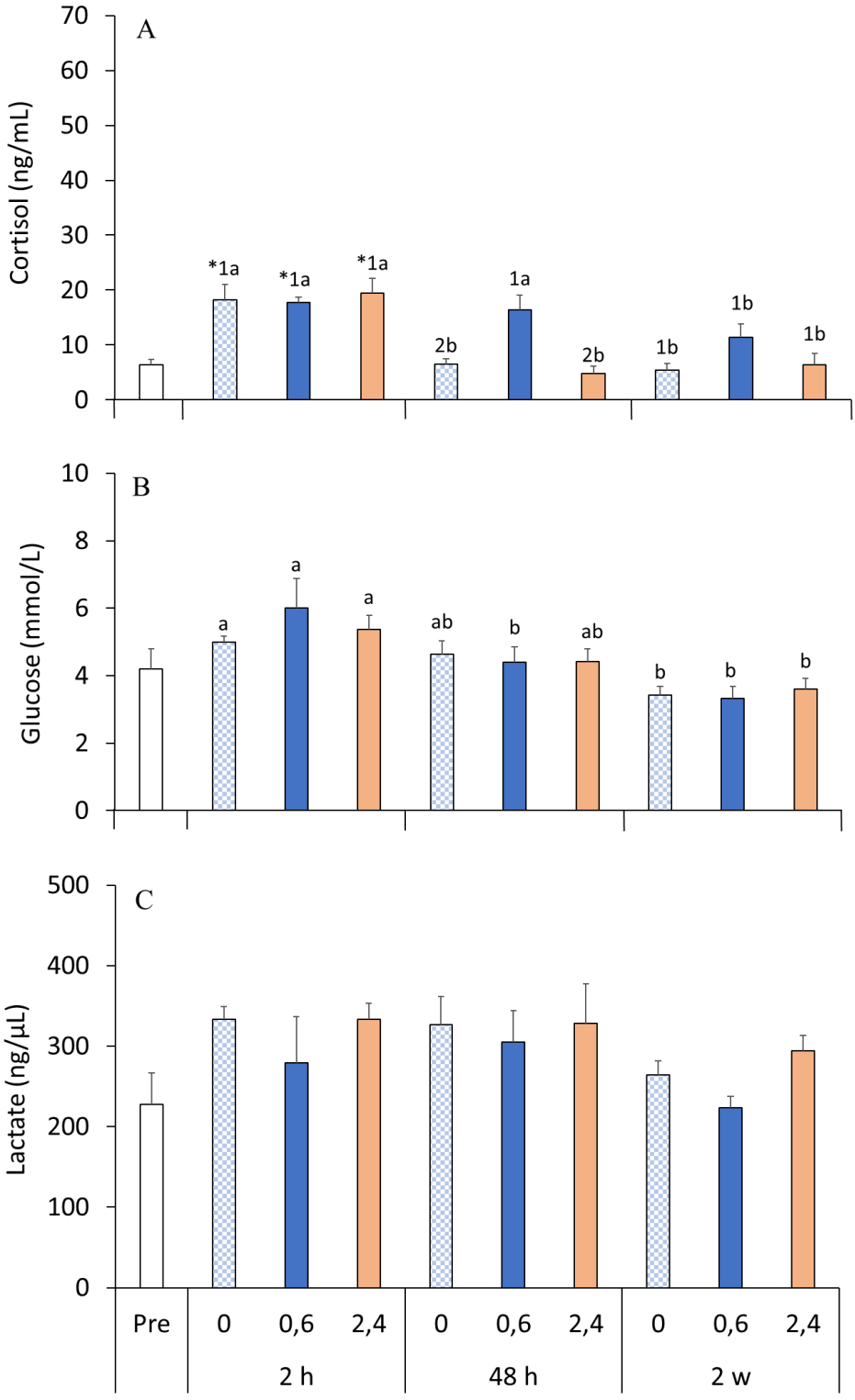

*Figure 8: Plasma A) cortisol, B) glucose and C) lactate levels before (pre), 2 h, 48 h and 2 w after first PAA exposure (0, 0,6, 2,4 ppm) in Trial 1. Values are mean ± SE. * notes significant differences between treatment groups and pre. Numbers denote significant differences between treatments within a sampling point, while letter notations indicate significant differences within a treatment through time. N = 8.*

3.1.4 Stress responses in plasma – Trial 1, Re-exposure

3.1.4.1 Cortisol

The average baseline plasma cortisol levels prior to exposure were 5,43 ± 1,22, 11,45 ± 2,41 and 6,35 ± 2,08 ng/mL for the *0, 0,6* and *2,4 ppm*, respectively. Two hours after re-exposure, all groups showed a significant increase (Figure 9A) in plasma cortisol compared with their respective baseline values. Particularly, the group exposed to *2,4 ppm* displayed almost a 9,5 times higher plasma cortisol level. Furthermore, the *2,4 ppm* group was significantly higher by at least 2,4-fold compared with the other two groups at this particular time-point. The average plasma cortisol levels returned to baseline in all groups 48 h after exposure and remained the same 2 w after.

3.1.4.2 Glucose

The fish exposed earlier to PAA had an average plasma glucose level of approximately 3,5 mmol/L (Figure 9B). The control group (*0 ppm*) showed a significant increase of 54 % in plasma glucose level 2 h after exposure compared with its respective pre-exposure value. The two other groups seemed to have an increase after 2 h, but the change was not statistically significant. After 2 w, the level of plasma glucose in the *0 ppm* group had decreased significantly compared with the level at 2 h, and was identical with the baseline value.

3.1.4.3 Lactate

The plasma lactate levels of treatment groups are shown in Figure 9C. The average plasma lactate level prior to exposure (pre) ranged from 264 – 294 ng/µL. Though it appeared that there were changes between groups and through time in the plasma lactate levels, the changes were not identified to be statistically significant.

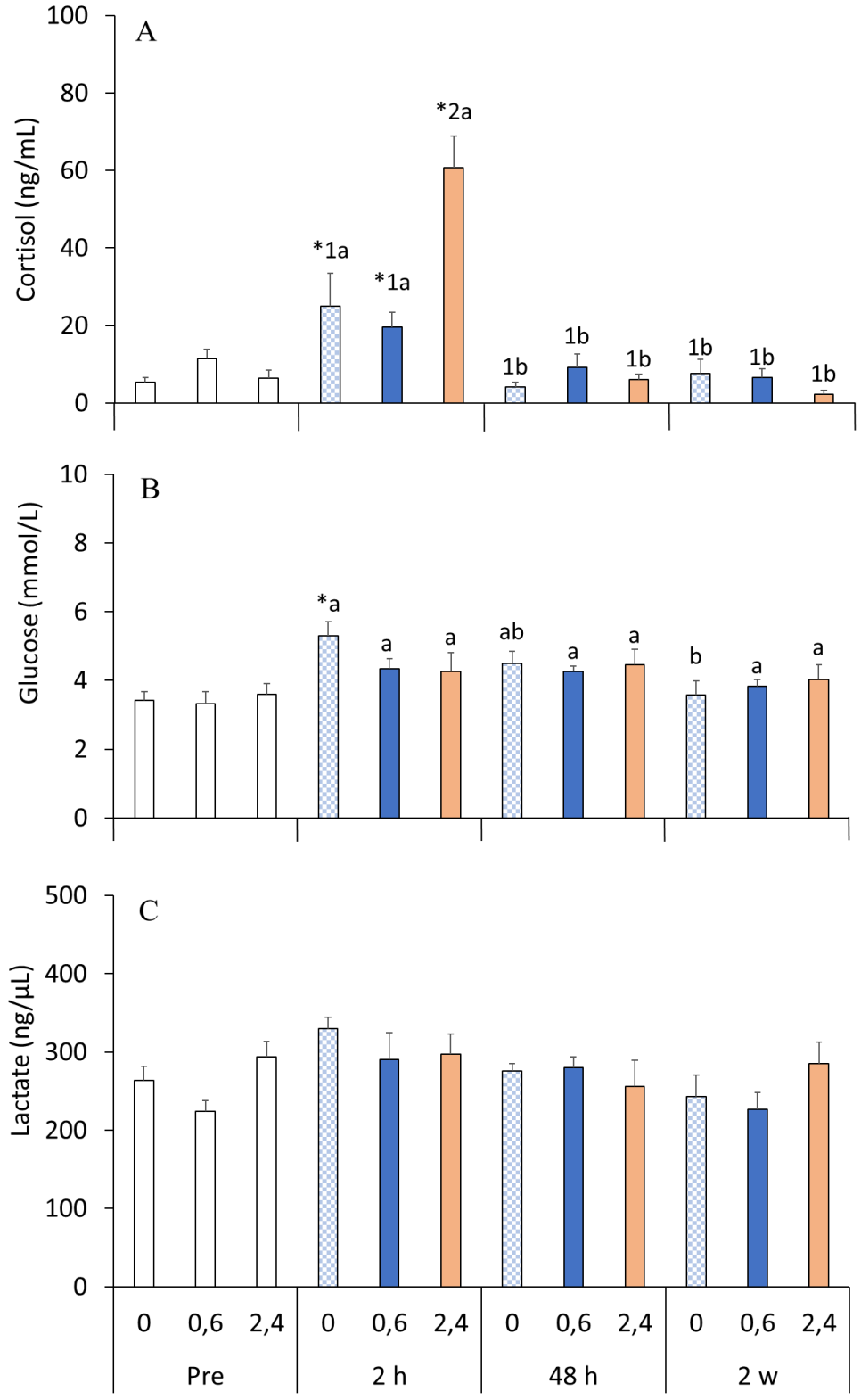

*Figure 9: Plasma A) cortisol, B) glucose and C) lactate levels before (pre), 2 h, 48 h and 2 w after PAA re-exposure (0, 0,6, 2,4 ppm) in Trial 1. Values are mean ± SE. * notes significant differences between treatment groups and pre. Numbers denote significant differences between treatments within a sampling point, while letter notations indicate significant differences within a treatment through time. N = 8.*

3.2 Welfare and stress responses of salmon – Trial 2

3.2.1 Growth of fish

The mean weight (g), length (cm) and condition factor (K) of all treatment groups are given in Table 5. Morphometric data appeared to be similar in all treatment groups.

Table 5: Growth measurement during Trial 2. Length (cm) and weight (g): mean ± SE. N = 8. K – condition factor.

Length	**1 h**	**4 h**	**2 w**
Pre	24,00 ± 0,50		
No stress control	23,89 ± 0,43	24,53 ± 0,42	25,38 ± 0,46
No stress PAA	24,13 ± 0,22	23,82 ± 0,47	24,74 ± 0,52
Stress control	23,48 ± 0,44	23,81 ± 0,55	24,71 ± 0,25
Stress PAA	24,46 ± 0,76	24,39 ± 0,26	25,41 ± 0,50
Mean	*23,99 ± 0,16*	*24,14 ± 0,19*	*25,06 ± 0,19*
Weight	**1 h**	**4 h**	**2 w**
Pre	126,10 ± 8,00		
No stress control	130,62 ± 6,74	142,32 ± 7,48	165,30 ± 10,86
No stress PAA	132,95 ± 4,15	130,57 ± 8,97	148,26 ± 9,14
Stress control	125,25 ± 6,39	125,83 ± 7,46	151,27 ± 5,82
Stress PAA	136,17 ± 5,29	131,35 ± 3,54	172,05 ± 10,36
Mean	*130,22 ± 2,06*	*132,52 ± 3,49*	*159,22 ± 5,66*
K	**1 h**	**4 h**	**2 w**
Pre	0,91		
No stress control	0,96	0,96	1,01
No stress PAA	0,95	0,97	0,98
Stress control	0,97	0,93	1,00
Stress PAA	0,93	0,91	1,05

3.2.2 Welfare scores

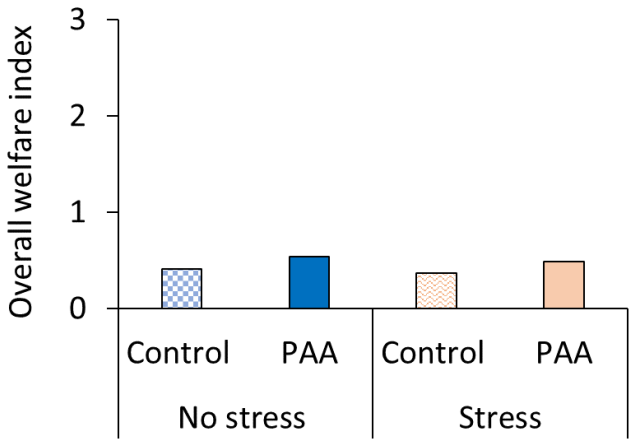

Figure 10: Overall welfare index in Trial 2, 2 w after PAA exposure (0 ppm - Control and 4,8 ppm - PAA). The 0 represents the "best" and 3 represents the "worst".

Welfare scores for each parameter are in Appendix iii.

The OWIs (average of all indicators evaluated) for the PAA exposed groups appeared to be higher than the control group 2 weeks after exposure in Trial 2. However, they were all scored below 1 (Figure 10). *No stress PAA* and *stress PAA* had OWIs of 0,54 and 0,49, and *no stress control* and *stress control* had OWIs of 0,41 and 0,37. Emaciation, eye damages, and vertebral deformities were not observed in any of the fish evaluated (Figure 11). Jaw deformities (average score 0,1), snout damage (0,2), pelvic fins (0,5) and operculum damage (0,9) were prevalent and scored the highest in *no stress PAA* among the treatment groups. Caudal fin damage was scored to 0,7 for the *stress control* group, 0,8 for both *no stress* groups and 1,2 for the *stress PAA* group. Pectoral fin, dorsal fin and skin damage were all scored to approximately 1, in which *no stress PAA* were scored highest for the fin damages and *stress PAA* had highest scores for skin damage, among the treatment groups.

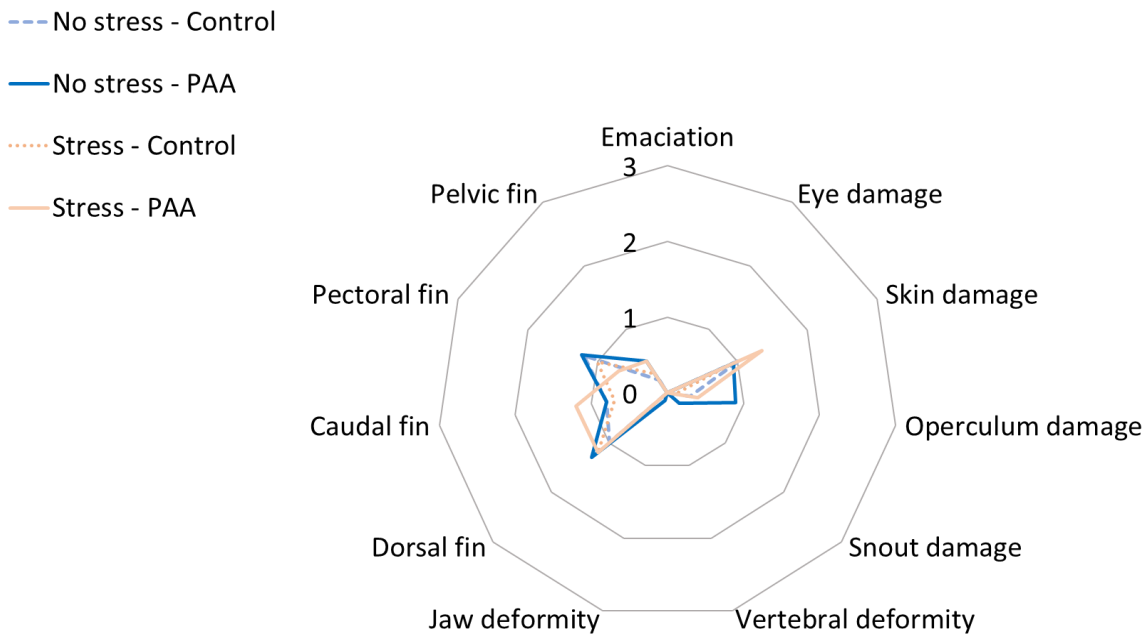

Figure 11: Welfare scores in Trial 2, 2 w after PAA exposure. The 0 represents the "best" and 3 represents the "worst". N = 8.

3.2.3 Stress responses in plasma – Trial 2

3.2.3.1 Cortisol

The baseline plasma cortisol had an average of 14,86 ± 6,79 ng/mL (Figure 12A). In the group that was not subjected to crowding prior to PAA exposure, the level significantly increased compared with the baseline value at 1 h after exposure and remained significantly elevated even after 4 h. No significant differences were observed between the *no stress control* and *no stress PAA* groups at both timepoints. After 2 w, the cortisol level in both groups was similar with the baseline value and both groups remained not statistically different from each other. The patterns of cortisol changes in the group that was exposed to stress before PAA treatment were almost the same with the group that was not stressed before PAA exposure: elevated levels in the first 4 h after exposure with no statistically difference between the *stress control* and *stress PAA* groups. The level at 2 w after exposure was similar to the baseline value.

3.2.3.2 Glucose

Figure 12B shows the change in plasma glucose levels over time. The baseline sample had an average plasma glucose value of 2,77 ± 0,29 mmol/L. The level significantly increased 4 h

after exposure compared with the baseline value in the *no stress* group and the sub-group exposed to PAA displayed a significantly higher level compared with the control. After 2 w, the glucose level in *no stress PAA* group remained significantly higher than the *no stress PAA*, so as with the baseline value. In the group that was subjected to crowding before PAA exposure, the glucose level remained similar with the baseline value in all groups and time points, except in the *stress control* group at 2 w after exposure where the level was significantly elevated relative to the baseline control. In all instances, no significant differences were documented between *stress control* and *stress PAA* groups. It was also observed that the groups subjected to stress before PAA exposure had significantly lower glucose level compared with their counterpart in *no stress* group and this pattern was found in all sampling points. The *stress control* group exhibited a similar tendency compared with its counterpart in *no stress* group, but a significant change was only observed 4 h after exposure.

3.2.3.3 Lactate

The baseline plasma lactate was 189,53 ± 17,41 ng/µL. In the *no stress* group, the sub-group exposed to PAA exhibited a significantly elevated plasma lactate level 1 h after exposure compared with the baseline value, however, it was not significantly different from the control group at that time-point (Figure 12C). Nonetheless, the lactate level in *no stress PAA* returned to baseline value in the succeeding time-points. Though there was an apparent increase in the lactate level of *no stress control* 1 h after exposure compared with the baseline control, the change was not statistically significant. Moreover, the lactate level of the *no stress control* group significantly decreased by at least 30 % 4 h after exposure when compared to its value 3 h earlier. For the *stress* group, the glucose level in all treatments in the early period post exposure (i.e. 1, 4 h after exposure) was not statistically different from the baseline value, as well as no significant difference between the control and the PAA exposed groups. At 2 w post exposure, both the *stress control* and *stress PAA* groups exhibited significantly elevated lactate levels compared with the baseline value. The lactate level in both *stress control* and *stress PAA* groups at 2 w post exposure was significantly higher than the level recorded at 4 h after exposure. Moreover, the level was significantly higher than their counterparts in the group that was not subjected to crowding prior to PAA exposure.

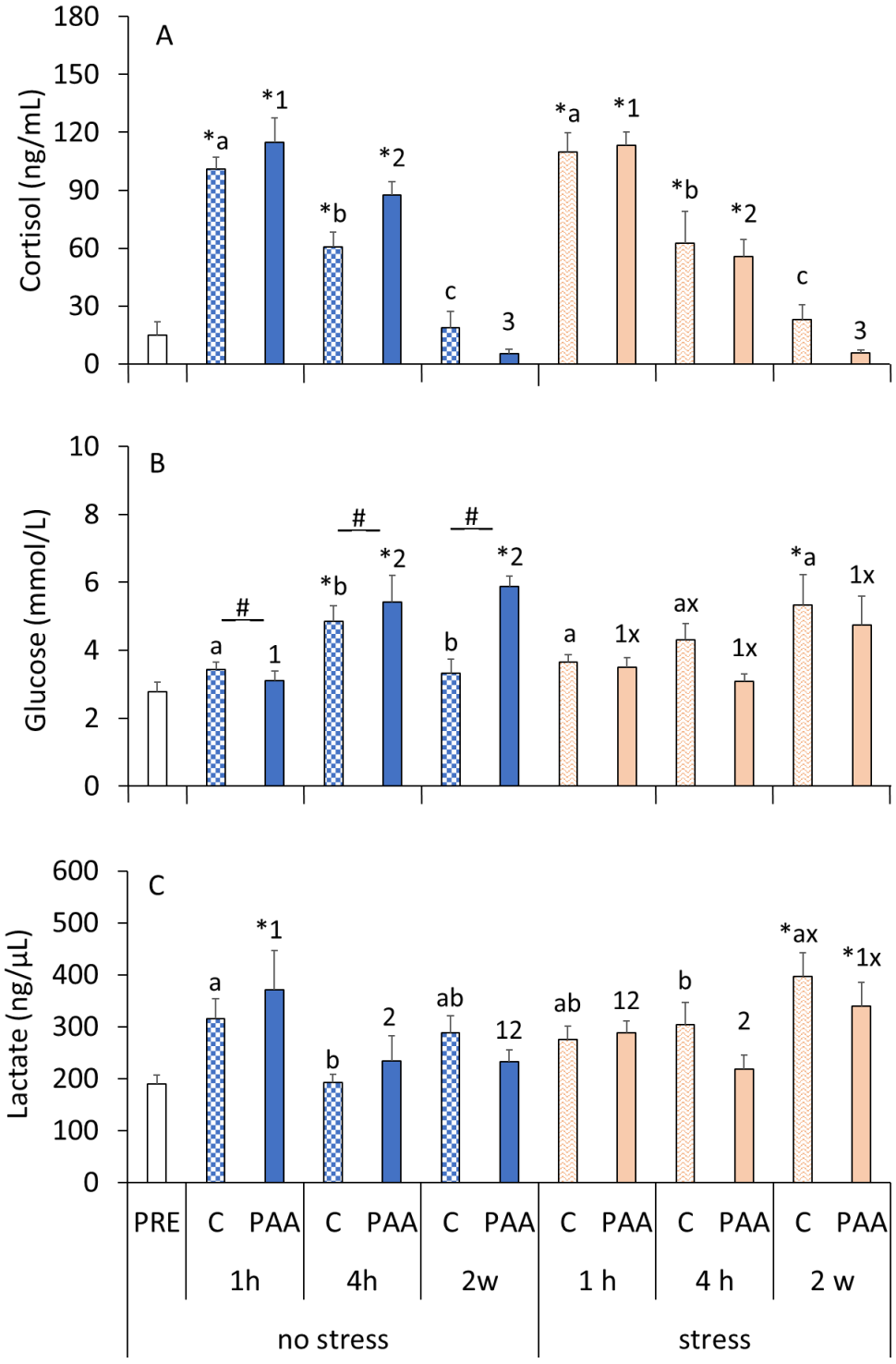

*Figure 12: Plasma A) cortisol, B) glucose and C) lactate levels before (pre), 1 h, 4 h and 2 w after PAA exposure (0 ppm - C and 4,8 ppm - PAA) and crowding stress (no stress or stress), in Trial 2. Values are mean ± SE. * notes significant differences between treatment groups and pre. Letters denote significant differences within control groups through time, while number notations indicate differences within PAA exposed groups through time. x indicates a significant difference between stressed and unstressed groups, whereas # notation indicates significant differences between the control and PAA-exposed group. N = 8.*

3.2.4 Expression of antioxidant genes in the gills

3.2.4.1 *glutathione peroxidase (gp)*

In the *no stress* group, the transcript level of *gp* in the gills 1 h after exposure did not significantly vary compared with the baseline control (Figure 13A). In addition, there was no significant difference between the *no stress control* and *no stress PAA* groups at this time-point. After 4 h, *gp* expression in *no stress control* was significantly higher compared with the pre-exposure level but such an increase was not observed in the *no stress PAA* group. Both the *no stress control* and *no stress PAA* groups displayed significantly elevated *gp* expression of around 40 % compared with the baseline control at 2 w after exposure. A similar *gp* expression pattern was likewise identified in the group that was subjected to crowding prior to PAA exposure, where increased transcript level compared with the baseline value was identified 2 w after exposure. There was also a conflicting dynamic in *gp* expression between *no stress* and *stress* groups immediately after exposure, where *gp* expression was significantly higher in *stress PAA* group compared with its counterpart in *no stress* group at 1 h after exposure. On the other hand, *stress control* group displayed significantly lower *gp* expression compared with its equivalent in the *no stress* groups at 4 h after exposure.

3.2.4.2 *mn superoxide dismutase (mnsod)*

The expression of *mnsod* in the gills showed a late regulation where significant increase in the transcript level was observed only 2 w after exposure. This trend was observed in both *stress* and *no stress* groups (Figure 13B). In addition, *mnsod* expression in *stress control* group was significantly lower compared with its counterpart in *no stress control* group at 4 h after exposure.

3.2.4.3 *cu/zn superoxide dismutase (cu/znsod)*

Figure 13C shows the relative expression of *cu/znsod* in the gills. The transcript level of *cu/znsod* in the *no stress* group did not display a significant difference from the baseline control post exposure. Nonetheless, a significantly elevated *cu/znsod* expression was observed in both *no stress control* and *no stress PAA* groups at 2 w post exposure when compared with their counterparts in the early hours after exposure. The expression pattern of *cu/znsod* in the *stress* group showed a similar tendency, particularly an elevated expression at 2 w post exposure. It was identified further that the *cu/znsod* expression in both *stress control* and *stress PAA* groups varied significantly in all time-points post-exposure, where significantly

higher *cu/znsod* transcript level was identified in the *stress control* group. Moreover, the transcript level of *cu/znsod* in the *stress* group at 4 h after exposure was significantly lower compared with its counterpart in the *no stress* group.

3.2.4.4 *glutathione reductase (gr)*

The temporal dynamics of *gr* expression in the gills is given in Figure 13D. Though it appeared that *gr* expression in the *no stress* group varied dramatically through time, the changes were not statistically significant when compared with the baseline control or between *no stress control* and *no stress PAA*. The expression of *gr* in *no stress control* at 2 w post-exposure however, was significantly higher compared with its expression in the early hours after exposure. The changes in *gr* expression in the *stress* group also remained unchanged following treatments. There were statistically significant differences between *stress* and *no stress* groups in the early hours after exposure. It was identified that the transcript level of *gr* in the *stress control* was significantly higher compared with its counterpart in *no stress* group at both 1 and 4 h after exposure. On the other hand, *gr* expression in *stress PAA* group was significantly lower compared with the *no stress PAA* group at both time points.

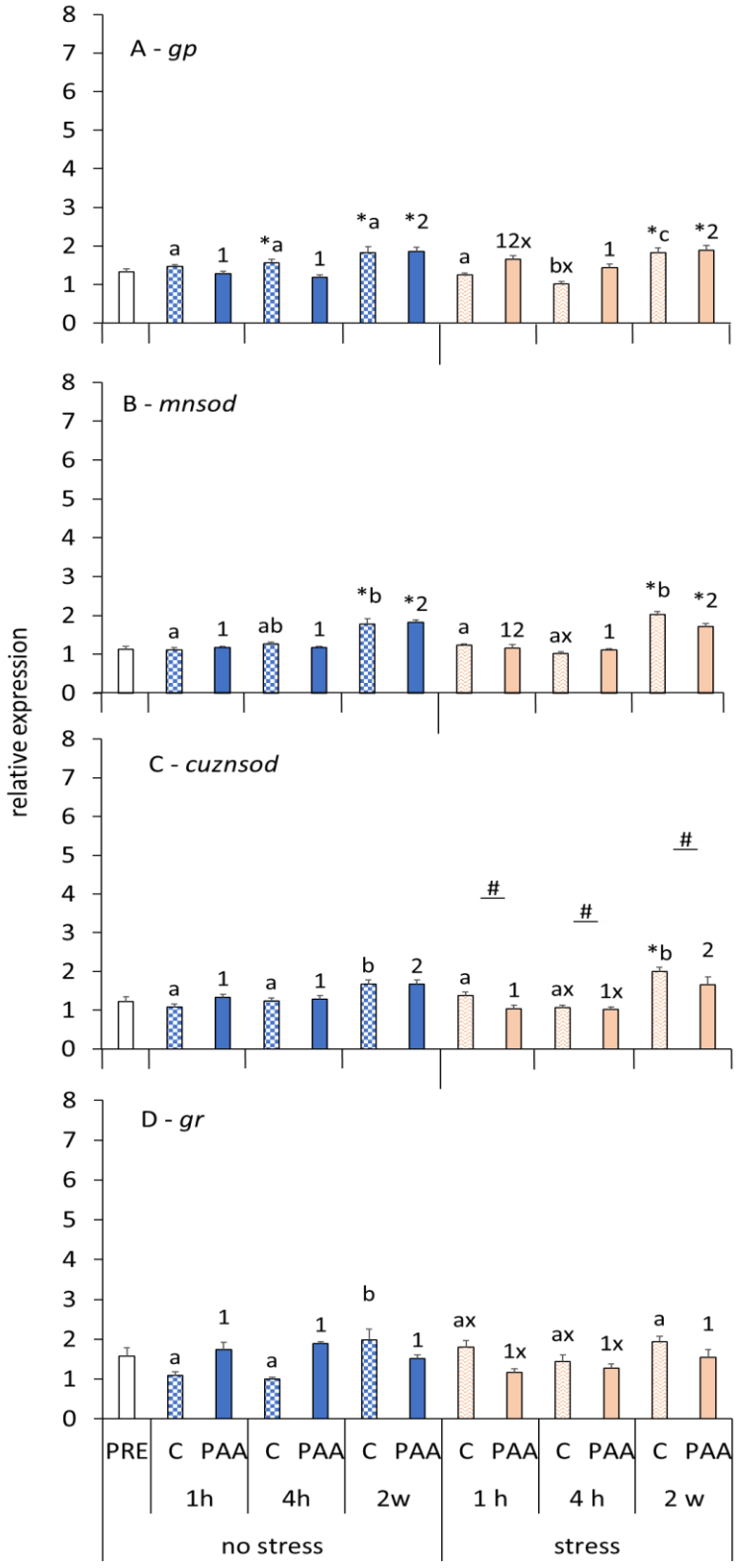

*Figure 13: Relative gene expression of A) gp, B) cu/znsod, C) mnsod and D) gr in gills before (pre), 1 h, 4 h and 2 w after PAA exposure (0 ppm or 4,8 ppm) and stress (no stress or stress) in Trial 2. Values are mean ± SE. * notes significant differences between treatment groups and pre. Letters denote significant differences within control groups through time, while number notations indicate differences within PAA exposed groups through time. x indicates a significant difference between stressed and unstressed groups, whereas # notation indicates significant differences between the control and PAA-exposed group. n = 8.*

3.2.5 Expression of antioxidant genes in the skin

3.2.5.1 *glutathione peroxidase (gp)*

Figure 14A shows the temporal dynamics of *gp* in the skin. Though it appeared that *gp* expression increased, especially in the *no stress* groups 4 h after exposure, the changes were not identified to be statistically significant when compared with the baseline control or with the other treatment groups. The expression of *gp* in the *stress* group remained unchanged all throughout the trial.

3.2.5.2 *mn superoxide dismutase (mnsod)*

The transcript level of *mnsod* in the skin for the *no stress* groups appeared to decrease through time, no significant difference was identified between the baseline control as well as with other timepoints (Figure 14B). A similar tendency was noted in the *stress* group except that the expression of *mnsod* in *stress PAA* group 2 w post exposure was around 75 % lower than the expression at the early hours after exposure.

3.2.5.3 *cu/zn superoxide dismutase (cn/znsod)*

The expression of *cu/znsod* in the skin at the early hours post exposure remained unchanged in both *no stress* and *stress* groups compared with the baseline value (Figure 14C). At 2 w post exposure, the transcript level of *cu/znsod* significantly increased in both sub-groups of *no stress* and *stress* groups relative to the baseline control. There was no significant difference however between control and PAA exposed fish in both *no stress* and *stress* groups.

3.2.5.4 *glutathione reductase (gr)*

The relative expression of *gr* in the skin is shown in Figure 14D. At the two first sampling points, the transcription level of *gr* remained unchanged in all groups. A significant increase of at least 4-fold was observed in both *no stress* and *stress* groups 2 weeks after exposure when compared with baseline values and their respective counterparts in the other timepoints. However, no significant difference was noted between control and PAA exposed fish in both *no stress* and *stress* groups.

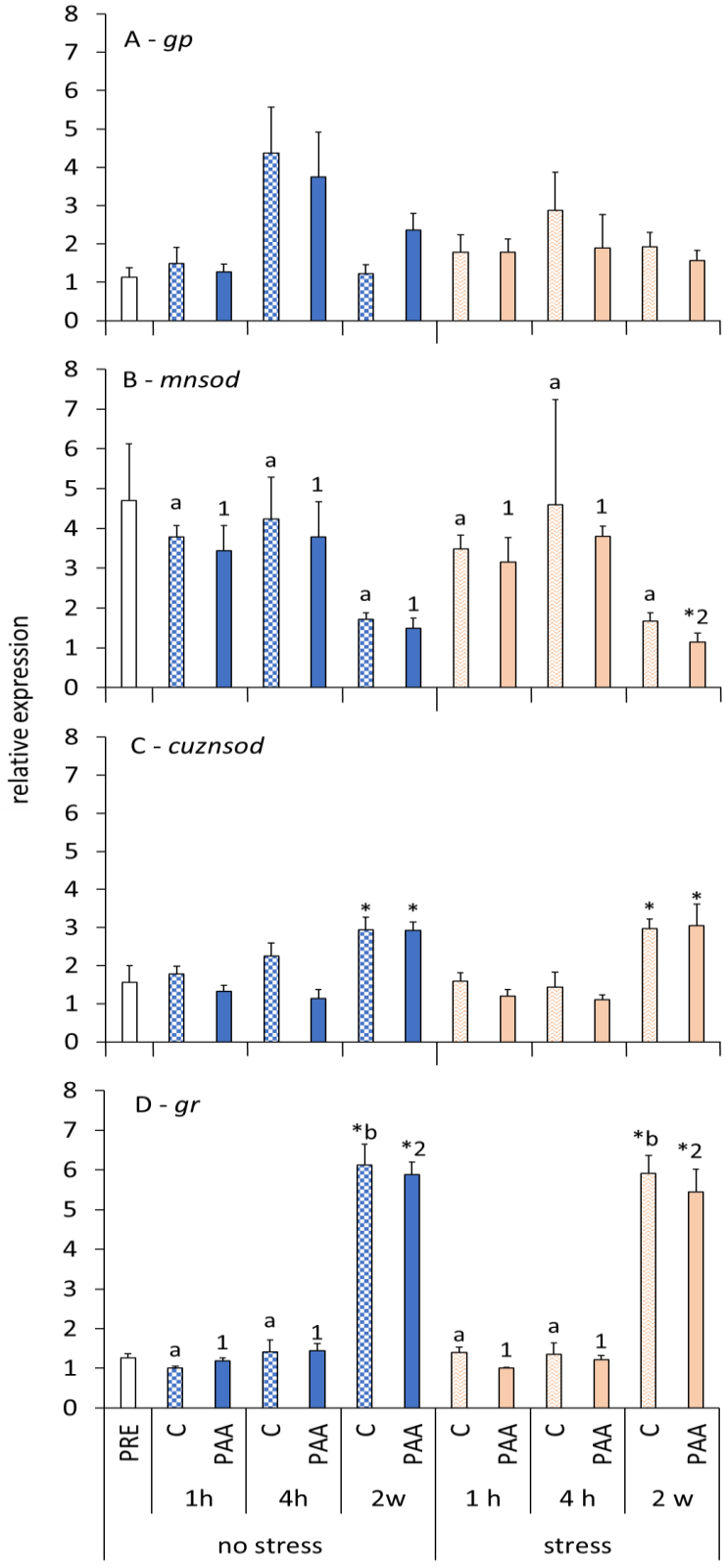

*Figure 14: Relative gene expression of A) gp, B) cu/znsod, C) mnsod and D) gr in skin before (pre), 1 h, 4 h and 2 w after PAA exposure (0 ppm or 4,8 ppm) and stress (no stress or stress) in Trial 2. Values are mean ± SE. * notes significant differences between treatment groups and pre. Letters denote significant differences within control groups through time, while number notations indicate differences within PAA exposed groups through time. x indicates a significant difference between stressed and unstressed groups, whereas # notation indicates significant differences between the control and PAA-exposed group. N = 8,*

3.3 *In vitro* PAA exposure of amoeba

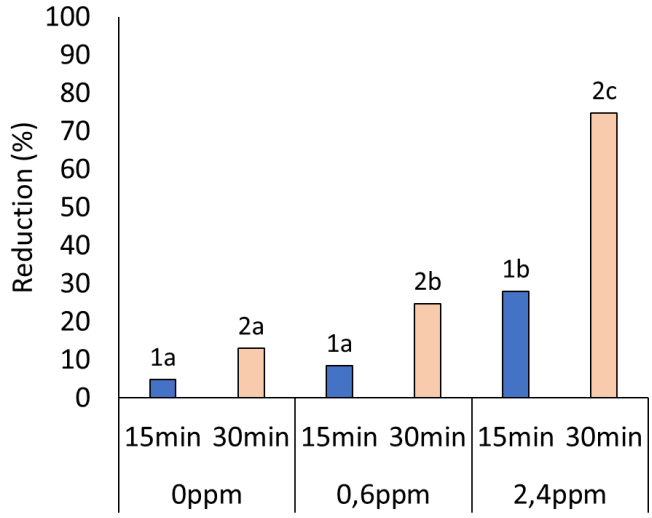

Figure 15: Reduction (%) in the viability of the amoeba exposed to 0, 0,6 and 2,4 ppm PAA with 15- or the 30-min exposure time, coloured with WST-1. The amoebas used in the study was 1 week old. Numbers denote significant differences between exposure times within a concentration, while letter notations indicate significant differences between concentrations

Figure 15 shows the reduction in the viability of amoeba when exposed to different PAA concentrations and at different exposure durations. Exposing the amoeba to *0, 0,6* and *2,4 ppm* for 15 min resulted in 4,78 %, 8,41 % and 27,94 % viability reduction respectively, where *2,4 ppm* displayed significantly higher viability reduction capacity than the two other concentrations. When the amoebas were exposed to the same nominal concentrations of PAA for 30 min, all tested concentrations were significantly different from each other. An exposure time of 30 min resulted in at least a 2-fold increase in the viability reduction potential compared with the tests carried out at 15 min. The reduction in viability after 30 min PAA exposure in % was 12,97, 24,69 and 74,74 for the *0, 0,6* and *2,4 ppm,* respectively.

4 Discussion

4.1 Welfare indicators of salmon exposed to PAA – Trials 1 and 2

4.1.1 Condition factor

Fish growth is one of the indicators used to asses performance (Balseiro *et al.*, 2018). At the beginning of Trial 1 and 2, the condition factor was 1,1 and 0,9, respectively. The control groups did not appear to differ from treatment groups, and the condition factor remained the same at termination. There is no exact value for condition factor to relate it to welfare since it varies after season and life stage of the fish, typically decreasing when the fish smoltifies ($K \approx 1$) and increasing ($K \approx 1,5 – 1,6$) in the post-smolt period (Stien *et al.*, 2013; Noble *et al.*, 2018). The values identified in the study indicate that PAA treatment did not affect fish performance. This result is supported by the routine visual inspection after exposure demonstrating that appetite was not affected, and fish resumed normal feeding after exposure.

4.1.2 External morphological welfare scores

The external welfare status was assessed through a morphology-based scoring of 11 key indicators with a 0 to 3 rating scale, where 0 means “best welfare condition” while 3 indicates “worst welfare condition”. The overall welfare status of the fish in both trials was considered in good-condition as all groups had overall welfare scores < 0,65, including the groups exposed to PAA alone and in combination with crowding. It appeared in Trial 1 that the OWIs in all groups after re-exposure were relatively lower than values obtained after the first exposure trial. This indicates that the former PAA exposure history did not cause any further damage or make the fish more susceptible to physical damage associated with the chemical or handling. There was a tendency for the PAA exposed groups in both Trials 1 and 2 to have higher OWI scores (+0,12 – 0,18), nonetheless higher PAA concentration did not appear to cause a dramatic increase in the cases of external morphological damage. Interestingly, the OWIs of fish exposed to *4,8 ppm* in this study was slightly lower than the OWIs for *0,6* and *2,4 ppm* groups, though this may likely be due to different fish stocks used and environmental conditions (i.e., temperature, from flow-through then to RAS in Trail 2).

Emaciation was one of the key indicators scored to 0 for all control and treatment groups, in both trials. This is in accordance with the result of the condition factor. Neither the fish exposed to crowding stress nor fish exposed to PAA displayed different feeding behaviour, compared to their control counterparts, indicating that the exposures did not affect the appetite. As for the operculum damage, fin damage (dorsal, caudal, pectoral and pelvic) and skin damage, they were the key indicators with the highest scores (≤ 2) in both trials, suggesting that there might be some external welfare changes.

The operculum has an essential role in respiration as it contributes to the water flow over the gill lamellas with oxygen absorption from the water (Noble *et al.*, 2018). Deformities have regularly been seen in farmed salmon, giving rise to reduced waterflow and increased susceptibility and vulnerability for diseases, and may lead to reduced growth, infections, mortality and compromised welfare (Kazlauskienė *et al.*, 2006; Noble *et al.*, 2012). Cases of operculum damage in both trials were below 1, and interestingly, the scores were higher for all PAA exposed groups compared with their respective control groups. It is difficult to firmly conclude at the moment that this striking change was a product of PAA exposure, though this is worth looking at in future trials. A recently concluded trial of the project conducted at the Havbrukstasjonen i Tromsø showed no signs of operculum damage, even the tested concentration was 1-fold higher than Trial 2 (Lazado, pers. communication). Therefore, it is very likely that this damage may be related to the stock rather than the treatment.

Fin damages is a well-known concern to fish welfare (Ellis *et al.*, 2008; Noble *et al.*, 2012; Noble *et al.*, 2018). The damages are often described as splitting, erosion or thickening (Noble *et al.*, 2012), and divided into active or healed damage. The fins play different roles for the fish, but their main function is to help control position and motion (Ellis *et al.*, 2008; Noble *et al.*, 2012; Stien *et al.*, 2013). Damages to the fins can affect growth, survival and swimming ability (Noble *et al.*, 2012), and furthermore, an active damage to the fins can be a potential portal of entry for pathogen that causes infection (Ellis *et al.*, 2008; Stien *et al.*, 2013; Noble *et al.*, 2018). In both trials, there was an apparent pattern in fin damage, in which the pelvic fins had the least cases of damage, followed by caudal then pectoral, and then the highest number of cases were found in the dorsal fins. Ellis *et al.* (2008) found that there is a general pattern in fin erosion in salmonids, with the dorsal and pectoral fins having the most damage, similar to this study. There are several factors (handling, crowding, chemicals, aggression, etc.) that can lead to fin damage among farmed fish. Salmonids appear to be particularly susceptible to fin damages, and this is often found in farmed salmonids (Ellis *et*

al., 2008; Stien *et al.*, 2013). When PAA is mixed in the water, the fish reacts on this new environment and may change their behaviour, for example leading them to be more active or aggressive (Liu, 2017) in which can lead to fin damages. However, there was no obvious aggression or any erratic behaviour during PAA exposure in both trials. Also, Trial 2 had a similar scoring trend for the different fins as Trial 1, even though the fish were exposed to a higher concentration of PAA, as well as confinement stress. Similar to this study, Folkedal *et al.* (2016) found in their study a high presence (above 70 %) of damages and splits of fish at fish farms. We can, therefore, speculate that the damages observed were likely due to farming conditions caused by one or the other, or an interaction between different factors in the production system prior to their transfer to the experimental facility.

The skin, with scales and mucus, represents the first barrier against the environment surrounding the fish (Noble *et al.*, 2012; Stien *et al.*, 2013; Noble *et al.*, 2018). They protect the animal from diseases, infections and pollutions, among other things (Noble *et al.*, 2012). Damage can lead to osmoregulatory changes, wounds, decreased growth and increased mortality (Noble *et al.*, 2012; Stien *et al.*, 2013; Noble *et al.*, 2018). Moreover, the skin has nociceptors, and if damaged, may lead the fish to feel pain (Noble *et al.*, 2012; Noble *et al.*, 2018). The skin condition can thus affect fish welfare. Common skin damage in salmon includes haemorrhage, wounds, and scale loss. The skin damage scores 2 weeks after each exposure in Trial 1 were almost similar, 1,1 – 1,8 after the first exposure and 1,25 – 2,0 after the re-exposure, where the highest number of cases were noted in PAA exposed groups. Farmed fish are exposed to several factors which can affect their skin, such as, mechanical handling (transport, sorting, netting etc.), bacteria and parasites, fungus, fish nets and ropes and high biomass (Vaagsholm and Djupvik, 1998; Noble *et al.*, 2012; Stien *et al.*, 2013). The skin damage observed in both trials, which was mostly scale loss, may be due to handling which included transport of fish from holding to exposure tanks, with a few seconds in the transportation container. This is similar to what Folkedal *et al.* (2016) found, where the majority of the farmed salmon had scale loss. There were also higher scores for the re-exposure fish compared with the first exposure in Trial 1, but this may likely be caused by that the fish did not fully recover from the last handling. Nonetheless, the scores were so low that to be considered a welfare issue.

As the fish in Trial 2 were exposed to two main stressors, i.e., 4,8 ppm PAA and crowding, in contrast to Trial 1 where the main stressor was PAA, one may expect that welfare issues would be likely more pronounced in the former. But, as described above, this was not the

overall picture indicated by the welfare scores. It appears that higher PAA concentration (within the tested levels) would not result in increased cases of morphological damages. Also, the fish exposed to crowding in Trial 2 did not differ from the control fish which indicates that it was PAA itself that affected the fish and not the crowding stress prior to exposure. However, there was little difference between the control groups and the treatment groups as well as between PAA concentrations. Farmed fish is exposed to many external factors which can affect the WIs, therefore further research should be done on the correlation between higher WI scores and higher PAA concentrations. In general, the changes observed in this study following PAA treatment and stress did not pose serious welfare issues to the fish, and the fish were able to recover quite fast.

4.2 Changes in the systemic stress indicators of PAA exposed fish – Trials 1 and 2

4.2.1 Cortisol

The results for plasma cortisol levels from Trial 1 and 2 followed the same pattern. They had a significant increase in the early hours after stress had been triggered, then followed by a decrease and return to the baseline values thereafter, which is the classical stress response in fish (Barton and Iwama, 1991; Pottinger and Moran, 1993). Moreover, this was in line with other studies on stress response in salmonids, including experiments on peroxide exposure (Bowers *et al.*, 2002; Liu, 2017; Chalmers *et al.*, 2018; Gesto *et al.*, 2018).

For the first exposure in Trial 1, the average cortisol levels 2 h after exposure was ~ 19 ng/mL for control and both PAA exposed groups. The response was rather low as cortisol levels after a stressor often are between 30 – 300 ng/mL (Barton and Iwama, 1991; Barton, 2002) and also in comparison with other studies were the values ranged from 120 ng/mL to above 1000 ng/mL (Einarsdóttir and Nilssen, 1996; Iversen *et al.*, 2005; Vera and Migaud, 2016). However, a rise in the average cortisol value indicates that the exposure triggered a stress response but not a substantial one. The *0* and *2,4 ppm* had values similar to baseline levels 48 h after exposure, but the *0,6 ppm* was significantly higher than the two other groups. The reason for this may be that this group was handled harder particularly during netting and transportation between holding and exposure tanks, or that the response to PAA at 5 min exposure may not be a linear relationship and that the peak-response might-be at *0,6 ppm*. Moreover, as the control group responded similarly to the stressor as the PAA exposed

groups, this indicates that it was not the PAA which triggered the response, but possibly the handling.

The response in plasma cortisol was higher in the *2,4 ppm* for the re-exposure in Trial 1, which reached 60,70 ± 8,23 ng/mL 2 h post-exposure. Barton *et al.* (1980) found that the rate of increase in plasma cortisol is affected by the severity of the stressor. Thus, a more intense stressor will give a faster increase and a higher peak. This indicates that 30 min exposure time, instead of 5 min, with *2,4 ppm* PAA was a more intense stressor. Still, the average plasma cortisol was low compared with results from other stress studies on Atlantic salmon (Einarsdóttir and Nilssen, 1996; Carey and McCormick, 1998; Bowers *et al.*, 2002; Vera and Migaud, 2016; Chalmers *et al.*, 2018), indicating that the fish did experience a stressful episode but its magnitude was not too high. The previous history of PAA exposure did not appear to have any negative effects on how the fish handled the same stressors, as the response was approximately equal despite a longer exposure period. The results indicated that the fish may recover fast as all groups were back at baseline 48 h post-exposure in the re-exposure. It is reported that repeated exposures to mild stressors, such as PAA exposure, can lead to habituation which is a process that desensitises the fish when the stressor is recognized to be harmless leading to matching or declined level in plasma stress response (Barton, 2002; Liu, 2017; Gesto *et al.*, 2018).

The response in Trial 2 for the average plasma cortisol level was higher (~ 110 ng/mL) for all groups compared with Trial 1, which indicates that the fish experienced a more intense stressor. This was likely because the PAA concentration was higher and the fish was exposed to crowding stress. However, the levels decreased 4 h after exposure and this showed that the fish were able to mount a classical response to a stressor. Furthermore, the peak was not high as average cortisol levels above 1000 ng/mL have been reported after peroxide exposure (Vera and Migaud, 2016). Interestingly, all groups regardless of treatment (control, PAA, and/or stress) had identical patterns in their average cortisol response. It can be speculated that this was perhaps caused by the transportation of the fish between holding and exposure tanks, which was in different halls. The peculiarities in the data highlight the different factors that may affect the cortisol response of salmon when exposed to PAA. These factors, predominantly related to handling, must be studied in depth in the future especially on how they contribute to the physiological responses during treatment and thus must be integrated in developing standardised chemotherapeutic protocols.

4.2.2 Glucose

In Trial 1 the control group had an increase in average plasma glucose. The other two groups (*0,6* and *2,4 ppm*) was not significantly different from baseline, and there were not observed any treatment-induced differences. This was similar to what Gesto *et al.* (2018) reported on rainbow trout exposed to PAA. Plasma glucose level usually increases after a stressor as a secondary stress response, to ensure energy to the muscles (Barton and Iwama, 1991). In Trial 2, both sub-groups in *no stress* group increased after 4 h, then 2 w post-exposure the *no stress control* returned to baseline, but in contrast, *no stress PAA* stayed elevated. The glucose level 4 h post-exposure was lower for the *stress* group compared with the *no stress* group. The level did not increase before 2 w post-exposure for the *stress* group. The difference in plasma glucose 4 h after exposure between the *no stress* and *stress groups,* indicates that the *stress* groups already had mobilised the stored glycogen during the crowding stress and during aerobic conditions, the glucose was catabolised by glycolysis (Polakof *et al.*, 2012). As the glycogen deposit in the liver is limited, no more glucose could be mobilised, as seen in Skjervold *et al.* (1999), thus the lower level for the *stress* groups. This also shows that prior crowding stress possibly interferes with the glucose stress response to PAA, as the *no stress PAA* group 4 h after exposure had significantly increased plasma glucose level compared with its counterpart in the *stress* group. The elevated glucose level 2 w post-exposure in *no stress PAA* and both *stress* groups indicated perhaps a delayed and prolonged effect of the stressors, and that the elevated glucose levels may be due to a heightened state of gluconeogenesis to supply the metabolic demands of PAA, crowding and their combination (Polakof *et al.*, 2012). This implies some metabolic consequences of PAA treatment that deserves future studies. The mechanisms that regulates glucose is intricate (Vera and Migaud, 2016). There is often a time-based delay between primary and secondary responses as seen in Veiseth *et al.* (2006) and Gesto *et al.* (2018). This was also observed in both trials as the increase in average plasma glucose was seen 2 – 4 h after exposure. The glucose levels in both trials were within the normal resting range of 3 – 6 mmol/L for salmon (Finstad, 1999; Normann, 2014), suggesting that the fish did respond to the stressors, but to an extent that an effective response is mounted without compromising other physiological systems. Other studies (Iversen *et al.*, 1998; Carey and McCormick, 1998; Bowers *et al.*, 2002; Chalmers *et al.*, 2018) have reported similar or higher levels (≤ 10 mmol/L) in Atlantic salmon after a stressor, indicating that the response seen here was not very high.

4.2.3 Lactate

The overall trend for plasma lactate was similar in both trials displaying an increase at the early hours after exposure followed by a decrease. Lactate is known to increase as a response to a stressful episode (Barton and Iwama, 1991). The elevated levels in the first sampling points suggest an increased activity in anaerobic metabolism thus more lactate was produced following exposure. In contrast to the *no stress* groups, both sub-groups in the *stress group* in Trial 2 had significant elevated lactate levels 2 w after exposure. This indicates that the crowding stressor before exposure was likely a compounding factor in the lactate response to an additional stressor, i.e., PAA. The late increase in the lactate level for both *stress* groups was probably in connection with the catabolism of the glucose at the early hours, as it goes through glycolysis, lactate increases due to the absence of oxygen (Polakof *et al.*, 2012). Espmark *et al.* (2015) reported that lactate levels for smolt should not rise above 5 mmol/L (≈ 450 ng/µL) after a stressor. None of the groups had values above this level. The plasma lactate level was slightly higher than the levels found for PAA exposed rainbow trout (Gesto *et al.*, 2018) and on the same level as the control group in the hydrogen peroxide study in Chalmers *et al.* (2018), suggesting that PAA is a milder stressor than H_2O_2.

4.2.4 Summary of the physiological response to PAA in plasma

Overall, the increase displayed in the plasma stress indicators in the early hours after PAA exposure indicates that PAA induced stress response. The stress indicators then returned to baseline at the end of the trials. Furthermore, the different treatment groups were all equally able to physiologically respond to PAA. The levels were also within what is considered normal values and, in some instances, lower compared with reported levels in other stress studies in salmon. These results indicate that systemic stress responses were not dramatically affected by PAA exposure. Neither repeated exposure of PAA nor crowding before PAA exposure appeared to induce stress beyond the salmon's capacity to respond, which was in agreement with Liu *et al.* (2017) and Gesto *et al.* (2018) studies on stress responses in rainbow trout after PAA exposure. These results are also by the results from the external morphological welfare. The fish did not display any difference in growth and the changes in the welfare indicators following PAA exposure did not pose serious welfare issues. The results of systemic stress indicators collectively suggest that PAA can be used for salmon as it does not impose a significant concern on the physiological stress response or affect the external welfare of the fish.

4.3 Antioxidant defence in mucosal tissue of salmon exposed to PAA – Trial 2

Trial 1 samples were not included in this thesis because they were a part of a different sub-study of the project.

Oxidative stress happens when the balance between ROS and antioxidant mechanisms is disturbed (Pedro *et al.*, 2018). H_2O_2, and perhaps PAA, may induce oxidative stress and provoke the defence mechanism against ROS, including the transcription of antioxidant genes like *gp*, *sod* and *gr* (Lesser, 2006). The gills and skin are mucosal tissues that function as the first line of defence and are highly responsive to the changes in the immediate environment, including the levels of ROS. They may provide interesting insight into how the antioxidant system is mobilised at the mucosa upon direct contact with a strong oxidant such as PAA.

Overall, the expression of *gp* in both gills and skin were not markedly affected by PAA treatment, especially in the skin where the expression remained unchanged regardless of the treatment and time. There were some interesting patterns, however, in the expression of *gp* in the gills. The expression was significantly higher at 2 w post exposure, regardless of the treatments. The late upregulation of *gp* expression relative to the pre-exposure value may be due to the temporal nature of *glutathione peroxidases* in fish (Lazado *et al.*, 2015; Lazado *et al.*, 2016), and may not likely be due to the treatments. The differential expression pattern at the early hours post-exposure in the *stress* groups was quite striking. It seemed that crowding stress before exposure may have altered the *gp*-mediated response. An increase in *gp* expression is associated with higher levels of ROS (Birnie-Gauvin *et al.*, 2017), and peroxidases are known to reduce H_2O_2 (Pedro *et al.*, 2018), therefore, protecting the cells from oxidative damage. Glutathione peroxidase requires several enzymes and co-factors for its activity, including the use of sulfhydryl form glutathione (GSH) during H_2O_2 reduction to H_2O and eventually generating glutathione disulphide (GSSG), to which Glutathione reductase regenerates (Weydert and Cullen, 2009). Superoxide dismutase scavenges superoxide radicals, hence, plays a central role in protection against oxidative stress. The different treatments significantly modulated the *sod* expression, but the differential expression was more pronounced in the gills indicating that the gill mucosa may have a central role in the superoxide-dependent response in salmon and thus, supporting further its role in oxidative stress in fish (Abdel‐Moneim *et al.*, 2012; Mozhdeganloo and Heidarpour, 2014). The expression of *mnsod* in both the gills and the skin was generally higher 2 w after exposure. A similar trend was observed in another form of *sod* with copper and zinc co-factors (i.e.,

cu/znsod). It is interesting to emphasize that crowding before PAA exposure appeared to influence the *sod* transcription in the gills in the early hours (i.e., 4 h post-exposure). In most cases, the expression in the *stress* group, regardless of whether it was from control or PAA, was low compared with their counterparts in the *no stress* group. Higher expression of *sod* is related with higher levels of superoxide radicals (O_2^-) (Pedro *et al.*, 2018). Superoxide dismutase breaks down O_2^- into H_2O_2, which then can be further catalysed to H_2O by Glutathione peroxidase (Weydert and Cullen, 2009). *Glutathione reductase* catalyses reduction of GSSG to GSH, which is critical in resisting oxidative stress, as GSH is used by *Glutathione peroxidase* (Chalmers *et al.*, 2018). The expression of *gr* in the gills was more dynamic compared with the skin. It was apparent that stress had a strong impact on *gr* expression and may interfere with the glutathione reductase-dependent oxidative response, since fish subjected to stress prior to PAA treatment exhibited strikingly lower *gr* expression in the gills compared to their counterparts in the *no stress* group in the early hours after exposure. The identical overall expression pattern of *gr* expression in the skin between the *no stress* and *stress* groups revealed that neither the stress nor the PAA exposure had a regulatory impact.

The overall results indicate that the antioxidant defense towards PAA was more responsive in the gills than the skin. Moreover, fish with stress history prior to PAA exposure exhibited a different oxidative response pattern to PAA compared with the non-stressed fish, highlighting the potential confounding and compounding roles of stress in the antioxidant defense. The gills have a large surface area in contact with the water and are less structurally complex in contrast to skin that has multiple layers (Peterson, 2015). Moreover, PAA and its intermediate products have a low molecular mass that might be gill-permeable and diffuse into the fish (Wilhelm et al., cited in Liu, 2017). Therefore, the striking regulation of antioxidant system in the gills may perhaps be due to its less complicated structure and higher surface-contact ratio to the aquatic environment.

4.4 Antiparasitic activity of PAA

An *in vitro* experiment was conducted to asses the viability of the amoeba exposed to different concentrations of PAA, with two different exposure durations. An exposure time of 30 min led to a higher amoebic viability reduction compared with a 15 min exposure. PAA concentration of *2,4 ppm* resulted in higher viability reduction compared with *0,6 ppm* and *0 ppm*. Several studies have shown that PAA is potent in controlling fish pathogens (Smail *et*

al., 2004; Meinelt *et al.*, 2009; Straus and Meinelt, 2009; Sudová *et al.*, 2010; Marchand *et al.*, 2012; Picón-Camacho *et al.*, 2012; Straus *et al.*, 2012; Meinelt *et al.*, 2015). H_2O_2 is the known chemical-based treatment of AGD (Adams *et al.*, 2012; McCarthy *et al.*, 2015; Hytterød *et al.*, 2017; Martinsen *et al.*, 2018). The potency of PAA as an antimicrobial agent is attributed to its ability to denature protein, disrupt cell wall permeability, and oxidize sulfhydryl and sulfure bonds in proteins, enzymes, and other metabolites quite rapidly because of chemical oxidation, in contrast to H_2O_2 which is dependent on enzymatic oxidation (Block, 1991; Freeman and Auer, 2012; Straus *et al.*, 2017). The potential of PAA as AGD treatment lies on the notion that we can use much lower concentration of the chemical to treat the disease, thus offer an alternative to the current use of H_2O_2 which requires high concentrations (≥ 800 ppm) and thus poses problems for fish welfare (Adams *et al.*, 2012; Hytterød *et al.*, 2017; Martinsen *et al.*, 2018). A PAA concentration around 1 ppm was reported to be effective against many pathogens (Pedersen *et al.*, 2013) and the results revealed antiparasitic activity of PAA below that concentration especially at 30 min exposure time.

The *in vitro* exposure indicates that PAA has an anti-parasitic activity against *P.perurans*, particularly at *2,4 ppm* PAA for 30 min, which resulted in approximately 74 % viability reduction. The interaction of PAA concentration and exposure time should be investigated further, as it was interesting to observe that exposing the amoeba to *0,6 ppm* for 30 min resulted in almost similar viability potential as with exposure to *2,4 ppm* for 15 min. This concentration of PAA (*2,4 ppm*) was shown in Trial 1 to result in an adaptive stress response which did not dramatically affect the welfare of the fish. Moreover, the results from the stress indicators in Trail 2, which tested PAA concentration doubled the highest dose in Trial 1, indicated that the PAA did not dramatically affect the physiological response to stress, thus, welfare was not compromised. Therefore, higher concentrations of PAA (i.e. 4,8 ppm) can probably be used against the amoeba, without compromising the health and welfare of fish. These results support the on-going approach on PAA as a treatment for AGD.

5 Conclusion

This study revealed how systemic and mucosal defenses were affected in Atlantic salmon (*Salmo salar*) when exposed to peracetic acid, a potential oxidative stressor. The results revealed that salmon were able to mobilise its systemic and mucosal stress responses to counteract the increased level of ROS (i.e. in the form of PAA in the water) in the environment. Previous exposure history to PAA did not interfere dramatically with the stress responses and the fish were able to recover quickly after re-exposure. Crowding stress before PAA treatment, however, did influence some of the stress indicators, i.e., glucose, lactate and the antioxidant genes in the gills. Nonetheless, the overall changes were considered to be classical responses to these conventional stressors. There were external morphological changes following the treatments, but the changes observed were not so dramatic to consider that PAA exposure resulted in welfare concerns. The physiological and morphological results correlate well and collectively suggest that PAA at tested concentrations did not substantially affect the normal biological functions of salmon, particular when prompted with a stressor. In addition, PAA was proven to have an amoebicidal effect on *Paramoeba perurans*, the causative agent for AGD. In conclusion, the results of this thesis revealed that PAA at the tested concentrations did not dramatically compromise the health and welfare of salmon. The fish were able to mount a strong adaptive response to different PAA doses, exposure time and a potential confounding factor. The preliminary data on the amoebicidal activity of PAA further supports the potential of PAA as a potential treatment for AGD.

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Appendix i

Residual plot, statistical evaluation

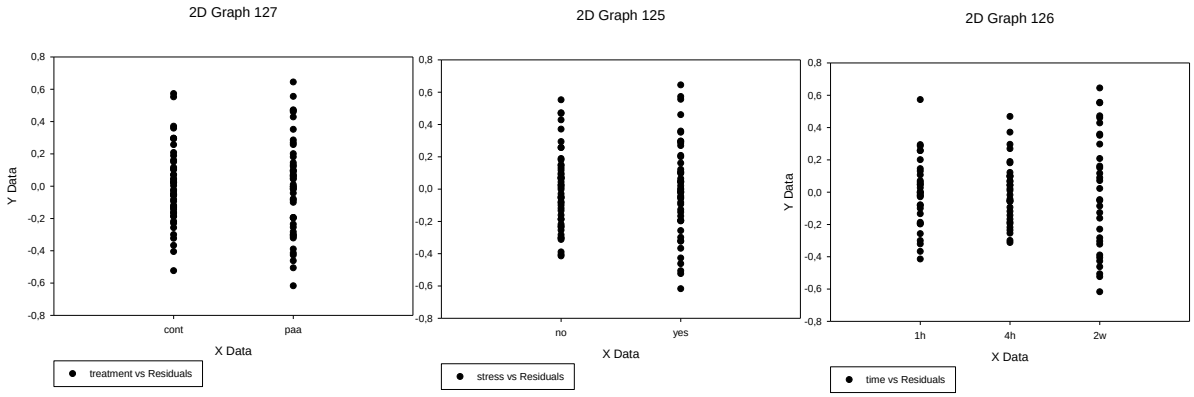

Figure 17: Example of visual inspection of the data showing residual plots of treatment, stress and time. If normality or equal variance test failed visual inspection was used to determine if the ANOVA could be used or not

Appendix ii

TURBO DNA-free Second Digest Buffer

Tris: 157,6 g/mol. Trizma hydrochloride $C_4H_{11}N$

$CaCl_2$: 147,02 g/mol. Calcium chloride dihydrate $CaCl_2 * 2H_2O$

$MgCl_2$: 203,30 g/mol. Magnesium chloride hexahydrate $MgCl_2 * 6H_2O$

$$n = \frac{m\ (g)}{Mw\ (\frac{g}{mol})} \qquad c = \frac{n\ (mol)}{v\ (L)}$$

100mL (0,1 L)

Tris, 200mM (0,2M)

0,2 mol/L * 0,1 L = 0,02 mol

0,02 mol * 157,6 g/mol = 3,152 g

$CaCl_2$, 5mM (0,005M)

0,005mol/L * 0,1 L = 0,0005mol

0,0005mol * 147,02 g/mol = 0,07351 g

$MgCl_2$, 100mM (0,1M)

0,1 mol/L * 0,1 L = 0,01 mol

0,01 mol * 203,30 g/mol = 2,033 g

The chemicals were weighed out and transferred to a glass by nuclease-free H_2O. A magnetic stirrer was used to dissolve the salts. pH was measured and adjusted to 7,5 with NaOH. Nucelase free water was added up to 100 mL. The buffer was sterile filtered (filtropur S 0.2 0,2 µm), aliquoted and stored at – 20 °C

Appendix iii

Welfare scores

Table 6: WIs and OWIs in Trial 1 and Trial 2

2 w after the first exposure in Trial 1												
	EMACIATION	EYE DAMAGE	SKIN DAMAGES	OPERCULUM DAMAGE	SNOUT DAMAGE	VERTEBRAL DEFORMITIES	JAW DEFORMITIES	DORSAL FIN	CAUDAL FIN	PECTORAL FINS	PELVIC FINS	overall welfare index
0 ppm	0	0	1,1	0	0	0	0	1,3	1,1	1,1	0,8	0,49
0,6 ppm	0	0	1,8	0,4	0	0	0	1,7	1,1	1,5	0,3	0,62
2,4 ppm	0	0	1,7	0,4	0	0	0	1,7	1	1,4	0,5	0,61
2 w after re-exposure in Trial 1												
	EMACIATION	EYE DAMAGE	SKIN DAMAGES	OPERCULUM DAMAGE	SNOUT DAMAGE	VERTEBRAL DEFORMITIES	JAW DEFORMITIES	DORSAL FIN	CAUDAL FIN	PECTORAL FINS	PELVIC FINS	overall welfare index
0 ppm	0	0	1,25	0,1	0	0	0	1,2	0,7	1,1	0,1	0,40
0,6 ppm	0	0	2	0,1	0	0	0	1,1	1	1,3	0,5	0,55
2,4 ppm	0	0	1,85	0,7	0	0	0	1,5	1	1,1	0,2	0,58
2 w after exposure in Trial 2												
	EMACIATION	EYE DAMAGE	SKIN DAMAGES	OPERCULUM DAMAGE	SNOUT DAMAGE	VERTEBRAL DEFORMITIES	JAW DEFORMITIES	DORSAL FIN	CAUDAL FIN	PECTORAL FINS	PELVIC FINS	overall welfare index
no stress control	0	0	1,05	0,3	0	0	0	1	0,8	1,2	0,2	0,41
no stress PAA	0	0	0,95	0,9	0,2	0	0,1	1,3	0,8	1,2	0,5	0,54
Stress control	0	0	0,8	0,1	0	0	0	1,2	0,7	1	0,3	0,37
Stress PAA	0	0	1,35	0,4	0	0	0	1,2	1,2	0,7	0,5	0,49