

**Stress and its impact on animal welfare during  
commercial production of Atlantic salmon (*Salmo  
salar* L.)**

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## Preface

This dissertation is submitted as part of the requirements for the degree of Philosophiae Doctor (PhD) in the Faculty of Bioscience and Aquaculture, University of Nordland, Bodø, Norway. The studies compiled in this dissertation represent original research carried out over a period of five years, from 2009 to 2013. All papers in this dissertation are part of the on-going research by Robert Eliassen and me regarding animal welfare and stress in commercial aquaculture industry. Most of the research has been funded by the Aquaculture industry and “SkatteFunn” governed by the Norwegian Research Council. Companies that have kindly contributed to this thesis are ACD Pharmaceuticals AS, ScanAqua AS and EWOS Innovation AS.

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*“There is pleasure in the pathless woods, there is rapture in the lonely shore, there is society where none intrudes, by the deep sea, and music in its roar; I love not Man the less, but Nature more”* George Byron (1788-1824).

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## List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-IV

- Paper I.** Iversen, M, & Eliassen, R. A. (2013). The effect of allostatic load on hypothalamic–pituitary–Interrenal (HPI) axis before and after secondary vaccination in Atlantic salmon postsmolts (*Salmo salar* L.). Fish physiology and Biochemistry (submitted)
- Paper II.** Iversen, M. H. & Eliassen, R. A. (2013). The use of Finquel vet (MS-222) and Benzoak vet (benzocaine) during vaccination of Atlantic salmon parr (*Salmo salar* L.) and its impact on water quality, efficacy and animal welfare. Aquaculture (submitted)
- Paper III.** Iversen, M., Eliassen, R. A. & Robb D. H. F. (2013). The effects of Aqui-S vet. sedation during vaccination on survivability, appetite, growth, immunological capacity, primary, secondary and tertiary stress responses in Atlantic salmon parr (*Salmo salar* L.). Aquaculture (submitted)
- Paper IV.** Iversen, M. & Eliassen. R. A. 2009. The Effect of AQUI-S® Sedation on Primary, Secondary and Tertiary Stress Responses during Salmon Smolt (*Salmo salar* L.) Transport and Transfer to Sea. Journal World of Aquaculture Society. 40 (2): 216-225

## Definitions

In the study on the relationship between stress and animal welfare in finfish production, several scientific terms are commonly used. Some key terms used may need a definition, and this thesis define the terms as:

**Stress:** A condition in which the dynamic equilibrium of animal organisms (homeostasis) is threatened or disturbed as a result of the actions of internal or external stimuli, commonly defined as stressors.

**Allostasis:** Achieving stability through changes. This is a process that supports homeostasis, as environments and/or life history stages changes.

**Allostatic state:** The altered and sustained activity levels of the primary mediators, which integrates physiology and associated behaviours in response to changing environments and challenges.

**Allostatic load:** The cumulative physiological burdens exacted on the body as the animal adjust their morphology, physiology and behaviour to adapt to more volatile factors.

**Allostatic overload type 1:** An adaptive response (acute stress response) triggered when the energy demands of an organism exceeds the available energy supplies. Thus glucocorticoid concentrations triggers an emergency life history stage (ELHS), which then results in a net decrease of energy demands below the available supply of energy, and a net decrease of allostatic load.

**Allostatic overload type 2:** A non-adaptive response (chronic stress response) which emerges when energy demands and glucocorticoid concentrations increase, but do not exceed the energy supplies, and thus they do not trigger an ELHS and the allostatic load is kept alleviated.

## Abstract

During a production cycle of Atlantic salmon from juvenile to slaughter the animal undergoes many critical production phases, which could elicit both grave stress responses and have major impact on animal welfare. In the present thesis the impact of stress and its consequences on animal welfare, during vaccination (**paper I, II and III**) and transport procedures (**paper IV**), has been studied.

Results from the present studies have shown that if plasma cortisol becomes elevated prior to vaccination it could instigate an allostatic overload type 2 (chronic stress response) with negative consequences on animal welfare. **Paper I** gives a plausible physiological causal relationship for allostatic overload type 2 scenarios in fish; which include oversensitivity to ACTH, reduced efficient negative feedback system, and elevated baseline levels of plasma cortisol, which eventually will lead to negative effects on wellbeing of the animal. During vaccination the fish have to be anaesthetised, and poor water quality in the anaesthesia tank affects the stress response (**paper II**) and the primary, secondary and tertiary stress response seems to be more aggravated during high stress group in poor water quality (high CO<sub>2</sub> and no air supply), thus emphasizing the importance of keeping the water quality under control during anaesthesia and large-scale vaccination, regardless of choice of anaesthetic. Failing to do so could have major impact on the animal welfare due to increased stress and mortality. Furthermore, **paper III** shows the potential benefit of reducing the overall stress response during vaccination, as a stress reducing measure, has positive effects by reducing the overall stress level, feed conversion, appetite and mortality. Most pronounced was this effect during sedation of AQUI-S vet. followed by Finquel Vet. anaesthesia (treatment II). Clearly, a combination of sedation with AQUI-S vet. and Finquel Vet. anaesthesia under vaccination could reduce the overall stress response and mortality, and thus improve both production cost and animal welfare during a critical production phase in Atlantic salmon.

**Paper IV** shows that sedation during on- and offloading sufficiently reduced the primary stress response with positive effect on animal welfare compared to an unsedated control group. None of the secondary stress responses measured in this

experiment could contribute to explain this phenomenon – except of plasma magnesium – which differed between the groups. While plasma magnesium in the AQUI-S® sedated group returned to pre-stress levels 12 hours after transport the unsedated group showed no such recovery even a week after transport. This may indicate a disturbance in the hydromineral balance, and gives a plausible explanation for delayed mortality in this group. AQUI-S vet. shows promises as a stress-reducing sedative for Atlantic salmon smolts, and if used properly it could improve animal welfare and survivability during and after common aquaculture related incidents.

This thesis also shows that mortality seemed to be closely linked to excessive release of plasma cortisol in vicinity of multiple stressors, both during vaccination and transport of salmon parr and smolt. Plasma cortisol seems to be a good candidate to assess the psychological aspect of regarding fish welfare. And further, the surge in plasma cortisol experienced in **paper II** and **III**, seems to be at some extend related to the fear response in fish.

As a summary, results from this thesis suggests, that to reduce the risk of compromising fish welfare during commercial vaccination of salmon parr, one should grade the fish minimum a week prior to vaccination, or grade simultaneously with vaccination, and keep the water quality in the anaesthesia bath under control. During transport one proposes to keep the stress response to a minimum during loading and off-loading, and if possible, use sedative during the hauling process. All these proposed measures can reduce the overall allostatic load during handling, vaccination and transport, and produce a healthy fish, with intact immune response and thus improve the fish welfare. In doing so, the salmon aquaculture industry could defend its reputation among consumers and the public in general, and at the same time improve the economical result by reducing overall mortality after transfer to sea.

## 1. Introduction

The overall aims of this study was to examine the effects of stress during common handling procedures (stressors) routinely used during commercial salmon aquaculture, and its possible impact on animal welfare. Thus, in this introduction the main focus will be on the theories regarding animal welfare, stress, allostasis and hypothalamic-pituitary-interrenal axis in teleost, and not on the commercial stressors (anaesthesia, vaccination and transport) used in the present study.

The earth faces multiple and interlinked challenges ranging from the impacts of the ongoing financial and economic crisis to human created climate changes and extreme weather events. At the same time, it faces the pressing food and nutrition needs of a growing population with limited natural resources. Fisheries and aquaculture make crucial contributions to the world's wellbeing and prosperity. In the last five decades, world fish food supply has outpaced global population growth, and today fish constitutes an important source of nutritious food and animal protein for most of the world's population (FAO 2012). This sector provides livelihoods and income, both directly and indirectly, for a significant share of the world's population. Fish and fishery products are among the most traded food commodities worldwide, with trade volumes and values reaching new highs in 2011, and this is continuing to rise in the future (FAO 2012). While capture fisheries production remains stable, aquaculture production keeps on expanding. Aquaculture is one of the fastest-growing animal food-producing sectors, and it is predicted that in the next decade the total production from both fisheries and aquaculture will exceed that of beef, pork or poultry (FAO 2012).

Capture fisheries and aquaculture supplied the world with about 148 million tonnes of fish in 2010 (with a total value of US\$217.5 billion), of which about 128 million tonnes was utilised as food for people (FAO 2012). In 2010, the top-ten producers of farmed aquatic animals were: 1. China (36.7 million tonnes), 2. India (4.6 million tonnes), 3. Vietnam (2.7 million tonnes), 4. Indonesia (2.3 million tonnes), 5. Bangladesh (1.3 million tonnes), 6. Thailand (1.4 million tonnes), 7. Norway (1.0 million tonnes), 8. Egypt (919 600 tonnes), 9. Myanmar (850 700 tonnes) and 10. The

Philippines (744 700 tonnes). These producers contributed 87.6 % of world production by quantity. World aquaculture production in 2010 consisted of 56.4 % of freshwater fishes (33.7 million tonnes), 23.6 % of molluscs (14.2 million tonnes), 9.6 % of crustaceans (5.7 million tonnes), 6.0 % of diadromous fishes (3.6 million tonnes), 3.1 % of marine fishes (1.8 million tonnes) and 1.4 % of other aquatic animals (814 300 tonnes) (FAO 2012).

However, aquaculture production is vulnerable to disease and adverse environmental conditions. Disease outbreaks in recent years have affected farmed Atlantic salmon in Chile, oysters in Europe, and marine shrimp farming in several countries in Asia, South America and Africa, resulting in partial or sometimes total loss of production (FAO 2012). In Norway the disease situation seems to be stable and under control. However in the recent years salmon industry have been under the public attention due to the negative impact the industry has on the wild salmon through escapees and salmon lice (IMR 2012). In the same context, concerns about the animal welfare and wellbeing of the salmon in industry has been raised by conscious consumers, official authorities, environmental organisations, researchers and the industry itself (Fisheries 2010; IMR 2012; Iversen and Eliassen 2012; Iversen 2005, 2008), as almost 1 in 5 salmons dies after transfer to sea (DOF 2012b).

## **2. Animal welfare**

Since the seventies, scientists and philosophers have tried to work towards a common goal of understanding and improving our relationship with other species (Arkow 1998; Fraser 1999). Although the goal is the same, the two professions have different approaches, assumptions and vocabulary towards the matter; this both alienate and create confusion between two professions.

### **2.1 Animal ethical philosophy and the science of animal welfare**

Man is considered a natural part of the world, since human beings is the only creature with rational thought and behaviour, the anthroposophist thinks that the animal world is a value we can use as we see fit. The philosopher Immanuel Kant (1724-1804) stated that humans have an innate value and have to be treated with

respect. All other living and non-living units are to be regarded as things. Things (animals, etc.) have only an instrumental value, and should be used by humans, and humans have no direct accountability to the animals, not even the responsibility not to be cruel to them (Kant 1977, 1980).

Since the sixties, traditional anthropocentric philosophies have been under attack from various quarters. Many of these attacks have been motivated by the growing environmental impact and destruction humans inflict on the world. The attacks have often been focused on the claim that humans are special, that makes us and only us worthy of moral consideration. Most critics of the traditional anthropocentric philosophy agree that to exclude animals from moral consideration is arbitrary and unnatural (Goodpaster 1978; Regan 1983; Singer 1990). Some eco-philosopher have attempted to reorient and convert humanity to understand the living world around us as a value, and thus it must be treated with the same respect as humans demand, and only that way will humanity survive (DesJardins 2006; Næss 1999).

Some philosophers and activists have taken the process a step further. They argue that if animals are more than an instrumental value to humans, and further if they should be respected as individuals, animals should not be regarded as a resource for man, and should only be used if absolutely necessary for human survival and health. For that reason, all people should become vegetarians, since the modern society does not rely on animal proteins to maintain a healthy lifestyle (Regan 1983, 2004; Singer 1990; Taylor 1986). There are two main directions in this extreme animal ethics philosophy. One direction is controlled by philosopher Peter Singer who with his book *Animal Liberation* is both an icon and role model for the animal rights movement. The great irony is that Singer is a utilitarian who has an ethical approach that rejects the idea that neither humans nor animals have rights. His argument is that since some animals seems to be thinking/feeling and since all things are being equal (no difference between humans and animals), one should not disparage the suffering of animals as less important than the suffering of people (Garner 2010). Another direction in the extreme animal ethical philosophy suggests that there is a resemblance between all species. The most prominent voice for the rights-based approach is Tom Regan (Regan

1983) and the abolitionist Gary Francione (Francione 1996, 2000, 2008). Regan's arguments for animals right for a life, is like building a protective fence around them so that their basic interests under no circumstances can be sacrificed for the general welfare or benefit of humankind (Regan 1983).

The science of animal welfare is of multidisciplinary nature. This science includes not only issues of animal science, but this disciplinary also have to take into consideration both ethical and philosophical issues (Dawkins 1980, 1990; Watanabe 2007). Scientists who study animal welfare as a subject have often started their scientific career in completely different disciplines. They have been driven into this topic because of public concern regarding how their own research animals were raised and treated (Arkow 1998). The public concern is not only represented by radical animal rights activists, but also by ordinary consumers with a love for animals like housewife Ruth Harrison (Harrison 1964), and the Swedish children's author Astrid Lindgren (Mejdell 2006). Often has this criticism been ruled by ethicists and social critics (Blatz 1991; Dawkins 1980, 1990), and public opinions have often been the driving force behind the new laws and legislations in animal welfare. This made researcher David Fraser to define animal welfare research as mandate driven research, and thus it could be said that animal welfare research is meaningful for the common people (Fraser 1999, 2003, 2008, 2009; Fraser et al. 1997).

## **2.2 Concept of animal welfare**

The concept regarding animal welfare is complex and a common definition is difficult to agree about. This is because the term is used in many different ways by people with different scientific and non-scientific backgrounds (Huntingford and Kadri 2008). The theme welfare creates both conflict and disagreements over inflamed political topics, such as what is the nature, do animals have emotions and do they feel pain? Science bases itself on rational empirical data, and could contribute to develop a common understanding of animal welfare, however scientist often strongly disagree with both the goal and purpose of the concept of animal welfare. Keeling (2004) stated that scientists tend to disagree with the interpretation of the results rather than to emphasise on the similarity in them. Fraser et al. (1997) tried to create a common



platform for a closer understanding of what animal welfare is, by identifying the three main scientific philosophies that have been used in addressing animal welfare. These were according to Fraser et al. (1997): Animals should be able to have natural life (eco-philosophy); Animals should not suffer (moral ethics) and animals should function well (biology, physiology). These points were then expanded and added over the past decade, but the definition and the science of animal welfare is based on how animals feel and not how people think animals feel or perceive a given situation (Fraser 2009; Fraser et al. 1997; Lassen et al. 2006; Rollin 1993). Thus, one could conclude that an animal has good welfare, when the following three conditions are met: 1) The animal is adapted to the environment, and have good health, with all their biological system function; 2) Animals are given the chance to live the natural life, with the opportunity to express their behaviour as the animal would have done in the wild (behavioural needs) and 3) The animal must not be exposed to negative experiences such as pain, fear and hunger, and have access to positive experiences such as social contact with other of its kind (Huntingford and Kadri 2008). Each section covers various important aspects of animal welfare, but this is an oversimplification of the matter/problem. Mellor & Stafford (2001) therefore proposed a more practical approach which could distinguish between good and poor welfare in practise. This approach identifies five areas or domains regarding the welfare of the animals. To ensure the animal's good welfare, animals shall be free from (the five degrees of freedom): 1) Hunger and thirst; 2) Harmful environmental changes; 3) Illness and injury; 4) Behavioural restrictions (including lack of space), and 5) Mental disorder. This approach is now used both legally in many countries and in the practical regulation of welfare related to animals (Fraser 2008, 2009; Huntingford and Kadri 2008; Keeling 2004; Mellor and Stafford 2001; Sandøe and Christiansen 2007).

### **2.3 Animal welfare and fish**

Some of the first studies on fish using the term welfare was done by Shelbourne (1975) whom discussed acclimation to captive of wild caught broodstock of plaice (*Pleuronectes platessa*) and sole (*Solea solea*). However, to apply the term animal welfare and the rules of five degrees of freedom to also include fish has been

somewhat controversial (Ashley 2007). Concepts of animal welfare have been applied to those animals which are considered to have the ability to experience pain, fear and suffering, and therefore have been associated with species implementing a higher level of cognition than compared to fish.

However, there is a scientific debate regarding the ability of fish to experience pain and fear (Arlinghaus et al. 2009). While some reviewers have argued that fish lack essential brain regions or any functional equivalent to the mammals, making not likely that they can experience pain and fear (Rose 2002), others suggest that there is anatomical, physiological, and behavioural evidence that make it conceivable that they have the potential to experience suffering in the form of pain and fear through different nociceptors (Chandross et al. 2004a, 2004b; Ellis et al. 2012; Lund et al. 2007; Sneddon 2006). It is unlikely that animals with a different brain structure than compared to humans (like the cortex) would experience the emotions that humans feel when experiencing pain and fear. Thus, it is impossible to know exactly what a fish experiences. However, in context of the theories of animal welfare and ethics, the nature of the pain or fear an animal experiences during suffering or discomfort should not be discarded as less important (Arlinghaus et al. 2009).

The aquaculture industry has more or less responded to public concern about fish welfare by participation in the debate, and by developing strategies for improvement (Huntingford and Kadri 2009). The industry has been active in developing internal guidelines for good practice (Dykes 2012; FEAP 2013) and working with regulators and policy makers to produce agreed standards (the European Food Safety Agency, the Norwegian Food Standards Agency). In Norway, the law of animal welfare was implemented in June 2009 (LOV 2009), and the animal welfare act for fish welfare in aquaculture fish is largely governed by regulations. This means that the law is regulated through a number of provisions on welfare for both establishments, operation of fish farms and slaughter of aquaculture fish (Mattilsynet 2012a).

### **3. Concept of stress and allostasis**

Stress is defined as a condition in which the dynamic equilibrium of an organism, called homeostasis, is threatened or disturbed as a result of the actions of internal or

external stimuli, commonly defined as stressors (Selye 1950, 1973; Varsamos et al. 2006; Wendelaar Bonga 1997, 2011). The physiological response to stressors has obtained great attention over the last decades and a great amount of data has attempted to complete, or even replace Selye's non-specific stress concept, also known as the General Adaptation Syndrome (GAS), which describes an alarm, a resistance and an exhaustion stage, without succeeding (Varsamos et al. 2006). In the later years the concept of allostasis has been introduced to complement the concept of stress, and more precisely to try to describe the role primary mediators (for example glucocorticosteroids) in response to an stressor (Goymann and Wingfield 2004; McEwen 1998, 2005; McEwen and Wingfield 2003; Wingfield 2005).

### **3.1 The overall stress response**

The definition of stress is characterised by controversies and debate (Barton 2002; Chrousos and Gold 1992; McEwen 2005; Wendelaar Bonga 1997). Nevertheless, the fact that the concept of stress has not been abandoned and is widely accepted by scientist of different subjects, demonstrates its vitality and indicates the attraction of describing a phenomena observed at different organizational levels as cells, organs, organisms, populations, and ecosystems. Stress however, plays a key role in the ability of vertebrates, to survive and perform necessary life functions during unfavourable situations (Schreck et al. 1993). The word stress has its roots in the physiological definition proposed by Selye, as he defined stress to be the nonspecific response of the body to any demand placed upon it (Selye 1950, 1973). A variety of definitions have been offered since that time (Schreck 2010). In any event, it needs to be recognized that stress is the physiological response to a demand, the stressor. More specific concepts tend to involve an endocrine cascade as part of the nonspecific response. However, Schreck (2010) favoured a broader view of stress, stress being the physiological cascade of events that occurs when the organism is attempting to resist death or re-establish homeostasis in the face of a threat. One therefore propose to define stress as a condition in which the dynamic equilibrium of animal organisms called homeostasis is threatened or disturbed as a result of the actions of internal or

external stimuli, commonly defined as stressors (Chrousos and Gold 1992; Selye 1950, 1973; Wendelaar Bonga 1997, 2011).

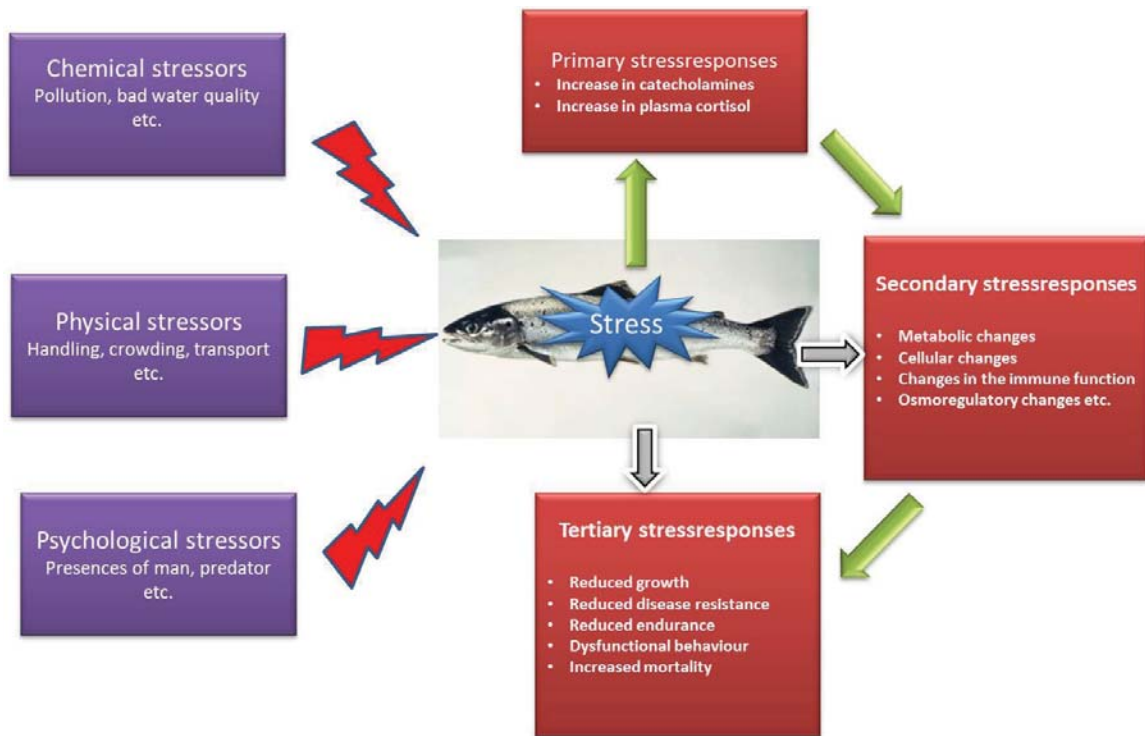
### **3.2 The stress response**

Physiological responses of fish to environmental stressors can be grouped broadly as primary, secondary and tertiary responses (**Figure 1**). Primary responses involve the initial neuroendocrine responses, including the release of catecholamines (CA) from chromaffin tissue, and the stimulation of the hypothalamic-pituitary-interrenal (HPI) axis culminating in the release of corticosteroid hormones. Secondary responses include among others changes in plasma and tissue ion and metabolite levels, and changes in haematological features. All of which relate to physiological adjustments such as in metabolism, respiration, acid-base status, hydro-mineral balance, immune function and cellular responses (Barton 2002; Chrousos and Gold 1992; Eddy 1990; Iversen et al. 1998; Lund and Tufts 2003; McDonald and Wood 2004; Milligan 2003; Mommsen et al. 1999; Selye 1950, 1973; Varo et al. 2002; Wendelaar Bonga 1997, 2011). Last and final, the tertiary responses occur when referred to as aspects of whole-animal performance such as changes in growth, overall resistance to disease, metabolic scope for activity, behaviour, and survival (**Figure 1**). This classification is simplified, as stress is depending on the magnitude and duration of the response, which may affect fish at all levels of organization, from molecular and biochemical to population and community (Wedemeyer 1996).

When fish are exposed to a stressor, the physiological stress response is initiated by the recognition of a real or perceived threat (psychological) by the central nervous system (CNS). The sympathetic nerve fibres, which innervate the chromaffin cells, stimulate the release of catecholamines (CA) via cholinergic receptors (Wendelaar Bonga 1997, 2011). The chromaffin tissue is located mainly in the anterior region of the kidney in teleost fishes (Wendelaar Bonga 1997, 2011). Because catecholamines, predominantly epinephrine in teleost fish, are stored in the chromaffin cells, the CA release is rapid and the circulating levels of CA hormones increase immediately with stress. However, because of the short biological half-life of circulating CAs, it is not feasible to use CA as an indicator on primary stress responses (Wendelaar Bonga 1997,

2011). In contrast, the release of cortisol in teleost and other bony fishes is delayed relative to CA release. Cortisol synthesis and release from interrenal cells have a lag time of several minutes, unlike chromaffin cells, and therefore, a proper sampling protocol can allow measurement of resting (baseline) levels of this hormone in fish (Barton 2002; Wedemeyer 1996; Wendelaar Bonga 1997, 2011). As a result, the circulating level of cortisol is commonly used as an indicator of the degree of stress experienced by fish (Barton and Iwama 1991; Wendelaar Bonga 1997, 2011).

Cortisol is often associated with the detrimental effects of stress including decreased growth rates, and reproductive dysfunction (Mommsen et al. 1999; Morgan et al. 1999; Schreck et al. 2001), increased incidence of disease and parasite resistance (Barton 2002; Davis et al. 2002, 2003; Einarsdottir et al. 2000a, 2000b; Finstad et al. 2007; 2012; Schreck et al. 1993; Weyts et al. 1999), reduced seawater tolerance (Iversen et al. 1998; Liebert and Schreck 2006; Sandodden et al. 2001) and survival (Finstad et al. 2003; Hasan and Bart 2007; Iversen et al. 1998, 2005; Portz et al. 2006). It has also been suggested that if the fish is not permitted enough time to recover completely after stress, a second, normally nonfatal, stressful occurrence can be fatal (Carmichael 1984). Thus, stress can be accumulated and this kind of response has been reported in fish to different type aquaculture related stressors. (Barton et al. 1986; Eliassen and Iversen 2009; Fevolden et al. 2003; Iversen et al. 2009; Maule et al. 1988; Mesa 1994; Waring et al. 1997). The knowledge of cumulative stress is important for fish farmers because while sub-lethal disturbances may not appear to be harmful, the accumulation of responses to such disturbances without a proper recovery period could be lethal for the fish (Barton and Iwama 1991; Finstad et al. 2007; 2012).



**Figure 1.** Physical, chemical and other perceived stressors act on fish to evoke physiological and related effects, which commonly are grouped as primary, secondary and tertiary responses. The arrows indicate the various response routs. Redrawn from Barton (2002).

### 3.3 Allostatics

The concept of allostasis was introduced in the late eighties by Sterling and Eyer (1988) who also described the endocrine pathways involved. McEwen (1998), and McEwen and Wingfield (2003) considered allostasis to be the ability to achieve stability through change. The origin to this concept is based on critics and new interpretation of Selye's General Adaptation Syndrome (GAS). McEwen and colleges felt that the third stage in GAS; exhaustion needed reinterpretation in light of newer knowledge of the stress mediators (CA and cortisol), which could have both a positive or malicious effect depending of the magnitude and duration of their secretion. Thus, rather than exhaustion of the defence mechanism being the problem, the hormones themself can turn on the body and cause problems (McEwen 2005).

### **3.3.1 Allostasis and allostatic state**

As stated above, allostasis is achieving stability through change. This is a process that supports homeostasis, as environments and/or life history stages change. This means that the set-points and other boundaries of control must also change. The primary mediators of allostasis are typical hormones as corticosteroids, catecholamines and cytokines. McEwen (2003) felt that allostasis clarifies an inherent ambiguity in the term homeostasis and distinguishes between the systems that are essential for life (homeostasis) and those that maintain these systems in balance (allostasis), as environment and life history stage change (Juster et al. 2010; Korte et al. 2005; McEwen 1998, 2005; McEwen and Wingfield 2003; Wingfield 2005).

Allostatic state refers to altered and sustained activity levels of the primary mediators (glucocorticosteroids), that integrates physiology and associated behaviours in response to changing environments and challenges such as social interactions, weather, disease, predators, pollution, and so on (Goymann and Wingfield 2004; Juster et al. 2010; Korte et al. 2005; Leong et al. 2009; McEwen 1998, 2005; McEwen and Wingfield 2003; Wingfield 2005). Thus, an allostatic state can result in an imbalance of the primary mediators, reflecting excessive production of some and inadequate production of others. Allostatic states can be sustained for limited periods of time if food intake and stored energy can fuel homeostatic mechanisms and keep them under control. If the imbalance continues for longer periods, and thus cannot be maintained by the energy reserves, symptoms of allostatic overload will appear (Juster et al. 2010; McEwen 2005; McEwen and Wingfield 2003).

### **3.3.2 Allostatic load and overload**

Allostasis separates between systems that are essential for life (homeostasis) and those that maintain these systems in balance (allostasis). Environmental and social perturbations can lead to an increase in the costs of maintaining homeostasis, that is, they increase allostatic load. Goymann and Wingfield (2004) refers to the allostatic load as the cumulative physiological burden exacted on the body as the animal adjust their morphology, physiology and behaviour to adapt to more volatile factors, such as season, environmental changes or reproductive status, and to more permanent

burdens as social status, parasites, wounds and ageing. Allostatic load, can thus be considered as the energy required of an organism to maintain the daily and seasonal routines (obtain food et cetera) plus the extra energy it needs to improve survival and propagation (Goymann and Wingfield 2004). This allows each individual to avoid or resist the potential risk for stress (McEwen 2005; McEwen and Wingfield 2003; Sapolsky et al. 2000). The rapid behavioural and physiological changes in response to perturbations have been collectively called the emergency life history stage (ELHS), which serves to enhance lifetime. Glucocorticosteroids seems to be important in orchestrating EHLS events and behaviours (Wingfield 2005; Wingfield and Sapolsky 2003).

The allostatic load concept unifies predictable and unpredictable events in the life cycle, so that there is no need to separate these events. An increase in allostatic load is typically followed by a rise in glucocorticoid concentrations, which then may trigger behavioural and physiological processes that help the organism to decrease allostatic load, allowing each to avoid or to resist the potential danger for chronic stress (Goymann and Wingfield 2004).

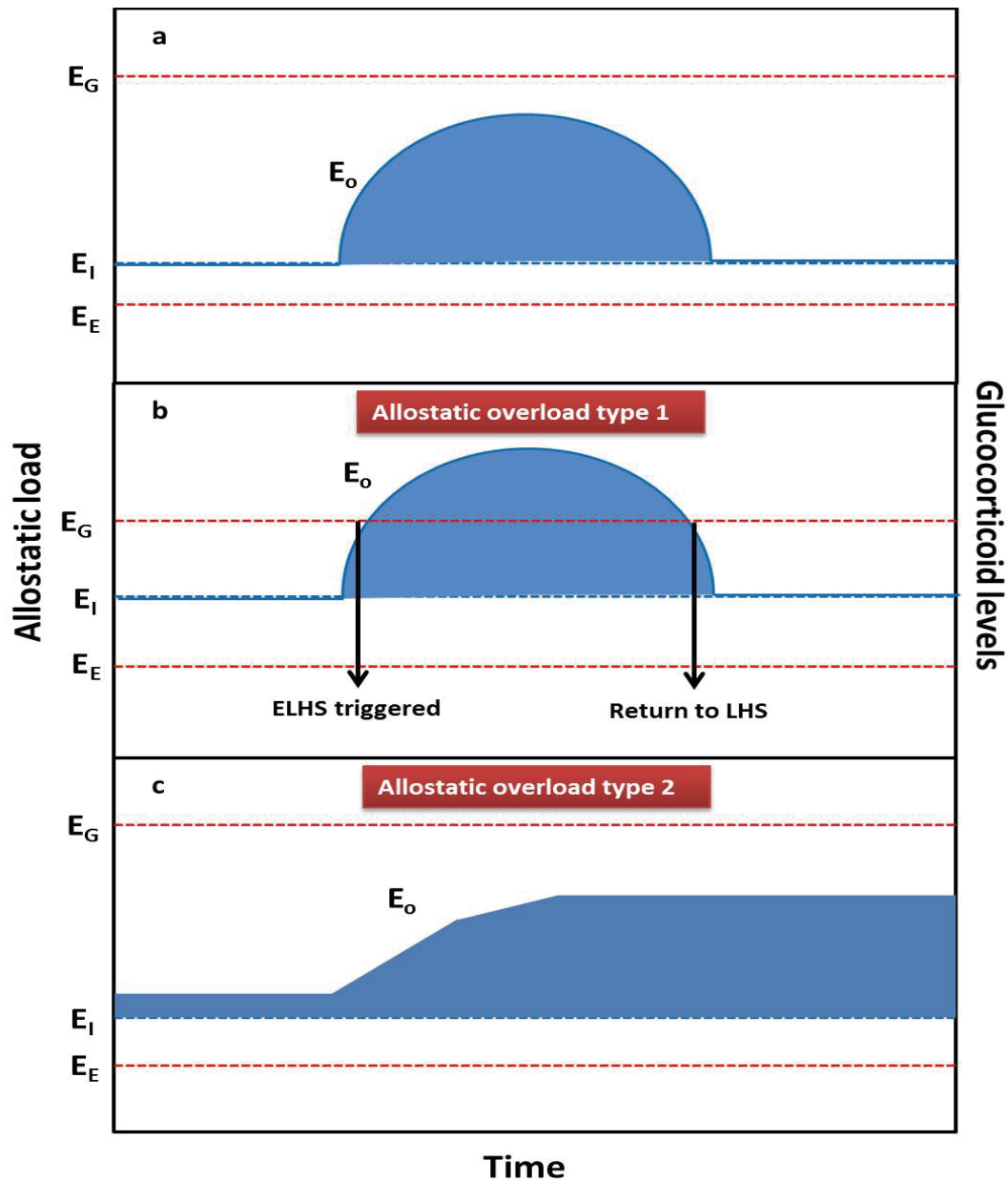
Within some boundaries, an increase in allostatic load represents an adaptive response to seasonal changes and other demands. However, if additional loads of unpredictable events such as disease, human disturbance and negative social interactions are introduced, allostatic load can increase dramatically (Wingfield and Sapolsky 2003). With such an increase in allostatic load, energy demands ( $E_o$ ) of the organism rise, but energy supplies ( $E_G$ ) remain constant or may even decrease. Increases in allostatic load are typically accompanied by an elevation of glucocorticoid concentrations (**Figure 2a**). If allostatic load increases dramatically, the energy demands of an organism ( $E_o$ ) may exceed the available energy supplies ( $E_G$ ), resulting in type 1 allostatic overload (**Figure 2b**). At this point, glucocorticoid concentrations reach the threshold where they trigger an emergency life history stage (ELHS, **Figure 2b**). The ELHS may suppress the expression of other life history stages (allocation of energy reserves), which then results in a net decrease of energy demands below the available supply of energy and a net decrease of allostatic load (**Figure 2b**). This type of



allostatic overload helps the organism to cope with unpredictable events that either increase the costs of obtaining available resources (as in **Figure 2b**) or compromise the availability of these resources (decrease  $E_G$ ). Thus, type I allostatic overload constitutes a mechanism to avoid and resist stress (Goymann and Wingfield 2004).

Social status may have influence on the allostatic load. Social support and cooperation may decrease allostatic load, while social conflict and competition for resources may increase it, which again could result in elevated glucocorticoid concentrations. The energy demands and glucocorticoid concentrations increase in this type of allostatic load, but in most cases they do not exceed the energy supplies, and thus they do not trigger an ELHS (McEwen 2005; McEwen and Wingfield 2003). If this kind of conflict is permanent it may result in a condition that has been termed type 2 allostatic overload (**Figure 2c**). It is characterized by chronically elevated glucocorticoid secretion (**Figure 2c**) with a high potential for disease. (Goymann and Wingfield 2004; Juster et al. 2010; McEwen 2005; McEwen and Wingfield 2003).

In a simplified and general way one can conclude that the type 1 overload could be associated with more acute stressors and the type 2 overloads being associated with more chronic stressors. However, as Schreck (2010) emphasised, is the difference between acute and chronic is quite blurred, as it is difficult to generalise about the effects of various stressors on different animals. As in aquaculture several stressors are often experienced at the same time, or in series, thus the effects of different stressors and severities are in reality more of a continuum than falling into two discrete categories. But using the terms acute and chronic is useful for understanding and simplify the effects of stress. Stress is also a individual experience as animals in different physiological states exposed to the same stressor might respond differently, and deviate in regard to which outcome the stress response have (acute or chronic). While the allostatic load associated with stress relates to increasing fitness in the short-term, its effects can reduce long-term fitness (Schreck 2010).



**Figure 2.** Schematic representation of allostatic load (—) and glucocorticoid secretion (blue shading).  $E_E$ =existence energy (resting metabolism),  $E_I$ =energy required to obtain food and process it under ideal conditions,  $E_O$ =additional energy required to obtain food and process it under non -ideal conditions,  $E_G$ =energy to be gained from the environment. **(a)** Allostatic load and glucocorticoid concentrations increase as the energy requirements of the organism ( $E_O$ ) increase. **(b)** Allostatic overload type 1: when  $E_O > E_G$ , glucocorticoids trigger an emergency life history stage (ELHS). The ELHS suppresses other life history stages (LHS), resulting in a net decrease in allostatic load and glucocorticoid concentrations below  $E_G$ . The animal can now survive the perturbation in positive energy balance. **(c)** Allostatic overload type 2: owing to permanent perturbations such as social conflict,  $E_O$  increases and remains high. Glucocorticoid concentrations increase and remain high, but since  $E_O$  does not exceed  $E_G$  no ELHS is triggered. Redrawn from Goymann and Wingfield (2004).

## **4. Hypothalamic-pituitary-interrenal axis in teleost**

During the stress response (more or less) three different endocrine axes are involved in the regulation and expression of the stress under the influence of the stressor. Those are the corticotropic, melanotropic and thyrotropic axes (Bernier et al. 2009). However, in this introduction one will focus on the corticotropic axis, as it is the main focus in the experiments conducted in this thesis.

### **4.1 Introduction**

An axis is an artificial and conceptual approach to describe and study how an endocrine system is controlled. Typical endocrine axis is divided in layers of controlling hierarchy. In mammals and teleost, the major neuroendocrine regulatory system consists of the hypothalamus (and adjacent brain areas), the pituitary gland (hypophysis) and periphery endocrine glands or cells that are regulated by pituitary secretion (Hadley 1992).

In the case of cortisol secretion in mammals, the hypothalamus secretes corticotropin-releasing hormone (CRH or corticotropin-releasing factor; CRF) stimulates adenohypophysial cells to release adrenocorticotrophic hormone (corticotropin; ACTH) into the general circulation. ACTH in turn stimulates cells in the adrenal cortex (zona fasciculata) to synthesise and release steroid hormones commonly known as glucocorticoids (cortisol and/or corticosterone). Cortisol will again stimulate protein catabolism in muscle, and convert amino acids and fatty acids to glucose (gluconeogenesis) in the liver. The steroid will also inhibit glucose uptake by other cells other than those cells of the nervous system, thus resulting in a marked elevation of blood glucose that is utilised by the brain during freeze, flight or fight syndrome of the stress response of mammals (Balment et al. 2006; Geslin and Auperin 2004). Cortisol (and other glucocorticoids) also acts as a negative feedback primarily at the brain level to repress the release of CRH and thereby reducing ACTH secretion, which again will stop or reduce the cortisol release from the adrenal cortex. There is also a shorter negative feedback loop in which cortisol has a suppressive role on ACTH secretion in adenohypophysis, thus there is also an impact on the cortisol release in

the head-kidneys of mammals (Greenspan and Gardner 2004; Hadley 1992). All of the components of the mammalian HPI axis are present in fish, however with some structural and functional modifications.

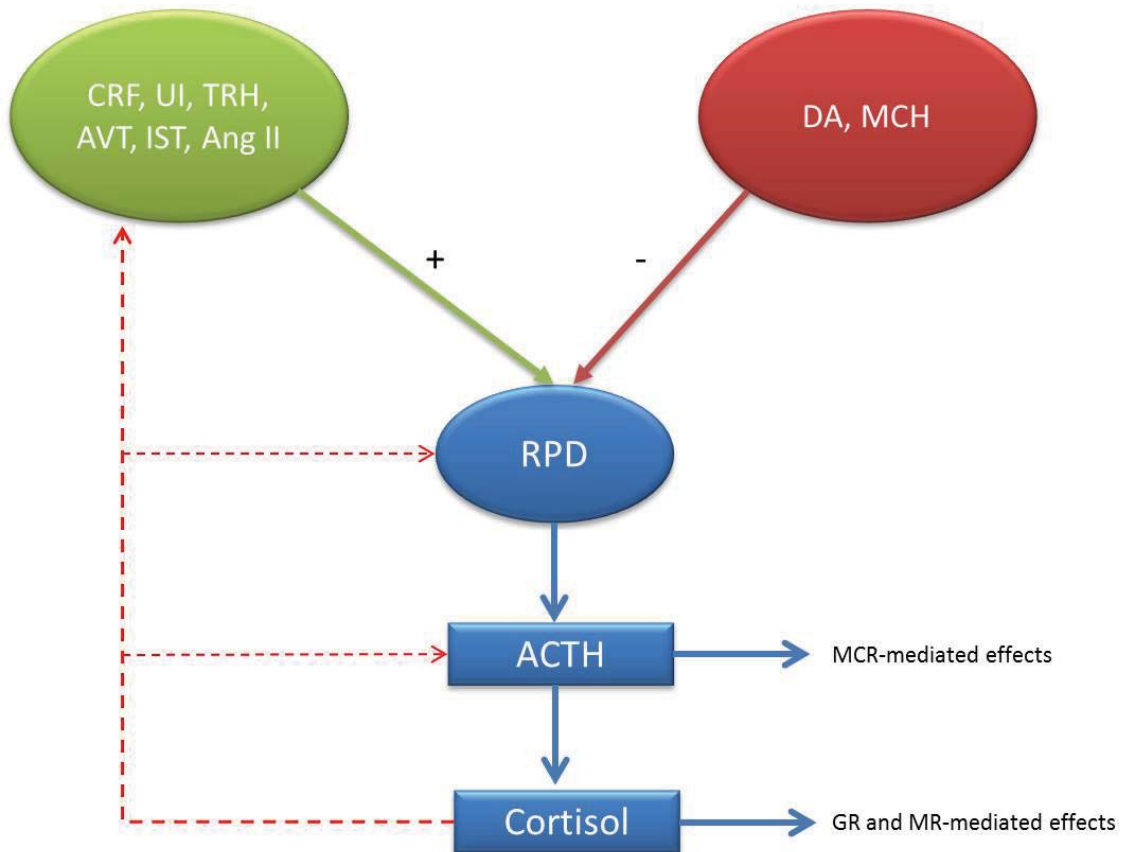
#### **4.1.1 The pituitary gland**

The pituitary gland is present in all vertebrates and is a pure neuroendocrine gland that secret a number of hormones under the influence of specialised nerve cells within the brain. The human pituitary is composed of a distinct adenohypophysis (AH) and a neurohypophysis (NH). The former is derived from an inward invagination of the oral ectoderm (Rathke's pouch), while the neurohypophysis arises from the neural ectoderm of the floor of the forebrain (Hadley 1992). While the communication between the AH and the brain in mammals are contributed by a local portal-hypophyseal vascular system, this system together with median eminence is absent in the teleost pituitary gland. In the teleost there is a complete interdiction between NH and AH, and by direct innervation of the endocrine cells in AH (Agulleiro et al. 2006; Pankhurst 2011; Schreck 2010; Singh et al. 1999; Wendelaar Bonga 1997). AH is divided in three sections in fish: rostral pars distalis (RPD), proximal pars distalis (PPD) and pars intermedia (PI).

#### **4.1.2 Control of the cortisol secretion**

The control of cortisol secretion in teleost fish is complex. Atrial natriuretic factor (Loretz and Pollina 2000; Ventura et al. 2011; Wendelaar Bonga 2011), angiotensin II (Arnold-Reed and Balment 1991, 1994), growth hormone (Björnsson et al. 2010; Ebbesson et al. 2008; Ojima and Iwata 2010), thyroxin (Ojima and Iwata 2010; Walpita et al. 2007), arginine vasotocin (Balment et al. 2006; Evans 2002; Gilchrist et al. 2000) and catecholamines (Rothwell et al. 2005; Wendelaar Bonga 2011) have all corticotropic actions, and cortisol have shown self-suppression by negative feedback of its own secretion at interrenal level (Bradford et al. 1992). Additionally interleukin-like factors have both inhibitory and stimulating effects (Holland et al. 2002; Wendelaar Bonga 2011; Yada and Nakanishi 2002). However, the effects from those above mentioned hormones/factors are probably only involved in modulating the corticotropic actions of hormones originated from the pituitary gland (Wendelaar

Bonga 1997, 2011). Studies on hypophysectomised fish have shown that the pituitary gland dominates the control of the cortisol secretion, as plasma cortisol levels dropped significantly in hypophysectomised fish (Ball and Hawkins 1976; Olivereau and Olivereau 1988; Young 1993).



**Figure 3.** Overview of major factors that affect the activity of HPI axis. Green arrows = stimulatory effect, Red arrows=indicates inhibition and dashed red arrows indicate negative feedback. CRF= corticotropin-releasing factor (CRH), UI=urotensin I, TRH=thyrotropin-releasing hormone, AVT= arginine vasotocin, IST= isotocin, Ang II= angiotensin II, Da=dopamine, MCH= melanin-concentrating hormone, MCR=melanocortin receptors, GR= glucocorticoid receptor, MR=mineralcorticoid receptor, and RPD=rotral pars distalis. Redrawn from Bernier et al. (2009).

#### 4.1.3 CRH and its hypothalamic control of ACTH

Off the multiple factors that affect the secretion of ACTH, CRH is considered to be the major regulator (Bernier et al. 2009). Corticotropin-releasing hormone (factor) (CRH) is 41-residue peptide, closely similar in the structure to urotensin I found in the urophysis of teleost fish (Hadley 1992). CRH neurons have been documented in preoptic nucleus (NPO) and in the nucleus lateralis tuberis (NLT) in Chinook salmon (*Oncorhynchus tshawytscha*) (Matz and Hofeldt 1999, Olivereau and Olivereau, 1988),

goldfish (*Carassius auratus*) (Bernier et al. 1999; Tran et al. 1990), rainbow trout (*Oncorhynchus mykiss*) (Ando et al. 1999; Doyon et al. 2003) and white sucker (*Catostomas commersoni*) (Lederis et al. 1994; McMaster and Lederis 1988). In several species CRH peptide is localised together with the peptide arginine vasotocin (AVT) and with urotensin I (UI) in NLT region of the brain (Norris and Hobbs 2006). It seems like CRH neurons in NPO and NLT have different functions, as lesion of goldfish NPO abolished plasma cortisol. This had no effect on the content of ACTH, as in contrast to NLT lesions reduced both the pituitary content of ACTH as well as plasma cortisol. This could indicate that these two brain regions have different physiological roles in respect to CRH control (Norris and Hobbs 2006).

### ***Stimulatory factors of ACTH***

The major ACTH regulator is CRH, and an overview of other factors/hormone for regulating HPI-axis is shown in **Figure 3**. Urotensin I (UI) stimulates ACTH in fish, as white sucker UI has shown to be more potent than ovine CRH to stimulate ACTH release from rostral pars distalis (RPD) in goldfish (Fryer et al. 1985), and when CRH receptors are blocked the corticotropic actions of both UI and CRH are reversed (Weld et al. 1987). In mammals urocortin (analogue to UI) has a potent ACTH releasing activity *in vitro*, however it does not seem to have similar effect in *in vivo* experiments (Oki and Sasano 2004). More studies are needed to determine the relative importance of endogenous UI in fish as corticotropic factor (Bernier et al. 2009; Flik et al. 2006). There is also good evidence that neurohypophysial hormones participate in the regulation of ACTH. *In vitro* experiments, both arginine vasotocin (AVT) and isotocin (IST) has shown to stimulate ACTH release from the pituitary gland from goldfish (Fryer et al. 1985) and rainbow trout (Backström and Winberg 2009). In goldfish the AVT and IST is not as potent as CRH/UI, as Lederis *et al.* (1994) showed that the maximum ACTH release activity of AVT and IST are about half of that of CRH and UI, and unlike in mammals (Aguilera et al. 2008). Neither AVT or IST enhance the corticotropic activity of CRH or UI (Fryer et al. 1985). In rainbow trout similar effects of AVT and IST on ACTH release is observed, and AVT did not potentiate the ACTH-releasing properties of CRH compared to goldfish (Bernier 2006; Bernier et al. 2009).

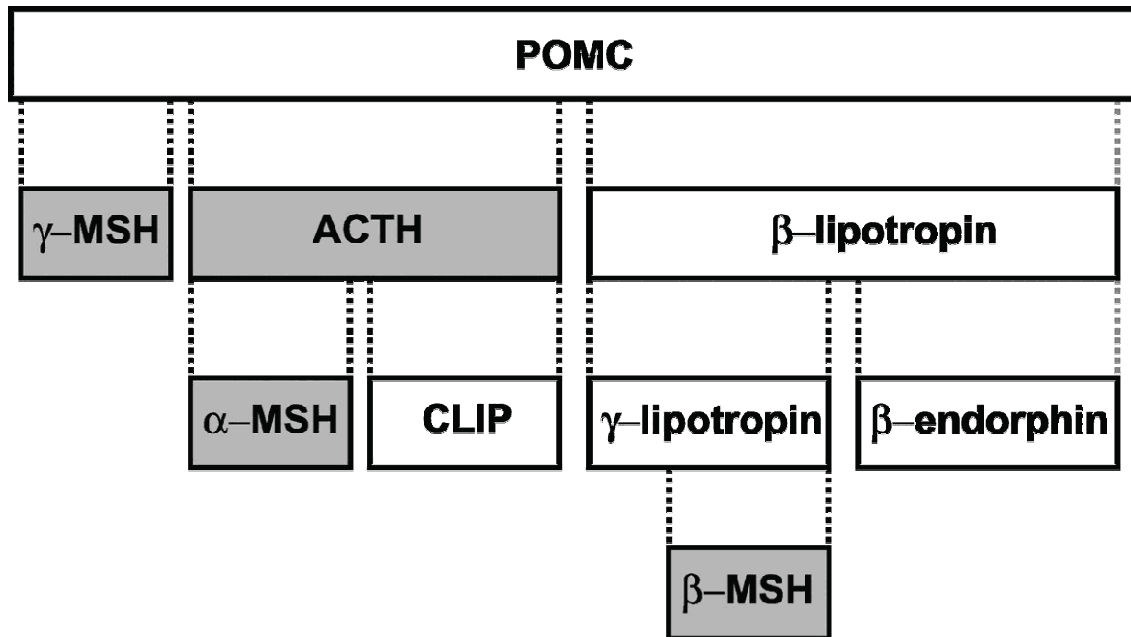
Angiotensin II (Ang II) and thyrotropin-releasing hormone (TRH) are also factors to be considered with plausible ACTH-releasing activity in fish and synergetic effect on CRH and UI. Angiotensin I and II can stimulate ACTH secretion *in vitro* in goldfish RPD, however they had no potentiated effect on the corticotropic activity of CRH or UI (Weld and Fryer 1987; Weld et al. 1987). Although TRH had a positive effect *in vitro* in gilthead sea bream (*Sparus aurata*) on ACTH release (Rotllant et al. 2000), it had no effect *in vitro* in ACTH-release in goldfish (Bernier et al. 2009).

### ***Inhibitory factors of ACTH***

Melanin-concentrating hormone (MCH) and dopamine (DA), which are both of hypothalamic origin, can inhibit ACTH release in the pituitary gland. MCH in rainbow trout has shown to be a potent inhibitor *in vitro* on ACTH secretion in rostral pars distalis (RPD) (Baker et al. 1985b; Barber et al. 1987). While most MCH fibers that end in the pituitary gland are neurohypophysial and thus are important in the background color adaption, there are suggested that some MCH nerve fibers innervate the RPD (Cerdá-Reverter and Canosa 2009; Moons et al. 1989). It has also been reported that rainbow trout (*Oncorhynchus mykiss*), eels (*Anguilla anguilla*) and red porgy (*Pargus pargus*) reared in light background have higher levels of MCH, and lower circulating ACTH and plasma cortisol compared to fish reared in darker background (Baker et al. 1985a; Baker and Race 1981; Rotllant et al. 2003). Additionally there is some physiological evidence that suggest that DA can inhibit basal ACTH secretion in fish. In common carp (*Cyprinus carpio*) *in vitro* superfusion studies suggest that *in vivo* release of ACTH is under DA inhibitory control and only CRH can stimulate ACTH in the presence of mild DA inhibitory tonus (Metz et al. 2004). However whether the inhibitory feature of DA on common carp ACTH secretion is a general feature in teleost corticotrope secretion, needs further investigations (Bernier et al. 2009).

#### **4.1.4 Adrenocorticotropic hormone (ACTH)**

Proopiomelanocortin (POMC) is a precursor of a number of peptides that can be divided into three groups: Adrenocorticotropic hormone (ACTH), endorphin-like and MSH-like products (**Figure 4**).



**Figure 4.** Opioid peptides derived from proopiomelanocortin (POMC) cleavage. Redrawn from Mosconi et al. (2006)

ACTH is the smallest peptide hormone in the anterior pituitary (AN) and consists of a single linear chain of 39 amino acids. The biological active amino acid sequence of the portion 1-24 ACTH, appears to be constant in mammals, whereas infrequent substitutions occur in this region in non-mammalians (Agulleiro et al. 2006; Hadley 1992). ACTH is being produced in cells in RPD region of the AN, and regulates cortisol release and is involved in both the stress response (Mormede et al. 2007; Wendelaar Bonga 2011), and in adaption to hypoosmotic environments (Björnsson et al. 2010, 2011).

The purpose of ACTH in fish is to regulate the steroidogenesis in the interrenal cells of the head kidney (Wendelaar Bonga 1997, 2011). ACTH and other POMC-derived melanocortins targets are the melanocortin receptors (MCR) in the head kidney. MCR is part of a superfamily of 7-transmembrane domain G-protein coupled receptors that stimulates adenylate cyclase and cAMP secondary messenger system (Bernier et al. 2009). In several species of fish (as in mammals) five MCRs has been identified (Flik et al. 2006; Logan et al. 2003; Schiöth et al. 2005). Both in fish and mammals ACTH is the only ligand that binds and activates the MC2R, and both ACTH and MSH bind to the four other MCR (Schiöth et al. 2005). However, unlike the in



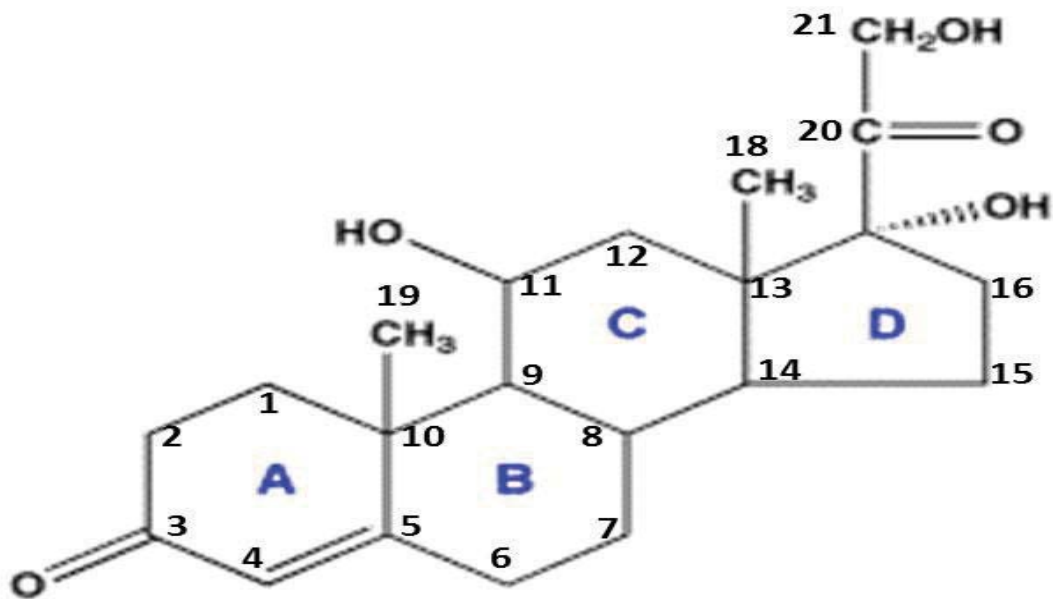
mammals, the four MCR (MC1R, MC3R, MC4R and MC5R) have greater affinity for ACTH<sub>1-24</sub> than for different MSHs. Therefore the binding properties of the different MCRs in fish (and chicken) suggest that ACTH is an older ligand than MSH. This indicates that the selective recognition of MSH by the MCRs, has evolved in higher vertebrates (Schiöth et al. 2005).

#### **4.1.5 Plasma cortisol**

While plasma cortisol is used as a valid indicator of stress in fish, few studies have actually measured the kinetics of cortisol in fish (Mommsen et al. 1999). Plasma cortisol reflects the net effect of production and clearance of the hormone. The clearance from the plasma is dependent on upon binding proteins, target tissue receptors and catabolism of cortisol (Hadley 1992; Mommsen et al. 1999).

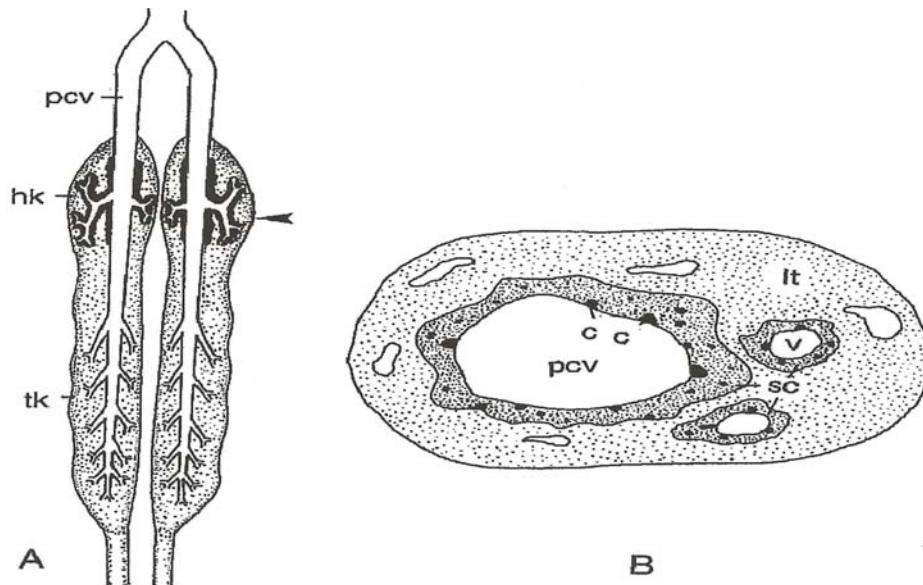
#### ***Biosynthesis of cortisol***

Fish do not possess a discrete adrenal gland as in mammals, and the cortisol producing cells called interrenal cells are scattered in the head-kidney region, mostly along the posterior cardinal veins and their branches. Close to the steroidogenic cells lie numbers of scattered chromaffin cells which could suggest a possible paracrine control or effect of the cortisol release and/or vice versa (Reid et al. 1998) (**Figure 5** and **Figure 6**).



**Figure 5.** The structure of corticosteroid, cortisol. The letters identify the A, B, C, D rings, and the numbers shows the position of the C-21 steroid structure. Redrawn from Greenspan and Gardner (2004).

In the steroidogenic cells ACTH binds to the MC2R receptors and activates the enzymatic pathways that convert cholesterol into cortisol (Aluru and Vijayan 2009). ACTH enhances the steroidogenic capacity by stimulating steroidogenic acute regulatory protein (StAR) (Aluru et al. 2005; Aluru and Vijayan 2009; Hagen et al. 2006), cytochrome P450 side chain cleavage enzyme (P450<sub>scc</sub>) (Aluru et al. 2005) and 11 $\beta$ -hydroxylase (P450<sub>c11</sub>) (Hagen et al. 2006). StAR facilitates the transport of cholesterol across the mitochondrial membrane, where P450<sub>scc</sub> converts cholesterol to pregnenolone (the rate limiting enzymatic step of corticosteroid synthesis) and finally (after several enzymatic processes) P450<sub>c11</sub> converts 11-deoxycortisol to cortisol (Mommsen et al. 1999). ACTH has a stimulatory effect on MC2R expression which also most likely contributes to enhanced steroidogenic effect after acute stressors (Hagen et al. 2006).



**Figure 6.** A. Principal drawing of head (HK) and trunk (TK) kidneys of teleost fish, showing postcardinal veins and their branches surrounded by interrenal tissue. Black areas show adrenal tissue. B. Cross section of the right head-kidney, SC=steroidogenic cells (adrenocortical tissue), C=cluster of chromaffin cells, PCV=postcardinal vein, V=small blood vessels and It= lymphomyeloid tissue (Wendelaar Bonga 1997). Reprinted with permission from the American Physiological Society © 2013.

The adrenocortical tissue of fish in most cases secretes a single corticosteroid; cortisol, while elasmobranchs secrete a unique 1  $\alpha$ -hydroxycorticosterone and lungfish primarily release corticosterone or deoxycorticosterone. In comparison to mammals only traces of aldosterone is sometime detected in teleost, and it seems like cortisol function both as a mineralocorticoid and glucocorticoid (Norris and Hobbs 2006).

### ***Plasma-binding hormone***

In mammals most of the cortisol in plasma is bound to a transporter protein named corticosteroid-binding globulin (CBG) or transcortin. The protein-bound cortisol is in equilibrium with free cortisol in plasma, and it is only free cortisol that is available to bind to membrane receptors on surface or more common to cytosolic receptors inside the target cell (Caldwell et al. 1991; Hosoya et al. 2007). Apparently, there are plasma proteins in all vertebrates that are capable of binding cortisol, although the percentage of bound cortisol is lower in fish than mammals. In terms of corticosteroids, cortisol binding globulin (CBG) and albumin are the major proteins binding to cortisol in mammals and birds (Siiteri et al. 1982). CBG has high affinity for

cortisol, but a low binding capacity, whereas albumin has a low binding affinity but high binding capacity (Aaron et al. 2004). While in most mammalian species, 67 to 87% of plasma cortisol binds to CBG, and 7–19% binds to albumin, (Gayrard et al. 1996), a specific cortisol binding protein has yet to be identified in fish. Caldwell et al. (1991) demonstrated that approximately 20% of cortisol in immature rainbow trout was bound to a cortisol binding globulin-like molecule (heat labile), and 40% was bound to a human albumin-like protein (non-heat labile).

### ***Glucocorticoid and mineralocorticoid receptors***

The targets of cortisol in teleost are ligand-activated transcription factors which include both glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). MRs and GRs evolved from an ancestral CR, following a genome duplication event in the early gnathostomes, and a second event dating back to some 335 million years ago led to two different GR proteins and one remaining duplicate of MR (Bury and Sturm 2007). Thus, in most teleost cortisol signal is achieved through two MR and one GR (Prunet et al. 2006). There is some variation in this rule, as zebrafish have a single GR and MR gene and a GR $\beta$  splice variant that lacks transactivational activity, but behaves as a negative inhibitor of GR $\alpha$  transactivation (Alsop and Vijayan 2009). Studies have demonstrated that the CRs of teleost are selective to cortisol and suggest that the multiple CRs in fish may mediate distinct physiological responses (Bury and Sturm 2007; Prunet et al. 2006). In rainbow trout MR is 10 to 100 times more sensitive to cortisol than GRs are, as a contrast in common carp the sensitivity of the MR is in between the sensitivity of the two GRs (Stolte et al. 2008). But both in rainbow trout and common carp GR2 isoform is more sensitive to cortisol compared to GR1 isoform (Prunet et al. 2006). This difference in CRs and MR sensitivity may allow to differential regulation by basal and stress-induced cortisol levels (Bernier et al. 2009). Prior to ligand binding both mammalian and teleost corticoreceptors are maintained in an inactive but high cortisol-affinity state by use of heat shock proteins (hsp) 40, 70 and 90 together with p23 and p60 (known as HOP) (Basu et al. 2003; Hutchison et al. 1996; Pratt and Dittmar 1998). The formation of this state is done by a two stage ATP-dependent process (Pratt and Dittmar 1998). Upon ligand binding GR undergoes a

conformational change, which disassociates the hsp and translocates to the nucleus where it can modify expression of multiple genes through a variety of mechanisms (Basu et al. 2003; Basu et al. 2001).

### ***Distribution of GR and MR receptors***

In fishes the universal distribution of GRs and MRs reveals the duplicity of the corticosteroids. Which have earned them the nickname dirty hormones because they seems to be everywhere and do everything (Norris and Hobbs 2006). Expression of GRs has been identified in the liver, gills, intestine, kidney, spleen, heart, skeletal muscle, gonads, leukocytes, and erythrocytes (Bernier et al. 2009; Mommsen et al. 1999; Takahashi et al. 2006; Vijayan et al. 2003). Both GR and MR expressing cells have also been located in many areas of the brain, for instance in dorsal telencephalon, NPO, NLT, the interior lobe of the hypothalamus and the caudal neurosecretory system, additionally they have also been detected in the ANs pars distalis and pars intermedia (Pepels et al. 2004; Stolte et al. 2008).

### ***Negative feedback***

**Figure 3** gives an overview of possible feedback routes of plasma cortisol on the HPI axis. Cortisol implants in the brain (intraperitoneal) inhibit CRH expression NPO in goldfish (Bernier et al. 2004; Bernier et al. 1999), and by removal of the negative feedback of cortisol by pharmacological adrenalectomy (metyrapone) lead to increase in NPO CRH production in white sucker (Okawara et al. 1992) and goldfish (Bernier and Peter 2001). While it is known that CRH expression in mammals is differentially regulated in discrete regions of the brain (Shepard et al. 2000, 2006; Shepard and Myers 2008; Skórzewska et al. 2008; Smith et al. 1997), it is uncertain if similar cell-type effects of cortisol on CRH expression exists in teleost (Bernier et al. 2009).

At pituitary level cortisol inhibits basal ACTH release and the ACTH-releasing activity of CRH and UI in goldfish (Fryer et al. 1985). Exogenous cortisol can also suppress ACTH levels in gilthead sea bream (Rotllant et al. 2000) and brown trout (Bernier et al. 2009), and a dose-dependent decrease in pituitary POMC expression and plasma ACTH levels in rainbow trout has also been documented (Bernier et al. 2009).

In the interrenal cells cortisol may suppress its own secretion as a paracrine feedback effect. This is debated as it has been documented in coho salmon (Bradford et al. 1992), but failed to be established in gilthead sea bream (Rotllant et al. 2000). Several papers in fish have also shown that cortisol could degrade GR receptor protein (Aluru and Vijayan 2009; Veillette et al. 2007; Wendelaar Bonga 2011). While there is clear evidence that cortisol can limit the magnitude and duration of the endocrine stress response in fish via several negative feedback loops, the molecular mechanisms behind this regulation is still unclear and poorly understood (Bernier et al. 2009; Wendelaar Bonga 2011).

### ***Metabolism and excretion of cortisol***

When inside the cell, cortisol will be bound and activated, or metabolised and consequently inactivated. The inactivation and reduction of cortisol are achieved through metabolic changes that make cortisol unrecognisable to their GR and MR receptors, and more water soluble (Greenspan and Gardner 2004). Hormone bound to the receptor will eventually be released from the hormone–receptor complex and will be disposed mainly through the liver with the hepato-biliary system as the main route for cortisol clearance (Mommsen et al. 1999; Vijayan and Moon 1992). The hepatic metabolism of cortisol in mammals involves a number of steroid-metabolising enzymes reductases, oxidoreductases and cytochrome P-450 dependent hydroxylases (Greenspan and Gardner 2004), and most of the same enzymes are present in fish (Davis and Small 2006; Mommsen et al. 1999). In short, the major reduction of cortisol is achieved by reduction of the 4.5 double bound of the A-ring and its keto-group in position 3 (**Figure 5**). Conjugation of the resulting hydroxyl group on carbon 3 with glucuronic acid or sulphate increase water solubility and decrease binding to CBG like protein in fish so the steroid now can pass through the renal glomerular capillaries and be excreted in the urine (Greenspan and Gardner 2004; Mommsen et al. 1999). Very little is known about factors regulating cortisol metabolism and excretion, however xenobiotics have shown to modulate the cytochrome P-450 in fish (Mommsen et al. 1999; Vijayan et al. 1997).

## 5. Aim of the study

The overall aims of this study was to examine the effects of stress during common handling procedures (stressors) routinely used during commercial salmon aquaculture, and its impact on animal welfare.

Sub goals:

- I. Identify and characterise different stressors commonly appearing in salmon smolt industry (**papers I, II, III and IV**)
- II. Study the effects of plasma cortisol on primary, secondary and tertiary stress responses (**paper I, II, III and IV**)
- III. Study the use of sedation as a stress reducing measure and its impact on the stress response and animal welfare (**paper III and IV**)
- IV. Study the effects of allostatic overload on the HPI axis and wellbeing of the animals (**paper I**).
- V. Investigate the relationship between stress and fish welfare (**paper I; II, III and IV**)

## 6. Summary of papers and abstracts

**Paper I.** Iversen, M, & Eliassen, R. A. (2013). The effect of allostatic load on hypothalamic–pituitary–Interrenal (HPI) axis before and after secondary vaccination in Atlantic salmon postsmolts (*Salmo salar* L.). Fish Physiology and Biochemistry (Under revision)

### Abstract

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



**Paper II.** Iversen, M. H. & Eliassen, R. A. (2013). The use of Finquel vet (MS-222) and Benzoak vet (benzocaine) during vaccination of Atlantic salmon parr (*Salmo salar* L.) and its impact on water quality, efficacy and animal welfare. Aquaculture (submitted).

### **Abstract**

During the vaccination process it is essential to anaesthetise the fish prior to handling and inoculation of the vaccine. Often the commercial smolt plant neglect to change the anaesthetic as often as recommended (by distributor) or do not sufficient aerate the anaesthetic bath, which could have possible dire consequences on the animal welfare. The purpose of this study was to study the efficacy and animal welfare during vaccination of stressed and unstressed Atlantic salmon parr (*Salmo salar* L.) using Finquel vet (MS-222) and Benzoak vet (benzocaine) as anaesthetics, with or without air supply. Both 200 and 400 mg/L Finquel Vet (MS-222) with no air supply gave a significant increase in carbon dioxide. At 400 mg/L Finquel Vet (no air) the CO<sub>2</sub> level reached 60 mg CO<sub>2</sub>/L. No such changes in CO<sub>2</sub> were recorded in the Benzoak Vet anaesthetics. In this experiment the time to enter anaesthesia was rapid and less than 2 minutes regardless of concentration, anaesthetic or air supply. Recovery time was reasonably short, ranging between 3.5 to 7.4 minutes. Water quality seemed to be an important factor on efficacy at the highest concentration of Finquel Vet. During no air supply at 400 mg/L Finquel Vet, both time to enter anaesthesia and recovery was reduced. Most likely is this caused by a combination of Finquel Vet and the buildup of carbon dioxide. The primary, secondary and tertiary stress response seemed to be more aggravated during high stress group in bad water quality (high CO<sub>2</sub> and no air supply) with Finquel Vet anaesthesia. This study shows the importance of keeping the water quality under control during anaesthesia and large-scale vaccination, regardless of choice of anaesthetic. This is however especially important during Finquel Vet anaesthesia, due to the risk of build-up of carbon dioxide, if the anaesthesia bath is not changed as often as recommended or aerated sufficiently. Failing to do so could have major impact on the animal welfare due to increased stress and mortality.

**Paper III.** Iversen, M., Eliassen, R. A. & Robb D. H. F. (2013). The effects of Aqui-S vet. sedation during vaccination on survivability, appetite, growth, immunological capacity, primary, secondary and tertiary stress responses in Atlantic salmon parr (*Salmo salar* L). Aquaculture (submitted).

#### **Abstract**

AQUI-S vet. has a very low stress action on the fish, especially in comparison to metacaine or Finquel (MS-222), which are currently used for pre-vaccination anaesthesia. Earlier studies have shown that AQUI-S vet. can be used as a sedative to keep the fish calm during handling and vaccination. By reducing the stress, it is anticipated that the fish will respond better to the vaccine, come back onto feed faster and increase the rate of survival at sea after transfer. In this experiment one looked at the use of the anaesthetic/sedation of AQUI-S vet. during vaccination, and monitored the effects on survivability, growth, appetite, immunological capacity, primary-, secondary- and tertiary stress responses. During the vaccination experiment, three different treatments were tested; control (anaesthesia 150 mg/L Finquel); treatment I (sedation 4 mg/L AQUI-S vet. and anaesthesia 30 mg/L AQUI-S vet.), and treatment II (sedation 4 mg/L AQUI-S vet. and anaesthesia 150 mg/L Finquel). Even though the handling and vaccination process gave no negative effects on the vaccine, the stress reducing measures had positive effects by reducing overall stress level, feed conversion and mortality, as well as increasing appetite. Most pronounced was this effect during sedation of AQUI-S vet. followed by Finquel anaesthesia (treatment II). Treatment I gave only minor positive effects on overall stress level, feed conversion, appetite and mortality compared to the control group. And the prolonged time (60 – 90 min) it took this group to reach stage 4 (anaesthesia), could make it difficult to implement this successfully as a commercial vaccine routine in the salmon smolt industry. Clearly, a combination of sedation with AQUI-S vet. and Finquel anaesthesia under vaccination could reduce the overall stress response and mortality. And thus these factors have potential to improve both production cost and animal welfare during a critical production phase of Atlantic salmon, which could lead to further benefits in terms of healthier and faster grow out later on.

**Paper IV.** Iversen, M. & Eliassen, R. A. 2009. The effect of AQUI-S® sedation on primary, secondary and tertiary stress responses during salmon smolt (*Salmo salar* L.) transport and transfer to sea. Journal of the World of Aquaculture Society. 40 (2): 216-225

**Abstract**

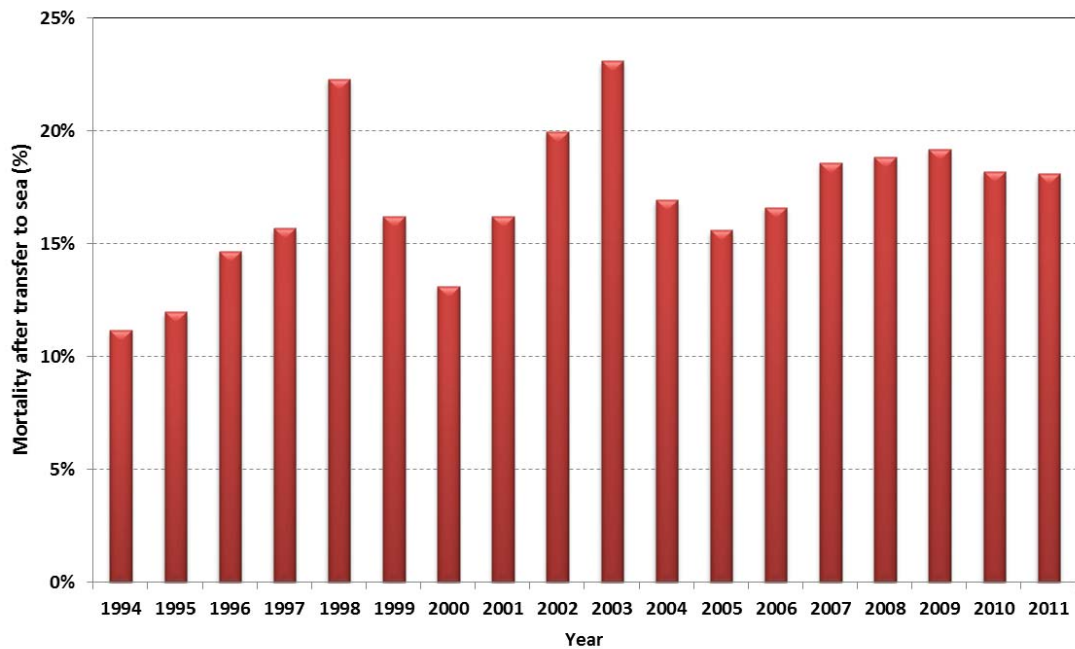
The purpose of this study was to determine the effect of AQUI-S® (5.0 mg/L) sedation compared to non-sedation on the primary (plasma cortisol), secondary (osmoregulation), and tertiary (mortality) stress responses in Atlantic salmon smolts during transport and transfer to sea. AQUI-S® sedation during on- and offloading sufficiently reduced the primary stress response to lower mortality (2.5 %) during transfer to sea compared to unsedated fish, which experienced a mortality rate above 11.5 %. The unsedated fish experienced an acute mortality which only stabilised 16 days after the transport. None of the secondary stress responses measured in this experiment could contribute in explaining this phenomenon, with the possible exception of plasma magnesium. While osmolality and chloride increased in both groups during the experiment, it did not seem to be related to the difference in the primary stress response documented in the sedated and unsedated group. It instead appeared to be an effect of the salinity change from freshwater to seawater after transport. Plasma magnesium differed between the groups, while plasma magnesium in the AQUI-S® sedated group returned to prestress levels 12 h after transport. The unsedated group showed no such recovery even 1 week after transport, which may indicate a disturbance in hydromineral balance, and give plausible explanation for the delayed mortality this group experienced. AQUI-S® shows promise as a stress-reducing sedative for Atlantic salmon smolts, and used properly it could improve animal welfare and survivability during and after common aquaculture related incidents

## 7. General discussion

Stress is unavoidable in the aquaculture environment and due to captivity the fish has no means of escaping the human imposed stressors. Exposure of fish to common stressors such as handling, netting, pumping, transport, vaccination and confinement, will to some degree activate the hypothalamo-pituitary interrenal (HPI) axis resulting in increased blood levels of catecholamines and cortisol (Barton 2002; Wendelaar Bonga 1997). Catecholamines induce rapid, short-term elevations in blood glucose primarily through the glycogenolytic pathway (Fabbri et al. 1998; Moon 2004; Vijayan and Moon 1992). Cortisol, however is involved in the longer-term mobilisation of non-carbohydrate energy stores from muscle protein and by increases in plasma free fatty acids in fish (Barton 2002; Wendelaar Bonga 1997). Cortisol has often been associated with the detrimental effects of stress including: decreased growth performance, reproductive dysfunction (Mommsen et al. 1999; Morgan et al. 1999; Schreck et al. 2001), increased risk of disease (Barton 2002; Davis et al. 2002, 2003; Einarsdottir et al. 2000a, 2000b; Weyts et al. 1999), impaired osmoregulation (Iversen et al. 1998; Liebert and Schreck 2006; Sandodden et al. 2001) and survival (Finstad et al. 2003; Hasan and Bart 2007; Iversen et al. 1998, 2005; Portz et al. 2006). Carmichael (1984) suggested that if the fish is not permitted enough time to recover after exposure to a stressor, a second, normally nonfatal, stressor could have grave impact on the wellbeing of the animal.

### 7.1 Stress and commercial salmon production

From 1994 to 2011 an average of 17% ( $\pm 3,2$ ) of the fish died after transfer to marine growth sites (the sea) (DOF 2012b) (**Figure 7**). Most of the mortality occurred the first three months after the transfer to sea (Farrell et al. 2010; Iversen and Eliassen 2009; Iversen et al. 2005). These observations do not give any good explanation of the losses experienced after transfer to sea. In 2011 a total of 50.970.000 smolts were lost after transfer. Seventy-seven percent of the loss was due to mortality, 4 % loss declassified at the slaughterhouses (not fit for human consumption), 1 % loss was escapees and 24 % loss was unexplained (not determined by cause).



**Figure 7.** The average mortality in Atlantic salmon in Norwegian aquaculture after transfer to sea in the period from 1994 to 2011. The data is based on the total number of loss in sea cages divided by the total number of fish put in the sea cages each year (DOF 2012b) .

During a production cycle of Atlantic salmon from juvenile to slaughter the animal undergoes several critical production phases, which could elicit both grave stress responses and have major negative impact on the animal welfare (Dykes 2012; Ellis et al. 2012; Farrell et al. 2010; Huntingford et al. 2006; Huntingford and Kadri 2008, 2009; Iversen 2008; Iversen and Eliassen 2009, 2012; Iversen et al. 2004, 2005; Turnbull et al. 2005).

### 7.1.1 Vaccination

One of the first challenges the salmon is facing during production is vaccination at the salmon parr stage. Vaccination of Atlantic salmon parr (*Salmo salar* L.) is a common and required procedure in aquaculture and fish management. In 2010, approximately 250.577.000 Atlantic salmon smolts went through the vaccination process in Norway, and with an average vaccination cost of NOK 1.03 per vaccinated fish, the industry used approximately NOK 250.600.000 (US\$ 44.800.000) on vaccines (DOF 2012a). Vaccination is an important tool to prevent outbreaks of a number of bacterial and viral diseases in farmed Atlantic salmon, and is largely responsible for reducing the use

of antibiotics and the continuing sustainable growth of Norwegian aquaculture in the 1990s (Drangsholt et al. 2011; Gudding et al. 1999). To maintain the health and welfare of farmed Atlantic salmon, individual fish are vaccinated prior to sea water entry. However the injection of these vaccines could lead to unavoidable stress and is often associated with short-term increases in plasma cortisol (Funk et al. 2004; Skinner et al. 2010).

To the author's knowledge no studies outlining the stress response before, during and after vaccination in Atlantic salmon have been done. During vaccination there are 3 common stressors that appear in a short time span. These stressors are loading, anaesthesia and vaccination (**paper III**). Plasma cortisol seems to increase rapidly after loading procedures in to the anaesthesia tank, and anaesthesia and inoculation just seem to aggravate this response. Plasma cortisol peaked 1 to 2 hours after the vaccination had ended (**paper III**). A similar pattern for plasma cortisol, exposed to different acute stressors, has been documented in a wide range of teleost species as three-spined stickleback (*Gasterosteus aculeatus* L) (Pottinger et al. 2002) paddlefish (*Polyodon spathula*) (Barton et al. 1998), gilthead sea bream (*Sparus aurata* L.) (Arends et al. 1999; Barton et al. 2005), green sturgeon (*Acipenser medirostris*) (Warren et al. 2004), coral trout (*Plectropomus maculatus*) (Frisch and Anderson 2000, 2005), brown trout (*Salmo trutta* L.) (Arnekleiv et al. 2004) rainbow trout (*Oncorhynchus mykiss*) (Pankhurst and Dedual 1994) and Atlantic salmon (*Salmo salar*) (Ellis et al. 2004; Iversen et al. 2009; **paper IV**).

Recovery back to resting plasma cortisol levels after an acute stressor of moderate intensity is normally obtained within 6 hours (Barton et al. 1980; Iversen et al. 1998; Pickering and Pottinger 1989), but there has been documented a great variation in the recovery time in plasma cortisol after an acute stressor (Barton and Dwyer 1997; Davis and Small 2006; Holloway et al. 2004; Iversen et al. 1998; Small 2004b). This is most likely due to the difficulty of getting both precise measurements of stress and the amount of factors that influences it. It has also been shown that the time needed for recovery is affected by the intensity and duration of the stress, physiological effects of

multiple stress events have for instance been shown to be cumulative with prolonged effect on the recovery time (Barton et al. 1986; Iversen et al. 1998; Maule et al. 1988).

As shown in **paper III** the plasma cortisol was kept elevated compared to pre-stress levels 24 to 72 hours after vaccination, and could be a result of the cumulative effect of three potent stressors applied in a short time span during vaccination. However, results in **paper I** indicate that there could be another plausible explanations for prolonged elevated plasma cortisol after vaccination as baseline level of plasmacortisol in the vaccine and stress group was kept elevated two weeks after secondary vaccination, where after it returned back to pre-stress levels (and control group). Similar and more aggravated increase in baseline levels of plasma cortisol after secondary vaccination was also detected in the stress and vaccine group, but in this experimental group no recovery was obtained. A possible explanation for this increase in baseline levels of plasma cortisol is due to the inflammatory response to oil-adjuvant vaccine, which activate different cytokines during inflammation. In mammals it has been shown that inflammation promotes the release of cytokines such as TNF, IL-1, IL-6 and IL-12 which results in the activation of a stress response (Calcagni and Elenkov 2006; Tort 2011). Especially IL-6 appears to have a significant role as a mediator of the HPI activation. IL-6 receptors have been detected in mammalian brain tissues and neuroendocrine glands, thus stimulating CRH, prolactin and GH secretion. ACTH secretion seems also to be under subject of IL-6 as high IL-6 levels have shown correlated with higher ACTH and cortisol levels (Zarkovic et al. 2008). In fish, several studies have shown a similar bi-directional communication between HPI-Axis and cytokines (Tort 2011). Holland et al. (2002) showed that recombinant IL-1 injection raises cortisol levels, and injection of lipopolysaccharide (LPS) raised plasma cortisol in yellow perch (*Perca flavescens*) (Haukenes and Barton 2004) and promoted StAR HK expression in gilt-head bream (*Sparus aurata*) (Castillo et al. 2008.). Similar an injection of LPS both in sea bream and rainbow trout have shown to promote increased cortisol secretion and increased cortisol receptor expression in most tissues 6 to 72 hours after the initial injection (Acerete et al. 2007).

In addition to the loading stressor during commercial vaccination, the required use of anaesthesia also could become a potent stressor due the effect of the chemical properties of different anaesthetics exerts on HPI-axis in fish during anaesthesia (Davis and Griffin 2004; Di Marco et al. 2011; Iversen et al. 2003; **paper II**; Zahl et al. 2010). While several studies reports that anaesthetics are effective in reducing the stress associated with confinement and handling (Davis and Griffin 2004; Iversen et al. 2003; Olsen et al. 1995; Small 2004b; Small and Chatakondi 2005), others have shown that the exposure to an anaesthetic agents in itself could induce an increase in plasma cortisol (Davidson et al. 2000; Davis and Griffin 2004; Iversen et al. 2003; Kiessling et al. 2009; Zahl et al. 2010). It seems that the choice of type regarding anaesthetics is important for the overall stress response during anaesthesia. For instance, **paper II** shows even closely related substances as benzocaine (Benzoak vet.) and tricaine (Finquel vet.), which have the same mode of action, differs in the stress response when fish were exposed to the two compounds of similar concentration with air supply. Plasma cortisol peaked two hours after vaccination in high stress group at 365 nmol/L and 487 nmol/L during 200 mg/L benzocaine and tricaine exposure (with air supply), respectively (**paper II**). In the same manner aggregated stress responses have also been reported for adult Atlantic salmon and Chinook salmon (*Oncorhynchus tshawytscha*) following tricaine (MS-222) exposure (Cho and Heath 2000; Kiessling et al. 2009). Further, a study with juvenile Atlantic salmon, showed the same trend as described in **paper II** with tricaine (MS-222) eliciting the highest increase in plasma cortisol concentration followed by benzocaine and isoeugenol, respectively (Zahl et al. 2010). However, poor water quality like elevated CO<sub>2</sub> level (200 mg/L benzocaine with 30 mg/L CO<sub>2</sub>) seemed to nullify this difference between the two anaesthetics. A plausible explanation for this could be the burden of the additionally stressor caused by high CO<sub>2</sub> level combined with the anaesthetic, are overruling the beneficial effect of Benzocaine on primary stress response. Carbon dioxide is not an ideal anaesthesia in regard of both efficacy and its negative effect on plasma cortisol. Several authors have noted that fish subjected to CO<sub>2</sub> tend to display severe hyperactivity, which can or will most likely increase stress (Erikson 2011; Ross and Ross 2008; Sanderson and Hubert



2007). Unfortunately, due to the time consumption, cost and efficiency the aspect regarding water quality during vaccination is often neglected. Often the commercial smolt plant neglect to change the anaesthetic as often as recommended (by distributor) or do not sufficient aerate the anaesthetic bath (Iversen and Eliassen 2005).

The last pronounced stressor during the vaccination process is experienced during and after the inoculation of the vaccine. Since the inoculation is done during deep anaesthesia, is not likely a source of origin for the last stressor. However, the vaccines used in **paper I, II and III** are all oil-adjuvanted vaccines, and these have been reported to cause post-vaccination mortality, reduction in growth rate, internal abdominal adhesions, and injection-site lesions (Berg et al. 2006; Midtlyng 1996; Midtlyng and Lillehaug 1998; Rønsholdt and McLean 1999). Thus, the prolonged recovery of plasma cortisol (**paper II and III**) and elevated baseline levels after vaccination in **paper I** could be a combination of bi-directional communication between HPI-Axis and cytokines (Tort 2011) and vaccine induced peritonitis, which is considered to be a very painful condition in mammals and most likely so in fish (Bjorge et al. 2011).

Salmon pancreas disease (PD) is an emerging disease in the Atlantic salmon farming industry in Europe. In a Norwegian study of outbreaks in 1999–2002, PD specific mortality has been reported to exceed 5% in 80% and above 15% in 33% of the sites with PD (Aunsmo et al. 2010; Kristoffersen et al. 2009). A vaccine has been introduced as a preventive measure to this disease, with variable success, and due both legal and practical issues the salmon parr needs to be vaccinated twice prior to transfer to sea; first with PD-vaccine and then 3 weeks later (240 degree days) with the combination vaccine protecting against common diseases as Furunculosis, Vibriosis, Coldwater vibriosis, Winter ulcer, IPN and ISA (MSD animal health, Norway). Thus, the allostatic load associated with handling and vaccination have increased in the later years in commercial salmon aquaculture industry due to pancreas disease (PD), as a secondary vaccination and handling procedure prior to transfer to sea is required to give the salmon smolt ample enough protection against new and old bacterial and viral diseases. The effect of allostatic overload before and after secondary vaccination

(**paper I**), indicate that the procedure with double vaccination could be a potential hazardous procedure which could jeopardise the animal welfare and inflict heavy mortality during and after the process. The experiment consisted of three experimental groups control, vaccine and stress, and stress and vaccine. All groups had previously been vaccinated prior to the start of the experiment. The vaccine and stress group was vaccinated for the second time with Pentium Forte Plus at start of the experiment and then given a daily stressor (crowding stressor, 267 kg/m<sup>3</sup> in 15 min) for four weeks, and the stress and vaccine group was given a similar daily stressor for four weeks, and then vaccinated for the second time. The results indicated that fish which entered an allostatic overload type 2 (as stress and vaccine) was oversensitive to ACTH, had a reduced efficient negative feedback system, elevated baseline levels of plasma cortisol, and reduced immune response with pronounced effects on wellbeing of the animal. The vaccine and stress group entered an allostatic overload type 1 response with oversensitivity to ACTH and transient reduced efficient negative feedback system, but at the end of the experiment the fish had recovered. Thus, the study in **paper I** showed that if plasma cortisol became elevated prior to vaccination it could instigate an allostatic overload type 2 with dire consequences on animal welfare.

### **7.1.2 Transport**

As described by Iversen et al. (2005), the salmon industry in Norway suffers heavy mortalities after transfer to sea, probably due to poor handling procedures prior to and after smolt transport to the sea sites. Transportation consists of several traumatic events (stressors): Capture, loading, transport, unloading, and stocking. Handling and transport may initiate a severe stress response in anadromous salmonids (*Salmo* spp. and *Oncorhynchus* spp.) (Forsberg et al. 2001; Iversen et al. 1998, 2004, 2005; Maule et al. 1988; Sandodden et al. 2001; Specker and Schreck 1980). In addition to the severe stressors during transport, Atlantic salmon smolts seems to be more responsive to acute handling and confinement stress compared to parr, partly due to the natural increase in plasma cortisol during smoltification (parr-smolt transformation) (Carey and McCormick 1998). The pre-stress levels prior to the onset of the experiment (**Paper IV**) were within the range of normal resting values for plasma cortisol in

smoltifying salmonids. During smoltification, plasma cortisol levels of Atlantic and coho salmon have been reported to range from 55 to 165 nM (Langhorne and Simpson 1981; Virtanen and Soivio 1985; Young et al. 1989), which is consistent with current observations in **paper IV**. Furthermore, the values for plasma cortisol after loading in our study were similar in magnitude to those measured in juvenile walleyes (*Stizostedion vitreum*) 1 hour after a 30 seconds handling stressor and post-loading (Barton et al. 2003; Barton and Zitzow 1995), and in Atlantic salmon smolts after loading (Finstad et al. 2003; Iversen and Eliassen 2005; Iversen et al. 1998, 2004, 2005, 2009; Nomura et al. 2009; Sandodden et al. 2001). Earlier studies have shown that the loading process elicited a far greater stress response than the well-boat and truck transport itself. Previous studies have shown that the initial moments of the capture-loading process is the major effector of the stress response (Barton et al. 1980; Iversen et al. 1998, 2005; Maule et al. 1988; Nomura et al. 2009; Robertson et al. 1987; Schreck et al. 1989; Specker and Schreck 1980; Weirich and Tomasso 1991).

Transport recovery did not materialise (**paper IV**) as plasma cortisol in the control group (no Aqui-S vet. sedation during loading and unloading) seemed to peak 1 hour after transport and did not recover back to pre-stress levels until 12-24 hours after end of the transport, and had a negative effect on mortality after transfer to sea. This is most likely due to the fact that the two hours transport was not long enough for recovery from the stress effect of the on- and off-loading procedures. These findings are in accordance with previous studies in Atlantic salmon and sea-farmed rainbow trout, in which better recovery from loading stress was shown after longer rather than shorter periods of transport (Iversen et al. 2005; Merkin et al. 2010). Several studies have shown that the transport in well-boat and truck has an important recovery function. Without this opportunity to recover (due to short transport, bad weather or bad road conditions) between the major stressors (on- and off-loading) the ability of the salmon smolts to handle additional stressors becomes reduced (**paper IV**). Multiple stressors are known to hinder basic performance in salmonids both in regards to immunological capacity (Ackerman et al. 2006; Maule et al. 1987; Schreck et al. 1993; Skinner et al. 2010) seawater tolerance (Iversen et al. 1998, 2009; Redding and

Schreck 1983), growth (Andersson et al. 2011; Beitinger 1990; Bernier and Peter 2001; Eriksen et al. 2007; Holloway et al. 2004; Mommsen et al. 1999) and survival (Barton and Iwama 1991; Iversen et al. 2005, 2009; Wendelaar Bonga 1997, 2011).

## **7.2 Plasma cortisol and secondary and tertiary stress responses**

The physiological responses to increased plasma catecholamines (CA) and cortisol are often categorised in secondary and tertiary stress responses (**Figure 1**). Secondary stress responses are often categorised as the result of the primary stress response on different measurable physiological parameters on cell and organ levels, while tertiary stress responses are often referred to the primary and secondary stress effects on the individual animal, populations of animals or whole ecosystems. This classification is simplified, as stress is depending on the magnitude and duration the response, which may affect fish at all levels of organization, from molecular and biochemical to population and community (Wedemeyer 1996). Because CAs release is rapid and due to the fact it has a short biological half-life, it is not feasible to use CA as an indicator on primary stress responses. In contrast, release of cortisol in teleost is delayed relative to catecholamine release. Cortisol synthesis and release from interrenal cells has a lag time of several minutes, unlike chromaffin cells, and, therefore, a proper sampling protocol can allow measurement of resting levels of this hormone in fish (Barton 2002; Wedemeyer 1996; Wendelaar Bonga 1997, 2011). As a result, the circulating level of cortisol is commonly used as an indicator of the degree of stress experienced by fish (Barton and Iwama 1991; Wendelaar Bonga 1997, 2011).

**Paper I to IV** discuss to some extent the relationship between the primary stressresponse (plasma cortisol) and secondary stress responses (plasma lactate, glucose, osmolality, chloride, magnesium and muscle pH) and its overall impact on tertiary stress responses as antibody response, appetite, growth and mortality in relationship to common aquaculture routines during the parr and smolt cycle of Atlantic salmon. In context to this discussion it is important to remember that since stress responses are widespread among all vertebrates, it seems likely that this response provides a survival benefit, which should not be neglected (Davis 2006) and recent studies have shown that plasma cortisol may have both a positive and negative

effect on the physiology of the animal and its survivability (Davis 2006; Davis and Small 2006; Dhabhar and McEwen 1997; Maule et al. 1989). This effect of plasma cortisol on an organism is perhaps due to the duration and magnitude of the hormone. As the duration and magnitude of cortisol in most studies can be directly related to the severity of the imposed stressor(s) (Carmichael et al. 1984; Davis and Small 2006). This effect is documented in **paper II** and **IV**, and shows that plasma cortisol is correlated to the duration and magnitude of the imposed stressors. For instance, in **paper II** one wished to simulate the difference in stressload during commercial vaccination the animals experience in a single unit (tank size: 8 -14 m<sup>2</sup>, containing in average 250-300.000 fish). Based on previous studies, the first group of fish into the anaesthetic bath are expected to have low level of stress (measured as plasma cortisol) while the last group (2-3 hours later) are expected to have a high level of stress (Iversen and Eliassen 2005; Iversen et al. 2005). To mimic this stressload (**paper II**) on the salmon parr, 50 % of the fish were hauled directly into the anaesthetic bath and vaccinated, and the other 50 % were given an additionally crowding stress by lowering the water level to approximately 5 cm (105.6 kg/m<sup>3</sup>), and kept crowded for 20 minutes before anaesthesia and vaccination. Consequently, the average plasma cortisol for low stress and high stress group was 416 ± 154.1 nM and 767.5 ± 202.7 nM, respectively two hours after anaesthesia and vaccination. Similarly the experiment in **paper IV** shows that the control group (no sedation during loading and off-loading) had significant higher plasma cortisol levels from 0 to 6 hours after transport, compared to the sedated group of salmon.

### **7.2.1 Plasma glucose, lactate and muscle pH**

#### ***Plasma glucose***

While plasma cortisol is the most commonly used indicator for primary stress responses in fish, plasma glucose has been the most used indicator for secondary stress responses (Cook et al. 2012; Mommsen et al. 1999). During the physiological stress response the animal provides the energy required to meet challenges associated with the stressor(s) (Barton 2002). As the energy resources are limited the overcoming of a stressor must occur at the cost of other energy-demanding processes, and will

result in life history trade-offs (emergency life history stage) for shorter and longer timespan (Wingfield 2005; Wingfield and Sapolsky 2003). Although the role of cortisol in regulating energy-demanding pathways is not clearly known, studies have shown cortisol related enhancement of liver metabolic capacity, through gluconeogenesis and increasing glucose output from this tissue (Aluru and Vijayan 2007; Mommsen et al. 1999). Indeed, this metabolic response is a key aspect of the stress recovery process, as glucose is the preferred fuel, especially for tissues such as gills and brain, to meet the increased energy demand (Moon 2004). No consistent changes in plasma glucose in relation to primary stress responses were observed during handling and vaccination (**paper III**). Significant hyperglycaemia can be evident within 16 to 32 minutes after stress (Laidley and Leatherland 1988), but no such stress related increase of plasma glucose was observed during the experiment. However, plasma glucose was elevated in all groups 24 and 72 hours after handling and vaccination, and most likely due to the increased plasma cortisol as part of the bi-directional communication between HPI-Axis and cytokines (Tort 2011) and vaccine induced peritonitis, and not as a sign of an acute stress response. This seemingly random effect of plasma cortisol effect on plasma glucose (**paper III**) is reflected in several studies as increases as well as decreases have been described after cortisol administration (Cook et al. 2012; Mommsen et al. 1999; Wendelaar Bonga 1997). The reason for this could be the metabolic status of the experimental animal prior to the onset of the experiment. Fasting or not could alter diet content and blood sugar concentration, and likewise fasting have shown both to affect their abilities to launch a glucose response and increase hepatic sensitivity to adrenergic stimulation (Barton 2002; Van Heeswijk et al. 2006). In this context one should notice that in **paper III** the experimental animals were fasted for 72 hours prior to the onset of the experiment, and could contribute to the randomly changes in blood glucose.

Additionally, it is important to remember that increased glucose levels immediately after stress, are likely maintained by glycogenolysis, whereas chronically elevated glucose concentrations are maintained by gluconeogenesis in the liver (Liebert and Schreck 2006). Glycogenolysis is governed by catecholamines (CA) as

stimulation of the  $\beta_2$  -adrenoceptor in the hepatocytes resulted in mobilisation of glucose, and adrenaline was more potent in doing so compared to noradrenaline (Van Heeswijk et al. 2006; Wendelaar Bonga 1997). Gluconeogenesis seems to be under the control of plasma cortisol, as cortisol treatment in fish significantly increased the activities of all key gluconeogenic enzymes, such as glucose 6-phosphatase, fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase (PEPCK). Activities of these gluconeogenic enzymes supported an increased liver capacity for gluconeogenesis in cortisol-treated fish, while the absence of an increase in plasma glucose concentration may result from elevated turnover of the metabolite (Mommsen et al. 1999). Thus, trying to correlate plasma glucose only to plasma cortisol could be troublesome without considering how the animal experiences the stress during an experiment, as allostatic overload type 1 (acute) or 2 (chronic) could lead to different outcomes of plasma glucose both in duration and magnitude.

However, one cannot rule out that plasma glucose is not a reliable indicator for secondary stress responses in a carnivorous fish as Atlantic salmon, as Mommsen et al (1999) emphasised that reliance on plasma glucose levels and liver glycogen content as a stress indicator, may not be a beneficial approach in fish. Fish plasma glucose is highly variable, and not well regulated as in mammals, and especially regarding carnivorous species it may not be a useful indicator of metabolic status at all (Mommsen et al. 1999).

### ***Plasma lactate and muscle pH***

The elevation of lactate concentrations immediately after stress is likely due to muscle glycolysis (Moon and Foster 1995), and lactate may be used for anaerobic gluconeogenesis in the liver (Vijayan and Moon 1992). In **paper II, III and IV**, plasma lactate peaked after loading (**paper IV**); anaesthesia and vaccination (**paper III**) and 2 hours after anaesthesia and vaccination with 20 minutes of additional crowding stress (**paper II**). The peak of plasma lactate ranged from 6,4 to 9,3 mmol/L, and this is not high compared to levels measured (>20 mM) after heavy physical exercise and air exposure in several species as salmonids (Liebert and Schreck 2006; Olsen et al. 1995; Pagnotta and Milligan 1991; Schreck et al. 1976; Wood et al. 1990), striped bass

(*Morone saxatilis*) (Cech et al. 1996), sablefish (*Anoplopoma fimbria*) (Davis et al. 2001), winter flounder (*Pseudopleuronectes americanus*) (Pagnotta and Milligan 1991) and lingcod (*Ophiodon elongates*) (Milston et al. 2006). However, the lactate peak experienced in the present experiment (**paper IV**) is similar to studies done by Nomura et al. (2009) on commercial land and sea transport of Atlantic salmon smolt. Regardless of the magnitude of the lactate peak in the different studies one should be careful to interpret that the fish in **paper II, III and IV** are less stressed. It could be an artefact of the applied method used to measure plasma lactate, as one used a portable handheld instrument Lactate Pro™ (Arkray, KDK Corporation, Japan). Wells and Pankhurst (1999) validated the efficacy of portable instruments for measuring blood glucose and lactate and compared it with established laboratory techniques. They concluded that portable instruments for measuring blood glucose and lactate could be used as a relative measure to evaluate responses to stressors, and care should be used when comparing to other methods.

It seems like the increase in plasma lactate documented in **paper II and III** is caused by a combination of handling stressor and deep anaesthesia. Plasma lactate significantly increased, both in treatment I- and II -smolt group after anaesthesia. The changes in lactate were more pronounced and clear during exposure of AQUI-S vet., compared to the control group using Finquel vet. only (**paper III**). Likewise plasma lactate was increased 0 and 2 hours after anaesthesia and vaccination of all high stress groups with exception to 200 mg/L Benzoak vet. and Finquel vet. supplied with air (**paper II**). Plasma lactate can be used to indicate more specific respiratory effects from anaesthesia (Olsen et al. 1995). Increases in plasma lactate levels during MS-222 anaesthesia have been documented by several authors (Houston et al. 1971; Soivio et al. 1977) and metomidate anaesthesia (Iversen et al. 2003; Olsen et al. 1995). Others have failed to establish this connection with lactate and anaesthesia (Wells and Pankhurst 1999). Lactate increases in blood occur when insufficient oxygen is available for aerobic cell metabolism. This could be due to reduced ventilation and circulation, which are common side effects of several anaesthetics (Barrento et al. 2011; Di Marco et al. 2011; Houston et al. 1971; Iwama et al. 1989; Wilkinson et al. 2008). Both



benzocaine (Mattson and Ripple 1989) and eugenol (Keene et al. 1998) caused reduced gill ventilation due to depression of medullary respiratory centres, with hypoxia as a consequence. The hypoxia is intensified by bradycardia and decreased blood flow through the gills (Mattson and Ripple 1989).

In addition, some anaesthetics could due to its own chemical properties (in combination with poor water quality) increase the lactic load in the animals, as the combination of 400 mg/L Finquel Vet (metacaine) together with poor water quality (no air supply) regardless of stress level gave reduced muscle pH two and six hours after treatment (**paper II**). It seems that the combination of the acidic Finquel Vet. and carbon dioxide (approximately 60 mg CO<sub>2</sub>/L) have a pronounced effect on acid-base balance in the salmon parr. Earlier studies have shown that that the use of MS-222 could elevate haematocrit, induce erythrocyte swelling, hypoxia, and hypercapnia (Di Marco et al. 2011; Ross and Ross 2008; Velisek et al. 2009, 2011), and exposure to carbon dioxide could cause both acidosis and a reduction in blood pH (Iwama et al. 1989; Ross and Ross 2008).

### **7.2.2 Effects on osmoregulation**

The release of cortisol into the blood is a primary stress response in fish, and it is established that one of the major physiological roles of cortisol are regulation of the hydromineral balance and energy metabolism in fish. Therefore, changes in plasma osmolality, chloride, and magnesium are all features of the secondary stress response (Veiseth et al. 2006). Cortisol promotes chloride cell differentiation and also stimulates Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in the gills, intestine and kidney during parr-smolt transformation (Wendelaar Bonga 1997). Similar is cortisol also involved in the adaptation to freshwater (Sakamoto and McCormick 2006). Studies have shown that cortisol treatment increases the transcription of NKA $\alpha$ 1a, which is believed to be involved in the ion uptake by chloride cells in fresh water (Kiilerich et al. 2007, 2011; McCormick et al. 2008), and Perry et al. (1992) discovered that cortisol injection increases the surface area of gill chloride cells (a characteristic of ion uptake cells) and elevated the branchial influx of Na<sup>+</sup> and Cl<sup>-</sup> in hypotonic environment. Further studies of the ion transport properties of cultured pavement cells from teleost gill epithelia

showed that cortisol also induces the active ion uptake of  $\text{Na}^+$  and  $\text{Cl}^-$  (Chasiotis and Kelly 2011; Kelly and Chasiotis 2011).

Plasma osmolality and chloride could therefore be useful indicators in assessing the excretion capacity in the gills, intestine and kidney in order to maintain a hydromineral balance both in freshwater and seawater (Mommsen et al. 1999; Wendelaar Bonga 1997). Earlier studies have demonstrated that plasma osmolality and chloride concentrations declines in freshwater adapted fish and increase in seawater adapted fish in response to handling or confinement (Iversen et al. 1998), while other studies have reported no changes in fish plasma osmolality (Barton and Zitzow 1995) or chloride levels (Barton et al. 2005) in response to crowding stress. While osmolality and chloride increased in both groups during the experiment (**paper IV**), it did not seem to be related to the difference in the primary stress response documented in the sedated and unsedated group. Instead it appeared to be an effect of the salinity change from freshwater to seawater after transport as both groups showed increases in osmolality and chloride levels with no significant differences between the stressed and unstressed group. Plasma chloride dropped in salmon parr in freshwater in all experimental groups during vaccination after the anaesthetic bath (**paper III**) and was kept suppressed until 24 hours after the vaccination. The reduction of plasma chloride seems to be part of the acute stress response, which is characteristic for fish exposed to stressors while held in freshwater (Liebert and Schreck 2006; Redding and Schreck 1983). However, osmolality in the same experiment (**paper III**) showed incoherent variation during the first 24 hours of the experiment and became significantly reduced only after 72 hours and did not recover back to pre-stress a week later. This delayed effect of stress on plasma osmolality could be due to the compensatory responses on cell volume as fish cells exposed to hypotonic media, rapidly swell but thereafter tend to return towards their original volume by eliminating cellular osmolytes and hence water. This regulatory mechanism is termed regulatory volume decrease (RVD) (Trischitta et al. 2005). In contrast, fish under hypersaline (seawater) conditions will try to use intracellular osmolytes in some part of the osmoregulation, because elevated plasma osmolality would cause cell shrinkage unless osmolytes are accumulated. In

most organisms, these solutes are not inorganic ions, but rather neutral free amino acids such as taurine and glycine, small carbohydrates such as myo-inositol, and methylamines such as trimethylamine oxide (TMAO). Organic osmolytes, unlike the common inorganic ions, do not perturb cell macromolecules and are thus called compatible solutes; in some cases they even protect macromolecules from perturbations such as temperature, urea, pressure and reactive radicals (Fiess et al. 2007; Trischitta et al. 2005).

Plasma magnesium differed between the groups in **paper I** and **IV**. While plasma magnesium in the AQUI-S vet. sedated group returned to pre-stress levels 12 hours after transport, the stressed (unsedated) group showed no such recovery even a week after transport to sea (**paper IV**). A similar result was observed in **paper I**, as the seawater adapted stress and vaccine group entered a chronic state (allostasis overload type 2) at week 5 (and lasted until the end of experiment) both plasma chloride and magnesium became elevated, and differed from the control group and the vaccine and stress group. Marine teleosts ingest seawater to compensate for osmotic water loss and prevent dehydration, and eliminate divalent ions such as  $Mg^{2+}$  and  $SO_4^{2-}$  from the body fluids during this process (Redding and Schreck 1983). Plasma magnesium ( $Mg^{2+}$ ) uptake and excretion are functions of the gut and kidney, respectively (Redding and Schreck 1983). However, Kirschner et al. (1974) concluded that the gill epithelium of rainbow trout was readily permeable to  $Mg^{2+}$ . In most cases, the total magnesium concentrations of blood plasma in fish do not exceed 2 mM, and the ionic concentration is normally less than 1 mM (Bijvelds et al. 1998). Both in **paper I** and **IV**, fish displayed a significant increase in magnesium concentrations above or close to 2 mM during the course of the experiment in the high stressed groups (indicated with high levels of plasma cortisol), and this may indicate a disturbance in hydromineral balance and give plausible explanation for the delayed mortality these groups experienced. Although reports concerning the effects of stress on  $Mg^{2+}$  in fish are limited, Staurnes et al. (1994) reported a significantly higher plasma  $Mg^{2+}$  concentration in Atlantic cod (*Gadus morhua*), compared to non-stressed fish, during simulated high-density transport. Coho salmon (*Oncorhynchus kisutch*) displayed

significantly higher plasma  $Mg^{2+}$  levels during stress in seawater compared to control fish (Redding and Schreck 1983), and plasma  $Mg^{2+}$  rapidly increased in gilthead sea bream (*Sparus aurata*) stressed by air exposure (Arends et al. 1999). In contrast to these studies and in the present studies (**papers I and IV**) steelhead trout (*Oncorhynchus mykiss*) exposed to a confinement stressor did not alter potassium, magnesium, and calcium levels, even though sodium and chloride levels were altered (Liebert and Schreck 2006). Although increased catecholamine concentrations may have a beneficial effect by stimulating oxygen uptake via gills, they may also result in increased permeability of the surface epithelia to water and ions (Arends et al. 1999). The concentration of  $Mg^{2+}$  in seawater is approximately 50 to 100 times greater than of fish plasma. Thus, relatively small increases in gill permeability during stress in seawater may be responsible for a large influx of  $Mg^{2+}$  (Redding and Schreck 1983).

### **7.2.3 Effects on antibody response**

The actions of stress or cortisol on the fish immune system can be described to be regulatory and not necessarily inhibitory. The effects on the immune system and the final outcome may depend on the severity and duration of the stressor, as it does in mammals (Tort 2011; Weyts et al. 1999). Recent studies have suggested that the observed differences in immune responsiveness and disease susceptibility in relation to elevated cortisol levels in fish are specific to species, strain, antigen type, and possibly to the timing and duration of the stressor (Engelsma et al. 2003; Funk et al. 2004; Skinner et al. 2010). In the experiment described in **paper I**, stress prior to vaccination affected negatively in the production of antibody titre, while vaccination before applying a daily stressor did not. The vaccine and stress group responded with a 2.5 times increase in antibody titre, 245 degrees days after the second vaccination, which is common during a second immunisation (Einarsdottir et al. 2000a; 2000b). After a boost injection, the secondary immune response emerged faster and resulted in a greater antibody titre than the primary immune response. This is in accordance with earlier findings involving brown trout (*Salmo trutta* L.), Atlantic salmon (*Salmon salar*) and Arctic charr (*Salvelinus alpinus*), where the initial humoral immune response produced antibody titre to SRBC, and second dose of SRBC produced higher serum

agglutinin (titre) compared the initial dose (Einarsdottir et al. 2000a; 2000b; Ingram 1985). A secondary immune response (boost) was not observed in the group stress and vaccine. It seems that applying the stressor before initiating the immune response could have dire consequences on the immunisation. Recent findings supports these observations, as studies shows that if plasma cortisol levels becomes elevated after initiation of the innate and adaptive immune responses, the overall disease susceptibility and the production of pathogen-specific Abs stays unaffected (Espelid et al. 1996; Funk et al. 2004; Lovy et al. 2008; Skinner et al. 2010).

It is considered that stressors induce suppressive or adverse effects on the immune system, but part of the responses could also be considered active or enhancing responses and therefore, the end result of this complex event will depend on the time course, the induced response and the stressor (Tort 2011). When a stress response develops the outcome will depend on the timing, intensity and its duration of the stressor(s). Thus, this can explain the different outcome of stress on the antibody titre represented in **paper I** and **III**. While the antibody titre response was suppressed in the stress and vaccine group (**paper I**) no such suppression was observed in **paper III**. However, the allostatic load experienced by the animals in **paper I** was more severe both in magnitude and duration compared to the animals in **paper III**. Acute stress, as observed in **paper III**, associated to allostatic overload type 1 (stress resulting in potential advantages), involving short-time challenges most likely resulted in immune-activation or immune-enhancing processes. Acute stress is characterised by a rapid physiological stress response mounted in presence of a stressor(s), but upon cessation of stress, the response experiences a rapid shutdown (Dhabhar 2002; Dhabhar 2008; Fridell et al. 2007). In contrast, chronic stress or allostatic overload type 2 as observed in **paper I** was repeated and prolonged, and may have resulted in uncoordination or suppression of immune function as observed in stress and vaccine group. An important characteristic of allostatic overload type 2 is that physiological response either persists long after the stressor has ceased or is activated repeatedly resulting in an overall integrated increase of exposure to stress hormones (Dhabhar 2002, 2008; Dhabhar and McEwen 1997; Tort 2011).

#### 7.2.4 Effects on appetite and growth

Vaccination of Atlantic salmon using oil-adjuvanted vaccines may affect growth of the fish due to loss in appetite or side effects (Melingen and Wergeland 2002). But also a characteristic behavioural response to stress in fish appears to cause a reduction in food intake (Wendelaar Bonga 1997). In fact, in addition to its appetite-suppressing effects, stress can disrupt many other aspects of the feeding behaviour in fish as, food searching, -finding, or -capturing can all be affected by stress (Beitinger 1990; Bernier 2006; Bernier and Peter 2001; Pankhurst 2011; Pankhurst et al. 2008; Tsigos and Chrousos 2002) and is most likely governed by the HPI-axis through the corticotropin-releasing factor (CRF) system (Bernier 2006). The stress reducing measures (sedation and anaesthesia) seemed to have a positive effect on appetite (**paper III**), and treatment II which gave the best stress reducing effects, also had significant higher feed intake and reduced feed waste production. However, this effect seemed to be short lived since no differences in growth between the groups were observed. Melingen and Wergeland (2002) found that the handling- and injection process seems to have little or no effect upon growth, but determined that the effect upon growth was caused by the antigenic component (*A. salmonicida*) in the vaccine, rather than the adjuvant and/or interactions between the two.

The effect on appetite observed (**paper III**), is most likely a cortisol effect, and not an effect of the vaccine in this experiment. Similar have experiments in which goldfish (*Carassius auratus*) were fed for 21 days with diets prepared to contain 0 (Control), 50 (Low) or 500 (High) cortisol/g of food. The feeding remained unchanged in the control group, and in fish fed the high cortisol diet group, as daily food intake gradually increased in the low cortisol diet group, and was significantly elevated between days 9 and 21. At the end of the feeding trial, specific growth rate was lowest in fish fed with the high cortisol diet, intermediate in those fed the low cortisol diet, and highest in the control group. Feed conversion efficiency, on the other hand, was significantly reduced in both groups of fish fed the cortisol diets (Bernier et al. 2004). Similar effects of cortisol on appetite and growth have also been documented in species such as rainbow trout (*Oncorhynchus mykiss*), matrinxa (*Brycon cephalus*), gilthead seabream

(*Sparus auratus*), channel catfish (*Ictalurus punctatus*), Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*) (Bernier 2006; Bernier et al. 2004; Bernier and Peter 2001; Bjornsson et al. 2002; Mommsen et al. 1999; Olsen et al. 2008; Pankhurst 2011; Pankhurst et al. 2008; Pickering 1990, 1992, 1993; Small 2004a; Sørensen et al. 2011; Tsigos and Chrousos 2002; Weil et al. 2001). In accordance to Sørensen et al. (2011) there are at least three likely explanations for how cortisol suppresses growth. First, cortisol treatments (injections or implants) have been found to cause an increase in basal metabolic rate (measured as oxygen consumption) in fish (Morgan and Iwama 1996), which could lead to reduction of the amount of energy that can be utilised for growth. Second; cortisol could directly reduce cellular growth, as [<sup>3</sup>H]-thymidine incorporation into DNA of cell cultures derived from fish are shown to reduce after cortisol treatment (Lee and Bols 1989; Pagniello et al. 2002; Van Oostrom and Bols 1991), and this effect is likely mediated through binding to glucocorticoid receptors (Lee and Bols 1989). And finally, Barton et al. (1987) suggested that oral cortisol treatment may suppress growth by altering gut morphology, reducing nutrient uptake from the food and thus restricting energy available for growth. In addition to these plausible explanations plasma cortisol could have a more direct effect on growth as increase in cortisol during stress have shown to reduce growth hormone and insulin-like growth factors (IGFs) produced by the liver, thus affecting both food intake and feed conversion rate (Peterson and Small 2004, 2005; Salas-Leiton et al. 2012).

However, as stated earlier, even with reduced appetite in the most stressed group (**paper III**) no effect on growth was observed and is most likely due to the short lived stress response during handling and vaccination. Liebert and Schreck (2006) observed similar effect, as feed intake was not disturbed by a single stressor that was applied before seawater transfer in yearling steelhead trout (*Oncorhynchus mykiss*). There is, however, a plausible and more intriguing explanation to the lack of reduced growth. In rats, most studies demonstrated that stress reduced food intake, unless access to food was given during the stress period then the danger of over-eating and the risk of obesity increased (Green et al. 1992; Zakrzewska et al. 1999). Stress-induced eating of food is also known as non-homeostatic eating. This eating behaviour has been

hypothesized to activate the brain reward system and decrease activity of the hypothalamo-pituitary-adrenal (HPA) axis, thus dampening the stress response, as expressed by lower cortisol and corticosterone concentrations (Dallman et al. 2006; Maniam and Morris 2012; Pecoraro et al. 2004; Tomiyama et al. 2011). However, it yet remains to establish if this over-eating behaviour exist or not in teleost.

All in all, the varied effects of stress on feeding behaviour, and the broad range of stressors that can suppress feeding, suggest that the interactions between the pathways mediating the stress response and the regulation of food intake in fish are complex and not fully understood (Bernier 2006; Bernier and Peter 2001).

### **7.2.5 Mortality**

Few stress studies have attempted to link stress directly to mortality outcomes, and in most cases, when it has been done, it has been conducted in relation to ecological studies (Anderson 2000; Davis 2010). Interpretation of different stress responses as signs of stress linked to mortality is complicated as they can be altered by factors like acclimation, size, species and individual-fitness which are not related to health status (Barton 2002). Mortality can occur from hours to weeks after exposure to stressors (Davis 2010; Davis et al. 2001). This is probably due to the concept of stress as the primary response results in rapidly and easily detectable increases in the catecholamines (CAs) and the corticosteroid cortisol, and the secondary response becomes a the physiological mirror of these hormones. The tertiary response again includes the physiological and behavioural changes occurring at the whole animal level, which can be both adaptive, as well as pathological. Only the start of the primary, secondary, and tertiary responses is sequential in time because the duration of each response is such that it creates temporal overlap once the stress response is underway (Wendelaar Bonga 2011). To identify factors associated with mortality and to measure true mortality rates, fish must be held for prolonged periods under controlled conditions to prevent that they do not contribute to the original stressors causing delayed mortality (Davis 2010), which was ensured and followed in all studies done in this thesis.



Iversen et al. (2005) concluded that that well-boat transports seemed to have an important recovery function. Without this ability to recover between the stressors, as on- and off-loading, the ability for salmon smolts to handle multiple stressors was reduced. Furthermore the study seemed to support the fact that some of the increased mortality experienced at sea sites in Norway may be explained by handling and transport prior to deliverance of smolts. They suggested that better planning of well boat routes, and avoidance of high seas could contribute to minimise mortality experienced after transports. Similar change in mortality after truck transport was observed in **paper IV**, as the unsedated fish (during on- and off-loading) appeared to enter a state of chronic stress, thus the unsedated fish experienced an acute mortality which only stabilised 16 days (11.3 % mortality) after transport.

All the studies conducted in this thesis, with exception of **paper II**, showed that mortality seemed to be closely linked to excessive release of plasma cortisol in vicinity of multiple stressors. Most pronounced was the link between the primary stressresponse (plasma cortisol) and the tertiary stress response (mortality) observed in **paper I** and **IV**. In both studies the mortality was significantly higher in the groups experience allostatic overload type 2, as observed in the stress and vaccine group (66% mortality; **paper I**), and unsedated group (11.3 % mortality, **paper IV**). The mortality seemed also to be correlated to the degree of allostatic load imposed on the other groups as observed in control (1% mortality) and vaccine and stress group (44% mortality) (**paper I**), and in the sedated group (2.5% mortality) (**paper IV**). In **paper II** the relationship between plasma cortisol and mortality do not seem to manifest itself, as even though all mortality originated from the Finquel Vet. groups. The highest mortality was recorded in 400 mg/L Finquel Vet. without air supply where mortality did not seem to be related to stress, as 7 out of 9 dead salmon was recorded in the low stress group. Due to the low number of fish in each treatment group (n=30; individually tagged) the significant differences in mortality should be interpreted with caution. Unpublished data presented for the authors from ScanAqua AS (distributor of Finquel Vet.) showed that during field surveillance between 2002-2011, where 110 groups (benzocaine) and 196 groups (Finquel Vet) were followed, no differences in 21

days mortality were recorded between the anaesthetics (Speilberg, pers. comm, Project leader ScanVacc AS).

### **7.3 Stress reducing measures and its effects on the stress response**

Chemical anaesthetics are often used in aquaculture research to ease handling and reduce stress. In addition to prevent physical injury, certain anaesthetics may reduce or block activation of the hypothalamo–pituitary–interrenal (HPI) axis associated with handling stress (Bosworth et al. 2007; Davis and Griffin 2004; Finstad et al. 2003; Iversen et al. 2003; Sandodden et al. 2001; Small 2004b). Failure to suppress activation of the HPI axis during stress can result in a release of cortisol which in turn causes various secondary and tertiary stress responses (Ackerman et al. 2000; Ellis et al. 2012; Wendelaar Bonga 1997, 2011).

The importance of controlling the stress response may differ depending on the acute or chronic nature of the stressor, and on the ultimate use of the fish (Davis 2006). However, during critical commercial handling routines as vaccination and transport, it could be beneficial to keep allostatic load under control, and **paper III** and **IV** suggest that sedation prior to and during handling has positive effects on primary, secondary and tertiary stress responses during both vaccination and transport. In **paper III** the control group (no stress reducing measures) used more than 72 hours to recover plasma cortisol after vaccination and handling, while the treatments groups (with stress reducing measures) returned to resting levels within 24 hours. The combination of sedation (AQUI-S vet.) and anaesthesia (Finquel vet.) during vaccination (treatment II), had the most positive effect on plasma cortisol, and fish in this treatment experienced only a mild increase in plasma cortisol. Treatment II showed also an overall positive effect both on appetite and mortality (**paper III**). The rapid increase in plasma cortisol in treatment I after loading, anaesthesia and vaccination is probably due to the prolonged time (60 to 90 minutes) it took this group to reach stage 4 (anaesthesia). The dosage used to anaesthetise the fish was 30 mg/L AQUI-S vet. Earlier studies have shown that only dosages of 40 mg/L AQUI-S vet. and above completely prevented plasma cortisol elevation above resting level (Iversen et

al. 2003). Thus, during the prolonged time in the anaesthesia bath the hypothalamic – pituitary – interrenal (HPI) axis may still be active continuing to produce plasma cortisol.

During truck transport (**paper IV**), AQUI-S vet. sedation during on- and off-loading sufficiently reduced the primary stress response (expressed as plasma cortisol) and lowered mortality after transfer to sea compared to unsedated fish, which experienced a mortality rate at sea similar to commercial salmon smolt transports in Norway (Iversen et al. 2005). This is most likely due to a general sedative effect of AQUI-S vet. In Atlantic salmon, Iversen et al. (2003) reported a reduction in plasma cortisol with AQUI-S vet. concentrations of 40 mg/L (20 mg iso-eugenol/L) and above, observing no improvement at lower concentrations. However, in channel catfish, a concentration of only 5 mg/L AQUI-S vet. (2.5 mg/L isoeugenol) reduced cortisol release compared to unsedated fish during both confinement and acute oxygen depletion (Small 2004b). Thus, since the sedative dosage of AQUI-S vet. (5 mg/L) is below an effective dosage to have a direct effect on the HPI-axis in salmon (Iversen et al. 2003), it cannot be ruled out that this is a more general effect of anaesthesia/sedation, which may reduce the cortisol stress response by affecting the perception of the stressor (Schreck 1981). AQUI-S vet. and other eugenol based anaesthetics (clove oil) have shown similar stress-reducing effects in several other species (Davis and Griffin 2004; Iversen et al. 2003, 2012; Small and Chatakondi 2005; Woods et al. 2008). It is not known how eugenol affects the plasma cortisol dynamics in fish. It is reasonable to assume, however, that the transmission of sensory information to the hypothalamus is blocked (Iversen et al. 2003; Small 2003).

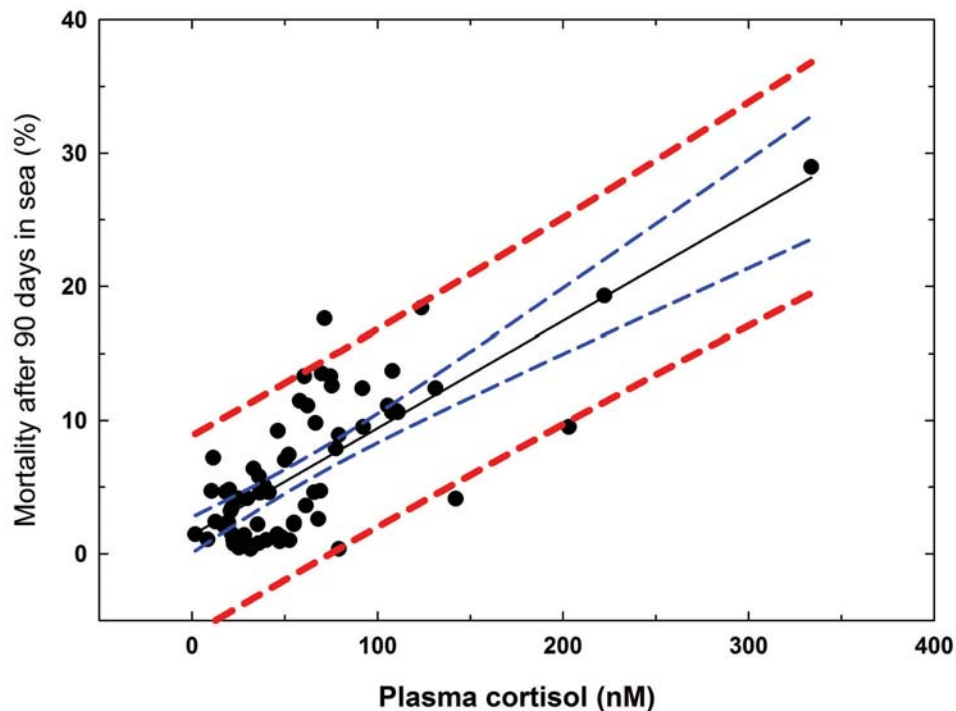
The sedation procedures described in **paper III** and **IV** can easily be replicated in many commercial aquaculture procedures as handling, vaccination and transport operations and it could participate to reduce the overall stress response and mortality. This could allow the fish to start feeding and growing sooner after the handling and transport, thus improve both production cost and animal welfare during a critical production phase of Atlantic salmon parr and smolts.

## 7.4 The concept of allostasis and fish

The concept of allostasis has been introduced to complement the concept of stress, and more precisely try to describe the role primary mediators (e.g. glucocorticosteroids) have in response to a stressor (Goymann and Wingfield 2004; McEwen 1998, 2005; McEwen and Wingfield 2003; Wingfield 2005). McEwen and Wingfield (2003) considered allostasis to be the ability to achieve stability through change, thus the stress response can be described in terms of allostasis, defined as the struggle to maintain homeostasis through changes in physiological systems. Allostasis allows the animal to actively adjust physiological systems (through set points) to meet predictable and unpredictable changes in the environment (Segner et al. 2012). An allostatic state refers to altered and sustained activity levels of the primary mediators (e.g. glucocorticosteroids) as a response to a stressor (McEwen and Wingfield 2003). The cumulative result of an allostatic state is allostatic load. Within limits an organism can cope with, adapt to, or tolerate stressors to keep homeostasis. However, when the system is not dealing well with the disturbing factor(s), the increased allostatic load results in allostatic overload, which either could be adaptive (overload type 1) or malicious for the animal (overload type 2) (Juster et al. 2010; McEwen 2005; McEwen and Wingfield 2003; Wingfield 2005).

Schreck (2010) stated that the concept of allostasis is relevant for fish. Several papers regarding fish and stress have incorporated the concept of allostasis into their studies (Anderson et al. 2011; Leong et al. 2009; Prunet et al. 2012; Schreck 2010; Segner et al. 2012; Varsamos et al. 2006). Mortality seemed to be closely linked to excessive release of plasma cortisol in vicinity of multiple stressors, which could signify a chronic stress response, commonly referred to as an allostatic overload type 2 (Schreck 2010). However, quantification of chronic stress conditions has proven to be complicated in fish. Many researchers reporting transient elevation of cortisol levels during crowding stress (Barcellos et al. 1999; Ruane et al. 2002), while others report no effect at all (Da Rocha et al. 2004; Fanouraki et al. 2007; Vijayan and Leatherland 1990) or even a reduction (Leatherland and Cho 1985). A baseline study of cortisol is a plausible way to document a chronic stressed fish and possible allostatic overload type

2 scenario (McEwen and Wingfield 2003). Iversen and Eliassen (2012) showed that there was a significant correlation between high baseline levels of plasma cortisol in regular smolt production and mortality after transfer to sea (**Figure 8**). If the baseline plasma cortisol are kept above 50 nM (18.1 ng/mL) during the smolt production, the relative risk for getting a mortality above 5 % after 90 days in sea increased to 3.1 times ( $p=0.001$ ), and the risk for a disease diagnose increased to 4.9 times ( $p=0.001$ ). Stress reducing measures in the smolt plants, such as improved water quality, predation control and reduced production density, done early in 2010 gave a positive effect on overall stress levels and mortality in 2010 and 2011 compared to 2009. The overall stress level was reduced with 66 % and the mortality with approximately 75 % after transfer to sea in 2010 and 2011 compared to 2009 (Iversen and Eliassen 2012). Similar, Varsamos et al. (2006) showed that variable water temperature triggered dramatic changes in terms of stress parameters and susceptibility to nodavirus in sea bass (*Dicentrarchus labrax*). This suggested a strong and prolonged activation of the HPI axis, which they concluded corresponded to an allostatic overload type 2. However, without high number of individual (n) during a survey of baseline cortisol, a single parameter related to the HPI axis could be insufficient to establish if the fish is chronically stressed. For instance different genetic strains of the same species may exhibit different resting levels (baseline) of cortisol due to the effects at any levels in the HPI axis (Fevolden et al. 2002; Pottinger and Carrick 1999; Tanck et al. 2001, 2002). Norris *et al.* (1997; 1999) showed that a population of brown trout exposed to heavy metals exhibited identical resting levels as a non-exposed group of brown trout in the same river living upstream of the pollution area. Each level of the axis (hypothalamus, anterior pituitary, interrenal cells) is subjected to opposing influences, trophic via their respective stimulating inputs (such as CRH to the pituitary or ACTH to the interrenal cells) and inhibitory via corticosteroid hormones (negative feedback) (Mormede et al. 2007).



**Figure 8.** The relationship between resting levels of plasma cortisol and mortality after 90 days at sea for groups of fish followed during spring and autumn production in Mainstream Norway AS's smolt plants in the period of 2009 to 2011 (n = 3684) (taken from SYSTAT 13.00 .05). Blue dashed lines indicate 99 % confidence limit while red dashed lines indicate 95 % confidence limit. Redrawn from Iversen and Eliassen (2012)

In mammals, several changes in the HPI-axis has been documented during a chronic stress-state such as weight loss, proliferation of the corticotrope cells in the anterior pituitary (trophic effect of CRH), inhibition of ACTH synthesis (by cortisol) and reduction of the feedback effect of GR agonists on ACTH release, increase of the size of the adrenal glands and of the response of the adrenals to ACTH (Mormede et al. 2007). This resetting of the HPI axis at a new level of activity (set points), that Selye (1973) described as the stage of resistance, is also known as allostasis (Goymann and Wingfield 2004; McEwen 1998, 2005; McEwen and Wingfield 2003; Wingfield 2005).

Different approaches could be used to detect these changes (as described above), including stimulation tests (by CRH, CRH/vasopressin, ACTH, insulin-induced hypoglycaemia) that measure the relative sensitivity of the pituitary and/or the interrenal cells, and the use of a inhibition test utilising dexamethasone (DEX) to

demonstrate the reduced efficiency of the negative feedback by corticosteroids (Mormede et al. 2007). In the experiment in **paper I**, both the vaccine and stress group and the stress and vaccine group became oversensitive to administration of a weight-adjusted dose of ACTH compared to the control group, and the oversensitivity increased with duration of the experiment. Few studies on ACTH sensitivity have been done on fish. However Pottinger and Carrick (2001) showed that two lines of rainbow trout selected for high (HR) and low (LR) responsiveness to a standard crowding test had different responsiveness to weight-adjusted dose of ACTH. The LR strain had significant lower production of plasma cortisol compared to HR strain. In domesticated mammals an injection of ACTH has shown an increased cortisol response in animals reared in poor conditions or subjected to repeated stressors. For instance, calves submitted to prolonged spatial and social restriction (Friend et al. 1985), repeated regrouping (Veissier et al. 2001), growing pigs in restricted space (Meunier-Salaun et al. 1987), and tethered sows (Janssen et al. 1994).

The dexamethasone (DEX) suppression test has been developed in humans to detect HPI (HPA) axis changes in melancholic patients (Banki et al. 1986; Kumar et al. 1986; Wilens et al. 1984). In humans, DEX have shown to reduce the morning peak of cortisol, but more so in healthy humans than in depressed patients, and the maximum post-DEX effects on plasma cortisol levels have shown to reflect the overall severity of depression during melancholia (Kumar et al. 1986). The response to DEX to chronic stress (**paper I**) seem to be similar to that of depressive humans and the severity of the response to DEX seems to be most severe in the stress and vaccine group, followed by the vaccine and stress group and the control group. Similar collapse as observed in the stress and vaccine group, with no changes in plasma cortisol response after injection in DEX, has also been documented in pigs submitted to a prolonged reduction in their space allowance (Meunier-Salaun et al. 1987).

To the best of our knowledge no such studies have been done on fish, combining baseline levels of plasma cortisol, sensitivity of the interrenal cells (ACTH), and efficiency of the negative feedback by corticosteroids (DEX) during prolonged stress. The results indicates that a fish who enters an allostatic overload type 2 (as the stress

and vaccine group) is oversensitive to ACTH, have a reduced efficient negative feedback system, and elevated baseline levels of plasma cortisol with pronounced effects on wellbeing of the animal (**paper I**). The vaccine and stress group represent an allostatic overload type 1 response with oversensitivity to ACTH and transient reduced efficient negative feedback system, but at the end of the experiment the fish recovered. However fish in the vaccine and stress group had elevated mortality compared to control group and experienced reduced animal welfare. In a simplified and general way one may conclude that the type 1 overload could be associated with more acute stressors with temporary changes in the sensitivity of the HPI-axis, and type 2 overload could be associated with more chronic stressors with severe and malicious effect on the HPI-axis and the welfare of the being. However, as Schreck (2010) emphasised, the distinction between acute and chronic stress is quite blurred. Thus, it is difficult to generalise about the effects of various stressors, since several stressors are often experienced simultaneously, sequentially, or in series during different life scenarios. Likewise, the effects of the different stressors and severities are more an ongoing process, where the outcome of stress is depending on the mental and physical fitness of the individual organism.

The studies conducted both in **papers I** and **IV** showed that if plasma cortisol becomes elevated prior to vaccination or during transport it could instigate an allostatic overload type 2 with dire consequences on animal welfare. To reduce the risk to compromise the animal welfare during commercial vaccination of salmon one propose to grade the fish minimum a week prior to vaccination, or grade simultaneously with vaccination. During transport one suggests to keep stress response to a minimum during loading and off-loading, and if possible, use sedative during the hauling process. All these proposed measures could reduce the overall allostatic load during handling, vaccination and transport, and produce a healthy fish, with intact immune response and improve animal welfare cultured species.

## **7.5 Stress and fish welfare**

With an average mortality rate at 17 % ( $\pm 3.2$ ) after transfer to sea in the last 17 years, there are some reasons to raise concern regarding animal welfare in the



Norwegian aquaculture. In 2011, over 50.970.000 smolts were lost at sea, mostly observed as mortality. This amounts to a first-hand price on smolt of approximately 411.837.600 NOK (71.605.584.27 USD; average smolt-price in Nordland was 8,08 NOK pr fish; 2011) (DOF 2012b), and if these animals had been able to survive until slaughter at an average weight of 5 kg, it would give a net profit of 1.276.798.500 NOK (222.089.155 USD, net profit in Nordland on slaughter fish was 5,01 pr kg in 2011) (DOF 2012b). As this study has shown, some of the mortality experience in Norwegian aquaculture industry is most likely due to chronic stress or allostatic overload type 2 as observed during vaccination (**paper I and II**) and transport (**paper IV**). All these studies have shown that mortality seemed to be closely linked to excessive release of plasma cortisol in vicinity of multiple stressors. However, mortality should not be used a measure on animal welfare alone, as my colleague Robert Eliassen elegantly put it: *“Mortality rate is a crude measurement for animal welfare in finfish production, and a lot of suffering has happened before this final endpoint”*.

The science of animal welfare is of multidisciplinary nature. This science includes not only issues of animal science, but this disciplinary also have to take into consideration both ethical and philosophical issues (Dawkins 1980, 1990; Watanabe 2007). Thus, it is important to remember that the definition and the science of animal welfare is based on how animals feel (viewpoint) and not how people think animals feel or perceive a given situation (Fraser 2009; Fraser et al. 1997; Lassen et al. 2006; Rollin 1993). Thus, one could conclude that an animal has good welfare, when the following three conditions are met: 1) Functionality, the animal is adapted to the environment, and have good health, with all their biological system function; 2) Behavioural freedom; animals are given the chance to live the natural life, with the opportunity to express their behaviour as the animal would have done in the wild (behavioural needs) and 3) Physiological feelings, the animal must not be exposed to negative experiences such as pain, fear and hunger, and have access to positive experiences such as social contact with other of its kind (Huntingford and Kadri 2008). Ellis et al. (2012) meant that a straightforward aid to understanding fish (animal) welfare is to view welfare as mental state and the mental state refers to the feelings of

the fish: in relation to the environment, husbandry conditions and practices to which they are exposed to.

The question which has been raised is how to measure fish welfare in a non-communicative species as teleost represent. Stress or stress responses are often mentioned as possible mean to assess welfare. The closely associated concepts of welfare and stress may be considered as opposites since welfare cannot be achieved under stress and vice versa (Veissier and Boissy 2007). In the present thesis it is focused on the functionality and feeling aspect of animal welfare during common commercial Atlantic salmon procedures in relation to the stress response, while the behavioural freedom aspect is not of concern in this thesis, as space requirements, water quality, predator protection etc. in Atlantic salmon industry is tightly regulated in Norwegian laws in respect to the different development stages as parr, smolt and postsmolt represent (Mattilsynet 2012b).

Plasma cortisol seems to be a good candidate to assess the psychological aspect of the feeling part of animal welfare. In **papers I** and **IV** there is a correlation between high levels of plasma cortisol with reduced functionality in osmoregulation which results in unnatural high mortality. Similarly the experiment in **papers II** and **III** shows that choice of sedation or not prior to anaesthesia (**paper III**) and control of the water quality during anaesthesia (**paper II**) had impact on primary, secondary and tertiary stress responses. Most of the studies regarding fish and feelings have focused on neurophysiology and behaviour (Chandroo et al. 2004a, 2004b; Lund et al. 2007; Rose 2002; Sneddon 2006). Kerstin (1994) was one of the first using endocrine stress responses together with neurophysiology to assess good welfare and not. Cortisol measured in serum and urine is widely used in human psychobiological studies as an indicator of stress, anxiety and depression together with psychological profiling (Broadbent and Koschwanez 2012; De Weerth et al. 2013; Lucas-Thompson 2012; Pluess et al. 2012; Taverniers et al. 2011). Similar to humans, the cortisol cascade in fish is triggered by the brain (**Figure 3**) thus surge in plasma cortisol could be linked to negative feeling response or the fear response (Ellis et al. 2012; Schreck 1981). A study by Iversen and Eliassen (2012) where baseline levels of plasma cortisol was followed in

four major smolt plants (n=3684) in Nordland during commercial production from 2009 until 2011. The study showed that fish in two neighbouring 14 m<sup>3</sup> tanks had significant different average baseline plasma cortisol levels and mortality rate during a production cycle. The first tank was totally covered with a plastic tent while the other tank was only covered by anti-bird of prey net, but they shared the same water supply and had the same origin (Aqua Gen strain). The average plasma cortisol levels and mortality after 30 days in sea was 22.8 ± 9.8 nM and 1.2 %, 64.4 ± 10.9 nM and 15.3% for fish in the covered and non-covered tank, respectively. As no obvious explanation was emerging, the tanks were kept under 24 hour video surveillance. The results showed that after working hour several grey herons (*Ardea cinerea*) turned up and settled on the anti-bird net of the uncovered tank. This clearly upset the salmon smolt in the tank, and even though the anti-bird net protected the fish from physical harm the pure presence of the bird of prey was enough to elicit increase plasma cortisol with dire consequences on the animal welfare. Other studies where there were no evident physic-chemical impact stressors, has shown similar cortisol responses, which may indicate fear feeling (response) in fish. Schreck (1981) showed that the pure presences of a dip-net was enough to elicit five-fold cortisol elevation in sockeye salmon (*Oncorhynchus nerka*), and gulf toadfish (*Opsanus beta*) showed similar response in plasma cortisol to the presence of feed-searching common bottlenose dolphins (*Tursiops truncatus*) (Remage-Healey et al. 2006). Studies that show that fish is able to learn for previous stress experiences (fear) through habituation, acclimation and conditioning indicates that cortisol could be utilised as an identification of mistreatment in commercial salmon industry (Moreira and Volpato 2004; Pickering 1992; Schreck 1981). **Papers III** and **IV** suggest clear signs of psychological controlled plasma cortisol increase, as sedation with 2.5 mg iso-eugenol/L seem to dampen the excessive plasma cortisol surge compared to the non-sedated groups, and similar the fish functionality is kept intact in the groups subjected to sedation. As discussed earlier (see section 7.3), 2.5 mg iso-eugenol/L is not enough to block plasma cortisol in salmon (Iversen et al. 2003), and this stress reducing effect of sedation is a more general effect

of sedation, which may reduce the cortisol stress response by affecting the perception of the stressor, thus reducing fear.

However the debate whether using cortisol as a single parameter to evaluate fish welfare is ongoing and one share the sentiment of Ellis et al. (2012) that concluded that cortisol has little value if welfare is viewed purely from a functional (or behavioural) perspective, as the cortisol response itself is a natural, adaptive response. Nevertheless, welfare could be considered in terms of mental health and feelings, and that stress in relation to welfare could be viewed as psychological, rather than physiological. Thus, cortisol can be used (with caution) as a tractable indicator of how fish perceive (and feel about) their environment. However, a total survey of the stress response from primary, secondary and tertiary responses could give enough information to make a risk assessment about different procedures during commercial salmon production. This is performed in **paper I** which showed that as the vaccine and stress group entered allostatic overload 2 (see section 7.4), it caused grave impact on the wellbeing of the individual salmon which both hampered with the functionality and feeling aspect of the subjected salmons. These kinds of studies done in this thesis could be used to reduce the risk of jeopardising the animal welfare during common handling procedures in aquaculture, as the fish showed response of negative feeling for various mistreatments subjected during the experiments in **papers I, II, III and IV**. With some caution one can conclude that excessive stress prior to vaccination, poor water quality in the anaesthesia bath, and transport without reducing loading stress prior to transfer to sea is part of poor management, which could compromise animal welfare with dire consequences for the domesticated salmon.

## 8. Conclusion

Results from the present study has shown that if plasma cortisol becomes elevated prior to vaccination it could instigate an allostatic overload type 2 (chronic stress response) with harmful consequences on animal welfare. A plausible physiological causal relationship for allostatic overload type 2 scenarios in fish seems

to involve a oversensitivity to ACTH, reduced efficient negative feedback system, and elevated baseline levels of plasma cortisol, as documented in **paper I**.

During vaccination fish has to be anaesthetised, and poor water quality in the anaesthetic bath affects the stress response, as the primary, secondary and tertiary stress response seems to be more aggravated during high stress group in poor water quality (high CO<sub>2</sub> and no air supply), thus emphasising the importance of keeping the water quality under control during anaesthesia and under large-scale vaccination, regardless of choice of anaesthetic. There is a potential benefit of reducing the overall stress response during vaccination, as stress reducing measures have positive effects by reducing overall stress level, feed conversion, appetite and mortality. Clearly, a combination of sedation with AQUI-S vet. and Finquel Vet. anaesthesia under vaccination could reduce the overall stress response and mortality during vaccination.

Sedation during on- and off-loading in regard to transport and transfer to sea sufficiently reduced the primary stress response with positive effect on animal welfare compared to unsedated control group. Plasma magnesium differed between the groups, while plasma magnesium in the AQUI-S vet. sedated group returned to pre-stress levels 12 hours after transport. The unsedated group showed no such recovery even a week after transport. This may indicate a disturbance in the hydromineral balance, and gives a plausible explanation for the delayed mortality this group experienced. AQUI-S vet. looks promising as a stress-reducing sedative for Atlantic salmon smolts, and if used properly, it could improve animal welfare and survivability during and after salmon smolt transports.

Results from this study have further shown that mortality seemed to be closely linked to excessive release of plasma cortisol in vicinity of multiple stressors both during vaccination and transport of salmon parr and smolt. Plasma cortisol seems to be a good candidate to assess the psychological aspect regarding fish welfare, and the surge in plasma cortisol documented in this study seem to some extent to be related to the fear response in fish.

To reduce the risk of compromising fish welfare during commercial vaccination of salmon parr, one should grade the fish minimum a week prior to vaccination, or grade

simultaneously with vaccination, and keep the water quality in the anaesthesia bath under control. During transport one proposes to keep the stress response to a minimum during loading and off-loading, and if possible use sedative during the hauling process. All these proposed measures could reduce the overall allostatic load during handling, vaccination and transport, and produce a healthy fish, with intact immune response, and thus improve the fish welfare. In doing so, the salmon aquaculture industry could be able defend its reputation among consumers and the public in general, and in the same time improve the economical result by reducing overall mortality after transfer to sea.

## **9. Further perspectives**

In the future, the Norwegian aquaculture cannot continue to lose 1 in 5 fish after transfer without jeopardising its credibility among the common public. As this thesis has shown, some of the losses are due to poor management and handling procedures, which are elicited by severe stress responses.

Stress management and focus on fish welfare could benefit the aquaculture as a recent study by Iversen and Eliassen (2012) has shown. In 2008 and 2009, Mainstream Norway AS smolt productions were tainted with high stress levels during production which affected both economical results and animal welfare of individual fish in the company. Due to these negative results, Mainstream Norway AS together with Faculty of Bioscience and Aquaculture, University of Nordland, initiated a program with survey of baseline plasma cortisol during regular smolt production, and during transport and unloading at the sea site. Different stress reducing measures performed in the smolt plants and at the sea sites early in 2010 gave a positive effect on the overall stress levels and mortality in 2010 and 2011 compared to 2009. The overall stress level was reduced with 66 % and the mortality with approximately 75 % after transfer to sea in 2010 and 2011 compared to 2009. But most important, Mainstream Norway AS now produces a healthy and a high quality smolt in accordance to good animal welfare practice.

During commercial aquaculture industry one cannot eradicate all common stressors associated with handling, vaccination and transport. However, one can reduce and somewhat control the stress response during these stressors by reducing the fear response through sedation. AQUI-S vet. shows promise to become an effective sedative, as the EU-committee approved the maximum residue limits (MRL) for isoeugenol in Commission regulation (EU) No 363/2011 of 13 April 2011. ScanAqua AS (Årnes , Norway) is now applying for a trade license for use of iso-eugenol (AQUI-S vet.) in EU including, Norway and Iceland. Thus, iso-eugenol could be used legal in aquatic animals in very short time span. However, safety protocols and protocols for use in large scale handling have to be implemented and tested prior to commercialisation of the product. Until an approved effective sedative is on the market, one should try to minimise the stress response, thus it do not turn into allostatic overload type 2 scenario with dire consequence on the animal welfare and production result.

Further studies on changes in the HPI-axis during allostatic overload type 1 (acute) and type 2 (chronic response) are needed to better understand when stress turns from an adaptive to a malicious component. And similar, further studies in the relationship between cortisol (stress) and fish welfare are needed to improve our communication and understanding about the psychological component in fish endocrine stress responses. This can be done by further exploration of cortisol as indicator of negative, and possibly positive, feelings and identification of associated behavioural states. Ellis et al. (2012) also emphasised the importance of understanding how cortisol influences the sensory input (how stimuli are perceived) and the central nervous system (where behavioural integration and processing takes place) as well as the third main element of behaviour—the interaction between cortisol and mood in fish.

All these tools can be important in the future aquaculture industry as fish welfare will have important role in defining and regulating the industry. Good animal welfare will only be fully obtained when we humans are able to communicate in a satisfactory way with the fish, thus ensuring that the fish's rights and welfare are maintained during commercial aquaculture. First step on the way is to reduce the overall stress

response, thus improving the fish welfare in the short term until new and better understanding emerges regarding stress and fish welfare.



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