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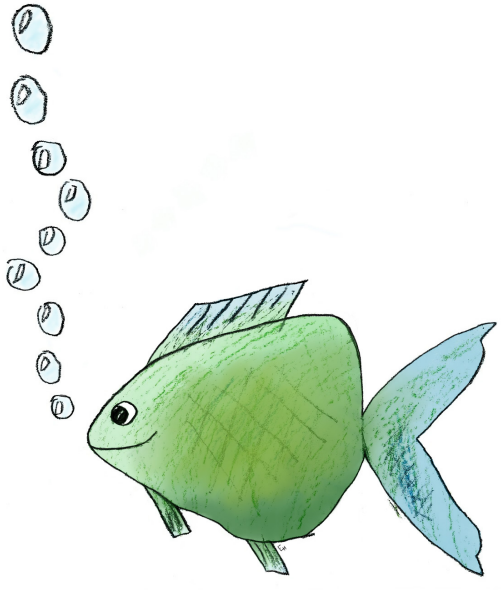
Safeguarding the Welfare of Fish in Aquaculture

Physiological Assessments of Stress and Welfare During Handling, Transport and Slaughter

Per Hjelmstedt



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Abstract

The increased demand of fish for human consumption has led to a rapid expansion of aquaculture. However, there are major knowledge gaps regarding species-specific needs, which entails an increased risk that fish are exposed to various aquaculture practices that have negative effects on their welfare. The purpose of this thesis has been to gain knowledge that can be used to assess the welfare of fish in aquaculture. To do so I have investigated various physiological indicators of fish welfare during handling, transport, and slaughter. First, the long-term effect of implantation of a heart rate bio-logger in the rainbow trout (*Oncorhynchus mykiss*) was investigated. I found no indications of impaired health three weeks after surgery, and concluded that these individuals can be assumed to represent a healthy population. The same type of bio-logger was then used, in combination with other biochemical stress indicators, to investigate the effect of repeated stress from handling, transport and slaughter in European whitefish (*Coregonus lavaretus*). The results clearly showed that the animals were stressed during crowding and brailing prior to transportation and during subsequent stunning before killing. To evaluate the reliability of a range of practical visual indicators of unconsciousness, measurements of Electroencephalogram was used, where changes in brain activity of rainbow trout before and after stunning with carbon dioxide, bolt-gun and electricity were investigated. Unfortunately, the result showed that the practical visual indicators used to assess unconsciousness was in poor agreement with the assessment based on brain activity. In summary, several important findings are presented here that can be used to improve fish welfare in aquaculture, and which can form the basis for future regulations and general advice on how fish should be handled, kept, cared for, and stunned and killed.

Keywords: EEG, stress, rainbow trout, European whitefish, stunning, cortisol, heart rate

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Vattenbruk och fiskvälfärd: Fysiologiska bedömningar av stress och fiskvälfärd under hantering, transport och slakt

Sammanfattning

Den ökade efterfrågan på fisk som livsmedel har lett till en snabb expansion av vattenbruket. Det finns dock stora kunskapsluckor om olika fiskarters behov, vilket innebär en ökad risk för att fisken i utsätts för olika moment som har negativa effekter på deras välfärd. Syftet med den här avhandlingen har varit att få ökad kunskap som kan användas för att bedöma välfärden för fisk i vattenbruket. Detta gjordes genom att undersöka olika fysiologiska indikatorer på fiskens välbefinnande under hantering, transport och slakt. Först undersöktes den långsiktiga effekten av implantation av en bio-logger i arten regnbåge (*Oncorhynchus mykiss*). Jag hittade inga tecken på nedsatt hälsa tre veckor efter operationen och drog slutsatsen att dessa individer kan antas representera en frisk population. Samma typ av bio-logger användes sedan, i kombination med andra biokemiska stressindikatorer, för att undersöka effekten av upprepad stress från hantering, transport och slakt hos arten sik (*Coregonus lavaretus*). Resultaten visade tydligt att djuren var stressade när de trängdes och håvades precis innan transport och under efterföljande bedövning före avlivning. För att utvärdera tillförlitligheten hos en rad lättillgängliga visuella indikatorer på medvetslöshet användes mätningar av elektroencefalogram, där förändringar i hjärnaktivitet hos regnbåge före och efter bedövning med koldioxid, bultpistol och elektricitet undersöktes. Tyvärr visade resultatet att de lättillgängliga visuella indikatorer som användes för att bedöma medvetslöshet inte stämde överens med bedömningen baserad på hjärnaktivitet. Sammanfattningsvis presenteras här flera viktiga fynd som kan användas för att förbättra fiskvälfärden inom vattenbruket och som kan ligga till grund för framtida föreskrifter och allmänna råd om hur fisk ska hanteras, hållas, skötas samt bedövas och avlivas.

Nyckelord: EEG, stress, regnbåge, sik, bedövning, kortisol, hjärtfrekvens

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Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Hjelmstedt, P., Sundh, H., Brijs, J., Ekström, A., Sundell, K., Berg, C., Sandblom, E., Bowman, J., Morgenroth, D. and Gräns, A., (2020), Effects of prophylactic antibiotic-treatment on post-surgical recovery following intraperitoneal bio-logger implantation in rainbow trout, *Scientific Reports*, Vol 10, 5583.

II. Hjelmstedt, P., Brijs, J., Berg, C., Axelsson, M., Sandblom, E., Roques, J., Sundh, H., Sundell, K., Kiessling, A and Gräns, A., (2021), Continuous physiological welfare evaluation of European whitefish (*Coregonus lavaretus*) during common aquaculture practices leading up to slaughter *Aquaculture*, Vol 534, 736258

III. Bowman, J., van Nuland, N., Hjelmstedt, P., Berg, C. and Gräns, A., (2020), Evaluation of the reliability of indicators of consciousness during CO_2 stunning of rainbow trouts and the effects of temperature, *Aquaculture Research*, Vol 51 (12), pp 5194-5202

IV. Hjelmstedt, P., Sundell, E., Brijs, J., Lines, Berg, L., Sandblom, J., Axelsson, M. and Gräns, A, Assessing the effectiveness of percussive and electrical stunning in rainbow trout: does an epileptic-like seizure imply brain failure?, accepted for publication in *Aquaculture*

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The contribution of Per Hjelmstedt to the papers included in this thesis was as follows:

I. Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, visualization

II. Conceptualization, validation, formal analysis, data curation, writing - original draft, visualization

III. Investigation, writing – review & editing

IV. Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, visualization

1. Introduction

The once seemingly endless food resource from capture fisheries is now facing great challenges with declining fish populations due to overfishing and poor management, resulting in that ~90 % of the wild fish stocks now are believed to be fully exploited, overexploited or depleted (FAO, 2020). Climate change, pollution and shifts in ecosystem structures also affect fish stock size, habitat availability and species distribution (Crook et al., 2015; Myers & Worm, 2003; Perry et al., 2005). To meet the demands of consumable fish for a growing world population with an increasing appetite for fish, food production from aquaculture is steadily growing. The rapid development and expansion of production systems in aquaculture has resulted in farmed fish today providing roughly half of all the consumed fish globally (FAO, 2020).

Aquaculture is defined as the farming of aquatic organisms such as algae, invertebrates and fish. Fish constitutes a large paraphyletic group of vertebrates, including more than 30 000 species and inhabits basically all aquatic environments, each with specific adaptations and lifestyles. Fish have for a long time been subjected to many misconceptions and viewed fish as non-intelligent creatures. Many of us have learned that the memory of a goldfish lasts for no longer than 3 s. However, this is far from the truth and during the last decades, researchers have found and advanced cognitive abilities of fish, including a well-developed ability for learning and memory (Laland et al., 2003), social networking (Griffiths & Magurran, 1997; Wilson et al., 2014), and complex co-operations both within and between species (Bshary et al., 2007). Two species, *i.e.* the bluestreak wrasse (*Labroides dimidiatus*) and the giant manta ray (*Manta birostris*) have even displayed behaviors indicative of self-awareness when presented to a mirror (Ari &

D'Agostino, 2016; Kohda et al., 2019). Together with the finding of nociceptors in fish and behavioral responses to noxious stimuli (Ashley et al., 2007; Correia et al., 2011; Sneddon, 2003; Sneddon et al., 2003), these finding have changed the human perception of fish and sparked attention to the treatment of fish in general. The increased understanding of cognitive abilities in fish has led to that fish now are considered sentient beings with the potential ability to experience pain and fear (EFSA, 2009). Even so, many challenges on how to safeguard the welfare of fish still exists, mostly because there are considerable knowledge gaps on how to rear, house and slaughter fish, some of which are addressed in this thesis. With respect to the animal welfare legislation, the framework of the Swedish Animal Welfare Act and Ordinance applies to all vertebrates kept by man – including fish – but no detailed regulations are in force with respect to fish in aquaculture, except some general aspects of emergency killing. Because the studies that are included in this thesis have been performed on salmonids, I have chosen to predominantly cite prior research that have investigated salmonid species. Thus, the reader is advised to recognize that statements regarding one species is not necessarily applicable for fish in general.

1.1 Aquaculture systems and fish welfare

Fish farming may have started as early as 8 000 years ago and archaeological findings show evidence of aquaculture activities for at least 2 000 years (Harland, 2019; Nakajima et al., 2019). One of the most ancient known aquaculture activities was a system where common carp (*Cyprinus carpio*) were kept in ponds and co-cultured with rice where the fish both fed on pest insects and at the same time fertilized the grains. Indeed, the same basic principle is used today in aquaponics, which are systems coupling aquaculture with hydroponics (cultivation of plants in water). The ancient pond systems were often completely extensive, *i.e.* the systems used photosynthesis to maintain a natural feed production and had low economic and labour input. The introduction of pelleted feed in the 1950s and 60s led to the development of modern semi-intensive (additional feed is provided) and intensive (commercial feed dominates) aquaculture production systems that enabled an increased output from a limited water source. This made it possible to substantially increase the number of animals kept in large sea or lake cages (*i.e.* net pens), ponds or indoors in recirculatory aquaculture

systems (RAS). These larger systems understandably increase the labor intensity and cost, and to further increase production efficiency, selective breeding, and domestication programs to develop fast growing strains suitable for aquaculture were introduced (Gjedrem, 1985; Gjøen & Bentsen, 1997). Today, more than 600 different species of aquatic animals, the majority being finfish species, are commercially farmed for human consumption (FAO, 2020), and the number of farmed fish slaughtered annually is estimated to be in the range of 50-167 billion individuals (*http://fishcount.org.uk*, Fig. 1).

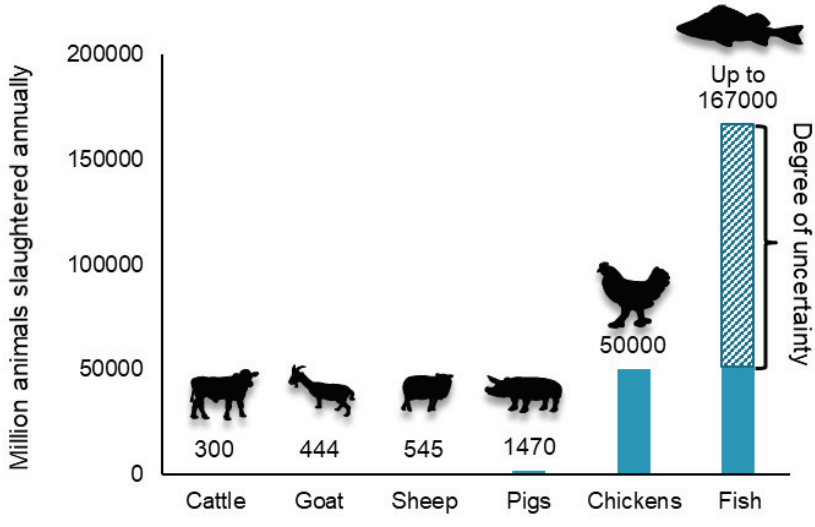

Figure 1. Number of farmed animals slaughtered for human consumption each year. It is estimated that between 51 and 167 billion fish are slaughtered in aquaculture annually, making it the largest group of farmed animals. This number is roughly equal to, or up to three times more, than all other farm animals combined. Estimated numbers of fish is taken from http://fishcount.org.uk while the number of other animals are from https://www.weforum.org. Note that the "Fish"-column includes all farmed fish species.

However, the intensification of aquaculture comes with its own set of problems. For example, wild-caught fish are used to produce fish meal and oil to feed the large quantities of farmed fish, which contributes to the

exploitation of the oceans' wild fish stocks (Deutsch et al., 2007; Naylor et al., 2000). Uneaten feed and the feces from the farmed fish can lead to eutrophication of the environments surrounding the sea- or lake based fish farms, which may negatively affect the ecosystem (Talbot & Hole, 1994). Escapees from the fish farms also risk spreading parasites and diseases, compete with wild fish populations for limited food resources, or impact offspring fitness if they breed with wild individuals which can cause conflict with *e.g*. wild fish populations and commercial fisheries (Glover et al., 2017; Naylor et al., 2005; Wiber et al., 2012). Beside these potential ecological problems, another urgent issue with fish farming is the effect it can have on the animal welfare. For example, off-shore rearing (where the fish are kept in lake or sea cages far away from land) result in limited control over environmental or anthropogenic disturbances such as extreme weather events, pollution, boat traffic and disease outbreaks that can negatively affect fish welfare. In combination with limited ability to monitor health and physical condition of the fish, a range of welfare hazards have been identified that are associated with aquaculture practices, an issue that have been highlighted and reviewed by numerous researchers, *e.g.* (Ashley, 2007; Braithwhite, 2014; Conte, 2004; Huntingford et al., 2006; Lines & Spence, 2012; Seibel et al., 2020).

Although a continuous development of rearing systems has reduced the environmental impact from aquaculture activities while production has increased, there are still many challenges remaining that are preventing us from safeguarding the welfare of fish in aquaculture. Animal welfare is a concept related to the well-being, feelings and perceptions of non-human animals. Animal welfare legislation can be found internationally and nationally for vertebrate animals (including fish) in human care, such as farm animals, laboratory animals, sports- and companion animals and wildlife in zoological gardens. The regulations for protection of farm animals are highly influenced by the five freedoms concept introduced in 1979 by the Farm Animal Welfare Committee (renamed to Animal Welfare Committee in 2019). The five freedoms refer to *freedom from* (1) *hunger or thirst*; (2) *discomfort*; (3) *pain, injury or disease*; (4) *freedom to express normal behaviour*; and (5) *fear or distress*. Although the incorporation of fish into the EU legislation regarding the welfare of farm animals, later implemented into Swedish national legislation, is an important step towards protection of

farmed fish, a recent review clearly pointed out that the lack of species-specific recommendations provide little guarantee for good fish welfare (Toni et al., 2019). In an analysis from 2020 evaluating what the extent of protection of farmed fish in Europe actually mean for the individual fish, the authors conclude that "'farmed' fishes are currently only protected by the very basic and general principles laid down in secondary EU legislation which leave room for interpretation and are partly not applicable or even contradictory to the welfare of fishes. The simple reason for this is that EU animal protection laws are designed, above all, for terrestrial 'farm' animals" (Giménez-Candela et al., 2020).

The underlying reason for these problems is not likely to be a lack of ambition from the competent authorities, or that the fish producers are indifferent to the welfare of their animals. The overarching problem is rather the enormous knowledge gaps that exist regarding optimal aquaculture practices for good welfare on a species level. During the course of my studies, I have focused on two welfare hazards that have been identified as especially concerning from an animal welfare perspective, namely stress during transport routines and at stunning during slaughter (European Commission, 2017).

1.2 The stress response in brief

When an animal is facing a physical or social challenge, *e.g.* a predator, hierarchical aggression, a change in external environment or a developmental step such as sexual maturation, a range of physiological and behavioral changes occur to temporarily optimize their performance and ultimately increase the chance of survival (see Information box). This adaptive physiological response is what is commonly referred to as the stress response and is found among animal groups. The word stress was first termed by Hans Selye who observed similar symptoms in chronically ill human patients and laboratory rats exposed to different types of aversive stimuli, and suggested a common response that is independent of the type of stressor (Selye, 1936, 1956). Based on the symptoms he observed, Selye identified three phases of the non-specific stress response, presented as a general adaptation syndrome (GAS), which consists of the *alarm phase* when the body reacts to the stressor by mobilizing resources, the *resistance phase* when the body depends on its reserves to cope with the stressor and

the *exhaustion phase* when the reserves are depleted as a result of chronic (*i.e.* long-term) stress and the body starts to become fatigued and more susceptible to disease. As our knowledge of neural and endocrine systems have expanded, Selye's somewhat simplistic definition of stress as solely a non-specific response has been revised. Yet, the importance of stress as a key element in the development of diseases or abnormal condition is perhaps more relevant today than ever. This holds true also for fish where much work has been done on their stress response system, which will be described briefly in the following section. For a detailed description the reader is referred to *e.g.* (Barton, 2002; Barton & Iwama, 1991; Wendelaar Bonga, 1997).

The concept of homeostasis, allostasis and allostatic load

All living organisms are constantly exposed to variations in their external and/or internal environment that affect their function and performance and ultimately threaten the survival of the individual. This is because most physiological functions are dependent on stability and the tolerance range for variations in the internal environment, *e.g.* ion concentration, is limited. Fortunately, the function of the endocrine and nervous systems is to handle such changes and to keep the internal environment within this functional range. The concept of an optimal internal milieu was first proposed by Claude Bernard, whose ideas were later named **homeostasis** by the work of Walter Cannon (Bernard, 1927, Cannon, 1932).This concept was later elaborated by Sterling and Eyer in 1988, arguing that stability is instead maintained by controlled physiological changes to cope with the perceived or anticipated threat or demand, a principle they called **allostasis** (Sterling, 1988). One example of this is that heart rate during the day vary depending on activity level, with low rates during rest and high rates when activity level is increased. This coping mechanism ensure that the antagonistic endocrine and nervous systems adjust to varying demands. There are, however, limitations to how severe or prolonged environmental disturbances the organism can cope with. If the animal is continuously forced to spend more energy into maintaining allostasis than it is able to absorb, the cost of maintaining allostasis has surpassed the limitation (**allostatic load**) which can have detrimental consequences for the individual (McEwen, 1993). Natural selection to different habitats and life-styles explain many of the species-specific sensitivity ranges for different environmental changes. Homeostasis and allostasis are used more or less interchangeably in the available literature, but I will from here on use the word allostasis to describe the state when the organism is within their functional limits in relation to the environment.

Information box

1.2.1 Primary stress responses

Changes in the internal or external environment, physical injury or other stimuli are detected via a range of different internal and external receptors, *e.g.* chemo-, mechano-, photo- and nociceptors. When the stimuli reaches a threshold, the neuron fire and the signal is processed in the central nervous system (CNS, *i.e.* the brain and spinal cord) eliciting the stress response. In fish, the stress response is primarily mediated via the fast-acting hypothalamic-sympathetic-chromaffin (HSC) pathway and the somewhat slower endocrine hypothalamic-pituary-interrenal axis (HPI-axis) (Wendelaar Bonga, 1997). When the HSC is activated, the chromaffin cells in the head kidney are stimulated by sympathetic innervation from the hypothalamus and release catecholamines (adrenalin and noradrenalin) into the blood stream (Reid et al., 1998). Activation of the HPI-axis cause hypothalamic release of corticotropin releasing factor that stimulates subsequent release of adrenocorticotropic hormone (ACTH) from the pituitary gland into the main circulation. ACTH reaches the interrenal tissue in the head kidney where cortisol is released into the blood stream (Barton, 2002). This is called the *primary stress response* (Fig. 2).

1.2.2 Secondary stress responses

The increase of stress hormones (cortisol, adrenalin and noradrenalin) elicits adaptive physiological alterations in a range of target organs, *i.e.* the *secondary stress response*. The main function of the stress response is to prepare the animal for increased activity by decrease functions that in short-term are non-vital, such as blood supply to the gastrointestinal tract for digestion and osmoregulation in fish, which means that blood flow can be redistributed to elevate metabolic activity in skeletal muscle, brain, heart and ventilation muscles (often called the fight-or-flight-response). In fish, heart rate, blood flow, blood pressure, gill permeability and ventilation rate increase to enable an increase in oxygen transport to the organs that are primarily active during the stress response (Wendelaar Bonga, 1997). Both catecholamines and cortisol trigger processes, such as the release of glucose and lipids into the blood stream, to mobilize substrates for the increased metabolic demand (Ashley, 2007). When the stressor is avoided, the acute phase of the stress response is over and down-regulated via the negative feedback system of the HPI-axis (*i.e.* cortisol inhibits further release of cortisol). A recovery period allow the fish to regulate and restore stress-

induced changes in their internal environment (Fig. 2). For example, aerobic metabolism is accompanied with anaerobic metabolism during intense physical activity which causes acid-base disturbances in the blood and tissue (Milligan & Wood, 1986a; Milligan & Wood 1986b; Wood, 1991). In fish, the recovery time from acidosis and down-regulation of cortisol is slow, and it may take several hours to reach pre-stress levels of cortisol and restore tissue and blood pH (Milligan, 1996).

1.2.3 Tertiary stress responses

If, however, the animal cannot cope with the stressor or if the stressor continues or is replaced by another stressor (commonly referred to as chronic stress), this can cause maladaptive *tertiary stress responses* due to the allostatic load. During chronic stress, both physiological responses and cognitive abilities can be reduced and the animal's ability to cope with the stressor becomes impaired (Braithwhite, 2014). Osmoregulation, for example, occurs actively in the gills and in the gastrointestinal organs of fish. During the stress response, gut function is inhibited and ion leakage across the gills increases, disrupting the ability to maintain stable internal ion concentrations, which can cause severe consequences in the long run with increased risk of disturbances in blood and cell chemistry. The allostatic load also make the animal more susceptible to disease or parasite infection due to decreased immune function (Nardocci et al., 2014; Pickering & Pottinger, 1989; Tort, 2011), cause reduction in growth (Gregory & Wood, 1999) and lower reproduction (Pankhurst, 2016). It has also been suggested that chronic stress is a contributing factor for the development of heart disease in farmed rainbow trout (Johansen et al., 2017) (Fig. 2).

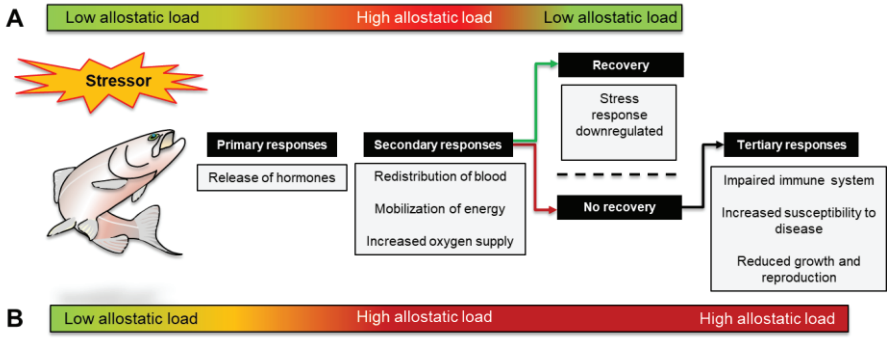

Figure 2. Schematic figure of the phases of a stress response. The primary response involves the release of stress hormones that in turn cause a range of secondary responses to enhance the individual ability to cope with the stressor, increasing the allostatic load. Following the stress response, the fish require a recover period to compensate for the increased metabolic demand and restore energy (A). The recovery period is dependent on the duration of the stressor. If, however, the stressor is maintained or is replaced by another stressor, the allostatic load is continuously high, which may induce maladaptive tertiary responses that suppress physiological functions and can impair fish welfare (B).

1.2.4 Stress as a welfare hazard in aquaculture

In aquaculture, fish are confined in an artificial or semi-natural environment with limited control over their external conditions and thus risk a limited possibility to *e.g.* perform natural behaviors or seek shelter from aggression or unfavorable changes in the ambient environment. Rearing conditions and husbandry routines can therefore become major threats to fish welfare as the fish risk being challenged with stressors from which they cannot escape, or stressors that are repeated. The fish must then spend more energy to maintain allostasis (Ashley, 2007; Conte, 2004; Madaro et al., 2020). Due to the different evolutionary adaptations among fish species, factors that contribute to acute and chronic stress are highly species-specific. For example, rainbow trout are sensitive to hypoxia while other species of fish *e.g.* the crucian carp (*Carassius carrasius*) are evolutionarily adapted to environments with extreme seasonal variations in oxygen levels (Arthur et al., 1992; Nilsson, 1990). Oxygen levels are maintained by exchange of water with the surroundings when fish are kept in sea cages and can be controlled in RAS,

but hypoxic conditions may occur when fish are transported *e.g.* before slaughter. Fish are commonly transported by boat, truck or helicopter depending on the site of the holding cage and slaughter facility, and they are normally held at high densities during transportation, which can last for several hours. Crowding risk to induce a rapid depletion of dissolved oxygen and lead to an accumulation of dissolved CO_2 in the transport tank water, which can lead to a decrease in water pH and result in acidosis in the fish (Brijs et al., 2018; Perry, 1982; Tang et al., 2009; Tovey & Brauner, 2018). It is therefore important to monitor, and prevent, deteriorating water quality during transport to avoid exposing the fish to an unnecessary stressor. Moreover, loading and unloading to the transport vessel may involve additional stress, such as air exposure and handling, which can further add to a cumulative stress response (Brijs et al., 2018). The transportation stage of the production hence risk exposing the fish to multiple stressors and cumulative stress, and it is known that mortality rates can be high post-transportation (Poppe et al., 2007).

It is thus vital to identify and prevent stressors in aquaculture that can lead to impaired welfare due to tertiary stress responses or risk of exposing the fish to severe and detrimental acute stress that they are not able to cope with. Understandably, it is challenging to monitor aquatic organisms as they inhabit an environment that is not immediately available to us. Moreover, sea cages are often located far away from land, and stocking densities in fish aquaculture is normally large which complicate health assessment on an individual level. Monitoring through advanced camera vision and hydro-acoustic equipment can be used to assess *e.g.* development, social behaviour and responses to treatments which can be used to interpret potential disturbances that may affect the animals (Føre et al., 2018). An alternative is to use a few number of selected individuals as representatives of the group, so called focal animals, which can be monitored to detect variations/changes in behaviour or physiological responses to environmental perturbations that indicates impaired welfare. This can be achieved via implantable bio-sensing tags that store and/or transmit physiological (*e.g.* heart rate), behavioral (*e.g.* swimming activity and depth distribution) or environmental (*e.g.* temperature) data that can provide continuous measurements over long periods (Brijs et al., 2021). This provide insight into the individual response to natural and anthropogenic disturbances on a relatively fine scale and can

be used to identify events that risk causing impaired welfare. One important consideration when using implants to measure natural responses and behaviours is to determine that the fish is not affected by the surgical procedure to insert the tag, or by the presence of the tag itself. It is therefore necessary to ensure that the fish has physiologically recovered from implantation and display "normal" behaviour before the collected data can be assumed to be representative of the whole group. Identifying reliable welfare indicators that do not rely on visual observations of fish or on the occurrence of detrimental tertiary stress effects are particularly important during the final days leading up to slaughter. This is because practices used immediately before and during slaughter are critical from an animal welfare perspective, and are also known to largely affect the quality of the final product (Ashley, 2007; Robb & Kestin, 2002). Stress-free slaughter is thus desirable from both a producer and a welfare point of view.

1.3 Slaughter of fish in aquaculture

The most common method to kill fish in European aquaculture is exsanguination by gill cutting or decapitation. This results in a sudden drop in blood pressure, leading to failure of oxygen transport to the brain tissue which ends in brain failure and subsequent death. Brain failure can occur before cardiac arrest, which is the definition of death of production animals at slaughter, but I will from here on discuss brain failure and its relevance when fish are stunned during slaughter. The time from cutting to brain failure varies among animal groups from seconds to a few minutes in mammals (see Holleben et al. 2010) to *e.g.* 7 min in Atlantic salmon, more than 15 min in African sharptooth catfish (*Clarias gariepinus*) and up to 30 min for turbot (*Scopthalmus maximus*) (Lambooij et al., 2004; Morzel et al., 2003; Robb et al., 2000) (Fig. 3). It has even been shown that the head of decapitated European eels (*Anguilla anguilla*) can display signs of life 8 h after the head is separated from the body (Verheijen & Flight, 1997). One explanation to this discrepancy is that fish are ectotherms and, compared to mammals, have a much lower metabolic rate. The brain of an ectotherm can thus survive for much longer than an endotherm brain when blood supply is inhibited. As metabolism in ectotherms is dependent on ambient temperatures, brain function can be maintained even longer if the water is cold. Temperature is thus a highly influential variable when it comes to

time until brain failure for ectotherm animals. Slaughter in aquaculture often include chilling of the fish to i) make handling easier and ii) improve the product quality (Skjervold et al., 2001). The consequence of this on fish welfare becomes evident reading how the time to brain failure by asphyxia in rainbow trout increases from just over 10 min to more than 3 h if the ambient temperature is decreased from 20 °C to 2 °C (Kestin et al., 1991). When determining the bleeding time to brain failure in salmon, Robb et al. further point out that they made sure to cut all four gill arches, while in reality under aquaculture conditions may be that not all vessels are separated, which the authors hypothesized would prolong the time to death even longer (2000). Moreover, as mentioned earlier, hypoxia-tolerance varies among fish species. This likely explains the temporal differences in time from cutting to brain failure between Atlantic salmon and the much more hypoxia-tolerant turbot (Morzel et al., 2003; Robb et al., 2000).

Exsanguination by cutting the throat or gills of a fish is inevitably a risk factor for impaired welfare as the fish is exposed to both stress and the procedure may cause pain. In Europe, farm animals must be humanely slaughtered, *i.e.* killing must be done without the animal experiencing unnecessary fear, pain, or anxiety. To achieve this, the fish should be stunned prior to exsanguination so that it is unable to perceive any of the negative effects of the slaughter. Stunning aims to render the animal i) unconscious by a method that is either immediate or non-aversive, and ii) so that it does not recover consciousness until blood loss cause irreversible unconsciousness and ultimately death. The long bleeding time until brain failure for fish must therefore be considered carefully when developing stunning methods in aquaculture, to ensure that the fish remains unconscious from stunning and unaware of its surroundings during the entire bleeding period. One of the biggest challenges to ensure good welfare at slaughter is how to determine that the fish has actually been rendered unconscious.

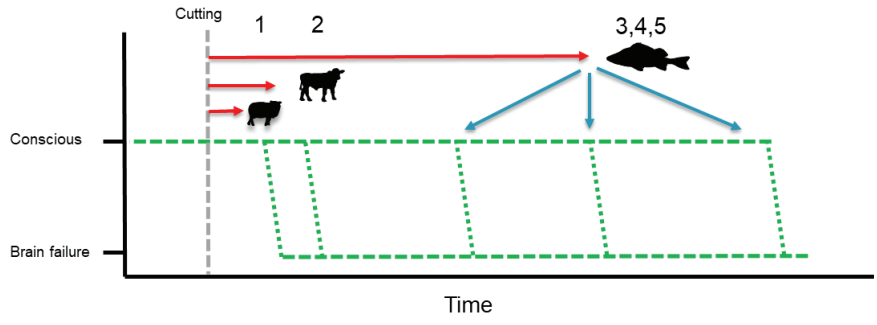

Figure 3. Schematic figure of time to brain failure from exsanguination. Brain failure have been reported to occur on average 14 s after exsanguination in sheep (1) (Gregory & Wotton, 1984) and up to 126 s in adult cattle (2) (Daly et al., 1988). For fish, the time until the brain stop responding after exsanguination is highly species-specific, ranging from 7 min in salmon (3) to 15 min in sharptooth catfish (4) and 30 min in turbot (5) (Lambooij et al., 2004; Morzel et al., 2003; Robb et al., 2000). Consequently, a fish must stay unconscious from stunning for a long period to avoid that it recovers consciousness during bleeding, indicated by the red arrow.

1.3.1 Indicators of unconsciousness

A key assumption when working with non-human vertebrates is that the neurophysiological basis shared among vertebrates is similar enough so that diagnosis of unconsciousness in humans can be used also for other animal groups (Lambooij et al., 2010; Lambooij et al., 2006). When validating different methods of stunning, in relation to slaughter, it is necessary to have reliable indicators of unconscious to determine if procedure had the intended effect. Normally, determination of stunning success in fish is done by looking for absence of certain behaviors and reflexes such as loss of equilibrium, ventilation, the vestibulo-ocular reflex, or response to handling (Sneddon, 2012). While loss of autonomous and voluntary muscle reflexes may coincide with loss of consciousness, certain stunning methods risk rendering the fish paralyzed and responsive to aversive feelings like stress, pain and distress (Bowman et al., 2019; Daskalova et al., 2015; Kestin et al., 2002; Retter et al., 2018; van de Vis et al., 2014; Van De Vis et al., 2003).

Many researchers have instead argued that a more reliable alternative to determine brain failure is to monitor brain activity via electroencephalogram (EEG) (Kestin et al., 1991; Lines & Spence, 2012; Robb et al., 2000; Van De Vis et al., 2003). This method may not always be practical to use out on a fish farm but is a useful tool when validating the effect of a stunning method, ensure proof of concept and proof of practice. EEG measures the weak electrical signals from depolarization in the brain tissue, and shifts in brain wave patterns can be used to determine when an animal is awake and responsive or not. EEG can also be used to determine if the brain of an animal is functional enough to respond to external stimuli such as sensory (*e.g.* a needle prick) audio or visual stimuli. When an animal no longer responds to external stimuli, the level brain failure is so severe that it can be assumed to be unconscious. Further description on how to record and assess brain activity can be found in section 3.3.2 and 3.4.4 and in **study III** and **IV**.

1.3.2 Methods to stun fish

In European aquaculture, fish are mainly stunned using carbon dioxide (CO_2), electricity, mechanical (percussive) stunning, or by being put in ice or in ice slurry (European Commission, 2017). Chilling and CO_2-stunning can be problematic from a welfare perspective as it does not induce immediate unconsciousness and can be stressful for the fish (Roth et al., 2006). Percussive and electrical stunning can, on the other hand, induce immediate unconsciousness and have been highlighted as promising methods to incorporate into humane slaughter protocols for some species of fish (Gräns et al., 2016). However, it is also known that an incorrectly executed stun can cause paralysis, a phenomenon observed during electrical stunning (Robb & Kestin, 2002), percussive stunning (Lambooij et al., 2010) and ice chilling (Roth et al., 2006; Skjervold et al., 2001). When using electrical stunning the fish can also recover during or even before bleeding (Brijs et al., 2020; Lambooij et al., 2013; Lines & Kestin, 2005; Robb et al., 2002). Therefore, combining electrical stunning with subsequent percussion or chilling has been suggested as a way to prevent recovery from electrical stunning for some fish species (Brijs et al., 2020; Daskalova et al., 2015; Grimsbø et al., 2014; Lambooij et al., 2013; Retter et al., 2018). There are pros and cons for all stunning methods, but efficacy is also species-specific as there are species-dependent differences in sensitivity to different stunning methods. I have focused on stunning with CO_2, percussion and electricity in

this thesis as these are the most commonly used stunning methods for salmonid species in European aquaculture. Below follows a more detailed description of these stunning methods.

CO_2-stunning

Although CO_2-stunning is not considered to meet the requirements for humane slaughter, it is still legal and used in several European countries including Sweden (European Commission, 2017). Here, the fish are placed in a tank containing CO_2-saturated water (Fig. 4), which causes an increase in dissolved CO_2 in the blood leading to long-lasting loss of consciousness and ultimately death from respiratory failure (Bernier & Randall, 1998; Kugino et al., 2016). This is a relatively cost-effective method that can be used for batch stunning of fish, but it is a slow process where the fish often display violent aversive behaviors during the stunning process. It has even been shown that brain failure is induced faster in Atlantic salmon using only exsanguination compared with a combination of CO_2-stunning followed by exsanguination (Robb et al., 2000), and other researchers have reported that exposing Atlantic salmon to CO_2 at cold temperatures (2 °C) may not induce unconsciousness at all (Roth et al., 2006).

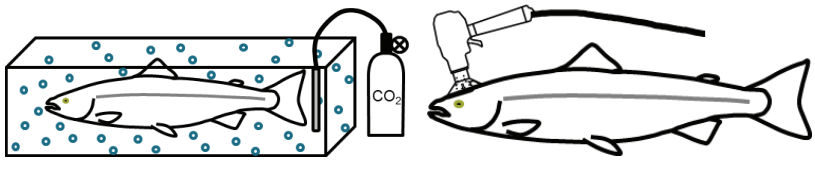

Figure 4. Stunning using CO_2 and a captive bolt-gun. On the left a fish submerged in CO_2-saturated water. On the right, the proper anatomical site for conducting euthanasia procedures in salmonids using a handheld captive-bolt gun.

Percussive stunning

The principle of percussive stunning is simply to enforce enough brain trauma to end normal brain function. Percussive stunning can be carried out manually with a special club (‘priest’ or ‘fish bonker’) or other similar tools. It can also be induced by using a specialized handheld captive-bolt gun or automated percussive stunners that are commercially developed for the slaughter of fish (Fig. 4). A powerful and correctly placed blow over the brain can induce an immediate and irreversible stun (Lambooij et al., 2010;

Robb et al., 2000; Roth et al., 2007). Although damages to the brain by force trauma is a reliable method to render a fish unconscious, there are some obvious practical considerations. Percussive stunning require that the fish are stunned individually, which is both time consuming and labour intensive when large numbers of fish are slaughtered. Moreover, percussive stunning often involves some degree of handling, which can be stressful for the fish, and it is also known that mis-stuns can occur using both manually, bolt gun or automated percussion equipment where the fish is either not stunned or recovers consciousness (Brijs et al., 2020; Kestin, 1995; Lambooij et al., 2010; Lambooij et al., 2007; Robb et al., 2000). Moreover, Lambooij et al. emphasize that percussive stunning sometimes merely paralyze the fish and that determination of consciousness cannot reliably be done using visual indicators of consciousness (2010).

Electric stunning

Electrical stunning is done by sending an electric current through the brain of the animal before slaughter. In mammals a current passing through the heart can produce an immediate cardiac arrest that also leads shortly to unconsciousness and death. However, in ectotherms like fish, a cardiac arrest is not guaranteed to shortly lead to unconsciousness as their brain can remain active and responsive long after being deprived of its blood supply. A current passing through the brain induces an immediate but non-fatal synchronous neural firing, causing a disruption of normal brain activity that render the animal unconscious (Terlouw et al., 2016). Fish are stunned with electricity by applying the current directly onto the head when the fish is transported along a conveyor belt, acting as one electrode, and passing a metal bar, acting as the other electrode, and the circuit is closed (Fig. 5). Alternatively, the fish can be submerged in water where an electric field is created between two electrodes where the fish is positioned (Fig. 5). In both cases the efficacy of the electric stun is dependent on a range of parameters such as whether using direct (DC), alternating (AC) current or a combination, the current frequency and strength. When submerged in water, electrode positioning and conductivity of the water also impact the strength of the electric field (Lambooij et al., 2008; Lines & Kestin, 2004). Electrical stunning can for some fish species be lethal if stun settings are high enough, but this increases the risk of carcass damages such as hemorrhages in the muscles and spinal injuries which lowers the product quality (Roth et al., 2003). Therefore, electrical stunning the way it is often used is a transient

stunning method where the fish risk recovering from the stun during or before bleeding (Brijs et al., 2020; Lambooij et al., 2013; Lines & Kestin, 2005). This risk can be further heightened during batch stunning in high conductivity water as high density clusters of fish have been modelled to decrease the strength of the electric field in the individual fish, which means that the stun efficacy can be unevenly distributed in the stunning tank (Lines & Kestin, 2004).

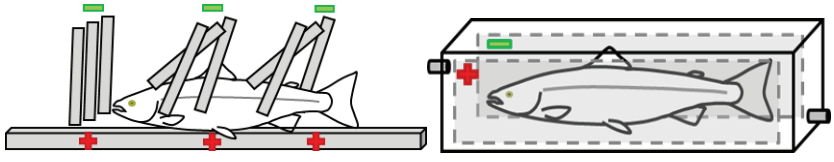

Figure 5. Stunning by exposure to electricity in air (dry stunning, left) or in water (wet stunning, right). Electricity is passed through the body of the fish when it touches the hanging electrodes during dry stunning. An electric field is created between the submerged plate electrodes where the fish is placed. The figure shows a side-to-side position of the electrodes, but they can also be positioned top-down or head-to-tail.

1.4 Concluding remark to the introduction

The aim of this introduction has been to describe some physiological, biochemical and behavioral characteristics that are shared, but also varies, within the animal group commonly referred to as fishes. I have also introduced how disturbances can cause physiological alterations which over time can challenge good animal welfare in an aquaculture context. Furthermore, the weaknesses of indicators of unconsciousness that are used to assess stunning of fish are highlighted. Next follows the methods section that describes the techniques that were used to obtain data for assessment of how common protocols for transportation and stunning of fish can affect fish welfare. In the discussion, I present the results and interpretation in both a general context and also how the findings can aid in improving both husbandry and slaughter routines of two farmed salmonid species.

2. Aims

The overall aim of this thesis was to achieve a deeper understanding on how to identify, assess and evaluate stress and unconsciousness when investigating fish welfare in aquaculture. This was made possible by investigating the following specific objectives;

- Evaluate whether a focal fish implanted with heart rate bio-loggers can be used as reliable representatives of the whole group, and to test if surgical protocol for bio-logger implantation can be refined.

- Investigate the stress response in European whitefish when exposed to a series of stressors during boat transportation and subsequent slaughter.

- Determine the effect of temperature on the time to loss of self-initiated behaviours and reflexes as indicators of consciousness in rainbow trout when exposed to water saturated with CO_2.

- Evaluate whether the abovementioned indicators can be used to determine loss of brain function.

- Examine the possibility to induce immediate loss of brain function in rainbow trout using percussive and electrical stunning, and further investigate the effect of increased stun application time and electric field strength on the time to recovery of brain function following electrical stunning.

3. Materials and Methods

This section gives a brief overview of the experimental animals and setups used in this project where strengths and limitations of the methods used are discussed. For detailed descriptions of experimental designs, methods and data analyzes the reader is referred to the methods sections in **paper I-IV**.

3.1 Animals

Rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) is a predatory salmonid species native to the northern Pacific parts of North America and Asia. It has been widely introduced to most parts of the world and is the most commonly farmed fish species in Sweden as well as an important species for commercial aquaculture in Europe (Stanković et al., 2015). Moreover, it is one of the most studied species in experimental fish research and thus a relevant species to investigate. In **study I, III** and **IV**, rainbow trout of mixed sexes were supplied by the commercial fish farm Vänneåns fiskodling, Halland, Sweden (56.53892189946023, 13.418006198877933) where the animals are kept in ponds or indoor tanks with an inflow of stream water. The three field parts of **study III** was conducted on the abovementioned commercial fish farm during autumn, winter and spring. The fish used in the laboratory part of **study I, III** and **IV** were brought to the animal facility at the Department of Biological and Environmental Sciences at the University of Gothenburg where they were kept in a recirculating freshwater system at 10 °C with a 12:12 h light:dark regime.

European whitefish

European whitefish (*Coregonus lavaretus*), used in **study II**, is a cold-water salmonid species native to central and northern Europe and is suggested to be a good candidate species for the expansion of aquaculture in the Nordic countries. To date, whitefish has received little attention from aquaculture related research activities and consequently their demands from a rearing and fish welfare perspective are poorly known. Fish farmers have reported that whitefish show signs of being sensitive to stress by displaying strong aversive reactions to routine practices at the farm such as transport. It is thus vital to scientifically investigate the impact of rearing conditions and aquaculture routines to achieve good animal welfare for this species. **Study II** took place on a fish farm at Brändö lax AB in Åland, Finland (60.455400327476916, 21.07838045149979) where fish are kept in sea cages in the Åland Baltic sea archipelago.

Ethical approval

All experiments in **study I, III** and **IV** were approved by the ethics committee for animal testing in Gothenburg (ethical permit no. 2013-177 and 2018-1873). Åland provincial government project approval committee approved the experiments in **study II** (ethical permit no 2/2016).

3.2 Physiological and biochemical stress assessment

Stress monitoring is an important part of aquaculture to ensure good animal welfare. An increased allostatic load from stress can cause changes in *e.g.* swimming activity, growth, behaviour and health which all can be used as indicators of compromised welfare (Conte, 2004; Huntingford & Kadri, 2014). Some of these indicators of impaired welfare can be monitored using computer imaging and tracking (Barreto et al., 2021; Martins et al., 2012). One advantage of such methods is that the animals can be monitored continuously without disturbances from human presence, but it can be difficult to define and quantify “normal” behaviours, and behaviours may also vary among species (Martins et al., 2012). An alternative or complement to monitor behavioral and physical indicators of poor welfare is measurements of physiological responses to stress. Although stress *per se* does not necessarily imply poor welfare, it can be used as an indicator of deteriorating welfare as the negative consequences of prolonged or repeated

stress are well known (Huntingford & Kadri, 2014). For stress assessment, a combination of physiological and biochemical markers of primary and secondary stress responses were evaluated in **study I** and **II** as described below (Fig. 6 and 7).

3.2.1 Sampling and analyses of stress indicators from whole blood

Blood was collected from the caudal vessels in the euthanized fish using a heparinized syringe in both **study I** and **II**. With this method only one sample is obtained from each animal which gives little temporal information. It is possible to conduct repeated blood sampling from the same individual fish using a catheter and cannulation techniques (Axelsson & Fritsche, 1994). Although, such techniques can give important information on the dynamics of *e.g.* a stress response, the fish needs to be confined in order to have access to the catheter making it unfit for the experimental setup used in my studies. On the other hand, there is large individual variations in susceptibility to stress, which also can introduce confounding variables when comparing measurements from different individuals (Sørensen et al., 2013). To minimize such effects, the investigated variables were instead compared using the means from multiple individuals sampled at the same time to detect changes between events in **study II**. Repeated sampling of heart rate was used to assess cumulative stress and recovery from stress when the fish is exposed to several succeeding stressors.

Primary stress response indicators

As the method of blood sampling used here requires handling that in itself is stressful for the animal this is something that needs to be considered when deciding what stress indicators to investigate in a study. For example, the catecholamine hormones adrenaline and noradrenaline are involved in the HSC pathway in fish and are quickly released into the circulation during a stressful situations, and it is thus difficult to avoid sampling-bias from circulating catecholamine levels (Ellis et al., 2011; Reid et al., 1998). The delay between the activation of the HPI-axis and the subsequent increase in plasma cortisol makes it possible to obtain baseline cortisol levels by blood sampling that is not affected by stressful handling, given that blood is sampled relatively fast before circulating cortisol levels increases. This have led to that cortisol has become the most common primary stress hormone to measure and is a widely used indicator for stress assessment in fish. Cortisol

levels have been extensively investigated both during rest and stressful situations for several fish species, including rainbow trout (Culbert & Gilmour, 2016; Pickering & Pottinger, 1989; Pottinger & Pickering, 1992; Sloman et al., 2001).

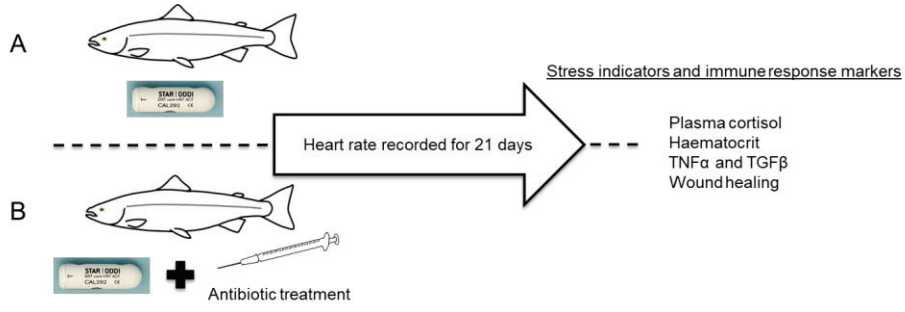

Figure 6. Experimental setup for study I. Rainbow trout implanted with a bio-logger were left to recover for 21 days while heart rate was recorded throughout the experiment. At the end of the trial, a range of stress indicators and immune response markers were sampled and analyzed and compared between a control group (A) and a group that had received an intramuscular injection of a broad-spectrum antibiotics prior to surgery (B).

Cortisol can be sampled using alternative methods that have little or no negative effects on fish welfare (Sadoul & Geffroy, 2019). The least invasive method is to use water samples to analyze cortisol levels, but this makes the individual response difficult to assess and is expensive to analyze (Ellis et al., 2004). Also, mucus, fecal and scale samples can be analyzed for cortisol (Cao et al., 2017; Simontacchi et al., 2008). Mucus samples provide lower cortisol concentrations compared to plasma, and there is a delay in feces excretion after the stress response so the temporal accuracy is lower using this method (Sadoul & Geffroy, 2019). Cortisol analyses from scale samples have been shown to be a good indicator of chronic stress in fish but is not suitable to determine an acute stress response (Aerts et al., 2015). As cortisol is released in the blood, blood sampling is a relatively easy and accurate method to assess circulating cortisol levels at a specific point in time. In **study I** and **II**, whole blood was centrifuged to separate the red blood cells from the plasma which was then analyzed for levels of cortisol using a radioimmunoassay (RIA) described by Young (Young, 1986).

Secondary stress response indicators

Increased levels of circulating cortisol and catecholamines typically result in a range of secondary physiological changes that ultimately prepares the fish for increased activity, *i.e*. to systemically enhance availability of aerobic substrates (*i.e.* energy and oxygen) to meet an increased metabolic demand. Therefore, the blood sampled in **study I** and **II** was also analyzed for various secondary stress response markers. Catecholamines stimulate the release of stored glucose into the blood plasma, and an increasing glucose level is a commonly used indicator of stress (Ackerman et al., 2000; Mazeaud et al., 1977). Plasma glucose levels was analyzed using a glucose assay kit. To elevate oxygen availability during exercise or when exposed to a stressor, fish can increase the blood oxygen carrying capacity by splenic release of red blood cells (RBC) which cause an increase in hemoglobin concentration [Hb] in the blood (Pearson & Stevens, 1991). Hemoglobin is the oxygen-binding protein in the RBC, and [Hb] was measured in whole blood using a hand-held Hb analyzer in **study II** (Clark et al., 2008).

Also, hematocrit (Hct) was measured in **study I** and **II** which represents the relative fraction of RBCs in the blood. An increase in Hct is indicative of stress as several mechanisms that are involved in the stress response can cause the relative RBC volume to a rise. The abovementioned splenic release of RBC is obviously one explanation, but Hct can also become elevated due to fluid transport from the blood to compensate for increased blood pressure, or by RBC swelling (Olson et al., 2003; Pearson & Stevens, 1991). Hct was measured by centrifuging whole blood in a microcapillary tube, thus separating the RBCs from the plasma, and calculate the % of RBCs in relation to the full volume. To estimate the contribution of increased RBCs to the overall increase in Hct in **study II**, the mean corpuscular hemoglobin concentration (MCHC, [Hb]/Hct × 100) was calculated, which is the correlation between an increase in hemoglobin and RBC volume.

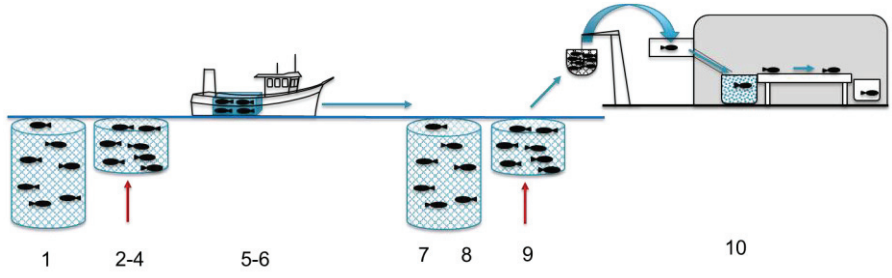

Figure 7. Schematic view of the fish transport in study II. European whitefish were exposed to a series of potentially cumulative stressors during transportation from the sea cage to the abattoir. Twenty focal animals (in a school of 5000 conspecifics) was implanted with a bio-logger that recorded heart rate throughout the transportation. Heart rate and blood samples were first analyzed in undisturbed fish (1), where after heart rates were analyzed during a range of crowding events (2-4). From the start of transportation (5) to the end of it (6), blood was sampled at regular intervals to detect the effect of transportation. When the fish had arrived at the dock, they were transferred to a recovery cage where blood was sampled every 12 min for 4 h (7). After 10 h of recovery (8), blood was sampled once again to assess for recovery from transportation. The fish were crowded and blood was sampled before brailing for transfer into the abattoir, (9). The last blood sampling was performed after exposure to CO_2 used for stunning (10).

3.2.2 Recording of heart rate using implantable bio-loggers

The use of bio-loggers in focal animals as representatives of a whole population can provide robust information on both acute and long-term effects of stressors from anthropogenic or environmental disturbances (Brijs et al., 2021). Heart rate varies depending on relative contribution of input from the antagonistic sympathetic and parasympathetic nervous systems (Taylor, 1992). During a stress response, the adrenergic (sympathetic) input increases which cause an increase in heart rate in many fish species. Heart rate loggers have been shown to provide reliable recordings of heart rate, and that heart rate is a good indicator to detect responses and recovery from exposure to different stressors related to aquaculture practices (Brijs et al., 2019; Svendsen et al., 2021; Warren-Myers et al., 2021). Compared to other methods to collect indicators of stress, such as blood samples or video

monitoring, bio-loggers can store continuous long-term data and provide high resolution data of sudden physiological responses (https://www.star-oddi.com/).

In **study I** and **II**, heart rate was sampled for 21 days to investigate temporal variation and responses to stressful events. Each sampling point consisted of a burst of measurements lasting for 6 s, where heart rate was calculated as the mean time of the R-R-intervals. The logger was programmed to sample every 10 min throughout 21 days in **study I** and for 19 days in **study II**. During the 2 final days in **study II**, the sampling rate was set to record every 2 min to get a higher resolution of changes in heart rate during the transportation events. Heart rate was calculated as the mean heart rate of all implanted fish for each sampling time point. The analysis of resting heart rate was based on a method used for determining standard metabolic rate in fish (Chabot et al., 2016), and consists of calculating the 20^{th} percentile of recorded heart rate values for each individual. To account for the stress caused by surgery, *i.e*. handling, air exposure, potentially painful stimuli and anesthesia, the time until the fish had recovered following implantation was carefully investigated (Altimiras & Larsen, 2000; Jepsen et al., 2001). If the experimental animal is exposed to additional stress before recovery, there is an increased risk of detrimental stress effects that negatively affect the welfare of the animal. Moreover, a fish that is not fully recovered may have inhibited adaptive responses compared to recovered fish, leading to that experiments performed on fish with an elevated stress response must be interpreted cautiously (Altimiras & Larsen, 2000; Brijs et al., 2019; Gräns et al., 2014).

3.2.3 Bio-logger implantation and the effect on fish health

It is necessary to recognize that logger implantation is an invasive procedure that will inflict short-term stress and immune responses on the investigated animals and thus allowing the animal to recover from surgery it critical in order to obtained unbiased data from the investigation. It is equally important to ensure that implantation does not cause long-term effects on fish health that may negatively affect the welfare of the experimental animal and alter behaviors or physiological responses. Consequently, the effect of bio-logger implantation on the inflammatory and stress responses in rainbow trout were assessed in **study I.**

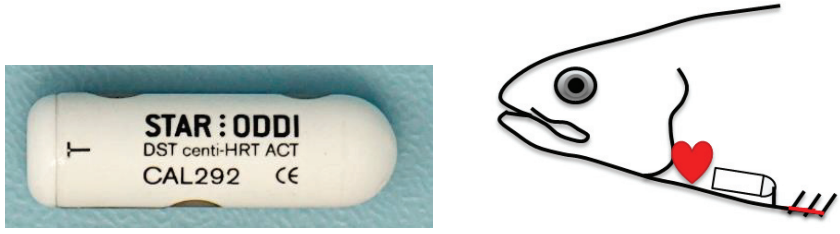

Figure 8. The Star-ODDI bio-logger used in study I and II. The logger was anchored to the abdominal muscles posterior to the heart to ensure that it stayed in optimal position throughout the trial and did not move or shift position, as described by Brijs et.al. 2018.

The bio-loggers were inserted into the abdominal cavity of the anesthetized fish via a ~4 cm mid-ventral incision between the pectoral and pelvic fins and anchored to the muscle tissue to ensure that it remained in proximity to the heart to get the strongest possible signal (Fig. 8). The surgical procedure was performed by two surgeons. An incision results in tissue damage that causes an innate immune response to facilitate wound healing (Richardson et al., 2013). The cells involved in the immune response communicate via a range of cytokines. Briefly, the tissue repair mechanism involves initiation of an inflammatory response which is primarily triggered by leukocyte release of pro-inflammatory cytokines (Schmidt et al., 2016). These cytokines upregulate the inflammation by promoting migration of leukocytes to the site of the damaged tissue. This phase is characterized by reddening and swelling of the inflamed tissue. The inflammatory response is down-regulated via negative feedback of anti-inflammatory cytokines that are released once the inflammatory phase has passed (Zou & Secombes, 2016). To investigate whether a prophylactic antibiotic treatment affect the inflammatory immune response, level of local inflammation and systemic inflammation markers three weeks after logger implantation were determined in **study I**.

Degree of inflammation of the incision wound and around the sutures were visually assessed from a photo that was taken after the fish had been euthanized, and scored for wound healing according to Wagner *et. al.* (2000) (Fig. 9). The wound was rated from 0 (incision closed, no inflammation) to 6 (wound completely open with severe inflammation), and the entry and exit

sites of the sutures were assessed for absence (0) or presence (1) of inflammation. Presence of inflammation was determined when the skin around the edges of the wound or around the suture points were red and swollen and the rating was performed by two investigators independently. Tissue from the head kidney, one of the primary organs involved in the immune system, was dissected out to determine mRNA transcript levels of expression of two key cytokines that act as systemic inflammation markers, the pro-inflammatory tumor necrosis factor alfa (TNFα) and anti-inflammatory transforming growth factor beta (TGFβ). TNFα expression increases during the acute phase and regulate inflammation by leukocyte recruitment indicating a systemic inflammation response. TGFβ act as an immune-suppressor that can downregulate some pro-inflammatory responses by *e.g.* inhibition of activated leukocytes (Reyes-Cerpa et al., 2012; Zou & Secombes, 2016). mRNA transcript levels of the cytokines were obtained using qPCR and gene expression determined using the ΔC_T-method.

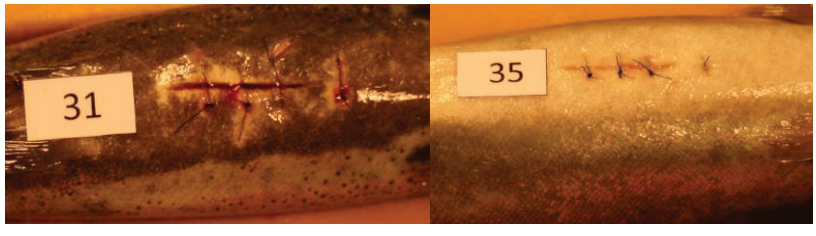

Figure 9. Examples of incisions and sutures in rainbow trout three weeks after implantation (study I). In fish no 31, the wound was not fully closed and suture entry and exit points display signs of inflammation, *i.e.* redness. In fish no 35 the wound was closed without signs of inflammation around the edges and no inflammation was seen on any of the suture points.

3.3 Indicators of unconsciousness

A range of behavioural, reflex and neurophysiological characters were used as indicators of unconsciousness following stunning in **study III** and **IV**. Presence and absence of these indicators was continuously monitored before and after stun application using visual observation and EEG-measurements. EEG has been used to investigate loss of brain function during stunning and

killing of fish since the beginning of the 90's when Kestin et. al. used it to determine onset of brain failure and loss of physical behaviour in rainbow trout killed by asphyxia (Kestin et al., 1991). Over the last 30 years, other research groups have successfully recorded EEG in several fish species to investigate the efficacy of different stunning methods (Lambooij et al., 2010; Retter et al., 2018; Robb et al., 2000). However, due to technical difficulties of invasive electrode-implantation the available literature on this subject is still sparse. In **study III** and **IV**, I have used modified non-invasive technique for EEG recordings in fish that I was involved in developing during my PhD studies (Bowman et al., 2019) (see 3.4.4).

3.3.1 Loss of visual indicators of unconsciousness

Loss of equilibrium, the vestibulo-optic response and ventilation has been proposed to indicate step-wise depression of different parts of the brain, representing different stages or planes of anesthetic depth (McFarland, 1959; Sneddon, 2012). The time it took for rainbow trout to lose visual indicators during CO_2-stunning in **study III** were determined by observation. The fish was placed in a glass aquaria during stunning and time to loss of equilibrium was thus possible to monitor without disturbing the fish. Presence or absence of the vestibulo-optic response was assessed every 30 s by carefully grab and hold the fish in the tank and slowly tilt it back and forth to determine if the eyes followed the movement. Ventilation was carefully observed throughout stunning and the time was noted when the opercular movements stopped.

In **study IV**, only loss of ventilation was monitored as a complement to the EEG. Here, also absence or presence of ventilation was determined from the raw EEG-signal rather than from visual observations. The reason for this is that all opercular movements could easily be observed on the raw EEG-signal while visual observation of ventilation was difficult the darkened experimental room (Fig. 10).

3.3.2 Neurophysiological indicators of unconsciousness

After the discovery of electrical activity in mammalian brain neurons in 1875 by Richard Caton, the first human EEG was recorded in 1924 by Hans Berger (mentioned in (Haas, 2003)). EEG measures electrical impulses (currents over the cell membrane) in the brain. An EEG is normally divided into different frequency bands; low frequency delta and theta brain waves (0.5-4

and 4-8 Hz, respectively), and high frequency alfa and beta brain waves (8-12 and 13-32 Hz, respectively). Low frequency brainwaves are, roughly, associated with drowsiness and sleep, while the high frequency activity is related to an awake to alert state in humans. EEG is also an important tool for clinical diagnosis of epilepsy and other brain disorders in humans. During an epileptic seizure (often referred to as a *grand mal* seizure, an epileptiform insult, generalized tonic-clonic seizure or general epileptic insult), abnormal brain wave fluctuations can be observed on the EEG and the person is unconscious during this stage. In fish a similar state can be induced and is from here on referred to as an epileptic-like insult. An EEG can be further analyzed for different changes in brain activity that is indicative of unconsciousness.

Median frequency and relative power of frequencies of the EEG-signal

A shift from high (8-32 Hz) to low (0.5-8 Hz) frequency brain waves has been used as an indicator of transition into unconsciousness in *e.g.* chickens, calves and fish (Gerritzen et al., 2004; Gibson et al., 2009; Lambooij et al., 2006). Changes in frequencies can be detected using either the median frequency of the EEG or by analyzing the relative power of frequencies, *i.e.* the contribution of each frequency band to the overall EEG. In **study III**, the EEG-signal was filtered using a 0.5-32 Hz band-pass filter to eliminate noise from disturbances such as movements of the fish and ambient electrical noise. The median frequency of the EEG (0.5 - 32 Hz) was determined for each minute the fish were exposed in the CO_2-stunning tank and compared to the median frequency before stunning to determine temporal changes in median brain wave frequency. To determine the relative power of high vs low frequency brain waves, the EEG was divided into separate alfa, beta, delta, and theta frequencies. The relative contribution to the overall signal of each frequency band was calculated for each minute the fish were in the stunning tank and compared to the relative power before stunning.

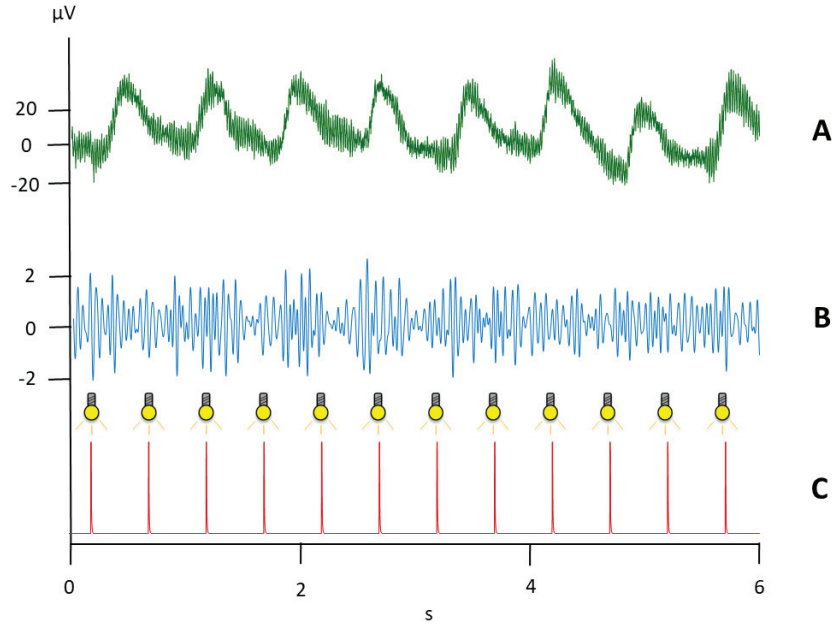

Figure 10. Signals from raw and filtered EEG as well as the light detector. The raw EEG-signal (A) was used to determine absence or presence of ventilation in **study IV**. The filtered beta-signal (13-32 Hz) was analyzed for neurophysiological indicators of consciousness and unconsciousness in **study III** and **IV** (B). The light flashes are time-locked to the light stimuli, triggered by signal from the light detector (C).

EEG-signal amplitude

A decrease in EEG-signal amplitude can also be used as an indicator of a transition into unconsciousness, and it has been suggested that unconsciousness is reached when amplitude is reduced to <50 % of pre-stun amplitude in calves, and profound brain failure occur when amplitude is <12 % (Gibson et al., 2009). Signal amplitude have also been used as indicator to determine loss of consciousness in anesthetized rainbow trout (Bowman et al., 2019). In **study III**, amplitudes were determined as the maximum – minimum amplitude of the filtered (0.5-32 Hz) EEG-signal for each minute after the fish had been placed in the stunning tank and compared to the amplitude prior to stunning to determine when amplitude decreased to <50 % and <12 % respectively.

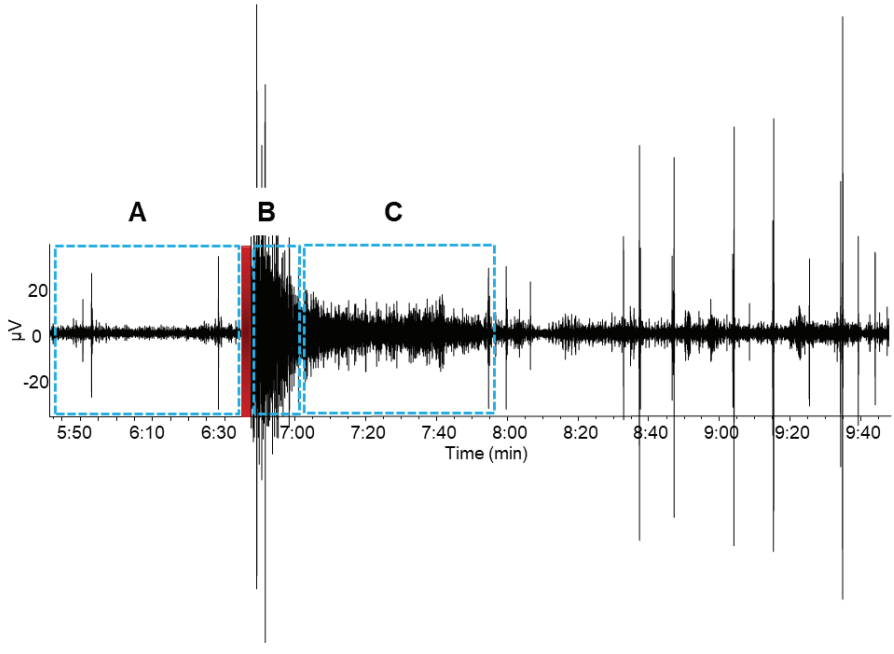

Figure 11. Image of an epileptic-like insult following a 1 s electrical stun. Compared to the pre-stun amplitude of the beta frequency EEG (A), the insult is easily distinguishable with a clear increase in amplitude (B) followed by a period with lower but still increased amplitude (C). The amplitude is then reduced to similar levels as before the stun. The occasional spikes seen after 8:30 are from gasps, which is most likely a reflex triggered by hypoxemia.

Epileptic-like insult

A tonic-clonic (*grand mal*) seizure in humans occurs when all parts of the brain are stimulated with rapid depolarization of brain cell membrane potential and involve both stiffening (tonic) and spasms (clonic) of muscles. This is characterized by an abnormal EEG with high amplitude polyspike activity during the tonic-clonic phase (Blumenfeld, 2012). A human is unconscious and unresponsive to stimuli during a *grand mal* seizure and this phenomenon is, by analogy, assumed to indicate unconsciousness also in other vertebrates (Lambooij et al., 2006). A similar tonic-clonic seizure (here referred to as an epileptic-like insult) can be induced in a various fish species, including rainbow trout, by passing an electric current through the brain

(Lambooij et al., 2008; Lambooij et al., 2006; Lambooij et al., 2007; Robb et al., 2002). In **study IV**, presence of an epileptic-like insult was determined visually as a period with increased EEG amplitude in the delta frequency band (13-32 Hz) immediately following a 1 s stun application (Fig 11). In addition, the duration of the epileptic-like insult was determined, with a first period with very high activity and amplitude and a second period where amplitude has decreased but remains considerably higher than pre-stun amplitude (Fig. 11).

Assessment of visually evoked responses (VERs)

Visually evoked responses (VERs) refer to electrical potentials, initiated by brief visual stimuli, which are recorded from the scalp and extracted from the EEG by signal averaging. In a healthy conscious animal VERs can easily be observed on the EEG. When this ability is abolished, *i.e.* the animal does not respond to external stimuli, it can be assumed that it is unaware of its surroundings and in an unconscious state. Also in fish, loss of VERs have been used as a indicator of unconsciousness following stunning of fish (Jung-Schroers et al., 2020; Kestin et al., 1991; Kestin, 1995; Retter et al., 2018; Robb et al., 2000; Robb & Roth, 2003). When the light hits the photoreceptors in the eyes, the signal from the stimuli is processed in the brain of the fish. When brain failure occurs, the brain becomes unable to process the stimuli and VERs are no longer distinguishable on the EEG (Fig. 12). It has been argued that VERs, in contrast to assessment of brainwave amplitude and frequency, are less subjective and "can be used to assess indirectly the level of brain function provoked by any slaughter method" (Kestin et al., 1991). Kestin et al. further emphasize that absence or presence of VERs is not a measurement of absence or presence of consciousness, but can be used as a strict indicator of complete loss of brain function (1991).

VERs were induced using a light flashing with a frequency of 2 Hz in **study III** and **IV**. One advantage of using visual stimuli over somatosensory stimuli (*e.g.* a repeated touch or a needle-prick stimulation) is that it can be delivered repetitively and continuously over a long time and thus evoke many responses. The flashing light also triggered a light detector that was connected to the recording equipment (Fig. 12). Every time the detector registered the light stimuli, a 500 ms time window (epoch) from the delta frequency EEG was stored (13-32 Hz). 120 epochs were averaged into an image which represent the average brain activity of 1 min of continuous

EEG-signal (Fig. 12). As each epoch is time-locked to the light stimuli, the brain response to the flashing light becomes visible on the image while the electrical impulses not related to the light stimuli is filtered out. This is also a practical way to confirm that the signal is in fact an EEG.

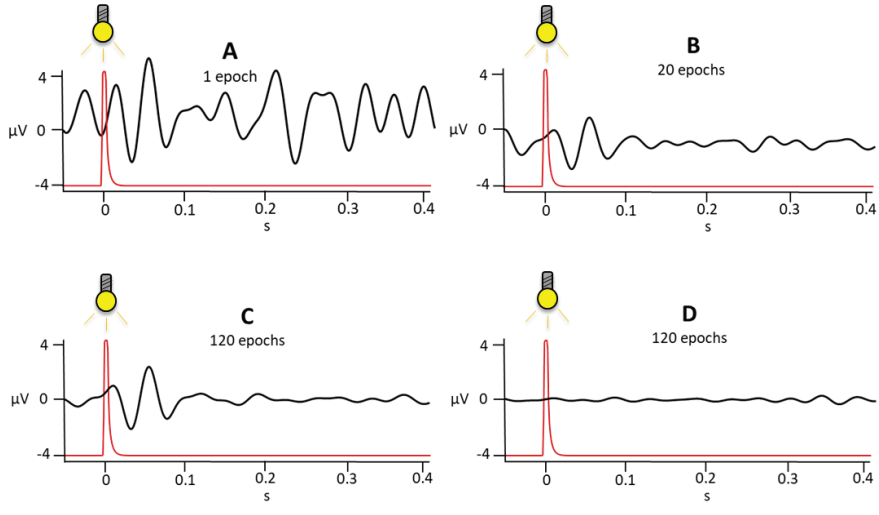

Figure 12. Determination of presence or absence of VERs. The red line is the signal from the light detector that time-locked each epoch. A single epoch (450 ms of EEG) prior to stunning (A). Here, the VER is present but cannot be distinguished from the rest of the signal. When 20 epochs are averaged VERs can be identified but the signal to noise ratio is often quite low (B). When all 120 epochs are averaged VERs can clearly be determined present and influence from other electrical impulses are effectively filtered out (C). When the fish is successfully stunned, no VERs are seen on the EEG (D).

Presence or absence of VERs following CO_2-stunning were determined visually from the averaged image for each minute in **study III**. VERs provide robust measurements of when an animal suffer from complete loss of brain function, but there are some important pitfalls that must be considered. For percussive and electrical stunning used in **study IV**, VERs were (often) lost immediately following stunning. The resolution of the averaged image is dependent on number of averaged responses, so while 120 averaged responses provided a clear image of VERs, the precision in time decreases

(Fig. 12). To increase accuracy of time to recovery of VERs in **study IV**, the images after stun application were then un-averaged and re-averaged with a new set of consecutive epochs to fine-tune the resolution.

3.4 Fish stunning and EEG recording

All animals were stunned individually using CO_2 (**study III**), percussive or electrical stunning (**study IV**). Loss and recovery of indicators of consciousness were monitored continuously for each individual.

3.4.1 Setup for carbon dioxide stunning

To induce CO_2 stunning in **study III**, rainbow trout was transferred by a dip-net to a 70 l tank that had been bubbled with CO_2 until saturation, determined by a decrease in pH to < 5 (Fig. 13). The fish was left in the tank for 12 min while the following visual indicators were noted; times to loss of equilibrium, vestibulo-ocular reflex and ventilation. When the stunning was over, the fish was transferred to another tank containing aerated water and signs of recovery were observed for 10 min. This protocol was then carried out during three different seasons which enabled me to explore the effect of different seasonal water temperatures (*i.e.* 2, 8 and 14 °C) on the induction time to loss of visual indicators. In the second laboratory part of **study III**, the protocol had to be slightly adjusted to enable EEG-measurements. The fish was first lightly anesthetized to facilitate EEG-electrode attachment, and subsequently placed in a recovery tank for 10 min while EEG was continuously recorded. Once the signal was clear, EEG was recorded for another 10 min but this time with a flashing light stimulus to induce VERs. The fish was then transferred to the 70 l tank containing fully CO_2-saturated water and kept there for 30 min. Here, time to loss of equilibrium, ventilation change and loss of ventilation was noted while EEG was continuously recorded.

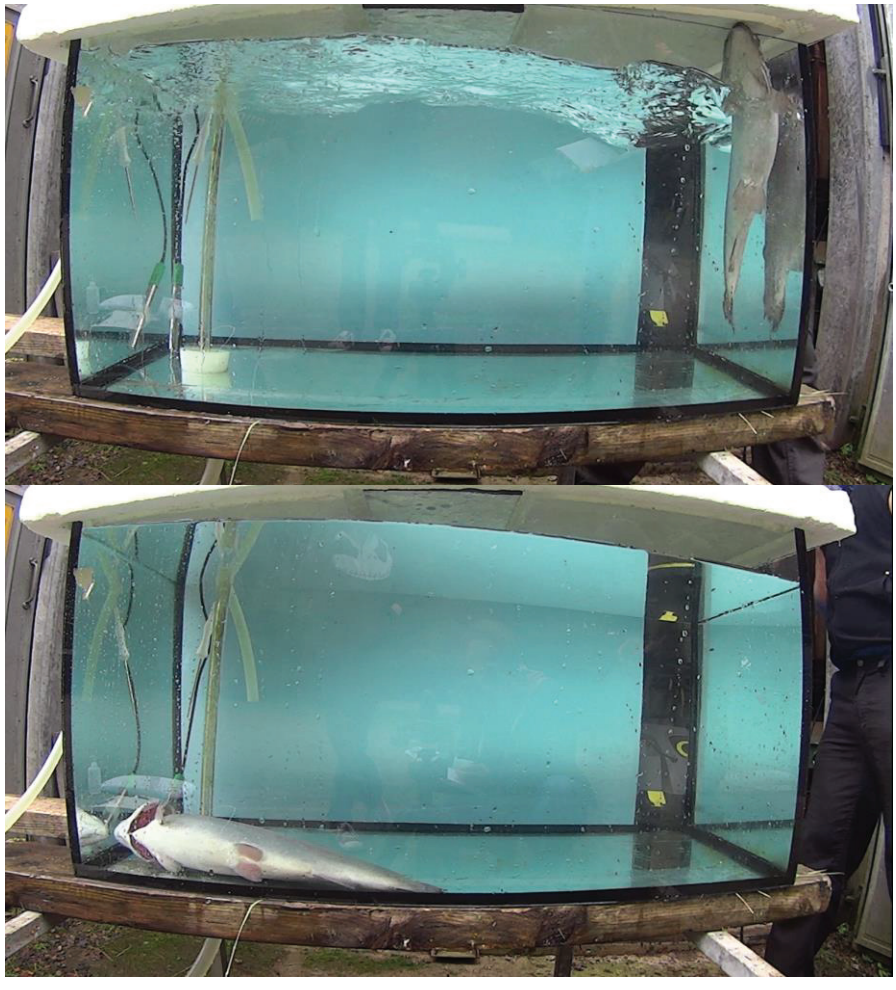

Figure 13. Fish in experimental tank during study III. The fish display strong aversive behavior immediately after being put into the tank saturated with CO_2 at a temperature of 14 C° with escape attempts, and the fish take deep gasps over the surface (top image). The fish eventually loses equilibrium, the vestibulo-ocular reflex and ventilation, but gasps was observed frequently for several minutes, which can be seen in the bottom image where gills are flaring during a gasp.

3.4.2 Setup for percussive stunning

The protocol for percussive stunning in **study IV** started with attaching the EEG-electrodes on the head of the fish. To ease handling and electrode placement, the fish was first lightly sedated. Once the electrodes were in position, the fish was transferred to an opaque 9 l flow through experimental tank that was gravity fed with aerated water. The EEG was monitored for presence of VERs to ensure that brain activity was recorded and that the fish was fully awake and responding to the flashing light before being stunned. When signal strength was stable and verified, the electrodes were removed and the fish was placed on a bench where it was stunned with a single blow to the head over the brain to inflict cerebral concussion or brain hemorrhages (Fig. 14). The blow was delivered using a commercially available non-penetrative captive bolt gun, driven by pressurized air (125 PSI). The equipment ensure that the percussive force does not vary between blows, which can occur when using *e.g.* a priest (Brijs et al., 2020; Kestin, 1995).The equipment is designed for commercial stunning of salmonids with a bolt made out of a hard plastic that shoots out and back in again in less than 5 ms. The firing end of the gun is designed to aid in aiming and keep the gun firmly in place during the blow (Fig. 14). Quickly following stunning (< 30 s), the electrodes were re-attached, the fish transferred to the experimental tank were EEG was recorded and movements was observed for 30 min.

Figure 14. The head of a rainbow trout after percussive stunning (left). The non-penetrative bolt gun used for percussive stunning in study IV (right).

3.4.3 Setup for electrical stunning

The electrical stunning in **study IV** was conducted by exposing rainbow trout to a side-to-side electric field when submerged in water in a 9 l flow through experimental tank. Before being placed in the experimental tank, the fish was lightly sedated to ease handling when EEG-electrodes were attached to the head. Once the fish had recovered from sedation and clear and steady VERs were present, the purpose built stunner was turned on which created an electric field between the electrodes that were positioned along the sides of the tank and the electric current passed through the fish. EEG was continuously recorded for 30 min following the stun to be able to analyze loss and recovery of VERs and ventilation (Fig. 10, 12). Water conductivity was kept at ~1000 µS cm^{-1} to ensure that the current density did not differ between the stuns. The first part of electrical stunning was to determine the minimum stun settings to induce an epileptic-like insult (see description above), used as a proxy of a successful stun, following a 1 s stun application. The second part aimed to prolong the time of brain failure which was done by i) increasing electric field strength and current density and ii), increasing the time of stun application, as both current magnitude and exposure time is known to affect the time to recover from an electrical stun in rainbow trout (Robb et al., 2002). As the unrestrained fish never had to be removed from the tank, EEG recordings could be done continuously except during the actual stun application (< 1 s recording was lost).

3.4.4 EEG-recording

Traditionally, EEG electrodes are attached to the skin of the animal, but they can also be surgically implanted directly on the brain or cortex, usually referred to as electrocorticography (ECoG). The skin properties of fish makes external electrode placement challenging, and previous studies on fish EEG have thus normally relied on surgically implanted electrodes (Kestin et al., 1991; Kestin, 1995; Lambooij et al., 2006; Retter et al., 2018; Robb et al., 2000). The non-invasive technique for EEG-recording that was developed for **study III** and **IV** instead uses electrodes fixed to a flexible silicone cup that was secured to the skin of the fish by creating a negative pressure using a vacuum pump (Fig 12, Fig 13, c1, c2). This technique has been validated and used to record EEG in both rainbow trout and African sharptooth catfish (Bowman et al., 2019; Brijs et al., 2020). For subsequent analysis of the EEG, the signal was amplified and filtered with a bio-

amplifier, sampled using an acquisition hardware, monitored and analyzed using a physiological data analysis software and saved on a PC (Fig. 15).

External electrode placement might give a somewhat lower signal strength and increase the risk of ambient interference or muscle activity in comparison with surgically implanted electrodes. However, the advantage of easy attachment on a lightly sedated fish that is able to move relatively freely outweighs the highly invasive alternative that require surgical anesthesia and surgical intervention which likely elicit a stronger stress response. This also enable quick removing and reattachment of the electrodes if the stunning method requires so (*i.e.* percussive stunning). However, the non-invasive technique may, in the current design, be limited by morphological differences among fish species. For example, we have experienced that the EEG-signal was very weak when investigating Nile tilapia (*Oreochromis niloticus*), likely affected by the thick scull bone and muscle tissue between the electrodes and the brain. Some modifications of the equipment are likely necessary to enable EEG-recordings in a wider range of fish species, but EEGs obtained in **study III** and **IV** prove that it can be used to evaluate several different stunning methods in rainbow trout.

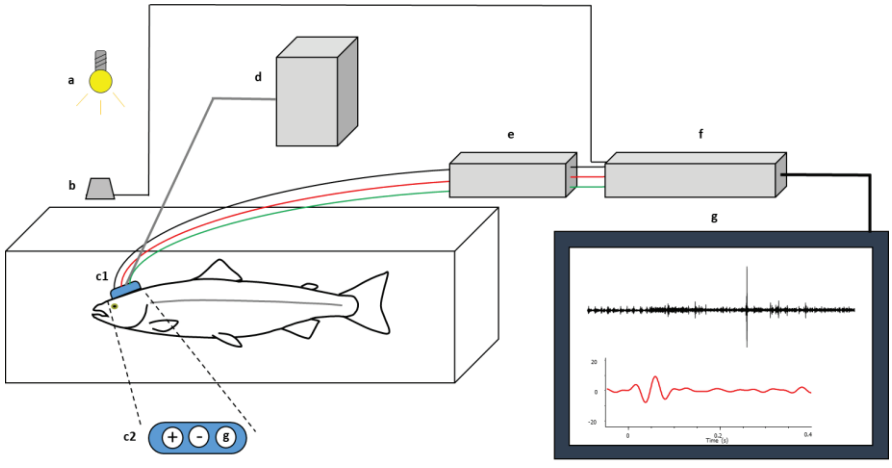

Figure 15. Schematic picture of EEG recording in study III and IV. The fish was placed in a flow-through tank filled with aerated water. The light stimulus was provided with a LED-light flashing with a frequency of 2 Hz (a) and a light sensor works as the recording trigger (b). A silicone cup (c1) mounted with electrodes (c2) was secured to the head of the fish by creating an under pressure using a peristaltic pump (d). The three electrodes were connected to a bio amplifier (e) and a data acquisition hardware (f) and the signal was recorded and analyzed on a PC using a physiological data analysis software (g).

4. Main results and discussion

4.1 Stress monitoring using heart rate bio-loggers

In **study I** and **II**, I used commercially available heart rate bio-loggers in combination with blood and tissue analyzes to identify and monitor welfare hazards of rainbow trout and European whitefish. My results validate that heart rate bio-loggers provide reliable data and can be used to both identify and quantify different stressors, information that provides novel insights on how to improve handling and rearing of these species in aquaculture. Moreover, I was involved in the development of a novel technique for EEG-recording of fish, which was used in **study III** and **IV** to measure brain activity before and after different stunning protocols. This resulted in a more complex picture on the effects of stunning and raised new questions on how to assess brain function following stunning in fish. The results from my studies are a mix of expected, unexpected and completely novel findings. In this section, I will in a general context present and discuss the importance of my findings. For details, the reader is referred to the corresponding articles and manuscript.

4.1.1 Post-surgical health

In all experimental research related to animal physiology and functions, it is essential to study healthy animals as stress may bias the results and cause poor animal welfare. This is true also for studies using tagged animals, as there is a risk that the monitored individuals are affected by the tagging procedure or by the tag itself, in a way that they are no longer reliable representatives of the population. Different lethal and sub-lethal effects related to tagging of fish in aquaculture was recently summarized by

Macaulay et al. (2021). They found that the mortality of tagged fish was substantially higher in sea cages (~25 %) compared with tanks (~2.5 %), and increased significantly in sea cage trials that lasted for more than 100 days. The reason for this difference is unknown, but it was suggested that a controlled tank environment enables better control over parameters such as water quality and external environmental disturbances. The authors further emphasized that sub-lethal physiological effects were only rarely reported in this kind of studies (Macaulay et al., 2021). Sub-lethal effects related to tagging of fish includes *e.g.* impaired depth distribution in Atlantic salmon with internally implanted tags and reduced swimming performance and tissue damage from externally mounted tags (Jepsen et al., 2015; Wright et al., 2018). Another reported side effect related to tagging of fish is impaired growth, or even weight loss. Is has been shown that Atlantic salmon implanted with heart rate loggers grow less compared to untagged conspecifics (Hvas et al., 2020), and that the weight difference between tagged and untagged salmon can be up to 20 % five months after implantation (Warren-Myers et al., 2021). Similarly, I observed significant weight loss (mean ~2%) following heart rate bio-logger implantation in both **study I** and **II**. The whitefish in **study II** were fasted the last week before transportation, which likely explains part of the weight loss in that study. The underlying reason for this, and if it affects the conclusions drawn is unknown. Yet, it is possible that implantation can cause altered feeding behaviours which is something that needs to be considered in order to avoid confounding tagging effects in future studies. However, considering that I found no indications of elevated stress indicators at the end of **study I,** and that the fish reacted to the stressors as anticipated at the end of **study II**, it seems unlikely that the observed weight loss significantly affected the investigated variables and biased the results of my studies.

I was encouraged to see that plasma cortisol levels were in the range expected for unstressed fish (<10 ng ml^{-1}), and that the heart rate had plateaued at a low resting level (~30-40 bpm) with clear diurnal fluctuations three weeks after implantation of the bio-loggers (**Study I**). In addition, the wounds and adjacent suture points showed good progress of healing with no clear signs of inflammation or infection. Treatment with a broad-spectrum antibiotic did not decrease the post-surgical immune response. Instead, individuals treated with antibiotics had both an increased mRNA expression

level of TNFα in the head kidney, which indicates a potential inflammatory response (Sigh et al., 2004), and a slightly elevated heart rate during the first week after surgery, compared to an untreated control group. Whether these effects were caused by the potentially painful antibiotics injection, the drug itself or some other unknown factor remains unknown. However, an upregulation of TNFα mRNA expression does not necessarily lead to translation of the protein, why this finding must be interpreted with caution (Teles et al., 2011).

The observed upregulation of TNFα does not likely influence the physiological state in a longer term, as both treated and untreated fish appeared to recover well as heart rate one week later had stabilized at the same level in both groups. Nonetheless, one must be cautious when translating results from laboratory studies to studies performed in uncontrolled environments such as in sea cages or in wild populations, and it is possible that prophylactic antibiotic treatment can have more pronounced beneficial effects in such situations. Of greater concern is perhaps the discovery of a long-lasting effect on heart rate that was dependent on which surgeon preformed the implantation. I found that the mean heart rate was ~5 bpm higher in fish implanted by one surgeon compared to fish implanted by a second surgeon, and this effect lasted throughout the entire three week trial (**Study I**). The underlying reason for this surgical effect is unknown, but as the difference in heart rate was not correlated to any differences in plasma cortisol, expression level of TNFα and TGFβ or wound healing, I have no reason to suspect that it biased the conclusions drawn in **study I**. However, this finding highlights the importance of randomizing treatment groups in experimental research so that confounding effects, *i.e.* the effects of different surgeons observed here, are not falsely mistaken for a treatment effect. Another potential consequence of this finding is that the increased variation in the investigated variable, here explained by two different surgeons, can mask moderate but potentially important treatment effects in the data, if unaccounted for.

4.1.2 Post-surgical recovery time

Interestingly, the recovery time (*i.e.* time until plateauing at a low resting heart rate) after implantation of a heart-logger was more than 4 times longer in **study I** (~144-168 h) compared to **study II** (~36 h), even though the

surgical protocols were comparable in all aspects between studies. The different species in **study I** and **II** can, at least partly, explain the difference in post-surgery recovery. However, to the best of my knowledge, heart rate in adult free-swimming European whitefish has not been measured before, and so the results for heart rate from **study II** cannot be compared to any previous studies. The 36 h recovery time in whitefish is markedly shorter than previously reported recovery times for other salmonids. Similarly, whitefish also seems to recover faster than rainbow trout following transportation (see section 4.1.3), at least if using plateauing heart rate as an indicator of recovery. Many fish species have a circadian rhythm (Boujard & Leatherland, 1992), and it has been suggested that emerging diurnal fluctuations in activity/heart rate is an additional sign of recovery following surgery and logger implantation in rainbow trout and Atlantic salmon (Brijs et al., 2018; Føre et al., 2021; Warren-Myers et al., 2021). In **study I**, a diurnal heart rate pattern was observed three days after surgery in rainbow trout, which is similar to the findings of Brijs et al. on the same species (Brijs et al., 2018). In contrast, the whitefish in **study II** never displayed any clear daily fluctuations in heart rate. This species may not have an obvious circadian rhythm or were affected by the long day length (~17 h) and short nights (~7 h) at this latitude in May when the experiments were performed. Interestingly, it has been shown that the diurnal swimming activity pattern that was present during a 12:12 h light:dark light regime disappeared when the light regime was changed to 24 h daylight in a closely related species of whitefish (*Coregonus clupeaformis*) (Scherer & Harrison, 1988). This is a good example of the importance of adopting a species-specific approach to physiological and behavioral responses when assessing post-surgery indicators of recovery, and that it is necessary to consider potential seasonal variations in indicators of post-surgery recovery.

It is also possible that the differences in experimental settings between **study I** (laboratory tank) and **II** (commercial sea cages) impacted on post-surgical recovery time. In a laboratory setting, the fish is normally kept undisturbed at constant water temperature and few environmental disturbances/stimuli compared to the complex environment at a sea based fish farm. The resting heart rate in rainbow trout in **study I** plateaued at roughly 30 bpm after 6-7 days, while the heart rate of rainbow trout implanted with the same type of bio-logger but released into a sea cage

plateaued at a higher 40-47 bpm after ~4 days (Brijs et al., 2018). Similar patterns can be found in Atlantic salmon, where the heart rate of tagged fish were reported to recover within 4 days when released into a sea cage (Warren-Myers et al., 2021), while heart rate recovery times in laboratory trials can range from 4-6 days and up to two weeks (Føre et al., 2021; Hvas et al., 2020). The faster recovery of rainbow trout in Brijs et. al (2018) compared to the same species in **study I**, can thus be hypothesized to be explained by a higher heart rate plateau due to underlying stimuli/stressors in the sea cage environment, *e.g.* higher frequency of environmental disturbances and/or more frequent social interactions, that are avoided in controlled laboratory studies. This could mean that the heart rate in fish in a sea cage may never reach "real" resting levels but instead plateau at a higher heart rate, which could explain part of the relatively rapid recovery of the European whitefish. To verify this assumption/explanation, further studies investigating heart rate in truly undisturbed whitefish are needed.

The rainbow trout in **study I** were acclimated to 10 °C, while the fish in Brijs et al. (2018) were acclimated to 15 °C. It is, however, unlikely that this difference explains the difference in plateauing heart rate as heart rate for rainbow trout acclimated to 10 and 15 °C has been reported to plateau at 30 and 32 bpm, respectively, in laboratory environments using a non-invasive method for heart rate recording (Altimiras & Larsen, 2000; Gräns et al., 2014). However, both studies emphasized that the reported heart rate in their studies are considerably lower than previously reported resting values of heart rate in rainbow trout. They further suggest that the underlying reason for this might be that fish used in lab experiments are seldom given enough recovery time from surgery, handling and/or anesthesia before the experiments are started (Altimiras & Larsen, 2000; Gräns et al., 2014). This is in line with the findings in **study I,** where it took 6-7 days for trout to recover and reach a stable resting heart rate of 30 bpm.

There can be practical consequences with leaving instrumented animals to recover for a week before experimentation. If, for example, the instrumentation requires that the fish be physically connected with cables to a data acquisition system or that the fish for some other reason needs to be kept in confinement, this will in itself affect the welfare of the fish. However, experimentation on a fish that is not fully recovered may result in

physiological and behavioral responses that are different from that of an unstressed fish. This is true also for heart rate where the response to a stressor can fundamentally vary depending on the condition of the fish (Brijs et al., 2019; Brijs et al., 2019; Thorarensen et al., 1996). For example, it was shown that free-swimming rainbow trout with a resting heart rate of ~50 bpm responded to a stressor with an increase in heart rate of 20 bpm. The same individual, at a later occasion, when its heart rate was already elevated to 90 bmp, responded to a similar stressor with a decrease in heart rate of 20 bpm (Brijs et al., 2019). Altered physiological responses like these ones are not only important to consider when collecting physiological data from a fish that is potentially recovering from stress, but can also have a negative effect on fish welfare when they are exposed to cumulative stressors in aquaculture, which will be described in section 4.1.3.

4.1.3 Identification of a potential chronic stressor

Many factors can cause stress in aquaculture. Yet, an unexpected discovery in **study II** was that the whitefish heart rate abruptly increased by ~ 10 bpm 24 h before they were going to be transported to the abattoir. It turned out that this response coincided with the introduction of second sea cage with rainbow trout, which was placed near the whitefish. Thus, this evidently elicited a rapid and long-lasting stress response in the whitefish as the heart rates remained elevated until the fish were moved from this location. As a consequence, the data obtained in **study II** must be interpreted cautiously as the fish displayed signs of being stressed even before the transport begun. However, it was clear that exposure to multiple acute stressors before transport caused further significant changes in several stress indicators, and it is likely that the physiological and biochemical responses can be used in a general context to confirm that transportation is indeed a source of stress in whitefish.

It is tempting to speculate that the strong stress response in whitefish subjected to the presence of rainbow trout was related to a perceived predator threat, as predator alarm cues have been shown to induce predator-avoidance behavior and increased cortisol levels in several species including salmonids (Barkhymer et al., 2019; Berejikian et al., 2003; Ferrari et al., 2007; Kopack et al., 2015). In any case, this previously unknown stressor for whitefish is an important finding and highlights that it is necessary to investigate species-

specific rearing routines to reduce welfare hazards when developing husbandry practices for novel aquaculture species. Keeping European whitefish in the vicinity of rainbow trout is thus a potential source of chronic stress for this species. Chronic stress is obviously unwanted as it can have negative effects on the health and welfare of the fish. With few exceptions, many farmed fish species have not undergone extensive domestication breeding (Teletchea & Fontaine, 2012), and many species are likely to experience subsequent breeding programs that may or may not increase sensitivity or coping ability to stress. Identification of both acute and chronic stressors is therefore beneficial from both a productivity and a fish welfare perspective, and the findings in **study II** show that heart rate bio-loggers are a useful tool to assess cumulative stress, but can also, identify sources of stress that may otherwise be missed.

4.1.4 Acute stress from handling and transportation

The recovery time following a stress response can have a considerable impact on a fish's ability to cope with additional stressors. Transportation in aquaculture inevitably involves several events that will expose the fish to severe and sometimes cumulative acute stress. The stress effects of transportation have been documented for several aquaculture species and highlighted as an important welfare hazard (Ashley, 2007; Brijs et al., 2018; Congleton et al., 2000; Lines & Spence, 2012; Robertson et al., 1988). There are reports of high mortality associated with transportation events where death caused by heart failure has been linked to prevalence of different forms of heart disease (*i.e.* maladaptive cardiac-hypertrophy, arteriosclerosis and abnormally shaped hearts) (Poppe et al., 2003; Poppe et al., 2007). It has been hypothesized that, if faced by additional stressors, when the heart is already working at or near its maximal capacity, the fish may not be able to cope with the situation and risk to develop heart failure (Mercier et al., 2008). It is thus important to allow the fish to recover from a stressful event during aquaculture practices that involve exposure to multiple stressors. This is highly relevant as there are indications that prevalence of heart disease in both rainbow trout and Atlantic salmon is linked to selective breeding and rearing strategies to improve growth rate, which is commonly used in aquaculture (Brijs et al., 2020; Frisk et al., 2020).

Sensitivity to different stressors are largely species-specific and likely reflects evolutionary adaptations to challenges the fish is facing in their natural habitats. Responses to different types of stressors in adult European whitefish are largely unknown, so it is highly relevant to investigate how a transportation routine affect cumulative stress effects and their ability to recover from multiple stressors as this species is being incorporated into the expansion of Nordic aquaculture. The findings in **study II,** where European whitefish were exposed to a range of potentially stressful events during transportation, will aid in the development of species-specific routines to decrease the risk of poor welfare for this species. The first event involved lifting the bottom of the sea cage to enable netting of fish for blood sampling for baseline levels of hematological parameters (Fig. 7 and 16). During this event, the available water volume for the whitefish was limited and the fish became lightly crowded. A reduction in water level has been shown to cause an increase in heart rate, cortisol, glucose and lactate in Atlantic salmon and rainbow trout (Brijs et al., 2019;Svendsen et al., 2021). The whitefish in **study II** responded similar to Atlantic salmon and rainbow trout to a reduction in water level with a rapid increase in heart rate of ~15 bpm when lightly crowded for 30 min.

After the recovery from the first crowding the bottom of the sea cage was lifted again for a second, much more severe, crowding to transfer the fish from the sea cage to the well boat. Swimming speed and frequency can increase from crowding, which increases the risk of exhausting the fish. Exhaustion has been shown to cause high mortality rates in rainbow trout, especially if it is followed by air exposure (Ferguson & Tufts, 1992; Wood et al., 2006). In **study II**, the fish were transferred by brailing from the sea cage into the well boat, exposing the fish to air for a maximum of 30 s, which caused a decrease in heart rate in 31 % of the focal fish while heart rate remained unchanged in the other 69 %, but no mortality was observed. Stress can intensify even more during the actual transportation if water quality deteriorates (Sampaio & Freire, 2016). This was likely not the case in **study II** as water quality and oxygen level remained high and the fish started to recover as indicated by gradually decreased heart rate during the last 40 min of the 70 min transportation. However, the transfer from the well boat to the holding cage near the abattoir caused heart rate to peak at 73 bpm and cortisol level to increase from 13 to 50 ng ml^{-1}. Although the increase in cortisol was

significantly elevated compared to baseline levels, the increase was considerably less pronounced in European whitefish compared to rainbow trout following a similar transportation event, where cortisol levels increased from 23 ng ml^{-1} after an initial crowding to 171 ng ml^{-1} after transportation (Brijs et al., 2018). On the other hand, changes in Hct, MCHC (indicative of RBC swelling) and plasma [K^+] were found to coincide with changes in heart rate in **study II**. Again, this contrasts with rainbow trout where ion concentrations and haematological parameters were poorly correlated with the stress-induced changes in heart rate (Brijs et al., 2018). This finding indicates that different stress indicators can be expressed differently among closely related species and that comparisons of physiological responses to stress can be species-specific. A study performed on four species of juvenile salmonids subjected to two types of stressors (short-term handling and long-term transportation) showed that lake trout (*Salvelinus namaycush*) had the highest response and rainbow trout the lowest when plasma cortisol levels were compared among the species (Barton, 2000). On the contrary, rainbow trout had the highest peak plasma glucose level and lake trout the lowest, and the author suggested that multiple stress indicators should be measured and that they cannot be directly compared among species. This is consistent with my findings.

After transport, the whitefish were kept in the sea cage for ~12 h to recover overnight. During this period, both heart rate, [K^+] and plasma cortisol decreased to significantly lower levels compared to 12 h earlier. In contrast, the rainbow trout in the study by Brijs et al. (2018) still had elevated heart rates and cortisol levels even though they were allowed to recover for a longer time (> 16 h). This is consistent with the faster recovery rate from bio-logger implantation in whitefish discussed earlier, and indicates that whitefish decrease heart rate and suppress the HPI-axis following a stressor much faster compared to rainbow trout (Brijs et al., 2018). Because of the known problem with high mortality associated with transport in aquaculture, it has been argued that fish should be allowed a long period to recover from stress after transportation (Iversen et al., 1998; Lines & Spence, 2012; Sandodden et al., 2001), and the findings in **study II** confirm that also European whitefish is experiencing transportation as a stressful event.

The next morning, the slaughter process started with crowding the fish before they were brailed from the holding cage to an air chute from where they were subsequently put in water bubbled with CO_2 for stunning. When fish were deemed unconscious by the slaughter personnel they were exsanguinated by cutting the ventral aorta. The crowding, brailing and CO_2 stunning caused a strong stress response indicated by an increase in plasma cortisol that peaked at 60 ng ml^{-1}, cell swelling and alterations in blood plasma levels of glucose and [Ca^{2+}]. Heart rate dropped rapidly during CO_2 stunning, similar to arctic char and rainbow trout (Brijs et al., 2018; Seth et al., 2013), indicating an acute response to hypoxia and/or hypercapnia (Perry et al., 1999; Randall & Shelton, 1963), potentially leading to irreversible heart failure from acidosis as suggested by (Seth et al., 2013).

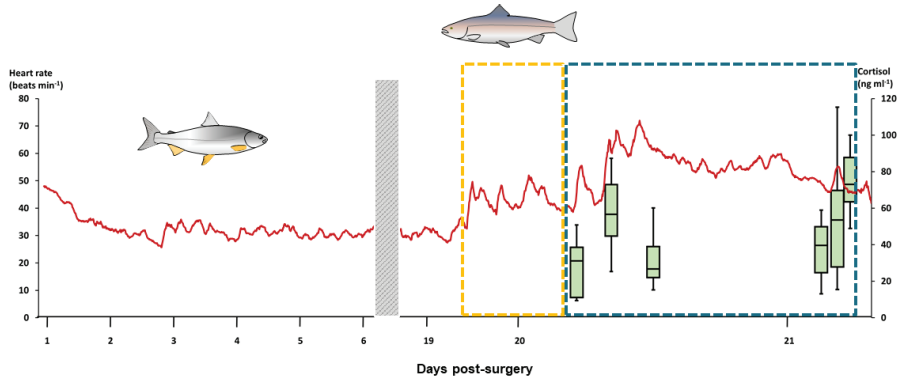

Figure 16. Using heart rate loggers in focal animals in combination with intermittent blood sampling of the surrounding fish to monitor stress in aquaculture. Red line is the mean heart rate during the first week after implantation, the introduction of rainbow trout on day 19 (yellow hatched box), and the final 24 h of transportation and slaughter (blue hatched box). Green bars represent plasma cortisol levels at six transportation events.

4.2 Stunning and killing of fish

Clear signs of severe stress was seen in both the behavior and physiology of the fish during the slaughter in **study II**. This is not in accordance with the requirements of humane slaughter, which states that slaughter methods that avoid and minimize reactions of fear and anxiety as well as pain, suffering

and distress among the animals should be used. To achieve this, the animal needs to be stunned (*i.e.* rendered unconscious) rapidly and with a minimum of negative experiences prior to killing. The results from **study III** and **IV** show that both CO_2, percussive and electrical stunning can induce unconsciousness prior to killing. However, as discussed in the upcoming sections, there are significant variations in both onset and duration of unconsciousness following stunning depending on the stunning method, environmental factors and the indicator used to assess unconsciousness.

4.2.1 CO_2 stunning

When used in fish, CO_2 is often described as a "slow" stunning method, in contrast to other more immediate methods such as percussive or electrical stunning. This is not a desirable character when it comes to stunning. Nevertheless, it may allow an evaluation of how loss of indicators of consciousness gradually decrease, and how the time to loss of these indicators correlates with changes in brain activity. Time to loss of equilibrium, ventilation and the vestibulo-ocular reflex during CO_2-stunning of rainbow trout were investigated during three different seasons and water temperatures (2, 8 and 14 °C) in **study III**. All fish displayed violent aversive behavior with escape attempts immediately after being placed in the stunning tank. Equilibrium was the first indicator to be lost at all water temperatures, but it took significantly shorter time at 14 °C compared to 2 °C. Temperature also had a significant effect on time to loss of both vestibulo-ocular reflex and ventilation in all water temperatures. The fish lost the vestibulo-ocular reflex after 60-120 s at 14 °C, 120-270 s at 8 °C and 180-330 s at 2°C. The same pattern was found for time to loss of ventilation, ranging from a mean of 205, 345 and 540 s at 14, 8 and 2 °C, respectively, and likely explained by decreased metabolism at the lower temperatures. Similar results with an increased induction time at lower temperatures has previously been reported for rainbow trout when asphyxiated out of water and when exposed to different anesthetic compounds (Kestin et al., 1991; Sneddon, 2012; Woolsey et al., 2004; Zahl et al., 2010). As exposure to high levels of CO_2 during stunning is known to cause a stress response in salmonid species such as whitefish in **study II**, rainbow trout (Brijs et al., 2018) and arctic char (Seth et al., 2013), stunning fish with CO_2 at lower water temperatures risks prolonging the time the fish is stressed before becoming unconscious.

In commercial on-site slaughter situations when fish are often stunned together in batches (Fig. 17), there are anecdotal reports that fish display behaviours indicative of consciousness for much longer periods than the maximum 540 s (9 min) recorded in **Study III**. One explanation can be that it can be difficult to create and maintain a sufficiently saturation of CO_2 during batch stunning, as decreasing partial pressure of CO_2 is known to prolong the time to loss of equilibrium in rainbow trout (Bernier & Randall, 1998). There is also large species-specific variation in sensitivity to CO_2 (Marx et al., 1997). For example, in a study by Roth et al. (2006) it was shown that Atlantic salmon never lost consciousness even when immersed for 40 min in 2°C water saturated with CO_2 (Roth et al., 2006). Collectively, the efficacy of CO_2-stunning is thus dependent on acclimation temperature as shown in **study III**, species and CO_2 saturation level, but in all situations the efficacy is a remarkably slow and low or insufficient.

It is believed that complete loss of ventilation indicates a decreased function of the *medulla oblongata*, the part of the brain that control *e.g.* breathing, and thus represents an anesthetic stage when the brain is rendered unable to process environmental stimuli (McFarland, 1959). Loss of ventilation and loss of VERs coincided relatively well when rainbow trout were anesthetized with MS-222 (Bowman et al., 2019). In contrast, time to loss of ventilation and loss of VERs was markedly different when rainbow trout were stunned with CO_2 in the second part of **study III**, as VERs could be present for up to ~3.5 min after the ventilatory movements had been lost. This suggests that loss of ventilation does not necessarily reflect brain failure that is consistent with a complete loss of neural responses to stimuli in CO_2-stunned rainbow trout (see section 4.2.4. for a more detailed discussion of this potential welfare hazard).

Taken together, the results confirm the conclusion that CO_2 is a method that does not meet the requirements of humane slaughter as it i) cannot be considered to induce immediate or even rapid brain failure, and ii) elicits a clear stress response during stunning. The poor reliability of visual indicators further highlight that muscle function may be lost before loss of consciousness during CO_2-stunning. Consequently, there is a risk that the fish may be awake during the subsequent killing by bleeding (Robb & Kestin, 2002). This is particularly problematic when slow stunning methods

are used as unconsciousness is determined by gradual loss of visual indicators, in contrast to stunning methods that induce immediate but transient unconsciousness when instead signs of recovery are monitored, which are likely easier to detect.

Figure 17. Arctic char (*Salvelinus alpinus*) during batch stunning with CO_2. The water is bubbled with CO_2 until deem saturated before a high number of fish are placed in the bath simultaneously. After a period of time the fish are deemed as stunned by the personnel and the fish exsanguinated. *Photo Lotta Berg*.

4.2.2 Percussive stunning

Different means of percussive stunning has been shown to efficiently cause brain failure indicative of unconsciousness in several species of fish (Brijs et al., 2020; Kestin, 1995; Lambooij et al., 2010; Lambooij et al., 2007; Robb et al., 2000). Likewise, the captive-bolt gun used for percussive stunning in **study IV** successfully induced immediate and irreversible brain failure in rainbow trout as indicated by immediate loss of VERs. However, in contrast to all of the above referred studies where instances of mis-stuns were reported*, i.e.* the hit is insufficient, or the blow misses the brain, all fish in **study IV** were successfully stunned when using the captive-bolt gun. Roth et al. have shown in Atlantic salmon that the efficacy of a percussive blow is dependent on the force that is delivered, although they did not identify a clear force threshold between a successful and a failed stun (2007). A disadvantage of manual percussive stunning with a fish priest (or other

hand-held clubs) is the unintentional but inevitable variable force that is delivered between hits, which increases the risk for failed stunning (Brijs et al., 2020; Kestin, 1995). Thus, an obvious advantage of the pneumatic bolt gun used in **study IV** is that it delivers equal force for each hit, and the shape of the adapter mounted at the firing end aids in aiming. The manufacturer also highlight that pneumatic non-penetrating captive-bolt stunning increase operator safety and minimize operator fatigue (http://www.bock-industries.com/zephyr-f.html).

One technical problem related to our method of measuring EEG during percussive stunning is that the electrodes need to be removed during the stunning intervention, which means that a short period (~ 30 s) of EEG immediately following the hit could not be recorded (see **study IV)**. Nonetheless, Robb et al. demonstrated that a percussive stun delivered by a pneumatic gun can cause immediate loss of VERs in Atlantic salmon, which simultaneously ceased all movements (2000). The reported mis-stuns occurred when the operator was unable to keep the salmon from moving when the shot was fired, and aversive behaviour was observed and VERs could then be recorded for up to 5 min after the stunning (Robb et al., 2000). All rainbow trout stunned with the bolt-gun in **study IV** remained still without presence of VERs after stunning for the remainder of the recovery period, and hence it is likely that VERs were absent also during the period when EEG was not recorded. This emphasizes the importance of good accuracy and proper restraint of the fish when performing percussive stunning which can be difficult when a fish is awake (Brijs et al., 2020). Fish are, from personal experience, difficult to handle out of water which likely interferes with percussive stun efficacy, especially when fish size increases. To ease handling, percussive stunning is sometimes combined with live chilling or short exposure to electricity prior to the application of the actual stun. It has, for example, been shown in common carp that farms that used a short electrical stun prior to percussive stunning, on average had a higher success rate (judged by absence of non-specified behavioral indicators) compared with farms that used percussive stunning only (Retter et al., 2018). Percussive stunning is, however, labour intensive as it requires manual handling of each individual fish. It is also known to cause product downgrading due to broken jaws (Lambooij et al., 2010), as well as eye-prolapses or burst eyes (Roth et al., 2007), which was observed in **study IV**

(Fig. 14). Today, many fish slaughtering facilities instead use only electrical stunning to render fish unconscious prior to exsanguination, as this requires less handling of the fish and reduces workload.

4.2.3 Electrical stunning

It is widely recognized that a range of electrical parameters affect the efficacy of electrical stunning in fish, like in other group of animals. It is also well known that electrical stunning of various salmonid species can cause hemorrhages and spinal damages, which downgrade the quality of the product (Roth et al., 2003). Unfortunately, for some key electrical parameters there is a positive relationship between improved stunning efficiency and impaired product quality caused by spinal injuries and muscle hemorrhages (Jung-Schroers et al., 2020; Robb et al., 2002; Roth et al., 2003). There is thus an obvious risk that insufficient stun settings are used to avoid carcass damages. Besides current frequency, the stun efficacy of fish stunned in water depends on a range of parameters including whether alternating or direct current (AC vs DC) is used, electrode position, stun duration, electric field strength and current density (Lambooij et al., 2008; Lines & Kestin, 2004; Lines et al., 2003; Robb et al., 2002; Roth et al., 2003; Roth et al., 2004). The field strength and current density are in turn dependent on water conductivity and distance between electrodes, as well as electrode area. Moreover, there are strong indications of substantial species-specific differences in sensitivity to electrical stunning, where rainbow trout has been suggested to be less susceptible to electrical stunning compared to Atlantic salmon, while African sharptooth catfish is known to require considerably greater currents and field strengths compared to the rainbow trout presented in this thesis (Brijs et al., 2020; Lines & Kestin, 2004). In **study IV**, the stun was delivered using a 50 Hz AC current with electrodes that were positioned side-to-side and a constant water conductivity of ~1000 μS cm^{-1} to ensure that these variables remained constant. The effect of different exposure times, field strengths and current densities on induction of an epileptic-like insult and time to loss of VERs and ventilation were assessed. The results from the electrical stunning in **study IV** should thus not be used as a "best-practice-stun-setting"-study as several variables were not investigated. Instead, some key findings in **study IV** rather motivate a discussion on the reliability of indicators of unconsciousness and can be read in section 4.2.4.

Induction of an epileptic-like insult is commonly used to determine unconsciousness in fish (Lambooij et al., 2008; Lambooij et al., 2010; Lambooij et al., 2007). In **study IV** this was achieved by a 1 s electrical stun, using an electrical field strength of ≥ 2.8 V_{RMS} cm^{-1} and a current density of ≥ 0.22 A_{RMS} dm^{-2}. The insult was clearly visible on the EEG as a period with abnormal voltage fluctuations with significantly increased amplitude for ~10 s followed by a second period where the amplitude was still significantly increased compared to pre-stun amplitude but significantly decreased compared to the first period (Fig. 11). This pattern resembles what is often referred to as a tonic-clonic seizure (Lambooij et al., 2008; Lambooij et al., 2010; Lambooij et al., 2007). The duration of the second (clonic) period could be prolonged with increasing current densities and electric field strengths (**study IV**). However, all fish stunned for 1 s recovered VERs and even when using the highest stun settings returned as early as after 20 s. This finding resembles what has been reported for African sharptooth catfish where VERs returned immediately after the epileptic-like seizure (Brijs et al., 2020), and common carp where 31 out 32 individuals recovered VERs within 30 s after being electrically stunned with an application duration of 1 or 5 min (Retter et al., 2018). Additionally, Retter et al. (2018) showed that recovery of VERs happened before recovery of ventilation and the vestibulo-ocular reflex. This is similar to the findings in **study IV**, where the trout did not display any visual signs of being awake when VERs reappeared, indicating that the fish remained immobile at the time VERs returned. There is thus a risk that fish exposed to an electric current is not stunned but merely immobilized by electrical stimulation causing the muscles to become exhausted (Robb et al., 2002), with the following exsanguination being performed in a paralyzed but potentially conscious fish (Fig. 18). Moreover, as the period of loss of VERs were transient in **study IV**, it is possible that electrically stunned rainbow trout can recover before they are bled to death. Similar concerns have been raised also for other species. For example, electrically stunned Atlantic salmon and African sharptooth catfish have both been shown to become responsive to noxious and light stimuli before they die from bleeding (Brijs et al., 2020; Lambooij et al., 2010). Taken together, these results show that the presence of an epileptic-like insult following electrical exposure cannot be used as a guarantee that the fish remain unconscious until killed by exsanguination.

In the second part of the electrical stunning trials in **study IV** the duration of the electrical exposure was prolonged. Here I showed that a prolonged stun application time increases the time until recovery of VERs, which supports previous findings describing a relationship between application time and time to recovery of equilibrium and ventilation in fish (Brijs et al., 2020; Retter et al., 2018; Robb et al., 2002). In **study IV** I showed that by increasing the period of electrical exposure to 30 and 60 s, it was possible to permanently abolish VERs when an electrical field strength of 10.2 V_{RMS} cm^{-1} and current density of 0.84 A_{RMS} dm^{-2} was used. However, both a 30-60 s stun application using the two intermediate electrical settings (≤ 5.1 V_{RMS} cm^{-1} and ≤ 0.4 A_{RMS} dm^{-2}), and a 15 s period of stun application with the highest stun settings, failed to abolish VERs completely. Instead, when using these settings, VERs re-appeared shortly after the exposure period and remained for ~ 1 min, in 13 out of 18 fish. Worth noting is that all fish remained motionless, and although ventilation recovered in eight of the fish that recovered VERs, ventilation generally re-appeared long after the recovery of VERs. In fact, VERs and ventilation were only present simultaneously in three individuals. This clearly shows that ventilation can be inhibited, while the brain is still able to respond to visual stimuli in electrically stunned rainbow trout, which similar to what I observed following CO_2-stunning in **study III**.

Taken together, these results clearly show how difficult it is to determine brain function and ability to respond to external stimuli using visual indicators, emphasizing that different indicators of unconsciousness may risk judging a conscious fish as unconscious and *vice versa*. In other words, the simple visual indicators have low specificity and low sensitivity, making it difficult both to correctly identify a properly stunned fish as unconscious, and for identifying a poorly stunned fish as (partly) conscious. Consequently, recordings of EEG is necessary to determine that brain activity have ceased. However, the relation between EEG indicators, such as VERs, and different stages of consciousness is not fully understood and this is a key distinction when it comes to the assessment of welfare of farm animals during slaughter (Kestin, 1995). It is assumed that loss of VERs indicate brain failure that is inconsistent with consciousness, but whether unconsciousness or the ability to experience pain, fear, anxiety or distress can occur before VERs are lost is still unknown. The complexity of this issue is further highlighted by the

results following electrical stunning in **study IV** where VERs could be observed at times when they in theory "should" be absent such as during an epileptic-like insult. This is discussed further in section 4.2.4.

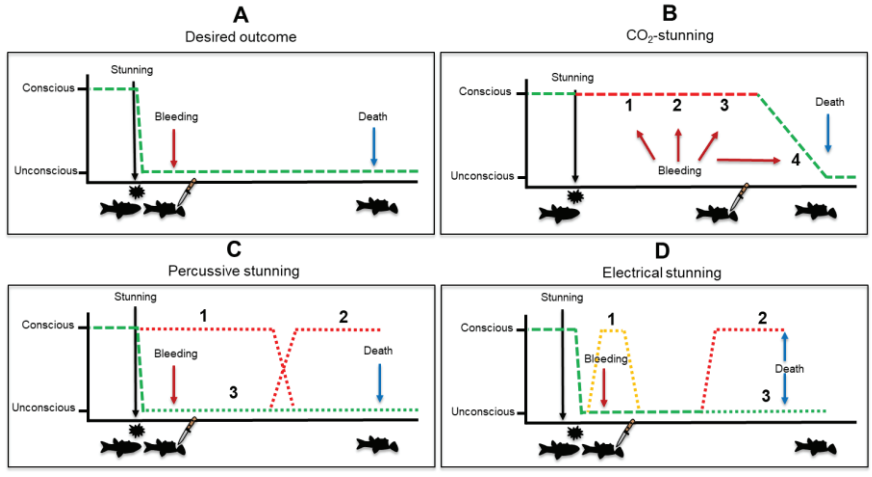

Figure 18. Schematic images of the impact of different stunning methods on induction time and duration of loss of visually evoked responses. (A) show the desired outcome of a stun, where unconsciousness is induced immediately and the fish dies from bleeding before consciousness is recovered. (B) show a fish entering CO_2 narcosis, where unconsciousness is not induced instantly but instead result in aversive behaviour and escape attempts. Depending on what indicator is used gill cut risk being done on a conscious animal, where 1) represent loss of equilibrium, 2) loss of the vestibulo-ocular reflex, 3) loss of ventilation and 4) loss of VERs in this hypothetical example. Percussive stunning (C) risk 1) that a mis-stun does not cause immediate brain failure (Robb et al., 2000), 2) that the fish recover brain function before death (Brijs et al., 2020) but 3), can also induce immediate and irreversible loss of consciousness in the rainbow trout as demonstrated in e.g. study IV. The outcome of electrical stunning (D) varied where 1) VERs were regained for a transient period after the stun, 2) recovered some time after the stun or 3), was both immediate and irreversible. The outcome of (D) was dependent on stun parameters and stun application duration and there is a risk that gill cut is performed when the fish is awake (1) or may recover consciousness before death occur (2) but it was also shown that it is possible to render the fish irreversibly stunned (3).

4.2.4 Reliability and contradictions of indicators of consciousness

Visual indicators of different stages of anesthesia in fish was suggested by McFarland already in 1959, and this work has influenced many researchers investigating anesthesia and unconsciousness in fish (McFarland, 1959). He suggested a gradual depression of different central nervous centers when the anesthetic level deepens, with a collapse of the part of the brain that control respiratory and ventilator function, as the deepest stage anesthesia. Visual stimuli is processed in the optic lobes in the mesencephalon (midbrain), and reduced activity in this area is suggested to occur earlier at somewhat lighter anesthetic depths (McFarland, 1959). However, the result from **study III** and **IV** indicate that this may be a too simplistic view on how the brain reacts to different types of stunning, as it was observed that a depression of ventilation can occur before responses to visual stimuli are lost. Other researchers have drawn similar conclusions with both electrical (Retter et al., 2018) and percussive stunning methods (Lambooij et al., 2010).

Because of this, it is suggested that disruption of brain functions, measured by EEG, can provide a more reliable estimate of absence of consciousness (EFSA, 2004; EFSA et al., 2018; Kestin et al., 1991; Lines & Spence, 2012; Robb et al., 2000; Van De Vis et al., 2003). This is the reason why we in our lab developed a novel technique to non-invasively measure EEG in rainbow trout (Bowman et al., 2019), which was used in **study III** and **IV**. Furthermore, the in-depth analyses in **study III** showed that the absence of VERs provides the most robust and reliable indicator of brain failure when assessing signals from external EEG-recordings. Alternative methods, such as the analyses of median frequency and relative power spectral density of the EEG, provide little information on brain function as no significant difference between pre- and post-stunning was found in these indicators. One explanation for this observation may be that ventilation frequency usually falls within the low frequency brain wave range (0.5-2 Hz) and thus skews the amplitude and proportion of this frequency in the EEG. Reduced signal amplitude, *i.e.* a reduction of EEG amplitude to less than 50 or 12 % of conscious amplitude, is another method that has been used to determine unconsciousness in calves and fish (Bowman et al., 2019; Gibson et al., 2009).This indicator was found to be highly variable among fish stunned with CO_2, and it took 4-15 min for the amplitude to reach < 50 % and 10 min – *not at all* to reach a 12 % reduction. An explanation for the

high variation in signal amplitude is that signals from muscle activity (electromyography, EMG) may also be picked up on the EEG. It is thus advantageous to analyze visual evoked responses (VERs) as interference from muscular activity is minimized. In **study IV**, the method to induce VERs was refined even further as a purpose-built LED-light that delivered a much shorter light stimulus was used to evoke responses.

However, the results of **study IV** revealed that also two of the most commonly used EEG-indicators of unconsciousness in fish; absence of VERs and presence of an epileptic-like seizure, can result in conflicting conclusions. A 1 s stun with sufficient current density and electric field strength induced an epileptic-like insult visible as abnormal EEG-voltage fluctuations, indicative of a hyper-stimulated brain. The EEG during the insults recorded in **study IV** resembles previously published images and descriptions of epileptic-like insults in earlier studies (Daskalova et al., 2015; Lambooij et al., 2008; Lambooij et al., 2012; Lambooij et al., 2008; Lambooij et al., 2010). Other research groups have instead used VERs to determine unconsciousness in fish, *e.g.* (Brijs et al., 2020; Jung-Schroers et al., 2020; Kestin et al., 1991; Retter et al., 2018; Robb et al., 2000). It was therefore both surprising and concerning to observe recovery of VERs before the epileptic-like insult had ended, *i.e.* VERs were present during the period when the brain was assumed to be hyper-stimulated and the fish unconscious (see Fig. 4 in **study IV**). The conflict between these indicators is puzzling and something that has, to the best of my knowledge, not previously been reported in fish. These findings would then suggest that either i) what was observed on the EEG was in fact not a hyper-stimulated brain or ii), absence of VERs is a too strict criteria to be used when determining the onset of unconsciousness.

If the first alternative is true, it would mean that the observed epileptic-like insult is not what in humans is referred to as a *grand mal* seizure but instead may be an absence seizure (formerly known as a *petit mal* seizure). The difference between these two types of seizures is, based on studies in humans, that the a *grand mal* seizure is indicative of unconsciousness but that unconsciousness may be very short lasting during an absence seizure (Bancaud et al., 1981; Panayiotopoulos, 2008; Sadleir et al., 2009). A similar phenomenon has been reported in chickens following electrical stunning,

where the authors propose that a *petit mal* seizure can occur following electrical stunning (Gregory & Wotton, 1987). The absence of an iso-electric phase and the rapid recovery of swimming following the tonic-clonic phase raises further questions regarding the actual nature of the epileptic-like insult that was observed in the electrically stunned rainbow trout. If what was observed in the rainbow trout in **study IV** in fact was not a *grand mal* seizure, it raises concerns also for other fish species, as the epileptic-like seizure I observed did in no obvious way differ from those described in previous studies, where they have been used to determine the onset of unconsciousness in fish (Daskalova et al., 2015; Lambooij et al., 2008; Lambooij et al., 2012; Lambooij et al., 2008; Lambooij et al., 2010).

The alternative explanation is that absence of VERs is an overly conservative indicator when determining the onset of unconsciousness and may be present even if the fish is unaware of its surroundings. This possibility has previously been mentioned by Kestin et al. (1995). A second surprising finding in **study IV,** when the fish were exposed to the electric field for a longer time (15-60 s), further complicate things. With such long stun application times, no epileptic-like insult was observed on the EEG. Instead, VERs were lost immediately after the stun but often returned within the first minute, only to disappeared again after approximately 1 min (Fig. 18). The same phenomenon was also observed in an unpublished study where I measured brain activity in rainbow trout using a head-to-body dry electrical stunning system. In those experiments, similar patterns were observed in some individuals, with VERs appearing during the first min post stun, only to disappear shortly after and the fish later died. In other individuals, VERs reappeared again after a few min with subsequent recovery of equilibrium and swimming movements (unpublished observations). The consequences of this phenomenon for fish welfare remains unknown, but it is possible that the short and transient period of VERs is not necessarily an indicator of consciousness. If the fish is insensible to stress, pain and fear during this transient period with VERs, the conclusions drawn in **study IV** may need to be revised.

Both explanations to the unexpected results of **study IV** highlight some of the knowledge gaps that currently prevents us from safeguarding the welfare of fish during time of slaughter. The million dollar question is still

what indicator provides the most accurate representation of unconsciousness in fish. Even vocal animals such as mammals cannot be asked what they feel, so we have to rely on assessment of physiological and behavioral responses. This may not be an issue when percussive stunning is used as the physical disruption or destruction of the brain is both instant and irreversible and VERs can thus safely be used to determine unconsciousness. However, using slow (*e.g.* CO_2) or reversible (*e.g.* electrical) methods require a reliable indicator to determine the moment when consciousness is lost or recovered. Absence of VERs can be used to determine unconsciousness in a laboratory for most, if not all, stunning methods, but the search for a reliable indicator that is also practical to use in the abattoir must continue. Nevertheless, the results from **study III** and **IV** clearly show a high level of complexity between self-initiated behaviours, reflexes and neurophysiological indicators following stunning, and that potential correlation between indicators seems to vary depending on the stunning method and potentially also among species.

5. Conclusions

The findings presented here provide novel insights on how to monitor and assess responses to some of the issues that pose a serious threat to the welfare of the billions of farmed fish that are reared and slaughter in aquaculture each year.

The conclusions drawn from **study I** and **II** show that physiological responses can provide quantitative information on how fish in aquaculture perceive their environment and can be used to identify welfare hazards. Heart rate bio-loggers are useful tools to investigate stress in fish and, if used correctly, they can be used without any major negative effects on the health and welfare of the experimental animals. Furthermore, it is emphasized that stress sensitivity and indicators of secondary stress responses may vary even between relatively closely related fish species, which must be considered when evaluating stress responses of different species in their respective aquaculture settings. For many other fish species beyond salmonids, little is known about how abiotic and anthropogenic factors affect the health and wellbeing of the individual, which is crucial knowledge to maintain good fish welfare. Thus, the introduction of new farming species to the expanding aquaculture industry will require extensive evaluation of rearing conditions and other farm-related routines to ensure that they are consistent with needs of that specific species and does not lead to detrimental chronic stress. It may sound like a cliché, but more research is necessary to safeguard the welfare for all the farmed fish species. To date, most research has focused on economically important species such as salmonids. Nonetheless, my findings show that there are existing knowledge gaps regarding welfare also for the well-investigated rainbow trout.

Study III reinforces that CO_2-stunning is an inappropriate method to induce unconsciousness in rainbow trout, as the induction time is long and lead to aversive behaviours, particularly at low ambient temperatures. Furthermore, it is clear that the loss of visual indicators occur before loss of VERs, indicating that the brain of the fish is able to process visual, and potentially other, stimuli when rendered immobilized. However, this was not an issue for the percussive stunning in **study IV**, as all fish immediately lost all indicators of consciousness following the blow to the head using a captive bolt-gun. Furthermore, it was found to be extremely challenging to determine stun efficacy following electrical stunning as the part of the brain processing the visual stimuli could be functioning although all other indicators of consciousness were absent. This warrants further investigation, as reliable indicators of consciousness/unconsciousness are vital for evaluating stun efficacy. Considering the species-specific sensitivity to different stunning methods, it is highly relevant to continue to measure EEG during stunning of fish to determine that the investigated stunning in fact reach the desired outcome.

In summary, monitoring stress and assessing consciousness in non-verbal animals that spend all of their life in water remains a challenge. Measurements of physiological responses or monitoring of behaviors are currently the only possible ways to get a glimpse of how they perceive changes in their surroundings. Luckily, the results presented in this thesis provide evidence that it is possible to use such measurements to identify welfare hazards, which will help to improve the welfare of farmed fish in the future.

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Popular science summary

Aquaculture has expanded rapidly during the last decades. It is estimated that 50-150 billion fish are slaughtered annually for human consumption in global aquaculture, which is more than any other production animal. Fish are today regarded as sentient beings and are protected like our other traditional agricultural animals, such as pigs, poultry and cattle, by the Swedish Animal Welfare Act (2018: 1192) and the Animal Welfare Ordinance (2019: 66).

Unlike the other production animals, the current regulations and general guidelines for how fish should be handled, kept and cared for or how slaughter or other killing should be performed are unspecific. This is due to the great lack of knowledge about the needs of different fish species, which entails an increased risk that fish are handled and killed with methods that negatively affect animal welfare. Today, about 400 different fish species are farmed in aquaculture around the world, and many of them have widely differing evolutionary adaptations to the environment in which they naturally occur. This places high demands on species-specific adjustments when it comes to rearing conditions and how they are handled and slaughtered. Today, only a few of the commonly farmed fish species have been studied to any great extent, but even for the most well-studied fish species, such as salmon and rainbow trout, there are large knowledge gaps when it comes to animal welfare.

Stress in connection with handling and transport as well as the risk of insufficient stunning during slaughter are elements in aquaculture that have been emphasized as particularly problematic from an animal welfare perspective. The overall purpose of this dissertation was to identify and evaluate physiological indicators of poor animal welfare linked to handling, transport and slaughter, which in the long run can be used to develop species-

specific regulations and general advice but also to increase general knowledge about the assessment of fish welfare.

In order to get an insight into how fish experience common situations in aquaculture, so-called focal animals were used in **studies I** and **II**, *i.e.* a few individuals who are allowed to act as representatives for the rest of the group. Each focal fish had a bio-logger implanted that has the capacity to save long-term measurements of heart rate. Specific changes in heart rate can be used as an indication that the fish have become stressed.

The purpose of study I was to ensure that the focal animals did not experience any long-term effects from the operation or presence of the bio-logger by evaluating a series of indicators of stress and immune responses in the fish species rainbow (*Oncorhynchus mykiss*) up to three weeks after surgery. For 21 days, the heart rate was stored to examine how long the fish needed to recover after anesthesia, handling and surgery. It turned out that the fish had an elevated heart rate for 6-7 days before stabilizing at a low level, which clearly shows that the rainbow needs a long recovery period after this type of procedure. At the end of the experiment, a number of indicators of stress and immune responses were measured, and satisfyingly, the results showed stress levels and the immune response of the focal fish were at levels that can be expected from a healthy and unstressed individual. Furthermore, it was also established that prophylactic treatment with an antibiotic do not have a positive effect on wound healing. However, a clear surgeon-effect was discovered, where the heart rate of the fish consistently differed throughout the trial period depending on which surgeon that had performed the surgery.

The same type of biologger was also used, in combination with several blood-borne biochemical stress indicators, in **Study II** to investigate how the species European whitefish (*Coregonus lavaretus*) is affected by repeated stress in connection with crowding, brailing, boat-transport to the slaughterhouse and during subsequent slaughter. When the information from the bio-loggers was analyzed, it was, to our surprise, discovered that the heart rate rose sharply without falling to previous levels already one day before the transport was carried out. It turned out that a sea cage containing rainbow trout had been placed near the whitefish, which caused a chronic elevation of heart rate in the whitefish. This indicates that the whitefish perceived some form of disturbance/stressor from presence of rainbow trout. It therefore seems inappropriate from a welfare perspective to keep whitefish close to

rainbow trout as the whitefish showed signs of prolonged stress under such conditions. The results clearly showed that the whitefish were stressed during crowding and hauling in connection with the transport. The fish also showed severe stress during stunning by exposure to water containing high levels of carbon dioxide prior to killing.

When animals kept by humans are transported for slaughter or when they are slaughtered, it must be done in such a way that they are spared from unnecessary suffering and discomfort (2018: 1192). Furthermore, an animal that is slaughtered must be stunned, and the stunning must be performed so that the animal quickly becomes unconscious and the consciousness must not return (2019: 66). Since some anesthetic methods used in aquaculture can be slow, only paralyze the fish or only cause temporary unconsciousness, it is of utmost importance to have reliable indicators to determine whether the anesthetic has had the desired effect.

To evaluate the reliability of a range of readily available indicators, e.g. breathing movements, a newly developed technique was used to measure changes in brain activity (EEG) in rainbows before and after carbon dioxide-, percussive- and electrical stunning in **study III** and **IV**. The results clearly showed that the readily available indicators used to assess unconsciousness do not agree with the assessment made based on the measurements of brain activity. This means that fish are at risk of being killed by diving and bleeding when they are still conscious.

In summary, several important findings are presented here that can be used to improve fish welfare in aquaculture, and which can form the basis for future regulations and general advice on how fish should be handled, kept, cared for, and stunned and killed.

Populärvetenskaplig sammanfattning

Vattenbruket har under de senaste decennierna expanderat kraftigt. Uppskattningsvis slaktas årligen 50-150 miljarder fiskar för humankonsumtion i det globala vattenbruket vilket är fler än något annat produktionsdjur. Fiskar betraktas idag som kännande varelser och skyddas likt våra andra traditionella lantbruksdjur, såsom grisar, fjäderfän och nötdjur, av Sveriges Djurskyddslag (2018:1192) och Djurskyddsförordning (2019:66).

Till skillnad från de andra produktionsdjuren så finns inga artspecifika föreskrifter för hur fiskar ska hanteras, hållas och skötas eller hur slakt eller annan avlivning ska gå till. Detta beror på den stora kunskapsbrist som finns kring olika fiskarters behov, vilket medför en ökad risk för att fiskar hanteras och avlivas med metoder som påverkar djurvälfärden negativt. Idag hålls cirka 400 olika fiskarter i vattenbruks-anläggningar världen över, och många av dem har vitt skilda evolutionära anpassningar till den miljö som de naturligt sett förekommer i. Detta ställer höga krav på artanpassningar när det kommer till anläggningar och hur de hanteras och slaktas. Idag är bara ett fåtal av fiskarterna som hålls inom vattenbruket studerade i någon högre utsträckning, men även för de mest välstuderade fiskarterna, exempelvis lax och regnbåge, finns stora kunskapsluckor när det kommer till djurvälfärd.

Stress i samband med hantering och transporter samt risk för otillräcklig bedövning under slakten är moment i vattenbruket som har uppmärksammats som speciellt problematiska ur ett djurvälfärdsperspektiv. Det övergripande syftet med den här avhandlingen var att identifiera och utvärdera fysiologiska indikatorer på djurvälfärd kopplade till hantering, transport och slakt, vilket i förlängningen kan användas dels

för att utveckla artspecifika föreskrifter och allmänna råd men också för att öka den generella kunskapen kring verktyg för bedömning av fiskvälfärd.

För att kunna få en inblick i hur fiskar upplever vanligt förekommande situationer inom vattenbruket, användes i **studie I** och **II** så kallade fokaldjur, det vill säga ett fåtal individer som får representera hela besättningen. Varje fokaldjur fick en bio-logger inopererad med kapacitet att spara långtidsmätningar av hjärtfrekvens. Specifika förändringar i hjärtfrekvens kan då användas som indikation på att fisken blivit stressad. Syftet med studie I var att säkerställa att fokaldjuren inte påverkas långsiktigt av operationen eller av närvaro av bio-loggern genom att utvärdera en rad indikatorer på stress- och immunresponser hos fiskarten regnbåge (*Oncorhynchus mykiss*) upp till tre veckor efter operationen. Under 21 dagar lagrades hjärtfrekvensen för att undersöka hur länge fisken behövde för att återhämta sig efter sövning, hantering och operation. Det visade sig att fiskarna hade en förhöjd hjärtfrekvens under 6-7 dagar innan den stabiliserade sig på en jämn nivå vilket tydligt visar att regnbåge behöver en lång återhämtningsperiod efter den här typen av ingrepp. När försöket var slut mättes en rad indikatorer på stress- och immunresponser, och glädjande nog visade resultaten att stressnivåerna och immunresponsen hos de opererade fiskarna låg på nivåer som kan förväntas av en frisk och ostressad individ. Vidare fastställdes också att förebyggande behandling med antibiotika inte verkar ha någon positiv effekt på sårläkningen. Däremot upptäcktes en tydligt kirurg-effekt, där hjärtfrekvensen konsekvent skiljde sig åt under hela försöksperioden beroende på vilken kirurg som hade utfört operationen.

Samma typ av bio-loggers användes även, i kombination med flera blodburna biokemiska stressindikatorer, i **studie II** för att undersöka hur arten sik (*Coregonus lavaretus*) påverkas av upprepad stress i samband med trängning, håvning, båttransport till slakteriet och under påföljande slakt. När informationen från bio-loggrarna analyserades upptäcktes till vår förvåning att hjärtfrekvensen steg kraftigt utan att sjunka till tidigare nivåer redan ett dygn innan transporten genomfördes. Det visade sig att en nätkasse innehållandes regnbåge hade placerats i närheten av sikarna vilket orsakade en kroniskt förhöjd hjärtfrekvens, något som indikerar att siken uppfattade någon form av störning från regnbågarna. Det verkar alltså vara olämpligt ur ett välfärdsperspektiv att hålla sik i närheten av regnbåge då siken uppvisade tecken på långvarig stress under sådana förhållanden.

Resultaten visade tydligt att fiskarna blev stressade vid trängning och håvning i samband med transporten. Fiskarna uppvisade också kraftig stress under tiden de bedövades genom att exponeras för vatten innehållande höga halter koldioxid innan avlivningen.

När djur som hålls av människan förs till slakt eller när de slaktas ska det ske på ett sätt så att de skonas från onödigt lidande och obehag (2018:1192). Vidare ska ett djur som slaktas vara bedövat, och bedövningen ska ske så att djuret snabbt blir medvetslöst och medvetandet får inte återkomma (2019:66). Eftersom vissa bedövningsmetoder som används inom vattenbruket kan vara långsamma, enbart paralysera fisken eller bara orsaka tillfällig medvetslöshet, är det av yttersta vikt att ha pålitliga indikatorer för att avgöra om bedövningen fått önskad effekt.

För att utvärdera tillförlitligheten av en rad lättillgängliga indikatorer, exempelvis andningsrörelser, användes en nyutvecklad teknik för att mäta förändringar i hjärnaktivitet (EEG) hos regnbåge före och efter bedövning med koldioxid, bultpistol samt elektricitet i **studie III** och **IV**. Resultaten visade tydligt att de lättillgängliga indikatorer som används för att bedöma medvetslöshet inte överensstämmer med bedömningen som gjordes utifrån mätningarna av hjärnaktivitet. Detta medför att fiskar riskerar att avlivas genom stupskärning och avblodning när den fortfarande är vid medvetande.

Sammanfattningsvis presenteras här flera viktiga fynd som kan användas för att förbättra fiskvälfärden inom vattenbruket, och som kan ligga till grund för framtida föreskrifter och allmänna råd för hur fiskar ska hanteras, hållas, skötas, samt bedövas och avlivas.

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I

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Effects of prophylactic antibiotic-treatment on post-surgical recovery following intraperitoneal bio-logger implantation in rainbow trout

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Bio-logging devices can provide unique insights on the life of freely moving animals. However, implanting these devices often requires invasive surgery that causes stress and physiological side-effects. While certain medications in connection to surgeries have therapeutic capacity, others may have aversive effects. Here, we hypothesized that the commonly prescribed prophylactic treatment with enrofloxacin would increase the physiological recovery rate and reduce the presence of systemic inflammation following the intraperitoneal implantation of a heart rate bio-logger in rainbow trout (*Oncorhynchus mykiss*). To assess post-surgical recovery, heart rate was recorded for 21 days in trout with or without enrofloxacin treatment. Contrary to our hypothesis, treated trout exhibited a prolonged recovery time and elevated resting heart rates during the first week of post-surgical recovery compared to untreated trout. In addition, an upregulated mRNA expression of TNFα in treated trout indicate a possible inflammatory response 21 days post-surgery. Interestingly, the experience level of the surgeon was observed to have a long-lasting impact on heart rate. In conclusion, our study showed no favorable effects of enrofloxacin treatment. Our findings highlight the importance of adequate post-surgical recovery times and surgical training with regards to improving the welfare of experimental animals and reliability of research outcomes.

Across a wide range of disciplines, approximately 11.5 million experimental animals were used for research purposes within the member states of the European Union in 2011[1]. While the vast majority of these animals are rodents (~80%), the proportion of ectotherms, including teleost fishes, is rapidly increasing[1]. One expanding area within experimental fish research concerns the use of novel implantable electronic tags (*e.g.* bio-logging and bio-telemetric devices). The recent technological developments and use of bio-logging and bio-telemetric devices in aquatic organisms has been proposed to open up a 'panoramic window into the underwater world'[2]. The use of these devices in freely swimming fish allow the continuous collection of high-resolution physiological and behavioural data (*e.g.* heart rate, blood flow and muscle activity) over long periods of time[3–9]. Moreover, data from implanted fish swimming amongst conspecifics (*i.e.* focal animals) can provide important insights into relationships between physiological and behavioral traits across different social contexts in both natural and aquaculture settings[3,10]. However, there are still challenges associated with the use of these implants in order to produce reliable high-quality data, as well as to safeguard the health and welfare of the experimental animal in accordance with the 3 R guidelines.

First of all, introducing a foreign body into an animal may lead to expulsion or encapsulation of the implant, and secondly, the protective barrier of the epithelium is breached during surgery where after the wound can act as an entry-point for pathogens, which will increase the risk of infection and immune reactions[11–14]. The wound repair process starts immediately, as the infliction of a wound initiates local inflammation and tissue repair mechanisms[15]. When a bacterial infection occurs, a systemic inflammatory response is induced. The pathogen

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is detected by the innate immune system, which elicits a cascade of adaptive immune responses in a complex network involving pro- and anti-inflammatory cytokines to neutralize the threat[16]. When a fish experiences such a systemic immune response it is generally linked to an activation of the hypothalamic-pituitary-interrenal axis (HPI-axis) and the release of corticosteroid stress hormones such as cortisol[17,18]. However, a fish undergoing a surgical procedure will additionally experience a combination of other stressors (*e.g.* noxious stimuli, handling, air exposure, anesthetics), which may also have aversive effects on the welfare of the fish and the quality of the obtained data.

In human and veterinary medicine, a wide range of drugs such as antibiotics, analgesia, general- or local anesthesia are commonly used for pain and wound healing management, as well as to facilitate a speedy recovery and minimize the aversive effects of surgery. In experimental fish research, the use of such drugs is still comparably low[19]. However, as the public concern regarding fish welfare is increasing and technical developments have made implantable electronic tags widely available, the use of post-surgical drug treatments is also increasing in fish research[20–22]. When using implantable devices, the fish is normally released back into the wild or into a large school of fish in an aquaculture setting for a relatively long period of time. In such studies, the assessments of wound healing, inflammatory responses or general health of the individual fish is difficult. Thus, treatment with antibiotics is sometimes used to reduce the risk of post-surgical infections when individual monitoring is impossible[20,23]. However, despite the common use of antibiotic treatment in mammals[24], little is known about the therapeutic efficiency, preferential administration routes and dosages for fish. Following the recommendation of veterinarians, the antibiotic enrofloxacin has frequently been used to prevent post-surgical infections in fish[3,7,25–27]. It is a broad-spectrum fluoroquinolone antibiotic that has been shown to efficiently reduce mortality (10-fold) in farmed adult and juvenile Atlantic salmon (*Salmo salar*) diagnosed with the bacterial disease furunculosis[28,110]. Despite the usefulness of prophylactic treatment with enrofloxacin to survive a bacterial infection, the effectiveness in fish subjected to experimental surgical protocols remains unknown.

Obviously, minimizing the risk of infections and allowing the fish an adequate recovery period to recover from the stress following surgery is important to safeguard fish welfare and to obtain unbiased data that are representative of the population. However, it can be difficult to determine when a fish is unstressed and no longer affected by the surgery. Traditionally, measurements of circulating levels of plasma cortisol from whole body- or blood sampling have been used as a proxy for measuring stress in fish, but this method requires the invasive collection of blood or tissue[29]. Novel alternative techniques are available and have been shown to provide robust data on cortisol levels, ranging from non-invasive sampling of faeces, urine or water-borne cortisol to more invasive sampling of fin tissue, mucous or scales[30,31]. However, all of these techniques require the collection of blood, water or tissue and will consequently only provide a "snapshot" of the recovery period or level of stress. As an alternative to measurements of cortisol, recent studies show a strong, significant relationship between cortisol and heart rate of fish responding to various acute stressors in aquaculture[3], as well as during recovery from stress[4]. Therefore, by analysing heart rate during recovery, we can determine when fish have fully recovered from post-surgical stress as a low, stable resting heart rate coincides with the low levels of circulating plasma cortisol commonly associated with an 'unstressed' fish[3,4,29].

The aim of this study was to investigate the effects of pre-surgical prophylactic treatment with enrofloxacin on post-surgical recovery in freely swimming adult rainbow trout implanted with heart rate bio-loggers. Specifically, we hypothesized that enrofloxacin-treatment would decrease the prevalence of infection and reduce the post-surgical recovery period. To address these hypotheses, we quantified local (visually assessed) and systemic inflammation markers (*i.e.* expression of key cytokines in the head kidney), as well as a range of primary and secondary stress indicators (*i.e.* heart rate, plasma cortisol and hematological variables) in rainbow trout with and without prophylactic enrofloxacin-treatment.

Materials and Methods

Animals. Rainbow trout (*Oncorhynchys mykiss*, Walbaum 1792) of mixed sexes were obtained from Vänneåns fiskodling (Knäred, Sweden) and transported to the Department of Biological and Environmental Sciences, University of Gothenburg. The fish were held in a 2000L tank supplied with recirculated, aerated freshwater maintained at 10 °C with a 12:12 hour photoperiod at a density of 15 kg m^{-3}. Fish were allowed to acclimatize for at least three weeks before the experiments. The experimental procedures were approved by the ethical committee on animal research in Gothenburg, Sweden (Gothenburg animal testing ethics committee, ethical permit 2013-177) and all experiments were performed in accordance with relevant guidelines and regulations.

Surgical procedure. Fish were individually anaesthetized in 10 °C freshwater containing MS-222 (150 mg l^{-1}, ethyl 3-aminobenzoate methanesulphonate) buffered with 300 mg l^{-1} $NaHCO_3$ in a 25 L bucket. When opercular movements ceased, the fish were transferred to a surgery table where they were placed on a water-soaked foam. Anesthesia was maintained during surgery by flushing aerated water containing MS-222 (100 mg l^{-1}) and $NaHCO_3$ (200 mg l^{-1}) over the gills. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Inc., St Louis, Missouri, USA.

Sterile Gammex PF (Ansell, Malmö, Sweden) gloves were used throughout surgeries and the instruments were thoroughly cleaned and rinsed in 70% ethanol and left to dry in air between surgeries. Iodine (Jodopax vet. Pharmaxim Sweden AB, Helsingborg, Sweden) diluted to 4 ml l^{-1} was applied to the skin of the fish before a ~4 cm mid-ventral incision was made between the pectoral and pelvic fins. A pit-tag (Passive Integrated Transponder, 12 mm, Oregon RFID, Portland, Oregon, USA) was first inserted into the abdominal cavity to allow individual identification. Bio-loggers (DST milli-HRT, Star-ODDI, Gardabaer, Iceland) were then placed into the abdominal cavity of 36 fish and anchored to their abdominal muscle with a 3-0 sterile monofilament non-absorbable Prolene suture (Ethicon, LLC, Puerto Rico, USA). The bio-logger enabled measurements of heart rate and body temperature, and was positioned in proximity of the pericardium to optimize signal strength and quality as described

previously[3]. As the levels of investigated blood-borne variables can be highly variable, six additional fish were implanted with identical dummy loggers to increase statistical power in the analysis of these variables. The edges of the wound were powdered with antibacterial (Bacibact, Orion pharma, Espoo, Finland) and antifungal powder (Pevaryl 1%, McNeil Sweden AB), where after the wound was closed with 3-4 interrupted sutures (Prolene 3-0 sterile monofilament) and covered with Orabase paste (ConvaTec Inc, Deeside, UK). The surgical procedure took approximately 15 minutes and was performed simultaneously by two surgeons with different levels of experience.

Experiment protocol and bio-logger configuration. Prior to surgery, half of the fish were randomly selected and given an intramuscular injection of enrofloxacin (10 mg kg^{-1} bodyweight, Baytril Vet. 25 mg ml^{-1}, Bayer Animal Health GmbH, Leverkusen, Germany) above the lateral line posterior to the anal fin[3]. This particular route of administration was selected as it allows for the quick administration of a tightly controlled dose of enrofloxacin without the need for additional implants (*e.g.* slow-releasing implants). This group is hereafter referred to as the *ab-treated* group (mass: 710 ± 75 g, n = 21). The other half of the fish were handled identically but did not receive any antibiotic treatment (*untreated* group; mass: 696 ± 78 g, n = 21). After surgery, all fish were placed in a tank similar to the holding tank (*e.g.* 2000L, fish density 15 kg m^{-3}) supplied with recirculating, aerated freshwater maintained at 10 °C with a 12:12 h photoperiod and left for 21 days. During the course of the experiment, fish were fed twice a week with commercial trout pellets (size 4, Protec Trout pellets, Skretting, Stavanger, Norway). Feeding was kept to a minimum to avoid a reduction of water quality due to leftover pellets, and faeces and unconsumed feed was flushed out of the aquaria once per week. To monitor the status of the wound and overall health of instrumented fish during the recovery period, a submersible camera (Sony Exmor R Steadyshot) was used to inspect the fish during the feeding events.

The bio-loggers sampled heart rate for 6 sec with a frequency of 100 Hz (*i.e.* 600 measurements) every 10 min. In addition, at 4, 11 and 17 days post-surgery, a 6 sec ECG recording was sampled (at midnight) to allow for subsequent evaluation of the signal quality and robustness of the heart rate recordings.

Sampling procedures, cortisol and mRNA analyses. After 21 days, the fish were quickly dip netted and anaesthetized in water containing 12 mg l^{-1} metomidate hydrochloride (Aquacalm, Western Chemical Inc, Ferndale, US). A blood sample of 1 ml was immediately drawn from the caudal vessels using a 1 ml heparinized syringe. Fish were then euthanized with a blow to the head, weighed, and the surgical wound was photographed for subsequent analysis of the wound healing (see below for details). The blood was analysed for haematocrit as the fractional red cell volume (%) following centrifugation in duplicate 80 µL microcapillary tubes at 10 000 rpm for 5 min. The remaining blood sample was immediately centrifuged in 1.5 ml Eppendorf tubes (5 min, 10 000 rpm) and the aliquot plasma was transferred to 1 ml tubes and stored at −80 °C for later cortisol analysis.

Blood plasma cortisol levels were determined using a radioimmunoassay described by Young[32] using cortisol antibody (dilution 1:3000, Code: S020; Lot: 1014-180182, Guildhay Ltd, Guildford, Surrey, UK) validated by Sundh[33]. 3H-cortisol hydrocortisone-[1,2,6,7-3H(N)] were used as tracer (NET 396, NEN Life Sciences Products, USA) and hydrocortisone (Sigma-Aldrich, St. Louis, USA) was used as cortisol standards. The radioactivity was determined with a β-counter, Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland).

The head kidney was dissected out and placed in RNAlater (Ambion, Austin, Texas), kept at 4 °C for 24 h and then stored at −80 °C until analysis. RNA from 15–20 µg of each sample was homogenized and extracted using RNeasy Plus Mini (Qiagen GmbH, Hilden, Germany) following the manufacturers protocol. Due to high amounts of DNA in the tissue, RNase-Free DNase Set (Qiagen) was used to avoid reduction of RNA yield and quality. RNA concentrations were quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, Massachusetts) and diluted to 1000 ng μl^{-1}, where after cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California) in a Bio-Rad MyCycler (RNA template concentration 2.5 ng μl^{-1}). mRNA transcript levels of the pro-inflammatory cytokine tumour necrosis factor alfa (TNFα) and anti-inflammatory cytokine transforming growth factor beta (TGFβ) was obtained using qPCR with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) (5 µl) using 0.5 µl primers (0.5 µM) and run in duplicates at 61 °C with a total amount of 2,5 ng cDNA (4 µl template, 10 µl final reaction volume) (Bio-Rad CFX Connect Real-Time System, Bio-Rad CFX manager 3.1) including NTC and control samples excluding iScript Reverse Transcriptase. The efficiency of the rainbow trout specific primers was determined using dilution series (t/2, 50 – 1.5625 ng) (Supplementary Table S1). Elongation factor 1 alfa (ELF1α) was used as reference gene (primer concentration 0.3 µM) and gene expression was determined using the ΔC_T-method. Furthermore, to enable the determination of how the relative expression of the cytokines related to that of completely uninstrumented fish, 10 fish housed in a separate similar tank were sacrificed, sampled in an identical manner as the two experimental groups and used as negative reference group.

Analytical method and calculations. The bio-logger heart rate data was retrieved using the associated Communication Box (Star-ODDI, Gardabaer, Iceland). The software Mercury v 4.28 was used to extract the heart rate data and to generate measurement points, where only the highest graded (*i.e.* grade 0) heart rate recordings on a four-grade scale (*i.e.* grades 0–4) were used in this study, which represented 63 ± 2% of recorded data. This ensured that subsequent analyses were based on highly accurate measurements, as the measurement error associated with grade 0 recordings has been demonstrated to be <1 beat per minute (bpm)[4]. The heart rate recordings were analyzed for the daily mean heart rate (*i.e.* includes periods of spontaneous activity and tachycardia following feeding) and resting heart rate. Resting heart rate was defined as the 20th percentile of the daily heart rate for each individual, which is the method suggested for determination of standard metabolic rate in fish[34] and a slight modification of the method used by Brijs *et al.*[3,4].

The site of incision and suture points were evaluated according to Wagner[35] where redness of the suture entry and exit points of the first and last stitches, as well as the anchoring points (a total of six points), were blindly and

independently rated by two evaluators using a binary scale 1 (inflammation) or 0 (no inflammation). In addition, the level of inflammation at the incision was evaluated on a 6 point scoring scale. Both scores were then summarized to obtain a final inflammation index score (maximum score: 12) as described by Wagner *et al.*[35].

Statistics. Statistical analyses were performed using SPSS version 24.0 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp) and all data are reported as means ± SEM. To describe the temporal changes in heart rate during the 3-week postsurgical recovery period, a linear mixed model with Toeplitz repeated covariance matrix (*i.e.* the lowest Akaike's Information Criterion) was used. The model was run separately on the three weeks post-surgery to avoid missing potential transient effects of the antibiotic treatment. For all models, individuals were set as subject variables and days post-surgery as the repeated variable. Each analysis was performed separately using the daily mean heart rate or the resting heart rate as the dependent variables. In the models, recovery time (day 1-21), experimental group (ab-treated and untreated), surgeon (1 and 2) and their interactions were included as fixed effects. If no interacting effects were observed, they were excluded from the models.

To further explore the general effects of treatment on recovery time, the daily mean and resting heart rates was compared to the heart rate of day 21. Day 21 was selected prior to experimentation as we assumed that at this time point all fish should have fully recovered and stabilized following the surgical implantation of the heart rate bio-loggers. For the non-repeated variables (*i.e.* cortisol, haematocrit, inflammation indices and the expression of TGFβ), independent samples t-tests were used to identify statistical differences. We also performed a paired samples t-test to explore metabolic state through weight differences before and after the trial for each group, followed by a one-way ANOVA to detect potential differences between groups. To meet the assumption of normal distribution, cortisol values were transformed using the natural logarithm (ln). As the data for the expression of TNFα did not meet the assumption of normal distribution, a non-parametric Kruskal-Wallis H-test was used to statistically analyze this variable. Statistical significance was accepted at $P \leq 0.05$.

Results

Visual observations of fish before and after feeding events revealed no obvious differences between the swimming and feeding behaviour of fish in the ab-treated and untreated groups. Furthermore, we did not observe the presence of any impaired or unusual behaviors such as elevated levels of aggression, which is further supported by the lack of wounds openings, fin/body damage or mortality during the course of the experiment. Statistically significant reductions in body mass of 16 ± 3.6 g (~1.4%, $t_{20} = 4.50$, $P < 0.001$) and 10.2 ± 5.8 g (~2.3%, $t_{20} = 1.78$, $P = 0.045$) were observed at the end of the experiment in untreated and ab-treated fish, respectively. However, the observed reductions in body mass did not statistically differ between the two groups ($F_{1,40} = 0.73$, $P = 0.396$).

Effects of surgery on heart rate, cortisol and hematocrit. The mean heart rate of both groups were initially ~60 beats per minute (bpm), which steadily decreased during the first week and plateaued around 30–40 bpm (Fig. 1A). A significant treatment effect on heart rate was found, however, with heart rate being on average 3.7 (resting) and 3.8 (mean) bpm higher in the ab-treated fish during the first week (resting; $F_{1,27.06} = 4.69$, $P = 0.039$ and mean; $F_{1,27.86} = 7.50$, $P = 0.011$ respectively; Fig. 1B,C). After approximately three days, a clear diurnal pattern emerged with a ~10 bpm difference between day and night for both ab-treated and untreated fish (Fig. 1A). Overall, the post-surgical recovery of heart rate was faster in untreated fish, as heart rate was significantly elevated relative to day 21 for six days in the untreated fish and seven days in ab-treated fish ($F_{20,87.74} = 35.46$, $P < 0.001$, Fig. 1B, $F_{20,86.03} = 29.66$, $P < 0.001$, Fig. 1C). Although, with respect to heart rate, it was deemed that both untreated and treated fish had fully recovered from surgery on day 21, both groups exhibited a transient increase in heart rate on days 9-10 when compared today 21 (Fig. 1C).

There was also a significant effect on heart rate depending on who performed the surgeries, whereby fish instrumented by the more inexperienced surgeon had heart rates 5.1 bpm (resting; $F_{1,32.50} = 12.28$, $P < 0.005$) and 4.6 bpm (mean; $F_{1,32.73} = 11.66$, $P < 0.005$) higher than fish instrumented by the more experienced surgeon (Fig. 2). This was not a transient effect, but lasted throughout the entire 21 day trial period and was present in both treatment groups. No interaction effect was found between treatment and surgeon in any of the analyses ($P > 0.9$). At the end of the 21 day recording period, both groups had plasma cortisol levels $< 10\,ng\,ml^{-1}$ (Fig. 3A), and there were no significant differences between the groups ($t_{40} = -0.839$, $P = 0.407$). Haematocrit levels were also similar between groups ($t_{39} = 1.87$, $P = 0.069$, Fig. 3B).

Inflammatory response. At the end of the trial, all fish were in seemingly good health, and no obvious fungal infections or signs of aggression (*i.e.* bite marks or fin damage) were found. Additionally, there were no signs of encapsulation or expulsion of the bio-loggers. The mean inflammation rating scored low in both groups and were not significantly different (1.71 ± 0.39 and 1.62 ± 0.38 for untreated and ab-treated fish, respectively, $t_{40} = 0.177$, $P = 0.86$). Similarly, the mRNA expression of TGFβ did not differ between the groups ($t_{12} = -0.177$, $P = 0.863$, Fig. 4B), but there was a significant, nearly doubled, upregulation of TNFα mRNA expression in ab-treated trout ($\chi 2_{1,12} = 5.545$, $P < 0.019$, Fig. 4A).

Discussion

Contrary to our hypothesis, prophylactic ab-treatment following the surgical implantation of heart rate bio-loggers did not decrease the prevalence of infection or reduce the post-surgical recovery time. Instead, the ab-treatment induced potentially aversive effects on both the gradual recovery of heart rate following surgery, as well as the mRNA expression of the pro-inflammatory cytokine TNFα three weeks post-surgery. Since fish are ectotherms, both bioavailability and half-life of enrofloxacin are affected by water temperature[36]. The half-life of an intramuscular injection of enrofloxacin ($10\,mg\,kg^{-1}$) was ~85 hours in juvenile Atlantic salmon at 10 °C, however, the tissue depletion time of this compound has been shown to be species-specific and even longer in rainbow

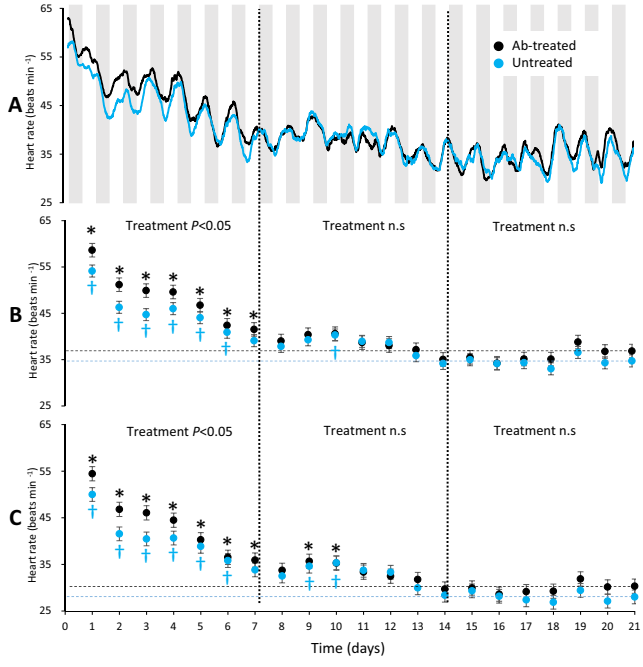

Figure 1. (**A**) Heart rate recordings for 21 days following the surgical implantation of heart rate bio-loggers (grey bars = lights off) in fish treated with antibiotics (ab-treated, black line) and untreated (blue line). (**B**) Post-surgical recovery assessed using daily mean heart rates and (**C**) resting heart rate (20th percentile of daily mean). The black asterisk (*) and blue dagger (†) represents statistically significant ($P < 0.05$) elevations in heart rate compared to values on day 21 for ab-treated and untreated fish, respectively. Dashed black and blue lines highlight the heart rates of day 21 in ab-treated and untreated fish, respectively.

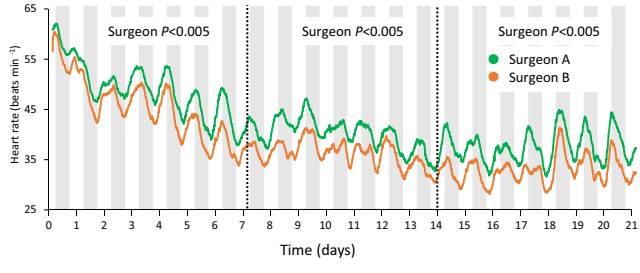

Figure 2. Recordings of heart rate showed a strong correlation to experience level of the surgeon. Throughout the entire three week trial period, the fish instrumented by the less experienced surgeon (surgeon A, n = 14, green line) had an elevated heart rate of 5.1 (resting) and 4.6 (daily mean) beats min^{-1} compared to the more experienced surgeon (surgeon B, n = 22, orange line).

trout than in salmon[37,38]. Thus, the relatively long depletion time of this substance might explain why ab-treated fish displayed a significantly higher mean heart rate (*i.e.* 3.7–3.8 bpm) throughout the first six days post-surgery, as well as a more prolonged overall recovery time as both mean and resting heart rates required an extra day to return to baseline levels when compared to untreated fish. Importantly, as the difference in heart rate between untreated and ab-treated fish was similar for both resting and daily mean heart rate, this implies that behavioural differences (*e.g.* swimming activity) between groups does not explain this effect. Interestingly, following the recovery from the permanent implantation of the bio-logger, both groups of fish exhibited a transient increase in

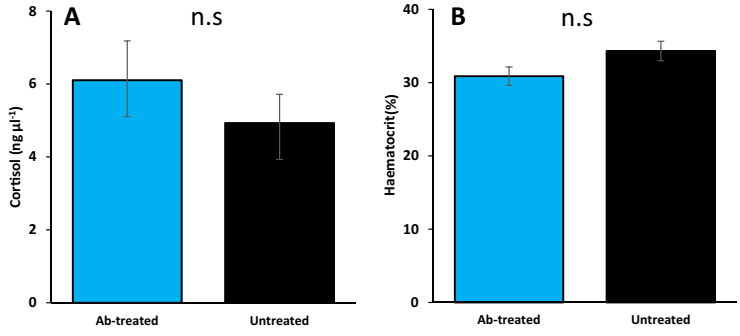

Figure 3. Haematocrit and blood plasma cortisol levels 21 days post-surgery. (**A**) Treatment with enrofloxacin had no effect on circulating blood plasma cortisol where levels were 6.1 ± 1.08 and $4.93 \pm 0.79\,ng\mu l^{-1}$ for ab-treated and untreated respectively 21 days post-surgery. (**B**) Haematocrit count was $30.9 \pm 1.26\%$ for ab-treated fish with no significant difference to the untreated group ($34.3 \pm 1.33\%$).

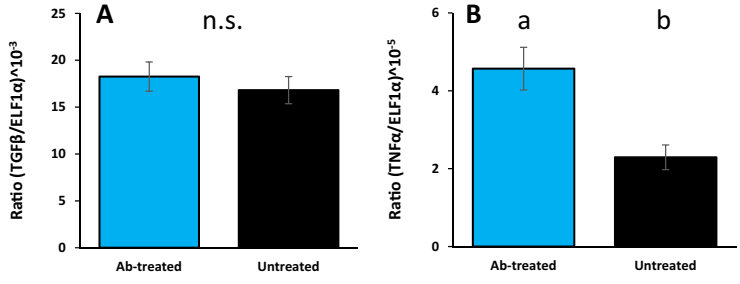

Figure 4. mRNA expression of key cytokines relative to housekeeping gene ELF1α from head kidney, sampled 21 days post-surgery. (**A**) No differences were found between groups for expression of TGFβ, n = 7, however, (**B**) the ab-treated group had a significantly increased expression of TNFα compared to the untreated group n = 6. (45.7 ± 5.48 versus 22.3 ± 3.17, (all values $*10^{-6}$) respectively.

heart rate on days 9–10. Although the underlying reason for this response remains unknown, the presence of this response in both groups demonstrates that fish were able to behaviorally and/or physiologically respond in a similar manner to the unknown stimuli. This unexplained transient elevation in heart rate has also been documented in a previous study on single-housed rainbow trout exposed to buprenorphine in the same aquaria facilities[39], and thus it may be beneficial for future studies to employ video and sound recording in experimental rooms to explain these seemingly random events.

An upregulation of the mRNA expression of TNFα in the head kidney was observed in ab-treated fish three weeks after surgery. Being a pro-inflammatory cytokine, TNFα is known to be involved in the acute phase reaction during infection, inflammation and/or vaccination[39,40]. This may indicate an inflammatory response. However, it should be kept in mind that mRNA will undergo post- transcriptional regulation to reach the functional protein. In rainbow trout, increased TNFα mRNA expression has been observed in absence of TNFα protein secretion[41]. Thus, the significance of increased TNFα mRNA levels should be interpreted with care. Furthermore, we found no evidence for a stress response of fish from either group three weeks after surgery, as levels of circulating plasma cortisol were within the expected range of unstressed trout ($<10\,ng\mu l^{-1}$ [29]). In addition, hematocrit levels were normal (30–35%[42,43]) and there was a low prevalence of visual signs of inflammation around the wound[44]. Overall, these results suggest that the welfare of the fish was not impaired.

During the period following surgery, mean and resting heart rates were both elevated for at least 6 days. Previous field studies in aquaculture settings have shown that it takes at least three days for heart rate to fully recover from the stress associated with implantation of the same type of bio-loggers[3,26]. This discrepancy between field and laboratory studies can partly be explained by higher ambient temperature in the field studies. Indeed, wound healing rate can be different, where the warm water zebrafish (*Danio rerio*) have been shown to heal at a much faster rate than cold water species such as rainbow trout[45,46]. The water temperature was roughly 5 °C colder in our study compared to the other field studies, which could explain why those fish recovered faster. In the

abovementioned aquaculture field studies both the daily mean and resting heart rate plateaued at higher levels in the field (~55–60 bpm, daily mean). This too could be an effect of the higher temperature but it might also be that fish in aquaculture environments are exposed to a higher level of general stress or increased activity levels compared to laboratory housed animals. At 15 °C, heart rate of rainbow trout was lower in laboratory environment (32 bpm)[47] compared to heart rates reported in field studies[3,26], suggesting a situation where the laboratory environment allow for "real" resting levels, *i.e.* possibly lower than what would be seen in the field. Similar to previous studies, a clear circadian rhythm in heart rate was absent during the first ~3 days following surgery, which has been suggested to be an indicator for post-surgical stress and potentially reflects behavioural disturbances in swimming activity[3,48,49].

Although the intraperitoneal implantation of a bio-logger is a relatively simple surgical procedure, the fish in our experiments are still subjected to a series of stressors, which include (i) capture of the fish by netting, (ii) exposure to an anesthetic agent[50], (iii) 15 minutes of surgery, (iv) the presence of a foreign body within the abdomen, and (v) the reintroduction with conspecifics in a new environment during the recovery period[4,51,52]. Previous studies have demonstrated that these stressors may contribute towards the relatively long period of elevated heart rate observed in the present study[3,4,39]. For example, under similar laboratory conditions (*e.g.* fish held in recirculating aerated freshwater maintained at 10 °C with a 12:12 hour photoperiod at a density of ~15 kg m^{-3}), heart rate of rainbow trout increased rapidly by ~25 beats min^{-1} following netting and took ~2 h to recover when alone[4]. In the same study, when trout were netted but instead grouped together at a density of ~15 kg m^{-3} during recovery, heart rate did not recover to pre-stress levels within 7 h[4]. Furthermore, in a separate study under the same conditions, when rainbow trout were anaesthetised using MS-222 and subjected to a similarly sized abdominal incision without the implantation of a bio-logger, heart rate of trout remained elevated by ~10 beats min^{-1} for 24 h after surgery[39]. In addition to the isolated effects of each stressor, repeated stress induced by multiple stressors have also been demonstrated to have a cumulative and long-lasting effect on heart rate of rainbow trout[3]. Thus, further refinement to the techniques associated with the implantation of bio-logging or bio-telemetric devices is warranted, as a reduction in post-surgical recovery time would be beneficial for both the wellbeing of the experimental animal and the outcome of the experiments. This is because stress compromises the ability for the fish to maintain homeostasis and potentially increases their vulnerability to infections which is somewhat problematic[53]. In addition, if a fish is exposed to a new stressor while recovering from an earlier stressor, their physiological or behavioural responses may not be representative of that of a healthy fish, which will consequently bias the results of the study[4,26].

Interestingly, our results also clearly show that surgical training and experience play an important role in improving post-surgical wound healing and the welfare of experimental animals, as less experienced surgeons often need more time to perform the surgery and may close the wound too tight[54]. Consistently, surgical times were noted to be approximately a few minutes longer for the inexperienced surgeon and it also resulted in somewhat longer suture ends. Indeed, the difference in surgical experience accounted for an elevation in heart rate of ~5 beats min^{-1}, which strongly suggests that refining surgical protocol may be more important than casual prophylactic use of antibiotics to facilitate fast recovery from instrumentation in fish[55]. However, prophylactic use of enrofloxacin may still be necessary in experiments conducted in environments where the risk of infection is significant and the possibility to monitor of fish welfare continuously is limited, and warrants further investigation. Our findings also highlight the importance that fish are given adequate post-surgical recovery times before the start of the experiments to avoid treatment bias.

Conclusion

The present study highlights the importance of researchers being aware of the potential side-effects when exposing animals to a drug as part of the experimental protocol. The purpose of medication should be to provide the best possible care for the animal by minimizing health problems or other welfare issues, which in turn should lead to more reliable data in accordance with the 3R concept. However, as this study demonstrates, the side effects of the medication need to be examined as they can potentially impinge on the health and welfare of the experimental animal, as well as the reliability of the data. Our findings are not only important from a 3R perspective but also from an antibiotic resistance viewpoint, as unnecessary use of antibiotics should be avoided. Thus, future improvements in surgical protocols and training may be more beneficial for the experimental animals and research outcomes, especially considering the growing number of both individuals and species of fish used in experiments.

Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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Writing original draft: P.H. Laboratory work: P.H., A.G., D.M., H.S., A.E. and J. Bo. Data processing: A.G., P.H. and J. Br. Conceptualisation: A.G., P.H., K.S., H.S., E.S. and C.B. Funding and project administration: A.G. All authors were involved in reviewing and editing of the manuscript.

Competing interests

The authors declare no competing interests.

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Continuous physiological welfare evaluation of European whitefish (*Coregonus lavaretus*) during common aquaculture practices leading up to slaughter

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ABSTRACT

European whitefish (*Coregonus lavaretus*) is an aquaculture species with the potential for expanded cultivation in the fresh and brackish waters of Northern Europe. Yet, relatively little species-specific information is available regarding the stress responses and associated welfare implications for this species in captivity. We addressed this knowledge gap by using a combination of implantable heart rate bio-loggers and a range of traditional stress indicators (*e.g.* haematological parameters and plasma concentrations of cortisol, glucose and ions) to comprehensively evaluate the physiological responses of freely swimming whitefish in captivity, as well as when subjected to aquaculture practices and stressors that commonly occur prior to and during slaughter. Whitefish appeared to recover rapidly from surgery, as resting heart rate decreased within 36 h to stabilize at ~25 beats min^{-1} for the next 18 days when fish were left relatively undisturbed (*i.e.* personnel were only present when feeding fish). In contrast with previous studies on farmed rainbow trout and Atlantic salmon, whitefish did not exhibit a clear circadian heart rate rhythm, which may be related to species-specific differences in diurnal locomotor activity. Whitefish also appear to have a well-developed capacity for thermal acclimation of heart rate, as daily resting heart rate did not change during the undisturbed period despite an increase in body temperature from ~6.8 to 11.2 °C. Following acute stressors such as crowding and transportation, the physiological response of whitefish typically involved transient elevations in heart rate, plasma cortisol and glucose, and red blood cell swelling, while plasma $[K^+]$ decreased. In contrast, the heart rate of whitefish plummeted following the combination of brailing (*i.e.* to haul in fish with a brail/net) and CO_2 exposure prior to slaughter, while plasma cortisol, glucose and $[Ca^{2+}]$ significantly increased. An unforeseen finding concerns the substantial and long-lasting physiological stress response observed in whitefish when held in close proximity (*i.e.* within ~10 m) to a rainbow trout net pen, as the mean heart rate of whitefish increased from ~32 to 43 beats min^{-1} (*i.e.* an increase of ~34%). This may represent an innate physiological response to the threat of predation, which consequently increases the allostatic load and energetic expenditure of whitefish when farmed alongside salmonids. To conclude, this study highlights the importance of performing long-term, species-specific evaluations of freely swimming fish in real aquaculture settings, and provides a platform for further research aiming to determine the welfare implications of simultaneously farming predatory and prey species in close proximity.

1. Introduction

Due to the ever-growing demand for safe and nutritious food, aquaculture continues to grow faster than other major food producing sectors (FAO, 2020). Alongside, and perhaps partly due to the rapid expansion of the aquaculture industry, the welfare of farmed fish has

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become increasingly important for consumers, producers, retailers, interest groups and the authorities (Ashley, 2007; Cooke, 2001; EFSA, 2009; Frewer et al., 2005; OIE, 2019). The concept of animal welfare is complex and takes into account an animal's subjective mental state, the capacity to express their natural behaviour, and their biological ability to adapt to their environment and remain in good health (Ashley, 2007; Segner et al., 2012). While the debate regarding whether or not fish possess the mental capacity to experience pain and suffering is still ongoing, the balance of evidence indicates that fish may indeed have such capabilities and should therefore be given the benefit of the doubt with regards to their welfare (Ashley, 2007; EFSA, 2009; FAO, 2020; Huntingford et al., 2006; OIE, 2019; van de Vis et al., 2012). Despite the growing awareness, understanding and concern for the welfare of farmed fish, an overall shortage of scientific data still exists, especially when one considers the high diversity of fishes currently being exploited for aquaculture purposes (*e.g.* ~369 species of finfish were estimated to be farmed world-wide in 2016; FAO, 2020; OIE, 2019).

Scientific approaches to assess the welfare of fish in aquaculture are continually developing and largely revolve around evaluating the responses of fish to environmental and/or anthropogenic stressors (Ashley, 2007; Segner et al., 2012). Stress can be defined as any condition or state that threatens an animal's homeostasis such that it needs to induce a range of responses to re-establish homeostasis (*i.e.* allostasis; Korte et al., 2007; Segner et al., 2012). The initial response to stress typically includes the release of catecholamines and the stimulation of the hypothalamic-pituitary-interrenal axis culminating in the release of corticosteroids such as cortisol (*i.e.* primary stress responses; Barton, 2002; Wendelaar-Bonga, 1997). This neuroendocrine stress response elicits subsequent changes such as an increase in cardiorespiratory activity, redistribution of blood flow according to altered tissue oxygen demands, splenic release of red blood cells and mobilization of energy stores. Collectively, this serves to enhance the probability of survival for an individual facing a threatening situation (*i.e.* secondary stress responses; Ashley, 2007; Barton, 2002). However, overly severe or long-lasting stress disrupts the negative feedback mechanism of the stress response, which ultimately increases the allostatic load on the animal and may result in detrimental tertiary stress responses such as impairments of appetite, growth rate, swimming ability, immune responses, behavioural repertoire, reproductive ability and cardiac function (Ashley, 2007; Eliason et al., 2013; Korte et al., 2007; Segner et al., 2012). Thus, from both an animal welfare and economic perspective, it is essential that the origin and/or causes of stress responses are identified early enough so that an intervention can take place before physiological mechanisms are compromised and become detrimental to the fish's health and well-being (*i.e.* state of distress or allostatic overload; Korte et al., 2007; Moberg, 2000; Segner et al., 2012; Huntingford et al., 2006; van de Vis et al., 2012).

Since neurohumoral factors released during the primary stress response (*i.e.* adrenalin, noradrenalin and cortisol) are known to increase heart rate and energy mobilization, the measurement of heart rate can be used as a proxy to assess the stress responses of fish (Cooke et al., 2016). Moreover, since heart rate is often correlated with metabolic rate in various fish species (Armstrong, 1986; Campbell et al., 2004; Clark et al., 2005; Eliason et al., 2008; Eliason et al., 2011), it can also provide insights into the relative energetic consequences of different environmental and/or anthropogenic stressors (Cooke et al., 2016). Recent advances in the development and miniaturization of heart rate bio-loggers and bio-telemetry systems have thus far made it possible to determine the magnitude and duration of stress responses induced by various aquaculture practices in species such as rainbow trout (*Oncorhynchus mykiss*; Brijs et al., 2018, 2019a, 2019b), Atlantic salmon (*Salmo salar*; Hvas et al., 2020), and Atlantic cod (*Gadus morhua*; Bjarnason et al., 2019). However, due to the wide variety of ecological adaptations and evolutionary histories of fishes, different species can react very differently to environmental and/or anthropogenic stressors. Thus, there is an urgent need for consideration of species-specific differences when developing guidelines, regulations and legislations intended to safeguard fish welfare in aquaculture (Barton, 2002; EFSA, 2009; FAO, 2020; OIE, 2019). To achieve this, further research is required for a vast number of species to identify situations in the rearing environment that may compromise fish health and welfare, as well as to quantify the potential negative impacts of common practices employed in aquaculture.

One of the species that warrants further investigation is the European whitefish (*Coregonus lavaretus*). This highly valued cold-water species has a relatively high growth rate and favorable feed conversion ratio, and thus has the potential to become an important commercial species in both fresh and brackish water in northern Europe (Jobling et al., 2010; Siikavuopio et al., 2012). Despite anecdotal reports stating that this species is highly susceptible to stress (*e.g.* fish have been observed to immediately reduce feed intake and/or lose equilibrium following common aquaculture practices), their ability to tolerate the varying environmental and/or anthropogenic stressors that they are regularly exposed to in aquaculture settings has not been systematically investigated. Therefore, the aim of the present study was to comprehensively evaluate the stress responses of European whitefish subjected to aquaculture practices that commonly occur before and during slaughter. Specifically, by using surgically implanted heart rate bio-loggers in focal fish, we aimed to continuously record heart rate and body temperature of whitefish for an extended period of time when left undisturbed in the sea cage to identify 'normal' heart rate patterns of this species, as well as to identify deviations from this pattern in response to a range of common aquaculture practices (*e.g.* crowding, well-boat transport, brailing and CO_2 exposure). In addition, we aimed to use 'early' physiological stress indicators such as plasma cortisol levels and a range of haematological and blood chemistry parameters to further assess the severity of the stress responses, as well as to validate the implications of the observed heart rate responses. Detailed species-specific information regarding the welfare of European whitefish in aquaculture is urgently required for the development of guidelines regarding the ethical production of this food source.

2. Materials and methods

2.1. Experimental animals

The experimental part of the study took place at Brändö Lax AB facilities, Brändö, Åland Islands, Finland between the 9th and 30th of May 2017 (hereafter referred to as day 0 to 21. European whitefish (n: 116, *Coregonus lavaretus*, hereafter referred to as 'whitefish') with body masses of 752 ± 255 g were used in the present study (all data in the materials and methods section are presented as means±s.d.). They were kept in a circular sea cage (diameter: 40 m, depth: 4 m) together with ~3000 conspecifics, which was located near the slaughterhouse facilities in the Djurholms Sound. All experimental protocols were approved by the Åland provincial government project approval committee (decision 2/2016).

2.2. Bio-logger details and surgical implantation

From the 116 fish used in the present study, a sub-sample of 20 individuals with body masses of 901 ± 310 g were individually implanted with a 12 mm Passive Integrated Transponder tag (PIT-tag, Oregon RFID, Portland, Oregon, USA) for future identification, as well as a DST milli-HRT bio-logger (Logger version 8 DM/CRC16/4800, STAR-ODDI, Gardabaer, Iceland). The bio-loggers (diameter: 13.0 mm, length: 39.5 mm, volume: 5 cm^3, mass in air: 11.8 g) monitor heart rate *via* a single channel electrocardiogram (ECG) amplifier that uses three measuring electrodes incorporated into the ceramic casing. Each logged heart rate value is derived from the mean RR-interval (*i.e.* time between two consecutive R waves in the ECG) from a burst of 600 measurements, which corresponds to a 6-s period when sampling at 100 Hz. For

validation purposes, all logged heart rate measurements are graded with a data verification quality index (QI) which range from 0 to 3 where 0 = Great, 1 = Good, 2 = Fair and 3 = Poor. A temperature sensor with a resolution of 0.032 °C and an accuracy of ±0.2 °C is also located within the casing of the bio-logger. Additionally, the sensor contains a real-time clock with an accuracy of ±1 min month^{-1}. The bio-loggers were programmed with the application software Mercury v 4.28 and the associated Communication Box (STAR-ODDI, Gardabaer, Iceland) to measure heart rate and body temperature every 10 min from 6:00 on day 0 till 6:00on day 20, after which measurements were taken every 2 min until 24:00 on day 21. All times are reported in Eastern European Summer Time (EEST or UTC + 2).

PIT-tags and bio-loggers were surgically implanted between 8:00and 15:00on day 0. To achieve this, fish were individually dip netted from the sea cage and placed in a bin containing seawater from the Djurholms Sound with 150 mg L^{-1} ethyl-3-aminobenzoate methanesulphonic acid (MS222, Sigma-Aldrich Inc., St. Louis, Missouri, USA) buffered with 300 mg L^{-1} $NaHCO_3$ at 5 °C to induce surgical anaesthesia. When anaesthetised (as indicated by loss of opercular movements), the fish was placed on an operating table and anaesthesia was maintained by continuously flushing aerated water containing MS-222 (100 mg L^{-1}) and $NaHCO_3$ (200 mg L^{-1}) over the gills. A 25–30 mm long mid-ventral incision was then made with a scalpel ~40 mm posterior to the pectoral fins. The DST milli-HRT bio-logger was subsequently inserted into the abdominal cavity and placed in close proximity to the pericardium, which is a position previously determined to be optimal for salmonids with regards to signal strength and quality (Brijs et al., 2018; Svendsen et al., 2021). The bio-logger was anchored to the abdominal muscle with a 3–0 sterile monofilament non-absorbable Prolene™ suture (Ethicon, LLC, Puerto Rico, USA). The PIT-tag was then inserted into the abdominal cavity *via* the same incision prior to closing the wound with interrupted non-absorbable Prolene™ sutures. To promote wound healing, a mixture consisting of a protective paste (Orabase®, ConvaTec, Bromma, Sweden), an antifungal agent (Pevaryl®, McNeil Sweden AB, Solna, Sweden) and an antibacterial agent (Bacibact®, Orion Corporation, Espoo, Finland) was applied on the surface of the wound while a broad-spectrum antibiotic (10 mg kg^{-1} Baytril®, Bayer Healthcare, Berlin, Germany) was injected intraperitoneally. To facilitate the retrieval of the bio-logger during slaughter, fish were tagged with blue dots in the area between the pectoral fins using Alcian Blue dye injected with a pressure injector (AKRA Dermojet Polymedical, Barthou, France). After the surgical procedure, which took ~10 min to complete, fish were released back into the sea cage with their conspecifics.

2.3. Monitoring of whitefish in sea cage and timing of events during aquaculture procedures

Whitefish remained in the sea cages with conspecifics during days 0–20. Fish were fed *ad libitum* once per day with commercial feed pellets (BioMar, Aarhus, Denmark) on days 0–13. Fish were fasted on days 14–20 in accordance with the pre-slaughter procedures employed at Brändö Lax AB. With the exception of the abovementioned daily feeding events, the fish used in the present study remained relatively undisturbed until 21:00 on day 19 when a sea cage containing ~5000 rainbow trout (individual body masses ranging between ~2 to 4 kg) was towed by a well-boat and secured in close proximity (*i.e.* within 10 m) to the sea cage containing the whitefish.

On days 20–21, the procedures associated with the slaughter of whitefish took place. Before and during a number of the aquaculture practices described below, sub-samples of randomly selected whitefish were carefully captured with a hand net, euthanised by a sharp blow to the head, sampled for blood (~1 mL) from the caudal vessels using a heparinised syringe, and then weighed for body mass and measured for fork length (Table 1). These blood samples were immediately placed on ice for further analyses (see *2.5. Blood analyses*).

Between 11:45 and 12:15 on day 20, whitefish were crowded by lifting the bottom of the sea cage during which the first sub-sample of fish (n: 10) were captured and sampled as quickly as possible in order to collect 'pre-stressor' blood samples (*i.e.* blood samples representative of event 1, see *2.4. Data retrieval from bio-loggers and analysis of heart rate*). The bottom of the sea cage was subsequently released to allow the whitefish to disperse until the well-boat arrived. Whitefish were then crowded between 13:45 and 14:20, and subsequently brailed (*i.e.* the act of hauling in fish with a brail/net) into the well-boat between 14:20 and 14:35. The fish were then transported around the Djurholms Sound in the well-boat between 14:35 and 15:45 during which the second sub-sample of fish (n: 30) were captured and sampled for blood (*i.e.* blood samples representative of event 4–6, see *2.4. Data retrieval from bio-loggers and analysis of heart rate*). Dissolved oxygen levels of the water within the well-boat were also monitored throughout the transportation event using a hand-held oxygen meter (HQ40D Portable Multi Meter, Hach Lange GmbH, Düsseldorf, Germany). Following transportation, whitefish were released from the well-boat into a holding cage near the slaughterhouse *via* a water chute. It was noted that this holding cage was also in close proximity to other holding cages containing rainbow trout. To investigate the recovery of whitefish from the procedures described above, a third sub-sample of fish (n: 19) were randomly captured from the holding cage and sampled for blood between 16:26 and 20:12 (*i.e.* a different individual was sampled every ~12 min). Fish were then left undisturbed overnight.

The slaughter of whitefish on day 21 occurred between 08:15 and 11:15. Between 06:20 and 06:40, the fourth sub-sample of fish (n: 10) were captured and sampled as quickly as possible in order to collect 'pre-slaughter' blood samples (*i.e.* blood samples representative of event 8, see *2.4. Data retrieval from bio-loggers and analysis of heart rate*). The slaughter procedure included the crowding and subsequent brailing of whitefish from the holding cage to an air chute, which led to a tank in the slaughterhouse containing CO_2-saturated seawater. Fish were then

Table 1
Description of the various events and when those events took place, as well as details regarding the sampling duration, times and sizes for the heart rate analyses and blood sampling associated with the various events.

Event	Event description	Day	Time	Mean heart rate sampling		Blood sub-sampling	
				Sampling duration (min)	*n*	Sampling time	*n*
1	Undisturbed	20	11:45–12:15	20	20	12:03–12:11	10
2	Peak response to first crowding	20	13:45–14:20	20	20		
3	Prior to second crowding	20	14:20–14:35	20	20		
4	Peak response to second crowding	20	14:35–15:45	20	20	14:23–15:35	30
5	Beginning of transportation			20	20		
6	End of transportation			20	20		
7	Peak heart rate during day 20	20	16:26–20:20	20	20	every 12 min	19
8	Prior to initiation of slaughter procedures	21	06:20–06:40	20	20	06:25–06:37	10
9	Peak response to final crowding	21	08:15–11:15	10	20	08:22–08:57	17
10	Response following brailing and CO_2 exposure	21		10	20	08:32–09:48	30[a]

[a] Consisted of sampling blood from 20 instrumented and 10 uninstrumented whitefish.

held in this tank until they could no longer maintain equilibrium and were deemed unconscious by the slaughterhouse personnel, whereupon they were mechanically lifted onto a grid designated as the 'gill cutting station'. Personnel then manually cut the gill arches and ventral aorta of each individual, and transferred the fish to an adjacent tank for exsanguination. The fifth and sixth sub-sample of fish were sampled for blood during the crowding phase (n: 17) and directly following brailing and CO_2 exposure (n: 30, which consisted of 10 uninstrumented fish and the 20 fish instrumented with bio-loggers), respectively. These blood samples were representative of event 9 and 10, respectively (see *2.4. Data retrieval from bio-loggers and analysis of heart rate*).

2.4. Data retrieval from bio-loggers and analysis of heart rate

The data was retrieved from the bio-loggers by placing the loggers in the associated Communication Box and the application software Mercury v 4.28 was used to extract the recordings. The data was then organised using Excel (Microsoft office 2016). To ensure that subsequent analyses were based on highly accurate heart rate measurements, only measurements with the highest grade (*i.e.* grade 0 which represented 57 ± 18% of the recorded data) were included in the analyses. The measurement error associated with grade 0 recordings has previously been demonstrated to be <1 beat min^{-1} (Brijs et al., 2019a).

Prior to procedures associated with slaughter (*i.e.* during days 0–20), mean heart rate and resting heart rate of whitefish were calculated from 12 hourly periods between days 0–3 (to investigate recovery from handling, anaesthesia, surgery and reintroduction with conspecifics in the sea cage), from 24 hourly periods between days 3–19 (to investigate the temporal dynamics of heart rate when fish were left undisturbed), and from 6 hourly periods between days 19–20 (to investigate the effects of the arrival of the sea cage containing rainbow trout). Mean heart rate includes periods of spontaneous activity and/or postprandial responses, whereas resting heart rate accounts for the theoretical temporal variations in the heart rate of individuals during periods of inactivity. The analysis of resting heart rate was based on a method used for determining standard metabolic rate in fish (Chabot et al., 2016), and consists of calculating the 20th percentile of recorded heart rate values for each individual. This method has previously been shown to provide a good approximation of heart rate of fish during periods of rest (Hjelmstedt et al., 2020).

To evaluate the severity of, and recovery from, the different acute stressors associated with slaughter, the mean heart rates of whitefish were calculated from ten specific time periods or 'events' during days 20–21 (Table 1). Mean heart rates were calculated from 20 min periods within events 1–8 (*i.e.* 1–10 measurements) and from 10 min periods within events 9–10 (*i.e.* 1–5 measurements). Finally, to assess the cumulative effects of the different stressors that whitefish were subjected to during the procedures associated with slaughter, mean heart rates during each of the abovementioned 'events' on days 20–21 were compared to the mean heart rates calculated for the corresponding time of day during days 16–18. These days were used for comparison as the fish were considered undisturbed and maximally recovered from anaesthesia and surgery, and best represented the conditions the fish were exposed to leading up to slaughter with respect to feeding status and water temperature (Brijs et al., 2018).

2.5. Blood analyses

Blood samples were analysed for haematocrit (Hct, %) and haemoglobin concentration ([Hb], g dL^{-1}). The Hct was determined as the fractional red cell volume after centrifugation of a subsample of blood in 80 μl heparinised microcapillary tubes at 10,000 rcf for 5 min in a Hct centrifuge (Haematokrit 210, Hettich, Tuttlingen, Germany). A handheld Hb 201+ meter (Hemocue® AB, Ängelholm, Sweden) was used to determine [Hb] and values were corrected for fish blood (Clark et al., 2008). Mean corpuscular haemoglobin concentration (MCHC, g dL^{-1}) was subsequently calculated as [Hb]/Hct × 100.

Following the haematological analyses, the remaining blood samples were centrifuged at 10000 rcf for 5 min in a microcentrifuge (Eppendorf 5415D, Eppendorf, Hamburg, Germany). The plasma was subsequently collected and frozen at −80 °C for analyses to determine the concentration of plasma glucose (mmol L^{-1}) and plasma cortisol (ng mL^{-1}). Concentration of plasma glucose was determined using a glucose assay kit (GAHK20, Sigma-Aldrich, St. Louis, Missouri, USA). Plasma cortisol concentration was determined by a radioimmunoassay (RIA) previously described by Young (1986) that uses a cortisol antibody (Code: S020; Lot:1014–180,182, Guildhay Ltd., Guildford, Surrey, UK) validated by Sundh et al. (2011). As a tracer, tritiated hydrocortisone-[1,2,6,7–3H (N)] (NET 396; NEN Life Sciences Products, Boston, Massachusetts, USA) was used and cortisol standards were prepared from hydrocortisone (Sigma, St. Louis, Missouri, USA). Radioactivity was determined with a Wallac 1409 liquid scintillation counter (LKB Instruments, Turku, Finland). Intra- and interassay coefficients of variation for this cortisol RIA has been shown to be 3.9% and 5.4%, respectively, with a detection limit of 0.7 ng mL^{-1} (Sundh et al., 2011). Plasma ion concentrations (*i.e.* $[K^+]$, $[Na^+]$, $[Cl^-]$, and $[Ca^{2+}]$ in mmol L^{-1}) were determined using a Convergys®ISE comfort Electrolyte Analyzer (Convergent technologies, Coelbe, Germany).

2.6. Statistical analyses

Statistical analyses were performed using SPSS Statistics 26 (IBM Corp., Armonk, New York, USA). All data were assessed to ensure that they did not violate the assumptions of the specific models outlined below. F-, t- and *p*-values obtained from the statistical analyses are reported throughout the text and all *p*-values of <0.05 were considered statistically significant. All data in the results section are presented as means±s.e.m. All data supporting the paper is readily available in the supplementary information (S1-S11).

To statistically analyse body temperature (*i.e.* days 0–20) and heart rate (*i.e.* days 0–3, days 3–19, and days 19–20) of instrumented whitefish prior to the initiation of slaughter procedures, we used one-way repeated measures ANOVAs with Bonferroni adjusted post-hoc tests. Linear regressions were used to assess the relationship between heart rate and body temperature for each individual whitefish during the entire undisturbed period (*i.e.* days 3–19), as well as separately during the fed (*i.e.* days 3–11) and fasted period (*i.e.* days 12–19).

To statistically analyse heart rate of instrumented whitefish during the ten specific 'events' that occurred during the slaughter days (*i.e.* days 20–21), as well as in comparison to the mean heart rates calculated for the corresponding time of day during the days leading up to slaughter (*i.e.* days 16–18), we used a two-way mixed ANOVA with a Bonferroni adjusted post-hoc test. This model used 'events' as the within-subjects factor (fixed factor, ten levels: event 1–10), the specific days as the between-subjects factor (fixed factor, two levels: slaughter day and days leading up to slaughter), and the interaction between these factors. Since a significant interaction was detected between the fixed factors of this model ($F_{6.33,240.42} = 48.67$, $p < 0.001$), the simple main effects of the model were determined by using one-way ANOVAs for each category of the within-subjects factor or by using one-way repeated measures ANOVAs for each category of the between-subjects factor. Since the assumption of sphericity was violated (assessed by Mauchly's test of sphericity), a Greenhouse-Geisser correction was applied to each of the abovementioned repeated measures analyses involving heart rate,

To statistically analyse the differences in body and blood/plasma parameters of whitefish that were representative of different events (*i.e.* event 1, 4–6, 8, 9 and 10), we used one-way ANOVAs with Tukey's post-hoc tests when no assumptions were violated (*i.e.* for body mass, $[Ca^{2+}]$ and plasma glucose).When the assumption of homogeneity of variance was violated, we instead used Welch ANOVAs with Games-Howell post-hoc tests (*i.e.* for body length, plasma cortisol, [Hb], Hct, MCHC, $[K^+]$, $[Na^+]$ and $[Cl^-]$). Despite significant differences in the body mass and

length of the sampled whitefish (*i.e.* body mass and length of whitefish sampled during event 10 > event 1, 4–6, 8 and 9; Table 2), neither of these body parameters were found to be significantly related to any of the measured blood/plasma parameters and were therefore not included as covariates in the abovementioned models. Following the combination of all the stressors that occurred on day 20 (*i.e.* after event 7), a logarithmic regression was used to predict the decrease in plasma cortisol over time since this model proved to be the best fit (as assessed by the R^2 value).

3. Results

3.1. Heart rate responses of whitefish before slaughter (days 0–20)

Visual inspection of the instrumented whitefish at the end of the experiment indicated that the incision made to insert the bio-logger had healed satisfactorily in all fish and no obvious signs of infection were detected. During the 21 days following the surgical implantation of bio-loggers, the instrumented fish had significantly increased in body length by 5 ± 1 mm (~1% increase in body length, $t_{19} = 4.22$, $p < 0.001$) and decreased in body mass by 22 ± 2 g (~2% decrease in body mass, $t_{19} = -10.58$, $p < 0.001$). The mean body temperature of instrumented fish significantly increased from ~6.0 to 11.2 °C throughout the experimental period ($F_{20,380} = 3660.55$, $p < 0.001$, Fig. 1A), as a result of similar increases in ambient water temperature.

Between days 0–3, the mean heart rate of whitefish was initially elevated following handling, anaesthesia, surgery and reintroduction with conspecifics in the sea cage, yet decreased rapidly during the recovery period (Fig. 1A). Temporal changes in mean heart rate (*i.e.* from ~44 to 30 beats min^{-1}) and resting heart rate (*i.e.* from ~38 to 24 beats min^{-1}) indicated that fish recovered from the stress associated with the surgical implantation of heart rate bio-loggers in less than two days (Fig. 1B). Between days 3–19, mean heart rate typically fluctuated between 25 and 45 beats min^{-1}, yet no clear circadian heart rate rhythm was observed (Fig. 1A). Despite a significant increase in body temperature from ~6.8 to 11.2 °C during this period, as well as changes in feeding state (*i.e.* fish were fed daily to satiation on days 0–13 and then fasted on days 14–20 in preparation for slaughter), resting heart rates did not significantly change (Fig. 1C). Visual inspection of scatterplots revealed no clear or significant relationships between heart rate and body temperature of individual whitefish during the entire undisturbed period or within the fed and fasted periods. At ~21:00 on day 19, mean heart rate rapidly increased and remained elevated thereafter (Fig. 1A). It was noted that this increase in heart rate coincided with the introduction of a sea cage containing ~5000 rainbow trout in close proximity (*i.e.* within 10 m) to the sea cage containing the whitefish. Statistical analyses of the temporal changes in heart rate revealed significant increases in both mean heart rate (*i.e.* from ~32 to 43 beats min^{-1}) and resting heart rate (*i.e.* from ~27 to 36 beats min^{-1}) shortly after the arrival of the rainbow trout (Fig. 1D).

3.2. Heart rate responses of whitefish during slaughter (day 20 to 21)

Prior to the commencement of slaughter procedures, the mean heart rate of whitefish was significantly elevated by ~11 beats min^{-1} ($F_{1,38} = 18.89$, $p < 0.001$) when compared to the mean heart rates observed at the same time of day during the days leading up to slaughter (*c.f.* black square and grey circle during event 1, Fig. 2A). The heart rate response to crowding was relatively consistent, as mean heart rate significantly increased by ~15 beats min^{-1} (*c.f.* black squares in event 1–2, 3–4 and 8–9, Fig. 2A). When the whitefish were allowed to recover within the sea cage following a crowding event, the mean heart rate significantly decreased to values observed prior to crowding within 1.5 h (*c.f.* black squares in events 1–3, Fig. 2A).

Following the second crowding event, the brailing of whitefish from the sea cage into the well-boat appeared to induce a slight decrease in the mean heart rate (between 14:20 and 14:35, Fig. 2A). However, closer examination of the data revealed that out of the whitefish with reliable heart rate recordings during brailing (n: 16) only 31% of fish experienced a clear reduction in heart rate (*e.g.* from ~51 to 23 beats min^{-1}), whereas heart rate remained unchanged in the other 69% of fish. When whitefish were transported around the Djurholms Sound in the well-boat, the mean heart rate significantly increased by ~13 beats min^{-1} during the initial 30 min, yet decreased during the last 40 min of transportation to reach values that were similar to those observed prior to transport (*c.f.* black squares in events 4–6, Fig. 2A). This decrease in heart rate was not related to the levels of dissolved oxygen in the water within the well-boat, as values always remained between 10.80 and 11.84 mg L^{-1} (*i.e.* ~100% air saturation). When whitefish were released from the well-boat into the holding cage, which was in close proximity to other holding cages containing rainbow trout, mean heart rate significantly increased to peak at 73 ± 2 beats min^{-1} (*c.f.* black squares in events 6–7, Fig. 2A).

Mean heart rate subsequently decreased overnight to reach values that did not significantly differ to that observed prior to the commencement of slaughter procedures (*c.f.* black squares in events 1 and 8, Fig. 2A). However, it remained significantly elevated ($F_{1,38} = 53.84$, $p < 0.001$) when compared to the values observed at the same time of day during the days leading up to slaughter (*c.f.* black squares and grey circles in event 8, Fig. 2A). Following the final crowding event,

Table 2
Body measurements, haematological parameters and plasma ion/glucose concentrations of European whitefish sampled at specific time points or events during the final 2 days leading up to slaughter. Statistical analyses were generated using a one-way ANOVA for each variable and significant differences between groups are represented by different letters ($p < 0.05$).

Measured variables	Events					Statistical summary
	Pre-stress (event 1)	Crowding/transportation (event 4–6)	Pre-slaughter (event 8)	Crowding (event 9)	Brailing/CO_2 narcosis (event 10)	
Body measurements						
Mass (g)	549 ± 45^{a}	741 ± 44^{a}	681 ± 75^{a}	714 ± 40^{a}	947 ± 36^{b}	$F_{(4,92)} = 9.253$, $p < 0.001$
Length (mm)	367 ± 10^{a}	392 ± 6^{a}	381 ± 12^{a}	389 ± 6^{a}	414 ± 3^{b}	Welch's $F_{(4,29.16)} = 8.38$, $p < 0.001$
Haematological parameters						
[Hb] (g dL^{-1})	11.3 ± 0.4^{a}	12.6 ± 0.5^{a}	11.4 ± 0.8^{a}	11.3 ± 0.4^{a}	12.3 ± 0.2^{a}	Welch's $F_{(4,27.72)} = 2.88$, $p = 0.041$
Hct (%)	33.3 ± 1.5^{a}	42.5 ± 2.0^{b}	34.6 ± 2.3^{a,b}	39.1 ± 1.5^{a,b}	54.6 ± 1.1^{c}	Welch's $F_{(4,30.57)} = 39.08$, $p < 0.001$
MCHC (g Hb dL^{-1})	34.0 ± 0.5^{a}	30.8 ± 1.4^{a,b}	33.0 ± 0.6^{a}	29.0 ± 0.5^{b}	22.7 ± 0.3^{c}	Welch's $F_{(4,30.31)} = 106.31$, $p < 0.001$
Blood plasma chemistry						
[K+] (mmol L^{-1})	2.6 ± 0.1^{a}	1.4 ± 0.2^{b}	2.4 ± 0.1^{a}	1.4 ± 0.1^{b}	1.4 ± 0.1^{b}	Welch's $F_{(4,28.84)} = 43.25$, $p < 0.001$
[Na+] (mmol L^{-1})	131.2 ± 0.8^{a,c}	134.3 ± 0.5^{b}	136.0 ± 1.0^{b}	128.9 ± 0.3^{a}	131.8 ± 0.4^{c}	Welch's $F_{(4,24.44)} = 28.50$, $p < 0.001$
[Cl-] (mmol L^{-1})	120.9 ± 0.9^{a}	122.4 ± 0.9^{a}	114.5 ± 0.5^{b}	121.0 ± 0.8^{a}	120.1 ± 0.5^{a}	Welch's $F_{(4,27.36)} = 26.75$, $p < 0.001$
[Ca2+] (mmolL^{-1})	1.00 ± 0.02^{a}	0.98 ± 0.02^{a}	0.65 ± 0.03^{b}	1.05 ± 0.03^{a}	1.2 ± 0.02^{c}	$F_{(4,71)} = 54.98$, $p < 0.001$
Glucose (mmol L^{-1})	4.3 ± 0.2^{a}	5.0 ± 0.1^{a,b}	4.8 ± 0.3^{a,b}	5.7 ± 0.2^{b}	6.7 ± 0.2^{c}	$F_{(4,83)} = 19.12$, $p < 0.001$

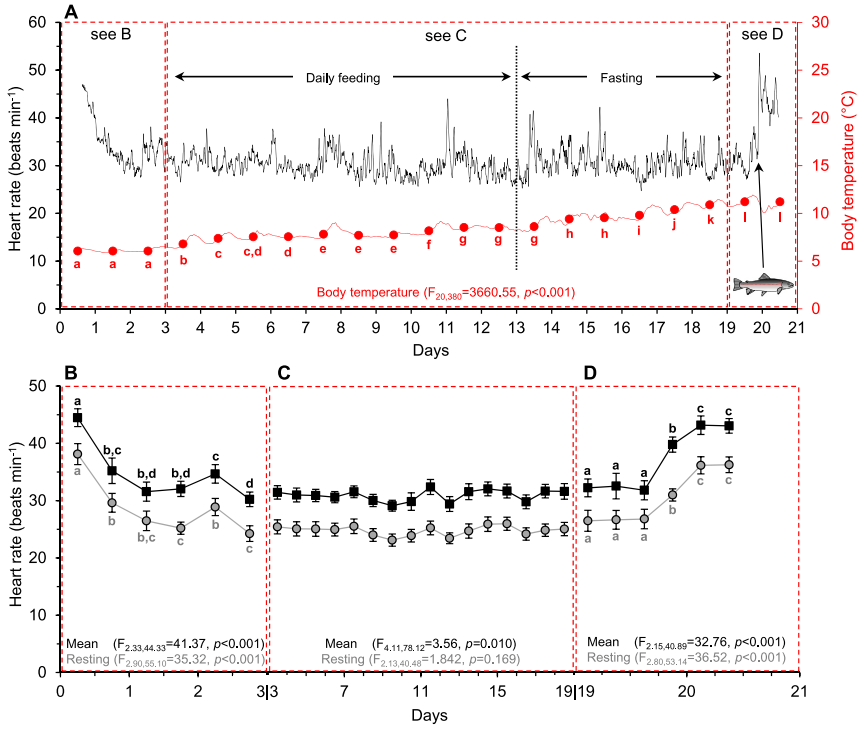

Fig. 1. (A) Hourly moving mean heart rate (black line) and body temperature (red line) of European whitefish (n: 20) from when all fish were reintroduced into the sea cage with conspecifics (~15:00 on day 0) until the slaughter procedures commenced (~24:00 on day 20). The fish symbol with the arrow illustrate the time when the cage with rainbow trout was introduced. (B-D) Detailed temporal changes in mean heart rate (black squares) and resting heart rate (grey circles, defined as the 20th percentile of recorded heart rate values) of European whitefish during (B) recovery from handling, anaesthesia, surgery and reintroduction with conspecifics, (C) the period when fish were left relatively undisturbed, and (D) the period during which a sea cage of rainbow trout were secured in close proximity (*i.e.* within ~10 m). Note that the time scale on the x-axis differ between A, B, C and D. Repeated measures ANOVAs were used to statistically analyse the temporal changes in (A) mean daily body temperature (red circles) and (B-D) mean and resting heart rates of European whitefish. The fixed effects of each model are reported and the different letters represent significant differences ($p < 0.05$) within each period. For the undisturbed period, minor significant differences in mean heart rate occurred sporadically (*e.g.* mean heart rate significantly differed on day 9 *vs.* day 7 and 14, and on day 12 *vs.* day 11,13 and 14), however, these differences are not highlighted in the figure for clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the mean heart rate was significantly elevated before dramatically decreasing by ~37 beats min^{-1} (from ~60 to ~23 beats min^{-1}) following brailing and CO_2 exposure (*c.f.* black squares in events 8–10, Fig. 2A), which was significantly lower ($F_{1,38} = 4.20$, $p = 0.047$) than the mean heart rate observed at the same time of day during the days leading up to slaughter (*c.f.* black squares and grey circles during event 10, Fig. 2A).

3.3. Blood and plasma chemistry of whitefish during slaughter (day 20 to 21)

The circulating levels of plasma cortisol in the 'pre-stressor' blood sample of whitefish was 13 ± 4 ng mL^{-1} (event 1, Fig. 2B). Following the combined stressors of crowding, brailing and transportation, plasma cortisol significantly increased to 50 ± 3 ng mL^{-1} (*c.f.* event 1 and 4–6, Fig. 2B). When examining the immediate recovery of whitefish following the combination of all the stressors that occurred on day 20, it was found that a logarithmic regression could significantly predict the decrease in plasma cortisol over time using $y = -8.184\ln(x) + 60.429$, where y represents plasma cortisol and x represents duration of recovery in minutes (Fig. 2C). Despite a significant decrease in plasma cortisol overnight (*c.f.* event 4–6 and 8, Fig. 2B), values tended ($p = 0.052$) to be elevated the next morning when compared to pre-stressor values (*c.f.* event 1 and 8, Fig. 2B). Following the combined stressors of crowding, brailing and CO_2 exposure, plasma cortisol significantly increased and peaked at 60 ± 5 ng mL^{-1} (event 8–10, Fig. 2B).

Crowding, as well as the combined stressors of brailing and CO_2 exposure, were observed to induce significant increases in Hct and significant decreases in MCHC, whereas [Hb] remained relatively similar throughout the slaughter period (Table 2). With regards to plasma ion concentrations, the greatest changes were observed for [K^+], which consistently decreased by ~44% in response to the different stressors (*c.f.* event 1 to 4–6, event 8 to 9–10 in Table 2). Both [Ca^{2+}] and plasma glucose peaked following brailing and CO_2 exposure, whereas only minor and relatively inconsistent changes were observed in [Na^+] and [Cl^-] throughout the slaughter period (Table 2).

4. Discussion

This is the first study to comprehensively evaluate the physiological

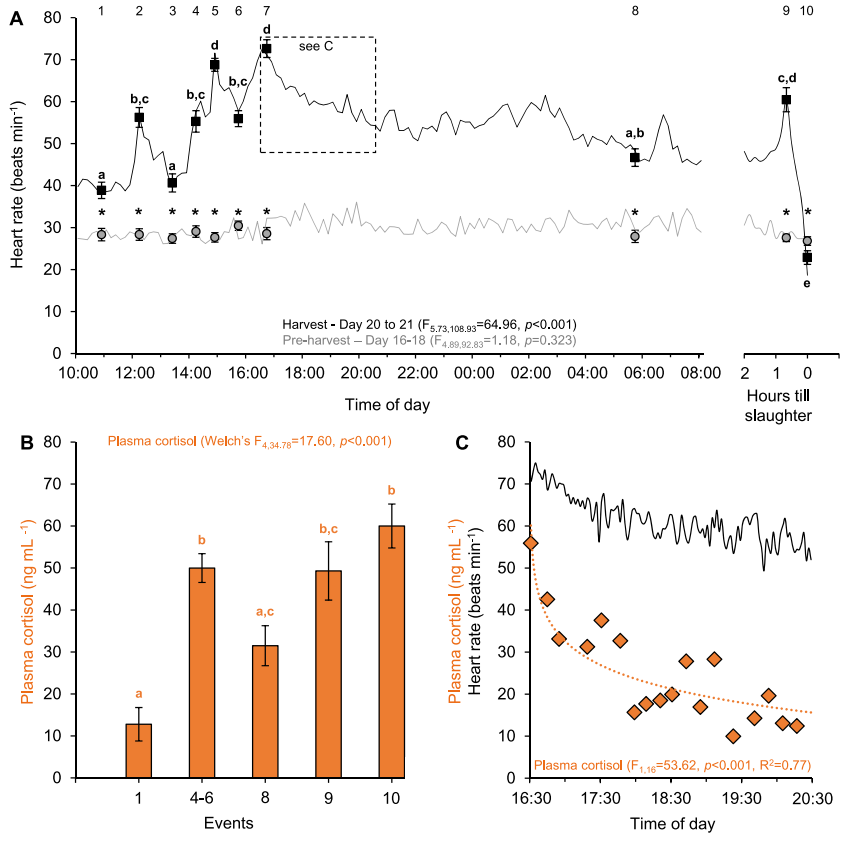

Fig. 2. (A) Mean heart rate of European whitefish (n: 20) during the final two days leading up to slaughter (black line, calculated from days 20–21) compared to the heart rate prior to slaughter in the same individuals (grey line, calculated from days 16–18, see materials and methods for details). Mean heart rate during this period was statistically analysed at specific time points or events (see black squares or grey circles within shaded bars) to evaluate the severity of, and recovery from, a range of different acute stressors. These time points or events represent (1) the period prior to the initiation of harvest procedures, (2) the peak response to the first crowding event, (3) the period prior to the second crowding event, (4) the peak response to the second crowding event, (5) the beginning of the transportation event, (6) the end of the transportation event, (7) peak heart rate during day 20, (8) the period prior to the initiation of slaughter procedures, (9) the peak response to the final crowding event, and (10) following brailing and CO_2 narcosis. (B) Circulating levels of plasma cortisol in whitefish representative of the abovementioned time periods or events (n: 10, 30, 10, 17 and 30 fish for event 1, 4–6, 8, 9, and 10, respectively). (C) The decrease in mean heart rate (black line) and plasma cortisol (orange diamonds) following the combination of all the stressors that occurred on day 20. The decrease in plasma cortisol over time could be significantly predicted using a logarithmic regression (orange dotted line). Statistical analyses were generated using (A) a two-way mixed ANOVA (within-subject and between-subject differences represented by different letters and *, respectively), (B) a one-way ANOVA (between-subject differences represented by different letters), and (C) a logarithmic regression analysis. All *p*-values of <0.05 were considered statistically significant.

responses of freely swimming European whitefish when left undisturbed in a commercial sea cage and in response to common aquaculture practices. The continuous recordings of heart rate combined with the 'snapshots' provided by the blood/plasma stress indicators revealed unique insights into the impact of, and recovery from, a range of acute and chronic stressors experienced by whitefish in aquaculture.

4.1. Heart rate responses of undisturbed and freely swimming whitefish (days 0 to 20)

Resting heart rate of whitefish decreased and stabilised within 36 h following the permanent implantation of the bio-logger. Thus, post-surgical recovery in whitefish appeared to be relatively rapid when compared to the time required for resting heart rates of rainbow trout to decrease and stabilize in response to an identical procedure at the same location (*i.e.* >72 h, Brijs et al., 2018). When fully recovered, whitefish did not display a clear circadian heart rate rhythm during the ~20-day period prior to slaughter, which is in contrast to the strong circadian heart rate rhythms previously demonstrated in other salmonid species such as rainbow trout, brown trout (*Salmo trutta*) and Atlantic salmon (Brijs et al., 2018; Føre et al., 2018; Hjelmstedt et al., 2020; Holliday et al., 1974; Hvas et al., 2020; Priede, 1978; Priede and Young, 1977; Young et al., 1972). Since the circadian heart rate rhythms observed in salmonids have previously been suggested to reflect daily changes in locomotor activity, the lack of a circadian heart rate rhythm in whitefish may be related to the less pronounced or complete lack of clear daily

changes in their locomotor activity. Indeed, when observing whitefish and rainbow trout swimming in the sea cages at the study site there appeared to be clear species-specific differences in circadian activity patterns. For example, rainbow trout were visibly more active during the day than at night (Brijs et al., 2018), whereas this did not appear to be the case for whitefish, which tended to continuously swim in a circular pattern within the sea cage regardless of the time of day. However, an alternative explanation for the lack of a circadian heart rate rhythm in whitefish could be related to the relatively long periods of daylight that the fish experienced in the present study (*i.e.* 17:7 light:dark cycle). For example, closely related Lake whitefish (*Coregonus clupeaformis*) displayed clear circadian swimming activity patterns in the wild and under controlled laboratory conditions when subjected to a 12:12 light:dark cycle, whereas these patterns were suppressed under constant light conditions (Anras et al., 1999; Bégout et al., 1998; Scherer and Harrison, 1988).

Acute warming in fish typically results in an increase in resting heart rate, whereas during more chronic seasonal temperature changes, compensatory physiological adjustments can be initiated to counteract the thermal effects (*i.e.* thermal acclimation) (Ekström et al., 2016; Sandblom et al., 2016; Seebacher et al., 2015). Here, we demonstrate the thermal acclimation capacity of whitefish for the first time, as whitefish maintained a resting heart rate of ~25 beats min^{-1} despite an increase in body temperature from ~6.8 to 11.2 °C during the undisturbed period (*i.e.* a ~ 1.6-fold increase). This indicates that, at least within the observed temperature range, whitefish were able to adjust physiologically to counter the effects of the temperature increase on resting heart rate. Similar to other fish species, this was most likely achieved *via* either an increased neural (vagal) inhibition of heart rate and/or a reduction in the intrinsic pacemaker rate (Sandblom and Axelsson, 2011; Ekström et al., 2016; Sandblom et al., 2016).

The processing and digestion of a meal coincides with an increase in energetic expenditure (*i.e.* specific dynamic action) and gastrointestinal blood flow in a diverse range of vertebrates (Secor, 2009; Seth et al., 2011). To meet the elevated metabolic and circulatory demands associated with feeding, fish typically increase cardiac output *via* an increase in heart rate and/or stroke volume (Seth et al., 2011). In the present study, the heart rate of whitefish did not differ with regards to feeding state, which contrasts with the pronounced post-prandial increase in heart rate previously reported in species such as rainbow trout (Eliason et al., 2008; Gräns et al., 2009), sea bass (*Dicentrarchus labrax*; Altimiras et al., 2008; Axelsson et al., 2002; Dupont-Prinet et al., 2009), northern pike (*Esox Lucius*; Lucas et al., 1991), shorthorn sculpin (*Myoxocephalus scorpius*, Seth and Axelsson, 2009), white sturgeon (*Acipenser transmontanus*; Gräns et al., 2010) and bald notothens (*Pagothenia borchgrevinki*; Sandblom et al., 2012). This finding could be due to a number of reasons such as i) surgical implantation of the bio-logger may have disrupted the feeding behaviour/activity of whitefish, ii) the amount of food ingested was not enough to induce a clear post-prandial increase in heart rate, iii) voluntary feeding and freely swimming fish may not exhibit similar post-prandial responses as those documented for gavage fed fish in the laboratory (Brijs et al., 2018, 2019b; Seth et al., 2011), and/or iv) whitefish may increase cardiac output *via* stroke volume instead of heart rate, which would be similar to the situation in Atlantic cod (Axelsson and Fritsche, 1991). Since body mass decreased by ~2% over the course of the study, it is likely that reasons i and/or ii underlie this finding and thus research on the effects of implanted devices on behavioural and physiological aspects of feeding is warranted. A potential recommendation for future research investigating the temporal dynamics of feeding in freely swimming fish is to allow longer recovery times prior to recording (*e.g.* several weeks before recording is initiated).

4.2. Physiological responses of whitefish during slaughter (days 20 to 21)

The physiological response of whitefish to acute stressors such as crowding and transportation typically consisted of increases in heart rate, cell swelling (as indicated by the reductions in MCHC; Nikinmaa, 1983) and circulating levels of plasma cortisol and glucose, while plasma [K^+] decreased. The heart rate response of whitefish to crowding and transportation (*i.e.* an increase of ~15 and 13 beats min^{-1}, respectively) were relatively similar to the responses observed in rainbow trout subjected to similar procedures (*i.e.* increase of ~13 and 9 beats min^{-1}, respectively; Brijs et al., 2018). However, whitefish appeared to recover relatively rapidly from these acute stressors, as heart rate recovered within 1.5 h following crowding or even began to decrease during transportation to reach pre-stressor levels by the end of the transportation event. The present study also highlights some species-specific differences in the usefulness of varying indicators for gauging the severity of acute stressors such as crowding and transportation. For example, changes in haematological (*e.g.* Hct and MCHC) and plasma stress indicators (*e.g.* [K^+]) were more pronounced in whitefish than in rainbow trout, as the latter exhibited no changes in these parameters following similar crowding and transportation events in the same location (Brijs et al., 2018). On the contrary, the plasma cortisol response to the combination of crowding and transportation was low in whitefish, as plasma cortisol only increased by ~37 ng mL^{-1} compared to ~148 ng mL^{-1} in rainbow trout (Brijs et al., 2018).

Another acute stressor that farmed fish are exposed to is brailing, which induced an immediate and transient decrease in the heart rate of rainbow trout that is likely due to inadequate oxygen availability during air exposure (Brijs et al., 2018). However, only 31% of the whitefish with reliable heart rate recordings experienced a clear and substantial reduction in heart rate during brailing (*i.e.* reduction of ~38 beats min^{-1}), while the heart rate remained unchanged in the other 69% of whitefish. The underlying reason for this finding remains unknown, but it could be related to variations in brailing duration or position of the fish within the brail during brailing. Consistent with previous studies on other fish species (Brijs et al., 2018; Gräns et al., 2016; Sandblom et al., 2013; Seth et al., 2013), CO_2 exposure induces a severe stress response in whitefish, as circulating levels of plasma cortisol, glucose and [Ca^{2+}] significantly increased along with substantial cell swelling. In addition, heart rate plummeted during CO_2 exposure, which is likely the result of a cardiac collapse caused by severe acidosis (Seth et al., 2013).

An unforeseen, yet extremely relevant, finding of the present study concerns the substantial and long-lasting physiological response of whitefish when held in close proximity (*i.e.* within ~10 m) to rainbow trout. Directly after a sea cage containing ~5000 large rainbow trout (~2 to 4 kg) was towed and secured in close proximity to the sea cage containing the whitefish, the mean heart rate of the latter increased from ~32 to 43 beats min^{-1} (*i.e.* an increase of ~34%) and remained elevated by at least this amount thereafter. Since whitefish are often among the dominant prey species in the wild for piscivores across their range (Jensen et al., 2008; Kahilainen and Lehtonen, 2003), this finding may represent an innate physiological response to the threat of predation. Indeed, previous studies have demonstrated an immediate and often maintained increase in heart rate following a predatory attack or threat in a range of fish species (Holopainen et al., 1997; Höjesjö et al., 1999; Johnsson et al., 2001; Sundström et al., 2005). This physiological response has been suggested to enhance the probability of escape and/or to maintain preparation for flight in the near future (Höjesjö et al., 1999; Ydenberg and Dill, 1986). While this response is crucial for survival in the wild, this sustained physiological response would most likely serve to increase the allostatic load on farmed whitefish, and could result in detrimental tertiary stress responses (Korte et al., 2007; Moberg, 2000; Segner et al., 2012). This may consequently have substantial economic implications, as less energy would be available for growth due to the potentially elevated metabolic demands of whitefish when held captive in close proximity to a potential predator (Fraser and Gilliam, 1992). Furthermore, there is a risk that the allostatic load imposed by the presence of rainbow trout may approach or turn into an allostatic overload when whitefish are simultaneously subjected to additional stressors such as those mentioned above. For example, the mean heart

rate of whitefish was ~44 beats min^{-1} higher than 'normal' due to the stress imposed by the presence of rainbow trout and the range of common aquaculture practices employed on day 20 (*c.f.* black squares and grey circles during event 7, Fig. 2A). The allostatic load imposed by the combination of these stressors most likely impacts the ability of whitefish to perform normal physiological processes (Brijs et al., 2019a) and may ultimately result in cardiac collapse, and even death, if conditions become more unfavourable (Eliason et al., 2013; Priede, 1977). Furthermore, the potentially high allostatic load imposed by the presence of rainbow trout may also explain why this species has previously been perceived by commercial fish farmers to be highly susceptible to the stress induced by common aquaculture practices, as it is quite common to have both species at the same farm with varying physical distances.

Finally, the present study demonstrates the advantages of using continuous heart rate recordings from bio-loggers to identify and quantify the impacts of both acute and chronic stressors when compared to the 'snapshot' provided by more traditional measures such as circulating plasma cortisol levels. Despite the introduction of rainbow trout on day 19 inducing a substantial and maintained elevation in heart rate, plasma cortisol of whitefish was within the previously reported unstressed range (<30 ng mL^{-1}; Lappivaara and Oikari, 1999; Lappivaara, 2001) on the morning of day 20. This may reflect a relatively rapid recovery of plasma cortisol in whitefish following a perceived stressor. For example, plasma cortisol returned to pre-stressor levels within 6 h after whitefish had been subjected to a combination of acute stressors on day 20 (see logarithmic regression equation in *3.3. Blood and plasma chemistry of whitefish during slaughter*). This result is not unexpected, as circulating levels of plasma cortisol has been reported to be a poor indicator of chronic stress in several fish species including Atlantic salmon (Sundh et al., 2010, 2019), brown trout (Pickering and Stewart, 1984), rainbow trout (Person-Le Ruyet et al., 2008) sea bream (*Sparus aurata*; Tort et al., 1996) and common carp (*Cyprinus carpio*; Aerts et al., 2015).

5. Conclusion

The present study highlights the importance of performing long-term, species-specific evaluations of freely swimming fish in real aquaculture settings when developing guidelines or regulations governing fish welfare in aquaculture. Although the acute stress responses of whitefish to a range of common aquaculture practices such as crowding, transportation, brailing and CO_2 exposure were comparable to those previously described in farmed rainbow trout (Brijs et al., 2018), there were several important species-specific differences. This information will be useful for safeguarding, as well as assessing, the health and welfare of farmed European whitefish in the future. The most pronounced differences in whitefish relative to other salmonid species included an increased sensitivity of some blood/plasma stress indicators to perceived stressors, the presence of a substantial physiological stress response when held captive in the proximity of a potential predator and the lack of a circadian heart rate rhythm in undisturbed whitefish. Thus, given the welfare and economic implications, further investigations are urgently needed to systematically evaluate the effects of farming both predatory and prey species simultaneously so that methods or techniques can be developed to reduce or prevent the physiological stress response of the prey species.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.736258.

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III

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ORIGINAL ARTICLE

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Evaluation of the reliability of indicators of consciousness during CO_2 stunning of rainbow trout and the effects of temperature

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Abstract

A two-part experiment was conducted to determine whether visual indicators of consciousness such as equilibrium, eye-roll reflex and ventilation are reliable for evaluating whether CO_2 stunning of rainbow trout (*Oncorhynchus mykiss*) is humane. In part 1, the time taken until the loss of visual indicators in rainbow trout during CO_2 stunning was monitored under field conditions at 14, 8 and 2°C. Here, we clearly demonstrate that it takes longer for visual indicators to disappear as temperature decreases, with significant differences in the time taken until the loss of equilibrium between 2 and 14°C, and significant differences between all temperatures in the time taken until the loss of eye-roll reflex and ventilation. In part 2, rainbow trout were equipped with external non-invasive electrodes for recording EEG prior to, and following, CO_2 stunning to assess the presence or absence of visually evoked responses (VERs), which are indicative of brain function and sensibility. The resulting EEG recordings during CO_2 stunning at 10°C demonstrated a poor relationship between visual indicators of consciousness and loss of sensibility, as VERs were present up to 3.5 min after ventilation was lost and up to 6.5 min after the fish lost equilibrium. Collectively, these results show that cold-water temperatures prolong the time taken until loss of consciousness and that visual indicators are insufficient for determining when sensibility is lost in rainbow trout during CO_2 stunning.

KEYWORDS
brain function, electroencephalography, sensibility, unconsciousness, welfare

1 | INTRODUCTION

Humane slaughter guidelines state that fish should be killed in a way that does not cause fear or pain (EFSA, 2004; OIE, 2018). This is generally accomplished by rendering the fish insensible with a stunning method followed immediately by killing. For a stunning method to be considered acceptable, loss of consciousness and/or insensibility must occur immediately and irreversibly so that slaughter personnel have enough time to kill the fish (EFSA, 2004; OIE, 2018). The only stunning methods proposed to meet the requirement for immediate

insensibility when used properly are percussive and electrical stunning (EFSA, 2004; OIE, 2018). However, there are many other methods of stunning still used in practice that do not meet the required standards (Gräns et al., 2016; Lines & Spence, 2012). One of these methods is carbon dioxide (CO_2) stunning, the use of which has declined in recent years but is still utilized in several European countries (Brijs et al., 2018; IBF, 2017). This method is easy and inexpensive to implement on large groups of fish, but does not meet OIE (2018) standards as it is known to cause severe aversive behaviours and requires long induction times before consciousness is lost in several fish species, including rainbow trout (*Oncorhynchus mykiss*; Kestin, Wotton, & Adams, 1995; Marx, Brunner, Weinzierl, Hoffmann, & Stolle, 1997; Robb & Kestin, 2002). CO_2 stunning is accomplished by bubbling CO_2 into water until the pH is reduced <5, which is a level associated with complete saturation (Anonymous, 1995). Fish submerged in this solution experience an increase in dissolved CO_2 in their bloodstream, which leads to narcosis, decreased ventilation and ultimately respiratory failure (Bernier & Randall, 1998; Kugino, Tamaru, Hisatomi, & Sakaguchi, 2016).

Verifying the functionality of a stunning method requires that consciousness, or lack thereof, is determined in a reliable way. As consciousness cannot be easily assessed, the presence or absence of visual indicators is often used by both researchers and farmers to determine whether a fish has lost consciousness (Table 1). The most commonly used visual indicators of consciousness such as loss of equilibrium, the eye-roll reflex and ventilatory changes can be difficult to observe and are easily misjudged, which results in the use of inadequate stunning methods that may cause unnecessary fear or pain (Lambooij et al., 2010; Robb & Kestin, 2002). Visual indicators are also unreliable when fish become paralysed or immobile without losing consciousness or sensibility. Loss of movements without loss of sensibility has been reported during CO_2 stunning of Atlantic salmon (*Salmo salar*; Robb et al., 2000). Currently, the only known method for accurately assessing a loss of sensibility in the case of paralysis is through electroencephalography (EEG) (Kestin, van de Vis, & Robb, 2002; Lambooij et al., 2010).

EEG is a method for recording and measuring the electrical activity of the brain through the use of electrodes that are either surgically implanted in the brain (Kestin et al., 2002; Kestin, Wotton, & Gregory, 1991; Quick & Laming, 1990), or attached externally to the head (Bowman, Hjelmstedt, & Gräns, 2019; Cho et al., 2017). EEG allows the investigator to detect changes in brain activity indicative of a loss of consciousness and/or sensibility. One such method involves assessing the presence or absence of averaged visually evoked responses (VERs), which in a conscious animal produces a distinct waveform in the EEG in response to visual stimulation from a flashing light (Kestin et al., 1991; Robb & Roth, 2003; Robb et al., 2000). The abolition of VERs is an objective and unequivocal indicator of brain dysfunction and hence, loss of sensibility, as the failure of this primary sensory pathway is one of the last responses to an external stimulus to be lost before brain death (Daly, Gregory, & Wotton, 1987; EFSA, 2004; Robb et al., 2000). While it is possible that the loss of consciousness occurs prior to the loss of VERs, the presence of VERs indicates that some level of rudimentary visual processing is still occurring in the brain, and thus from an ethical perspective, it must be assumed that there is a possibility the animal is still conscious (Kestin et al., 1991; Robb et al., 2000).

Other methods for assessing changes in consciousness include measurements of EEG signal amplitude and changes in brain frequencies. A ≥50% reduction in total signal amplitude compared to pre-treatment values has been cited as the stage at which calves transitioned from conscious to unconscious (Gibson et al., 2009). An even further reduction, such as when the amplitude of the EEG is less than 12% of pre-treatment values (sometimes defined as iso-electric EEG), has been used as an indicator of profound brain failure in calves and chickens (Gibson et al., 2009; Mcilhone, Beausoleil, Johnson, & Mellor, 2014).

A shift in consciousness can also be assessed by separating the EEG signal into beta, alpha, theta and delta frequency waves, and measuring their relative contribution to the overall signal. A transition from high-frequency beta and alpha brain waves to low-frequency theta and delta waves has been used as an indicator that a fish is no longer conscious (Lambooij et al., 2006, 2010, 2013). The shift from high- to low-frequency waves has also been assessed by using the median frequency of the total EEG signal in calves and chickens (Gibson et al., 2009; Martin & McKeegan, 2017).

While the effects of CO_2 stunning on fish are well documented, there has been little investigation of how different seasonal

TABLE 1 Description of visual indicators used to determine loss of consciousness in fish during stunning and slaughter (table modified from Readman, 2015; McFarland, 1959; Ross & Ross, 2008; Sneddon, 2012)

Visual indicator	Description	Sign of	Estimated comparative depth of anaesthesia
Loss of equilibrium	Unable to maintain upright swimming[1]	Depression of the midbrain[2]	Light anaesthesia[1,2]
Change in ventilation	A change from regular opercular movement to slowing and irregular movement[1]	Depression of the medulla[2], thought to be first sign of transition to an unconscious state	Light to deep anaesthesia[2]
Loss of eye-roll reflex	Unable to adjust eyes to compensate for tilt[1]	Loss of visual reflex and processing[1]	Deep to surgical anaesthesia
Loss of ventilation	No opercular movement[2]	Impending medullary collapse[2], thought to indicate unconsciousness	Surgical anaesthesia/ Medullary collapse[2]

Note: References: [1]Kestin et al. (2002); [2]McFarland (1959).

temperatures affects the efficacy of the method. Therefore, we investigated the effects of acclimation temperatures on the time to loss of visual indicators (i.e. loss of equilibrium, eye-roll reflex and ventilation) of consciousness in rainbow trout stunned in CO_2 saturated water. Part 1 was conducted at a commercial rainbow trout farm in southern Sweden in summer, autumn and winter with water temperatures of 14, 8 and 2°C respectively.

The aim of part 2 of the study was to test whether the loss of visual indicators used in part 1 of the study can be considered accurate indicators of a loss of consciousness. To do this, we fitted rainbow trout with three non-invasive electrodes to monitor brain function. EEG was recorded simultaneously with visual indicators when trout were submerged in CO_2 saturated water at 10°C using the same experimental setup as the field portion of the study. By investigating the relationship between the visual indicators and brain function, we aimed to determine if the external visual indicators used in commercial farm and slaughter situations during CO_2 stunning are accurate indicators of a loss of sensibility.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

This study was performed in accordance with Swedish animal welfare legislation. Animal care and experimental procedures were approved by the ethical committee of Gothenburg, ethical permit number: 177-2013. No protected species were used during the experiment.

2.2 | Part 1: Field experiments

2.2.1 | Animals and husbandry

Rainbow trout of mixed sex with a mean weight ± SE of 1,087 ± 27.9 g were maintained in freshwater at Vänneåns fiskodling AB. Tests were conducted on three different occasions over a year to entail the effects of different seasonal temperatures on the stunning efficiency.

2.2.2 | CO_2 stunning

To measure the time to loss of visual indicators of consciousness during CO_2 stunning under field conditions at a commercial Swedish fish farm, rainbow trout (n = 30) were submerged in CO_2 saturated water at acclimation temperatures of 14, 8 and 2°C. Ten rainbow trout were used per acclimation temperature. CO_2 saturated water was circulated between a 50-L barrel and a 70 L aquarium using a hydraulic pump and kept at a consistent temperature by a refrigeration unit (CB 8-30E, Heto-Holten A/S). CO_2 was continuously bubbled into the barrel until the pH reduced to a steady state < 5, indicating the water was fully saturated. Temperature and pH were measured in the aquarium, before each fish was submersed, using a digital thermometer (Testo 108−-2, Testo North America) and a multifunctional pH meter (HANNA Instruments, HI981901 pH-ORP-OWE. Three point calibrated: 4.01, 7.01 and 10.01) to ensure that temperature and pH were consistent throughout testing. Fish were individually and quickly transferred from their holding tanks using a dip net to the 70-L tank containing CO_2 saturated water where they remained for 12 min while being monitored for loss of visual indicators. After treatment, fish were transferred by hand to a tank containing aerated water for 10 min and monitored for recovery, followed by euthanization via a percussive strike.

2.3 | Data analysis and statistics

Experiments were filmed for verification purposes. Time until loss of equilibrium, eye-roll reflex and ventilation was recorded during experiments. The eye-roll reflex was checked every 30 s until it was lost. A one-way ANOVA followed by Tukey's post hoc test was used to analyse difference between acclimation groups in SPSS (version 2017).

2.4 | Part 2: Laboratory experiments

2.4.1 | Animals and husbandry

Rainbow trout (n = 9) obtained from Vänneåns fiskodling AB, Sweden, of mixed sex and ranging in size between 560 and 905 g (mean ± SE: 673.9 ± 32.9 g) were housed in the animal facility at the Department of Biological and Environmental Sciences, University of Gothenburg between April and June 2018. Water was maintained at 10°C on a 12h:12h light:dark photoperiod.

2.4.2 | Placement of electrodes and recording of VER on the EEG

Fish were individually netted and submerged a tank containing 150 mg/L MS-222 (ethyl 3-aminobenzoate methanesulphonic acid, $C_{10}H_{15}NO_5S$) (Sigma-Aldrich Inc.) buffered with 300 mg/L sodium bicarbonate ($NaHCO_3$) dissolved in 12 L of water to induce light anaesthesia. A silicone cup with integrated electrodes was secured to the head of the fish using a peristaltic pump, and consistent low suction was maintained during the experiment to ensure the cup remained in place. For details of cup design and placement, see Bowman et al. (2019).

Fish were transferred to a 20 L tank containing 16 L of 10°C water and allowed to recover for 10 min. EEG was confirmed and recorded for 10 min without light stimulus to serve as a reference signal, ensuring that the fish was fully awake and the EEG signal was clear. EEG was then recorded for 10 min with light stimulus delivered

as two 250 ms light flashes per second using an LED strobe light and detected using an in-house designed light detector connected to a PowerLab system (ADInstruments Pty Ltd.) sampling at 1 kHz. The peristaltic pump was turned on high for 30 s before fish were gently manually transferred to a 70-L tank of 10°C water that was fully saturated with CO_2, as indicated by a pH meter (HANNA Instruments, HI981901 pH-ORP-OWE). EEG was recorded with light stimulation for 30 min in the treatment tank followed by 10 min without light. If the cup moved or became dislodged after transfer, it was reattached after the fish lost equilibrium. Visual indicators of loss of consciousness were determined during testing and times were recorded. Visual indicators included loss of equilibrium, change in ventilation and loss of ventilation. A transition from regular opercular movement to irregular and gradually slowing was deemed a change in ventilation. Fish were euthanized following treatment with a percussive blow to the head. Room lights were turned off for the entirety of the experiment.

2.5 | Data analysis and statistics

EEG recordings were assessed using LabChart 7 (version 7.3.8, AD Instruments), and a bandpass filter of 0.5–32 Hz was used to reduce signal noise. LabChart Scope View was set to record 500 ms time windows of EEG with the strobe light acting as the input trigger to begin recording. A 1-minute representative window of EEG signal was created for each minute or recording by averaging 120 consecutive, non-overlapping windows. To ensure that the signal accurately represented electrical activity in the brain and was not skewed by electrical artefacts caused by movement or gasping, LabChart was set to exclude windows where the EEG signal exceeded 10 μV from the averaging process. If representative minutes of EEG signal contained less than 60 averaged 500 ms windows (<50% of windows were included), they were removed from analysis.

For analysis of VERs, the EEG signal was filtered into beta waves (13–32 Hz) using a bandpass filter. The presence or absence of the VER waveform for each fish was determined through observations of measurable changes in the beta wave of the brain in response to a visual stimulus (i.e. a flashing light turning on and off) over time. When the VER wave amplitude became indistinguishable from the amplitude of the rest of the beta wave, the VER was considered absent (Figure 1).

Amplitude was measured as the maximum minus the minimum point of the total EEG signal (0.5–32 Hz) for each representative minute. The time to reduce the EEG signal amplitude to <50% of pre-treatment values was calculated using Microsoft Excel (version 2016). EEG signal median frequency and relative power were calculated using data that was transformed in LabChart using Fast Fourier transformation with a Hann (cosine-bell) window with 50% overlap. Relative power was analysed for changes between high-frequency (8–32 Hz) beta and alpha waves and low-frequency (0.5–8 Hz) theta and delta waves indicative of a loss of consciousness. SPSS (version 2017) was used to analyse EEG signal amplitude, median frequency and relative power using a linear mixed model with AR(1) as the covariance structure and time as the repeated variable. Amplitude and median frequency data were log-transformed to meet the assumptions of the model.

3 | RESULTS

All data are reported as mean (min-max value) unless otherwise stated.

3.1 | Part 1: Field experiments

There was a significant difference in weight between the temperature groups [$F_{(2,27)} = 10.96$, $p < .001$] where fish acclimated to 14°C

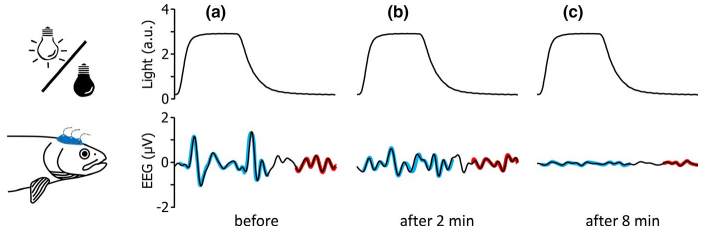

FIGURE 1 Changes in the visually evoked responses (VERs) in the EEG of rainbow trout during submersion in CO_2 saturated water. VERs represent the measurable changes in the electrical potential of the brain in response to a visual stimulus (i.e. a flashing light). In the present study, each VER represents the averaged response to 120 consecutive flashes delivered at one flash per 500 ms. (a) Directly prior to submersion in CO_2 saturated water, VERs can be observed in this specific individual as distinct waveforms in the EEG recordings milliseconds after the light flashes on and off (marked in blue). These waveforms have an amplitude ~3 times greater than the EEG recordings during the dark period (marked in red). (b) After 2 min of CO_2 exposure, VERs can still be observed as the waveform in the EEG repetitively appears following the light flashes. However, the amplitude of the waveform has decreased and is now only ~1.7 times greater than the EEG recordings during the dark period. (c) After 8 min of CO_2 exposure, VERs were abolished as no distinguishable waveform in response to light flashes could be identified within the EEG recordings (i.e. the amplitude of the EEG signal is the same before and after the light flash, ~1:1 ratio)

were smaller, 945.5 g (805–1,090 g), compared to fish acclimated to 8°C, 1,168.5 g (985–1,315 g) and 2°C, 1,148 g (990–1,355 g). However, no interacting effects were found on any of the investigated variables; thus, mass was not included as a covariate in the final statistical analysis.

3.1.1 | Visual indicators

Overall, the time taken until the loss of visual indicators of consciousness increased as the temperature of CO_2 saturated water decreased (Figure 2). It took significantly longer for fish to lose equilibrium after submersion in CO_2 saturated water at 2°C compared to 14°C [$F_{(2, 27)} = 4.81$, $p = .016$]. The eye-roll reflex was lost shortly after equilibrium, taking 1.30 (1–2) min, 3.15 (2–4.5) min and 4.15 (3–5.5) min at 14, 8 and 2°C, respectively, and the time taken for the eye-roll reflex to be lost was significantly different between all temperature groups [$F_{(2, 27)} = 53.5$, $p < .001$]. Ventilation was the last indicator lost, taking nearly four times longer to cease than the time taken until the loss of equilibrium at all temperatures tested. Ventilation ceased 3.61 (2.90–4.55) min at 14°C, 5.74 (4.01–7.33) min at 8°C and 9.00 (7.33–11.33) min at 2°C, and the time taken for ventilation to cease was significantly different between all temperature groups [$F_{(2,27)} = 69.5$, $p < .001$].

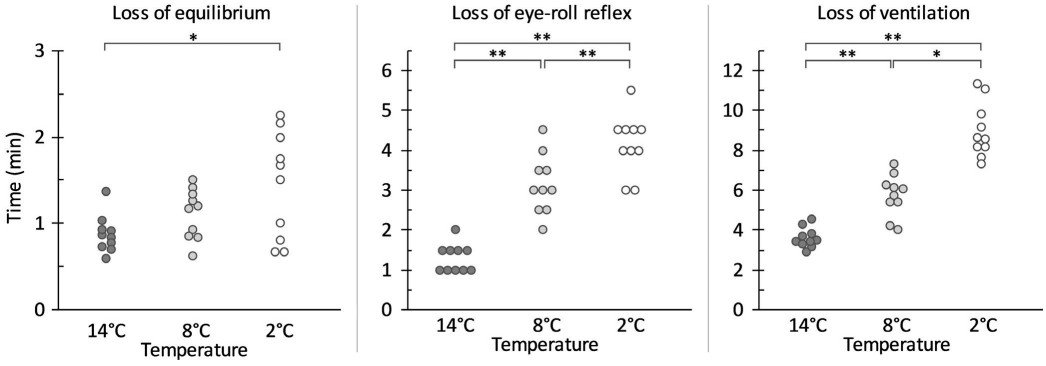

FIGURE 2 Differences in time (min) to loss of equilibrium, the eye-roll reflex and ventilation at 14, 8 and 2°C of individual rainbow trout during progressive loss of consciousness ($n = 30$). The eye-roll reflex was assessed every 30 s. Statistical significance indicated $^{*}p < .05$, $^{**}p < .001$

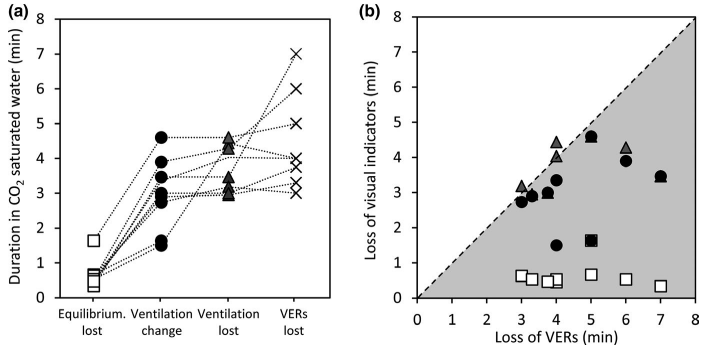

FIGURE 3 Relationship between the loss of VERs and loss of visual indicators of consciousness following submersion in CO_2 saturated water. (a) Time taken for trout ($n = 8–9$) to lose equilibrium (open squares), exhibit changes in ventilation (black circles), cease to ventilate (grey triangles) and lose VERs (X) following submersion in CO_2 saturated water. (b) The time taken for trout ($n = 8–9$) to lose VERs compared with the time taken to lose equilibrium (open squares), exhibit changes in ventilation (black circles) and cease to ventilate (grey triangles). The black dashed line is a 1:1 line and has been included to demonstrate the welfare implications of using visual indicators of consciousness to identify when an animal is insensible. Considerable variation exists between the loss of the visual indicators and the loss of VERs, which from an individual welfare point of view can be acceptable (markers white section, VERs are lost before the visual indicators) or unacceptable (markers in grey shaded section, visual indicators are lost before the VERs)

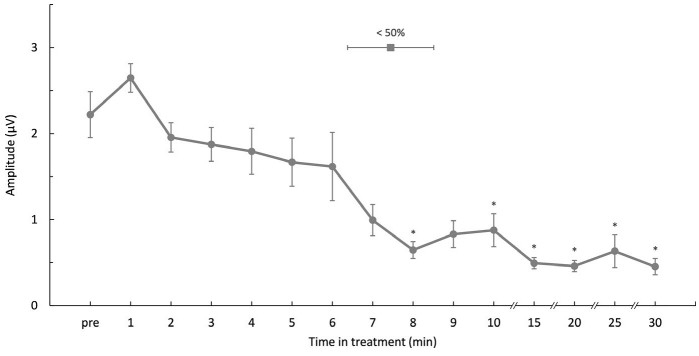

FIGURE 4 Amplitude ± SE of filtered EEG (0.5–32 Hz) during progressive loss of consciousness in rainbow trout ($n = 9$) for the averaged pre-treatment signal (pre) and each minute of treatment. '*' indicates that the amplitude of the EEG signal is significantly different ($p < .05$) from the amplitude of the pre-treatment mean. The marker and horizontal bars above the amplitude data indicate the average time ± SE it took for EEG signal amplitude to reach < 50% pre-treatment amplitude

3.2 | Part 2: Laboratory experiments

3.2.1 | Visual indicators and VERs

After submersion in the CO_2 saturated water, loss of equilibrium occurred after 0.64 (0.33–1.63) min (Figure 3a). Ventilation changed and ceased 3.01 (1.50–4.60) min and 3.74 (2.95–4.6) min, respectively, following submersion in CO_2 saturated water (Figure 3a). Ventilation loss failed to be recorded for one fish. VERs were lost 4.56 (3.30–7.00) min after submersion in the CO_2 saturated water (Figure 3a). Considerable variation was observed between the time to loss of different visual indicators of consciousness and the loss of VERs (Figure 3b). All trout lost equilibrium and altered their ventilation before the VERs were lost with a disparity of up to 6.7 and 3.5 min respectively (Figure 3b). Five out of eight trout ceased their ventilation before losing their VERs with a disparity of up to 3.5 min (Figure 3b).

3.2.2 | Signal amplitude

EEG signal amplitude increased immediately after transfer to the treatment tank compared to pre-treatment amplitude measurements. Time until amplitude declined to <50% pre-treatment amplitude was 7.44 (4–15) min after transfer. Time until amplitude reached <12% pre-treatment amplitude ranged widely, with one fish reaching <12% after 10 min and one fish not reaching < 12% at all during treatment.

Results of the linear mixed model (Figure 4) showed a significant decrease in amplitude during min 8 and min 10–30 min 7 and 9 were close to achieving significance ($p = .056$ and $p = .071$) but ultimately did not.

3.2.3 | Median frequency and relative power

No significant difference was found between pre-treatment mean and treatment values for EEG signal median frequency. Mean pre-treatment median frequency was 7.74 (2.53–22.3) Hz. After transfer and until min 10 mean median frequency stayed below pre-treatment mean, with the lowest frequencies occurring at min 4 and 7, measuring 3.87 (1.93–7.91) and 3.86 (1.27–11.1) Hz respectively. After this, median frequency fluctuated between 5.52 and 13.33 Hz for the remainder of the experiment.

Results of the linear mixed model showed no significant difference between the pre-treatment means and the relative power of beta and alpha (B/A) and theta and delta (T/D) frequencies over treatment. B/A frequencies decreased during the first minute after transfer from 46.8% to 32.9% of the total signal power, while T/D increased from 53.0% to 67.0%. B/A increased and T/D decreased slightly over the next min before reversing over the next 2 min. The lowest B/A and highest T/D powers occurred during min 4, comprising 18% and 81% of the total signal power respectively.

4 | DISCUSSION

This is the first study to use a newly developed, non-invasive technique to monitor brain function and sensibility via EEG recording in a fish to evaluate the effectiveness of a stunning method. The results of this study show great promise on how to validate stunning efficiency in fish using a non-invasive method. We have demonstrated that this method can be a useful tool for filling in the knowledge gaps needed for safeguarding the welfare of farmed fish at the time of slaughter. Furthermore, our results show that stunning rainbow trout with CO_2 clearly does not meet the OIE standards for humane slaughter and that cold-water temperature exacerbate the poor effectiveness of the method even further.

The results of part 1 clearly demonstrate that the time it takes until rainbow trout lose visual indicators of consciousness following CO_2 stunning is strongly dependent on temperature. Even at 14°C, the highest acclimation temperature tested, it took >30 s before the first visual indicator was observed (i.e. loss of equilibrium). Prior to the loss of equilibrium, all fish showed strong aversive behaviours in the form of repeated attempts to escape the tank and vigorous swimming. The

longest time it took for the last visual indicator of consciousness to be observed (i.e. loss of ventilation) increased from around 4.5 min at 14°C to more than 11 min at 2°C. CO_2 narcosis clearly does not induce immediate unconsciousness and triggers intense aversive behaviours before stunning is effective, with cold acclimation temperatures significantly prolonging the induction time of the narcosis, which ultimately leads to substantial animal welfare problems.

Similar negative effects of cold temperatures on stunning and killing efficiency have previously been demonstrated for other methods and can be explained by a decreased metabolic rate in fish at low water temperatures (Kestin et al., 1991; Robb & Kestin, 2002). For example, when asphyxiated in air it took around 11 min for rainbow trout to lose ventilation at 14°C, whereas at 2°C it took >3 h, more than 17 times longer, for ventilation to cease (Kestin et al., 1991). Another factor that plays into the strong effect temperature has on stunning efficiency is the decreased diffusion rate of gases at lower temperatures (Ott, Heisler, & Ultsch, 1980). This will potentially prolong the time it takes before enough CO_2 is absorbed into the bloodstream to reach the levels needed for the gas to induce unconsciousness.

The analyses of EEG signals showed that there was no defining minute in which consciousness was lost following CO_2 stunning. The analysis of changes in EEG signal amplitude indicates that the transition to unconsciousness occurred between 6.5 and 8.5 min after submersion, whereas the analyses of the lowest median frequencies and the period when high-amplitude, low-frequency waves comprised the largest portion of the signal indicate that the transition to unconsciousness occurred between 4 and 7 min. Thus, by collectively examining the time it takes for VERs to disappear and for changes to occur in the EEG signal (amplitude and median frequency), it seems that the transition to an unconscious state occurred 4.5–8.5 min after submersion in CO_2 saturated water. Unfortunately, these results confirm that visual indicators of consciousness may be unreliable from an ethical perspective (Lambooij et al., 2010; Robb & Kestin, 2002; Robb & Roth, 2003), as brain function was observed to continue in some individuals for up to 3.5 min after ventilation ceased and up to 6.5 min after the loss of equilibrium in rainbow trout.

Previous studies have shown that loss of VERs indicates a degree of brain failure indicative of insensibility in fish (Bowman et al., 2019; Bullock, Hofmann, New, & Nahm, 1991; Kestin et al., 1991, 1995; Readman, 2015; Retter et al., 2018; Robb & Roth, 2003; Robb et al., 2000; van de Vis et al., 2003), chickens and ducks (Gregory & Wotton, 1986) and livestock (Verhoeven, Gerritzen, Hellebrekers, & Kemp, 2014). While visual indicators of a loss of consciousness in fish are susceptible to misinterpretation from subjective visual observation, the use of EEG to assess VERs allows for a more definitive method of determining when sensibility is lost. The presence of a waveform as a response to flashing light indicates that there is some level of processing occurring in the brain, and so in the absence of a direct measure of consciousness, the presence of VERs must represent the possibility that the fish is not insensible (Kestin et al., 1991). When VERs are lost, it indicates that the insult to the brain was sufficient enough to inhibit a primary sensory pathway, which may represent a degree of brain failure that is inconsistent with sensibility (Daly et al., 1987; Kestin et al., 1991).

Taken together, these results highlight that visual indicators of a loss of consciousness in rainbow trout during CO_2 stunning are insufficient, and the prolonged time of induction for fish in cold-water temperatures increases the risk of misjudgements of the state of consciousness. To safeguard the welfare of farmed fish, it is critical to evaluate stunning methods using EEG to determine sensibility (or lack thereof) rather than relying on visual indicators alone and to do so at all possible water temperatures.

5 | CONCLUSION

A successful stun should induce insensibility in as short a time as possible, ideally immediately, and not cause any adverse reactions. In the present study, we found no evidence in support of this for CO_2 stunning as the long induction time and aversive behaviour indicate that fish are subjected to avoidable stress. Therefore, we recommend that CO_2 stunning continue to be considered an inhumane stunning practice. Additionally, we showed that there is a poor relationship between the loss of VERs and loss of visual indicators of consciousness, which suggests that when visual indicators alone are used fish risk being misjudged as insensible before sensibility is actually lost. Colder temperatures extend the time it takes for visual indicators of consciousness to be lost and aggravates the problem of determining when a fish loses brain function and sensibility. Our results highlight that stunning methods, like CO_2 stunning, that rely on human observation to determine when a fish loses sensibility are welfare hazards in aquaculture.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at http://doi.org/10.6084/m9.figshare.11356283

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This thesis presents novel findings on the welfare of rainbow trout (*Oncorhynchus mykiss*) and European whitefish (*Coregonus lavaretus*) during handling, transport and slaughter in aquaculture. I discuss how physiological stress responses can be used as welfare indicators. Furthermore, a newly developed technique for EEG-recording was used to determine onset and duration of unconsciousness using carbon dioxide, percussive and electrical stunning. The knowledge gained here will aid in the development of species-specific regulations on handling and killing of fish in aquaculture.

Per Hjelmstedt received his doctoral education at the Department of Animal Environment and Health, Swedish University of Agricultural Sciences. His undergraduate degree was received at the University of Gothenburg.

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