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Internal tagging in Atlantic salmon (*Salmo salar L.*): A study of welfare, wounds, and stress

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Preface

This dissertation is submitted in fulfilment of the requirements for the Degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University, Bodø, Norway. The original research presented in this thesis was part of the industrial PhD- project funded by The Research Council of Norway (Project No.297525) in collaboration with Arctic Seafood Group AS and Nord University.

The PhD project team consisted of the following members:

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Monica F. Brinchmann: Professor, FBA, Nord University: co-supervisor

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Bodø, August 15th, 2023

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Lastly, my deepest thanks go to my partner, Elin, for her love, support, and understanding. Without her grounding influence, I might have lost myself in a world of work.

"The mind is a powerful place, and what you feed it can affect you in a powerful way." - NF

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Paper II **Virtanen, M.I.**, Iversen, M.H, Patel, D.M., and Brinchmann, M.F. Daily crowding stress has limited yet detectable effects on skin and head kidney expression in surgically tagged Atlantic salmon (*Salmo salar*). Manuscript.

Paper III **Virtanen, M.I.**, Brinchmann, M.F., Patel, D.M., and Iversen, M.H. Transcriptomic response of wounded skin under daily chronic stress in Atlantic salmon (*Salmo salar*). Manuscript.

Abstract

Annually, the loss of an average 17% of Atlantic salmon during the grow-out phase in Norway represents not only an animal welfare catastrophe but also an economic strain, a detriment to food security, and a decline in public perception. While increasing production remains a central focus, the frequent loss of life during the production cycle and the effect of our methods on each animal's well-being cannot be overlooked. One strategy to address this substantial loss of lives involves gaining a deeper understanding of the animals and promptly responding to welfare issues. The desire to document and monitor fish has led to the development of technologies such as underwater cameras connected to machine learning models and individual monitoring systems such as real-time smart-tags. Importantly, it is essential to understand whether these methods affect the individuals in anyway, as we cannot consciously document fish welfare while at the same time affecting it. Moreover, smart tags in aquaculture require the use of sentinel fish, which are used as representative samples of the whole population and therefore require them to not be affected by tagging. As such, this thesis aimed to investigate how Atlantic salmon with internally implanted dummy smart-tags respond in terms of stress reactions and wound healing capability, and to explore how additional chronic stress influences these dynamics.

The thesis comprised a single experimental study that resulted in three scientific papers. **Paper I** examined the visual internal and external wound healing and physiological stress response. **Paper II** used RT-qPCR to investigate skin and head kidney tissue transcriptional responses associated with immunity, wound healing, and stress. **Paper III** employed RNA-seq to provide an in-depth understanding of transcriptional responses in the skin tissue. The study design utilized triplicate tanks with triplicate sampling each week from three different groups: an unaffected control group, a wound group that had a dummy smart-tag surgically implanted on day 0, and a wound + stress group (referred to as Stress^{+}) that, in addition to having a dummy smart-tag surgically implanted on day 0, was subjected to daily crowding stress for the entire 8-week duration of the study

Although the process of implanting sensor tags was not seen to prompt chronic stress, it was noted that chronic stress arising from daily crowding stress led to a type two allostatic overload response. This was evidenced by elevated ACTH and cortisol levels, and increased fin erosion. It was also observed that chronic stress slows down the wound healing process and heightens the inflammatory response, with minor but significant alterations in the expression of associated genes. The RNA-seq analysis identified markers for wound healing and chronic stress, along with indicating modifications in the skin healing process. Changes were observed in the molecular and cellular mechanisms of skin tissue subjected to wounding and subsequent chronic stress conditions. Additionally the idea of a cutaneous stress axis was supported. This thesis concludes that while tagging can be successful in an unstressed environment, chronic stress adversely impacts fish welfare, alters wound healing, and modifies gene expression, with implications for long-term monitoring of fish welfare using smart tags.

Abstract in Norwegian - Sammendrag på norsk

Gjennomsnittlig dør 17% av atlantisk laks i vekstfasen i løpet av kommersiell lakseproduksjon. Dette representerer ikke bare en katastrofe for dyrevelferden, men er også en økonomisk belastning; en risiko for matsikkerheten, og gir dårlig offentlig omdømme for oppdrettsindustrien. Selv om økt produksjon er et sentralt fokus i næringa, kan man ikke overse det hyppige tapet av liv under produksjonssyklusen og betydningen våre metoder har på hvert dyrs trivsel. En strategi for å forsøke å løse problemet med forringelse av livskvalitet for fiskene, er å få en dypere forståelse av dyrenes adferd slik at man raskt kan respondere på ulike velferdsproblemer. Et ønske om å kunne dokumentere og overvåke fisk har ledet til utviklingen av teknologier som undervannskameraer koblet til maskinlæringsmodeller. Og videre til individuelle overvåkingssystemer som sanntids smart-tags. Det er viktig å forstå om disse metodene på noen måte påvirker individene. da vi ikke med viten og vilje kan dokumentere fiskevelferd, samtidig som vi påvirker den. I tillegg krever smart-tags i akvakultur bruk av bevisst og tilstedeværende fisk, som skal være representative individer for hele populasjonen. De må dermed ikke påvirkes av merkingen. Denne avhandlingen hadde blant annet som overordnet mål å undersøke hvordan atlantisk laks med internt implanterte dummy smart-tags responderer i henhold til stressreaksjoner og sårhelingskapasitet, og å utforske hvordan tilført kronisk stress påvirker disse dynamikkene.

Avhandlingen besto av en eksperimentell studie som resulterte i tre vitenskapelige artikler. **Artikkel I** undersøkte den synlige indre og ytre sårhelingen, og den fysiologiske stressresponsen. **Artikkel II** brukte RT-qPCR for å undersøke hud- og hodenyrevev-transkripsjonsresponsene forbundet med immunitet, sårheling og stress. **Artikkel III** brukte RNA-seq for å gi en dypere forståelse av transkripsjonsresponsene i hudvevet. Studiedesignet benyttet tre kar med triplikatprøvetaking hver uke fra tre forskjellige grupper: en urørt kontrollgruppe, en gruppe som hadde fått en dummy smart-tag kirurgisk implantert på dag 0 («Wound»), og en stressgruppe («Stress$^+$»). Den sist

nevnte gruppen fikk en dummy smart-tag kirurgisk implantert på dag 0, og utsatt for daglig trengselstress i hele studiens varighet på 8 uker.

Selv om prosessen med å implantere sensortagger ikke førte til kronisk stress, ble det vist at kronisk stress oppstod i gruppen stress$^+$ eksponert for daglig trengselstress. Dette førte til en type to allostatisk overbelastningsrespons. Dette ble observert som økte ACTH- og kortisolnivåer, og økt grad av finneerosjon. Det ble også observert at kronisk stress forsinker sårhelingsprosessen og øker den inflammatoriske responsen, med en lavere, og signifikante endringer i uttrykket av tilknyttede gener. RNA-seq-analysen identifiserte markører for sårheling og kronisk stress, i tillegg til å indikere at det skjer endringer i hudhelingsprosessen. Det ble observert endringer i de molekylære og cellulære mekanismene i hudvev hos fisk som var påført sår for så å bli utsatt for kroniske stress. I tillegg ble ideen om eksistensen av en kutan stressakse støttet. Denne avhandlingen viser med at mens merking kan være vellykket i et ustresset miljø, påvirker kronisk stress fiskevelferd negativt. Da påvirkes sårheling, og genuttrykket, med negative implikasjoner for langsiktig overvåking av fiskevelferd ved bruk av smart-tags.

1 Introduction

A growing population is a hungry one, and the increasing need for seafood as an affordable protein source and a desirable food item has contributed to the rapid growth of the aquaculture sector (FAO, 2022b). Yet, the global economy suffers and prices of produce increase, so cheap food sources may become scarce, affecting small-scale farmers and those who rely on them. In Norway, Atlantic salmon (*Salmo salar*) accounts for 80% of aquaculture production, making food security vulnerable to unexpected challenges if not managed effectively and promptly (FAO, 2022a). Additionally, the increased production through the intensification of aquaculture has given rise to various challenges, including increased susceptibility to diseases, parasites, environmental stressors, and welfare concerns (Olaussen, 2018).

In Norway, from 2010 to 2021, the production of Atlantic salmon increased by 51%, and the value of the produce skyrocketed by 207% (DOF, 2022). Although in decline, this rapid growth can be attributed to the adoption of aquaculture innovation, policy-making, public perception and scientific research, yet there is still much room for improvement in each area (Bailey and Eggereide, 2020; Hersoug et al., 2021; Hersoug, 2022). A new saying may stay until the needed change is made: “More salmon, more problems.” This is highlighted by the fact that, on average in Norway, 17% of Atlantic salmon have failed to reach the slaughtering stage during their grow-out phase each year over the last 21 years, as shown in Figure 1 (DOF, 2022). This trend raises significant economic and, more importantly, welfare concerns.

To address these challenges, the Norwegian government is promoting new developments in the aquaculture industry by tying production to innovative solutions while regulating production with the traffic light system and using different licensing systems (Olaussen, 2018; Føre et al., 2022). These solutions drive some aquaculture development towards overly complicated and expensive megastructures that may never be adopted into the average fish farmer's budget as they lack the same government funding as the creators had to build it (Greaker et al., 2020). Considering the above, one hopes government funding focuses on beneficial and adoption-friendly innovations for the entire Norwegian aquaculture industry. This should not be mistaken for expecting cheap solutions but scalable solutions that can be implemented across the sector.

Regardless, as we pursue innovation and increased production, we must remain mindful of the welfare of the animals we subject to these new advancements. If these innovations do not effectively reduce mortality rates or contribute to their accurate assessment, they merely represent improved production iterations of past systems. We are increasing production knowing that the mortality rate is stable, subjecting the lives of individual farmed fish as a cost of doing business. To decrease mortality rates and accomplish the anticipated production of approximately 5 - 6.3 million tons of salmon in Norway by 2050, it is critical to take into account our understanding of fish welfare and the application of welfare measures in the industry (Olafsen et al., 2012; PwC, 2021).

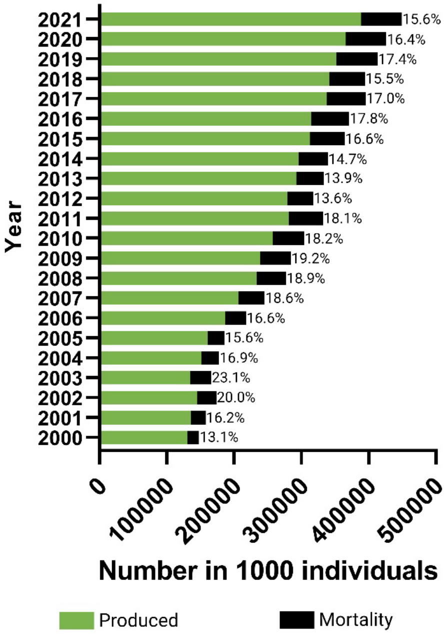


Figure 1. The number (in thousands) of Atlantic salmon produced (green) and mortality (black) during the grow-out phase of production in Norway. The percentage of individuals lost is also depicted. Data from DOF (2022).

1.1 Animal welfare

Animal ethics is a branch of philosophy examining the moral questions regarding how animals are utilized and treated, whether for food, apparel, scientific study, or leisure. Conversely, animal welfare focuses on the tangible aspects of an animal's life, exploring their physical and emotional well-being and constitutes a rapidly evolving, multidisciplinary field (Kristiansen and Bracke, 2020). This field incorporates three core components: welfare science, welfare ethics, and welfare standards (Huntingford et al., 2006; Main, 2010). The welfare standards of animals, the science behind welfare, and ethics are intricately interwoven. Understanding the welfare standards is essential for assessing animal welfare's scientific and ethical aspects.

Furthermore, ethical considerations dictate which actions should be taken concerning animal welfare, and understanding the science is imperative for practically applying these ethical stances (Broom, 2011). This complicates the ability to define "*Animal Welfare*", as the definition may vary depending on one's perspective and specialization. Generally, animal welfare is approached through one of three lenses: physiological functioning, natural living, or feelings (Fraser, 2008).

In my opinion, the definition of animal welfare should be described from the perspective of the individual animal's perceived well-being (subjective welfare) (Dawkins, 1990; Fraser, 2009a; Stien et al., 2013; Browning, 2022b). The approach to animal welfare has evolved in various ways throughout history (as reviewed by Duncan (2019)). The first known notion originated with Aristotle (384 – 322 BC), who postulated that humans, equipped with the ability to reason, are superior to animals. Thus animals exist for human utilization, as rain exists for plants (Duncan, 2019). This ideology was echoed in various forms until Bentham (1823) wrote, "*The question is not, Can they reason? Nor, Can they talk? But, Can they suffer?*" This perspective evolved into the philosophy of Utilitarianism by Mill (1910), where the moral worth of an action revolved around whether it contributes to the overall happiness of all involved through the presence of pleasure and absence of pain.

In the middle of these philosophical developments, the works of Darwin (1872) and Romanes (1883) played important roles in advancing animal welfare theories by providing empirical evidence and theoretical frameworks that challenged the current notions of the time. Darwin explored the concept that humans, like animals, can suffer and experience well-being, while Romanes investigated mental capacities and cognition in both animals and humans. Over the next century, the scientific information gathered for animal welfare was primarily confined to the scientific community, as a divide emerged between those who embraced science and those often involved in government and legislation-making, who were apprehensive about it (Broom, 2011). This changed when Ruth Harrison published Animal Machines in 1964, critiquing confinement systems for denying animals any pleasure in life (Harrison, 2013). Following this, Peter Singer's book Animal Liberation published in 1975 advocated that pain and pleasure are the fundamental criteria for moral judgments, and all involved parties should be evaluated accordingly (Singer, 1975). Harrison's book catalysed the Brambell Committee's agenda in 1965 to develop the Five Freedoms model, employing scientific evidence from veterinary medicine, stress physiology, animal science, and animal behaviour to comprehend animals' feelings (Hemsworth et al., 2015). The establishment of the Five Freedoms was a landmark development in creating a framework for understanding and assessing the treatment of animals.

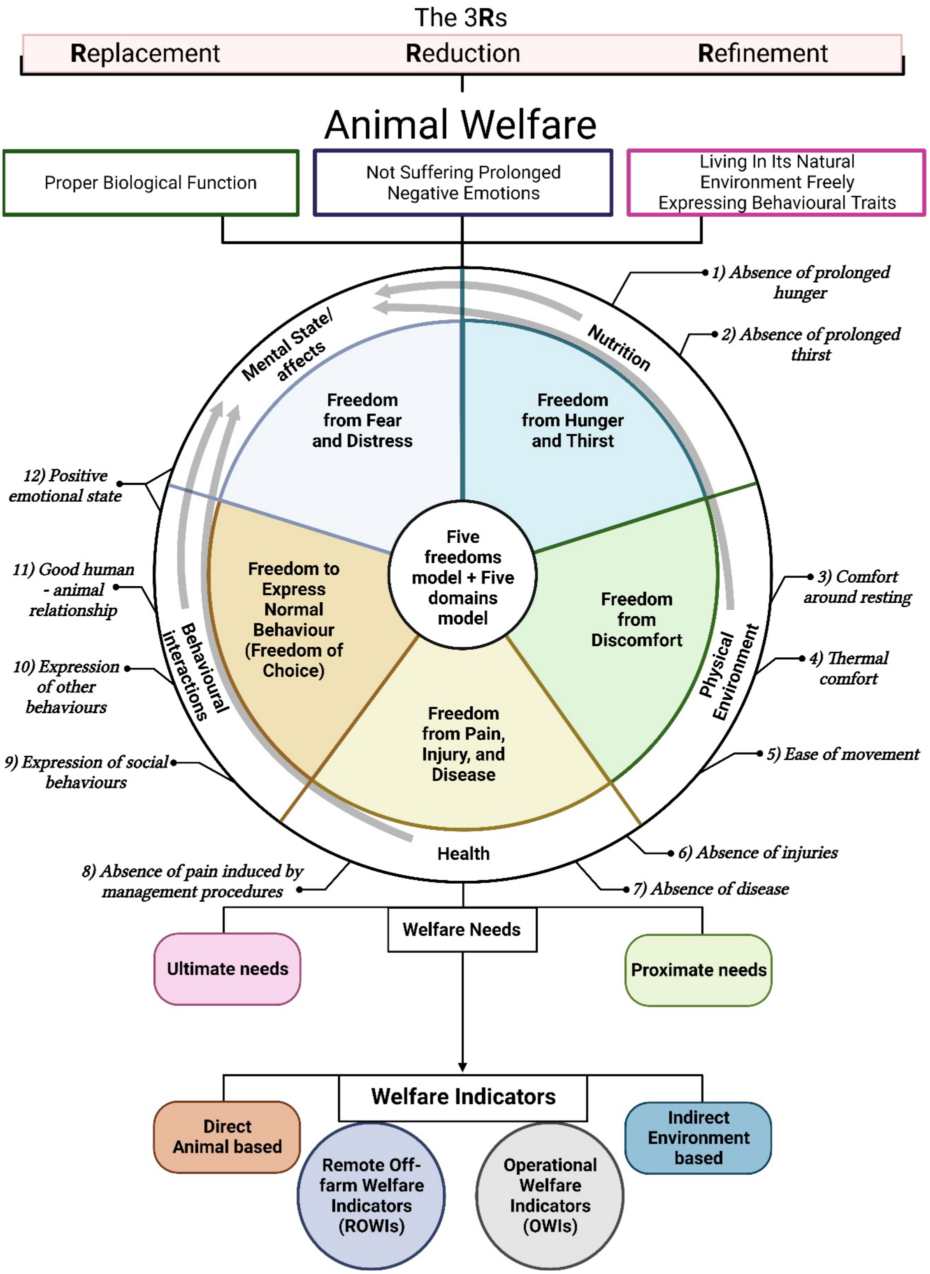


Figure 2. Diagram illustrating the interplay between the 3Rs, animal welfare, and assessment models. The three ideal principles of animal welfare are shown. The inner circle embodies the Five Freedoms, while the outer circle showcases the Five Domains model for welfare assessment. Surrounding this, the 12 factors from the Welfare Quality® programme are depicted. The lower part of the figure demonstrates how animal welfare is connected to fulfilling welfare needs and the methods employed to measure them.

Jumping through history, Figure 2 describes the concept of animal welfare through the Five Freedoms, introduced by the Brambell Committee (Council, 1993), and the modified Five Domains concept (Mellor, 2016; Mellor et al., 2020). Additionally, it presents the 12 key animal welfare criteria set out by the Welfare Quality® program (Botreau et al., 2009). The Five Domains aim to figure out how the surroundings, including the physical spaces and the social interactions, affect the emotions and feelings of animals that can experience sensations. On the other hand, the Five Freedoms serve as a practical method to identify and evaluate the efficacy of measures taken to enhance an animal's well-being (Webster, 2016). These concepts are grounded in three interconnected conceptual frameworks: biological functioning, affective state, and natural living (Fraser, 2008; 2009b; Diggles et al., 2011). The 3Rs framework (Replace, Reduce, Refine) also indirectly belongs to the discussion of animal welfare, as it fundamentally impacts animal well-being. Moreover, the welfare needs concept asserts that good welfare is achieved when needs, such as ultimate (immediate survival) and proximate (long-term success), are fulfilled (Noble et al., 2018). Welfare indicators assess whether these needs are met and can be measured through on-site Operational Welfare Indicators (OWIs) or Remote Off-farm Welfare Indicators (ROWIs), which require external facilities like labs for sample processing. These welfare indicators can either be direct animal-based indicators or indirect environmental-based indicators (Noble et al., 2018). Regardless of one's perspective on animal welfare or the methods employed to measure it, the objective should be to improve the animal's current state as much as possible.

Throughout the history of animal welfare, perceptions have been influenced by individuals, societies, scientific communities, governments, producers, and consumers. In recent years, the perception of a divide between individuals, society, and animal well-being is increasingly considered an artificial construct, according to Colonius and Earley (2013). They contend that these three elements are inherently interconnected, originating from the same scientific measures. This interconnection has paved the way for developing the One Health concept, an integrative approach that unites the domains of animal, human, and environmental health and welfare through collaborative and interdisciplinary efforts (Losada-Espinosa et al., 2020). However, the practical application of the One Health concept poses challenges, especially in the context of animal welfare. For example, implementing measures to improve animal welfare might incur additional costs, potentially causing financial strain for individuals or communities. This poses an ethical conundrum: Is it justifiable to enhance animal welfare at the expense of human, financial well-being? Conversely, is it ethically acceptable to compromise animal welfare to alleviate economic pressure on human communities? Addressing these dilemmas necessitates careful consideration and critical evaluation of the trade-offs involved.

To conclude this introduction to animal welfare on a positive note, the approach of evaluating animal welfare primarily through negative aspects, such as suffering, which historically led to the establishment of minimal standards for animals, is evolving to embrace the consideration of positive welfare – characterized by a fulfilling and meaningful life (Yeates and Main, 2008). This evolution is essential for the genuine betterment of animals, as the absence of observable signs of distress does not necessarily imply the presence of positive emotions. Moreover, the conception of positive welfare has gained traction with the increased awareness of animal sentience and their capacity to experience positive emotions (Boissy et al., 2007; Held and Špinka, 2011; Serrapica et al., 2017; Lawrence et al., 2018; Rault et al., 2020; Browning and Veit, 2022). According to Yeates and Main (2008), taking care of animals in a positive way influences multiple factors, such as making society happier, helping both humans and animals feel better when they bond through compassionate bonding, lowers negative conditions through positive reinforcement, motivates animal caretakers to provide better care, and makes it easier for policy-makers to adapt new legislations. Notably, an example of a positive welfare aspect that has yielded mutual benefits for both humans and animals includes gentle brushing and petting of dogs, sheep, cattle, and pigs (Rault et al., 2020). While incorporating positive welfare assessments represents a progressive advance in animal welfare, it is important to recognize that this does not take away from the fundamental importance of mitigating negative welfare elements.

1.1.1 Fish welfare

Much of the early work in the 1980s on fish welfare was associated with angling, as the idea that all vertebrate animals are capable of suffering led to discussions on methods to alleviate pain caused by hooks in fish (Kristiansen and Bracke, 2020). Alongside this, the increasing interest and increased production in aquaculture during the 1990s brought to light issues associated with fish farming, such as poor animal husbandry, diseases and parasites. This led to growing concerns about fish welfare (Kristiansen and Bracke, 2020). Consequently, studies on fish stress physiology were conducted. While the concept of fish stress was established, the ability of fish to feel pain remained unproven. However, the idea that fish can experience poor welfare through stress was addressed (Pickering and Pottinger, 1989; Schreck, 1990; Wendelaar Bonga, 1997). Nevertheless, welfare considerations for terrestrial animals, and the public's interest in them, progressed faster than fish welfare. This difference is likely due to the disconnect between humans and aquatic animals, which are not only farmed in large quantities but also inhabit a biome unfamiliar to us (Stien et al., 2020). This disconnect, coupled with the ongoing debate on whether fish are capable of experiencing pain and suffering, creates challenges in ensuring the best care for fish under human cultivation.

The belief that fish cannot feel pain stems from their lack of a human-like cortex involved in human pain processing (Rose, 2002; Diggles et al., 2011; Rose et al., 2014; Key, 2015; 2016). However, many participants in this debate strongly oppose this notion, contending that fish are capable of experiencing pain, fear, and possibly sentience (Braithwaite, 2010; Brown, 2016; Merker, 2016; Sneddon et al., 2018; Brown and Dorey, 2019; Kristiansen and Bracke, 2020; Sneddon and Roques, 2023). The argument that two species must have identical anatomical or physiological features to process information in the same matter is flawed. For instance, fish perceive visuals differently than humans, but this does not imply that they are incapable of vision (Sneddon, 2020).

Whether we believe fish can or cannot feel pain and suffer should ultimately not matter, as fish pain sceptics like Diggles et al. (2011) confirm, "*empirical science is unable to prove that fish are capable of awareness.*" With this in mind, we cannot confirm that fish do not feel pain either, and if one accepts the notion that fish do not feel pain, one accepts that all efforts to reduce pain regarding fish welfare are meaningless. Which leads us back to the question: What is animal welfare? Which we described as the current well-being as perceived by the animal, and given the fact that we are unable to prove (agree upon) or disprove fish sentience, it is advisable to adopt a precautionary approach as described by Birch (2017): "*Where there are threats of serious, negative animal welfare outcomes, lack of full scientific certainty as to the sentience of the animals in question shall not be used as a reason for postponing cost-effective measures to prevent those outcomes*." However, Browman et al. (2019) argue that the precautionary approach can lead to humanising fish (biologically), which can be problematic when making scientific inferences. Striking a balance between precautionary approaches to aid animal welfare in assuming they can feel pain like humans and objective interpretation of data which does not outright assume fish behave like humans is crucial.

According to Mason and Lavery (2022), future research in fish welfare should focus on operant learning (behaviour modified by consequences), as it could potentially indicate sentience and is empirically testable. Additionally, while seeking new methods to show sentience is important, there is a simultaneous focus on improving fish welfare by promoting positive experiences within existing frameworks, as described in the animal welfare section above. Several studies have aimed to provide positive experiences for fish and the absence of negative ones. Promising results have been demonstrated, for example, through environmental enrichment (Näslund and Johnsson, 2016; Brunet et al., 2022; Zhang et al., 2022; Zhang et al., 2023).

1.1.2 Fish welfare indicators

Welfare indicators have become instrumental in assessing the welfare of fish, especially considering that fish cannot communicate how they feel. These indicators should comprehensively address various welfare needs, which for fish include adequate nutrition, appropriate water quality, good health, behavioural freedom, and safety which are parts discussed prior in Figure 2 (Stien et al., 2020). The welfare indicators are broadly categorized into two types: *input-based* indicators, which measure environmental factors that have the potential to impact the well-being of fish. These include factors like water quality parameters such as temperature and pH levels. The second type of welfare indicator is the *output-based* indicator, which assesses the effects of environmental conditions on fish. These can include measures of behaviour and health status (Noble et al., 2018; Stien et al., 2020). The evaluation of these welfare indicators requires the application of diverse sources, including physiological metrics, behavioural patterns, observable signs, and, in some cases, assessments based on psychological perspectives (Barton, 1997; Schreck and Tort, 2016; Noble et al., 2018).

Highlighting the recent evolution of how to asses welfare through welfare indicators relating to Atlantic salmon, the development of the farmer-oriented Salmon Welfare Index Model (SWIM) 1.0 protocol by (Stien et al., 2013) was a significant development. This protocol uses direct measures (such as health and morphology) and indirect measures (such as the rearing environment) to assess welfare by adding multiple welfare indicator scores. Each indicator has its relative influence taken into account during scoring. It was later extended into SWIM 2.0, which incorporated more specific indicators for assessing health and morphological welfare to be used by fish health professionals at farm sites. These protocols were then used to produce other encompassing protocols. (Pettersen et al., 2014; RSPCA, 2021). Presently, two of the most comprehensive protocols for Atlantic salmon are the *Welfare Standards for Farmed Atlantic salmon* (RSPCA, 2021) and the *Welfare Indicators For Farmed Atlantic salmon* (Noble et al., 2018). The latter incorporates physiological parameters and updating prior protocols with current knowledge. However, it is important to recognize that these protocols should serve as guidelines rather than definitive standards as the scope of welfare indicators continues to expand with the development of new research and technological tools (Barreto et al., 2022).

One part of measuring fish welfare is the assessment of stress response measurements, which is considered effective in monitoring animal welfare with the ultimate goal of minimizing adverse states. This thesis places significant emphasis on the use of stress responses as a means to identify potential welfare issues with the internal tagging of smart tags. Nevertheless, it is crucial to recognize that an animal may not exhibit stress but could still be experiencing sub-optimal welfare. Conversely, exposure to mild stress may sometimes yield beneficial effects (Schreck and Tort, 2016; Sloman et al., 2019).

1.1.1 Fish welfare: Immense species conundrum

Approximately 34,000 species of teleosts exhibit various unique traits, behaviours, and capacities to cope with stress (Guinot and Cavin, 2015; Balasch and Tort, 2019; Fernö et al., 2020). Consequently, tailoring fish welfare assessments and stress indicators to the specific species being studied is important. Over the past two decades, there has been a notable increase in the number of farmed species, now estimated at 400, with countries like China having 25% of their farming being non-native species (Lin et al., 2015; FAO, 2022a). Figure 3 illustrates the introduction of new species and the expansion of aquaculture-producing countries within continental zones. However, this rapid progression in species farming and or new countries starting aquaculture has not been matched by the development of species-specific welfare indicators. However, new technology may accelerate the study of species-specific welfare as behavioural changes and large amounts of data become easier to track and handle.

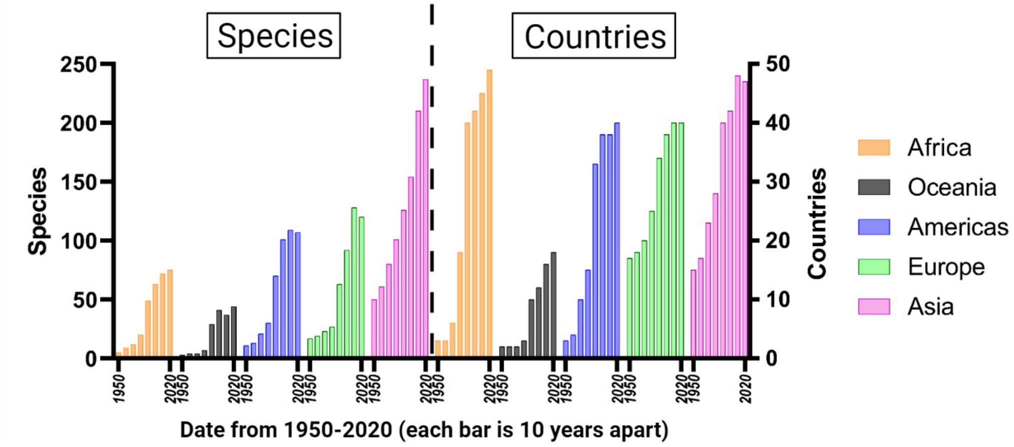


Figure 3. The increase in aquatic species farmed by continent and countries involved over time. The figure represents on the left half the total number of species farmed in each continent in 10-year gaps. The right side similarly shows the number of countries within each continent that have farmed at least one species. Data to make the graph was obtained from FAO (2022a).

Franks et al. (2021) revealed that only 25 species of farmed aquatic animals had been the subject of five or more published species-specific welfare studies, which could suggest a welfare crisis. The number of publications is limited, but each species is unique, suggesting that existing literature on other species should be considered only as an initial reference and not be directly applied unless demonstrated to be adaptable. Furthermore, the extent to which the limited welfare information available for these 25 species is being used by farmers, regulatory bodies, or even researchers is uncertain.

Since stress responses and other welfare indicators differ significantly between species due to various factors, this leads to many variances in the data gathered and highlights the importance of understanding welfare at the species level. The reliability of indicators will be discussed in detail in a later section. Still, to briefly introduce this critical aspect with varied examples, Brydges et al. (2009) observed that when exposed to the same stressor, the opercula beat rate increase differed in three distinct fish species – rainbow trout, three-spined sticklebacks, and Panamanian bishops – and the duration taken to return to normal levels also varied among these species. Further, it is noted that hormonal reactions such as cortisol levels exhibit significant differences among species when subjected to identical stimuli. Moreover, variations have also been documented among different strains of the same species and between wild and hatchery-bred fish (Congleton et al., 2000; Barton, 2002). Considering a specific species, fluctuations in stress responses can be attributed to daily and seasonal changes and differences based on sex and maturity stage. Additionally, even within the confines of a single experimental tank, factors like “stress-coping styles” can come into play - for instance, the hierarchy established among fish in a tank can modify the intensity of stress responses recorded (Culbert and Gilmour, 2016; Johansen et al., 2020; Madaro et al., 2020). This diverse response of characteristics and traits of individual species has led to operational welfare indicators being established and published for lumpfish, ballan wrasse, Atlantic salmon, and rainbow trout in Norway (Espmark et al., 2019).

1.2 Stress and welfare

Stress naturally arises in living organisms due to interactions with their environment and fellow organisms and as an integral part of normal physiological processes. Moreover, human intervention can heighten stress, including environmental modifications, routine aquaculture practices, and scientific research (Adams et al., 2007; Iversen, 2013; Sneddon et al., 2016). Fish stress has seen considerable attention in research since it is a critical protective mechanism for aquatic animals. Investigations into stress offer invaluable insights into the impact of altered environments and various treatments to which these animals are exposed (Braithwaite and Ebbesson, 2014; Sneddon et al., 2016; Stevens et al., 2017). The relationship between stress and animal welfare becomes crucial as suboptimal practices can induce stress in individuals, which may subsequently diminish their quality of life if the stressors are intense or persistent, often leading to pain and suffering (Ashley, 2007; Schreck and Tort, 2016; Sneddon, 2019; Kristiansen and Bracke, 2020). The primary objective of the stress response in fish is to reconfigure energy allocation, immune functions, and neural and endocrine processes to effectively manage and adapt to stressors (Samaras et al., 2018).

Direct physiological stress indicators, such as cortisol, are released from the hypothalamic-pituitary-interrenal (HPI) axis in response to stressful events. These events include factors that have a detrimental effect on an individual, such as perceived pain, temperature changes, crowding, poor water quality, exposure to pathogens, and handling, among others (Santurtun et al., 2018; Hoem and Tveten, 2020; Tang et al., 2022; Sneddon and Roques, 2023; Virtanen et al., 2023). However, for a comprehensive animal welfare assessment, it is critical not to rely solely on one indicator, which could provide only a partial perspective. Instead, a more holistic approach that encompasses multiple aspects of the animal's well-being should be adopted (Browning, 2022a; Browning, 2023; Sneddon and Roques, 2023).

Cortisol measurements, for instance, supply essential information but should be complemented with other assessments. These include but are not limited to, the examination of primary, secondary, and tertiary stress responses. While relying on a single, proven indicator can yield insights into welfare, there is no reason not to take into consideration more than one; as C.S Lewis said, “*Two heads are better than one, not because either is infallible, but because they are unlikely to go wrong in the same direction.*”

1.2.1 Concept of stress

The earliest known concept of stress was metaphorically presented by Pythagoras (570-510 BC) in his *harmony of the cosmos*. He put forward the idea that the universe operates in a harmonious balance. Here we can extend this to infer that so do living organisms, in that challenges faced by an organism or the universe are diminished through internal harmony (Agorastos and Chrousos, 2022). In the early 20th century, Walter Bradford Cannon studied the body's responses to various stimuli. He observed that during stressful situations, the body either increases or decreases its energy conservation functions to mobilize greater energy for escape, attack, or defence. He termed this the "fight-or-flight" response, now called the freeze-fight-or-flight response, and introduced the concept of homeostasis, laying the foundation for stress research (Godoy et al., 2018). The concept of homeostasis was termed as the process the body goes through via constant regulation to maintain a stable internal environment. Hans Selye expanded upon previous ideas by introducing The General Adaptation Syndrome, which outlines the non-specific responses of the body to stress in three stages: 1) the alarm reaction stage, 2) the resistance stage, and 3) the exhaustion stage. He defined stress as "*a nonspecific response of the body to any demand made upon it*" (Selye, 1936; 1950).

Stress today can be defined as the disruption or threat placed on an individual's dynamic balance, including both homeostatic and homeodynamic states, caused by stressors. Stressors can be real or perceived, internal or external factors that disrupt equilibrium, triggering physiological and psychological responses. These responses are part of an adaptive process known as allostasis, which aims to restore homeostasis (McEwen and Wingfield, 2010; Schreck and Tort, 2016; Agorastos and Chrousos, 2022).

It should be mentioned that a deviation from homeostasis should not be immediately defined as stress, as homeostasis is in a constant state of change. This distinction has confused the terminology and assessment of stress, stressors, and stress responses. While this thesis adopts the allostatic perspective, it is crucial to understand that the activation of a stress response does not necessarily indicate the presence of stress (Koolhaas et al., 2011; McEwen and McEwen, 2016). Additionally, one should be aware that mild stress can have beneficial effects (eustress). In contrast, more severe stress can lead to adaptive responses to overcome the stress or maladaptive responses (distress) when adaptation is impossible (Schreck and Tort, 2016).

1.2.2 Allostasis

Allostasis, known as "*stability through change*," has evolved as a concept in conjugation with the definition of stress in recent years. It implies non-linear, adaptable set points for maintaining physiological balance (Sterling, 1990; McEwen and Wingfield, 2003; McEwen, 2005; Wingfield, 2005; McEwen and Karatsoreos, 2015). Allostasis represents an organism's ability to respond effectively to changes in its environment by integrating prior knowledge with current needs, which facilitates the adjustment of physiological set points accordingly (Samaras et al., 2018).

Figure 4 illustrates the concepts of allostasis. An *allostatic state* represents a chronic deviation of the regulatory system from its original state. In this state, primary mediators such as glucocorticoids are sustained or altered in response to an event (McEwen, 2004; Ramsay and Woods, 2014). The allostatic state is depicted as the baseline physiological equilibrium in Figure 4. For example, if an individual is relocated from an area without predators to an area with predators, its allostatic state increases, leading to a new equilibrium within its new environment. *Allostatic load*, on the other hand, is the cumulative physiological stress experienced by an individual, comparable to the chronic toll on the body resulting from continued stress (McEwen and Stellar, 1993). Building on our previous example, if a fish moves into an area with predators (representing an altered allostatic state) and is subjected to a steady water current, its allostatic load increases through fluctuations in homeostasis and allostasis until a new allostatic state is achieved. If allostatic load persists with additional unpredictable events, *allostatic overload* may occur. Allostatic overload has two forms: type one (adaptive), which is the response to acute stress, and type two (maladaptive), which is the response to chronic stress. Using the fish example, if a predator attempts to catch the fish, it will experience *allostatic overload type I*, where a surge in catecholamines and glucocorticoids leads to a higher energy expenditure than is available to escape the predator. This triggers emergency life history stages (ELHS), which suppress other life history stages, such as reproduction, to facilitate recovery from stressful events if the predator is successfully evaded (Goymann and Wingfield, 2004; Iversen, 2013). However, if an individual cannot overcome a stressor as it did in the acute predator attack, it enters *allostatic overload type II*, which is detrimental to survival and often leads to irreversible harm if the stress persists (Goymann and Wingfield, 2004; Sadoul and Vijayan, 2016; Schreck and Tort, 2016). For instance, if the fish is subjected to unpredictable and constant aggression from conspecifics, the cumulative energy expenditure to resist the aggressive behaviour will persist just below the available energy supply, drawing energy away from other aspects of living until only the most vital functions are supported. If further energy is required beyond this point, death is usually the outcome. This scenario represents a non-adaptive state with chronic glucocorticoid secretion, high energy demands, reduced growth, impaired immune function, and reduced reproduction (Goymann and Wingfield, 2004; Iversen, 2013; Sneddon et al., 2016).

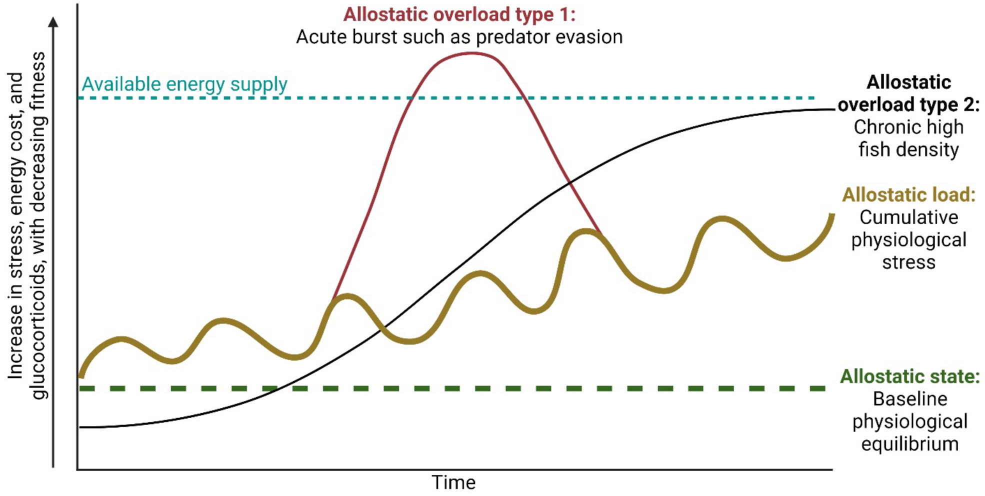


Figure 4. Illustration of the allostatic response to stress. Shown is the allostatic state, which represents an organism's baseline physiological equilibrium. The allostatic load refers to the cumulative physiological stress experienced by an organism in the face of chronic stressors. Allostatic overload Type 1 represents an acute spike in energy consumption through the increase of glucocorticoids, which activates the emergency life history stage and subsequently reduces energy levels and glucocorticoid concentrations to regain stability (adaptive). Allostatic overload Type 2 illustrates a chronic increase in energy usage aimed at achieving stability, which fails to recover and results in the diversion of energy from other life processes, such as growth, to compensate for the increased energy demand (non-adaptive). Inspired by Goymann and Wingfield (2004).

1.2.3 Metabolism and stress

The allostatic response to stress is dependent on the available energy supply. Therefore, it is important to understand how stress, by increasing allostatic load, can deplete the available energy, accelerating the onset of allostatic overload situations. Energy is mainly generated by breaking down carbohydrates, fats, or proteins through catabolic reactions to produce adenosine triphosphate (ATP), the universal cellular energy currency. An organism's ability to adapt to environmental changes and stress is determined by how much energy it has, how quickly it can take in and use it, and its ability to store energy (Sokolova et al., 2012). Although these shares similarities with the previously discussed concepts of allostasis, Figure 5 shows how in terms of ATP supply and demand, stress can alter an organism's response through energy management.

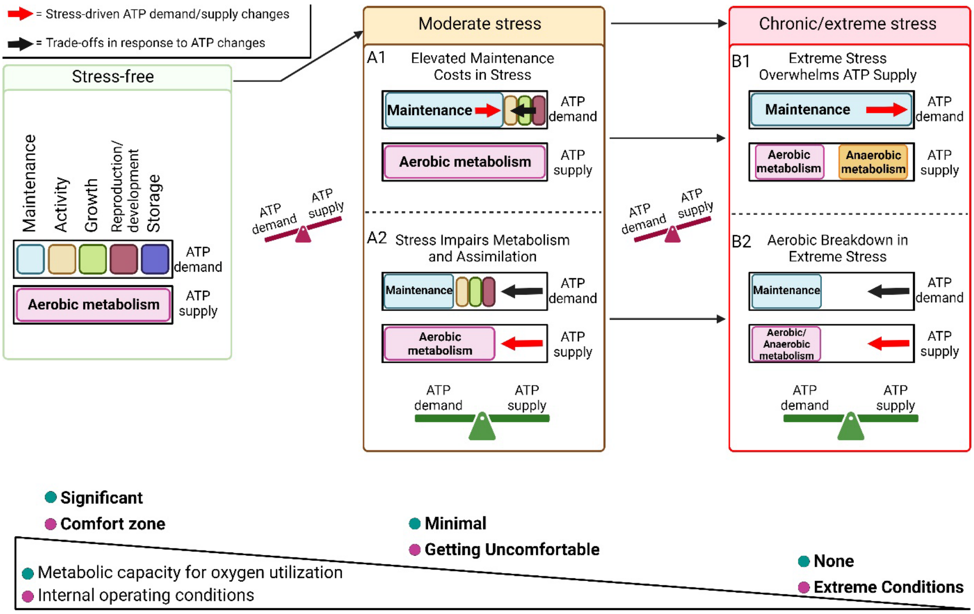


Figure 5. This figure illustrates how an organism manages its energy in response to stress. Under normal conditions, enough energy (ATP) is needed for maintenance, activity, growth, reproduction, and storage. During moderate stress, energy is reallocated: maintenance takes priority, and growth or reproduction may be reduced (A1: increased maintenance costs by energy trade-offs; A2: stress affecting metabolism causing energy reallocation). In extreme stress, the organism struggles to produce enough energy. It switches to a less efficient energy source for survival, which can't be sustained for long periods (B1: maintenance needs exceed energy supply, shift to short-term survival anaerobic metabolism; B2: the breakdown in normal energy production, aerobic metabolism impairment with partial anaerobiosis to sustain maintenance at the expense of long-term survival). Redrawn and modified from Sokolova et al. (2012), with permission from ELSEVIER with license no. 5584180869337.

When confronted with stress, individuals must initiate a series of processes to reestablish their internal equilibrium, or homeostasis, which demands substantial energy. These processes include ion regulation, ensuring the availability of metabolic substrates, and the synthesis of proteins essential for coping with stress (Sadoul and Vijayan, 2016). In response to stress, fish activate pivotal metabolic pathways such as glycolysis/gluconeogenesis, the citric acid (TCA) cycle, and mitochondrial oxidative phosphorylation. Furthermore, they may metabolize carbohydrates, free amino acids, and lipids to produce ATP via aerobic or anaerobic oxidation. This production of ATP is vital for maintaining homeostasis and supporting essential functions (Sokolova et al., 2012; Hu et al., 2015). However, a critical threshold is reached when stress becomes chronic or exceedingly intense. Thus, this leads to an extremely high ATP demand, or when ATP production is impeded due to a decreasing supply, as illustrated in Figures 5 B1, B2. Beyond this point, maintenance can no longer be sustained after the reduction of all other non-essential functions for survival. This leads to a decline in ventilation, heart rate, cellular ion pumping, and protein synthesis, which, in turn, can result in severe consequences, including death (Sokolova et al., 2012).

1.2.4 Mechanism of stress

While some aspects of the stress axis have been described briefly above, the focus in this section will be on the corticotropic endocrine axis. Figure 6 summarizes simply the main neuroendocrine processes activated in response to a threat to homeostasis. These processes trigger stress responses aimed at restoring an allostatic state. It should also be noted that the allostatic state has a direct implication on the performance capacity in the sense that in an ever-changing environment, eustress increases the capacity to perform during mild stress, while the capacity to perform under distress would decrease (Schreck and Tort, 2016; Balasch and Tort, 2019; Hou et al., 2019). Building from the basic concept of stress, one can classify the stress responses into three distinct categories, primary, secondary, and tertiary (Barton et al., 2003; Braithwaite and Ebbesson, 2014).

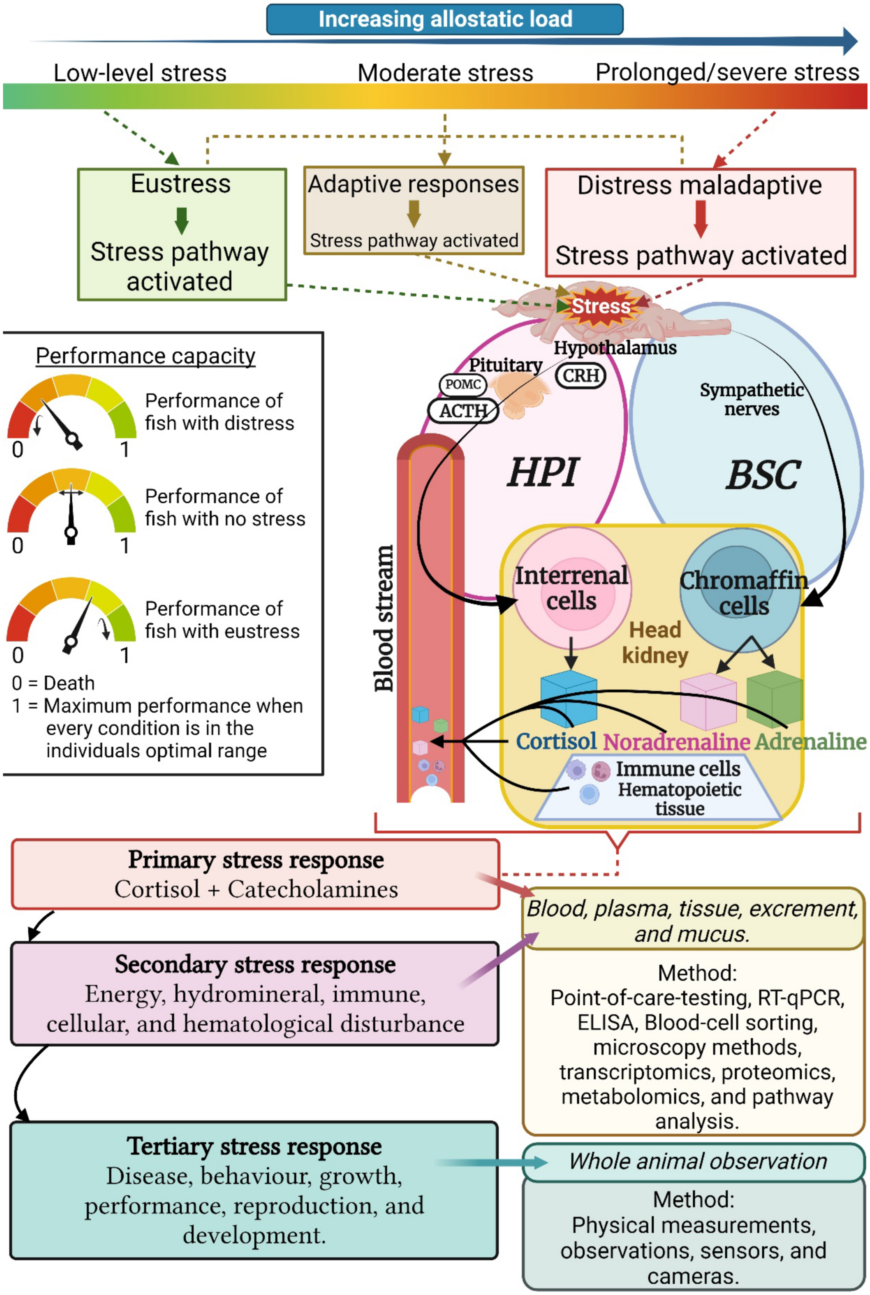


Figure 6. Illustration of the simplified stress pathway demonstrates the relationship between increased allostatic load and stress severity and its impact on individual performance capacity. The figure also presents primary, secondary, and tertiary stress responses, including their respective methods of analysis and sampling. Redrawn and modified from Iversen (2013), Seibel et al. (2021), Martorell-Ribera et al. (2022), and inspired by (Schreck and Tort, 2016).

Primary stress response

Brain-sympathetic-chromaffin cell axis

The first system to be initiated to a stressor through the primary stress response is the adrenergic system which is initiated in a fraction of a second to provide instant mobilization of energy to the "freeze-flight-or-fight" mechanism, which is signalled by the brain-sympathetic-chromaffin cell (BSC) axis (Gorissen and Flik, 2016; Schreck and Tort, 2016). This activation occurs when an organism perceives stress through sensory inputs such as sight, smell, hearing, and touch, which in turn prompt the nucleus preopticus (NPO) within the hypothalamus to stimulate the sympathetic nerves via the brain stem and spinal cord (Madaro et al., 2020). This response is principally responsible for the release of catecholamines into the blood, specifically noradrenaline, adrenaline, and their precursor dopamine, which are stored in chromaffin cells within the head kidney (Reid et al., 1998; Perry and Bernier, 1999; Gorissen and Flik, 2016). In teleosts, adrenaline is the main catecholamine. It plays a vital role in regulating cardiovascular and respiratory functions to ensure adequate oxygen levels in the blood through β-adrenoreceptors. It also mobilizes energy reserves to meet the increased energy demands during stress (Reid et al., 1998; Madaro et al., 2020). As the BSC axis depends on how an individual reacts to a sudden event, coping styles play a significant role. For example, shy and reactive individuals typically exhibit higher stress hormone levels and less active sympathetic systems, showing cautious “freeze-and-hide” behaviours, whereas bold individuals tend to have lower stress hormone levels and more active sympathetic systems, displaying aggressive and risk-taking behaviours; these traits are consistent across fish and mammals (Johansen et al., 2020).

Hypothalamic-pituitary-interrenal axis

Subsequently, minutes to hours after the release of catecholamines, glucocorticoids are released through the activation of the hypothalamic-pituitary-interrenal (HPI) axis, with cortisol being the primary factor (Wendelaar Bonga, 1997; Schreck and Tort, 2016).

In teleost fish, the hypothalamus plays a central role in the stress response by sending signals through two distinct neuroendocrine pathways: the corticotropin-releasing hormone (CRH) pathway to pars distalis, and the pathway carrying arginine vasopressin (AVP), isotocin (IST), and CRH to pars nervosa and melanophore-stimulating hormone (MSH) cells (Gorissen and Flik, 2016). The MSH pathway controls MSH cells in the brain and regulates other bodily functions by releasing hormones directly into the bloodstream from the pars intermedia (Gorissen and Flik, 2016). Focusing on the first pathway, CRH is released from preoptic neurons, which stimulates the pituitary gland through the activation of the CRH receptor to produce adrenocorticotropic hormone (ACTH) via the secretion of the prohormone pro-opiomelanocortin (POMC), a precursor to peptides from three groups: ACTH, endorphin-like, and MSH-like peptides (Iversen, 2013; Gorissen and Flik, 2016). ACTH subsequently stimulates the production of cortisol through the melanocortin 2 receptor (MC2R), which is found in the interrenal cells within the head kidney (Wendelaar Bonga, 1997; Madaro et al., 2020).

Cortisol synthesis in interrenal cells involves a process termed steroidogenesis, in which cholesterol is converted to pregnenolone, then to 11-deoxycortisol, and ultimately to cortisol via specific enzymes, including P450 side-chain cleavage and 11β-hydroxylase (Mommsen et al., 1999; Diotel et al., 2018). The conversion of active cortisol into inactive cortisone is facilitated by the enzyme 11β-hydroxysteroid dehydrogenase 2 (11βHSD2), which prevents the binding to glucocorticoid receptors and inhibits further stress response (Mommsen et al., 1999; Madaro et al., 2020). In addition to the brain, fish uniquely possess a caudal neurosecretory system (CNSS) at the end of the spinal cord, which also releases CRH, contributing significantly to the regulation of the pituitary gland and stress response. Furthermore, the chromaffin cells in the head kidney are also implicated in the production of CRH (Wendelaar Bonga, 1997; Bernier et al., 2008; Pankhurst, 2011; Gorissen and Flik, 2016; Schreck and Tort, 2016).

Mineralocorticoid (MR) and glucocorticoid (GR) receptors are intracellular proteins to which cortisol binds, initiating a cascade of genetic responses to stress. While MR receptors play more elusive roles in teleosts than GR receptors, they have been associated with activity in the HPI axis, osmoregulatory functions, and behavioural changes in response to stress (Faught et al., 2016; Faught and Vijayan, 2018). GR receptors, as the primary receptors for glucocorticoids, are involved in modulating functions such as metabolism, growth, immune responses, stress management, and osmoregulatory functions by influencing gene activity (Vijayan et al., 2010; Faught et al., 2016). Both receptor types have been implicated in the negative feedback of cortisol (Best et al., 2023)

Negative feedback on HPI-axis

During a stress event, the Hypothalamus-Pituitary-Interrenal (HPI) axis, which includes the hypothalamus, pituitary, and interrenal tissue, is activated (Wendelaar Bonga, 1997). This activation leads to the synthesis and release of stress hormones, cortisol being the primary one. Once the stressor is no longer present, the fish must reduce the production of these hormones through negative feedback, which operates at all levels of the HPI axis (Bernier et al., 2009). This is essential to prevent energy waste and chronic stress as these adversely affect the organism's physiological well-being. Cortisol modulates the stress response through feedback effects on different signalling factors and at different levels of the HPI axis. For instance, cortisol has been found to inhibit the expression of CRH in certain fish species, such as goldfish (Bernier et al., 1999). This inhibition of CRH expression leads to a decrease in adrenocorticotropic hormone (ACTH), which regulates cortisol production. ACTH can also be inhibited by corticotropin-releasing hormone-binding protein (CRH-BP), which binds to CRH and decides its bioactivity (Faught et al., 2016; Gorissen and Flik, 2016; Schreck and Tort, 2016; Madaro et al., 2020).

As previously described, the termination process of cortisol involves an enzyme called 11βHSD2, which under chronic stress, might lose its ability to effectively regulate cortisol levels (Best et al., 2023). Concurrently, adaptations in the glucocorticoid (GR) and mineralocorticoid (MR) receptors may occur, potentially altering the baseline for cortisol regulation, indicative of a recalibrated 'normal' state (Best et al., 2023). These processes can alter the negative feedback and lead to chronic stress.

The secondary and tertiary stress response

Secondary stress responses encompass a spectrum of physiological adjustments. These responses are triggered and controlled by primary stress responses and become evident through changes in energy levels, fluid and mineral balance, immune function, cellular activity, and haematological aspects as the organism combats stress (Wendelaar Bonga, 1997; Barton, 2002; Iversen and Eliassen, 2014; Schreck and Tort, 2016; Sopinka et al., 2016; Urbinati et al., 2020). When the primary and secondary stress responses efficiently mitigate the stressor, maintaining the allostatic balance, the organism can revert to homeostasis.

Nonetheless, an allostatic overload may occur if the stressor is excessively severe or prolonged and the allostatic load surpasses the organism's ability to adapt. In such cases, tertiary (whole-organism) stress responses are activated. These responses are primarily maladaptive when observed at the whole-organism level. While primary responses, mediated by hormones, generally enable the organism to allocate energy effectively in response to stress, the activation of tertiary responses often indicates a detrimental impact on the organism. For example, health and resistance to diseases (immune function), reproductive capabilities, survival rate, growth, learning, behaviours like evading predators, swimming performance, and cardiac activity are all negatively affected (Barton, 2002; Ellis et al., 2012; Schreck and Tort, 2016; Sopinka et al., 2016).

1.2.5 Stress habituation

Habituation, a form of non-associative learning, occurs when an animal gradually reduces its response to a repetitive, non-threatening stimulus (Grissom and Bhatnagar, 2009). The habituation of the HPI axis activity to repeated stimuli can be characterized by four main themes according to Grissom and Bhatnagar (2009): its occurrence in response to repeated stimuli, its reversibility, its enhancement by certain parameter modifications, and its unpredictable progression. The habituation mechanism in terms of stress serves as a protective measure, preventing continuous activation of stress responses from various inputs, thereby conserving energy, and facilitating effective risk management. While habituation represents the diminishing response over time to a recurring stressor, it is not a simple, linear process. Long-term stress studies have shown that habituation can sometimes "fail", resulting in an uncontrolled stress response (Iversen and Eliassen, 2014; Hou et al., 2019; Virtanen et al., 2023).

Variability in stress responses due to habituation poses challenges for scientific research. Factors such as the experimental animal's origin and initial state, study designs, stress protocols, and sampling methods can significantly influence stress responses. Cyr and Romero (2009) suggest that habituation to repeated stressors may result in diverse outcomes, reflecting seasonal variability, alterations in stress perception, decreased physiological response without changes in perceived stress, or exhaustion due to chronic stress. Furthermore, the choice and severity of stressors and the timing of stress application and response measurement are crucial factors affecting data interpretation (Sopinka et al., 2016). The interpretation of habituation in a study ultimately leads to how we describe chronic and acute stress. If chronic stress is considered in the context of increasing allostatic load without habituation to stress, it can be definitively stated that it is taking place. However, if the experiment lasts only two weeks and the accumulation of allostatic load is gradual without exceeding a critical threshold, it could be assumed that habituation to stress occurred. This is where the confusion arises. Habituation is contingent on the stressor's strength and the measurement timeframe. It can manifest as reduced glucocorticoid production over repeated stress exposure.

Still, there may be a point where repeated stress increases production after an extended period of habituation, especially if the stressor is potent. Therefore, it is crucial to elucidate the distinction between chronic and acute habituation to stress when stating that "habituation" has occurred. Furthermore, it is important to consider whether habituation is an ongoing or transient event that may change over time. Conde-Sieira et al. (2018b) showed that repeated acute stress does not cause habituation in Senegalese sole, while Madaro et al. (2016) showed that habituation can occur in Atlantic salmon. Interestingly, in another study, Madaro et al. (2015) showed that unpredictable stress does not lead to habituation. Care must be exercised when designing studies to identify habituation, as accurately timing habituation can be challenging concerning the strength of stressors and the duration necessary to conclude that habituation has occurred confidently. To add to the confusion, habituation may have occurred at one point in a stress study but failed throughout the whole study.

1.2.6 Reliability of stress as an indicator of welfare

A more pressing concern in the design of experiments is the consideration of the reliability of stress indicators. As this is due to the intrinsic link between stress responses and fish welfare, which influences policymaking and farming decisions related to the species. As such, bearing in mind the future conditions and well-being of the entire population of an aquaculture species, it becomes a moral obligation to understand and employ reliable measurement indicators. To recap, the most commonly used indicators for stress responses include primary stress responses (e.g., cortisol), secondary stress responses (e.g., glucose, lactate, osmolality, and ion concentrations), and tertiary stress responses (e.g., fin erosion, morphological changes, growth, performance, behaviour, survival, reproduction, disease resistance, and condition) (Sopinka et al., 2016; Noble et al., 2018). While general concepts of reliability have been introduced in previous sections, this section highlights a few specific cases to underscore the importance of considering the reliability of chosen indicators within a study, as illustrated in Figure 7.

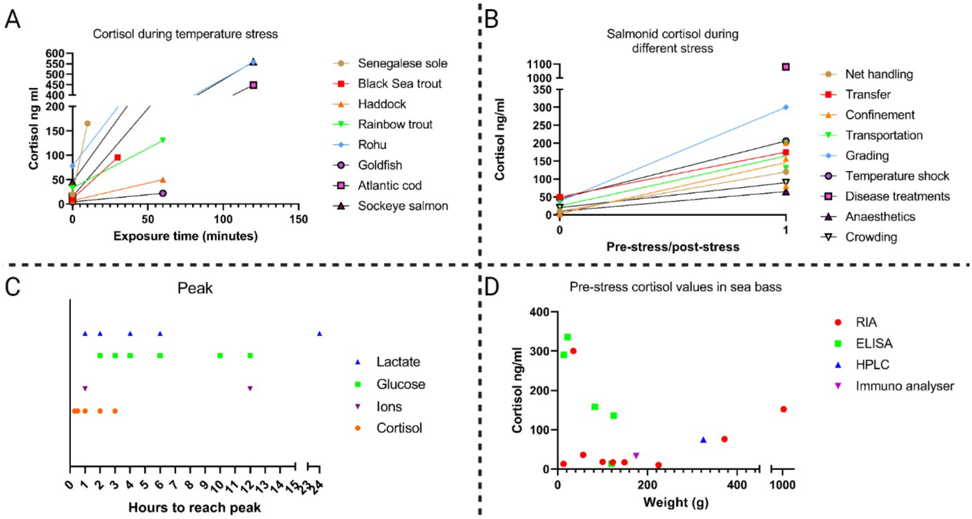


Figure 7. Cortisol response and physiological changes under stress across species, time, and condition. (A) Selection of data extracted from Table 1 in Alfonso et al. (2021) depicting the increase in cortisol levels from pre-stress to post-stress conditions under similar stressors (temperature) in different species. (B) The increase in cortisol levels from pre-stress to post-stress situations across various Salmonid species subjected to different stressors, with various analysis methods. The data was obtained from multiple sources, including (Flos et al., 1988; Schreck et al., 1989; Sandodden et al., 2001; Tort et al., 2002; Weber et al., 2002; Ellis et al., 2004; Steinhausen et al., 2008; Basrur et al., 2010; Pottinger, 2010; Vera and Migaud, 2016). (C) Shown are peak lactate, glucose, ions, and cortisol levels in various species under different stressors. The data was collated from the same sources as previously mentioned, along with additional studies from (Pottinger, 1998; Barton, 2000; Bracewell et al., 2004; Barton et al., 2005; Jentoft et al., 2005; Biswas et al., 2006). (D) The pre-stress cortisol values in seabass are displayed after analysis using various methods. The data was obtained from Table 5 in Ellis et al. (2012).

In Part A of Figure 7, the response of cortisol to varying temperature stress across different species is shown. Variability between species is attributed to individual tolerance levels and the severity of temperature changes relative to this tolerance, or in other words, the intensity of the temperature deviation from the species' normal range. For instance, considering the two lowest cortisol responses in the figure, observed in haddock and goldfish, the experimental protocol for both species involved a 1-hour treatment. However, the temperature for haddock suddenly changed from 10 to 15°C (Afonso et al., 2008), whereas the temperature for goldfish gradually changed from 19 to 31°C (Cockrem et al., 2019). As a result, haddock exhibited a greater stress response to a smaller temperature change, which can be attributed to the sudden change and species-specific tolerance limits. When examining one of the highest responding species, sockeye salmon, which was subjected to a gradual temperature change from 15 to 24°C at a rate of 2°C per hour while also being fatigued (Steinhausen et al., 2008), it becomes evident that multiple factors influence the response of species to a single stressor applied in different ways. There are even instances where certain temperature changes have shown no elevation in stress levels in fish (Alfonso et al., 2021). This highlights the challenges associated with comparing stress effects across different species and experiments and underscores the need for considering species-specific stressors.

The response of a species to stress varies not only between species but also according to the nature of the stressor and the species-specific tolerance towards each stressor. Figure 7, Part B, illustrates how reactions among similar species (salmonids) differ based on the type of stressor applied. While the figure depicts varying cortisol release responses to different stressors, it's important to note that it does not display the duration or the time of measurement, two critical components in stress research. Generally, acute stress studies tend to exhibit rapid elevations in cortisol levels, whereas chronic stress studies may show habituation followed by eventual breakdown.

However, Cockrem et al. (2019) demonstrated that specific stressors can elicit more pronounced responses in the same species, measured at the same time intervals. In their study involving goldfish, three stress protocols were used to examine the stress response through cortisol measurement: air exposure, chasing, and temperature change. All three stressors elicited a spike in cortisol levels at the 15-minute mark, but the response to air exposure and chasing was five times as pronounced as to temperature change. Interestingly, the temperature protocol yielded the highest cortisol levels at four hours. Consequently, the choice of stressor significantly impacts stress responses, as does the timing of its administration and measurement. In the study by Koakoski et al. (2012), the stress response of yellow catfish (*Rhamdia quelen*) was examined, and peak cortisol levels in fingerlings occurred 5 minutes after the onset of stress. In contrast, juveniles experienced peak levels 5 minutes post-stress, albeit at half the intensity. In contrast, peak cortisol levels were observed 60 minutes post-stress for adult fish.

To summarize, factors that affect the measurement outcome of stress responses include species, type of stressor, the timing of stressor administration, intensity of the stressor, and the initial health of the fish before stress. These factors compound the complexity of identifying peak response times. Moreover, what further complicates this is the addition of multiple stress responses, as illustrated in Part C of Figure 7. As best practices dictate the selection of multiple responses to stress, it is imperative to understand the different peak intervals and choose sampling times that capture these peaks. If sampling times do not coincide with these peak periods, the impact of stress could be understated or potentially obscured when statistical analyses are applied.

In Part D of Figure 7, various methods used to measure cortisol are depicted for the pre-stress values of seabass (Ellis et al., 2012). Cortisol levels in fish, which play an essential role in maintaining homeostasis and metabolic functions, can exhibit daily or seasonal variations and, as demonstrated above, vary significantly between species and within a species. The differences observed in Part D may be attributed to individual conditions, genetic factors, environmental conditions, unrecognized stressors, or the analytical method employed (Ellis et al., 2012). However, regarding analytical differences, it is advisable to standardize the adoption of species-specific assays where possible. Although hormonal kits designed for human use are acceptable for fish species, preference should be given to fish-specific kits when available.

Given the above information, reliability depends on our comprehension and control of the situation. Consequently, the reliability of stress as an indicator depends entirely on the study design and the research question being posed. For instance, if we compare a chasing stressor to a confined space stressor, we might expect a significantly higher increase in lactate levels in the former due to activity. However, concluding that the fish in the confined space was less stressed would be incorrect based on this. Additionally, the challenges associated with the laboratory environment, where conditions are more tightly controlled, differ from those in the field, where multiple variables must be considered. For example, netting a fish in a tank trial can be executed relatively quickly, whereas, in an aquaculture facility at sea, this involves multiple stages and can elicit a stress response.

Consequently, the choice of samples for indicators depends on the study design; for instance, measuring scale cortisol, accumulating over time, can be more appropriate than blood for sea trials. The reliability of indicators is a topic with boundless scope for discussion. Figure 8 presents foundational considerations for a laboratory study design, which researchers can adapt and build upon depending on their research objectives.

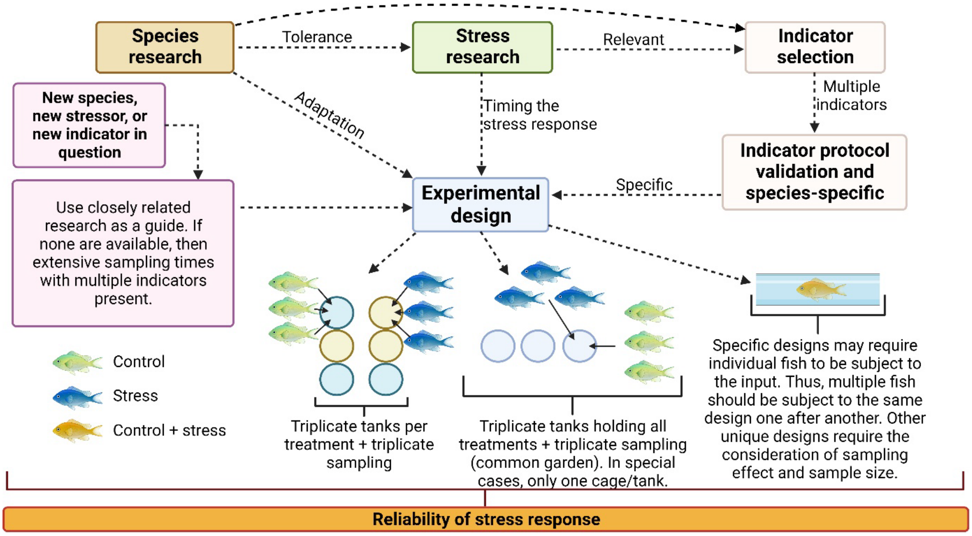


Figure 8. A conceptual diagram highlighting factors to consider when preparing a hypothetical experimental design concerning reliable indicators to assess the stress response.

1.3 Reeling in the future: Merging tech and aquaculture

The journey has been remarkable, from merely attaching a worm to a hook to catch fish to today's advanced systems encompassing spawning, hatcheries, and grow-out facilities. These systems involve a range of advancements including medical innovations, engineering feats, welfare assessments, and innovative feed products, among others. The aquaculture industry's rapid growth and the increasing demand for its products have significantly fuelled the need for advancements in this sector. Norway is a leading provider of novel technology for the aquaculture industry and is consistently working toward enhancing various aspects of its current practices.

Føre et al. (2022) demonstrated that, among the applications for technological advancements submitted to the Norwegian government, the top three sustainability measures were: the prevention of sea lice infection (90% of applications), escape prevention (70% of applications), and promotion of fish welfare (60% of applications). Gladju et al. (2022) highlighted the intelligent solutions currently available for commercial use in the aquaculture industry, encompassing categories such as feed monitoring and control, water quality monitoring and control, growth and biomass monitoring, disease outbreak prediction and evaluation, behaviour tracking, farm site monitoring, and automation of fish processing systems.

Individuals will variably adopt, adapt to, innovate, or dismiss new technologies as we progress. Nonetheless, technological disruptions penetrate our daily lives, whether we actively engage or not. As Kumar et al. (2018) reviewed, adopting technology within the aquaculture industry is not as easy as adopting or not adopting. Recent advancements in artificial intelligence (AI), particularly machine learning (ML) through neural networks, can be harnessed to process massive amounts of data, making the data more accessible and manageable (Holzinger et al., 2023).

We can amass extensive data from fish farms and research using cameras, sensors, smart-tags, and comprehensive biological analyses (Yue and Shen, 2022). This data is subject to interpretation by various individuals with different objectives, which could inadvertently sway the data due to their biases, despite our best efforts to remain objective. Machine learning offers the advantage of making studies accessible to novices and seasoned researchers. It establishes an unbiased, data-driven reference point, provided the initial data used for model training is unbiased. However, some have begun questioning the ethical aspects of AI concerning animal welfare, which might create a hurdle to widespread acceptance (Singer and Tse, 2022). Nevertheless, one should recognize that AI has already demonstrated its utility in multiple scientific domains (Fang et al., 2019; García et al., 2020; Cui and Zhang, 2021; Moingeon et al., 2022; Holzinger et al., 2023).

Regarding fish welfare, employing systems to monitor behavioural and physical changes and transcriptional and physiological modifications can pave the way for a novel model to understand when an individual is experiencing negative, neutral, or positive welfare. This is significant as conventional methods do not account for dynamic shifts in welfare over time and often only offer snapshots of welfare during specific life stages under certain physiological and environmental conditions (Brijs et al., 2022). However, data models are only as effective as the information they are trained on; therefore, understanding the fundamental principles on which to base your models is crucial for developing useful and reliable tools, such as those that examine animal environment interactions for each species separately (Føre et al., 2022).

1.3.1 Technology in animal farming (precision livestock farming)

While many forms of technological advancements exist in both farming and aquaculture, this section focuses on technologies that enable assessments of welfare at an individual level. The desire for individual welfare assessments extends beyond the farm, encompassing research facilities and zoos, where historically, environmental and housing considerations have been of focus. Recently, this focus has been put on animal-based measures assessing captive animals physical and psychological state (Whitham and Miller, 2016). As the global population expands and demand for animal products increases, the need to understand the living conditions of individual animals within farming systems has led to the development and implementation of Precision Livestock Farming (PLF) (Schillings et al., 2021; Aquilani et al., 2022). However, as production intensifies and fewer farmers are tasked with managing larger numbers of animals, existing challenges such as environmental impacts, space utilization, disease control, effluent management, and animal welfare become more pronounced (Werkheiser, 2020). Nonetheless, there is reason to remain optimistic, as optimizing animal welfare through increased production does not necessarily call for reducing the workforce. Instead, it calls for reorganising the workforce to create positive experiences for animals and focus on proactive problem-solving rather than reactive measures currently in place.

The technology behind PLF is designed to assist farmers in livestock management by continuously, automatically, and in real-time tracking and regulating animal productivity, assessing environmental impacts, and providing health and welfare indicators (Berckmans, 2014). There is, however, a pitfall in how the user approaches PLF. On the one hand, there is the risk of undermining the human-animal relationship through the industrialization of farming, which could have detrimental effects as farmers may lose their connection with the animals and begin to view them solely as commodities (Bos et al., 2018; Werkheiser, 2018). On the other hand, PLF can be used to strengthen the human-animal relationship by better understanding what promotes positive outcomes and enabling swift responses to negative welfare incidents (Norton et al., 2019). One specific challenge in aquaculture is that fish farmers might not have as strong a connection with their animals as terrestrial livestock farmers do, partly because fish's expressions of stress or discomfort are less obvious and more difficult to observe underwater. This is where PLF can play a pivotal role in bridging the gap between fish and farmers by providing real-time tools that offer insights into the well-being of fish (Antonucci and Costa, 2020).

Several authors have reviewed use cases for PLF. For example, Tzanidakis et al. (2023), Zhang et al. (2021), and King (2017) provide a good introduction to various use cases. Some noteworthy examples of PLF applications in agriculture include recording chicken vocalizations to detect respiratory diseases by analysing sneezing behaviour (Carpentier et al., 2019) and monitoring distress calls in commercial chicken flocks

(Mao et al., 2022). Cameras have been deployed in innovative ways to support PLF. For example, analysing the leg swing of dairy cattle through video footage has been used to predict lameness (Zhao et al., 2018), and thermal cameras have proven effective in detecting temperature changes associated with mastitis. This disease affects the udders of dairy cattle (Xudong et al., 2020).

In Atlantic salmon farming in Norway, PLF is beginning to gain presence through the advent of systems such as CageEye (https://www.cageeye.com/), which adjusts feeding schedules based on fish behaviour, and AKVAfusion (https://www.akvagroup.com/), which incorporates multiple factors to give farmers better control over various aspects of their operations. Similarly, ScaleAQ (https://scaleaq.com/) offers forward-looking innovations to aquaculture, while iFarm (https://www.biosort.no/) is developing an all-in-one health, lice and growth monitoring system. Aquabyte (https://aquabyte.ai/) have developed a fish welfare monitor and automatic lice counter. These companies represent a few of the vast emerging technological advancements in aquaculture. A common theme currently is the use of cameras to identify individuals meaningfully and gather data, which is subsequently processed by machine learning algorithms to deliver actionable insights to farmers. Furthermore, though more challenging to implement and develop, tagging in teleosts is garnering interest as a precise method for monitoring individual welfare, with its ability to provide a reliable examination of behaviour (Endo and Wu, 2019; Muñoz et al., 2020; Macaulay et al., 2021; Brijs et al., 2022).

1.3.2 Tagging along: Aquaculture's future with tag integration

The principal challenge in tracking individuals and monitoring their welfare in aquaculture comes from the total number of fish, often ranging from 500,000 to 1,000,000, within a single grow-out cage. These submerged cages and exposure to weather, waves, and currents make repeatedly identifying the same individuals difficult (Føre et al., 2022). Acoustic telemetry, using real-time smart-tags, has been investigated as a promising solution for assessing individual welfare (Macaulay et al., 2021), and its implementation within an aquaculture grow-out setting is illustrated in Figure 9. Smart-tags can be equipped with various features, such as activity monitors (Hjelmstedt et al., 2020; Hvas et al., 2020; Muñoz et al., 2020; Kolarevic et al., 2021; Svendsen et al., 2021; Li et al., 2022), pressure and temperature sensors (Yang et al., 2022), and positional data accompanied by behaviour monitoring (Leclercq et al., 2018; Ulvund et al., 2021). Utilizing these features, comprehensive fish welfare assessments can be developed. Moreover, when smart-tags are combined with environmental sensors, underwater cameras, and machine learning models, large amounts of real-time data can be transformed into visualizations that effectively represent fish welfare status (Føre et al., 2022).

However, implanting smart tags in every fish is impractical. Therefore, the concept of "sentinel fish" has been introduced as a representative approach, wherein data from a statistically significant portion of individuals can be used to reflect the conditions of the larger population (Føre et al., 2017; Føre et al., 2018; Warren-Myers et al., 2021). For this approach to be effective, the tagging process and the presence of the tag should not adversely affect the individual fish, as the behaviour and condition of the tagged fish must genuinely represent that of the untagged population (Macaulay et al., 2021). If this aspect is not thoroughly investigated, there is a risk of adopting false or damaging management practices based on altered rather than natural behaviour. Regarding animal welfare, it is ethically unjustifiable to knowingly subject animals to a potential negative welfare infliction to study and say they are improving the total welfare of a farm. In this context, understanding the tagging and implantation process and its potential effects on the fish is equally as crucial as developing the physical tag. This understanding must not be compromised or overlooked in the race to produce advanced technological solutions.

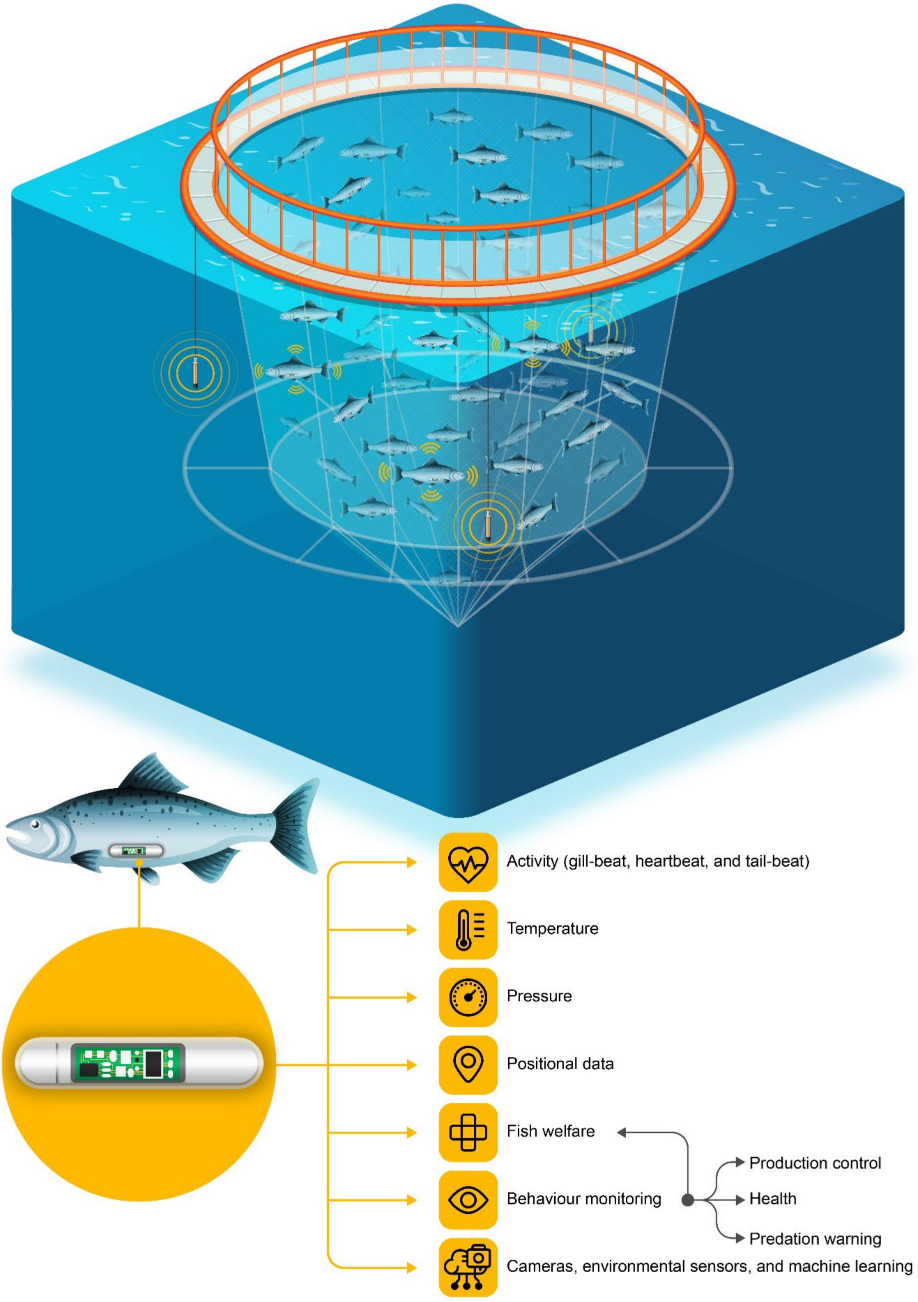


Figure 9. Illustration of real-time transmitter smart-tag usage in an Atlantic salmon grow-out facility. Data is transmitted via hydroacoustic communication from individually tagged fish, providing farmers with valuable insights such as health status, swimming patterns, and environmental conditions. © Miiro Virtanen

As tags are being developed, the initial size of these tags may be larger than the desired final size due to the costs associated with miniaturization. It can be assumed that if larger tags, which result in bigger wounds, do not adversely affect the fish, smaller tags creating smaller wounds would be even less invasive. Additionally, gaining insights into wound healing in the context of aquaculture practices is essential for optimizing tagging processes and establishing guidelines for the minimum fish size and maximum tag size. Currently, a "2% rule" is generally accepted, wherein the mass of the tag should not exceed 2% of the total fish mass, though this guideline is highly variable and some have even proposed up to 10% as being acceptable in salmonids (Cooke et al., 2011b; Thiem et al., 2011; Brownscombe et al., 2019; Wright et al., 2019; Vollset et al., 2020).

As tagging studies gain traction, understanding the challenges associated with tagging is vital. Current challenges include optimizing tag size, extending battery life, ensuring that the tag and implantation process do not cause harm or behavioural changes in the fish, achieving high tag retention, minimizing mortality rates associated with tagging, adhering to the three R’s (replacement, reduction, and refinement) in animal testing, developing standardized protocols for on-farm tagging, and establishing best surgical practices. Additionally, these considerations must be tailored to be species-specific and account for different size ranges (Brownscombe et al., 2019; Macaulay et al., 2021). Regarding the 3Rs, intelligent camera systems currently in use to assess features that tags also measure present an alternative, less invasive method. However, camera systems typically measure aggregated data and are highly dependent on fish positioning relative to the cameras, which often results in individual behaviour being masked by group averages. Simultaneously utilizing camera systems and tagging, a group's collective movement patters can be captured alongside individual behavioural data, providing a more robust and comprehensive overview of the activities within a cage (Ulvund et al., 2021).

It should also be noted that while tagging studies can be conducted in controlled laboratory settings, validating these results in real-world field conditions is necessary before proceeding with large-scale implementation. This is particularly evident in wound healing; while laboratory settings can maintain controlled stress levels and cleanliness, grow-out facilities are exposed to multiple stressors and pathogens. Therefore, ensuring that wounds heal properly to maintain acceptable welfare standards under varying conditions is fundamental to the successful adoption of telemetry (Andersen et al., 2023).

1.4 The immune system in fish

A diverse array of microorganisms and pathogens constantly surrounds fish in aquatic environments. Their immune system acts as a defensive barrier, establishing a critical boundary between the organism's internal setting and the external world to shield against infections and potential threats. The immune system is categorized into two systems, the innate and adaptive. These systems collaborate and complement each other to sustain the fish's overall health and well-being (Rivera et al., 2016).

Lymphoid tissue, an essential immune system component, comprises reticular cells and leukocytes called lymphoid organs when arranged into a functional unit (Bjørgen and Koppang, 2022). These organs are further divided as primary lymphoid organs, where lymphocytes (T and B cells) mature from precursor cells. In contrast, secondary lymphoid organs are sites of lymphocyte proliferation which produce immune responses by antigen encountering. Unlike mammals, fish do not possess lymph nodes or bone marrow. In fish, the primary lymphoid organs include the thymus and head kidney, whereas secondary lymphoid organs encompass mucosa-associated lymphoid tissue (MALT), salmonid bursa, spleen, and kidney (Flajnik, 2018; Bjørgen and Koppang, 2022; Makesh et al., 2022; Zapata, 2022). The MALT in fish includes skin-associated lymphoid tissues (SALT), gill-associated lymphoid tissues (GIALT), gut-associated lymphoid tissues (GALT), nasopharynx, as well as buccal and pharyngeal-associated lymphoid tissues (Reverter et al., 2018). This thesis primarily focuses on the skin, directly impacted by smart-tag implantation, and the head kidney, which serves a dual role as an endocrine and immune organ (Tort and Balasch, 2022).

1.4.1 Innate immunity

The innate immune system is the body's fist defence against infections in previously infected and naive fish (Dalmo and Bøgwald, 2022). It recognises pathogen-associated molecular patterns (PAMPs) through germline-encoded pattern recognition receptors (PRRs) in immune cells. Upon recognising PAMPs, the innate immune system activates myeloid and lymphoid cells and stimulates the release of molecules such as cytokines and complement factors. This leads to initiating inflammatory responses and chromatin modifications, often co-stimulating the adaptive immune response (Abós et al., 2022; Dalmo and Bøgwald, 2022). The innate immune response involves various cells, including macrophages, dendritic cells, granulocytes (neutrophils, eosinophils, and basophils), red blood cells, thrombocytes, and possible B and T cell subtypes. A comprehensive review of the functional roles of these cells in the innate immune response can be found in Dalmo and Bøgwald (2022).

1.4.2 Adaptive immunity

When the innate immune system cannot eliminate a pathogen or in cases where the pathogen recurs, the adaptive immune system is signalled to assist. However, one should note that immunological memory is not exclusive to the adaptive immune system, as it can also be observed in the innate immune system through a phenomenon known as trained immunity (Abós et al., 2022). Yet, the immunological memory of the adaptive immune system forms the basis for vaccine development and is of significant interest in aquaculture, given the prevalence of various diseases (Adams, 2019).

Adaptive immunity comprises both humoral components (involving B cells) and cellular components (involving T cells). These cells enable the formation of immunological memory and the identification of specific antigens through antigen receptors on B cells (membrane-bound immunoglobulins: B-cell receptors or BCRs) and T cells (T-cell receptors or TCRs), which are generated by recombination-activating genes (RAG1 and RAG2) (Smith et al., 2019; Abós et al., 2022).

B cells play a central role in mounting defence against extracellular pathogens. When B cells encounter antigens that bind to their BCRs, they become activated and, with the help of helper T cells, differentiate into plasma cells and memory B cells. Plasma cells act as antibody factories, producing antibodies that circulate in the blood and lymphatic vessels to mark pathogens for destruction. Memory B cells remain in the body long-term and can rapidly produce antibodies upon re-encountering the same antigen (Firdaus-Nawi and Zamri-Saad, 2016; Abós et al., 2022; Cao et al., 2023).

T cells target intracellular pathogens and play a central role in orchestrating the immune response. The TCRs on T cells recognize antigens presented by major histocompatibility complex (MHC) molecules on cells. Helper T cells (Th cells) produce cytokines that stimulate other immune cells, including B cells. Cytotoxic T cells (Tc cells) directly destroy infected cells by recognizing antigens presented on MHC class I molecules and inducing apoptosis in these cells (Nakanishi et al., 2015; Abós et al., 2022; Cao et al., 2023).

B and T cells produce memory cells, ensuring a rapid and effective response upon re-exposure to a pathogen. The coordinated efforts of B and T cells, and the regulation of their cytokine responses, are essential for the adaptive immune system's ability to protect the organism against infections and diseases (Secombes, 2022; Cao et al., 2023).

1.4.3 Head kidney

Unlike mammals, which possess bone marrow that provides hematopoietic stem cells, teleost fish rely on the head kidney as their major hematopoietic organ (Makesh et al., 2022). The head kidney contains interrenal cells that produce cortisol and chromaffin cells that generate catecholamines. These cells within the head kidney are surrounded by hematopoietic tissue responsible for producing antibodies, cytokines, and leukocytes among others (Geven and Klaren, 2017). This illustrates that the head kidney has multiple functions, including hematopoiesis, immunity, and endocrine signalling, with varying degrees of paracrine interactions. During the stress response, not only are cortisol and catecholamines activated, but corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), alpha-melanocyte-stimulating hormone (α-MSH), as well as modulators of stress, inflammation, and immunity such as cytokines are also involved (Tort and Balasch, 2022; Madaro et al., 2023). This interplay of cells and regulatory substances within the head kidney facilitates a local neuroimmunoendocrine regulatory feedback system (Tort and Balasch, 2022).

1.4.4 Skin

Fish skin, a notable organ involved in immunological activity, plays a critical role as the primary shield against infections and parasites, featuring many functions and cellular components (Esteban and Cerezuela, 2015). The skin of teleost fish possesses a unique ability to secrete mucus, which is vital for their immune system and facilitates adaptation to their watery surroundings (Schmidt, 2013; Salinas et al., 2022). The skin functions not only as a mechanical barrier but is also essential for numerous processes ranging from immune defence, communication, sensory perception, locomotion, respiration, ion regulation, and thermal regulation (Esteban and Cerezuela, 2015; Akat et al., 2022). The main cellular immune components include natural killer cells, macrophages, neutrophils, blood cells (eosinophils, basophils, thrombocytes, and monocytes), dendritic cells, lymphocytes, mast cells, eosinophilic granule cells, rodlet cells, and mucus mainly consisting of mucins (Mokhtar et al., 2023). The epidermis comprises living cells with ongoing cell division, forming a stratified epithelium of varying thickness beneath a cuticle or mucus layer (Akat et al., 2022). These live cells in fish are instrumental in maintaining homeostasis and offering a barrier against the infiltration of pathogens. Besides cellular components, the immune function and adaptability of the skin also rely on several proteins, among which immunoglobulins (antibodies) and cytokines are key players. Notably, cytokines have diverse functions, with a particular emphasis on cell signalling (initiate and organize immune responses) (Turner et al., 2014; Mendoza and Magadán, 2022; Secombes, 2022).

1.5 Skin structure and wound healing

1.5.1 Structure of skin

Several studies have outlined the structure of the fish's skin, particularly focusing on Atlantic salmon (Elliott, 2011; Sveen et al., 2020; Akat et al., 2022). Figure 10 highlights the Atlantic salmon skin's structure and functional roles for the mucus matrix. The skin is composed of two layers: the epidermis, which is the outermost layer consisting of a multilayered epithelium, and the dermis, which is fibrous (Elliott, 2011).

Epidermis

The epidermis consists of keratocytes, also known as epithelial cells, present in three distinct structural forms. The surface cells containing microridges are pentagonal, the inner cells are round, and the basal layer takes on a cube-shape (Sveen et al., 2020). Tonofilaments are fibers in keratocytes that strengthen the skin by forming bundles (tonofibrils) and connecting to adjacent cells (Elliott, 2011). Keratocytes are mobile cells with phagocytic ability, which is thought to be facilitated by the cells consuming as many foreign particles as possible before they die and are shed from the epidermis (Dalmo and Bøgwald, 2022). Among the living epidermis, mucus-producing cells are also present; and in Atlantic salmon, goblet cells are the primary type. They produce a constant layer of mucus, the composition of which varies depending on the body part of the individual, as well as environmental and physical factors (Sveen et al., 2020). Mucus mainly consists of proteins called mucins combined with a mixture of various molecules and water. Mucus is associated with immunity, microbial community colonization, protection against pathogens, tissue repair, swimming dynamics, and osmoregulation, among other functions still being discovered (Salinas et al., 2022).

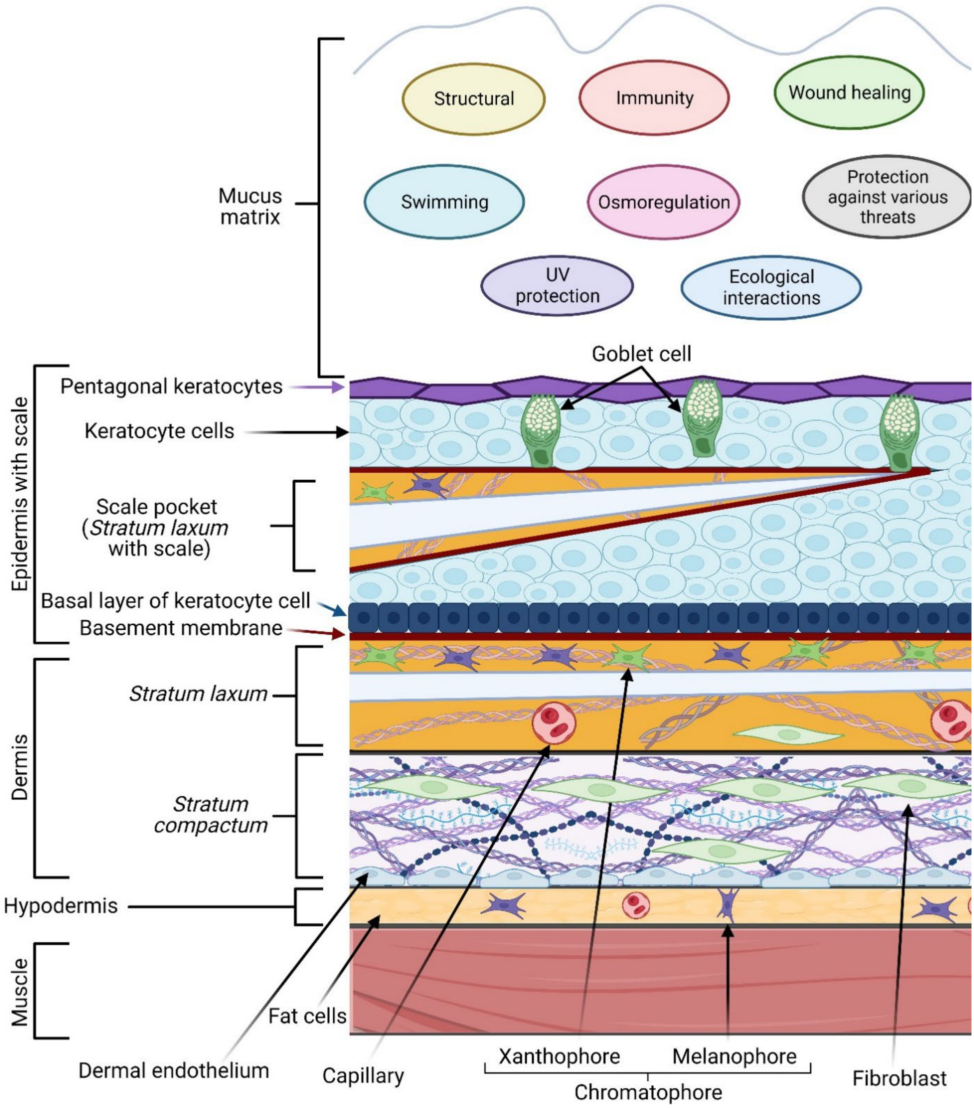


Figure 10. Illustration of the Atlantic salmon skin, showcasing the principal cell types, structural morphology, and the mucus matrix with its functional roles. Immune cells are not shown. Redrawn and modified from Elliott (2011), with permission from ELSEVIER with license no. 5584180668303.

Dermis

The epidermis and dermis are divided by an extensive basement membrane complex, with the epidermis draped over scales embedded within the dermis (Fontenot and Neiffer, 2004; Elliott, 2011). The dermal layer is situated immediately beneath the epidermal layer. It is subdivided into two layers: the outer segment, the stratum laxum, also termed stratum spongiosum, housing nerve cells, chromatophores, blood vessels, and loose connective tissue, and is the primary supportive layer for scales. Conversely, the inner segment, which gives strength to the skin, referred to as the stratum compactum, is characterized by dense connective tissue composed of collagen-rich fibers synthesized by fibroblasts (Fontenot and Neiffer, 2004; Elliott, 2011; Sveen et al., 2020; Akat et al., 2022).

Hypodermis

Located directly beneath the dermal endothelium is the hypodermis. This layer is a separator between the dermis and the underlying skeletal muscles. It contains a layer of connective tissue with a top layer of pigment cells surrounded by blood vessels throughout the layer, with the bulk of the layer being adipose tissue (fat) (Elliott, 2011; Sveen et al., 2020).

1.5.2 Skin injuries during aquaculture

While the skin, mucus, and scales serve as a physical barrier to protect fish from the conditions they are subject to, injuries can still occur. Figure 10 illustrates several factors that can cause wounds, some of which act independently, while others result from a combination of factors. In the case of Atlantic salmon in grow-out sea aquaculture, fish are subjected to handling, for instance, during monthly sea lice count or weight measurements. This handling process involves using large nets followed by smaller nets and, ultimately, the fish's physical handling, which can damage their physical barriers.

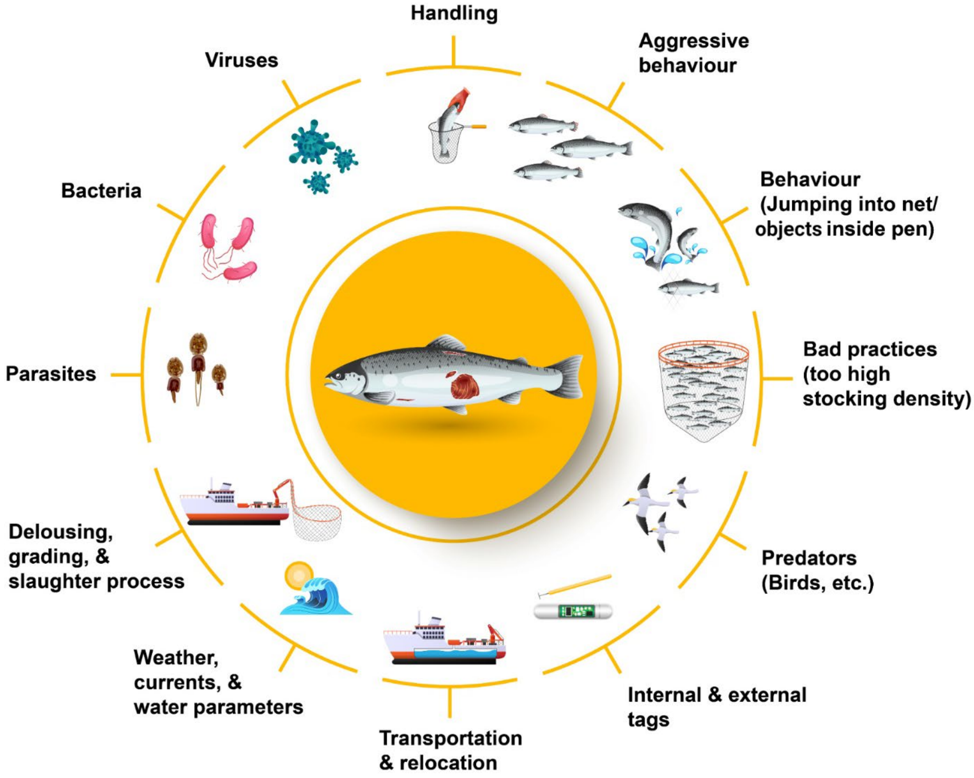


Figure 11. Potential causes of wounding in Atlantic salmon during the grow-out stage in Norwegian aquaculture. The illustration highlights direct sources of injury, such as handling and tagging, and factors that may contribute to wound development over time, such as environmental conditions. Understanding these causes is vital for developing strategies to minimize wounding and improve fish welfare. © Miiro Virtanen

Aggressive behaviour is another factor for wounds, as fin nipping can lead to fin erosion (Latremouille, 2003). Behaviour can also contribute to skin damage. For example, when Atlantic salmon jump out of the water, they may collide with the sides of the net or objects within the cage. Additionally, if the bird net is damaged or lowered, it may result in fish being entangled within the net.

Improper practices, such as high stocking densities, can lead to stress, aggression, poor water quality, and other factors that harm the fish and cause delayed wounding (Sveen et al., 2016; Sveen et al., 2018).

Predators can also cause wounds if they enter the cage or even from the outside, such as when newly stocked lumpfish struggle to swim in strong currents and are pushed to the side of the net where predators may try to attack them. Even if they do not capture the fish, they may still injure them.

Applying internal and external tags such as RFID, PIT, and smart tags directly cause wounds.

Standard industry practices like transportation in well-boats, delousing, and grading, which require various tools and often involve the fish being crowded and coming into contact with surfaces and nets, can also cause injuries (Overton et al., 2019).

Environmental factors such as temperature, waves, currents, and other water parameters can contribute to direct and indirect skin damage (Dempster et al., 2008; Jensen et al., 2015). Parasites like salmon lice directly injure the skin and may cause indirect damage through preventative measures (Bowers et al., 2000).

In Atlantic salmon, three common bacteria strains include those of the genus Tenacibaculum, which cause fin erosion, mouth erosion, and skin lesions (Småge et al., 2018); *Moritella viscosa* (winter ulcer), which causes skin lesions that may become chronic deep ulcers (Lunder et al., 1995); and *Aliivibrio wodanis*, which may interact with *Moritella viscosa* as a secondary pathogen (Karlsen et al., 2014). Additionally, viruses such as the infectious hematopoietic necrosis virus (IHNV) (Chong, 2022) or pancreas disease (Thorarinsson et al., 2021) can cause skin damage directly or indirectly. Thus, it is important to understand the interplay of factors that cause injuries. For example, tagging fish in winter may worsen injuries by causing handling wounds and creating open wounds that are more susceptible to infection by *Moritella viscosa*, which is more prevalent in lower water temperatures as well as having slower wound healing times in colder water (Jensen et al., 2015).

1.5.3 Regeneration and wound healing

Organisms possess two primary strategies for wound healing: *repair* and *regeneration*. Repair often leads to changes in tissue configuration and potential functional impairment, while regeneration restores tissue integrity, functionality, and configuration (Reviewed in (Godwin, 2014)). It should be noted that repair and regeneration can function together and are highly species and tissue specific. Figure 12 illustrates the inverse relationship between the sophistication of an immune system and regenerative capabilities. Although an advanced immune system often correlates with diminished regenerative capacity, this relationship is not absolute and exhibits considerable variability (Adhikary and Hui, 2021). The ability to regenerate varies significantly among species, ranging from full-body axis regeneration in sea stars to complete limb regeneration in amphibians and organ regeneration in certain fish species (Arenas Gomez et al., 2020; Adhikary and Hui, 2021). The skin is the first line of defence, so its injuries must be rapidly repaired to restore barrier functions. However, repair mechanisms can differ among vertebrate species, life stages, recurring injuries, and location of the injury (Murawala et al., 2012; Pfefferli and Jaźwińska, 2015; Richardson, 2018; Vieira et al., 2020).

Upon sustaining an injury, the distinct properties of the epithelium at the wound site determine whether regeneration or repair will occur. In the regeneration of salamander limbs or zebrafish fins, the epidermis seals the wound site without scar formation and transforms into a secretory epithelium known as an apical ectodermal cap, which is instrumental in blastema formation and subsequent regeneration (Murawala et al., 2012; Pfefferli and Jaźwińska, 2015; Seifert and Muneoka, 2018). In contrast, scarring typically characterizes adult human wound healing, with exceptions observed in shallow cuts on the palms of the hand and buccal mucosa within the mouth (Murawala et al., 2012). Interestingly, mammalian early embryonic stages also can regenerate epidermal wounds (Hu et al., 2018).

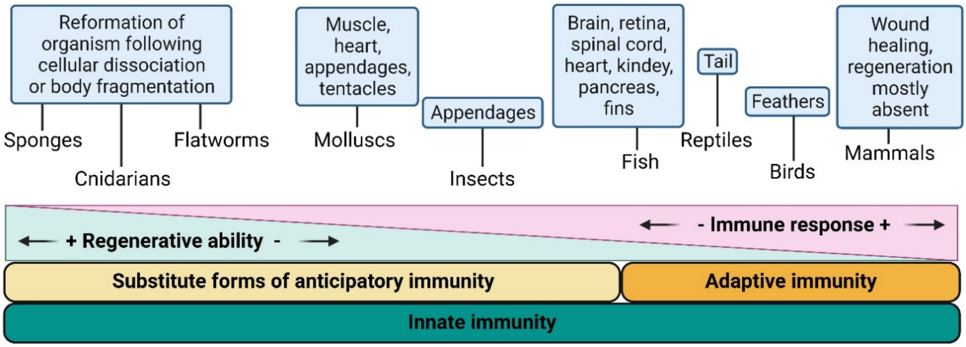


Figure 12. Comparative illustration of the regenerative and healing abilities among organisms and with respect to their immune systems. Redrawn and modified from Adhikary and Hui (2021), with permission from ELSEVIER with license no. 5584181097186

Although it is commonly believed that regeneration in adult mammals is impossible, there is variability in regenerative capacity observed across different species, including adult mammals. For example, the African spiny mouse can regenerate skin, including ear holes, through a mechanism known as skin autotomy. Another example is the regeneration of antlers in deer (Seifert et al., 2012; Li et al., 2014; Maden and Brant, 2019). Notably, even among fish, there are differences; for instance, zebrafish can regenerate heart tissue, while medaka cannot, a phenomenon largely attributed to a dampened immune response and neutrophil presence in medaka (Lai et al., 2017; Arenas Gomez et al., 2020). Moreover, species renowned for their regenerative capabilities, such as newts, exhibit differences within closely related species, with newts possessing more regenerative abilities than axolotls (Joven et al., 2019).

As our understanding of wound healing and regeneration continues to evolve, it is important to recognize the classical model of immunity-regeneration interplay as dynamic rather than static, given the considerable species-specific variations that have yet to be fully understood. Furthermore, the use of model species, such as zebrafish, with regenerative abilities can aid in the discovery of future therapeutics and potentially enable the application of regenerative techniques to mammalian tissues, such as in the treatment of burn victims (Richardson, 2018; Adhikary and Hui, 2021; Arenas Gómez and Echeverri, 2021).

1.5.4 Wound healing

When regeneration is not the primary mechanism, wound healing seals the wound and protects the inner tissues from external elements. This process is crucial in preventing further injury and infections. The wound must be sealed before regenerative processes commence for the reconstruction of the fish skin architecture in a scar-free manner. There have been numerous studies on fish wound healing. To name a few, Sveen et al. (2019) reviewed the wound healing process in Atlantic salmon, Richardson (2018) in zebrafish, Schmidt (2013) in rainbow trout and common carp, Ceballos-Francisco et al. (2021) in gilthead seabream, while Fontenot and Neiffer (2004), and Yun et al. (2021) focused on a variety of species. Common techniques for studying wound healing in fish include punch biopsies, scalpel incisions, and lasers (Sveen et al., 2020). Like the issues discussed in the "1.2.6 Reliability of stress as an indicator of welfare" chapter, several factors need to be considered when examining wound healing in fish, including species-specific healing rates, environmental factors, mucus secretion levels, scales, skin structure, wounding methods (size, depth), and wound location.

Regardless, wound healing generally follows four distinct but overlapping phases: re-epithelialization, inflammation, granulation tissue formation, and tissue remodelling. It is important to note that in mammals, the process varies slightly as inflammation and forming a platelet/fibrin-rich clot occur before re-epithelialization (Richardson, 2018). Figure 13 presents the wound healing process and some principal cells involved. It is essential to understand that the timelines for the appearance of cells in Figure 13 are illustrative and not concrete. Cells such as macrophages play a significant role throughout all phases of wound healing. Still, they are particularly crucial in concluding the inflammation phase to facilitate the transition to the subsequent phase (Kloc et al., 2019).

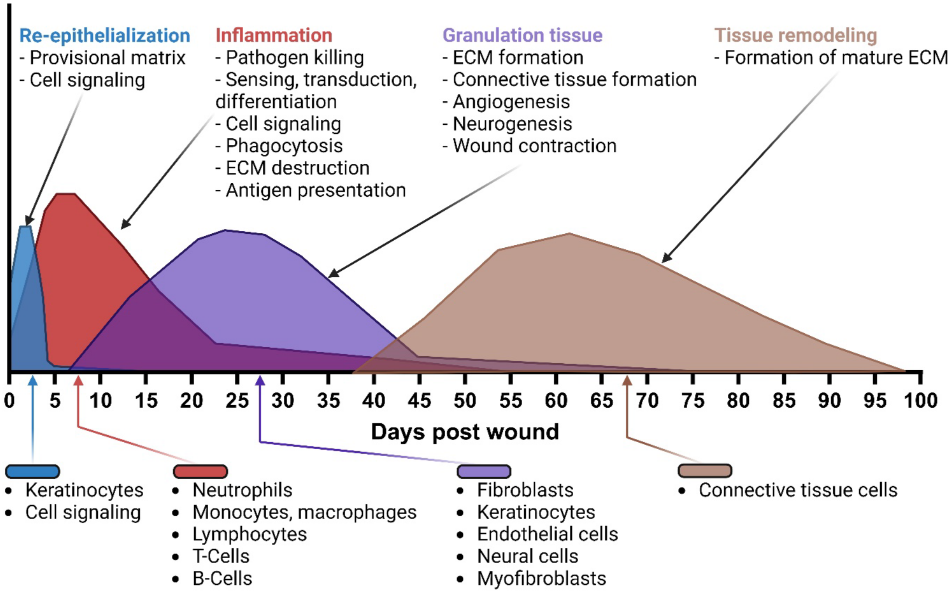


Figure 13. Illustration of the four phases of wound healing, their function, and the main cells involved for Atlantic salmon. Redrawn and modified from Krasnov et al. (2012) and Sveen et al. (2020). (ECM = extracellular matrix)

Re-epithelialization

The rapid onset of re-epithelialization in fish, which establishes a barrier between the internal tissues and external environment following skin injury, is vital due to their aquatic habitat being exposed to various pathogens and osmotic gradients (Schmidt, 2013). Notably, fish do not form blood clots in their skin when injured despite bleeding and the presence of genes involved in the clotting process, as observed in the early stages of wound healing (Sveen et al., 2019). Instead of clotting, a substance fills the wound, presumably serving as a base layer upon which the recruited keratinocytes migrate to form an initial barrier (Richardson et al., 2016; Sveen et al., 2019). Following keratinocytes proliferation and thickening of the epidermis, a mucus line formed by mucus cells develops across the wound. This line of mucus, which is denser and more adherent, is hypothesized to support the structural defence of the newly formed epidermis (Sveen et al., 2019).

Inflammation

A well-regulated inflammatory response is crucial for effective wound healing, as excessive inflammation can lead to chronic wounds, while controlled inflammation is essential for tissue repair and regeneration (Richardson et al., 2013). The inflammatory phase has three sequential stages (Cain and Cidlowski, 2017). Firstly, the alarm phase, during which inflammatory mediators are released to signal the injury, followed by the mobilization phase, where leukocytes, especially neutrophils, are recruited to the wound site for defence and degradation. Neutrophils have specialized granules containing antimicrobial agents pivotal in infection control and phagocytosis (Rodrigues et al., 2019). The final stage is the resolution phase, during which macrophages, derived from monocytes, clear away cellular debris, including dying neutrophils, paving the way for healing (Cain and Cidlowski, 2017; Rodrigues et al., 2019; Campos-Sánchez and Esteban, 2021). The transition from inflammation to proliferation is a critical phase in the wound healing cascade, as failure to resolve inflammation properly can result in chronic wounds (Zhao et al., 2016; Campos-Sánchez and Esteban, 2021). For an in-depth review of the inflammatory response in fish, refer to Campos-Sánchez and Esteban (2021).

Granulation and Tissue remodelling

Granulation or repair tissue comprises connective tissue, fibroblasts, myofibroblasts, immune cells, and capillaries (Richardson et al., 2013; Sveen et al., 2020). In Atlantic salmon, granulation tissue becomes visible 14 days after the wound is inflicted and continues to develop until 36 days post-injury. In contrast, this tissue forms much faster in zebrafish, between 2 to 4 days after the wound occurs, underscoring the variation in healing timelines between species (Richardson et al., 2016; Sveen et al., 2019). During this phase and the subsequent remodelling phase, damage signals at tissue injury sites initiate new blood vessel formation through growth factors like vascular endothelial growth factor-A (VEGF-A), essential for healing. Recent studies have emphasized the significant role of pro-inflammatory macrophages in angiogenesis, as they deliver VEGF-A and regulate other cellular components (Gurevich et al., 2018; Richardson, 2018). Restoring neuronal networks, particularly cholinergic neurons, is critical for heart and skin healing and post-injury regeneration (Richardson, 2018).

After the granulation phase, the wound-healing process transitions into the remodelling phase. During these stages, there is a shift from type III to type I collagen, which fortifies the skin despite a slower formation rate (Rodrigues et al., 2019; Sveen et al., 2019). Concurrently, the granulation tissue experiences a decline in both cell density and metabolic activity during remodelling (Sveen et al., 2019). Zebrafish skin nearly regenerates to its original state within a month, including scales, adipocytes, and pigmentation (Richardson et al., 2013; Bergen et al., 2022). Conversely, in salmonids, scales and pigmentation (skin darkening) have alterations in deep wounds and may not regenerate completely (Sveen et al., 2019; Sveen et al., 2020). However, neither zebrafish nor salmonids exhibit complete regeneration of muscle tissue in cases of deep wounds. For example, the epidermis of rainbow trout regenerates, but the dermis and muscle tissue only partially recover, even a year after injury (Richardson et al., 2013; Schmidt, 2013; Schmidt et al., 2016; Sveen et al., 2020).

2 Main objectives

This research was conducted as part of a project collaboration between the Research Council of Norway, Arctic Seafood Group AS, and Nord University, focusing on using live smart-tags in Atlantic salmon to understand behaviour and welfare. It is essential to understand the implications of the tag implantation process to ensure the reliability of the data obtained, particularly regarding stress levels, wound healing post-implantation, and negative welfare effects. The ideal scenario assumes the population under study to be representative of a grow-out cage without adversely being affected by the implantation procedure or the capsule itself. An in-depth exploration of this process enables the development of methodologies to mitigate any negative impact and justify the possible use of smart tagging. The primary aim of this thesis was to employ physical, physiological, and transcriptomic data to analyse the chronic stress response of wounded Atlantic salmon subjected to daily crowding stress, as depicted in Figure 12. Furthermore, the study investigated the impact of chronic stress on the healing process of wounds inflicted by the implantation of a dummy smart-tag. The research was conducted through a single experiment and has been divided into three interconnected papers, each addressing specific objectives:

1) The first paper aimed to determine whether the wounds induced a chronic stress response in the Atlantic salmon and assessed how chronic stress influenced the healing of both internal and external wounds. Moreover, it evaluated the overall effects of daily chronic stress using primary, secondary, and tertiary stress indicators (**paper I**).
2) The second paper utilized RT-qPCR to examine the expression of genes associated with wound healing, immunity, and stress in the skin and head kidney (**paper II**).
3) The third paper employed RNA-seq to analyse the skin's response to wounding and chronic stress at three different time points: week 1, week 4, and week 8 (**paper III**).

3 Experimental design

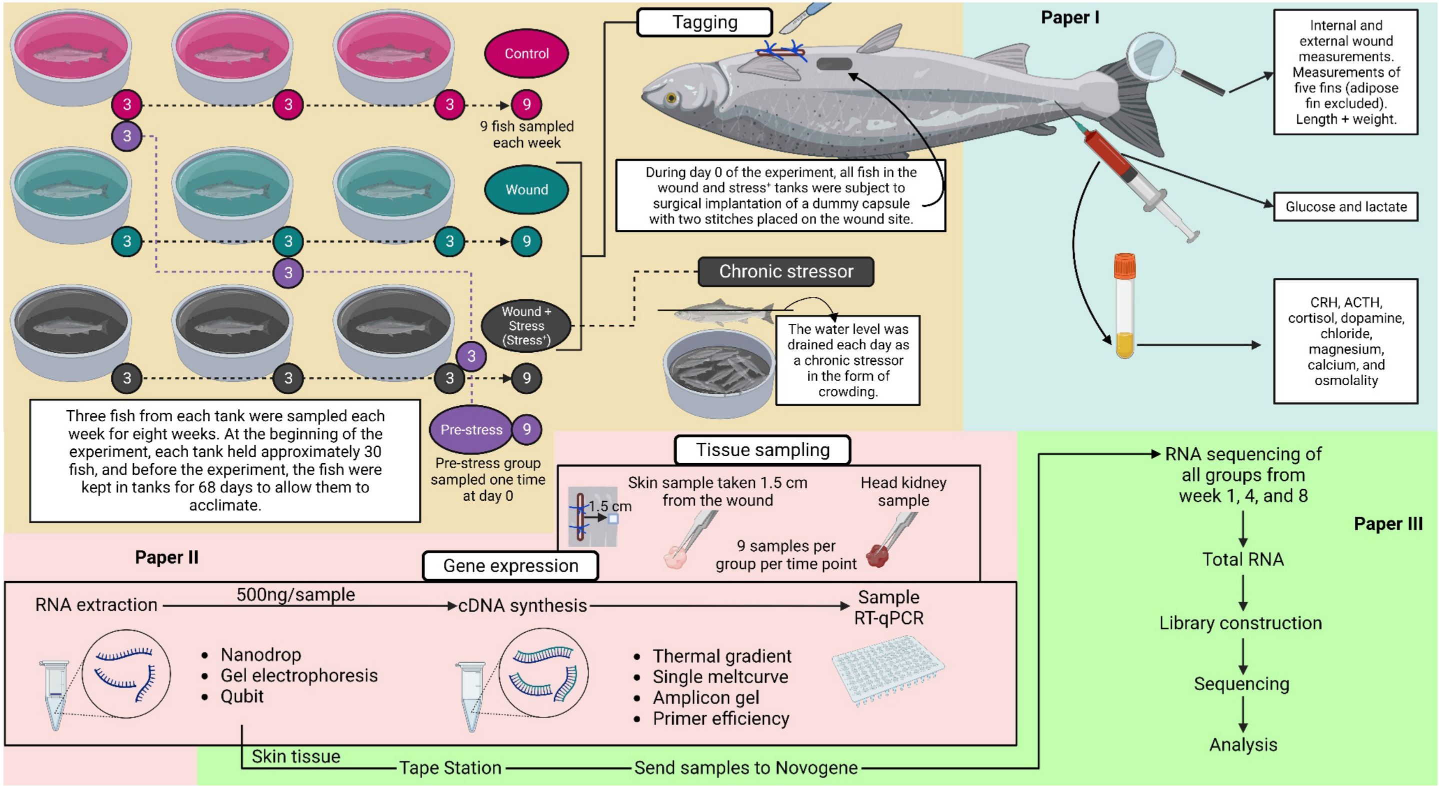


Figure 14. Illustration of the experimental design employed in Papers I, II, and III. The background colors indicate the respective papers: blue for Paper I, pink for Paper II, and green for Paper III. The upper left panel illustrates the experimental design common to all three papers. Modified from experimental figure in paper II. Specific protocols for each analysis can be found in the corresponding material and methods section of the paper in question.

4 Summary of papers: Abstracts

Paper I

Virtanen, M.I., Brinchmann, M.F., Patel, D.M., and Iversen, M.H. (2023). ***Chronic stress negatively impacts wound healing, welfare, and stress regulation in internally tagged Atlantic salmon* (*Salmo salar*).** Frontiers in Physiology 14. doi: 10.3389/fphys.2023.1147235.

Abstract

The desire to understand fish welfare better has led to the development of live monitoring sensor tags embedded within individuals for long periods. Improving and understanding welfare must not come at the cost of impaired welfare due to a tag's presence and implantation process. When welfare is compromised, the individual will experience negative emotions such as fear, pain, and distress, impacting the stress response. In this study, Atlantic salmon (*Salmo salar*) underwent surgical implantation of a dummy tag. Additionally, half of this group was introduced to daily crowding stress. Both groups and an untagged group were followed for 8 weeks using triplicate tanks per group. Sampling took place once a week, and where stress was given, it was conducted 24 h before sampling. Stress-related measurements were taken to understand if tagging caused chronic stress and explore the chronic stress response and its impact on wound healing. Primary stress response hormones measured included CRH, dopamine, adrenocorticotropic hormone, and cortisol. Secondary stress response parameters measured included glucose, lactate, magnesium, calcium, chloride, and osmolality. Tertiary stress response parameters measured included weight, length, and five fins for fin erosion. Wound healing was calculated by taking the incision length and width, the inflammation length and width, and the inside wound length and width. The wound healing process showed that stressed fish have a larger and longer-lasting inflammation period and a slower wound healing process, as seen from the inside wound. The tagging of Atlantic salmon did not cause chronic stress.

In contrast, daily stress led to an allostatic overload type two response. ACTH was elevated in the plasma after 4 weeks, and cortisol followed elevation after 6 weeks, highlighting a breakdown of the stress regulation. Fin erosion was elevated alongside cortisol increase in the stressed group. This data suggests that tagging previously unstressed fish in a controlled environment does not negatively affect welfare regarding stress responses. It also indicates that stress delays wound healing and increases the inflammatory response, highlighting how continued stress causes a breakdown in some stress responses. Ultimately, the tagging of Atlantic salmon can be successful under certain conditions where proper healing is observed, tag retention is high, and chronic stress is not present, which could allow for the possible measurement of welfare indicators via smart tags.

Paper II

Virtanen, M.I., Iversen, M.H, Patel, D.M., and Brinchmann, M.F. ***Daily crowding stress has limited yet detectable effects on skin and head kidney expression in surgically tagged Atlantic salmon (Salmo salar). Manuscript.***

Abstract

To ensure welfare-friendly and effective internal tagging, the tagging process should not cause a long-term burden on individuals given that tagged fish serve as representatives for the entire population in telemetry research. Stress to some extent is inevitable within regular aquaculture practices and thus the consequences of long-term stress should be described in terms of their effects on internal tagging. In fish, stressors activate the Hypothalamus-Pituitary-Interrenal (HPI) and Brain-Sympathetic-Chromaffin Cell (BSC) axes, leading to neuroimmunoendocrine communication and paracrine interactions among stress hormones. The interrelation between wound healing and stress is complex, owing to their shared components, pathways, and energy demands. This study assessed 14 genes (*mmp9*, *mmp13*, *IL-2*, *IL-4*, *IL-8a*, *IL-10*, *IL-12*, *IL-17D*, *IL-1β*, *TNF-α*, *IFN-γ*, *Leg-3*, *IgM*, and *CRH*) in the skin (1.5 cm from the wound) and head kidney over an eight-week period. These genes, associated with cell signaling in immunity, wound healing, and stress, have previously been identified as influenced and regulated by these processes. Half of a group of Atlantic salmon (n=90) with surgically implanted dummy smart-tags were exposed to daily crowding stress. The goal was to investigate how this gene panel responds to a wound alone and then to the combined effects of wounding and daily crowding stress. Our observations indicate that chronic stress impacts inflammation and impedes wound healing as seen through matrix metalloproteinases expression in the skin, but not in the head kidney, likely attributable to the ongoing internal wound repair in contrast to the externally healed wound incision. Cytokine expression, when significant in the skin, was mainly downregulated in both treatments compared to control values, particularly in the study's first half. Conversely, the head kidney showed initial cytokine downregulation

followed by upregulation. Across all weeks observed and combining both tissues the significantly expressed gene differences were 12% between the Wound and Stress$^+$ groups, 28% between Wound and Control, and 25% between Stress$^+$ and Control. Despite significant fluctuations in cytokines, sustained variations across multiple weeks are only evident in a few select genes. Furthermore, Stress$^+$ individuals demonstrated the most cytokine correlations, which may suggest that chronic stress effects cytokine expression. This investigation unveils that the presence of stress and prolonged activation of the HPI axis in an eight weeklong study has limited yet detectable effects on gene expression within immunity, wound healing, and stress, with notable tissue-specific differences.

Paper III

Virtanen, M.I., Brinchmann, M.F., Patel, D.M., and Iversen, M.H. ***Transcriptomic response of wounded skin under daily chronic stress in Atlantic salmon (Salmo salar). Manuscript.***

Abstract

RNA-seq was employed to investigate wound healing and identify the effects of chronic daily stress on wound healing in Atlantic salmon. Surgical incisions of 1.5 cm were made on the ventral surface, where a dummy smart-tag was inserted into the cavity, and two stitches were sewn to aid in wound closure. Three groups were used: a Control group that was undisturbed, a Wound group that was tagged, and a Wound + Stress group that was tagged and subjected to daily crowding stress by lowering and then refilling the water in the tank. Skin samples were taken from the same area in all three groups from three-time points at weeks 1, 4, and 8. The transcriptomics analysis revealed markers for wound healing, supported the concept of a cutaneous stress axis, and identified changes in skin healing and the molecular and cellular mechanisms affected by chronic stress within the wounded skin. Chronic stress amplified the expression of inflammation-associated genes, dysregulated tissue repair, and induced cellular and metabolic stress within the wounded skin tissue.

5 General discussion

5.1 Synopsis

Aquaculture encompasses the rearing of many aquatic organisms in large quantities. Evaluating the welfare of such large populations is challenging, requiring the involvement of innovative technologies. One such technology, the real-time smart tag, can obtain live data from individual fish within the aquaculture environment. However, the implantation of these smart tags is invasive, raising concerns regarding the potential negative effects on fish welfare. Thus, this presents a potential paradox: the method intended for welfare assessment may inadvertently compromise the well-being of the fish. Consequently, the data gathered might not reflect the welfare status accurately, especially if the implantation procedure has already adversely affected the subject fish.

This thesis is based on a single study divided into three distinct yet interconnected papers. Collectively, they explore aspects of fish welfare, chronic stress, and wound healing associated with the smart tag implantation in Atlantic salmon. Not only are the processes independently explored, but also how they impact each other, offering unique insights into the physiological and genetic interplay between chronic stress and wound healing.

Though the three papers are rooted in the same experiment, they each delve into different aspects and levels of detail. **Paper I** was used to investigate the possible physiological stress response resulting from the tag implantation and to describe how chronic stress influences this response. Furthermore, it investigated the visual indicators of wound healing on the exterior and interior surfaces, aiming to determine the timeline of the wound healing process and evaluate whether daily crowding stress impacted these timelines. **Paper II** used RT-qPCR to examine a selection of genes associated with cell signalling, immunity, wound healing, and stress in both the head kidney and skin. Thus, this aimed to discover the change in localized responses (at the skin level) and whole-body responses (in the head kidney) following tag implantation and daily stress. **Paper III** utilised RNA-Seq, influenced by the timeline and tissue samples analysed in **Papers I** and **II**, to uncover a more comprehensive transcriptional response in the skin than what was touched upon in **Paper II**. This enabled a deeper exploration into the molecular mechanisms underlying the responses to smart tag implantation and daily stress within the skin tissue.

5.2 Fish welfare and tagging

In this section, we refer to comparisons between the non-stressed tag-implanted group (wound) and the control group (untagged) unless specifically mentioning the stressed group (referred to as Stress$^+$) to highlight the effect of tagging on the fish.

Two critical factors must be considered when discussing fish welfare and tagging. The first is whether the tag implantation leads to any short-term or long-term negative impacts on the fish. The second is how the process can be improved to ensure optimal treatment, regardless of whether any adverse effects are observed. To address the first factor, **Paper I** did not identify any measurable stress response seven days post-tagging, nor was any negative prolonged indicator of stress found throughout the eight-week study. Furthermore, no adverse long-term effects were evident in non-healing wounds, as the healing of wounds was documented in **Paper I** visually and using transcriptomic analysis in **Paper II** and **III**. It must be acknowledged that the lack of sham control (a procedure in which surgery is performed without tag implantation) in the papers means that the specific effects of the tag alone cannot be established. However, the effects of tag implantation combined with surgery can be determined.

5.2.1 The effect of tagging

The short-term stress induced by tagging fish is hard to dispute. It could arise from a combination of factors, including the netting process, anaesthesia bath, surgical operation, presence of a foreign object in the body, and recovery bath (Hjelmstedt et al., 2020). For example, studies have demonstrated stress-related gene activation in the brains of Senegalese sole two days post-tagging, which was not observable eleven days later (Carballo et al., 2018). The Gulf killifish also exhibited elevated cortisol levels two hours post-tagging, disappearing after a week (Reemeyer et al., 2019). Similarly, the findings from **Paper I** suggest no stress response to surgical internal tagging when the first sampling occurs seven days post-tagging and extends over eight weeks.

Additionally, Føre et al. (2021) found an increase in heart rates for tagged individuals, which took an average of 4 to 6 days for Atlantic salmon to return to baseline post-internal tag surgery. These results are similar to those found by Hvas et al. (2020), Zrini and Gamperl (2021), and Yousaf et al. (2022). Yousaf et al. (2022) also confirm the initial cortisol increase from surgery. Moreover, there is no visible long-term stress response, as evidenced by Alfonso et al. (2020) study, which found no stress response in sea bream 46 days post-tagging and European sea bass 95 days post-tagging.

Semple et al. (2018) found no significant increase in cytokine expression in the spleen and muscle for rainbow trout, similar to our findings for Atlantic salmon in papers **II** and **III** for the skin. Although we found some elevated cytokines in the head kidney, Semple et al. (2018) found multiple inflammatory cytokines upregulated in the peritoneal immune cells 70 days post-surgery. Notably, only interleukin-2 was consistently upregulated from weeks 6, 7, and 8 in the head kidney in **Paper II**. Produced by activated T-cells, interleukin-2 is crucial for the immune system, driving T-cell proliferation, T-helper cell development, CD8+ T and natural killer cell activity, T-cell differentiation regulation, and immune tolerance via T regulatory cells (Malek, 2008; Boyman and Sprent, 2012; Liao et al., 2013). Thus, this might suggest a potential later-stage inflammatory response to the tag or termination of inflammation and a switch to immune memory (Hoyer et al., 2008). However, with only one pro-inflammatory cytokine being consistently upregulated, the evidence from our studies remains inconclusive. The phenomenon of an inflammatory response to implants, known as the foreign body response, is well-established (Anderson et al., 2008; Mooney et al., 2010; Christo et al., 2016; Chandorkar et al., 2019). If an inflammatory response does occur due to the tag, it will likely affect the metabolic demand and performance capacity of the "sentinel" fish (Wargo Rub et al., 2020). Despite no long-term stress responses detected in tagging studies and proper wound healing occurring, a more comprehensive examination is needed, including the internal environment. Generally, tagging studies on fish have overlooked the internal environment. Focus could be made on identifying materials that minimize harm to the internal milieu in long-term studies and explore the long-term inflammatory response to these materials (Bian et al., 2023). For example, Kieffer and Kynard (2012) found using inert elastomer coating of internal tags improved tag retention in shortnose sturgeon.

Behavioural changes due to tagging could be equally as detrimental to its viability as the presence of a long-term stress response. Our studies did not consider changes in documented movement. Still, we did measure physical aspects linked to behaviour, such as fin erosion and growth in **Paper I**. Within the wounded only group compared to control, significant differences in the weight of the subjects were only observable at week 8, and these disparities were no longer significant when considering the extra unsampled fish. Similarly, fin erosion was only significant in week eight in one out of five fins measured, specifically the caudal fin, which was generally the most eroded in all three groups (Control, Wound, and Stress$^+$). Regarding the impact of tagging on weight, Zale et al. (2005) suggest that up to a certain point (2% rule), tag size relative to fish size has no impact. However, exceeding this limit affects weight and swimming stamina. This is an oversight made in **Paper I**, where weight changes from previous wounding studies were discussed but not those from tagging studies. The insignificant weight changes observed might be attributed to the small size of the tag (maximum 0.2% of the weight of the fish) or the insufficient timeframe of the study to impact the weight significantly.

5.2.2 Tag retention

While **Paper I** reported a 100% tag retention rate across all treatments, this is not always true. Tag retention can vary significantly from 45% to 100%. This variation depends on several factors, such as rearing conditions, species, sex differences, fish size, tag size, suture material, surgeon experience, metabolic state, stressed state, and the location of tagging (Panther et al., 2011; Luo et al., 2015; Liss et al., 2016; Hadden et al., 2018; Byrd et al., 2019; Schumann et al., 2020; Zrini and Gamperl, 2021; Gong et al., 2023; Lawrence et al., 2023; Virtanen et al., 2023). The 100% tag retention observed in **Paper I** could be attributed to the small size of the capsule used in the study, which matches the intended size of the future miniaturized commercial product. However, the placement of the tag could offer a possible alternative explanation. The tag was positioned slightly away from the actual wound site to evaluate internal wound healing without disruption. As observed in **Paper I**, the internal healing of wounds takes considerable time, a factor not previously described in prior studies. This delay in healing might allow tags to be expelled from the unhealed wound. This observation carries significant implications for enhancing tag retention and improving animal welfare. Simply repositioning or gently massaging the tag away from the incision site can improve tag retention, thereby increasing the viability of tagging studies. However, this approach may not always be suitable. As demonstrated in a study by Lawrence et al. (2023) on Atlantic salmon, tag expulsion occurred away from the incision site. They also observed the encapsulation of the tags into the body wall mesentery. Interestingly, their study reported only a 45% tag retention rate, a figure significantly lower than what is typically documented in most tagging studies. In contrast to their findings, our study did not observe tags being encapsulated in the walls. We did, however, discover tags in the pyloric caeca.

5.2.3 Improving the tagging process

In **Paper I** we observed complete healing of the external wound between weeks 4 and 5 for all individuals, indicating an ideal timeframe for the sutures to be no longer present. As identified visually in **Paper I**, sutures can cause additional visual inflammation at the wound site and have also been described to contribute to fungal growth and secondary injuries (Wagner et al., 2011; Deng et al., 2017; Wargo Rub et al., 2020). Therefore, to improve fish welfare during tagging of Atlantic salmon, effort should be made to find or develop an absorbable suture that can maintain its tension for approximately 4-5 weeks under aquaculture conditions. Some previous studies have begun to explore the best suture materials and cyanoacrylate adhesives (Wagner et al., 2000; Deters et al., 2010; Ivasauskas et al., 2012; Jepsen et al., 2017; Schumann et al., 2020; Kientz et al., 2021).

Moreover, incorporating additives like silver into suture materials and cyanoacrylate may be beneficial. Given its antimicrobial effects, silver could potentially reduce fungal growth and inflammation (Zhang et al., 2014; Baygar et al., 2019). Silver has been shown to accelerate wound healing in zebrafish, as evidenced by a study using silver nanoparticles applied topically or through water (Seo et al., 2017). Other additives, such as bioavailable zinc, which has been shown to promote wound healing in salmon and other species could be beneficial (Batool et al., 2021; Haghniaz et al., 2021; Hassan et al., 2021; Chen et al., 2022). In **Paper III** many zinc-associated genes, including zinc transporters (*slc30a-5, 6, 7, 9 and slc39a-1, 7, 11, 12*), *metal regulatory transcription factor 2* which controls metallothionein transcription (Wang et al., 2014), zinc-dependent enzymes (matrix metalloproteinases), and zinc-finger associated genes were found to be upregulated in tagged individuals. Transcription factors associated with zinc have been previously described as contributing to wound healing (Kogan et al., 2017; Lin et al., 2018). Interestingly, a recent study has shown that using zinc nano particles at 4 mg/kg in the feed of *Pangasianodon hypophthalmus* mitigated multiple stress responses (Kumar et al., 2023).

Feed additives present unique opportunities for preventative measures, especially given the significant impact of stress on energy production related genes in the skin tissue, as indicated in **Paper III.** This finding could inspire the testing of feeds that support rapid energy utilization. One example being the possible addition of synthetic ATP (or compounds aiding in ATP production) to alleviate or prepare for stressful situations. Such feeds could be given before extreme events such as grading, sea lice treatment, transportation, vaccination, long-term handling, and operations such as tagging. In humans, a two-week pre-exercise oral ATP supplementation helped maintain ATP levels, prevented the decline in muscle excitability during later exercise, and enhanced peak power in repeated high-intensity sprinting (Purpura et al., 2017). Energy requirements are also concerning since there is a significant difference in mortality rates between tagged fish kept in tanks versus those in sea cages, with the latter experiencing a substantially higher mortality rate that increases with the length of the study (Macaulay et al., 2021). Alongside additives to support high energy demands, it may be beneficial to include substances such as silk fibroin that promote wound healing, as shown in a study by Albaladejo-Riad et al. (2023) on gilthead seabream. This approach would be particularly relevant, given that most stressful events carry the risk of tissue damage.

Though the primary focus here is the outcomes found within the three papers, there is much to consider when internally tagging fish and many opportunities for improvement to enhance fish welfare and, in turn, the reliability of data collected from sentinel individuals. Therefore, conducting a pilot study before a full-scale experiment is recommended. This preliminary research, if none has been conducted prior, should seek to understand species-specific tagging needs and any adverse effects of tagging as well as provide training to personnel on the best surgical practices. In addition, effort should be made to document the tag implantation and extraction procedures (Cooke et al., 2011a; Brownscombe et al., 2019; Clemens et al., 2023; Leroy et al., 2023). Lastly, the focus should be extended to the long-term impacts of tags, including the internal environment.

5.3 Effects of altered fish density

The study design selected, which involved the removal of individual fish for sampling each week, unavoidably altered the density within the tanks and potentially affected any established hierarchy (Winberg and Sneddon, 2022). Alternative options, such as reducing the water level to maintain equal density or substituting fish, could have disrupted individual behaviour and group hierarchy. An ideal design would have involved separate tanks for each sampled group at every time point, but this approach was impractical; thus, the current design was chosen. This design may have exerted an influence on certain data across the studies.

Two examples illustrate the possible impact. First, plasma dopamine (**Paper I**) increased in all three experimental groups as time progressed. However, to our knowledge, the plasma dopamine levels in fish have not been documented until now (**Paper I**), so it is challenging to draw definitive conclusions about the reasons behind this increase. Nevertheless, it could be a natural progression related to time and growth, or a response to reduced density, as all groups exhibited a similar pattern. In fish, dopamine is associated with various functions, such as its roles in behaviour, learning capabilities, movement, stress responses, reward systems, and social interactions (Øverli et al., 2001; Gesto et al., 2013; Höglund et al., 2017; Soares et al., 2018; Alfonso et al., 2019; Staven et al., 2022). Therefore, the change in plasma dopamine levels from the start to the end of the experiment might influence study outcomes in various ways.

Acute stress typically increases dopamine activation, and chronic stress suppresses it (Baik, 2020). In **Paper I**, the dopamine levels in the Stress$^+$ group were generally lower, although non-significantly in most cases. Notably, a similar timely increase, as seen with plasma dopamine, was observed for most of the genes studied in the head kidney across all three treatment groups in **Paper II**. In addition, the cortisol levels in **Paper I** for the control fish but not wounded fish decreased as time progressed, where the lowest levels were found during the end of the experiment.

The second notable example of a possible density-related effect was discovered in **Paper II** (carried over to **Paper III**), where elevated levels of *mmp9* and *mmp13* in the skin were seen in the control group, decreasing as the experiment proceeded. Sveen et al. (2016) reported that high fish density increased *mmp9* transcription and compromised skin health. While the initial density in our study was already on the low end (30 kg/m^3), reducing it further might have affected the transcriptional data observed in **Papers II** and **III**. Additionally, since the genes regulated in the skin due to increased density are involved in wound healing, it is possible that the elevated levels observed in the control samples could reduce the observed intensity of the healing response as the treatment is compared to the control.

While not directly related to the study design (fish density), but more so the chosen methods of study analysis, one must recognize that mRNA transcription levels, as determined by RT-qPCR (**Paper II**) and RNA-seq (**Paper III**), do not necessarily correlate with protein abundance. Therefore, while we present transcriptional data and use this to hypothesize about biological systems, these hypotheses might not hold during protein-level analyses.

5.4 Stress response

The discussion of stress responses requires an understanding of the stress design used, as the reliability of a stress response depends on multiple factors, as described in the introduction (1.2.6). The design used to assess chronic stress incorporated a daily crowding stress given during an 8-hour time window for eight weeks. The stress was applied 24 hours before the sampling time on the day before sampling, providing sufficient time for any acute stress responses to subside. The first sampling was taken on day 7 of the experiment, except for the pre-sampled individuals that were sampled before the start of the experiment. As previously discussed, the surgery of the tag should be considered an additional acute stressor.

In **Paper I**, the wounded-only group showed no chronic stress response to the presence of a tag and implantation process. In contrast, the daily stressed group showed a breakdown of the HPI axis, starting at week four for ACTH and week six for cortisol (possible involvement of CRH at week three). Additionally, there was severe dysregulation of genes in the skin tissue by week eight (**Paper III**). Had the experiment continued longer, the allostatic overload type II response would have become more visible, with evident changes in secondary and tertiary responses. However, it would be ethically and morally inappropriate to knowingly subject individuals to extreme conditions such as severe chronic stress with a high chance of mortality. The harsh conditions in this study guided our decision to conduct a single investigation. This approach aimed to reduce the number of fish used while obtaining the most relevant data for the objectives. The gill, brain, and mucus were also collected, which have yet to be analysed. However, one should consider running long-term studies on only wounded fish as cortisol levels in wounded fish did seem to rise in the latter half of the study, although not significantly compared to the control group in **Paper I.** The presence of the tag may also have a late phase effect currently shadowed by experiments running for short time frames (**Paper II** but inconclusive) and without sham groups.

5.4.1 Primary

Cortisol is a commonly accepted method of measuring stress in fish due to its central role in the stress response (Wendelaar Bonga, 1997). Studies identifying acute stress responses in Atlantic salmon typically document peak cortisol values between 1 – 3 hours post-stress, with basal levels being reestablished within 24 hours (McCormick et al., 1998; Fast et al., 2008; Djordjevic et al., 2021; Madaro et al., 2023). However, Robinson et al. (2019) underlined that returning to basal cortisol levels does not imply full stress recovery. Previous long-term sampling and chronic stress studies have revealed that the stress response becomes deregulated around the four-week mark (highly dependent on the stressor, amount of stress, and life history, among other factors), with cortisol levels seen to rise (Iversen and Eliassen, 2014; Aerts et al., 2015; Sveen et al., 2018; Patel et al., 2022). This is similar to the findings found in **Paper I**. However, this is not always the case as Madaro et al. (2015) noted that subjecting fish to multiple stressors more than once per day resulted in diminished cortisol production within 23 days. Similarly, Carbajal et al. (2019) found comparable results when stress was applied continuously over 30 days.

Most chronic stress studies measure the stress response shortly after the stressor has been applied, thus tracking the individual's chronic response to the recently given stressor. It is also possible that many studies ending after only a few weeks do not observe a breakdown in the HPI-axis. In this thesis's experimental design, where sampling occurred 24 hours post-stressor, the resulting plasma levels reflect upkeep levels. Therefore, the individual fish could regulate the HPI axis during the first three to four weeks. It should be highlighted that a high standard deviation was observed within the stressed group in the analysis of plasma levels for primary stress responses. Thus, this indicates that although a general stress response occurs, certain individuals cope better than others (Martins et al., 2011; Sørensen et al., 2013; Castanheira et al., 2017; Vindas et al., 2017).

Within the Stress$^+$ group, the significant plasma CRH recorded at week three might have triggered the loss of ACTH regulation, which initiated the deregulation of cortisol by week six. However, cortisol elevations began to appear during week four. This pattern possibly resulted from the overwhelming CRH production, which led to the overproduction of ACTH. Through negative feedback, ACTH likely regulated CRH levels. The eventual rise in cortisol due to ACTH overactivation then reduced ACTH levels, again likely through negative feedback (Gorissen and Flik, 2016; Schreck and Tort, 2016). The elevated and sustained cortisol levels even after 24 hours post-stress suggest that normal cortisol regulation and negative feedback mechanisms are no longer functioning properly.

While the explanation mentioned above is the endocrinological perspective suggesting that daily stress applications lead to chronic stress through HPI-axis breakdown, lack of negative feedback, and Allostatic Overload Type II, other factors could also be at play. In humans, persistently elevated cortisol can result from various factors, including Cushing's syndrome, medication intake, pregnancy, mental disorders (such as depression), physical conditions (like injuries, alcoholism, and dietary habits), as well as life experiences (such as post-traumatic stress disorder, examinations, financial issues, relationship struggles, etc.) (Adinoff et al., 2003; Lacroix et al., 2015; Cherian et al., 2019; Speer et al., 2019; Rinne et al., 2023). Many of these issues can cause and be a result of chronic stress.

Reliably measuring, for instance, depression or post-traumatic stress in fish without being able to communicate with the individual can be challenging, if not considered impossible. Nevertheless, recent research regarding depression has started to use fish as model species (de Abreu et al., 2018; Hubená et al., 2022). With this in mind, one must remain open to the possibility that the fish in our study may have other underlying biological conditions, potentially induced by the chronic stress and tagging experience, that would affect the response of primary stress mediators.

5.4.2 Secondary

In **Paper I**, we identified only one consistent effect of the stress on secondary stress responses. This likely occurred because sampling was conducted 24 hours after applying the stressor. Interestingly, although the primary responses and fin erosion are activated, no intense significant differences were observed within the secondary responses. Thus, this suggests that critical survival functions such as osmoregulation and metabolism continue to operate effectively even under severe stress. Nonetheless, should the experiment have been extended, we anticipate seeing a more pronounced shift in secondary responses as the body approaches a state of allostatic overload type II and survival mechanisms start to falter due to resource depletion.

A potential difference exists between the whole body's secondary stress responses (**Paper I**) and transcriptomic effects in the skin tissue near the wound (**Paper III**). This difference relates to the regulation of metabolism (particularly mitochondrial health) and solute transporters with potential roles in osmoregulation. It may result from the crucial role that the skin plays in osmoregulation, as even scale loss has been associated with osmotic dysfunction (Olsen et al., 2012; Mateus et al., 2017). The absence of sustained or early severe osmotic changes confirms that the surgical procedure was efficient and minimally disruptive to the osmotic balance. At week eight, it is important to note the significant differences between the Control and Stress$^+$ groups regarding osmolality, chloride, calcium, magnesium, and lactate. These differences support the theory of a potential late-phase breakdown of essential regulatory functions. Additionally, in **Paper I**, we observed that the average chloride level in the Stress$^+$ group over eight weeks was consistently higher than in the Control or Wound group, with significant differences occurring during weeks 3, 4, 5, and 8. Thus, this may be caused by cortisol's role in stimulating ion transport and maintaining water balance by enhancing sodium excretion, boosting the activity of Na+/K+-ATPase enzyme in the gills, promoting water absorption in the intestine and urinary bladder, and an increase in the size of chloride cells in the gills (Perry et al., 1992; Wendelaar Bonga, 1997; Barton et al., 2003; Olsen et al., 2012; Wood and Eom, 2021).

This study highlights the complex influences of sampling time on stress responses and the variation between a dysregulated primary stress response and a seemingly intact secondary stress response within the study's parameters. Significant results may have been overlooked within the secondary stress responses if these occurred closer to the actual daily stress events. It is also important to consider other influencing factors such as the nature and frequency of stressors, duration and type of anaesthesia, whether the anaesthesia is aerated (or if hypoxia is a contributing factor), and time spent netting individuals, among other factors.

5.4.3 Tertiary

Growth

Paper I demonstrated that the Stress$^+$ group showed no significant changes in weight or length compared to the control or wound group. Food intake depends on the current energy and nutrient availability, along with the palatability of the food. Stress can affect an individual's growth through changes in metabolic needs, nutrient absorption and allocation, suppression of muscle development promoters, and feeding behaviour (McCormick et al., 1998; Sadoul and Vijayan, 2016; Volkoff, 2019). It has been reported in fish that components of the stress axis, primarily CRH and cortisol, directly influence food intake decisions (Conde-Sieira et al., 2018a). While one did not observe a growth effect due to stress, this is likely because no substantial dysregulation in the secondary stress response occurred either. The individuals exposed to daily stress showcased a breakdown of the primary stress responses. Still, their whole-body responses had not yet been dysregulated, as these responses are typically tied to survival. However, this interpretation of the results does not provide a complete nor convincing explanation for the results seen, as growth is less necessary to survival compared to keeping an osmotic balance.

Typically it would be expected that to cope with a stressor, an individual increases their ATP production to meet the heightened energy demand for maintaining bodily processes, thereby diverting energy from growth (Sokolova et al., 2012; Faught et al., 2016; Sadoul and Vijayan, 2016). However, since our study lacked a group consisting of only stressed individuals (untagged), we have a highly manipulated scenario where processes involved in wound healing share genes with the regulation of growth. Further complicating this issue, in **Paper III**, during week eight in the Stress$^+$ vs Control groups, upregulation of autophagy, insulin signalling pathway, and mTOR signalling pathway were documented. This suggests that the stress and response to injury were promoting cellular recycling and increased growth signalling. The mTOR pathway has been described to inhibit autophagy, and having these pathways present indicates complex interactions at play (Sadoul and Vijayan, 2016). Additionally, comparing the Stress$^+$ vs Wound groups, we found downregulation of protein synthesis and energy metabolism, indicating wound healing mechanics, response to stress, and the possible effect on growth.

Interestingly, it was observed that the apelin signalling pathway was slightly upregulated in the Stress$^+$ vs Wound group at week eight. In a study on goldfish injected with mammalian apelin, an increase in food intake was reported (Volkoff and Wyatt, 2009). This increase in apelin may suggest hedonic (pleasure-driven) regulation of food intake, where stress can escalate consumption, as noted in mammals (Conde-

Sieira et al., 2018a). Undoubtably increased energy expenditure occurs within the skin near the wound in stressed fish, coinciding with heightened cell death and growth. This cycle of cell death and growth is essential for muscle growth. As Sadoul and Vijayan (2016) indicate, cortisol shifts this balance towards protein breakdown, resulting in growth suppression. Our results agree with this increased breakdown pattern, yet no observable growth suppression is seen during eight weeks. However, since we have introduced a wound, pure stress-related changes cannot be distinguished from injury-related ones. It could also be plausible that other factors, such as reproductive capacity and swimming activity, were reduced as trade-offs before reducing growth.

Fin erosion

In **Paper I**, fin erosion emerged as a key indicator in the Stress$^+$ group compared to the Control and Wound groups, starting from week 3 onwards. The exact causes of fin erosion remain speculative. Still, it is often associated with factors such as friction against objects, aggressive behaviour (bites or nips), suboptimal water conditions, feed management, and overcrowding (Ellis et al., 2008; Jones et al., 2010; Noble et al., 2018; Weirup et al., 2021). In all treatment groups, the caudal fin was the most eroded. In contrast, the most significant differences in fin erosion in the Stress$^+$ group compared to the other groups were observed in the pectoral, pelvic, and anal fins. Given that these fins are all located on the ventral side of the fish, one might question whether this is purely a case of tank abrasion, with no impact from the actual stress response but rather a response to the choice of a stressor. While tank abrasion undoubtedly plays a role, it is uncertain if it is the primary cause. Previous studies have demonstrated that feeding frequency (leading to increased aggregation) and high stocking density can increase fin damage, even in the absence of direct tank abrasion (Ellis et al., 2002; Ellis et al., 2008; Person-Le Ruyet et al., 2008; Calabrese et al., 2017; Stejskal et al., 2020; Weirup et al., 2021). Stress-induced aggressive behaviour has also been documented in fish reared at high density (Latremouille, 2003; Hoyle et al., 2007). Additionally, juvenile rainbow trout with cortisol implants experienced increased fin erosion (Gregory and Wood, 1999), and scale cortisol in rainbow trout is positively correlated with fin erosion (Weirup et al., 2021).

Paper I found a positive yet non-significant correlation (rs = 0.66, *p* = 0.085) between plasma cortisol and fin erosion of the pectoral, pelvic, and anal fins. Fin erosion was evident from week three, while increased plasma cortisol only became visible at week six. This suggests that fin erosion might be an early indicator of poor conditions and an additional stressor that individuals must overcome. However, this is complicated further by the fact that fins are regenerative and nociceptive, and the stress response can impact the healing process. Thus, the healing of fins may be disrupted by increasing HPI-axis activity, which in turn can become an additional source of stress, intensifying the stress response (Roques et al., 2010; Pfefferli and Jaźwińska, 2015; Sveen et al., 2018; Sneddon and Roques, 2023). In light of this, fin erosion on a farm site could potentially serve as an early indicator of fish welfare, and individuals with high fin erosion should be excluded from tagging to enhance the welfare and success of the tagging study.

With the above in mind, it would be interesting to explore the impact of various stress methods (density, chasing, air exposure) on fin erosion and length, incorporating the differentiation of active erosion and healing erosion into the study through hyperspectral imaging (Lindberg et al., 2023). Additionally, in **Paper II**, *mmp 9* and *13* were upregulated in the head kidney due to the injury resulting from the tagging process and wound healing. Considering the role the head kidney plays in these wound healing-related genes, it could be interesting to study these genes among other significant wound healing biomarkers found in **Paper III** to explore whether fin erosion is regulated through the head kidney. If found to play a significant role, this could be used as a welfare indicator of whole-body fin erosion and whole-body skin condition.

5.4.4 Chronic stress in the skin?

The existence of a cutaneous stress response system, known to occur in humans and various mammals, is hypothesized in fish. However, the specifics of this local system, particularly whether the skin self-synthesizes or receives transported stress hormones, remain uncertain (Kulczykowska et al., 2018; Gozdowska et al., 2022). In mammals, the synthesis of cortisol within skin cells has been documented (Vukelic et al., 2011; Slominski et al., 2022). Research has highlighted chronic stress's impact on cutaneous skin repair in gilthead seabream, finding that elements involved in the stress axis are present as well as associated with impairing the healing process, such as *steroidogenic acute regulatory protein* (*star*) and *glucocorticoid receptor* (*gr*) (Mateus et al., 2017). Additionally, Uren Webster et al. (2020) discovered increased cortisol levels in the skin of juvenile Atlantic salmon following two weeks of daily confinement stress.

In **Paper III**, the presence of the HPI-axis in the skin was found to be active, though whether local hormone synthesis occurs remains to be determined. Week 1 displayed an increased activity of some stress pathway-related genes. However, by week 8, chronic stress and elevated plasma cortisol had not significantly influenced these genes in the Stress$^+$ group relative to the Control group, though significant differences were observed when comparing the Stress$^+$ with the Wound group. Thus, this can partly be explained in the non-uniform expression within the Control group at week 8, where the principal component analysis (**Paper III**) displays individuals from the Control group within both treatment groups and separated (other influencing aspects are discussed in the future text). It may suggest that skin sampling is highly variable and becomes increasingly so as the experiment progresses. However, many other studies have described fairly equal control levels. One factor to mention here is the possible site influence, as the location was on the ventral surface, which is not normally studied in fish wound healing experiments. Importantly, no tank bias was detected, as the three individuals from each tank did not cluster together, indicating that the variation was not due to the tank effect. Investigating genes linked to skin damage that impact stress-related genes can pose challenges. It remains speculative to indicate any one reason for the cause of variation in the control group, but it profoundly influences the results. Establishing a control group with undamaged skin (regarding gene expression) may be difficult and highly dependent on conditions and the tissue sampling site. However, this is further complicated by even loss of scales provoking an upregulation in skin-healing genes. Additionally, even when sampling areas are selected carefully, subtle changes in healing genes may still be expressed in parts of the skin tissue that are not close to the original wound (Ingerslev et al., 2010; Mateus et al., 2017; Eslamloo et al., 2022). Thus it would be wise for future studies to incorporate not only an external control as present in the current thesis but also use internal controls sampling from a section of skin opposite the wound where possible (different side of an individual).

Nevertheless, when comparing the Stress$^+$ group to the Wound group at week 8, one observed an upregulation of stress response-related genes (such as *glucocorticoid receptors, mineralocorticoid receptors, serine/threonine-protein kinase, cytochrome P450, Rho GTPase, p53, mitogen-activated protein kinase, nuclear receptor subfamily, members of tumour necrosis factor receptor, melanocortin receptor, and heat-shock proteins*). Crucial in stress response and expressed in leukocytes, adrenergic receptor-like genes were also upregulated. Chronic stress, particularly mediated by beta-adrenergic receptors, can modify immune responses and has been linked to cancer progression (Eng et al., 2014; Rains et al., 2017; Mravec et al., 2020; Hong et al., 2021). This unexplored domain of adrenergic receptors expression in Atlantic salmon skin may present an interesting research avenue. Interestingly, certain stress-related genes are present at week one but not at week eight, and vice versa, and may indicate divergent regulation at the skin level between injury, injury accompanied by one week of stress, and eight weeks of stress. From both a physiological and energetic perspective, this is to be anticipated. Cumulative stress effects disrupt the regulation of the HPI-axis (**Paper I**), which theoretically diminishes the available energy supply, bringing the individual closer to allostatic overload type II. In this overload stage, bodily changes should manifest at large as the energy reserved for maintenance decreases.

One key stress-related hormone highlighted in all three papers is CRH. In **Paper I**, CRH assessed from the plasma was upregulated in the Stress$^+$ group during week 3 and in the control group in weeks 6 and 7. In **Paper II,** we could not establish standard curves for the skin tissue due to low expression of CRH. In **Paper III,** while CRH expression was visible, it was insignificant. However, in **Paper II**, the CRH expression within the head kidney was documented, with no significant increase found in the Stress$^+$ group. The low expression of CRH in the skin has previously been documented in fish and mice (Slominski and Wortsman, 2000; Mazon et al., 2006).

In contrast, RNA-seq revealed CRH-BP to be significantly downregulated in the wound group but not the Stress$^+$ groups during week 1. Similar findings have been found by Mazon et al. (2006) in common carp gills and skin stressed for 24 hours. This suggests a local stress response to injury may occur, with reduced CRH-BP allowing more available CRH to bind to its receptors. This response to acute stress (injury) appears disrupted by the addition of daily stress. CRH-BP expression typically increases to bind CRH during repeated stress, thereby contributing to negative feedback within the pituitary and brain (Ketchesin et al., 2017). The role of CRH and, consequently, CRH-BP in the skin is complex due to CRH having multiple roles, including the regulation of keratinocyte growth and differentiation, protection against apoptosis in various skin cells, and influencing inflammation management (Slominski et al., 2013; Slominski et al., 2022).

Paper III suggested that genes associated with skin colour could be used as markers for late-phase healing, which is based on the observation that hyperpigmentation (characterized by melanocytes migrating to healing tissue) has been linked to wound healing in fish (Lévesque et al., 2013; Sveen et al., 2019; Sveen et al., 2020). **Paper III** highlighted the four genes *melanin-concentrating hormone*, *opsin-1*, *phosphodiesterase 6D*, and *retinoid isomerohydrolase*. Additionally, stress has been observed to cause either paling or darkening of the skin, depending on the species (Vissio et al., 2021). Thus, there is an influence of healing and stress-related pigmentation in response to injury. Svitačová et al. (2023) provide an interesting take on fish welfare associated with pigmentation, which after reading, leads to the speculative idea that if chronic stress can change the pigmentation significantly enough, this may further influence other factors such as behaviour and social hierarchy within fish communities. Future studies could incorporate changes in colour as an animal welfare indicator, utilizing machine learning models such as support vector machines or convolutional neural networks for analysis. This approach could be significantly improved by researchers freely contributing images from their experiments to an open-source platform to train the model for everyone to benefit.

5.5 Wound healing

Research on wound healing in fish has been focused mainly on full-thickness wounds (penetrating muscle) and surface wounds. However, this thesis examines wounds inflicted entirely through the posterior body cavity, manipulated further with suture material, and potentially influenced by tagging. Existing tagging studies have not reported extensively on wound healing or tag effects with attention to biological detail, with only a few examples available for histology (Yousaf et al., 2022) and transcriptomics (Semple et al., 2018). One would assume that ensuring feasibility and optimizing insertion and healing processes before proceeding with on-site live tagging would be done on a species-by-species basis.

The physical parameters of wound healing were studied in **Paper I**. It took up to five weeks for all individuals to show no visible open wounds. However, finding existing wound healing measurements in studies is challenging. Many do not report them, and those often report them as a score or refer to wounds as healed without further explanation. Nevertheless, in previous tagging studies, wound healing times were documented taking four weeks in big head carp (Luo et al., 2015), two weeks in round goby (Behrens et al., 2017), 4-6 weeks depending on wound closure method in rainbow trout (Kientz et al., 2021), 13 weeks in recaptured walleye (Schoonyan et al., 2017), and 3 weeks in Atlantic salmon (Yousaf et al., 2022). Some of these studies have reported long periods of inflammation (redness around wound or raised surface around wound), similar to our findings in **Paper I**.

In the Wound group, visible inflammation deviation was low in week one but increased in variability in the subsequent week. This may be due to individual differences in wound coping, although it is intriguing to observe a large effect in week two and not in week one. Healing differences can be extremely large among individuals. For instance, by week one, an individual from the Stress$^+$ group had already healed their wound. In the Wound group during week two, five individuals had healed wounds compared to four in week three. Visible inflammation is also complicated, as multiple sources from the wound, suture, and tag may occur. Complete internal wound healing was only recorded in the Wound group, observed in one individual in week seven and two in week eight. Further variability in wound healing and inflammation can come from factors expressed in the tag retention effects (5.2.2).

While we might have missed the initial surge in cytokines (**Paper II**) from acute inflammation, which according to findings for Atlantic salmon wound healing by Sveen et al. (2018), was highest during day three post-wounding, the presence of *mmp*s along with other inflammation-related genes (**Paper III**) implies a persistent inflammation from week one, which gradually subsides by week four and is faintly present in week eight. This is consistent with the visual observations throughout the study (**Paper I**) (Pedersen et al., 2015; Kim et al., 2023). *mmp*s are also involved in granulation tissue formation (proliferative phase), indicating that while they contribute to degradation, they also facilitate new cells to migrate to the wound and initiate tissue remodelling (Nguyen et al., 2016; Kandhwal et al., 2022; Chi et al., 2023).

Paper II likely highlights a correlation in the *mmp*s in the skin tissue to the outer wound healing timeframe (**Paper I**). However, the samples were taken 1.5 cm away from the wound, and results from the wound itself might differ. This correlation suggests using *mmp*s as indicators of wound severity and healing stage. However, consistent upregulation of head kidney *mmp*s was noted, likely due to ongoing internal wound healing. Interestingly, no significant upregulation was found in cytokines examined in the skin tissue in **Paper II**. Instead, one found significant downregulation in the Wound group compared to the Control group, especially in week one (*il-8a, tnf-a, il-10*) and week four (*il-8a, il-12, il-1b, il-4/13a*). A finding from this is that during week one in the skin tissue for the Wound group, we observed no upregulation of *mmp9* and downregulation of *il-8a*, a pattern not present in the Stress$^+$ group. Similarly, in week 4, *mmp9* was significantly expressed in the Stress$^+$ group, with concurrent significant expression of *il-8a* (Stress$^+$ compared to Wound). The finding supports the results in human neutrophils by Chakrabarti and Patel (2005) that demonstrated upregulation of *il-8* leads to its interaction with the CXCR2 receptor on neutrophils, stimulating the release of *mmp9* and its absence when *il-8* is downregulated, leading to an absence of *mmp9*. However, the observed pattern of *il-8* upregulation coupled with *mmp9* expression, as seen in the Wound vs Stress$^+$ groups, was not evident in the Stress$^+$ vs Control group. This lack of similarity could potentially be explained by previously described *mmp* upregulation in the Control group (5.3).

Paper III supports the idea that initial cytokine presence is already being dampened, as highlighted by an example such as the significant upregulation of *tumour necrosis factor alpha-induced protein 6* (*TSG-6/tnfaip6*) during week one. Consistent with previous research, *TSG-6*, a protein known for its anti-inflammatory effects and primarily secreted by mesenchymal stem cells (MSCs), may suppress the release of pro-inflammatory molecules such as tnf-a (Day and Milner, 2019; Zhu et al., 2023). MSCs, which are highly associated with production from bone marrow, are not exclusive to bone marrow (not present in fish) and can be expressed in multiple tissue (Lund et al., 2014). The *TSG-6*-mediated suppression could potentially facilitate wound healing, as indicated by its ability to reduce tissue fibrosis and the secretion of other inflammatory molecules (Zhu et al., 2023).

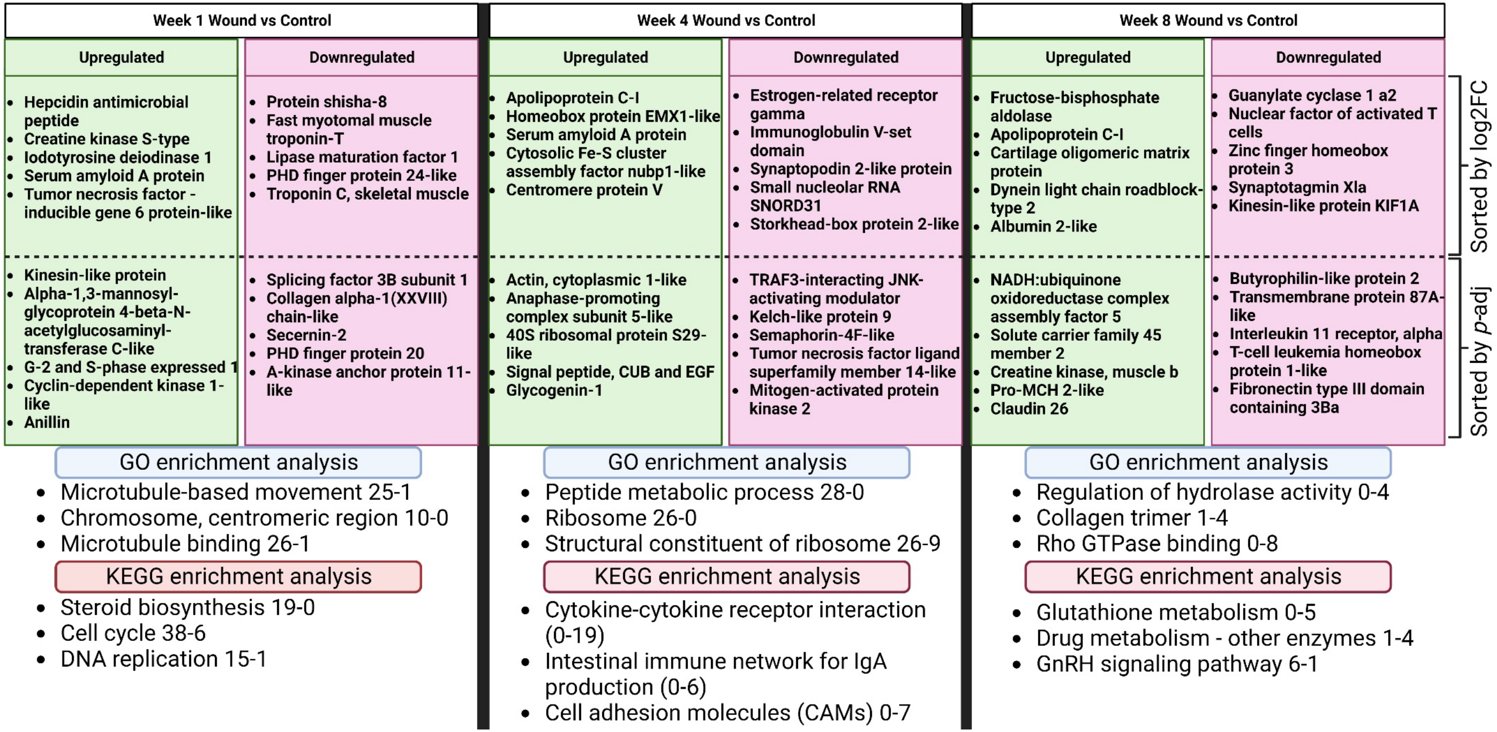
In **Paper II** an interesting result from the head kidney is that of *IgM* being significantly downregulated from the second to the eighth week in the Wound group, compared to the Control group. This indicates a possible effect of the wound and tag on the *IgM* levels, and could be a consequence of B-cell migration to the wound site, but no corresponding increase in *IgM* was observed in the sampled skin tissue. It's possible that high expression of *IgM* in the Control group occurred or that B-cell migration was not observable 1.5 cm away from the skin, instead being concentrated within the internal wound or directed towards the tag location due to a foreign body response. To my knowledge, no published studies discuss *IgM* transcriptional levels in the head kidney concerning wound healing or tagging in fish. However, Heath's (2023) PhD thesis reports that internal surgical tagging in rainbow trout led to elevated peritoneal IgM levels (measured by ELISA). Their studies also revealed that there were minor differences between the sham surgery and tagging on IgM levels within the peritoneal cavity, suggesting that the IgM response is more likely tied to the wound than the tag.

Paper III provided a large amount of wound healing genes. Figure 15 only begins to scratch the surface of some of the genes present, providing a selective summary. The genes highlight different phases of wound healing, while additionally, in **Paper III** many wound healing markers are suggested, including wounds with stress-associated markers. Although this thesis does not directly examine the different phases of wound healing by using select gene markers, as presented by Costa and Power (2018), Figure 15 offers insights into potential phase shifts. Furthermore, the study of *mmp 9* and *13* in **Paper II** provides significant information.

To summarize Figure 15 and the findings of **Paper III** regarding the Wound group, several wound healing and functional processes were observed. During the first week post-wounding, an inflammatory and proliferative response was noticed, alongside tissue remodelling and altered energy metabolism. A decrease in muscle function and signalling processes was also evident. By week 4, gene expression patterns indicated an ongoing wound-healing process involving metabolic, neural, and immune responses. New aspects such as lipid metabolism, neural development, and immune response modulation became apparent. By week 8, gene expression data showed a clear transition from active wound healing towards tissue remodelling and cellular processes' normalisation. There was an increased focus on tissue remodelling, energy metabolism, and cellular transport and a reduced emphasis on immune responses and cell division.

5.5.1 Reflections on improvements to the wound healing study

Should this experiment be conducted again, multiple adaptations to the protocol and sampling procedures could be considered. First and foremost, histology samples of both external and internal wounds would be obtained. Skin samples would be collected from both the immediate vicinity of the wound (0 cm) and a bit farther away (1.5 cm, as done in this study), enabling a comparison of the wound's direct and peripheral effects. An internal control, such as a sample from a different part of the individual (opposite side), could also be incorporated. Moreover, sampling of the internal wound tissue could be introduced, potentially providing novel insights into the healing process at the wound's core. The physical strength of the wounds could be evaluated using tensile strength assessments. These assessments would yield important data about the structural integrity of the wounds over time and between the two treatments. In conjunction with tensile strength measurements, an analysis of collagen types I and III in the skin tissue could offer additional information about the differences in skin structure and transition between the two treatment groups (Mathew-Steiner et al., 2021). Finally, digital image analysis could be employed to record wounds, overlaying the wound size and inflammation. This technique would offer a more accurate representation of the actual wound condition.



	Week 1 Wound vs Control		Week 4 Wound vs Control		Week 8 Wound vs Control	
	Upregulated	Downregulated	Upregulated	Downregulated	Upregulated	Downregulated
Sorted by log2FC	• Hepcidin antimicrobial peptide • Creatine kinase S-type • Iodotyrosine deiodinase 1 • Serum amyloid A protein • Tumor necrosis factor - inducible gene 6 protein-like	• Protein shisha-8 • Fast myotomal muscle troponin-T • Lipase maturation factor 1 • PHD finger protein 24-like • Troponin C, skeletal muscle	• Apolipoprotein C-I • Homeobox protein EMX1-like • Serum amyloid A protein • Cytosolic Fe-S cluster assembly factor nubp1-like • Centromere protein V	• Estrogen-related receptor gamma • Immunoglobulin V-set domain • Synaptopodin 2-like protein • Small nucleolar RNA SNORD31 • Storkhead-box protein 2-like	• Fructose-bisphosphate aldolase • Apolipoprotein C-I • Cartilage oligomeric matrix protein • Dynein light chain roadblock-type 2 • Albumin 2-like	• Guanylate cyclase 1 a2 • Nuclear factor of activated T cells • Zinc finger homeobox protein 3 • Synaptotagmin XIa • Kinesin-like protein KIF1A
Sorted by *p-adj*	• Kinesin-like protein • Alpha-1,3-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyl-transferase C-like • G-2 and S-phase expressed 1 • Cyclin-dependent kinase 1-like • Anillin	• Splicing factor 3B subunit 1 • Collagen alpha-1(XXVIII) chain-like • Secernin-2 • PHD finger protein 20 • A-kinase anchor protein 11-like	• Actin, cytoplasmic 1-like • Anaphase-promoting complex subunit 5-like • 40S ribosomal protein S29-like • Signal peptide, CUB and EGF • Glycogenin-1	• TRAF3-interacting JNK-activating modulator • Kelch-like protein 9 • Semaphorin-4F-like • Tumor necrosis factor ligand superfamily member 14-like • Mitogen-activated protein kinase 2	• NADH:ubiquinone oxidoreductase complex assembly factor 5 • Solute carrier family 45 member 2 • Creatine kinase, muscle b • Pro-MCH 2-like • Claudin 26	• Butyrophilin-like protein 2 • Transmembrane protein 87A-like • Interleukin 11 receptor, alpha • T-cell leukemia homeobox protein 1-like • Fibronectin type III domain containing 3Ba

Week 1 Wound vs Control

GO enrichment analysis
- Microtubule-based movement 25-1
- Chromosome, centromeric region 10-0
- Microtubule binding 26-1

KEGG enrichment analysis
- Steroid biosynthesis 19-0
- Cell cycle 38-6
- DNA replication 15-1

Week 4 Wound vs Control

GO enrichment analysis
- Peptide metabolic process 28-0
- Ribosome 26-0
- Structural constituent of ribosome 26-9

KEGG enrichment analysis
- Cytokine-cytokine receptor interaction (0-19)
- Intestinal immune network for IgA production (0-6)
- Cell adhesion molecules (CAMs) 0-7

Week 8 Wound vs Control

GO enrichment analysis
- Regulation of hydrolase activity 0-4
- Collagen trimer 1-4
- Rho GTPase binding 0-8

KEGG enrichment analysis
- Glutathione metabolism 0-5
- Drug metabolism - other enzymes 1-4
- GnRH signaling pathway 6-1

Figure 15. Representation of the top five most highly expressed and the top five most significantly altered genes (as determined by padj) from the RNA-seq analysis conducted over weeks 1, 4, and 8. This analysis compares the Wound group to the Control group. Genes are illustrated within boxes, where green signifies upregulated genes, and pink signifies downregulated genes. Below the gene list, the most significant pathways derived from GO and KEGG enrichment analyses are shown. Numbers following each pathway represent the count of significantly upregulated genes versus downregulated genes within that pathway.

5.6 Stress, wound healing, and welfare. Final words.

All three papers in this thesis come from the same experiment and thus illustrate various aspects of chronic stress's impact on wound healing. **Paper I** identified that chronic stress increased visual inflammation severity and delayed internal wound healing, although it did not affect the complete outer wound healing timeline in all individuals. In **Paper II**, transcriptional data revealed more prolonged expression of *mmp*s in the skin tissue of the stressed group, along with a possible suppression of the early immune response in the head kidney. **Paper III** outlines extensive differences between the Stress^{+} and Wound groups at week eight, as shown in Figure 16. The escalation from zero differentially expressed genes at week one to nearly 12,000 at week eight is likely due to the chronic deterioration of the HPI-axis regulation, as evidenced in **Paper I**. This data is an additional point of reference to indicate that the effects of stress on the primary, secondary, and tertiary stress responses would have possibly been more pronounced and additionally observable in the transcriptional data. Key differences identified in **Paper III** in the Stress^{+} vs Wound comparison at week eight include increased reactive oxygen species with diminished protective mechanisms, reduced cellular processes, decreased ATP-encoding genes and mitochondrial function, heightened cell death and inflammation, and less evidence of late-phase healing.

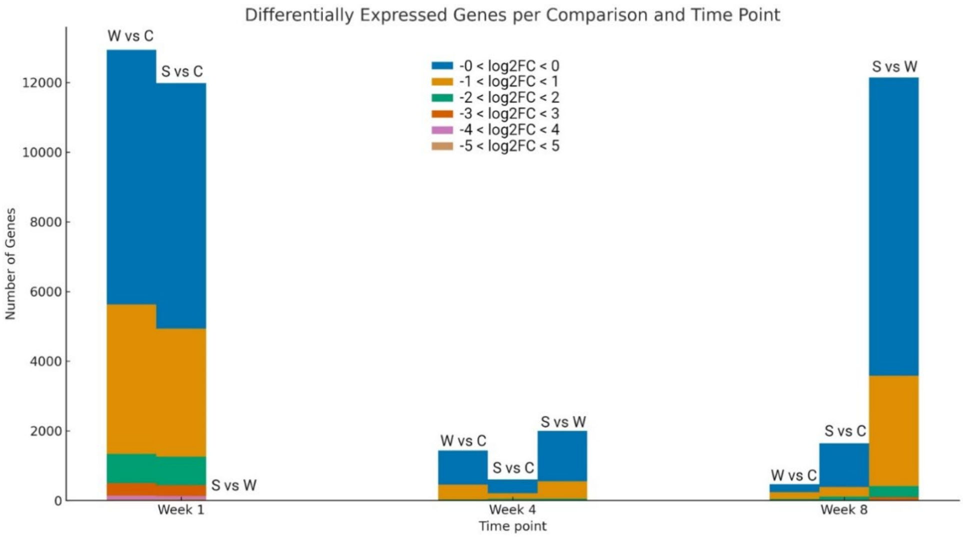


Figure 16. The number of genes that display significant differences between the Control (C), Wound (W), and Stress$^+$ (S) groups at three distinct time points. Each gene's significance is represented through log2 Fold Change (log2FC), with colour coding used to depict the range of log2FC values as presented in the provided legend.

In mammalian studies, it is well-documented that stress typically correlates with delayed wound healing (Kiecolt-Glaser et al., 1995; Marucha et al., 1998; Padgett et al., 1998; Kiecolt-Glaser et al., 2005; Christian et al., 2006; Vileikyte, 2007; Gouin and Kiecolt-Glaser, 2011; Fayne et al., 2020). Fewer studies exist on wound healing processes associated with stress response in fish. Sveen et al. (2018) researched the impact of chronic high fish density on wound healing in Atlantic salmon, while Mateus et al. (2017) investigated how stress (crowding) exposure four weeks before injury affected wound healing in gilthead sea bream. Both studies noted a stress-induced reduction in wound healing time, but the nature of the interaction varied. When stress coincided with the start of the experiment, it generally induced an enhanced transcriptional response regarding inflammation, similar to our findings. Pre-exposure to stress, however, elicited a suppressive response. As suggested in our study, individuals initially reacted to what may be an overactive immune response, which subsequently transitioned to chronic stress under continued stress exposure (Tort, 2011; Nardocci et al., 2014; Tort and Balasch, 2022).

While not directly focused on wound healing response, a study by Mateus et al. (2021) should be noted as it revealed that skin injuries from scale loss in sea bream can induce intestinal inflammation, a condition further exacerbated by chronic overcrowding stress. Skin injuries in fish have been associated with intestinal barrier dysfunction, emphasizing there is an impact beyond the immediate wound site, which may have profound effects on individuals (Chen et al., 2020).

The prolonged wound healing period following chronic stress added to the activation of stress responses and their tissue impacts, justifies serious welfare considerations. When conducting tagging studies, where deliberate wounds are made, it is vital to ensure the highest survival chances for the subjects. This requires increased oversight and stressor reduction during a time frame sufficient for substantial healing and would minimize risks such as mortality, tag loss, infections, and adverse welfare consequences. Extended recovery baths, careful pre-tagging planning, and strict post-tagging stressor management (avoidance of delousing, grading, handling, etc.) could be beneficial. Other factors, such as the time of year (with wounds healing slower in colder water) and water quality (algal blooms, run-offs, bacterial/viral presence) also influence wound healing and, by extension, individual welfare. Ongoing research into best practices for surgical incisions and healing aids should be a constant undertaking, alongside ensuring extensive training for those performing surgical procedures. This approach will help reduce potential challenges and ensure the best welfare outcomes, providing the most reliable data from the smart tags.

6 Concluding remarks

This doctoral research establishes that internal tagging does not induce a stress response in unstressed fish, whereas chronic stress triggers a breakdown of primary stress responses, fin erosion, and modifications to wound healing and skin tissue. Specific remarks concluded from the research are as follows:

- The surgical implantation of a dummy smart-tag did not cause any detrimental impact on the individuals concerning the measured stress responses and wound healing. Furthermore, internal wound healing is a slow-paced process, hence, tags should not be implanted directly adjacent to the healing surface to avoid potential complications and tag loss.
- Chronic stress has significant impacts on the HPI-axis due to the overproduction of ACTH, starting at week four, with cortisol following at week six. The chronic stress group also exhibited more severe fin erosion, supporting the reliability of fin erosion as a stress indicator.
- Chronic stress was found to influence wound healing, evidenced by increased internal wound size and external inflammation. Despite no changes observed for external wound closure, chronic stress demonstrated substantial effects on wounded skin tissue, featuring increased expression of inflammation-related genes in the initial weeks, and severe effects on multiple gene groups by the eighth week.
- A limited yet noticeable difference was observed in the expression of select genes related to wound healing, stress, and immunity between the wounded skin tissue and the head kidney.

Given that chronic stress is detrimental to fish welfare and wound healing, mitigating stress before and after tagging is important. This approach ensures the tagging of an individual without compromised welfare and provides the best sentinel fish to represent the population at a farm site.

7 Future perspectives

This thesis builds upon the foundations examining a tags impact on Atlantic salmon's endocrine response and wound healing process. This section highlights potential areas of interest for future research and reflects on oversights that emerged while completing the current study. By bringing these points to light, subsequent studies should be able to prevent similar oversights, thereby enhancing their design and outcomes.

Research perspectives and considerations:

Practical application:

- Practical analysis of wound strength, measured through tensile strength, could provide insights into easy-to-measure potential stress effects on wounds.
- Including general behaviour monitoring, for instance, through open-source tracking software such as idTracker (Pérez-Escudero et al., 2014).
- Feeding strategies could be optimized to provide a supplemental energy boost (more available ATP), during stressful events, such as transportation or de-lousing/grading procedures. The development of feeds to enhance wound healing or promote healthier mucus production is occurring. For instance, dietary changes during winter months could be a proactive measure to combat winter ulcers.

Technological tools:

- Including histological samples of the outer and inner wound to highlight the structural differences and the differences caused by stress. AI-based tools can assess these objectively (Sveen et al., 2021).
- The possible use of computer vision tools to measure the area of the wound and overlay each individual to get a realistic model of inflammation and wound sizes.

Transcriptional studies:

- Transcription study of the inside wound to discover what limits internal growth and if it is the same as the outside wound.
- Investigating the transcription of single cells within wounds in stressed individuals may help pinpoint the origins of issues and potential treatment aids.
- Taking samples of the skin at varying positions away from the wound could better highlight the exact processes taking place (such as directly at the wound and in 0.5cm increments away from it), as in this study, we highlight mainly processes taking place, not directly at the wound location but 1.5 cm away from it.

Tagging studies:

- Considering different size groups with varying tag sizes and the resulting impact on behaviour and swimming could validate or challenge the 2% rule, identifying a problematic tag size for the fish (initial tagging, how small can fish be?).
- Tag material and is there a beneficial coating which can be applied to reduce the foreign body response along with aiding healing.
- Introducing a sham surgery group to highlight changes from only the tag.
- Investigation into absorbable suture materials and their tensile strength. Could silver-zinc sutures be an option? The application of external closing glue, possibly imbued with silver or zinc, could also be explored. Additionally, using a cautery pen could be tested alone and in conjunction with sutures and glue.
- Temperature baths and diffusible substances in the water to aid healing of wounds in tagging studies.

Stress and injury response:

- Considering real-life situations, including trials on farm sites, or utilizing varied pre-stress scenarios in laboratory trials, obtaining a fish in the grow-out facilities that have experienced no previous stress may be difficult to determine and obtain. Thus, studies that explore different levels and forms of stress both prior to and during the tagging process could provide a more encompassing understanding of wound healing timelines.

- Including an un-wounded stressed control group will also highlight the difference associated with the wound's involvement in the stress response, as the wound undoubtable takes away from the available energy supply bringing allostatic overload closer and ATP demand up.
- Exploration of the acute stress response post-injury and its differing effects on skin and head kidney compared to chronic stress responses.

8 Practical implications

Insights for the industry on the application of smart tagging based on the outcomes of this thesis, despite all results being derived from controlled laboratory settings, including the following:

- Implementing smart tags on fish that have not been previously subjected to stress does not elicit chronic stress responses (primary, secondary, or tertiary stress responses). Thus, it could be considered a monitoring tool that does not adversely affect the fish via stress. The timing of the tagging procedure can influence the healing ability of individuals. Specifically, tagging after the fish has experienced stressful environmental or physical conditions may alter their immune response and energy availability, potentially increasing their susceptibility to diseases, delaying wound closure, and reducing tag retention. Thus, optimal timing should be considered to increase fish welfare and the success of tagging.
- Efforts should be made to relocate the tags from directly being in contact with the internal wound site post-tagging, either by using a tool such as tweezers to push the tag away or massaging the capsule further away from the wound area. Internal wounds from tagging take considerable time to heal; thus, the tag should not be placed directly above the wound to enhance tag retention and fish welfare.
- Absorbable sutures may be warranted to improve fish welfare and wound healing, as non-absorbable sutures may lead to tissue damage during the growth of individuals in long-term applications.
- Extensive fin erosion may be a reliable marker of chronic stress in fish. This observation can determine whether tagging should proceed at the grow-out site. If tagging is necessary and cannot be postponed, placing tags in fish exhibiting minimal fin damage is advised.

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Chronic stress negatively impacts wound healing, welfare, and stress regulation in internally tagged Atlantic salmon (*Salmo salar*)

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The desire to understand fish welfare better has led to the development of live monitoring sensor tags embedded within individuals for long periods. Improving and understanding welfare must not come at the cost of impaired welfare due to a tag's presence and implantation process. When welfare is compromised, the individual will experience negative emotions such as fear, pain, and distress, impacting the stress response. In this study, Atlantic salmon (*Salmo salar*) underwent surgical implantation of a dummy tag. Additionally, half of this group was introduced to daily crowding stress. Both groups and an untagged group were followed for 8 weeks using triplicate tanks per group. Sampling took place once a week, and where stress was given, it was conducted 24 h before sampling. Stress-related measurements were taken to understand if tagging caused chronic stress and explore the chronic stress response and its impact on wound healing. Primary stress response hormones measured included CRH, dopamine, adrenocorticotropic hormone, and cortisol. Secondary stress response parameters measured included glucose, lactate, magnesium, calcium, chloride, and osmolality. Tertiary stress response parameters measured included weight, length, and five fins for fin erosion. Wound healing was calculated by taking the incision length and width, the inflammation length and width, and the inside wound length and width. The wound healing process showed that stressed fish have a larger and longer-lasting inflammation period and a slower wound healing process, as seen from the inside wound. The tagging of Atlantic salmon did not cause chronic stress. In contrast, daily stress led to an allostatic overload type two response. ACTH was elevated in the plasma after 4 weeks, and cortisol followed elevation after 6 weeks, highlighting a breakdown of the stress regulation. Fin erosion was elevated alongside cortisol increase in the stressed group. This data suggests that tagging previously unstressed fish in a controlled environment does not negatively affect welfare regarding stress responses. It also indicates that stress delays wound healing and increases the inflammatory response, highlighting how continued stress causes a breakdown in some stress responses. Ultimately, the tagging of Atlantic salmon can be successful under certain conditions where proper healing is observed, tag retention is high, and chronic stress is not present, which could allow for the possible measurement of welfare indicators *via* smart-tags.

KEYWORDS

chronic stress, wound healing, inflammation, welfare, tagging

1 Introduction

Global salmon production in 2019 reached 3.8 million tons worldwide; on average, 15% of the salmon produced is lost, which is of significant concern for the producer, government, and the public (Ellis et al., 2012; Bang Jensen et al., 2020). The push to explain the underlying cause of mortality has been accompanied by the increasing need to understand and document fish welfare (Noble et al., 2018; Oliveira et al., 2021). Fish welfare has no universally defined definition or way of measurement. Still, one common consensus is to use The Farm Animal Welfare Committee (FAWC) guidelines for the Five Freedoms, which represent a framework of animal welfare, and to follow three types of welfare approaches; function-based, nature-based, and feelings-based (FAWC, 1996; Ashley, 2007; Kristiansen and Bracke, 2020). Regardless of how animal welfare is defined, one standard agreement can be made that it is the quality of life felt from the eyes of the animal itself that ultimately must be considered (Stien et al., 2013; Noble et al., 2018). To measure fish welfare, one integral component associated closely is the stress response, which can have both an improving or malicious effect on the wellbeing and survival of the individual, depending on whether the nature of stress is either adaptive or maladaptive (Wendelaar Bonga, 2011). The concept of stress introduced by Selye (1950) has been altered and modified over the years, and in recent years the notion of allostasis has been submitted to complement the concept of stress. Thus, more precisely describing the intricate role of primary mediators (e.g., glucocorticosteroids) (McEwen, 1998; McEwen and Wingfield, 2003; Iversen and Eliassen, 2014). Figure 1 summarises the complexity of the stress response in fish, focusing on the endocrine response.

While measurements of stress and welfare offer insight into the wellbeing of aquatic fish, the need how to measure them has led to the adoption of new methods. One method uses innovative tagging technology to provide real-time data on farmed fish's environment, behaviour, and physiology (Macaulay et al., 2021). Tagging gives fish a possible voice to communicate behavioural and physiological responses within a population through the tags that analyse and interpret multiple forms of data. Tagging technology has been implemented in terrestrial animal agriculture for real-time monitoring of individual animals to improve the response to compromised animal welfare in an approach termed; "precision livestock farming" (PLF) (Berckmans, 2014). The number of individuals farmed in aquaculture and the hardships of studying behaviour in the aquatic environment present a challenging task for farmers. Some of the issues that have been associated with decreased welfare and increased mortality are; sea lice infestation, diseases, water quality, temperature, salinity, predators, and algal blooms, (Ellis et al., 2012; Bang Jensen et al., 2020; Hvas et al., 2021; Oliveira et al., 2021). Tagging offers a possible tool for understanding these welfare challenges. However, tagging every individual within a grow-out pen at sea becomes economically and logistically unrealistic; thus, using a percentage of individual fish to represent the whole population is advisable (Føre et al., 2018). The invasive nature of tagging is a paradox, for, in the context of aquatic welfare, one should not compromise welfare when assessing it. As such, the implantation and presence of the tag cannot influence the expected behaviour, physiology, and welfare of individuals for them to be considered a viable representative of the population (Alfonse et al., 2020; Macaulay et al., 2021). Thus, it is essential to build scientific knowledge on the interplay between the invasive tagging process and any adverse welfare effects.

The process of tagging with larger internal tags requires an incision, which leads to the formation of an open wound. The wound must heal normally under stressful aquaculture conditions for precision livestock farming to be considered viable in aquaculture. Previous studies on salmonid wound healing show that the healing cascade consists of the immediate re-epithelialization of the wound coinciding with a longer than 2-week inflammation period (Sveen et al., 2019). Further tissue repair and remodelling can last several months, while scale regeneration can take over a year when the underlying muscle is damaged, even though the skin pigmentation resembles pre-wounding (Fontenot and Neiffer, 2004; Schmidt et al., 2016). This wound-healing cascade for humans has been shown to slow down significantly with stress (Christian et al., 2006). In Atlantic salmon, high fish density delays the epidermal and dermal repair of the wound site (Sveen et al., 2019). Surgical tagging, however, creates a manipulated wound site consisting of sutures and a deep wound that penetrates through the individual, which has seen limited research considering the effects on fish welfare. Therefore, the outcomes of tagging in aquaculture require more transparency, as outlined by Macaulay et al. (2021). Clear and consistent reporting of results will allow faster governmental and industrial adoption of tagging to ensure better welfare during the tagging process and provide unaffected welfare data from tagged individuals, ultimately providing live welfare status to farmers.

This study aims to document and explore how surgically tagged fish under unstressed and stressed conditions affect their wound-healing ability and fish welfare. Aquaculture practices consist of fish held at high stocking density to increase yield and profit. Thus, daily crowding stress was utilised as a chronic stress condition. Atlantic salmon (*Salmo salar*) was chosen due to its importance in aquaculture and its significance as the most researched farmed fish species with behaviour monitoring tags (Macaulay et al., 2021).

2 Materials and methods

The Norwegian Food Safety Authority approved the experiment on 24.05.2019 and it is registered under FOTS ID 19447.

2.1 Fish and housing

The 225 (270 total, including unsampled) Atlantic salmon sampled to conduct the study were of the strain AquaGen QTL-Innova SHIELD, hatched on 08.12.2018 at Cermaq hatchery department in Hopen, Norway. The fish arrived at the Mørkvedbukta research station (Nord University; Bodø 67.2804° N, 14.4049° E) on 09.05.2019, where they were smoltified and then transferred (21.04.2020) to nine isolated off-white circular indoor tanks (30 fish per tank) ($1.0\ m^{-3}$) with a continuous flow of seawater with salinity 33.5‰, the temperature of 7.3 ± 0.3°C, and oxygen level of 97.7% ± 5.2%.

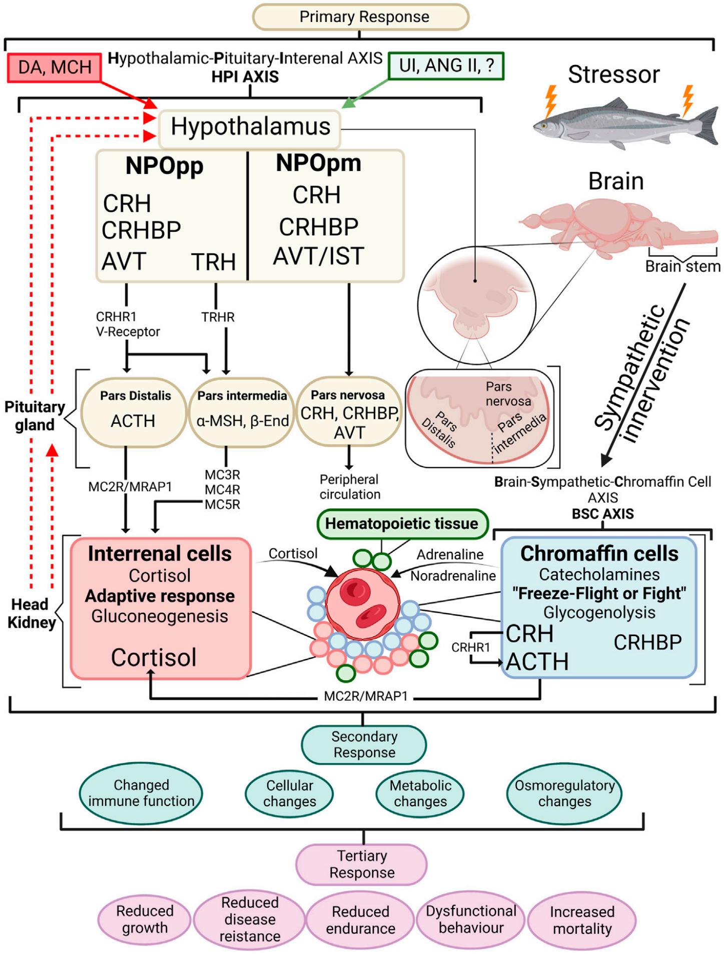

FIGURE 1
An overview of the stress response in fish with emphasis on the endocrine response. When a stressor is perceived, the primary stress response is activated by the BSC axis in the chromaffin cells of the head kidney where the initial freeze-fight or flight response releases adrenalin and noradrenalin in the circulation. Along with chromaffin cells the head kidney contains haematopoeitic tissue and interrenal cells. The HPI axis is activated starting within the hypothalamus where cortisol releasing hormone (CRH) containing axons are sent from the nucleus preopticus (NPOpp) to cells of adrenocorticotropic hormone (ACTH) within the pituitary. Simultaneously CRF-binding protein (CRFBP) is brought to the ACTH cells where CRF/CRFBP decides CRF bioactivity. CRF/CRFBP along with thyrotropin-releasing hormone (TRH) control the α-melanophore stimulating hormone (α-MSH) which has a role in stimulating cortisol release from the interrenal cells. ACTH through the melanocortin 2 receptor (MC2R)-melanocortin 2 accessory protein 1 (MRAP1) complex induces cortisol synthesis at the interrenal. Negative feedback is visible by dashed red lines showing negative feedback on the pituitary gland and hypothalamus. Stimulatory effects (green box) produced include urocortin I (UI) angiotensin II (ANG II) as well as arginine vasotocin (AVT) and isotocin (IST) among others, and negative feedforwards (red box) include dopamine (DA) and melanin-concentrating hormone (MCH). Primary stress responses lead to secondary stress responses which are physiological adjustments to a stressor. Tertiary responses are whole-animal responses and occur after secondary responses take place. β-End (beta-endorphin). Redrawn and modified from Gorissen and Flik (2016) and Khansari et al. (2017a).

The fish were kept under a 24-h light regime, with dry feed dispensed automatically in excess (Supreme, Skretting AS). The acclimation lasted 68 days until the start of the experiment on 28.06.2020. 30 fish with a mean weight of 1.01 ± 0.3 kg and a mean length of 43.1 ± 3.3 cm were held in nine tanks (in triplicate) at the start of the experiment.

2.2 Experimental design

To determine the effect of chronic stress and the impact of a wound after tagging, three experimental groups (in triplicate, a total of nine tanks) were used: (1) Control, (2) Wound, and (3) Wound + Stress. Control groups were undisturbed healthy individuals (Figure 2). The

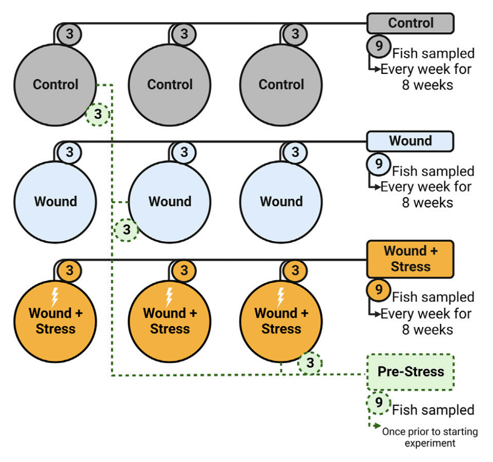

FIGURE 2
Experimental setup showing the nine tanks, three groups (Control, Wound, Wound + Stress), and the pre-Stress group. Numbers represent how many fish were taken from each tank on a sampling day (n = 9).

wound group had a dummy tag surgically implanted according to Section 2.2.2 and was used to show the effect of wound healing on unstressed fish. The wound + stress group underwent the same surgical procedure as the wound group but was also exposed to a daily crowding stressor to study the effect of chronic stress on stress responses, wound healing, and welfare. The experiment lasted 8 weeks, and sampling was conducted once per week on each experimental group for 8 weeks. Additional sampling was done 1 day before the start of the experiment, considered the pre-stress group.

2.2.1 Chronic stress

The experimental group "Wound + Stress" was subject to daily crowding stress. Stress was achieved by lowering the water level in the tank (triplicate) until only half the fish's body was submerged. The water was allowed to be at its lowest level for 30 s, and then the tank was filled up to normal levels. From when the water started to drain out of the tanks to when the water was back to normal took approximately 30 min (±30 s). The total fish density changed abruptly from 30 kg/m^3 to 315 kg/m^3 in these 30 min. The crowding stress was applied daily (starting on day one) at random times during an 8-h window. The last stress would always be used 24 h before sampling. Earlier studies by Iversen and Eliassen (2014) have shown that this crowding stressor was enough to elicit chronic stress after 4 weeks of exposure.

2.2.2 Surgical implantation

The implantation of a dummy smart tag with size 0.4 × 2 cm made with a 3D printer using high-density polyethene provided by Artic Seafood Group was placed into the fish in the wound group and the wound + stress group on day 0 of the experiment. All the fish in one tank were moved to a holding tank from which two fish at a time were moved into a small tank containing a dose of 60 mg/L of Finquel vet (MSD Animal Health Norge AS), with an aerator and a water pump. Once the fish were under the effect of general anaesthesia (stage 4), as described by Iversen et al. (2003), they were placed on the surgery table. The water pump connected to the anaesthesia bath continuously pumped the same 60 mg/L of Finquel vet water over the gills while the procedure took place. An incision of 1.5 cm was made with a scalpel (No 23, Swann-Morton, Sheffield, United Kingdom) and a tool that only allowed the blade to move 1.5 cm on the ventral surface, located between the pectoral fins and 1.5 cm behind the base of the fins. The dummy tag was cleaned with 75% ethanol and dried before inserting it into the abdominal cavity, where it would be next to the pyloric caeca. Two stitches were sewn with superficial interrupted knots tied on opposite sides of the incision using a non-absorbable 4/0 monofilament suture (www.resorba.com). Sutures were made to be sewn through the muscle not to affect the internal wound surface. The operation took between 60–90 s; once operated, the individual would be placed into a wake-up bath before putting it into its main tank, where it would be held for the duration of the experiment. Miiro Virtanen did all surgical procedures to avoid differing surgeon effects.

2.3 Sampling

Sampling occurred once per week for 8 weeks for each experimental group. Three fish from each tank belonging to the same group were randomly taken and placed in anaesthetic 10 L bucket baths containing Finquel vet (120 mg/L). One by one, fish were sampled for blood and then euthanised by cutting their spinal cord near the brain with precise scalpel placement. The fish was then measured for its weight, length, fin scores, and all wound parameters. Two blood samples were taken from the caudal vein complex with a 3 mL heparinised syringe. The blood was measured for glucose and lactate and placed in 1.5 mL Eppendorf tubes (VWR, Norway). It was then centrifuged at 5,000 rpm for 5 min. After this, the plasma was removed into new Eppendorf tubes and stored at −40°C pending further analysis. 72 fish were sampled for each experimental group, totalling 216 fish plus the nine pre-stress fish for the whole experiment.

2.4 Measurements and analytical methods

2.4.1 Plasma cortisol

Plasma cortisol levels were measured using the Enzyme-Linked Immunosorbent Assay (ELISA) method, using a DRG Cortisol ELISA kit (EIA-1887, DRG Instruments GmbH, Germany, 2020). The antibody-coated 96-well microplate provided works based on the principle of competitive binding. The manufacturer's instructions were followed, and the absorbance of each well was read by a 450 nm microtiter plate reader (Tecan Sunrise Remote, Bergman diagnostics, Austria) and corrected for optical imperfections by subtracting from 540 nm. Standards were run in triplicates, while samples, negative control, and positive control (also used to determine plate-to-plate variation) were run in duplicates. The assay has a dynamic range between 1.3–800 ng/mL. The intra- and interassay coefficients were 8.1% and 7.7%, respectively (EIA-

1887, DRG Instruments GmbH, Germany, 2020). Plasma cortisol is expressed in nmol/L (nM).

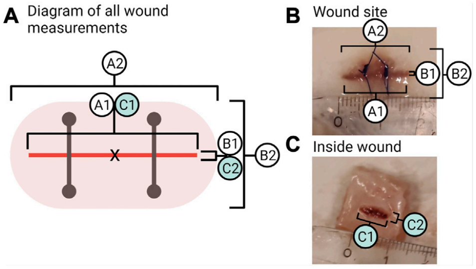

FIGURE 3
Wound measurements. **(A)** Shows all points measured of the wound where A1 is the length of the incision and B1 is the width of the incision. A2 is the length of inflammation, and B2 is the width of inflammation. C1 is the inside wound length, and C2 is the inside wound width. **(B)** Photo of a sample from week 2 in the group “wound + stress” showing the measurements of the outside wound for A1, B1, and A2, B2. **(C)** Photo of a sample from week 2 in the group “wound + stress” showing the inside measurements for C1 and C2.

2.4.2 Plasma ACTH

Plasma adrenocorticotropic hormone (ACTH) levels were measured by utilising the Enzyme-Linked Immunosorbent Assay (ELISA) method, using a Cusabio ACTH ELISA kit adapted for fish (CSB- E15926Fh, Cusabio Houston, TX, United States). The antibody-coated 96-well microplate provided works based on the principle of competitive binding. The manufacturer’s instructions were followed, and the absorbance of each well was read by a 450 nm microtiter plate reader (Tecan Sunrise Remote, Bergman diagnostics, Austria) and corrected for optical imperfections by subtracting from 540 nm. Standards were run in triplicates, while samples, negative control, and positive control (also used to determine plate-to-plate variation) were run in duplicates. The assay has a dynamic range between 75–1,200 pg/mL. The intra- and interassay coefficients were <15% and <15%, respectively (CSB-E15926Fh, Cusabio Houston, TX, United States). Plasma ACTH is expressed in pmol/L (pM).

2.4.3 Plasma CRH

Plasma corticotropin-releasing hormone (CRH) levels were measured by utilising ELISA method, using an Abebio CRH ELISA kit adapted for fish (AE64596FI, Wuhan Abebio science, Wuhan, China). The antibody-coated 96-well microplate provided works based on the principle of competitive binding. The manufacturer’s instructions were followed, and the absorbance of each well was read by a 450 nm microtiter plate reader (Tecan Sunrise Remote, Bergman diagnostics, Austria) and corrected for optical imperfections by subtracting from 540 nm. Standards were run in triplicates, while samples, negative control, and positive control (also used to determine plate-to-plate variation) were run in duplicates. The assay has a dynamic range between 0.8–20 ng/mL. The intra- and interassay coefficients were <8% and <10%, respectively (AE64596FI, Wuhan Abebio science, Wuhan, China). Plasma CRH is expressed in ng/mL.

2.4.4 Plasma dopamine

Dopamine (DA) levels were measured by ELISA method, using a Cusabio DA ELISA kit adapted for fish (CSB-EQ027496FI, Cusabio Houston, TX, United States). The antibody-coated 96-well microplate provided works based on the principle of competitive binding. The manufacturer’s instructions were followed, and the absorbance of each well was read by a 450 nm microtiter plate reader (Tecan Sunrise Remote, Bergman diagnostics, Austria) and corrected for optical imperfections by subtracting from 540 nm. Standards were run in triplicates, while samples, negative control, and positive control (also used to determine plate-to-plate variation) were run in duplicates. The assay has a dynamic range between 62.5–1,000 pg/mL. The intra- and interassay coefficients were <15% and <15%, respectively (CSB-EQ027496FI, Cusabio Houston, TX, United States). Plasma dopamine is expressed in pg/mL.

2.4.5 Plasma ions

Plasma was analysed for ions using the analytical instrument Respons 910 (DiaSys, Holzheim, Germany) with 1:2 sample dilution in ion-free water. Ions included for analysis were; chloride (Cl−, Chloride 21 FS, 40–170 mM), magnesium (Mg^{2+}, Magnesium XL FS, 0.08–3.00 mM) and calcium (Ca^{2+}, Calcium P FS, 0.22–4.00 mM). The interassay coefficient between the Response 910 analysis and the previously described analysis by Iversen and Eliassen (2014) for fish using 20 samples regarding chlorine and magnesium showed a variation of 8.3% and 2.1%, respectively.

2.4.6 Plasma osmolality

Plasma osmolality was analysed using a Fiske One-Ten Osmometer (Fiske Associates, Norwood, MA, United States).

2.4.7 Blood glucose

Blood glucose concentrations were measured from whole blood using the handheld Freestyle Freedom Lite^{TM} (Abbott Diabetes Care Inc., United Kingdom) and test strips from Ascensia Diabetes Care. Whole blood was applied to the test strips immediately after sampling. Glucose concentrations were read between 1.1–27.8 mmol/L (mM). Values below the detection limit were set to 1.1 mM (lower range limit).

2.4.8 Blood lactate

Blood lactate concentrations were measured using the handheld Lactate Scout + ^{TM} with its test strips (EKF Diagnostics for life). Whole blood was applied to the test strips immediately after sampling. Lactate concentrations were read between 0.5–25 mmol/L (mM). Values below the detection limit were set to 0.5 mM (lower range limit).

2.4.9 Fin erosion

Visual scores were given for five fins of the fish. These included the pectoral, pelvic, dorsal, anal, and caudal fins. Fin scores were given based on a compressed version of a scoring system introduced by (Hoyle et al., 2007) with minor modifications utilising an ordinal scale of 0, 1, 2, and 3, corresponding to erosion (0% of fin eroded), mild erosion (1%–24% eroded), moderate (25%–49% of fin eroded) and severe erosion (>50% of fin eroded), respectively. To reduce subjective variation, the scoring of fin erosion was done by Miiro

Virtanen, and fish was provided randomly and blindly throughout the different experimental groups by another person.

2.4.10 Wound parameters

To measure visible wound healing, six points of measurements of the wound region, as seen in Figure 3, were taken (to the nearest millimetre) from each fish during sampling. The incision length and width were measured (A1 and B1; Figure 3). The wound inflammation (red area) was measured by taking the length and width of inflammation (A2 and B2; Figure 3) from the incision point to the maximum point where inflammation can be seen. Inflammation is not static; the place and distribution between the sides of the incision will vary as the cause of inflammation can be due to the wound, the suture, abrasion, or all combined. Due to this and the fact that inflammation was random in its orientation, an oval shape of the mean width and length of inflammation was used to create an area showing the mean distribution of inflammation around the incision. The centre point is marked X in Figure 3A, where the measurements cross-section pass. Inside wound healing was measured by taking the length and width of the visible wound (C1 and C2; Figure 3). As the injury was a narrow incision, the length for A1 was measured as the visual line. Thus, even if healed, the line would still be measured as the visible disruption of the skin. While for the width of the incision (B1), a healed wound was determined as the presence of no measurable open wound. The inflammation length (A2) was measured until it matched the length of the incision (A1), after which the reported inflammation would be the same as the incision length.

2.5 Statistical analysis

All data were tested for normality using Shapiro-Wilk's test and for homogeneity of variance using Levene's test. One-way ANOVA was performed from the start of the experiment (pre-stress) to each sampling time point and within each sampling point for each physiological and morphological parameter measured to identify the difference within groups and the pre-stress. Tukey's *post hoc* multiple comparisons test was carried out to determine if the F-values were significant. When data did not follow Gaussian distribution, Kruskal–Wallis ANOVA (non-parametric) with Dunn's multiple comparisons test was conducted. To study the relationship between plasma cortisol (primary stress response) and fin erosion (tertiary stress response), one performed a Spearman's rank correlation coefficient (rho or r). Wound measurements, inflammation area and inside wound area were compared using an unpaired *t*-test with Welch's correction, and if not following normal distribution unpaired Mann-Whitney test was done. The triplicate tanks were compared for all parameters with the method previously described for group analysis to identify any tank effect. No such tank effects were discovered. Statistical analysis and graphs were performed and created with GraphPad PRISM v9.3.1 (GraphPad Software, Inc., California, United States). The significance of the results was determined at a $p < 0.05$. Results are represented as mean with standard deviation (SD). Statistical significance in figures and tables within a group at different times compared to pre-stress was indicated by *, and the difference between the experimental groups at the same sampling time was indicated by superscripts a and b in tables and graphs at a significance level of 0.05.

3 Results

During the experimental period, mortality and tag retention was recorded. The control group's survival rate was 98.6%, while a survival rate of 97.2% and tag retention of 100% were registered for the wound group, and a 98.6% survival rate and 100% tag retention were recorded for the wound + stress group. There were no significant differences between the groups.

3.1 Primary stress responses

The primary stress responses measured include CRH, dopamine, ACTH, and osmolality, as shown in Table 1.

3.1.1 CRH

In week 3, the average plasma CRH in the wound + stress group was significantly higher than in the control and wound groups ($p = 0.0033$). The control group's plasma CRH was significantly higher than the wound group at week 7 ($p = 0.016$), and the wound + stress group at week 6 ($p = 0.0012$). Significant differences in the pre-stress group were observed in the wound + stress group at weeks 3 and 4 and in the control groups at weeks 6, 7, and 8 (Table 1).

3.1.2 Dopamine

The changes in levels of plasma DA during the experiment are shown in Table 1. The average plasma DA in the wound group was significantly higher compared to the wound + stress group during weeks 1 ($p = 0.027$), 5 ($p = 0.049$), 6 ($p = 0.019$), and 7 ($p = 0.011$). The average plasma DA in the wound group was significantly higher compared to the control group in week 7 ($p = 0.012$). Significant differences compared to the pre-stress group were observed in the control group at weeks 3, 5, 6, 7, and 8, at all-time points for the wound group, and during weeks 3, 5, 6, 7, and 8 for the wound + stress group (Table 1).

3.1.3 ACTH

The changes in levels of plasma ACTH during the experiment are shown in Figure 4. The average plasma ACTH in the wound + stress group was significantly higher than in the control group during weeks 4 ($p = 0.0007$), 5 ($p = 0.012$), and 7 ($p = 0.009$). Additionally, the wound + stress group had significantly higher average plasma ACTH than the wound group during weeks 4 ($p = 0.001$) and 5 ($p = 0.005$). Significant differences compared to the pre-stress group were observed in the control group at weeks 1, 4, and 5, while for the wound group, it was observed during weeks 1, 2, 4, 6, 7, and 8, and for the wound + stress group during all weeks after week 1(Table 1).

3.1.4 Cortisol

The changes in plasma cortisol levels during the experiment are shown in Figure 4. The average plasma cortisol in the wound + stress group was significantly higher than in the control group during weeks 6 ($p = 0.0005$), 7 ($p = 0.0001$), and 8 ($p < 0.0001$), and for the wound group during weeks 6 ($p = 0.0001$), 7 ($p = 0.0037$), and 8 ($p = 0.0026$). The stress + wound group also had significantly higher cortisol levels in weeks 6 and 8 than pre-stress values. The wound

TABLE 1 Mean ± SD (n = 9) for CRH (ng/mL), Dopamine (DA) (pg/mL), ACTH (pmol/L) and cortisol (nmol/L) in control (C), wound (W) and wound + stress (WS) during an 8-week experimental timespan (primary stress response).

	Group	Pre-stress	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
		M ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*	*M* ± *SD*
CRH (ng/mL)	C	1.48 ± 0.29	1.73 ± 0.51	1.75 ± 0.53	1.51 ± 0.36^{a}	1.60 ± 0.43	1.36 ± 0.57	2.49 ± 0.86^{*a}	3.13 ± 2.46^{*a}	3.70 ± 3.18*
	W		1.62 ± 0.24	1.77 ± 0.25	2.01 ± 1.09^{ab}	1.65 ± 0.41	1.78 ± 0.77	1.65 ± 0.56^{ab}	1.91 ± 1.91^{b}	3.46 ± 3.41
	WS		1.78 ± 0.28	1.78 ± 0.28	5.44 ± 6.10^{*b}	2.71 ± 1.63*	1.83 ± 0.99	1.28 ± 0.44^{b}	1.71 ± 0.66^{ab}	2.56 ± 1.34
DA (pg/mL)	C	243.4 ± 18.0	292.2 ± 62.7^{ab}	267.5 ± 41.0	278.3 ± 26.4*	292.7 ± 61.4	303.5 ± 48.0^{*ab}	329.0 ± 48.8^{*ab}	301.5 ± 21.1^{*a}	427.1 ± 109.8*
	W		302.6 ± 39.8^{*a}	293.5 ± 22.5*	285.6 ± 21.0*	305.3 ± 57.4*	324.7 ± 37.7^{*a}	347.9 ± 30.9^{*a}	365.7 ± 68.8^{*b}	391.8 ± 80.2*
	WS		253.7 ± 45.5^{b}	272.8 ± 45.9	281.4 ± 26.2*	277.8 ± 33.4	282.0 ± 16.5^{*b}	304.1 ± 17.2^{*b}	300.2 ± 22.6^{*a}	385.6 ± 49.0*
ACTH (pmol/L)	C	34.46 ± 4.14	44.83 ± 3.69*	39.28 ± 9.35	41.99 ± 6.48	52.84 ± 8.50^{*a}	55.93 ± 12.37^{*a}	47.63 ± 9.32^{a}	55.22 ± 14.01^{a}	50.35 ± 4.38^{a}
	W		44.6 ± 6.91*	45.53 ± 7.98*	43.73 ± 10.10	52.94 ± 9.04^{*a}	47.99 ± 6.13^{a}	76.64 ± 29.00^{*b}	83.42 ± 36.66^{*ab}	62.48 ± 12.84^{*ab}
	WS		39.93 ± 6.66	51.09 ± 9.30*	48.53 ± 4.35*	198.95 ± 81.55^{*b}	124.39 ± 78.72^{*b}	79.31 ± 6.10^{*b}	132.5 ± 98.76^{*b}	72.33 ± 25.40^{*b}
Cortisol (nM)	C	15.12 ± 14.87	18.66 ± 38.77	16.91 ± 28.29	9.74 ± 16.21	9.50 ± 14.02	17.14 ± 19.64	3.46 ± 3.60^{a}	1.68 ± 0.00^{a}	1.68 ± 0.00^{a}
	W		4.54 ± 7.08	5.08 ± 10.19*	11.26 ± 28.75	8.97 ± 18.12	5.46 ± 11.11	9.54 ± 15.08^{a}	20.67 ± 30.37^{a}	10.52 ± 14.60^{a}
	WS		31.92 ± 40.60	5.07 ± 10.15*	11.19 ± 28.53	39.44 ± 69.90	40.14 ± 43.61	80.89 ± 36.81^{*b}	71.51 ± 62.77^{b}	93.38 ± 98.58^{*b}

C = Control, W = Wound, WS = Wound and Stress. Values represent means ± SD, n = 9 per treatment/week. Means in a column (week) within the same measurement that have differing superscript letters a-b indicate significant differences ($p < 0.05$). Asterisks* show a significant difference compared to the Pre-stress group ($p < 0.05$).

and stress + wound groups had significantly lower cortisol levels in week 2 compared to the pre-stress values (Table 1).

3.2 Secondary stress responses

The secondary stress responses measured include glucose, lactate, magnesium, calcium, chloride, and osmolality, as shown in Table 2.

3.2.1 Glucose

Blood glucose had no difference between the three groups at any time point, while all groups and time points were significantly higher than pre-stress values (Table 2).

3.2.2 Lactate

Blood lactate was higher only during week 8 ($p = 0.0046$) in the control group compared to the wound group. Plasma lactate differed from the pre-stress levels in the control group during weeks 4, 5, 6, 7, and 8, for the wound group during weeks 3, 4, 6, 7, and 8, and for the wound + stress group during weeks 3, 4, 6, and 8 (Table 2).

3.2.3 Magnesium

The average plasma magnesium in the control group was significantly higher at week 2 compared to the wound ($p = 0.014$) and wound + stress group ($p = 0.035$). The wound group ($p = 0.0017$) and wound + stress group ($p = 0.036$) were significantly higher at week 6 compared to the control group. Additionally, the wound group had significantly higher magnesium values in week 8 ($p = 0.0089$) compared to the wound + stress group. Plasma magnesium differed from the pre-stress values for the wound group during weeks 6 and 8 (Table 2).

3.2.4 Calcium

The average plasma calcium in the control group was significantly lower at week 6 ($p = 0.0086$) compared to the wound group and significantly higher at week 8 ($p = 0.046$)

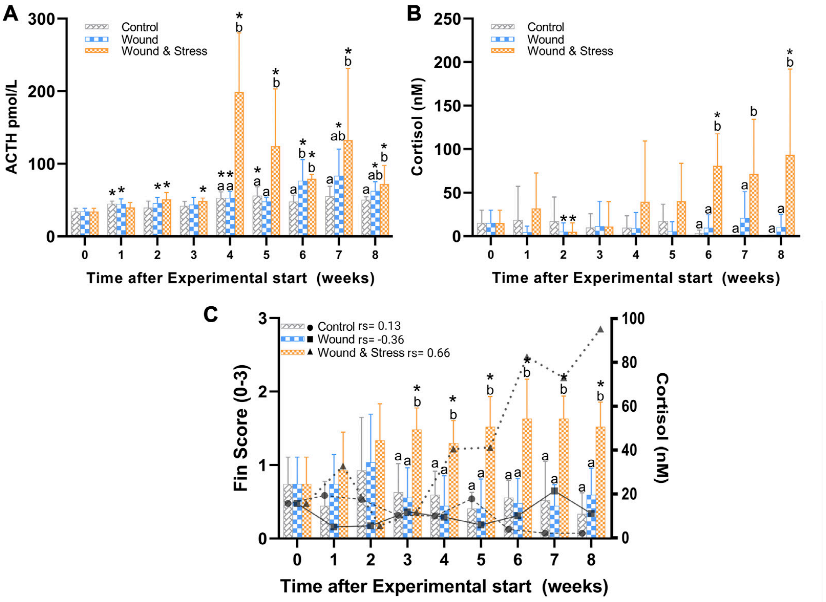

FIGURE 4
The average (±SD) changes in plasma ACTH **(A)**, cortisol **(B)**, and a combined average of pelvic, pectoral, and anal fin erosion scores with an overlay of cortisol **(C)** in the control, wound, and wound + stress group (n = 9) during an 8-week experimental time. * Indicates a significant difference ($p < 0.05$) from each group to the pre-stress value and letters a-b represent the significant difference ($p < 0.05$) within a single time point for all three groups where sharing letters means no significance between the groups. In **(C)** the individual rho or rs is represented in terms of a Spearman's rank correlation coefficient indicated next to the group legend.

compared to the wound + stress group. Additionally, the wound group had significantly higher average plasma calcium values in week 8 (p = 0.0008) than the wound + stress group. Significant differences compared to the pre-stress group were observed in all groups only at week 8 (Table 2).

3.2.5 Chloride

The average plasma chloride in the control group was significantly higher at week 1 (p = 0.0015) compared to the wound group and significantly lower at week 3 (p = 0.028), 5 (p = 0.0006), and 8 (p = 0.0016) compared to the wound + stress group. Additionally, the wound group had significantly lower average plasma osmolality values in week 1 (p = 0.003), 3 (p = 0.0002), 4 (p = 0.0041), 5 (p = 0.027), and 8 (p = 0.037) compared to the wound + stress group. Significant differences compared to the pre-stress group were observed in the control group at weeks 1, 3, 4, 5, 6, 7, and 8, at weeks 1, 3, 4, 5, 7, and 8 for the wound group and during weeks 1, 3, 4, and 7 for the wound + stress group (Table 2).

3.2.6 Osmolality

The average plasma osmolality in the control group was significantly lower at week 1 compared to the wound group (p = 0.0074) and wound + stress group (p = 0.0028) and significantly higher compared to the wound + stress group at week 8 (p = 0.026). Significant differences compared to the pre-stress group were observed in the control group at weeks 1 and 7 and week 6 for the wound group. In contrast, no differences were found in the pre-stress and wound + stress groups (Table 2).

3.3 Tertiary stress responses

The growth is shown in Table 3. A significant decrease in weight was observed for the wound group compared to the control group for week 8 (p = 0.035). Additionally, the control group showed a significant increase in length compared to pre-stress values at weeks 7 and 8. At week 8, the control group was significantly longer than the wound and wound and stress group.

3.3.1 Fin erosion

Table 3 summarises the average fin score for all experimental groups. Significant differences were found for all five fins when comparing the wound + stress group to the control group and four fins, excluding the caudal fin for the wound group, compared to the wound + stress group. From week 3 until the end of the experiment,

TABLE 2 Mean ± SD (n = 9) of glucose (mM), lactate (mM), magnesium (mM), calcium (mM) and osmolality (mOsm/kg) in control (C), wound (W) and wound + stress (WS) during an 8-week experimental timespan (secondary stress responses).

	Group	Pre-stress		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
		M ± SD	*M ± SD*	*M ± SD*	*M ± SD*	*M ± SD*	*M ± SD*	*M ± SD*	*M ± SD*	*M ± SD*	
Glucose (mM)	C	2.71 ± 0.13		3.66 ± 0.38*	3.78 ± 0.82*	3.43 ± 0.37*	3.32 ± 0.47*	3.52 ± 0.39*	3.49 ± 0.22*	3.58 ± 0.39*	3.68 ± 0.43*
	W			4.07 ± 0.72*	3.32 ± 0.32*	3.44 ± 0.34*	4.06 ± 1.14*	3.27 ± 0.30*	3.63 ± 0.43*	3.57 ± 0.48*	3.48 ± 0.16*
	WS			4.03 ± 0.84*	3.54 ± 0.49*	3.58 ± 0.32*	3.44 ± 0.38*	3.43 ± 0.30*	3.64 ± 0.26*	3.69 ± 0.64*	3.82 ± 0.41*
Lactate (mM)	C	3.23 ± 0.69		3.67 ± 0.97	3.71 ± 1.02	3.99 ± 0.74	4.60 ± 0.71*	4.39 ± 0.97*	4.62 ± 1.09*	4.79 ± 0.81*	5.73 ± 0.90^{*a}
	W			4.56 ± 1.87	3.71 ± 1.17	5.08 ± 1.35*	4.34 ± 1.05*	3.72 ± 0.65	5.12 ± 2.04*	5.14 ± 1.45*	4.27 ± 0.65^{*b}
	WS			4.33 ± 1.31	4.20 ± 1.29	4.89 ± 1.53*	4.14 ± 0.51*	3.41 ± 0.58	5.29 ± 1.27*	4.54 ± 1.57	4.87 ± 0.99^{*ab}
Magnesium (mM)	C	1.20 ± 0.35		1.18 ± 0.17	1.43 ± 0.41^{a}	1.12 ± 0.28	1.02 ± 0.24	1.05 ± 0.13	0.96 ± 0.31^{a}	1.45 ± 0.47	1.44 ± 0.38^{ab}
	W			1.41 ± 0.35	1.01 ± 0.23^{b}	1.29 ± 0.34	1.17 ± 0.28	1.14 ± 0.28	1.60 ± 0.30^{*b}	1.50 ± 0.33	1.75 ± 0.25^{*a}
	WS			1.34 ± 0.49	1.07 ± 0.16^{b}	1.40 ± 0.36	1.14 ± 0.20	1.06 ± 0.20	1.40 ± 0.41^{*b}	1.22 ± 0.43	1.17 ± 0.34^{b}
Calcium (mM)	C	3.17 ± 0.29		3.42 ± 0.25	3.49 ± 0.36	3.29 ± 0.20	3.24 ± 0.37	3.31 ± 0.15	2.97 ± 0.65^{a}	3.54 ± 0.27	3.53 ± 0.30^{*a}
	W			3.32 ± 0.36	3.19 ± 0.39	3.40 ± 0.31	3.29 ± 0.25	3.23 ± 0.43	3.61 ± 0.19^{b}	3.49 ± 0.26	3.76 ± 0.22^{*a}
	WS			3.22 ± 0.33	3.19 ± 0.21	3.46 ± 0.23	3.33 ± 0.24	3.32 ± 0.17	3.39 ± 0.25^{ab}	3.10 ± 0.59	3.18 ± 0.35^{*b}
Chloride (mM)	C	133.1 ± 5.9		128.1 ± 8.7^{*a}	131.4 ± 20.8	115.4 ± 13.2^{*a}	120.4 ± 12.3^{*ab}	106.9 10.2^{*a}	117.9 ± 11.5*	112.8 ± 7.0*	108.9 ± 12.4^{*a}
	W			108.8 ± 10.7^{*b}	116.8 ± 10.4	103.8 ± 12.3^{*a}	109.3 ± 12.8^{*a}	114.7 ± 13.5^{*a}	119.9 ± 14.4	119.6 ± 7.0*	116.4 ± 15.3^{*a}
	WS			126.8 ± 11.2^{*a}	125.2 ± 10.1	130.9 ± 10.2^{*b}	131.3 ± 14.0^{*b}	128.4 ± 6.4^{b}	125.8 ± 12.1	117.6 ± 13.8*	131.3 ± 6.7^{b}
Osmolality (mOsm/Kg)	C	408.3 ± 25.7		348.8 ± 10.3^{*a}	381.3 ± 41.6	403.3 ± 37.9	416.6 ± 30.1	429.7 ± 30.9	433.9 ± 38.0	448.1 ± 39.3*	437.8 ± 27.1^{a}
	W			389.1 ± 28.7^{b}	399.7 ± 40.2	427.6 ± 37.1	417.6 ± 30.7	441.8 ± 81.0	466.7 ± 56.3*	432.0 ± 26.5	418.6 ± 29.0^{ab}
	WS			393.9 ± 32.2^{b}	402.3 ± 28.1	400.0 ± 30.7	406.4 ± 30.9	398.2 ± 29.9	435.7 ± 34.3	413.8 ± 32.2	403.2 ± 21.8^{b}

C = Control, W = Wound, WS = Wound and Stress. Values represent means ± SD, n = 9 per treatment/week. Means in a column (week) within the same measurement that have differing superscript letters a-b indicate significant differences ($p < 0.05$). Asterisks* show a significant difference compared to the Pre-stress group ($p < 0.05$).

there was a significant increase in the erosion of pectoral, pelvic, and fin regions in the wound and stress group compared to the control and wound group. For the wound + stress group, the severity of fin damage ordered from most damaged at week 8 compared to week 1 is pectoral, pelvic, anal, caudal, and dorsal. Figure 4 highlights the positive correlation between the aggregated fin score of the pelvic, pectoral, and anal regions and plasma cortisol in the wound and stress group (rs = 0.66, $p = 0.085$). A weak correlation was shown in the control group (rs = 0.13, $p = 0.76$) and a negative correlation in the wound group (rs = −0.36, $p = 0.38$) groups (Table 3).

3.4 Wound healing

The visually observed effect of internal tagging and internal tagging with daily stress with a focus on the size of the incision, inflammation, and inside wound was determined by measurements at weekly intervals starting at week one and ending on week 8 (Figure 5, where explanations of A1, A2, B1, B2, C1, C2 can be found in Figure 3 A). The incision length (A1) was not significantly decreased for the two groups and stayed within the 5% range of the original 1.5 cm incision. The width of the incision (B1) for both groups and all individuals at and after week 5 was 0.0 cm, thus, defined as a completely closed wound. Before week 5, the number of fish with completely closed wounds in the wound group compared to the wound + stress group is as follows (n = 9); week 1–0:1, week 2–5:2, week 3–4:3, week 4–6:4, and 9:9 for weeks 5 and onward.

The length of inflammation (A2) gradually declined from weeks 1, 2, and 3 for both groups, and from week 4 onward, it matched the wound incision length. The length of inflammation for the wound + stress group was significantly higher in week 1 ($p = 0.001$) compared to the wound group in week 1. The width of inflammation (B2) gradually decreased from week 2 onward, as both groups had an increase in inflammation width from week 1 to week 2; however, there was no significant difference between the groups at any time. When taking the area of inflammation (A2, B2) and calculating it as an ellipse, the wound + stress group had a significantly larger area of inflammation ($p < 0.05$) in weeks 1, 2, 3, 4, 5 and 8 compared to the wound group while the opposite was true for week 6. No differences between the two groups were seen in week 7.

The inside wound length (C1) gradually decreased in both the wound and wound + stress groups. However, the wound + stress group had higher length values in seven of the 8 weeks. The inside wound width also gradually decreased in both groups and was higher in six of the 8 weeks in the wound + stress group, whereas in weeks 3 and 6, the wound group had higher values. The wound width was significantly higher in week 1 ($p = 0.003$) and 8 ($p = 0.04$) for the wound + stress group compared to the wound group. When taking the area of the inside wound (C1, C2) and calculating it as an ellipse, the wound + stress group had a significantly higher area of inside wounding ($p < 0.05$) in weeks 1, 2, 4, 5, 7, and 8 compared to the wound group while the opposite was true for week 3 and no differences between the two groups were seen at week 6. Additionally, the wound group contained one individual at week 7 with 0 cm inside wound width (healed) and two individuals with 0 cm inside wound width at week 8, while the wound + stress group had no individuals with closed inside wounds during the trial.

4 Discussion

While partly overlooked by fisheries biologists, terrestrial biologists and statisticians have given considerable attention to tagging studies to improve techniques that estimate animal population size and mortality (Pine et al., 2003). However, using tags to monitor wild fish has recently become crucial in understanding otherwise hard-to-observe behaviours (Lucas and Baras, 2000; Cooke et al., 2004). Due to the development of aquaculture and technology, using tags to monitor fish has seen increasing interest as providing better welfare through live monitoring can secure healthier animals (Macaulay et al., 2021). While the development of tags proceeds in aquaculture, the effects that the initial insertion causes inflammation and the long-term impact on fish should not be overshadowed. The present experiment suggests that tagged Atlantic salmon post-smolts may not experience chronic stress from the tagging itself. However, tagged fish under daily crowding stress respond with an altered allostatic state and wound healing compared to unstressed fish.

4.1 The stress response

Aquaculture-produced fish will encounter stressful situations ranging from mechanical to environmental stressors (Eissa and Wang, 2016). During these stressful events, the fish responds by activating its stress responses, to which the HPI axis is a significant contributor. The HPI axis end product is the release of corticosteroids to redistribute energy utilisation into various organs to combat the altered metabolic demand of stress (Faught et al., 2016). Corticosteroids come in two classes, glucocorticoids (GR) and mineralocorticoids (MR) which can affect metabolism, immunity, and ion regulation (Krasnov et al., 2012; Faught et al., 2016). In a variety of fish species, it has been found that neuroendocrine factors increase rapidly (minutes) after exposure to acute stress and can last for hours. Comparatively, chronic elevations take longer (days/weeks/months) to be visible as controlling and regulating factors within the HPI axis breakdown (Vijayan et al., 2010).

4.1.1 Primary stress responses

CRH (alternatively named CRF; Corticotropin-releasing factor) is a neuropeptide hormone. In fish, it is the main regulatory factor of the stress axis while also having roles in immune response modulation and suppressing appetite, reproduction, and locomotion (Wendelaar Bonga, 1997; Bernier and Peter, 2001; Flik et al., 2006). Chronic stress effects on plasma CRH in fish to our knowledge, is yet to be studied. However, Pepels et al. (2004) showed that acute stress in tilapia caused plasma CRH to peak 11 min after the applied stressor and then decline to pre-stress levels. Findings from Pepels et al. (2004) corroborate similar findings found for humans that suggest plasma CRH has a half-life ranging from 4–20 min (Schürmeyer et al., 1984; Stalla et al., 1986). In mammals, circulating CRH stems from hypothalamic secretion into the hypothalamic-pituitary portal systems. While fish lack this portal vascular system and use direct innervation, it is believed that circulating CRH secretion in fish is associated with the caudal neurosecretory system (CNSS), as well as the lateral part of the ventral telencephalon (brain) and may be produced locally in organs such as the head kidney (Lu et al., 2004; Pepels et al., 2004; Sower, 2015; Gorissen and Flik, 2016).

In the current experiment, a significant increase of CRH within groups occurred during week 3 for the wound + stress group compared to the control group and in weeks 6 and 7 for the control group compared to the wound + stress group. There was, however, no consistent increase within a time point for all individuals within a group. Where increases in plasma CRH in individuals did occur, it did not correlate with any other primary or secondary stress response. Since the crowding stressor in the current experiment was given 24 h before sampling, the peak in circulating CRH produced could not be visible due to its short half-life in the plasma. Interestingly, Pepels et al. (2004) study showed that confinement stress of 48 h eliminated the plasma CRH and cortisol response to a novel acute stressor.

In comparison, the control groups experiencing the same acute stressor showed high plasma CRH and cortisol values. Desensitisation of the HPI axis to a stressor may happen; however, prolonged stressors have been shown to become maladaptive, and this can be seen with the increase of ACTH and cortisol in the latter half of the current study for the wound + stress group but not for CRH (Kristiansen and Bracke, 2020). It has been described that the HPI axis can be activated without CRH in mice *via* CRH-like hormones as long as the CRH receptors are present, and it is also known that fish CRH gene expression in the brain varies greatly between species and types of stressors given (Weninger et al., 1999; Lai et al., 2021). Additionally, it has been described that CRH expression in the brain increases following immune stimulation in goldfish (Volkoff and Peter, 2004). In our study, plasma CRH does not have a similar buildup within the plasma as seen with ACTH and cortisol during the 8-week experiment when sampling is taken 7 days after tagging and 24 h after stressor application.

The significant increase in the wound + stress group for ACTH during weeks 4 and 5, followed by elevated baseline levels of plasma cortisol from week 6, possibly represents HPI-axis changes that may lead to a chronically stressed state described as allostatic overload type 2. To enter this overload state, individuals will have an altered allostatic state that activates primary mediators to help maintain stability through change, where cumulative effects from the allostatic state result in allostatic load. Should allostatic load become a cumulative burden through prolonged exposure to stress, the individual experience allostatic overload. An acute adaptive response (allostatic overload type 1) is initiated when the energy needed exceeds the energy available. Therefore, the release of glucocorticoids causes a decrease in the energy demand of an individual by avoiding normal life history stages, decreasing the allostatic load. When energy needed does not exceed energy available, a chronic non-adaptive response (allostatic overload type 2) is present that increases the level of glucocorticoids, and allostatic load is not reduced (McEwen, 1998; McEwen and Wingfield, 2003; Goymann and Wingfield, 2004; McEwen and Wingfield, 2010). If allostatic overload continues, this results in damage instead of protection to the individual (McEwen and Wingfield, 2010). Baseline plasma cortisol levels in previously unstressed fish can be as low as 13.8 nM, while chronically stressed fish show values above 27.5 nM (Pickering and Pottinger, 1989; Van Zwol et al., 2012; Iversen and Eliassen, 2014). The applied stress used in the current study yielded chronically stressed values ranging up to a mean of 93 nM in week eight. Repeated acute stress has been shown to have higher cortisol values compared to permanent chronic stress. The sampling is taken 24 h after the stressor allowing for a chronic accumulative response to be monitored (Tort et al., 2004). The entering into this chronic stress state is supported not only by primary stress response parameters but also by the increase in the wound + stress groups fin erosion, wound inflammation, and internal wound healing, as they were seen to be significantly increased compared to the wound and control groups at some time points. Chronic stress and the prolonged increase in cortisol have been associated with several tertiary stress responses such as; decreased growth rates (Sadoul and Vijayan, 2016), reproductive dysfunction (Schreck, 2010; Pankhurst, 2016), increased susceptibility to disease (Yada and Tort, 2016), decreased survival (Gomes et al., 2003; Schreck and Tort, 2016), and disruption in osmoregulation (Sampaio and Freire, 2016; Vargas-Chacoff et al., 2021).

At all levels of the HPI-axis, the release of cortisol due to stress is regulated by negative feedback and inducing and inhibiting factors (Wendelaar Bonga, 1997; Mommsen et al., 1999). The negative feedback regulation in the wound + stress group possibly functioned from week one to three, but after that, the cortisol within the wound + stress group continued to increase gradually. A gradual decrease in HPI-axis reactivity has been previously observed in chronically stressed fish, where habituation and resistance to stress occur (Madaro et al., 2016; Moltesen et al., 2016). In the current study, habituation is not seen. It can be attributed to either a high stressor level or length of the experiment where ending the investigation too early might result in the conclusion of habituation, as could be assumed to be the case for the first half of this study. While the negative feedback regulation becomes dysregulated, its effect on dopamine is shown by lower levels in the stress + wound group compared to the wound group. Brain monoaminergic systems have been shown to increase dopaminergic activity, however, the available data is limited, and data for plasma dopamine regarding chronic fish stress is undocumented (Weber et al., 2012; Gesto et al., 2013). However, it has been shown that within the brain, dopamine activity is reduced by pro-inflammatory cytokines in Senegalese sole (Weber et al., 2015). In the current study, plasma dopamine was highest in all groups at week eight, where the holding tanks contained the lowest number of fish. If confirmed, increasing plasma dopamine by reducing tank density could impact study outcomes not currently considered in experimental designs.

When considering internal tagging, the implantation process for the wound group was shown to cause no chronic stress when the first sampling was taken 7 days post-wounding. Additionally, inflammation during wound healing did not activate the stress response; therein, no detectable bidirectional communication between the HPI-axis and the immune system was seen regarding the wound group within the framework of the study. However, bi-directional communication with interactions between the immune-and endocrine network, cannot be dismissed as a possible influence on the chronic stress response in the wound + stress group through elevated stress hormones, higher inflammation and slower wound healing in inside wounds (Stolte et al., 2008; Pérez-Casanova et al., 2010; Tort, 2011; Wendelaar Bonga, 2011; Tort and Balasch, 2022). The introduction of a wound is followed by an inflammation response which will produce inflammatory cytokines (discussed in 4.2) that are under glucocorticoid control.

TABLE 3 Mean ± SD (n = 9) of weight (kg), length (cm), pectoral, pelvic, anal, caudal, and dorsal fins (scored 0–3) in control (C), wound (W), and wound + stress (WS) during an 8-week experimental timespan (tertiary stress responses).

	Group	Pre-stress	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
		M ± SD	*M ± SD*	*M ± SD*	*M ± SD*	*M ± SD*	*M ± SD*	*M ± SD*	*M ± SD*	*M ± SD*
Weight (kg)	C	1.01 ± 0.27	0.96 ± 0.26	0.98 ± 0.28	1.20 ± 0.33	1.10 ± 0.22	1.20 ± 0.17	1.32 ± 0.31*	1.54 ± 0.22*	1.67 ± 0.43^{*a}
	W		0.97 ± 0.25	1.07 ± 0.30	1.09 ± 0.31	1.07 ± 0.11	1.25 ± 0.32	1.32 ± 0.30*	1.42 ± 0.23*	1.24 ± 0.27^{b}
	WS		1.16 ± 0.31	0.87 ± 0.21	1.14 ± 0.22	1.15 ± 0.26	1.25 ± 0.39	1.31 ± 0.23	1.38 ± 0.28*	1.43 ± 0.32^{*ab}
Length (cm)	C	43.14 ± 3.33	42.62 ± 3.22	42.67 ± 3.09	43.07 ± 3.48	43.00 ± 2.57	43.72 ± 2.22	44.70 ± 3.61	46.84 ± 1.86*	47.67 ± 3.93^{*a}
	W		42.21 ± 2.88	42.16 ± 3.59	42.34 ± 3.68	43.52 ± 1.54	44.21 ± 3.78	44.38 ± 2.70	45.73 ± 2.14	43.63 ± 2.87^{b}
	WS		44.12 ± 3.90	40.63 ± 2.91	43.74 ± 2.49	43.82 ± 2.44	44.29 ± 3.92	44.79 ± 2.46	45.60 ± 3.27	46.11 ± 3.14^{ab}
Pectoral fin	C	0.67 ± 0.71	0.33 ± 0.71	1.11 ± 0.60	0.67 ± 0.71^{a}	0.67 ± 0.50^{a}	0.33 ± 0.50^{a}	0.56 ± 0.73^{a}	0.33 ± 0.71^{a}	0.11 ± 0.33^{a}
	W		1.00 ± 0.71	1.56 ± 0.88	0.56 ± 0.53^{a}	0.33 ± 0.71^{a}	0.22 ± 0.44^{a}	0.22 ± 0.44^{a}	0.22 ± 0.44^{a}	0.56 ± 0.53^{a}
	WS		1.00 ± 0.87	1.56 ± 0.73	1.89 ± 0.78^{*b}	1.67 ± 0.87^{*b}	1.78 ± 0.44^{*b}	2.00 ± 0.71^{*b}	2.00 ± 0.50^{*b}	1.78 ± 0.83^{*b}
Pelvic fin	C	0.89 ± 0.60	0.56 ± 0.73	0.67 ± 1.00	0.56 ± 0.53^{ab}	0.22 ± 0.44^{*ab}	0.11 ± 0.33^{*a}	0.44 ± 0.73^{a}	0.56 ± 0.88	0.22 ± 0.44^{*a}
	W		0.44 ± 0.53	0.89 ± 0.78	0.22 ± 0.44^{*a}	0.00 ± 0.00^{*a}	0.00 ± 0.00^{*a}	0.44 ± 0.53^{a}	0.44 ± 0.53	0.33 ± 0.50^{a}
	WS		0.67 ± 0.50	1.22 ± 0.67	1.00 ± 0.00^{b}	0.78 ± 0.44^{b}	0.89 ± 0.60^{b}	1.22 ± 0.44^{b}	0.78 ± 0.44	1.11 ± 0.60^{b}
Anal fin	C	0.67 ± 0.50	0.33 ± 0.50^{a}	1.00 ± 1.00	0.67 ± 0.50^{a}	0.89 ± 0.60	0.78 ± 0.44^{a}	0.67 ± 0.50^{a}	0.67 ± 0.50^{a}	0.67 ± 0.50^{a}
	W		0.78 ± 0.67^{ab}	0.67 ± 0.50	0.89 ± 0.60^{ab}	1.00 ± 0.71	1.00 ± 0.87^{a}	0.78 ± 0.44^{a}	0.67 ± 0.50^{a}	0.89 ± 0.78^{a}
	WS		1.11 ± 0.78^{b}	1.22 ± 0.67	1.56 ± 0.53^{*b}	1.44 ± 0.53*	1.89 ± 0.78^{*b}	1.67 ± 0.71^{*b}	2.11 ± 0.60^{*b}	1.67 ± 0.71^{*b}
Caudal fin	C	1.44 ± 0.88	1.00 ± 0.87^{a}	2.00 ± 1.00	1.78 ± 0.67	1.33 ± 0.71	1.44 ± 0.73	1.00 ± 0.87^{a}	1.11 ± 0.60	1.00 ± 0.50^{a}
	W		1.67 ± 0.50^{ab}	2.22 ± 0.44	2.33 ± 0.87*	1.56 ± 0.53	1.78 ± 0.67	1.56 ± 0.53^{ab}	1.78 ± 0.67	2.22 ± 0.67^{b}
	WS		2.00 ± 0.71^{b}	2.22 ± 0.83	2.22 ± 0.67	1.89 ± 0.78	1.89 ± 0.60	2.11 ± 0.33^{b}	1.78 ± 0.44	1.67 ± 0.71^{ab}
Dorsal fin	C	0.78 ± 0.44	0.22 ± 0.44^{a}	0.33 ± 0.71	0.33 ± 0.50	0.56 ± 0.88	0.33 ± 0.50	0.33 ± 0.50	0.11 ± 0.33^{*a}	0.11 ± 0.33*
	W		0.56 ± 0.73^{ab}	0.33 ± 0.71	0.67 ± 0.50	0.11 ± 0.33*	0.33 ± 0.50	0.33 ± 0.50	0.11 ± 0.33^{*a}	0.56 ± 0.73
	WS		0.89 ± 1.05^{b}	0.44 ± 0.53	0.56 ± 0.53	0.56 ± 0.53	0.89 ± 0.60	0.78 ± 0.44	0.89 ± 0.78^{b}	0.67 ± 0.71

C = Control, W = Wound, WS = Wound and Stress. Values represent means ± SD, n = 9 per treatment/week. Means in a column (week) within the same measurement that have differing superscript letters a-b indicate significant differences ($p < 0.05$). Asterisks* show a significant difference compared to the Pre-stress group ($p < 0.05$).

Yet the exact role of cytokines within the stress axes is ill-defined (Tort and Balasch, 2022). What has been described is mainly the up and downregulation of pro and anti-inflammatory cytokines by glucocorticoids in various fish species (Verburg-van Kemenade et al., 2011; Nardocci et al., 2014; Philip and Vijayan, 2015; Yarahmadi et al., 2016; Khansari et al., 2017b; Reyes-López et al., 2018). The increase in cytokines following inflammation has the potential to activate the stress response, as is apparent with interleukin-6, where it has a role as a stimulating factor of CRH, prolactin, growth hormones, and ACTH, which in turn will increase cortisol (Calcagni and Elenkov, 2006; Žarković et al., 2008). Therefore, although the wound group showed no increase in stress responses, the cumulative effect of additional load on the HPI-axis through a daily stressor and the possible increase in cytokines should be considered.

4.1.2 Secondary stress responses

Secondary stress responses are reactive changes within the individual's physiology depending on the stressor it has encountered and is affected by primary stress responses. The most commonly

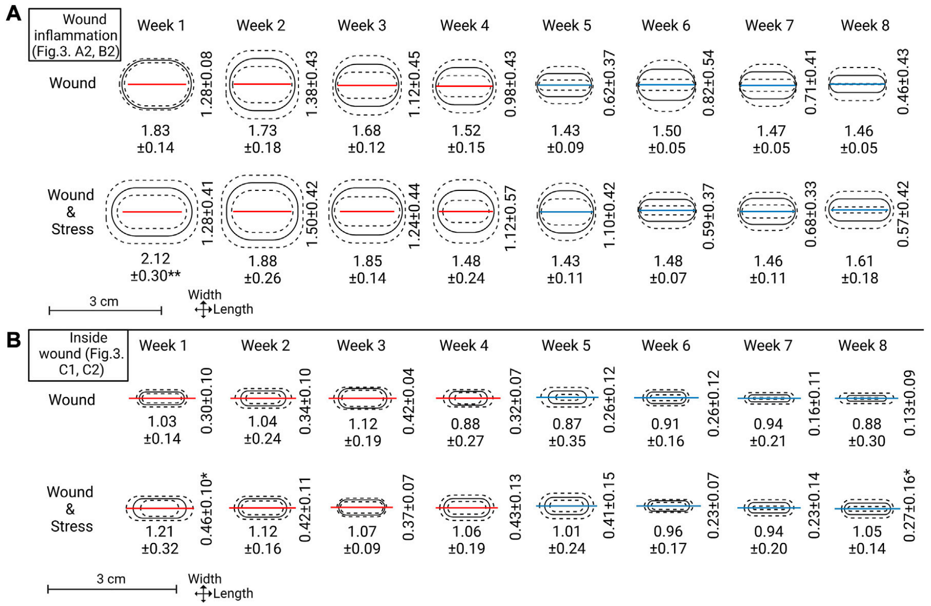

FIGURE 5

Top section **(A)**: Wound inflammation width and length measurements for each week are represented as means (solid line) ± standard deviation (dashed lines). The red or blue line within the ovals represents the mean length of incision (A1 in Figure 3). A red line indicates not all replicates within that week had wound width (B1 in Figure 3) = 0.0 cm and a blue line indicate all replicates within that week had wound width = 0.0 cm. Lower sections **(B)**: Inside wound healing width and length, represented the same as the top section. Mean incision length lines are carried over to represent scale and show a comparison point to the outside wound (note: section **(B)** is slightly zoomed see the length of scale). Significance between groups for length or width is represented as * = $p \leq 0.05$.

measured secondary stress responses include; (1) glucose which is influenced by increased catabolism as well as hormonal-stimulated glycogenolysis and gluconeogenesis; (2) lactate which fluctuates due to muscle glycolysis that produces ATP through anaerobic metabolism; and (3) ionic or osmolality changes resulting from the increase in catecholamine and cortisol release (Mommsen et al., 1999; Weber and Shanghavi, 2000; Liebert and Schreck, 2006; McEwen and Wingfield, 2010; Pankhurst, 2011; Sopinka et al., 2016; Weber et al., 2016; Noble et al., 2018). The effects of chronic stress on the secondary stress responses occurred during week eight, as magnesium, calcium, chloride, and osmolality were dysregulated in the wound + stress group. Additionally, the wound + stress group had significantly higher chloride levels in weeks 1, 3, 4, 5, and 8 compared to the wound group. This shows chronic stress and the later increase of cortisol through the breakdown of the HPI-axis *via* over-sensitivity of ACTH. However, this had few effects on secondary stress responses in this study. The stress + wound group showed changes in all four ionic parameters at week eight, which could signify the start of the breakdown of the osmoregulatory systems. Catecholamines have been shown to affect gill permeability and thus can disrupt the regulation of internal ions (Barton et al., 2003).

With the sampling protocol used, one can conclude that surgery does not cause a long-term increase in secondary stress responses and that the instigation of chronic stress in the wound + stress group does not seem to affect the secondary stress responses severely. However, care should be taken in being too conclusive, as the onset of changes in the HPI-axis only manifested itself after week 6 in the wound + stress group, as plasma cortisol became elevated 2 weeks before the end of the experiment. Notably, Iversen and Eliassen (2014) and Patel et al. (2022) previously showed that a group of Atlantic salmon and lumpsucker (*Cyclopterus lumpus L.*) that entered allostatic overload type 2 created only pronounced changes in plasma magnesium while other secondary responses (glucose, lactate, osmolality, chloride) were unaffected by the altered state of the HPI-axis during a six to 8-week trial.

Understanding the time frames during active stress responses should be considered. Experimental design and measurement times can describe different situations as the recovery of primary and secondary stress responses are fast (minutes to hours) after acute stress, while the effects of chronic stress can take weeks to develop, as seen in the current study (McDonald and Milligan, 1997; Wendelaar Bonga, 1997; Mommsen et al., 1999). Concerning animal welfare, it is imperative to remember that severe acute and prolonged chronic stressors can be shown not to affect the fish at a specific time when they are out of the peak zones and when studies are not carried out with long enough duration to develop the negative impacts. With this in mind, we acknowledge that the current study only focuses on chronic buildups and can miss any acute issues arising immediately after stress or those regulated before the sampling 24 h after applied stress.

4.1.3 Tertiary stress responses

4.1.3.1 Weight and length

When a prolonged stressor threatens homeostasis, the organism must distribute energy to the most vital processes to survive the stressor and neglect energy input into processes such as growth and reproduction due to the energetic cost of initiating the stress response (Wendelaar Bonga, 1997; Ashley, 2007; Wendelaar Bonga, 2011). In the current study, all groups showed a steady rise in growth. While stress and reduced growth rates are commonly associated, no significant growth reduction in the wound + stress group suggests that energy partitioning and inhibition of muscle growth promoters did not substantially occur (Faught et al., 2016; Sadoul and Vijayan, 2016).

Research into salmonid wound healing where stress is not considered has shown no difference between wounded groups compared to control groups in growth (Jensen et al., 2015; Liss et al., 2016; Schmidt et al., 2016; Sveen et al., 2018). When stress is present in the absence of wounds, it is shown to slow growth, likely from moving towards structural protein breakdown and inhibiting myogenesis and hormonal growth regulation, such as the impact cortisol has on the growth hormone/insulin-like growth factor I system (Sadoul and Vijayan, 2016; Vargas-Chacoff et al., 2021). Sveen et al. (2018) showed that chronically stressed Atlantic salmon with wounds did not significantly differ in body weight compared to control fish and that the control fish were slightly larger at the end of the experiment (57 days); these results are similar to our findings. This leads to the possible theorisation that wounded fish under stress cannot completely shift energy out of growth and reduce cytokine signalling, which happens when wounds are not present (Philip and Vijayan, 2015). This may be due to the need for growth factors and cytokines in the wound-healing process. Severe wounds allow the external environment to contact the internal, which can ultimately lead to death. As such, it is essential to allow repair to occur for survival. However, the current study found that internal wounds heal slower in stressed than in non-stressed individuals. While data to support this theory of wounds interfering with growth suppression through stress is lacking, it is interesting to find other systems where overall adverse effects can cause minor beneficial effects. One such system is the immune system which can benefit from otherwise debilitating factors, where acute stress, to some extent, has short-term positive effects on immunity compared to chronic effects (Khansari et al., 2017a; Khansari et al., 2017c). Had the current study run more extended, a decrease in growth could have been seen, as the HPI-axis broke down in the later part of the study, and wounds began to be completely healed. Having a group of only stressed fish without wounds and a group stressed before wounding could have helped to clarify some more.

4.1.3.2 Fin erosion

Fin erosion can be defined as the erosion of the epidermis, dermis, and fin rays which can be seen as damage to the fins in the form of fraying, splitting, size reduction, and loss of standard shape (Latremouille, 2003; Ellis et al., 2008). Fin erosion has been seen extensively with the onset of aquaculture, as it is found much less in the natural environment. Thus, due to its direct association with aquaculture and being externally visible, it has been accepted as a meaningful operational welfare indicator in fish (Ellis et al., 2008; Stien et al., 2013; Noble et al., 2018). Fins of fish are nociceptive, contain nerve cells, and have been shown by Roques et al. (2010) to have an acute response to fin clipping. This highlights the seriousness of fin erosion when discussing fish welfare. Fin erosion has been shown to have the capacity to heal, even to the extent that whole fins can regenerate, as seen in Zebrafish, suggesting that under good welfare conditions, fins should heal even after stressful events (Ellis et al., 2008; Pfefferli and Jaźwińska, 2015).

The experiment shows an increase in fin damage which appeared to be generally higher for all fins in the wound + stress group and had a significant increase towards the latter half of the experiment, starting at week three for the pectoral, pelvic, and anal fins when compared to the control and wound groups. This pattern is faintly present in the caudal and dorsal fins. When averaging the pectoral, pelvic and anal fins together, a positive correlation between cortisol and fin erosion was seen in the wound + stress group compared to the control and wound group. A positive correlation between fin erosion and scale cortisol has been described previously in rainbow trout (Weirup et al., 2021). Gregory and Wood (1999) also showed that cortisol-injected rainbow trout had significantly higher fin erosion than untreated fish. The current study shows that a daily chronic crowding stressor lasting 30 min causes fin erosion with differing rates and severity on each fin. While the most damaged fins appear on the ventral surface and the stressor applied was to crowd the fish, it can be assumed that tank and conspecific abrasion had a role in increased fin erosion along with aggressive behaviour (Noble et al., 2012). Interestingly, fin erosion is not seen in all species of farmed fish, leading to theorising that species with similar rearing practices and morphology must sustain damage from behavioural differences such as the absence or presence of aggressive fin nipping (Ellis et al., 2008). In future studies, a different stress method, such as chasing, can be used to assess how much impact tank abrasion had on fin erosion.

Fin erosions aetiology is not fully understood and is mainly theorised to be from abrasion with surfaces, aggressive behaviour in the form of nipping and biting, poor water quality, feeding regime, and in some cases, increased density (Ellis et al., 2008; Jones et al., 2010). Fin erosion can be viewed as a stressor that activates the HPI axis, which in turn will release glucocorticoids which have been shown to disturb the routine healing of wounds (Sveen et al., 2019). Øverli et al. (2002) demonstrated that 48-h cortisol treatment inhibited aggressive behaviour in rainbow trout, while 1-h short-term treatment did not inhibit nor significantly increase aggression. This may suggest that initial fin erosion can be caused by aggressive behaviour in stressed fish due to the resulting acts of causing the stressor, while prolonged erosion may be due to abnormal healing of fins by sustained HPI-axis activation or a possible culmination of both working simultaneously. Understanding the effects and relationships that cause fin erosion on each specific fin with biological factors, such as cortisol, or farming factors, such as feeding practices, can significantly increase our capacity to respond to harmful effects on welfare. The ease with which one can assess fin erosion should be appealing, as it can be a complementary observation to mandatory handling processes as well as possibly measured non-invasively with emerging imaging technology used for salmon lice counting (Cvetkovikj et al., 2015; He et al., 2016; Guragain et al., 2021).

4.2 Wound healing and tagging

When considering tagging fish, the main concerns are the effects on welfare, the healing process, survival rate, and retention of tags. Using a surgical tagging procedure and manipulating wounds by sutures, the current study provides insight into how healing proceeds in a controlled environment and how a daily crowding stressor can influence the visible healing process on the outside and inside of Atlantic salmon skin. One observed more significant inflammation area of wounds and slower wound healing closure on the inside for the wound + stress group compared to the wound group. Stress has slowed wound healing in humans, mice, reptiles, and several fish species (Kiecolt-Glaser et al., 1995; Marucha et al., 1998; Padgett et al., 1998; French et al., 2006; Sveen et al., 2018). A comprehensive review of stress and the immune system in fish has been established by Tort and Balasch (2022), while assessments for stress and wound healing have been established for humans (Christian et al., 2006; Vileikyte, 2007). The effect of chronic stress on deep cutaneous wounds in Atlantic salmon has been described by Sveen et al. (2018), as well as the impact of hydrocortisone implants on wound repair described by Roubal and Bullock (1988). The current study also confirms the expectation of wound healing slowing when the fish are chronically stressed.

The immune system and stress system have a location of significant crosstalk in the head kidney, where chromaffin cells, interrenal cells, and hematopoietic tissue are located to form a system known as the neuroimmunoendocrine regulatory feedback system (Tort and Balasch, 2022). The effects of stress on healing are mainly through the influence glucocorticoids have on pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-1α, IL-6, IL-8, and tumour necrosis factor-α (Christian et al., 2006; Guo and DiPietro, 2010; Serra et al., 2017; Tort and Balasch, 2022). In the current study, an increased inflammation exaggerated in the first 2 weeks for the stress + wound group can be identified compared to the wound group. Similar results to the current study of increased inflammation are presented by Sveen et al. (2018), who concluded that high fish density increased the transcription levels of inflammatory genes in Atlantic salmon within the first-week post wounding in the wound site. However, Hou et al. (2019) showed that in unwounded stressed rainbow trout, cortisol inhibits the release of pro-inflammatory cytokines IL-1β and TNF-1α, where slight activation of the HPI-axis can increase anti-inflammatory cytokines. There is a difference in the effect acute and chronic stress have on the immune system, and while cortisol regulation broke down at week 6 in the current study, inflammation in cold-water species of fish is most severe in the first 2 weeks (Tort, 2011; Sveen et al., 2020). Therefore, the inflammation occurs under daily stress where the chronic breakdown has not happened yet. In mice, an acute stressor given 24 h before another acute stressor showed accelerated cytokine production. Yet, the amplitude of increase was unaffected compared with only acutely stressed mice (Cheng et al., 2015). Thus, pro-inflammatory cytokines are released as a normal part of the inflammation process in wounds, and stress increases the time for wounds to heal. Stress can also reduce and increase the expression of specific pro-inflammatory cytokines, where concurrent stress possibly increases the rate of release post-stress but not amplitude (Johnson et al., 2005; Kiecolt-Glaser et al., 2005; Christian et al., 2006; Vileikyte, 2007; Tort, 2011; Manikowska et al., 2014; Cheng et al., 2015).

Despite its shortcomings, inside wound healing presents a unique method to isolate wound healing to muscular healing with an internal medium where the surface is scaleless, consists of no outer mucus and is free from abrasion that might be experienced during studies. In the inside, wound healing progressed slower for fish suffering from stress. They also presented zero completely healed wounds at the end of the experiment, whereas the wound-only group contained two fish with completely healed inside wounds at week eight. Inside wound healing times are often unconsidered and are vital when considering tag retention, as various internal tagging studies on multiple species have shown varying levels of tag retention (Hadden et al., 2018; Gerber et al., 2019; Walton-Rabideau et al., 2019; Macaulay et al., 2021; Marsden et al., 2021; Matthew, 2021). The current study highlights the importance of placing internal tags away from the incision site as much as possible to eliminate the protrusion of unhealed internal wounds by tags. The current study also highlights that the incision held by sutures closed on the surface within 4 weeks at 7.3 ± 0.3°C in stressed and unstressed Atlantic salmon. As Deters et al. (2012) suggested, suture retention is only beneficial up to the point of complete healing. After that, it becomes a point of increased inflammation and a source of infection. Thus, as telemetry tagging becomes more common in aquaculture, tagged fish should be maintained as stress-free as possible to ensure well-healed wounds and to identify sutures that can hold retention up to the point of healing. The measurement of internal healing times for target species should be considered and conducted as increased understanding can increase the success of tag retention, wound healing, survival, and ultimately the welfare of the individuals. While this study offers insight into tagging previously unstressed fish, this can be an unrealistic goal to achieve in the grow-out phase of aquaculture. Therefore, tagging fish subject to previous acute and chronic stress will allow further insight into possible harmful effects on wound healing and welfare and can help to contribute to the successful use of tags.

5 Conclusion

The main observations from the study are: (1) Wounded individuals do not suffer from chronic stress or chronic adverse welfare effects; (2) Wound + stressed individuals suffer from allostatic overload type 2 with an increase of ACTH starting at week four to an increase in baseline levels of plasma cortisol starting at week six; (3) Fin erosion is significant in stressed fish where damage to fins occurs well before the allostatic overload, indicating the possible use of fin erosion as a pre-indicator of chronically stressful conditions; (4) Stressed individuals suffer a more extensive inflammation period in weeks 1-2 while their inside wounds heal slower than unstressed wounded fish; (5) Due to the outside wound being sealed at week 4, the use of absorbable sutures should be considered with 4 weeks retention times to improve welfare and reduce unwanted damage; (6) Tag retention can be improved by placing tags away from the site of incision as internal wound healing was seen taking place in weeks 7 and 8 for a few unstressed individuals. Altogether, the results indicate that internal tagging, assuming a stress-free environment, does not compromise welfare within this study's selected parameters. While on the other hand, chronic stress disrupts the healing process and dysregulates the HPI axis, compromising welfare. Thus, tagging in grow-out facilities should prepare for a period of minimized stress post-tagging to provide the best welfare and wound healing.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by The experiment was approved by the Norwegian Food Safety Authority on 24.05.2019 and is registered under FOTS ID 19447.

Author contributions

MV: Helped conceive study design, performed sampling, analysed and interpreted results, and wrote the manuscript. MB: Helped conceive study design, writing-review, and edit DP: Helped conceive study design and performed sampling MI: Supervised the experiment, conceived the study design and interpretation of results and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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While fish might be in the dark when it comes to expressing their own well-being, with the use of real-time smart tags, they are now ready to spill the beans, creating a path to improve fish welfare in aquaculture. But wait, not so fast! We must pause and ponder whether implanting these smart tags inadvertently introduces stress, altering their behaviour so that measurements no longer reflect the broader population they are meant to represent. This thesis explores the physical, physiological, and transcriptomic responses to surgical tagging in stress-free and stressful environments. The findings suggest that tagging, in general, does not trigger long-term stress responses and that wound healing occurs. However, when a daily stressor is introduced, dysregulation of the stress axis is observed, profoundly affecting stress indicators, wound healing, and skin health. Therefore, it is important to consider when and how to tag fish since aquaculture facilities are not immune to stressful conditions.

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