Evaluating the Interaction between Isoeugenol and Nutraceuticals on Anaesthesia Efficiency in Salmonids:

Implications for Aquatic Welfare

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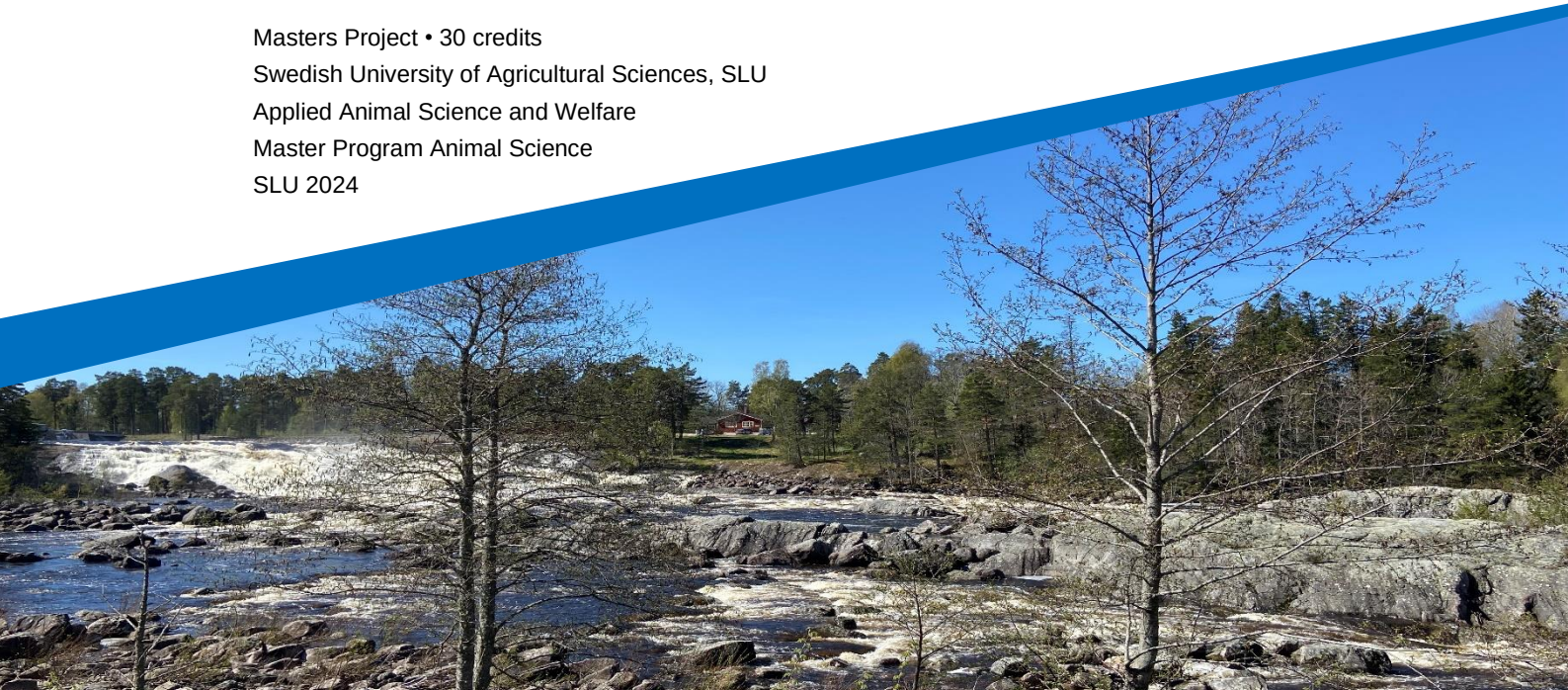

Evaluating the Interactions between Isoeugenol and Nutraceuticals on Anaesthesia Efficiency in Salmonids: Implications for Aquatic Welfare

Michelle Warren

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Abstract

The rapid growth of the aquaculture industry has brought increased attention to fish welfare, especially with rising demand for ethical and sustainable aquaculture practices. Traditional anaesthetics, like MS-222 (Tricaine), are effective but raise concerns related to fish welfare, environmental impact, and humane consumption due to their chemical nature. This study addresses these concerns by evaluating the efficacy of a novel herbal formulation combined with isoeugenol, a plant-derived anaesthetic, in promoting welfare outcomes for salmonids, specifically in rainbow trout and brown trout. The research involved a detailed assessment of the anaesthetic's impact on welfare parameters, examining stress biomarkers (cortisol) and tissue integrity through histological analysis of gills. Preliminary trials compared induction times, recovery rates, isoeugenol dose responses, and sedation depth of the herbal-isoeugenol formulation against MS-222. High-performance liquid chromatography (HPLC) was employed to analyse skin-on-fillet tissue concentrations, providing data to determine safe withdrawal periods for human consumption of isoeugenol-treated fish. Results showed that MS-222 achieved faster induction than lower doses of isoeugenol, while higher isoeugenol concentrations improved induction times. Nutraceutical additions had minimal impact on recovery times and anaesthesia depth but were associated with increased structural changes in *S. trutta* gills and elevated cortisol levels at higher doses. HPLC analysis detected residual isoeugenol in tissues 48 hours post-exposure, indicating potential influence of nutraceuticals on retention. The findings suggest that the herbal formulation combined with isoeugenol could offer a more sustainable, welfare-friendly alternative to conventional anaesthetics, with potential benefits for reducing stress, supporting recovery, and enhancing overall fish health. However, it is essential for future research to further investigate and refine optimal dosing and formation impacts. This research provides valuable insights into humane and environmentally responsible aquaculture practices, aligning with consumer demand for ethically produced seafood.

Keywords: Isoeugenol, Nutraceuticals, Anaesthesia, Recovery, Rainbow Trout, Brown Trout, Welfare, Stress, Aquaculture, HPLC, Histology, Cortisol

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Abbreviations

CV	Coefficient of Variation
DO	Dissolved Oxygen
DNR	Did Not Recover
ELISA	Enzyme-Linked Immunosorbent Assay
EO	Essential Oil
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
HPI	Hypothalamic-Pituitary Interrenal (axis)
HPLC	High-Performance Liquid Chromatography
ISO	Isoeugenol
mg/L	Milligrams per Liter
mL/L	Milliliters per Liter
MS-222	Tricaine Methanesulfonate
MSB	MS-222 with 25% Buffer
NUTRA	Nutraceutical
PAFS	Polyfluorinated Alkyl Substances or ‘Forever Chemicals’
SLU	Swedish University of Agricultural Sciences
µg/g	Micrograms per Gram
USDA	United States Department of Agriculture

1. Introduction

Global aquaculture production hit a record high of 130.9 million tonnes in 2022, marking a 6.6% increase from 2020 (FAO, 2024). This growth is attributed to transformations over the past few decades, including expansion, intensification, and technological innovations. However, the rapid growth of the sector has raised concerns about the welfare of farmed fish among consumers, researchers as well as policymakers. Historically, fish welfare has often been neglected based on the misconception that fish lack sentience or the ability to experience pain and suffering (Dawkins, 1998; Huntingford et al., 2006; Arlinghaus et al., 2007; Diggles et al., 2011; Diggles et al., 2024). However, over the past few years, this dogma that fish do not feel pain has been increasingly challenged by scientific research. Accumulating evidence suggests that fish possess nociceptors, and they exhibit physiological, behavioural, and biochemical responses to stress response and adverse conditions (Iwama et al., 1998; Sneddon, 2003; Xu et al., 2012; Ma and Lu, 2020; Zhang et al., 2023). These suggest that fish can feel pain in a way comparable to higher vertebrate-like mammals, with many researchers urging for objective evaluations of fish welfare based on the measurable indicators like stress hormones (Barton and Iwama, 1991; Arlinghaus et al., 2007; Diggles Et al., 201; Schreck and Tort, 2016).

Stress responses in fish are now widely recognised as reliable indicators of their welfare. Prolonged or acute stress can impair immune function, stunt growth, and increase susceptibility to diseases, all of which highlight the need for better welfare practices in aquaculture (Barton & Iwama, 1991; Schreck & Tort, 2016). Consumers demand for welfare-certified seafood has also risen significantly, with studies showing that a large proportion of consumers are willing to pay for more products produced under high welfare standards (Stubbe Solgaard & Yang, 2011). This demonstrates the growing importance of not only ensuring food Safety and quality, but also addressing the ethical concerns surrounding fish farming practices.

In aquaculture, anaesthetic agents play a crucial role in reducing the stress associated with handling, transport, and other procedures. Traditional synthetic anaesthetics, such as MS-222 (tricaine methanesulfonate or TMS), have widely been used as fish anaesthetics or sedatives. It acts by blocking sodium ions from

entering cells, silencing action potentials and preventing signal exchange between the brain (Wayson et al., 1976). MS-222 and other anaesthetics are commonly used in handling, transport, research, spawning practices, weighing, and other procedures. Traditional synthetic anaesthetics, such as MS-222, have been effective, but there are concerns regarding their environmental impact, toxicity, and consumer safety issues (Liu et al., 2018; Bavumiragira and Yin, 2022). Additionally, the lack of FDA-approved immediate-release sedatives, which do not require withdrawal periods, poses significant challenges to fisheries and aquaculture operations, particularly in public hatcheries and commercial farms (Trushenski et al., 2013). These issues have prompted researchers and aquaculturists to search for alternative anaesthetic agents or formulations that are both environmentally and consumer friendly. Plant-based products have emerged as promising alternatives due to their natural origin, lower toxicity, and broad biological activities, including sedative, antioxidant, and immunostimulant properties (Reverter et al., 2014).

Over the past years, natural essential oils like isoeugenol have gained significant interest for being used as natural anaesthetics, alternative to the synthetic ones, for application in aquaculture because of their availability, reduced toxicity, and potential benefits in reducing stress and improving fish welfare (Ross and Ross 2008; Ross and Ross, 2009; Sneddon, 2012). Isoeugenol, a natural phenylpropanoid commonly found in clove oil and other essential oils is highly effective in causing anaesthetic effects in fish (Small, 2003; Wagner et al., 2003; Small, 2004; Small & Chatakondi, 2005; Ross and Ross, 2009). It has been widely studied for its sedative and analgesic biological activities for use in aquaculture animals (Saydmohammed and Pal, 2009; Soltani et al., 2004). In fact, it has been shown to induce rapid sedation, offering a balance between effectiveness and safety. Additionally, isoeugenol is categorised as a Generally Recognized as Safe for a flavouring agent and Food Preservation by the Food and Drug Agency (Schroeder et al., 2021). However, it is noteworthy to mention that there are certain limitations to using isoeugenol alone in anaesthetizing fish. This includes a longer recovery period, which might also lead to inducing stress in fish. Additionally, the use of isoeugenol alone may not fully address the stress responses associated with fish handling. Both these can further compromise the health, welfare and performance of the fish. Combining isoeugenol with nutraceuticals possessing anti-stress effects presents a novel strategy to mitigate the stress experienced by fish during both handling and anaesthetic procedures. However, the anaesthetic effects of such a combination in fish, particularly in salmonids remain underexplored.

At the Aquaculture unit of the Department of Animal Science and Welfare, SLU, extensive studies have been performed over the past few years to develop anti-stress

formulations based on nutraceuticals derived from various plant sources. This formulation contains bioactive compounds combined in an optimized proportion. Preliminary studies conducted in the brine shrimp animal model and rainbow trout showed that the formulation generates robustness in the test animals when they are exposed to an optimum dose of the formulation. As mentioned above, to enhance its anaesthetic effects and reduce potential side effects, combining isoeugenol with an anti-stress nutraceutical formulation could be a potential strategy, however, that warrants exploration.

Salmonids, particularly rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*), play important roles in the aquatic ecosystems in Sweden, and hold significant ecological, economic, and cultural value (Jonsson and Jonsson, 2011; Stanković et al., 2015; Svanberg et al., 2020). Rainbow trout is the most farmed food fish species in Sweden, contributing substantially to the country's aquaculture industry. Meanwhile, brown trout, with its prominent role in recreational fisheries, is deeply valued for its ecological importance and cultural value. However, over the past few years, brown trout populations have been declining considerably, due to both human and anthropogenic activities, such as the construction of hydropower plants, habitat destruction, water pollution, and the growing impacts of climate change (Kovach et al., 2019). These factors have disrupted natural habitats, limiting the ability of brown trout to thrive in their native ecosystems. Swedish streams generally have low fish diversity, with salmonid species being more frequent and abundant in extreme environments where other species cannot thrive, suggesting their sensitivity to biotic interactions (Degerman and Sers, 1992). To address this, various conservation efforts, including restocking programs, have been implemented across Sweden to protect and restore these populations. Given the significant importance of both rainbow trout, from an aquaculture perspective, and brown trout, from a fisheries and conservation standpoint, it is essential to focus on stress and welfare management for these species. Proper management will not only improve the growth and health status of the fish but also will ensure the sustainability of both farmed and wild populations in the face of ongoing environmental and anthropogenic challenges.

1.1 Objective

The primary aim of this master thesis was to investigate the effects of a nutraceutical formulation in combination with isoeugenol on the anaesthesia of two important salmonid species, rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta* as well as to evaluate the gill histology, residual impact of the test formulations and the oxidative stress status of the fish exposed to the test formulation, compared to the most commonly used chemical anaesthetic, MS222.

In this thesis, we focused on rainbow trout and brown trout because of their commercial and ecological significance as described above.

1.2 Literature Review

1.2.1 The Welfare Debate – Can Fish Feel Pain

The aquaculture industry, due to experiencing substantial growth in recent decades, has gained increased attention from policymakers, scientists, and consumers regarding the welfare of farmed fish and the associated husbandry practices. Historically, the concept of fish welfare was often overlooked due to the misconception that fish lacked sentience and mental capabilities. Within these discussions were disagreements on behavioural and physical aspects of fish welfare. Huntingford et al. (2006) carried out an extensive review to explore whether or not fish and other lower-level vertebrates 'suffer' in adverse conditions and is context dependent. Within the review they find that fish welfare is highly context dependent because of the influence of species-specific needs, environmental conditions (captive, laboratory, wild, etc.) and the nature of human interactions, underscoring the need for tailored welfare approach. They also point out critical gaps in our understanding of mental capabilities, health indicators and practical welfare assessments, which are essential for improving aquaculture. There is an unresolved and controversial issue about animal welfare and views surrounding whether non-human animals can experience suffering (Dawkins, 1998). The connection between health and welfare is complex. When an animal or fish display disease symptoms, it's reasonable to conclude that it is in a state of poor welfare. For example, stress can lower immune function and increase infection which could be an indication of the fish's environment. This could imply the problem is human caused due to the mismanagement of the fish within captivity. However, this could be due to unavoidable stress caused by challenges when living within captive environments as unavoidable situations could invoke spikes in stress due to natural prey behaviour. Therefore, it is important to have a broad stroke of definitions that can be objectively used to define and describe welfare.

In terms of animal welfare three broad definitions are objectively used across animal welfare fields:

(1) **Feeling-based approach**: subjects mental state or psychological state, where welfare is achieved when an animal is free from suffering, such as pain or fear and has access to positive experiences (Huntingford et al., 2006; Arlinghaus et al., 2007; Diggles et al., 2011)
(2) **Function-based approach:** The welfare of the animal is gauged by its capacity to thrive in its environment. This includes maintaining good health, demonstrating robustness, and effectively coping with challenges without being overwhelmed (Huntingford et al., 2006; Diggles et al., 2011).
(3) **Nature-based approach:** Animal welfare based on the idea that animals have a natural instinct to express certain behaviours. Good welfare is achieved when animals in captivity can exhibit behaviours typical of their species in the wild (Huntingford et al., 2006; Diggles et al., 2011).

However, Arlinghaus et al. (2007) challenged 'feelings-based' approach to fish welfare, considering that many aspects are subjective. *(1) the feelings-based approach to fish welfare; (2) the artificial divide between human beings and nature; and (3) ways in which stakeholders can address fish welfare issues.* They urge more objective evaluations based on biochemical, physiological, and behavioural indicators (Dawkins, 1998). The idea being the neocortex, which in humans is an important part of the neural mechanisms that subject experience of suffering, lacks in fish and non-mammalian animals. Therefore, it has been argued that it's absence in fish indicate they therefore cannot perceive or experience suffering. Even in recent years, arguments continue encouraging sceptics in welfare policymakers regarding sentience in aquatic animals. Diggles et al. (2024) encourages win-win scenarios for both aquatic animals and stakeholders. Although they identify as a supporter of animal welfare, they emphasize that it's not an issue about choosing between welfare and no welfare for fish and aquatic invertebrates, but rather to ensure that important decisions about their welfare are based on scientifically robust evidence. Those who argue that fish are not sentient base their position on the absence of a neocortex, which, in mammals, handles emotion, sensory perception, and cognition. This Cartesian view (traditionally associated with the view that animals lack minds) suggests that while fish may physically react to injury, these responses are unconscious (nociception). Cartesian philosophy argues that to be capable of experiencing pain and suffering, an animal must have a conscious awareness of the painful experience beyond mere reflexive reactions.

Regardless of whether fish can feel pain, it's clear that studying non-mammalian animals, particularly in aquatic environments, presents challenges. Technology is often limited by the need for waterproofing and systematic application. Fish prey-response to human presence and intervention is difficult to assess in a non-stressful

way. Additionally, there may be challenges facing preventive action and welfare surrounding individual fish when there is a high colony population. Assessing fish welfare involves a multifaceted approach combining behavioural, physiological, and biochemical analyses. Therefore, it's important to observe changes in behaviour, such as increase avoidance responses, jumping activity and alterations in normal swimming activities, while also monitoring physiological and biochemical indicators like stress hormone, environments and vital signs. Long-term studies can track growth, reproduction, and survival rates, while pain and stress management research explore the effectiveness of analgesics. These comprehensive methodologies enable a thorough evaluation of fish well-being, identifying potential sources of distress or discomfort within a colony.

1.2.2 Consumer Views on Welfare Practices and Industry Standards

Consumers view animal welfare as a marker of food safety, quality, and healthiness and continue to voice an increasing desire to improve. Stubbe Solgaard and Yang (2011) conducted a study to investigate Danish consumers' willingness to pay for farmed rainbow trout with a quality label certifying good fish welfare, driven by retailer and consumer demands. Using a contingent valuation method and binomial logit model, data were collected from an online survey of 1,000 Danish consumers in 2009. Findings showed that 48% of respondents were willing to pay 25% extra for welfare-certified trout, with higher willingness among women, older consumers, those with longer education, higher income households, and those valuing eco-friendly production, freshness, and animal welfare.

The use of chemicals in aquaculture and their release into the environment are an increasing concern. Chemicals used in aquaculture, including anaesthetics, should be harmless and rapidly biodegradable with small withdrawal periods. One of the main anaesthetics used, Tricaine (MS-222), despite being the only FDA-approved aesthetic for fish, has a 21-day withdrawal period before human consumption (Jenkins et al., 2014). Global legislation exists covering a wide range of on-farm compounds and pharmaceuticals including the discharge limits and groundwater standards. Once pharmaceuticals reach the aquatic environment, they may degrade through a series of processes until completely metabolized. They and their intermediate metabolites may also accumulate in the aquatic environment and/or bioaccumulate in the ecosystem, where they often negatively affect many non-target organisms (Liu et al., 2018; Bavumiragira and Yin, 2022). Several studies are focusing on examining the fate and risk of pharmaceuticals in aquatic environments (Xu et al., 2021; Bavumiragira and Yin, 2022; Wang et al., 2020b). For instance, Wang et al. (2020b), observed environmental toxicology caused by NSAID's (anti-inflammatory drugs), such as ibuprofen and aspirin, caused

chloroplast deformation, reduced chlorophyll content and down-regulated photosynthetic genes in green algae, *Scenedesmus obliquus.*

With growing concerns, The European Green Deal sets out how to make Europe the first climate-neutral continent by 2050. It maps new sustainable alternatives to boost economy, improves people's health and quality of life, care for nature and leave no one behind. Within lies the Farm to Fork Strategy, central to the European Green Deal, which seeks to create a sustainable food system that benefits the environment, mitigates climate change, protects biodiversity, ensures food security and public health, and promotes fair, affordable and competitive food production (Schebesta, and Candel, 2020). In the aftermath of the COVID-19 pandemic, the economic downturn not only affected the livelihood of the people, health and well-being but also highlighted the importance of a robust and resilient food system that ensures access to an adequate supply of affordable food in all circumstances and disasters. Additionally, COVID-19 also has made the public abundantly aware of the value and interaction between our health, ecosystem, supply chains and consumption patterns. Therefore, the creation of a favourable food environment makes it easier to choose healthy and sustainable diets with the benefit of consumer health and quality of life. As part of the Farm to Fork Strategy, The European Commission plans to revise EU animal welfare legislation, including regulations on animal transport, with the revision being part of the Commission's 2023 work programme. EU animal welfare laws are aiming to enhance animal welfare and support the internal market with the current Transport Regulation (Regulation EC) No1/2005) adopted in 2004.

The growing emphasis on fish welfare reflects increasing consumer demand for ethically sourced seafood and humane treatment of farmed aquatic animals. Improved welfare standards address ethical concerns and contribute to enhanced product quality and safety, aligning with consumer preferences for sustainable and responsibly produced seafood. The United Nations' Food and Agriculture Organization (FAO) has been instrumental in advancing fish welfare on a global scale, developing guidelines, and promoting best practices to ensure ethical treatment throughout the aquaculture supply chain.

Global aquaculture production continued its increasing trend in 2020, 2021 and 2022, undisrupted by the COVID-19 pandemic. World aquaculture production in 2022 achieved a record of 130.9 million tonnes, up by 8.1 million tonnes from 122.8 million tonnes in 2020 (FAO, 2024). Over the past 50 years, this industry has been the most prominent sector, growing at an average rate of 5.3% per year with a projected global growth estimate of 46.3% by 2030 (Maia et al., 2024;). In 2022, global fisheries and aquaculture production hit a record high of 223.2 million

tonnes, with Asian countries leading the way, producing 70 precent of the total, and China alone contributing to 36 percent. Aquaculture, valued at USD 313 billion reached 130.9 million tonnes, marketing the first time farmed aquatic animal production surpassed captured fisheries. This growth is mainly driven by finfishes, with Asia accounting for nearly 88 percent of the increase, followed by Latin America and Europe. Captive Fisheries produced 92.3 million tonnes, with China Indonesia and India as the top producers (FAO, 2024; Maia et al., 2024)

Scientific approaches to assessing fish welfare in farmed conditions have continued to evolve, recognising health, mental state, and natural living conditions as key welfare components. However, the diversity of fish species and production systems, coupled with a lack of comprehensive scientific data, presents challenges in this field. Despite this, in the European Union (1998), legislation such as Council Directive 98/58/EC has been established to set minimum standards for the protection of animals, including fish, bred or kept for farming purposes. Additionally, fish welfare in aquaculture can be evaluated using The Five Freedoms, which were formulated in the early 1990s by the UK Farm Animal Welfare Council (1992), which states: *(1) freedom from hunger or thirst; (2) freedom from discomfort; (3) freedom from pain, injury or disease; (4) freedom to express normal behaviour; and (5) freedom from fear or distress* (Council, 1992; Webster, 2001; Mellor, 2016). This, in turn, helped influence legislation for aquaculture welfare and in 2005, the Council of Europe adopted a recommendation on the welfare of farmed fish and in 2008 the World Organisation for Animal Health (OIE) adopted guiding principles as well. Subsequently, Directive 2010/63/EU (European Union, 2010) on the protection of animals used for scientific purposes covers vertebrate animals including cyclostomes and cephalopods, as there is scientific evidence of their ability to experience pain, suffering, distress and lasting harm. These recommendations establish that all animals, including fish, must be provided with environment, food, and care appropriate to their health and well-being. Moreover, the environmental conditions the animal are bred must be checked daily to prevent avoidable pain, suffering, distress or lasting harm (National Research Council [NRC], 2010).

1.2.3 Stress in Fish

While the debate over whether fish feel pain in the same way humans do is ongoing, the evidence for fish experiencing stress is robust and widely accepted. *Stress is a state caused by a factor, or stressor, that deviates from a normal resting or homeostatic state* (Barton and Iwama, 1991). This stress can have significant impacts on their health and behaviour, indicating that they do respond adversely to negative stimuli in a meaningful way. In modern commercial fish farming, fish are subjected to various handling practices and confinement in the environment and

transportation, leading to acute stress and triggering physiological responses. Commercial fish farming often involves practices that can be highly stressful for fish, such as frequent handling, netting, grading, sorting, and moving them between different holding and grow-out tanks. Additionally, fish health management may have preventative disease treatments such as vaccination (injection, oral, and immersion) or bath treatments for pathogen or parasite treatment (delousing). These stressful practices can negatively impact the fish's immune system, growth, and overall welfare, making them more susceptible to diseases and reducing their quality of life (Barton and Iwama, 1991; Schreck and Tort, 2016). On farms, fish may become accustomed to stress, and stress responses can vary from one individual to another within a population.

External Signs of Stress

The external signs of stress in fish can include changes in colouration, ataxia, and tachyventilation. Ataxia is evident in high speed and short bursts, frequent changes in swimming patterns, body-twisting, and even swimming upside-down. Often, a response to small stimuli by the Mauthner cell, the largest neuron known in the vertebrate brain and, in fish, mediates rapid escape behaviour (Ross and Ross, 2008). Hecker et al. (2020) demonstrated that removing both the soma and the giant axon results in the permanent loss of rapid escape responses, directly impacting survival in predator-prey interactions. Tachyventilation, or rapid and shallow gill movement, is another clear indication of stress in fish, as it suggests the fish is attempting to increase its oxygen intake to cope with the stressful situation. Fish exposed to high concentrations of drugs, metals, pesticides, or pH may display rapid and repeated flaring of the opercula, accompanied by a "coughing" reflex to expel the chemical irritant from the gill cavity (Carlson & Drummond, 1978; Ross and Ross, 2008, Ross and Ross, 2009, Neiffer et al., 2009). Improper crowding, pumping, oxygen and handling before slaughter can cause gill injuries, haemorrhages, fin splitting, crush injuries, snout injuries, and bruising. Damage to the skin or gills, indicated by floating scales and blood in the water ("red water"), becomes more noticeable when post-smolts are crowded in small or white containers. Red water in euthanizing baths signals reduced water quality, fish damage, or depleted anaesthetic dosage (Noble et al., 2020).

Internal Signs of Stress

Stress incurs a metabolic cost, and the inability to reestablish homeostasis can lead to death. Moreover, prolonged stress can cause a deterioration in the fish's health condition and performance (Cyr & Romero, 2009; Soltanian et al., 2018). Stress responses also serve as defensive reactions to environmental challenges, with

varying durations, and addressing stress aims for complete relief, adaptation, or acceptance of mortality (Figure 1-1.). Pickering et al. (1985) found that fish under conditions of chronic stress period of 2-4 weeks sufficiently increased the mortality rate due to susceptibility to furunculosis (deep infection) caused by *Staphylococcus aureus*, *Saprolegnia* infection and bacterial fin-rot.

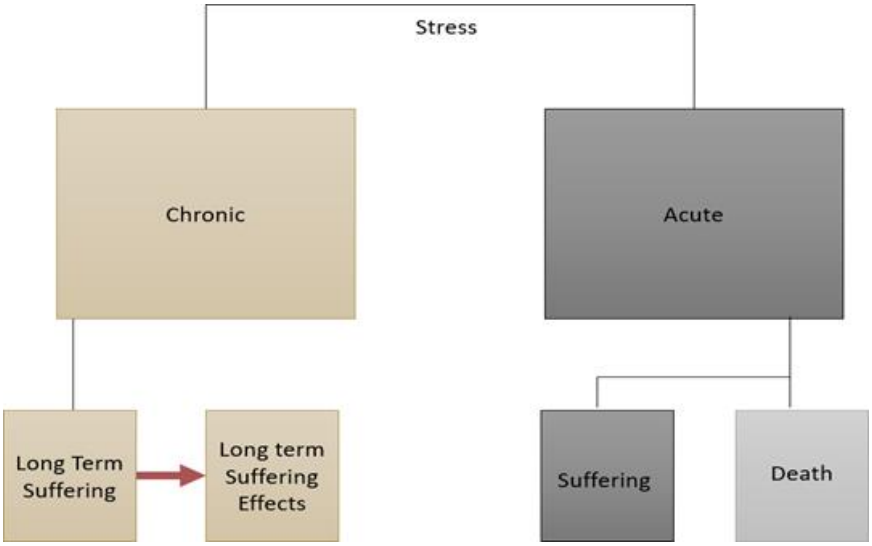

Figure 1-1 Acute and chronic types of stress. Acute stress, while capable of causing intense suffering and potential fatality, typically allows for recovery and restoration of homeostasis. Chronic stress poses a more prolonged threat, often leading to enduring suffering and potential long-term consequences.

Initially, neuroendocrine pathways activate, releasing significant amounts of hormones like catecholamines (adrenaline) and corticosteroids (cortisol), two endocrine hormones (Pankhurst, 2011). At different levels, the hypothalamus-pituitary-internal (HPI) axis medicates a primary response to deal with these stressors, increasing the glucocorticoid cortisol (Iwama et al., 1998; Rotllant et al., 2001). This hormonal surge mobilizes energy reserves, supporting cardiovascular and respiratory functions to overcome stress (Figure 1-2.).

Along with cortisol, heat shock proteins (HSPs), including the highly conserved Hsp70, act as biomarkers for stress, responding rapidly to environmental challenges (Ma and Luo, 2020). Xu et al. (2012) reported that the molecular chaperone 70-kDa heat-shock proteins (Hsp70s) play essential roles in maintaining protein homeostasis. They also highlight the upregulation of Hsp70 under stress, emphasizing its critical role in the stress response of aquatic organisms.

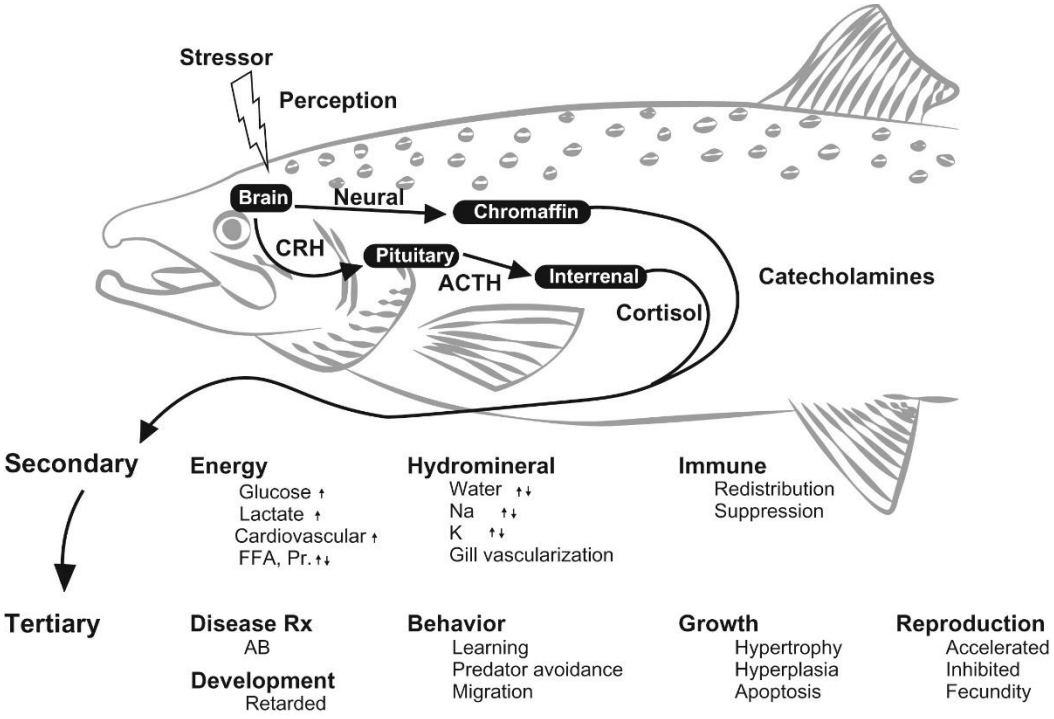

Figure 1-2 The concept of stress in fish physiological pathways. The figure illustrates the primary, secondary, and tertiary responses of fish during distress. The primary response (depicted inside the fish) involves the release of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH). Secondary responses include changes in hormone levels, such as cortisol, leading to mobilization of energy reserves like free fatty acids (FFA) and proteins (P), as well as alterations in immune function, indicated by antibody (AB) levels. Tertiary responses encompass broader physiological and behavioural changes resulting from these hormonal and metabolic adjustments (Source: Schreck and Tort, 2016),

1.2.4 Anaesthesia – Stress Management Intervention

A common challenge in aquaculture when working with aquatic species is managing the unique stressors they face. Ironically, we often become the "fish out of water" in these situations, inadvertently introducing stress factors such as netting, handling, harvesting, and other human interference. Low stress harvesting of fish, also known as "Rested Harvest", is widely known to improve the meat product quality post-harvest. For rested harvest to occur, fish are typically sedated or anaesthetised during the harvest process thus completely removing the stress during the harvest process and escape response or other prey-like behaviours. The effects of pre-harvest stress and harvest method on the stress response, rigor onset, muscle pH and drip loss in barramundi *Lates calcarifer* (Bosworth et al., 2007; Wilkinson et al., 2008; Matos et a., 2010). By reducing escape response, the animal's energy is retained in the muscle which increases the time for rigor mortis and sequent increases shelf life. The muscle from rested harvest fish has a better texture, less scraping, less bruising and less blood spotting and where the fish is sold whole, the

overall appearance is improved with less scale loss and other evident physical damage (Tonghao et al., 2023). All these result in a higher consumer acceptability and profit margin. But individual responses to sedation or anaesthesia is challenging due to the numbers of fish captive or captured for harvest. Different harvest methods commonly used in aquaculture tend to reflect the value of the species. For example, high value or physically larger species such as salmon and tuna require harvest procedures, that pay individual attention to the fish to aim to maximise muscle quality. Smaller species such as perch, carp, tilapia, catfish or brood stock of salmon or tuna do not have the same level of individual care and are processed in larger numbers with subsequent impact on the final product (Bosworth et al., 2007; Wilkinson et al., 2008; Matos et a., 2010).

Farmers and researchers utilize anaesthetics to alleviate stress in fish, long-term and tank-to-tank transport or handling as well as treatment of disease, surgery or experimental procedures. Components of anaesthesia include sedation, immobilization, unconsciousness, amnesia, and analgesia. The ideal anaesthetic should be water-soluble, easy to prepare and administer, chemically stable for a reasonable period, and biodegradable. Anaesthetics are also used for invasive procedures such as routine veterinary care, blood sampling, or research on the migratory patterns of wild populations such as radio tagging. Knowing which anaesthesia to use for your procedure is crucial as each may vary in the degree of consciousness and analgesia they provide, influencing cortisol release and stress response. The common way to immobilize fish is by dissolving chemicals in the water for absorption over the gills (inhalation), and the stage of anaesthesia is normally assessed by the behaviour and activity of the fish (Table 1) (Kiessling, et al., 2009; Zahl et al., 2010; Sneddon, 2012; Zahl and Kiessling, 2012).

Anaesthesia is crucial for tasks like vaccinating millions of fish annually, counting sea lice on millions of fish, and tagging broodstock. MS-222 (Tricaine), chemically similar to benzocaine, and isoeugenol, synthesized from eugenol, are commonly used to sedate trout for routine stress-inducing transport or procedures (Haya, 2005). In Norway, the top salmon exporter worldwide, has approval for four anaesthetics for fish: benzocaine, isoeugenol, metomidate, and tricaine. Synthetic or herbal anaesthetics prevent physical injury and reduce metabolism (Soltanian et al., 2018). However, prolonged exposure can lead to adverse effects such as decreased immunity, toxic shock, and reduced survivability, necessitating careful management. Anaesthetics are crucial in mitigating cortisol impact and investigating serum cortisol's role in the adaptive stress response. These agents can alleviate pain, reduce bodily movement, and dampen neural activity, but typically target only one aspect. For example, analgesics mitigate pain without hindering the primary fibers' response to harmful stimuli, while paralytic agents inhibit synaptic

transmission at the neuromuscular junctions, resulting in skeletal muscle paralysis (Ross and Ross, 2009; Sneddon, 2012). Therefore, while analgesics and paralytics each achieve a specific outcome, it is difficult to achieve or address effects alone. However, Ramlochansingh et al. (2012) found that MS-222 and benzocaine caused a dose-dependent, reversible blockade of extraocular motor and sensory nerve activity, indicating their effectiveness as single-drug anaesthetics.

Tricaine and benzocaine are primary anaesthetics used in farms as well as in research laboratories to handle animals, with tricaine effective at up to 135 mg/L, often used at higher doses for rapid induction during vaccination. However, in the United States, tricaine (MS-222) is the only licensed anaesthetics for fish used for food production and has a withdrawal period of 21 days before human consumption. Similarly, Norway implements the same withdrawal period for benzocaine and tricaine. Despite being used often, benzocaine, effective up to 40 mg/L, can unfortunately cause higher mortality at temperatures above 15°C for salmonids. Metomidate is another example of an anaesthesia used, however, not approved for human consumption. Metomidate, is mainly used in research due to its fast induction and recovery at 5 mg/L and its ability to block cortisol synthesis in interrenal cells (Ross and Ross, 2008; Ross and Ross 2009; Schroeder et al., 2021).

Clove oil (eugenol), has been used as an essential oil alternative to chemical anaesthetics for quite some time with even studies providing that clove oil and isoeugenol have less stress elevation than MS-222 (Small, 2003; Wagner et al., 2003) Building on the success of clove oil, isoeugenol, widely used in aquaculture as AQUI-S helps mitigate the harmful effects of stress on fish during handling and transport procedures. Although found to have a wide safety margin at low concentrations, there are conflicting results on its effectiveness on different species of fish. Small and Chatakondi (2005) found isoeugenol (AQUI-S) induction times to be similar to MS-222, however reported recovery times taking longer, but had stress reduction properties.

Table 1 Anaesthesia depth and stages in fish, including associated behaviour characteristics and recommended procedures. The table categorises stages from light sedation to deep anaesthesia, describing behaviours like decreased swimming activity, loss of equilibrium, gill ventilation, reactivity, heart rate and muscle tone. The table outlines appropriate procedures for each stage to ensure safe and effective anaesthesia. Source: adapted from Sneddon (2012).

Stage	Plane	Level of Anaesthesia	General Demeanour	Activity	Equilibrium	Gill Ventilation Rate	Reactivity	Heart Rate	Muscle Tone	Examples of Procedures
0		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	
I		Lightly sedated	Disorientated	Reduced	Normal	Normal	Reduced	Normal	Normal	
II		Excitation	Agitated	Increased	Difficulty	Increased	Increased	Increased	Normal	
III	1	Light anaesthesia	Anesthetized	None	Loss	Decreased	Reflex responses†	Regular	Decreased	Weight; close visual inspection; external noninvasive tags, gill scrape
	2		Anesthetized	None	Loss	Shallow	None	Reduced	Decreased	Invasive tags; tissue removal; injection; blood sampling; gill biopsy, lesion dressing, recovery surgery‡
		Surgical*								
	3	Deep	Anesthetized	None	Loss	Rare movements	None	Reduced	Relaxed	Nonrecovery surgery‡
IV		Overdose	Apparently dead	None	Loss	None	None	Cardiac failure		

1.2.5 Aquatic Pharmacological Concerns and Essential Oil Alternatives

In recent years, there has been a growing concern among many consumers for chemicals in the water, particularly Per- and polyfluorinated alkyl substances (PAFS) or ‘forever chemicals’, which can take a very long time to break down. PFAS are organic chemical compounds where the hydrogen atoms have been entirely (in the case of perfluorinated) or predominately (in the case of polyfuorinated) substituted with fluorine atoms. These compounds are non-aromatic (Brunn et al., 2023). The aquaculture industry prioritizes the use of chemical anaesthetics that are both approved and biodegradable. Extensive monitoring is done to ensure toxicological exposures are excreted before human consumption (withdrawal periods). Additionally, in successful high-quality indoor fish rearing, water quality is one of the most important conditions for the success of food fish. Recirculating Aquaculture Systems (RASs), which allow cleaning of the water for reuse through fish culture tanks, maintain the water quality and positive bacteria cultures. They also are a sustainable approach as they minimize

water input and decrease the discharge of waste (chemical or organic) into the environment. Typically, RAS consists of many beneficial factors to clean and disinfect suspended organic waste. In such intensive aquaculture systems, MS-222 is often used to control stress during handling. Once it is present in the RAS or other aquaculture systems it can reach the environment by the discharge of contaminated influence, which can also influence non-target organisms (Ferreria et al., 2017). Typically, anaesthesia baths are given separately from the system, however, the chemicals used within are still flushed down into the drainage system, which ends up in the environment.

Given these challenges and the growing concern over chemical residues in the environment, the search for safer, non-toxic alternatives is an increasing concern. The emergence of food-born threats, exacerbated by the social and economic impacts of the COVID-19 pandemic, has created an urgent need for safer food solutions. One potential approach is the development of novel, non-toxic, nature-based formulation with antistress qualities. Essential oils have the potential to offer widespread benefits, particularly in aquaculture. In nature, the specific aromas and chemical properties of essential oils have the potential to offer widespread benefits, particularly in aquaculture (Falleh et al., 2020). In nature, the aromatic and chemical properties of essential oils serve several crucial functions for plants: (1) attracting insects and pollinators, (2) providing protection from environmental challenges such as extreme temperatures, and (3) safeguarding against pests and pathogens (Dhifi et al., 2016; Falleh et al., 2020). These natural roles of essential oils suggest they possess significant biological properties including antimicrobial, antifungal, antioxidant, antiviral, antimycotic, antiparasitic, and insecticidal effects (Friedman et al., 2002; Emir et al., 2013; Dhifi et al., 2016; Cui et al., 2018; Falleh et al., 2020)

In recent years, nutraceutical components obtained from different plants species have begun to take their place as an option for aquatic anaesthesiology. Essential oils (EO) are often considered as a sustainable and eco-friendly option compared to synthetic chemicals. EOs also exhibit pronounced antibacterial and food preservative properties that represents a real potential for food industry practices, such as meat, meat products, vegetables and fruits as well as dairy products (Falleh et al., 2020).

Clove oil, belonging to the Myrtaceae family has been shown to be a powerful anaesthesia due to its content of high eugenol and lesser isoeugenol concentrations. Because of these ingredients, clove oil is a good organic commercial anaesthesia and in addition, novel extracts from some plants may offer more economical alternatives. Recent studies have highlighted the anaesthetic effects of various plants from several families, including Myrtaceae (Bodur et al., 2018), Lamiaceae (de Lima et al., 2012; Toni et al., 2014; Metin et al., 2015; Silva et al., 2015;

Pedrazzani & Neto, 2016; Ribeiro et al., 2016; Cunha et al., 2017), Verbenaceae (Cunha et al., 2010, 2011; Parodi et al., 2012; Cárdenas et al., 2016; Fogliarini et al., 2017), Lauraceae (Kizak et al., 2018; Pedrazzani & Neto, 2016; Silva et al., 2013), Asteraceae (Can et al., 2017), and Geraniaceae (Can et al., 2018). Peppermint oil (*M. piperita*) has been reported to have an anaesthetic effect in rainbow trout (Metin et al., 2015), while thyme oil (*Origanum sp.*) provides deep anaesthesia in European sea bass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*) (Bodur, et al., 2018). Silva et al. (2013) noted that the essential oil of *Hesperozygis ringens* caused deep anaesthesia in silver catfish (*Rhamdia quelen*) within 2 minutes, with recovery durations ranging from 5 to 15 minutes. Additionally, the volatile oil of *Ocotea acutifolia* induced anaesthesia in Nile tilapia within 13–18 minutes (Silva et al., 2013). Clove basil has proven to be an effective sedative and anaesthetic for juvenile giant river prawns, especially in mildly alkaline water (de Souza Valente et al., 2024). The Camphor tree (*Cinnamomum camphora)* essential oil showed an anaesthetic effect in clownfish (*Amphiprion ocellaris*) within 10–11.5 minutes, with recovery times after deep anaesthesia varying between 3 and 5 minutes (Pedrazzani & Neto, 2016). Another study on goldfish (*Carassius auratus*) demonstrated the anaesthetic effects of essential oils from the camphor tree (*Cinnamomum camphora*) and rosewood (*Aniba rosaeodora*) (Kizak et al., 2018). Additionally, clove oil has anaesthetic properties in crustacean species such as *Macrobrachium rosenbergii* (Saydmohammed and Pal, 2009) and *Penaeus semisulcatus* (Soltani et al., 2004) and has been seen significantly boosting glutathione-S-transferase (GST) activity and improved the antioxidant system against reactive oxygen species (ROS) (Parodi et al., 2012).

1.2.6 Clove Oil: Uses, Benefits and Challenges in Aquaculture

Clove oil is a deep brown liquid characterized by a rich, aromatic odour and flavour. Historically, clove oil has been employed as a mild topical anaesthetic, particularly for the relief of toothaches, headaches, joint pains and is commonly used as an anaesthetic in dentistry (Liñán-Atero et al., 2024) Although clove oil allows for easy control of anaesthesia and sedation in aquaculture, one of its drawbacks is its potential flavouring properties, affecting harvestability.

The principal active ingredient in clove oil is eugenol, which constitutes 70-90% of its weight. Isoeugenol and methyleugenol make up 5 to 15% of the remaining ingredients. Eugenol is also known by several synonyms, including allylguaiacol, caryophyllic acid, eugenol, p-allylguaiacol, eugenic acid, p-eugenol, 4-allylguaiacol, and 4-allyl-2-methoxyphenol. The IUPAC name for eugenol is 2-methoxy-4-prop-2-enyl-phenol, and its molecular formula is $C_{10}H_{12}O_2$ (Ross and Ross, 2008; Ross and Ross 2009; Schroeder et al., 2021).

Clove oil has been classified as a “Generally Recognized As Safe” (GRAS) substance by the United States Food and Drug Administration (FDA) when used at levels not exceeding 1500 ppm in all food categories (21 CFR 184.1257; Elbestawy et al., 2023). Eugenol additionally is GRAS in animal feed (21 CFR 582.60) and isoeugenol is cleared for use in human food (21 CFR 172.515). Although clove oil and eugenol have been employed off-label as fish anaesthetics due to their effectiveness and cost efficiency, their use is not officially approved by the FDA. Therefore within the United States, they are not authorised for use in fish intended for human consumption or those that may be released into public waterways. Eugenol may not be used in any form on animals (fish) that could possibly be consumed by humans, even if the treatment occurs in a laboratory setting.

Antimicrobial and Antioxidant Properties of Clove Oil

Clove oil has been shown to exhibit many beneficial effects like antiviral (Siddiqui, 1996), antimicrobial (Stecchini et al., 1993), and antifungal (Karapinar, 1990) properties. Essential oils and eugenol have shown antimicrobial efficacy on various agar media, effectively inhibiting pathogens such as *Listeria monocytogenes*, *Campylobacter jejuni*, *Salmonella Enteritidis, Heliobacter pylori*, Escherichia *coli*, and *Staphylococcus aureus* (Friedman et al., 2002; Emir et al., 2013; Cui et al., 2018; Elbestawy et al., 2023). These antimicrobial effects make clove oil a valuable natural preservative in food safety applications.

In the context of vacuum-packed smoked seafood, lactic acid bacteria often produce undesirable sour odours and flavours. Dimitrijević et al. (2007) used the agar-well diffusion method to test the effectiveness of essential oils against *Listeria monocytogenes* and found that lactic acid enhanced the antimicrobial activity of thyme and rosemary oils, though this synergy decreased at higher oil concentrations. Microbiological tests confirmed that clove oil treatments extended the shelf life of hot-smoked rainbow trout (*Oncorhynchus mykiss*) fillets in vacuum and modified atmosphere packaging (Oğuzhan Yıldız, 2015). Furthermore, clove oil also extended the shelf life of sliced smoked rainbow trout fillets by three weeks by delaying lipid oxidation (Emir et al., 2013).

Eugenol and isoeugenol have exhibited significant antioxidant activities and DNA damage protection, likely due to their ability to trap free radicals and inhibit radical chain reactions. Isoeugenol has shown higher antioxidant and DNA protective effects than eugenol, attributed to subtle structural differences. The phenolic oxygen's lone pairs delocalize over the aromatic ring, facilitating hydrogen ion dissociation, and the ortho-methoxy groups enhance antioxidant activity. Isoeugenol's closer carbon–carbon double bond to the benzene ring may contribute

to its stronger biological activity (Zhang et al., 2017). Similarly, polyphenols have been shown to enhance antioxidant defence mechanisms, immune parameters, and growth performance in fish, as demonstrated in common carp (Cyprinus carpio) juveniles fed polyphenol-enriched diets, where catalase and peroxidase activity increased alongside improved serum and mucus immune responses (Jahazi et al., 2020). These shared mechanisms underscore the role of phenolic compounds in reducing oxidative stress and improving overall health. Additionally, water quality and biological factors, such as species, length and weight, sex, time of year, condition, disease, and stress, can alter and/or amplify physiological responses (e.g., cortisol production) to anaesthetics and the handling or surgical procedures (Carter et al., 2011). Given these preventative properties, essential oils used for anaesthesia could be highly useful in laboratory settings where disease management is critical.

Toxicity, Stress Response, and Gill Health in Aquatic Species Exposed to Clove Oil

The information on the toxicity of clove oil on vital organs particularly the surface organs of the fish due to effects of anaesthesia is meagre. Eugenol is also regarded as a toxic and aversive substance to aquatic organisms and mammals (Davidson et al., 2000; Yousefi et al., 2018, 2019). Clove oil is known to be an irritant when applied topically to laboratory rodents, rabbits, and dogs, causing inflammation and local cellular necrosis (Sladky et al., 2001). Additionally, in humans, clove oil is classified as an irritant and can cause harmful effects if swallowed, inhaled or absorbed through the skin, irritation to the eyes, skin and respiratory tract and may need to minimize direct contact. Additionally, it has been scrutinized for causing inconsistent results under stress conditions and may be more challenging to control its toxicological properties at higher concentrations or long-term maintenance dosages. Clove oil has been observed to decrease respiratory rates, potentially due to the inhibition of the respiratory centre in the medulla oblongata, which is associated with central nervous system depression (Anderson et al., 1997). An alternative hypothesis posits that ventilatory failure and medullary collapse in some fish may result from the neurotoxic and hepatotoxic properties of eugenol, as observed in mammals (Sladky et al., 2001). This toxicity can cause swelling that obstructs the gill lamellae, leading to reduced oxygen uptake.

Additionally, under long-term exposure, eugenol can cause stress, leading to significantly increasing stress biomarkers, such as plasma cortisol, glucose, lactate, and malondialdehyde or lipid peroxidation, while decreasing catalase, an enzyme that protects cells from oxidative damage (Yousefi et al., 2018, 2019). A study conducted by Barbas et al. (2021) explored the neurogenic effects of eugenol in

freshwater fish, tambaqui (*C. macropomum*), comparing it with pentylenetetrazole (PTZ), a central and respiratory stimulant. The authors found that eugenol induced body immobilization and neuronal excitability similar to PTZ without depressing the central nervous system, making it unsuitable for general anaesthesia in *C. macropomum* due to its potential for seizurogenesis and brain toxicity. These findings underscore the need to carefully reconsider protocols involving eugenol for short-term anaesthesia or euthanasia in fish to ensure ethical and welfare standards.

Clove and its derivatives have been reported to mitigate stress in fish by serving as effective anaesthetics that elicit lower stress responses compared to traditional methods. Clove oil anaesthesia in rainbow trout elevates plasma cortisol levels less than MS-222 anaesthesia, suggesting a reduction in short-term handling stress (Wagner et al., 2003). Additionally, in other species such as Senegal sole, the use of clove oil at 1000 mg/L effectively prevented pre-mortem increases in cortisol, lactate, and glucose (Ribas et al., 2007). Faster anaesthesia induction or higher concentrations of eugenol in clove oil reduces stress response, while slower induction at lower eugenol concentration can increase stress response (Hoseini and Nodeh, 2013; Mirghaed et al., 2018). Mirghaed et al. (2018) observed that rainbow trout exposed to varying concentrations of eugenol showed no significant differences in serum lactate or liver values, though longer induction times increased serum glucose and certain enzyme levels. Similarly, Hoseini and Ghelichpour (2012) demonstrated that Beluga sturgeon exposed to clove solutions had varied induction and recovery times, with lower concentrations over extended periods being more stressful. Da Paz et al. (2024) reported that eugenol is an effective anaesthetic for Nile tilapia at specific concentrations, though higher doses may cause hemodynamic changes and affect cardiac function. Lastly, Sintuprom et al. (2024) observed that low concentrations of clove oil reduced transport stress in betta fish, while higher concentrations increased stress markers, suggesting that appropriate dosing is crucial for maximizing the welfare benefits of clove oil during stressful conditions.

There is evidence of gill damage in fish caused by exposure to eugenol oil (Velisek et al., 2005a; Velisek et al., 2005b; Singh, 2021). Histological examinations revealed capillary ectasia of gill filaments immediately upon exposure to clove oil anaesthesia. However, the gill filaments recovered to the normal state within 24 hours (Velisek et al., 2005a; Velisek et al., 2005b). Singh, (2021) reported toxicopathological effects of clove oil anaesthesia, including damage and sloughing of the gill epithelium, lifting of the gill epithelium, and bleeding from damaged blood channels in dendritic organs. However, other studies have shown that both MS-222 and eugenol adversely affected the gills of Japanese sea bass, with the optimal assessment period for anaesthetic effects on the gills being 6 hours after

recovery. The study concluded that eugenol caused less gill damage compared to MS-222 (Wang et al., 2020). These welfare concerns may not be unique to eugenol but could also apply to other organic and non-organic anaesthetics. Further studies emphasize the variable responses to different anaesthetics. For instance, Sladky et al. (2001), observed that fish anesthetized with eugenol exhibited greater reactions to needle punctures than those anesthetized with tricaine methanesulfonate (MS222). Both anaesthetics, however, were associated with hypoxemia, hypercapnia, respiratory acidosis, and hyperglycemia in red pacu, highlighting the physiological challenges posed by these substances. For a size of 41 g Nile tilapia (*O. niloticus*) juveniles, Ferreira et al. (2021) found that between 90 and 150 mg/L concentrations of essential oil of *Ocimum gratissimum* (EOOG) are ideal for anaesthesia. The authors found that 90 mg/L EOOG prevented elevated plasma glucose. However, they also suggested that it may lead to kidney lipid damage and alter antioxidant defences by increasing hepatic and brain reactive oxygen species levels and reducing brain TBARS activity. Chance et al. (2018) found that unbuffered MS-222 in post-smolt Atlantic salmon (*S. salar*) reduced peripheral neutrophils and increased tnfα3 (tumour necrosis factor α 3) in the head kidney as well as Na–K-ATPase β1 and cftr2 in the gills. Chance et al. (2018) suggested it is likely that by buffering the MS-222-based anaesthetic solution, the observed effects could be mitigated. Additionally, he found that the commercial-grade iso-eugenol-based AQUI-S has been reported to cause upregulation osmoregulatory genes in the gills, caused minor epithelial lifting, increase plasma cortisol secondary response and had longer induction times compared to MS-222 and metomidate (Chance et al., 2018; Jerez-Cepa et al., 2021).

1.2.7 AQUI-S™ and Isoeugenol: A Versatile and Safe Anaesthetic

AQUI-S, developed at the Seafood Research Laboratory in New Zealand, contains isoeugenol as its active ingredient. Due to its active plant-derived ingredients and solvent, disperses quickly in water and degrades in natural sunlight. AQUI-S is a versatile anaesthetic that has been developed to give flexibility and control in animal husbandry, transportation and harvesting operations.

Isoeugenol is the active ingredient of AQUI-S and offers more effective and controlled anaesthesia compared to the mixture of compounds in clove oil. AQUI-S consists of 50% isoeugenol and 50% emulsifiers, including polysorbate 80 (sorbitan mono-9-octadecanoate poly(oxy-1,2-ethanediyl) derivatives). Polysorbate is commonly used in various proprietary pharmaceuticals, and both isoeugenol and polysorbate are categorised as GRAS (Generally Recognized as

Safe) for food use by the FDA, with a withdrawal period of 2 days (Schroeder et al., 2021). Commercial preparations containing eugenol or isoeugenol as major active compounds, such as AQUI-S (20E) (higher isoeugenol and lower eugenol) and AQUI-S (Isoeugenol only), have been reported as safe, low-cost, and highly effective fish anaesthetics (Iversen et al., 2003; Silbernagel and Yochem, 2016), They are widely used to assist in fish handling in Australia and European Union countries, especially Norway (European Medicines Agency, 2011). In countries where AQUI-S is registered with a 'zero withholding period', authorities have determined fish harvested in combination with AQUI-S is safe to eat due to the active ingredient, isoeugenol. Currently, AQUI-S is approved in Australia, Chile, New Zealand and Vietnam with a zero withholding period, making it the only anaesthetic that can be used for harvesting fish in those countries. However, it is also approved in Norway, Iceland and the Faroe Islands, but not currently with a harvest claim. However Norway, in 2016, isoeugenol was used on about 100 million fish, rendering them suitable for human consumption after a two-day withdrawal period (Aqua AS, 2013; Schroeder et al., 2021).

AQUI-S also does not affect the flavour or smell of harvested fish. Residue studies have shown that isoeugenol does not adversely affect the taste of fish fillets. For example, rainbow trout exposed to AQUI-S did not develop undesirable taste responses (Meinertz et al., 2006). This contrasts with eugenol, which has been found to alter the flavour of silver catfish fillets, making it unsuitable for anesthetizing fish intended for human consumption (da Cunha et al., 2010; Jiang et al., 2023). Studies indicated that isoeugenol exposure results in minimal activation of the hypothalamic-pituitary-interrenal (HPI) axis and subsequent cortisol secretion in various fish species subjected to stress conditions such as confinement and acute oxygen deprivation (Small, 2003; Small, 2004; Small & Chatakondi, 2005). Additionally, there are also reports suggesting isoeugenol to exhibit a favourable pharmacokinetic profile that follows a two-compartment open model, characterized by rapid distribution to tissues from the plasma and a slower elimination process. This slower elimination, compared to agents like MS-222, can be advantageous for maintaining therapeutic effects over extended periods, necessitating careful dosage management to avoid accumulation and potential toxicity (Kiessling et al., 2009). Additionally, temperature increases have been reported to shorten induction and recovery times for various anaesthetic agents in teleost species, suggesting that the effectiveness of isoeugenol could be enhanced in warmer conditions (Stehly and Gingerich, 1999).

The ability of isoeugenol to block nociceptive pathways makes it suitable for procedures that might cause pain, ensuring the welfare of fish during invasive procedures (Sneddon et al., 2003). Fish possess the neural systems necessary for perceiving painful stimuli, and anaesthetic agents like benzocaine, MS-222, and

isoeugenol effectively block these pathways (Dunlop and Laming, 2005; Sneddon et al., 2003). Comparative studies further support isoeugenol's efficacy. For example, Iversen et al. (2003) found that AQUI-S at concentrations ≥30 mg/L effectively anesthetized Atlantic salmon smolts, preventing cortisol elevation and demonstrating its stress-reducing capabilities. Iversen and Eliassen (2009) also reported that AQUI-S at 5.0 mg/L significantly reduced mortality rates during the transport of Atlantic salmon smolts, from over 11.5% in unsedated fish to 2.5%. These findings underscore the effectiveness of isoeugenol in reducing stress and enhancing survivability during and after transport.

Challenges and Considerations in the Use of Isoeugenol

Despite being safe for fish and humans and cost-effective, a noted limitation is the slow induction of anaesthesia at the recommended concentrations, posing a challenge for commercial fishing users. A dose of 2.5 mg/L of isoeugenol for light sedation reduces swimming activity, oxygen consumption, carbon dioxide production, and anxiety within 5–15 minutes without notably increasing cortisol levels. An anaesthetic dose of 12.5 mg/L has more pronounced effects (Ross and Ross, 2009), and at 50 mg/L, it can be lethal without significantly elevating cortisol levels (Iversen et al., 2003). Conversely, it's also been reported to not alleviate stress with or without crowding and causes a biphasic cardiac response with depression of the heart (Davidson et al., 2000; Rothwell and Forster, 2005; Rothwell et al., 2005). When used commercially, the dosage of isoeugenol is often administered at half the rate of AQUI-S, a factor that should be highlighted in studies and literature on concentration. Additionally, isoeugenol commonly has a purity level that varies depending on the supplier and intended use. This variability can affect the concentration and intended dose levels. Unfortunately, this information is challenging to find in the literature, but it should be cited in literature and research findings.

Optimization and Safety Evaluation of Herbal Anaesthetics in Aquaculture

Balancing the concentration of drugs in combination therapies is crucial to optimize efficacy and minimize adverse effects. Optimization also improves pharmacokinetics and pharmacodynamic profiles ensuring optimal absorption, distribution, metabolism, and excretion. These practices in return additionally address the chemical stability of the drug and formulation to maximize bioavailability and therapeutic efficacy. It is therefore imperative to explore drug optimization to develop safe and effectively viable therapeutic agents, particularly in the context of anaesthetics used in fish. Eugenol and isoeugenol, for example, exhibit a quicker onset, prolonged recovery, and a narrower safety margin due to

its potential to cause ventilatory failure at high concentrations (Sladky et al., 2001), along with biphasic cardiac response with depression of the heart (Davidson et al., 2000; Rothwell and Forster, 2005; Rothwell et al., 2005).

An example of optimizing aquatic anaesthetics involves combining sodium bicarbonate with MS-222. This combination is used because MS-222 alone lowers the pH of the water, which negatively impact aquatic species (Welker et al., 2007). Bona et al. (2024) assessed the anaesthetic effectiveness and toxicity of various essential oils on fish. They found that clove oil at 200 µL/L caused toxic effects and oxidative stress, whereas tea tree and cinnamon oils at 75 and 100 µL/L were less effective, inducing anaesthesia more slowly and not achieving deep anaesthesia efficiently. However, combining these oils with clove oil reduced the toxic effects, suggesting a potential strategy to minimize physiological damage during anaesthesia in aquaculture. Martins et al. (2024) further demonstrated the efficacy of combining clove oil with salt for the long-term transport of common carp (*Cyprinus carpio*), finding that a combination of 5 mg/L clove oil and 3 g/L salt was effective and safe, mitigating the oxidative stress and ammonia increase associated with clove oil alone. In another study conducted on *S. aurata* (Gilt-head bream), Tchobanov et al. (2024) evaluated the anaesthetic effects of clove oil and 2-phenoxyethanol combined with lidocaine. They found that lidocaine reduced both induction and recovery times and decreased lactate levels, though it also raised alanine aminotransferase and (aspartate aminotransferase) levels, indicating potential toxicity within the liver. The combination of 2-phenoxyethanol and lidocaine emerged as the most effective, balancing anaesthesia with fewer negative impacts compared to clove oil alone, which had more pronounced negative effects indicated by higher heat shock protein hsp70 expression in the gills and elevated oxidative stress biomarkers.

Due to bioactive compounds derived from plant sources still being limited in toxicological response, there is a continued interest in using them in anaesthetic formulation for aquaculture usage. Plant-derived nutraceuticals can vary in their anaesthetic effects due to their complex structures, which may lead to unknown toxicological interactions and long-term effects (Zhang et al., 2022). Some plants or plant parts, such as mullein, walnut leaf, and spurge, can cause death or immobility in fish, despite their use in commercial fishing (Neuwinger, 2004; Alagöz et al., 2021; Zhang et al., 2022) Alagöz et al. (2021), explored the plant spurge (*Euphorbia rigida*), known for its high content of terpenoid and steroid compounds, as an alternative anaesthetic for rainbow trout. The results showed that at concentrations of 40,000 - 70,000 µL/L, spurge effectively served as an anaesthetic without altering plasma cortisol levels in the fish. They collected different parts of spurge during and after the flowering stage, dried it and later used it to examine its potential anaesthetic effects. In the preliminary study, 5 fish were

placed in 1 L of aerated clean water for each trial, and the fresh spurge stem was dripped into the water. However, the fish died within 2-3 minutes, exhibiting behaviours indicative of asphyxia, such as hyperactivity and swimming towards the surface. Additionally, a gel-like layer was found on their gill lamellae. In a subsequent study, the aqueous methanolic extract from all aerial parts and roots of the plant was tested in concentrations from 20 µL/L to 10,000 µL/L for up to 30 minutes in aerated water, but none of the fish exhibited sedation or anaesthesia. When returned to clean water, they behaved normally. Similarly, an aqueous macerate from the dried whole plant was tested at concentrations from 1,000 µL/ L to 80,000 µL/L for 30 minutes, resulting in only infrequent erratic swimming. Upon being placed back in clean water, the fish returned to normal behaviour within 10-15 seconds. However, an aqueous macerate of the spurge stem successfully induced anaesthesia, which led to its use in the main study and underscored the importance of preliminary work in determining non-lethal concentrations.

While essential oil or plant-derived nutraceuticals offer promising avenues for use as aquatic anaesthesia due to their biodegradable nature and economic benefits, their complex chemical structures can lead to varying anaesthetic effects and potential toxicological interactions to target and non-target organisms. The variability in efficacy and safety, as demonstrated by studies on different plant families, species and plant parts, underscores the necessity for thorough preliminary research to identify non-lethal and effective concentrations. Additionally, it would be beneficial to test essential oils in RASs in a captive laboratory environment to see if low dosing may benefit water quality, and the antiparasitic and antimicrobial properties of the system. The example of spurge highlights the critical importance of detailed experimental evaluations to ensure the safety and welfare of aquatic organisms not only during anaesthesia procedures but secondary effects the plant may have at different concentrations.

1.3 Rainbow and Brown Trout

Salmonids are one of the most widely studied groups of fish worldwide due to their high value, growth, market-value, food source and history of translocation across many different continents (Molony and Molony, 2001). Brown trout, *Salmo trutta* is one of the most widely used and sought-after, studied, introduced, and actively managed species across the world, rivalled only by rainbow trout, *Oncorhynchus mykiss*. Both species are valued as recreational and food resources and include a global interest and passion by rod-and-line anglers who generate robust direct and derivative economies, leisure and other social interactions and subsequent management efforts (Lobón-Cerviá and Sanz, 2017). In Sweden, *Salmo trutta*, is

incredibly widespread species found throughout lakes, rivers, streams and the sea. They thrive in Northern parts of the country in cold, clean and oxygen-rich streams. Unfortunately, due to hydropower plants, population numbers have been declining due to the lack of being able to roam freely and return to their birthplace. Unlike, *S. trutta*, *O. mykiss* is not a native species to Sweden and has been planted throughout Swedish water systems as a popular sport fish for spinners and anglers. Their natural habitat is in North America and despite the growing quickly in Swedish waters if conditions are favourable, the vast majority cannot naturally reproduce in Sweden. Given the commercial and ecological importance of these two species, this thesis will focus on these two salmonid species to evaluate the effectiveness of a herbal anaesthetic formulation on their physiological and behavioural responses.

2. Materials and Methods

2.1 Experimental Sites and Ethical Statement

The study on rainbow trout was conducted at the Fish Larvae Lab, Department of Applied Animal Science and Welfare, SLU, Sweden. Experiments on brown trout were conducted at the Fisheries Research Station in Älvkarleby, Sweden. The experiment was carried out in full compliance with laws and regulations on procedures and experiments on live animals in Sweden are overseen by the Swedish Board of Agriculture (Registration number: DNR: 5 .2.18- 17992/2022 valid until 2027). The permit for the facilities in Älvkarleby is 5.2.18-02612/2022. The permit for using animals for scientific purposes at the facilities in Älvkarleby: 5.2.18-07827/2020

2.2 Chemicals, and Reagents

The isoeugenol (2-methoxy-4-propenyl phenol; molecular weight, 164.20 g/mol, ≥99% purity) was obtained from Arora Aromatics PVT. Ltd (Sambhal, India). The nutraceutical formulation (concentration: 4.35 mg/ml) used in this study was developed at the Department of Animal Science and Welfare, SLU under the supervision of Assoc. Prof. Kartik Baruah and colleagues. The formulation was prepared by blending a few selected plant-derived extracts, each chosen for their potential health benefits. The extracts were obtained through industry-standard extraction procedures to ensure the consistency and potency of the bioactive components. All processes were carried out under strict hygienic conditions in compliance with Good Manufacturing Practices guidelines. MS-222 was provided from the Älvkarleby facility. Before use, it was combined with sodium bicarbonate buffer at a 2:1 ratio (Sodium bicarbonate:MS-222).

2.3 Experimental Animals and Conditions

In total, two separate experiments using two different salmonid species reared under two environmental scenarios were conducted. The first experiment consisted of three independent trials that were conducted on farmed rainbow trout under a laboratory condition. The second experiment was performed on brown trout, which are used for restocking purposes. This experiment was conducted on a semi-industrial scale at the Fisheries Research Station of SLU in Älvkarleby, Sweden.

2.4 Video Recording and Camera System

The video camera was an essential aspect of the experiment, particularly in monitoring behaviour, induction time, sedation depth and recovery time. During the preliminary research phase, web cameras (Logitech V-U0004 and Logitech C920e HD 1080p) were positioned in the front of the tanks, pressed up to ensure a clear view of the fish. One of these cameras were dedicated to recording the recovery process, while the others focused on capturing the anaesthesia process, including the depth of anaesthesia (Figure 2-1; Figure 2-3).

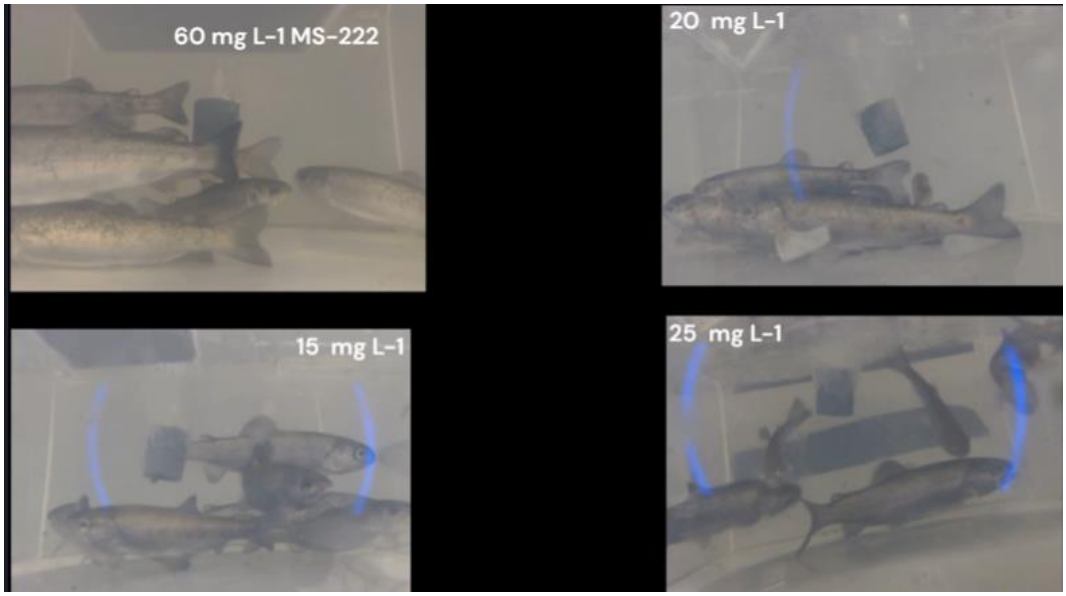

Figure 2-1 Camera system capturing experiment 1, dosage trial of isoeugenol.

During hatchery experimentation, two Time Lapse Camera (Brinno TLC200Pro HDR; Figure 2-2) were employed. One camera was dedicated to recording the recovery process, while the others focused on capturing the anaesthesia process, including induction and the depth of anaesthesia. Unfortunately, during replicate 3, footage was destroyed due to an unforeseen technical error, resulting in data unable to be captured.

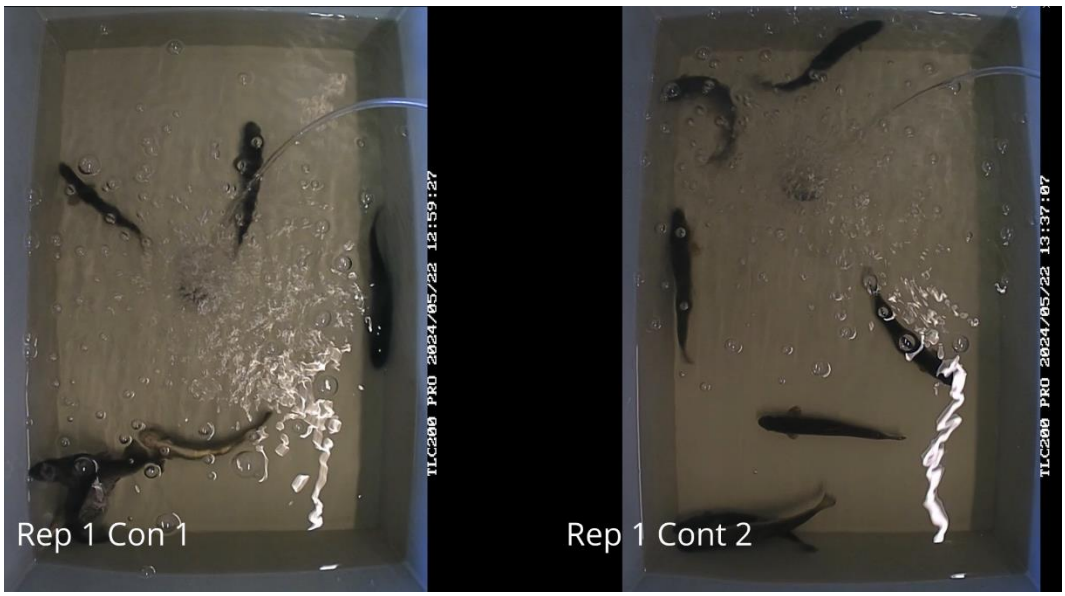

Figure 2-2 Camera system looking at recovery tanks of experiment 2, May 22nd, 2024 (Replicate 1)

Figure 2-3 Example of experiment 1 camera recording system.

2.5 Experiment 1: Rainbow Trout Anaesthesia Trials under Lab Conditions – Preliminary Study

The aim of experiment 1 was to evaluate the anaesthesia effects of a natural anaesthetic formulation on farmed rainbow trout fingerlings. To this end, three independent trials were conducted aimed at optimizing the dose combinations of the iso-eugenol with the nutraceutical formulation. The studies involved thirty rainbow trout fingerlings (average weight: approx. 25 g; length: between 8 and 14 cm). The fish have been maintained at the Fish Larvae Lab of SLU in a recirculatory aquaculture system (RAS) for over a year. The water temperature of the tanks was set to 11℃ but varied between 11 and 14 ℃ across individual tanks during the trial. The dissolved oxygen (DO) level before treatment was 9.5 mg/L, while in the recovery tanks, the average DO was 10.3 mg/L. The anaesthetic tanks were 15-L plastic containers, each containing nine litres of water. Before exposure to anaesthesia, the fish were fasted for 24 hours. In the first trial, the fish were exposed to increasing doses of iso-eugenol: 10, 15, 20 and 25 mg/L. Six fish were randomly collected from the RAS tanks, placed in the anaesthetic tanks, and exposed to each test dose. The time taken for each fish to reach sedation level III (see, Table 1 for sedation level) was recorded using a digital monitoring system (Fig 2-1). After the anaesthesia, fish were netted and placed into a recovery tank, and the time taken for recovery was recorded for all the experimental groups using the monitoring system.

Based on the outcome of trial 1, two doses of iso-eugenol (10 and 20 mg/L) were selected and taken forward for optimization of dose combinations. In trial 2, a fixed dose of iso-eugenol (10 mg/L) was combined with increasing concentrations (250 uL/L, 500 uL/L and 1 ml/L) of a nutraceutical formulation to prepare the anaesthetic formulation (Table 2A). In Trial 3, the increasing doses of the nutraceutical formulation were mixed with 20 mg/L of iso-eugenol to prepare different anaesthetic formulations (Table 2B). Groups that were exposed to MS222 (positive control) or only iso-eugenol (10 mg/L) served as controls. A group of six rainbow trout fingerlings was exposed to the anaesthetic formulation in a similar fashion as described in Trial 1. The time taken for each fish to reach sedation level III (see, Table 2 for sedation level) and the time required for them to recover were recorded using a digital monitoring system (Fig 2-1).

The anaesthesia tanks were disconnected from the RAS systems and were cleaned before anaesthesia to remove any faecal matter in the rearing water. The recovery tanks were connected to the water from the RAS system and were well-oxygenated with air stone. Disconnection did increase the anaesthesia tank temperature up to 2 °C in comparison to the recovery tank's temperature. In this experiment, fish were not sacrificed at the end of the trial for sample collection as the aim of the

preliminary experiment was the optimization of dose compositions focusing on behavioural response and induction time.

Table 2 Experimental groups for Anaesthesia trials using MS-222 (positive control) and varying concentrations of isoeugenol or combination with nutraceutical formulations. Group A. tested isoeugenol at 10 mg/L, and group B tested 20 mg/L, each combined with increasing doses Nutra (Nutraceuticals).

Table	**Experimental groups**	**Positive control**	**Treatment 1**	**Treatment 2**	**Treatment 3**	**Treatment 4**
A	MS222 (positive control)	+	-	-	-	-
	Only Isoeugenol (10 mg/L)	-	**10 mg/L**	**10 mg/L**	**10 mg/L**	**10 mg/L**
	Nutraceutical formulation	-	-	**250 uL per Liter**	**500 uL per Liter**	**1 mL per Liter**
B	MS222 (positive control)	+	-	-	-	-
	Only Isoeugenol (10 mg/L)	-	**20 mg/L**	**20 mg/L**	**20 mg/L**	**20 mg/L**
	Nutraceutical formulation	-	-	**250 uL per Liter**	**500 uL per Liter**	**1 mL per Liter**

2.6 Experiment 2: Brown Trout Anaesthesia Trial Under Semi-Industrial Conditions

The aim of the second experiment was first to validate the laboratory findings. Secondly, it also aimed to evaluate whether such an anaesthetic formulation showed effectiveness in fish that are used for restocking programs since the fish passes through a number of handlings before being use for restocking. A total of 150 one-year-old hatchery-reared brown trout fingerlings were used in this experiment. These fish had an average length of 13.5 cm and a weight of 26.72 g. Natural water obtained from the Dalälven river was used in the experiment. The water quality parameters in the experimental tanks during the experimental period showed temperatures ranging between 16.1℃ and 19.4℃, the pH between 6.04 and 6.45, and dissolved oxygen levels between 8.16 and 8.56 mg/L.

The second experiment was performed between late May and early June 2024. Here the doses used in Trial 3 of the experiment 1 (see Table 2B) were selected to perform this experiment. A total of 150 fingerlings were randomly distributed into five different experimental tanks, each maintained at three replicates. Each of the five experimental tanks consisted of ten fish. Two days before exposure to the anaesthesia, the fish in all the groups were provided with no feed. A group of ten fish were collected from the tanks and placed as a group in the anaesthetic tank. Video recording of sedation start, induction time and recovery period, with parameters such as dissolved oxygen, pH and temperature of the anaesthesia tank were recorded. Following sedation fish were weighed and total length was collected prior transfer to recovery tanks. Once fish were fully recovered, they were then transferred back to the experimental tanks where they were monitored for 7 days. During the experiment, gill bleeding was observed in the isoeugenol-treated groups, both during the trial and upon reviewing video footage. To rule out the influence of low pH levels and rising temperatures, an additional group (n=10) was added with three replicates: MS-222 25% Buffer, characterized by low pH conditions. This group used a 25% buffer solution (0.12 g Buffer, 0.24 g MS, 4 L water).

Figure 2-4 Brown trout (S. trutta), which showcases pigment discolouration, possibly bacterial infection, staring approximately past dorsal fin to caudal fin.

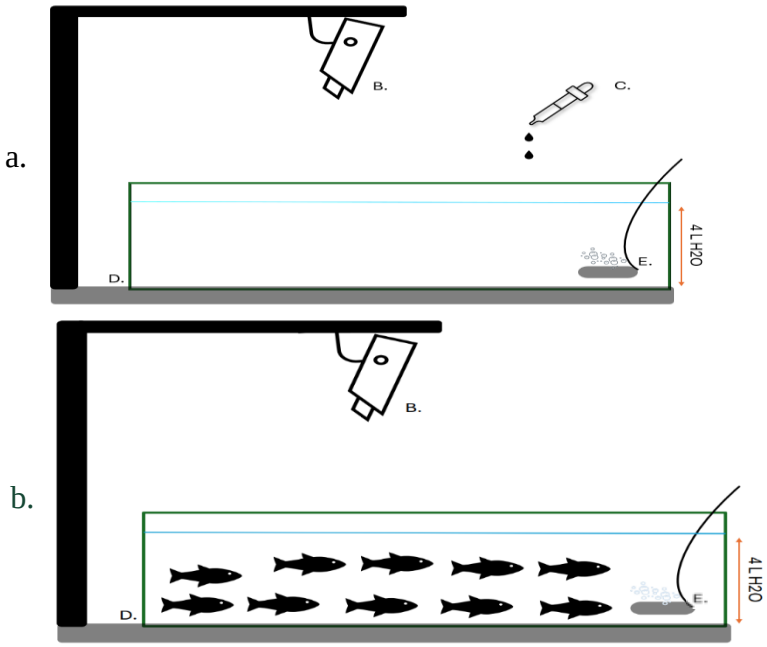

Figure 2-5 Schematic of the experimental setup used during experiment 2 anaesthesia trials (a.) Pre-fish exposure stage where anaesthetic is added to the tank water. (B) Overhead camera system used to monitor and record fish behaviour during anaesthesia exposure. (C) Anaesthetic delivery device. (D) Experimental tank containing 4-L of water for each trial. (E) Aerator to maintain adequate oxygen levels, mixing and gill transfer of sedative. (b.) After fish have been introduced into the anaesthesia bath solution.

2.6.1 Tissue Sample Collection and Storage

Plasma and tissue samples were collected over three days: Day 1 (Start of experiment), 48 hours and day 7 post-recovery. After recovery at respective timepoints, three fish from each replicate were randomly sampled and euthanized by head blunt trauma. Plasma samples were drawn from caudal vein, and the fish were dissected to collect liver, heart, head kidney and second gill arches (left side of fish). All samples were collected on the left side of the fish and were immediately flash-frozen in liquid nitrogen and stored at -80 ℃.

Skin-on-fillet and additional plasma samples were collected 48 hours post-recovery and at the end of the 7-day recovery period. All incisions and tissue samples were taken from the left side of fish to ensure consistency. Samples were transported using a vehicle equipped with liquid nitrogen and dry ice and stored in a designated department freezers at the university to maintain their integrity for future biochemical analyses. Organ tissues are planned fro

future analysis, while skin-on-fillet samples were preserved at -20 ℃ for future High-Preformance Liquid Chromatography (HPLC) analysis.

Figure 2-6 Example of tissue dissection and sample collection on brown trout

2.6.2 Hatchery Experiment Blood Collection and Analysis

2.6.3 Plasma Collection and Storage

A total of 45 blood samples were collected during anaesthesia, using a 25 g needle and pre-hepatized syringe via the caudal ventral vein. The blood was then centrifuged for 5 minutes at 7000 rpm, the plasma was collected, frozen in liquid nitrogen, and then stored in a -80 ℃ freezer for further analysis.

Cortisol Analysis by ELISA

Cortisol, a glucocorticoid hormone, as previously stated plays a crucial role in stress response, metabolism, and immune function. Measuring cortisol levels in brown trout can provide valuable insights into their physiological state, stress and overall health. Tecan Cortisol Elisa Kit (RE52061) is a widely utilized tool for measuring cortisol levels in biological samples such as serum and plasma in various research and clinical settings. This ELISA kit was recommended based on its successful application in previous cortisol studies conducted at SLU. The following section describes the methodological approach.

The TECAN cortisol ELISA kit involves several critical steps to ensure accurate measurements of cortisol levels in plasma samples. Samples were prepared with

standards and controls, serving as a reference to quantify the cortisol levels in the samples. Following this, 20 µL of each standard, control, and sample is pipetted into a designated well of the Microtiter Plate, labelling each well. Next, 200 µL of enzyme conjugate was added to each well. This enzyme conjugate contains a horseradish peroxidase-label anti-cortisol antibody, which binds to the cortisol present in the sample. The plate was then covered with adhesive foil to prevent evaporation and contamination. Samples are then thoroughly mixed by gently tapping the plate or using a plate shaker and then incubated for 60 minutes at room temperature (18-25 °C) to allow the antibody-cortisol complex to form. After the incubation period, the adhesive foil is carefully removed, and the incubation solution is discarded from each well, which is crucial for removing unbound substances and minimizing background noise within the assay. The plate was washed three times with 300 µL of dilute wash buffer to ensure only the antibody-cortisol complexes remained, removing any non-specific bound substances. Subsequently, 100 µL of TMB Stop Solution was added to each well. This stop solution causes the colour to change from blue to yellow, enhancing the assay's sensitivity and allowing for precise quantification of cortisol levels (Figure 2-7). The optical density (OD) of each well was measured using a photometer at 450 nm, with a reference wavelength of 600-650 nm. This measurement was performed within 10 minutes after adding the Stop Solution to prevent any fluctuation and sensitivity. The OD reading was then obtained from the photometer to generate a standard curve by plotting the known cortisol concentrations of the standards against their corresponding OD values. The cortisol concentrations of the unknown samples were then interpolated from the standard curve, including controls and replicates.

Figure 2-7 ELISA Cortisol Kits showcasing, the stop solution colour to change from blue to yellow.

2.6.4 Gill Histology

Sample Collection and Storage

A total of 45 gill samples were collected and 17 samples were sent to histology for analysis. The left gill, second arch was collected and placed into labelled cassettes for each treatment groups and fixed in 4% formaldehyde solution for 48 hours. The cassettes were then rinsed in PBS, 3 times for 5 minutes each rinse, before being placed in 70% ethanol for extended storage.

Dehydration and Paraffin-embedding

The processing cycle involved gradual dehydration through increasing ethanol concentrations, clearing with xylene, and infiltration with liquid paraffin, running overnight for approximately 14 hours. The following day, the tissues were embedded in liquid paraffin and solidified on a cooling plate. The paraffin-embedded tissues were then sectioned using a rotary microtome. After trimming and cooling on ice, the samples were sliced into 4-micrometer sections. These sections were transferred to untreated slides and dried before proceeding with staining.

Histology Staining

The process begins by incubating the slides at 60°C for 40 minutes, preparing them for deparaffinization and rehydration. Deparaffinization is performed through immersion in Xylene I and Xylene II for 15 minutes each, followed by rehydration with a series of ethanol solutions: Absolute Ethanol I and II for 5 minutes each, 95% Ethanol for 5 minutes, and 70% Ethanol for 5 minutes. The slides are thoroughly rinsed in running tap water. Staining is carried out by immersing the slides in Mayer's Hematoxylin for 5 minutes, rinsing them in running tap water for 20 minutes, and then staining with Eosin Y (0.2% with 1 mL concentrated acetic acid) for 30-60 seconds. Dehydration is conducted through agitation in 95% Ethanol I and II for 20-30 seconds each, followed by immersion in Absolute Ethanol I and II for 2 minutes each, and Absolute Ethanol III for 5 minutes. The slides are then treated with Xylene I and II, stirring for 10-20 seconds, and Xylene III for 2 or more minutes. The slides are finally mounted using Pertex and coverslips, resulting in blue nuclei and red, pink, or orange connective tissue, erythrocytes, and cytoplasm.

2.6.5 Determining Concentration of Isoeugenol Residue using HPLC

Extraction of Isoeugenol from Skin-on-Fillets

The left skin-on-fillet was stored in a -20 °C freezer before extraction. The day of extraction the frozen tissue was thawed at room temperature on a laboratory work bench. A precise amount of tissue (5.0 ± 0.2 g) was weighed into a 50 mL Falcon tube, followed by the addition of 10 mL of acetonitrile (ACN). The falcon tube was then placed on crushed ice for several minutes to cool and maintain the sample. For the first extraction, the sample was homogenized using an Ultra-Turrax T25 homogenizer in three 30-second intervals, ensuring the tube remained on ice for one minute between each homogenization step. The sample was then vortexed for five minutes to promote thorough mixing. Following vortexing, the tube was centrifuged at 960 g for five minutes at ambient temperature. The resulting supernatant was carefully transferred into a rotary evaporation flask. For the second extraction, 5 mL of ACN was added to the remaining pellet in the falcon tube, which was vortexed for five minutes and centrifuged at 2600 g for five minutes. The supernatant was combined with the first extract in the same rotary evaporation flask. This process was repeated twice more, each time adding 5 mL of ACN, vortexing, centrifuging, and combining the supernatant.

The combined supernatant (approximately 25 mL) is evaporated under vacuum using a rotary evaporation system (Figure 2-8) with a water bath set to 45-50 °C, reducing the volume to about 5 mL. After evaporation, 40 mL of distilled water was added to the concentrated extract in the rotary evaporation flask. The solid phase extraction (SPE) process was prepared by placing a Strata phenyl SPE column in the SPE manifold. The column was then conditioned with 1 mL of methanol/distilled water (9:1, v/v), followed by equilibration with 1 mL of distilled water. The sample was then loaded into the SPE column, which was subsequently rinsed with 3 mL of distilled water. The column is dried under the vacuum for five minutes. Finally, the analyte was eluted from the column with 5 times with 1 mL MeoH/ distilled water (9:1, v/v). The eluate is collected, vortexed and filtrate using a syringe with a 0.45 µm PTFE filter direct into a 2 mL HPLC vials for further analysis (Meinertz et al., 2008).

Figure 2-8 Rotary Evaporator system for HPLC isoeugenol extraction

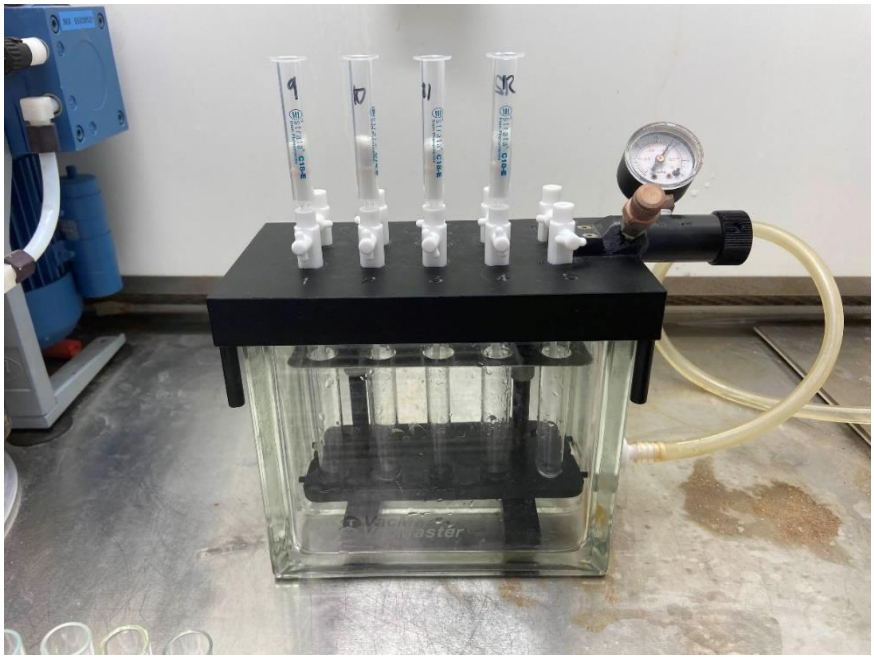

Figure 2-9 The solid phase extraction (SPE) process in the SPE manifold vacuum.

High-Performance Liquid Chromatography Analysis of 48-hour Isoeugenol Residue

The HPLC analysis was conducted using an Alliance 2795 Separations Module equipped with a Temperature Control Module II (operating range: 0-150 ℃) and a PDA 2998 detector (Waters Associates, USA). The separation was performed on a Phenomenex Luna C18 column (5 µm, 100 A, 250 x 4.6 mm). The mobile phase consisted of 51% acetonitrile (CAN) and 49% water, delivered at a flow rate of 1.0 mL/min, with an injection volume set at 40 µL. UV detection was carried out at a wavelength of 261 nm.

System Calibration was achieved using solutions with known concentration of isoeugenol. Calibration followed the peak height method, establishing a linear calibration curve for quantitative analysis.

Isoeugenol concentration in unknown samples were determined based on the established calibration curve, using the equation:

$$x = \frac{y - b}{m}$$

Where,
x = sample analysis result (concentration of Iso-eugenol measured)
y = raw data (mV- sec.)
b = y intercept (mV- sec.)
m = slope or calibration factor (mV-sec./
mM)

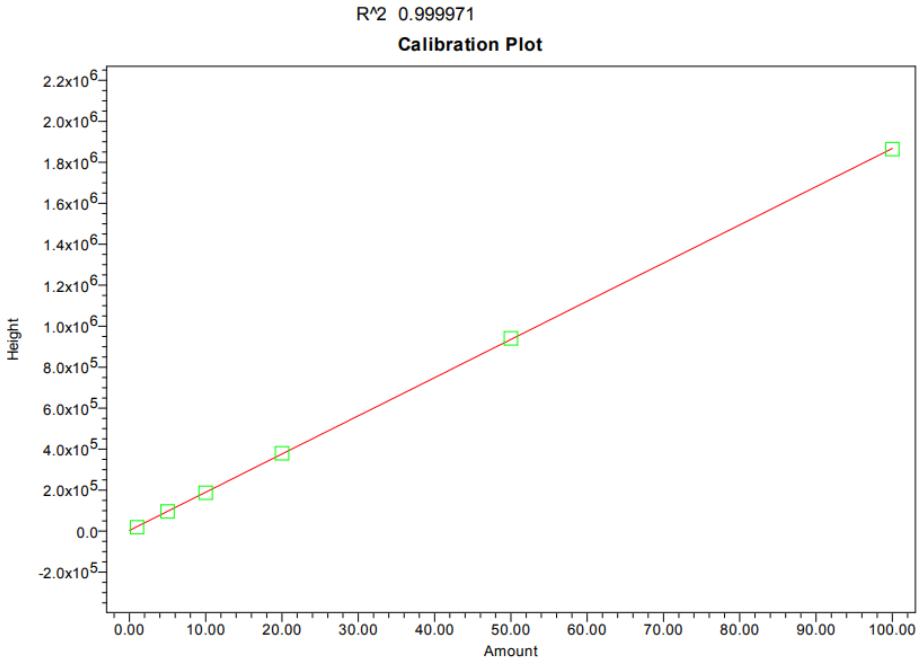

Figure 2-10 Calibration curve for Iso-Eugenol with data points at six concentration levels (1, 5, 10, 20, 50, and 100 µg/mL). R^2: 0.999971, indicating an excellent and successful calibration.

The detection limit represents the minimum concentration of each organic acid and alcohol that the HPLC instrument can reliably measure. Essentially, the MDL is the analyte concentration needed to produce a signal that exceeds the noise level by at least three times.

$$MDL = \frac{3 \times Noise(mV)}{Tophigh(mV)} \times [analyte(\%)]$$

The limit of quantification (LOQ) is the point at which we can reliably distinguish between two distinct values. Since the LOQ can vary significantly across laboratories, another threshold, known as the practical quantification limit (PQL), is often applied. The PQL is generally defined as approximately five times the MDL.

$$PQL = 5 \times MDL = 5 \times \left(\frac{3 \times Noise(mV)}{Tophigh(mV)} \times [analyte(\%)]\right)$$

2.7 Statistical Analysis and Results Methodology

The study design and statistical evaluations were followed according to recommendations and guidelines by study supervisor. The following mathematical models were applied throughout the thesis: Variance analysis: One-Way ANOVA was used with descriptives, along with Tukey Test using the software IBM SPSS (McHugh, 2011; Kim, 2017). Histology slides were read by Lena Holm, a Senior Lecturer at the Department of Anatomy, Physiology and Biochemistry (AFB); Division of Anatomy and Physiology.

3. Results

3.1 Experiment 1: Rainbow Trout Anaesthesia Trials under Lab Conditions: Preliminary Study

3.1.1 Behavioural Observations: Mortality, Anaesthesia and Recovery Times

Experimental Trial 1 Dose Comparison Test

The doses of isoeugenol required to induce anaesthetic effects vary depending on the size of the organism and the environmental conditions of the rearing environment (Zahran et al., 2021). In trial 1, a dose-response test using increasing doses of isoeugenol was conducted to evaluate the anaesthesia induction and recovery times of the iso-eugenol on rainbow trout fingerlings reared under an RAS system (Figure 3-1). The dose comparison identified the most effective dosage for rapid, safe anaesthesia induction with minimal recovery time. By examining 10, 15, 20, and 25 mg/L isoeugenol alongside MS-222 as a positive control, the analysis reveals a significant dose-dependent differences, providing insight into efficiency and welfare outcomes.

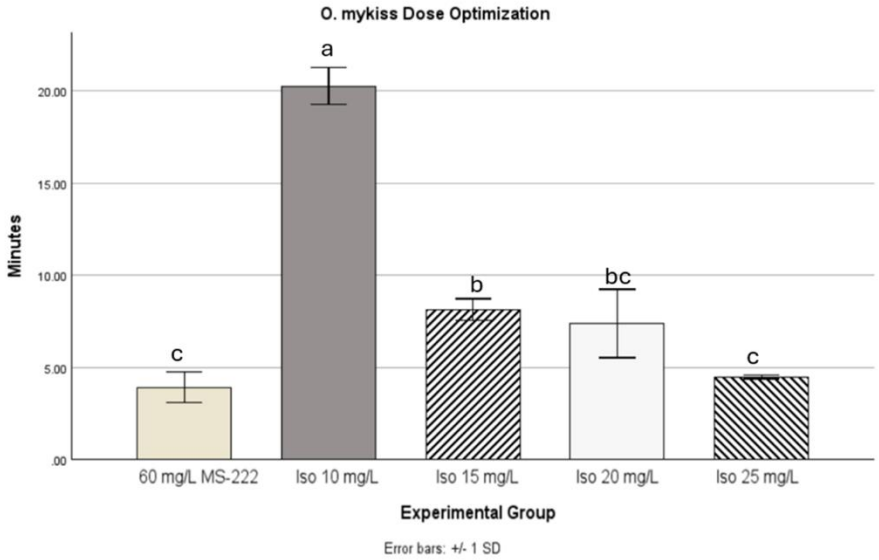

Figure 3-1 Effect of isoeugenol on the time (minutes) needed to reach stage III anaesthesia on rainbow trout fingerlings. Fingerlings were exposed to varying doses of isoeugenol (10, 15, 20 and 25 mg/L) as described in the methodology section. Fingerlings exposed to 60 mg/L of MS-222 served as a control. Error bars represent the mean ± standard deviations.

MS-222 had the shortest mean anaesthesia time at 3.93 minutes, followed by 25 mg/L of isoeugenol (4.49 minutes), 20 mg/L (6.57 minutes), 15 mg/L (8.14 minutes), and 10 mg/L of isoeugenol with the longest induction time at 19.96 minutes. A one-way ANOVA showed a significant effect of treatment type on anaesthesia time ($p < 0.001$), indicating that both the type and concentration of anaesthesia had a significant influence on induction time. The Tukey HSD post hoc analysis grouped the doses into subsets, showing that MS-222 and 25 mg/L were not significantly different, while 20 mg/L, 15 mg/L, and 10 mg/L differed significantly in anaesthesia times. Notably, the 10 mg/L dose of isoeugenol required significantly more time for anaesthesia induction than the other concentrations. These results indicate that lower doses of isoeugenol, particularly 10 mg/L, lead to considerably longer induction times compared to MS-222 and higher concentrations of isoeugenol.

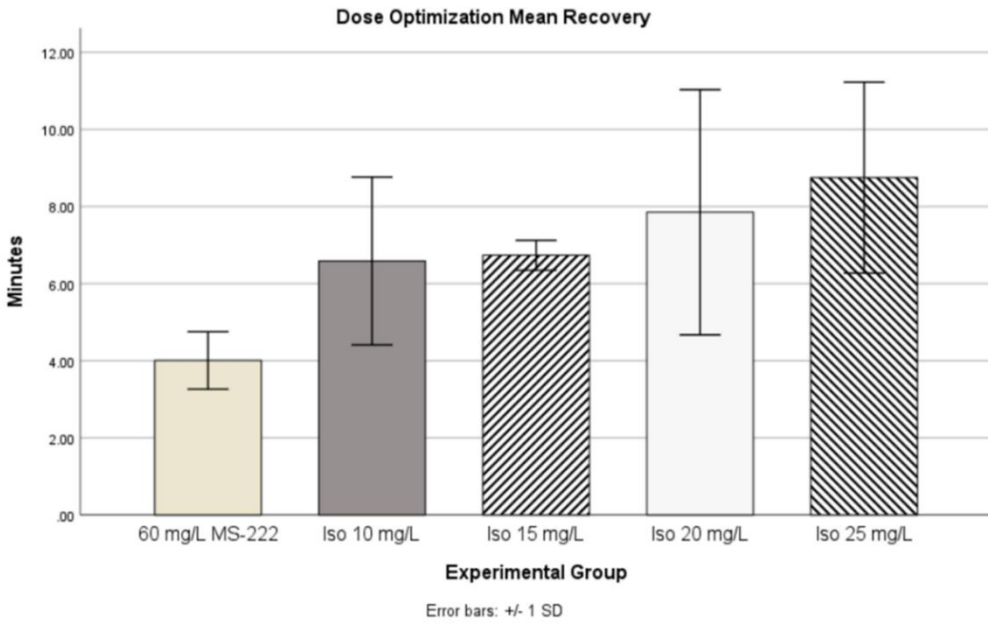

Figure 3-2 Effect of isoeugenol on the recovery time (minutes) of rainbow trout fingerlings. Fingerlings were exposed to varying doses of isoeugenol (10, 15, 20 and 25 mg/L) as described in the methodology section. Fingerlings exposed to 60 mg/L of MS-222 served as a control.

No mortalities were recorded during or at the end of the trial. The shortest recovery time was observed in the MS-222 group, with a mean of 4.04 minutes, and the longest in the 25 mg/L isoeugenol group, averaging 8.75 minutes. Intermediate recovery times were observed at 6.59 minutes for 10 mg/L isoeugenol, 6.74 minutes for 15 mg/L isoeugenol, and 7.85 minutes for 20 mg/L isoeugenol (Figure 3-2). However, the differences in recovery times were not statistically significant among the experimental groups, as indicated by a Tukey HSD test ($p = 0.192$). While the MS-222 group showed a quicker recovery, the lack of significant differences across groups suggests that variations in isoeugenol concentration do not substantially impact recovery duration. These findings indicate that, although anaesthesia induction times differ by dose, recovery times remain similar across different isoeugenol concentrations.

Experimental Trial 2

Based on the outcome of Trial 1, two doses of isoeugenol were selected and taken forward to evaluate the interactive effects with an anti-stress nutraceutical formulation. To this end, first, a dose of 10 mg/mL of isoeugenol was combined with different nutraceutical doses and their effects on anaesthesia induction and recovery times were investigated. A One-Way ANOVA was carried out using SPSS and the outcomes revealed a significant difference in the anaesthesia time between the experimental groups (Figure 3-3; $p = 0.008$). Fingerlings exposed to isoeugenol

at 10 mg/L (Control) had significantly longer mean anaesthesia time than that of those exposed to 60 mg/L of MS-222 (positive control group). Fingerlings that received exposure to the different combination of natural anaesthetic formulation had a higher induction time compared to the positive control, but the difference was not significant ($p > 0.5$). In comparison to the control group that received exposure to only isoeugenol (10 mg/L), the natural anaesthetic formulations, regardless of the combination of isoeugenol and nutraceutical formulation, had a shorter induction time for stage III anaesthesia. However, the difference in the response was not significant ($p > 0.05$).

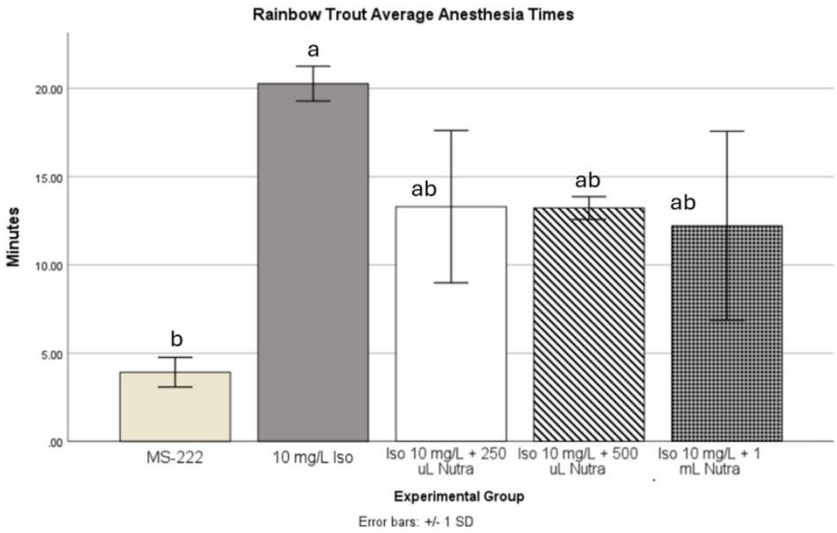

Figure 3-3 A) Effect of isoeugenol alone and in combination with different nutraceutical formulations on the time (minutes) needed to reach stage III anaesthesia on rainbow trout fingerlings. Fingerlings exposed to 60 mg/L of MS-222 served as a positive control.

An additional analysis of variance was conducted to assess the impact of the treatment groups on the recovery times of the anaesthetized fingerlings (Figure 3-4). The results showed no significant differences in the recovery time among the experimental groups ($p = 0.481$).

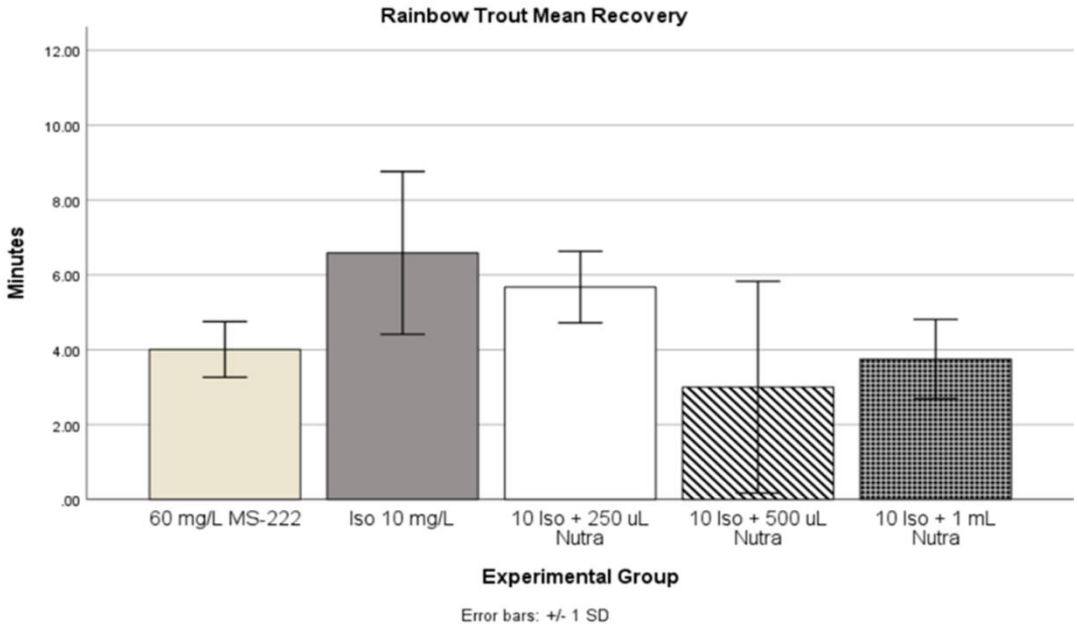

Figure 3-4 Mean recovery times across treatment groups

Experimental Trial 3

In Trial 3, the interactive effects of isoeugenol at 20 mg/L with various doses of the nutraceutical formulation (250 µL, 500 µL, and 1 mL) were examined on anaesthesia and recovery times in fingerlings (Figure 3-5). A one-way ANOVA indicated a marginal effect of treatment type on induction time ($p = 0.099$). Observable differences in group means were noted, with anaesthesia times ranging from 3.93 minutes for MS-222 to 7.39 minutes for 20 mg/L isoeugenol alone, and intermediate times for the combined treatments: 5.97 minutes (250 µL), 5.52 minutes (500 µL), and 4.78 minutes (1 mL) of the nutraceutical formulation. Tukey HSD did not reveal significant differences among the groups ($p = 0.170$), though there was a trend toward increased anaesthesia times for 20 mg/L isoeugenol without the nutraceutical formulation. These results suggest that while anaesthesia induction times vary with different combinations, statistical significance was not established.

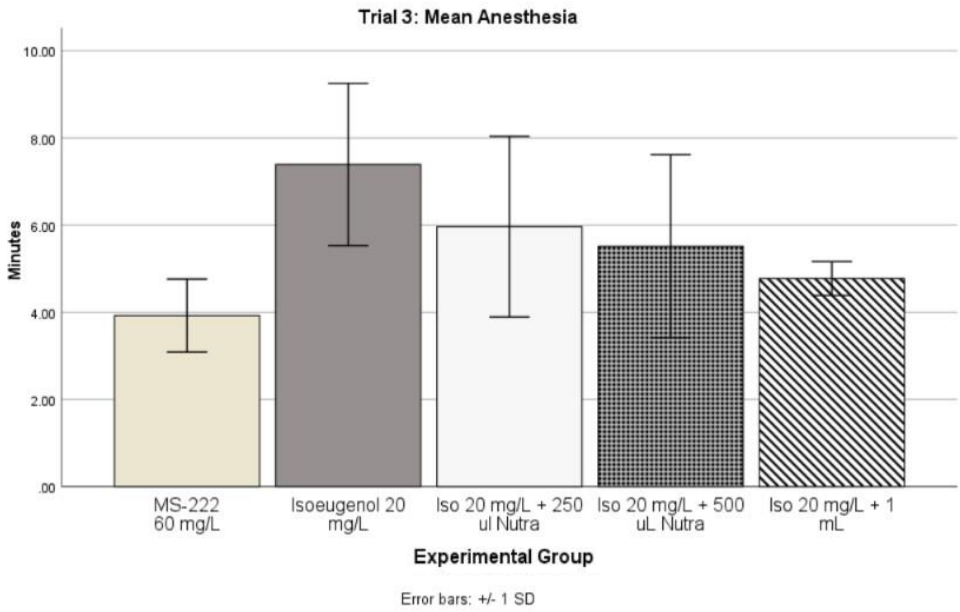

Figure 3-5 Trial 3 Average Anaesthesia and Nutraceutical additions to isoeugenol show impact on anaesthesia times.

While recovery times show greater variability, MS-222 had the shortest mean recovery time at 4.01 minutes, while the control group with 20 mg/L isoeugenol had the longest at 7.85 minutes (Figure 3-6). The addition of nutraceuticals to isoeugenol resulted in mean recovery times ranging from 6.72 minutes for 1 mL/L to 7.56 minutes for 500 µL/L. The one-way ANOVA showed no significant differences in recovery times among the groups ($p = 0.224$), and Tukey HSD further indicated no statistically significant differences between treatments ($p = 0.379$). This also suggests that if there were more preliminary replicates conducted, perhaps the treatment experimental groups may have an ability in producing optimal recovery and shortening recovery times.

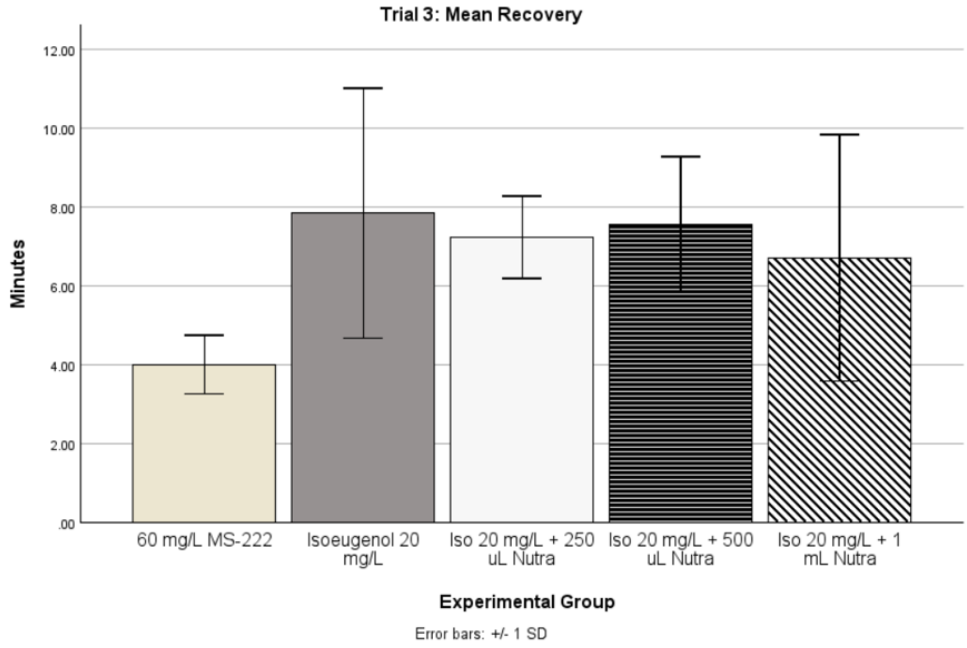

Figure 3-6 Trial 3 Average Anaesthesia and Recovery times. Nutraceutical additions to isoeugenol show reduced recovery times compared to just Isoeugenol 20 mg/L.

Summary of Anaesthesia and Recovery Results for Experiment 1

Summary of experiment 1's anaesthesia and recovery rates showed there was a significant effect of treatment on anaesthesia times across different doses of isoeugenol and MS-222 ($p < 0.001$). MS-222 had the shortest mean anaesthesia time (3.93 minutes), followed by 25 mg/L isoeugenol (4.49 minutes) and the 20 mg/L + 1 mL nutraceutical combination (4.78 minutes). In contrast, 10 mg/L isoeugenol had the longest time (20.27 minutes), with nutraceutical combinations at this dose also resulting in prolonged times (12.22–13.30 minutes). Tukey HSD post hoc analysis grouped MS-222 and higher doses of isoeugenol in shorter anaesthesia subsets, while 10 mg/L isoeugenol and its combinations fell in the longest subset. Higher doses of isoeugenol, particularly with nutraceuticals, approached MS-222's anaesthesia efficiency, while lower doses led to significantly longer induction times. Additionally, this conclusion justified not using 25 mg/L Isoeugenol, to avoid any unnecessary toxicological effects of upregulation from the higher dose with nutraceuticals, due to unknown effects (Figure 3-7).

Additionally, there was no significant differences in recovery times across treatments ($p = 0.124$) (Figure 3-8). MS-222 had one of the shortest recovery times (average = 4.01 minutes), while 10 mg/L isoeugenol combined with 500 µL nutraceutical had the shortest overall (average = 3.00 minutes). In contrast, higher

doses, such as 25 mg/L isoeugenol, resulted in longer recovery times (average = 8.75 minutes). Tukey HSD post hoc analysis indicated that while lower isoeugenol doses with nutraceutical combinations tended toward shorter recovery times, these differences were not statistically significant. These findings suggest that lower doses of isoeugenol, particularly when combined with nutraceuticals, may facilitate faster recovery, but further research is needed to confirm these trends.

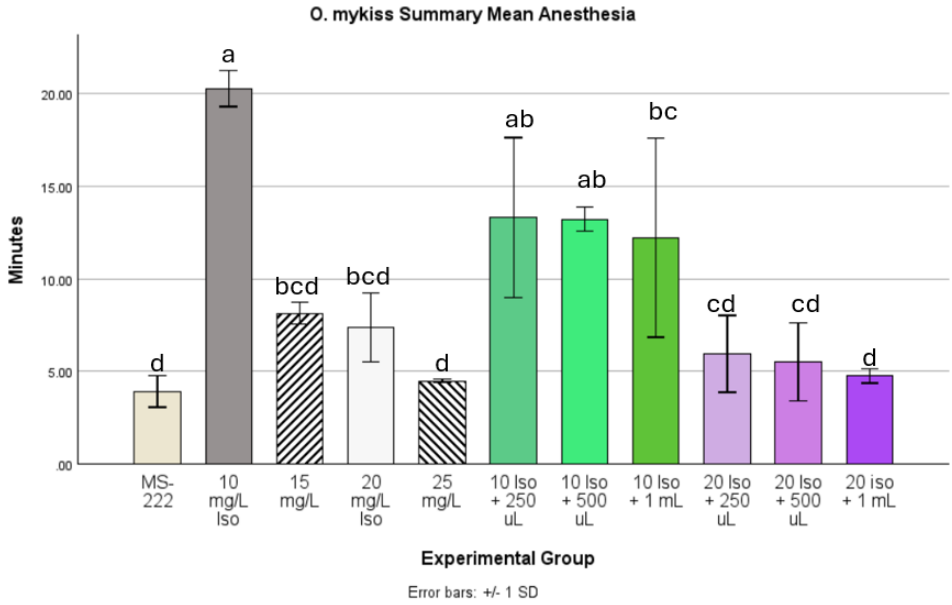

Figure 3-7 Mean anaesthesia times for different doses of isoeugenol and MS-222 across experimental trials, illustrating variations in anaesthesia duration based on dose and combination with nutraceuticals, highlighting significant differences between.

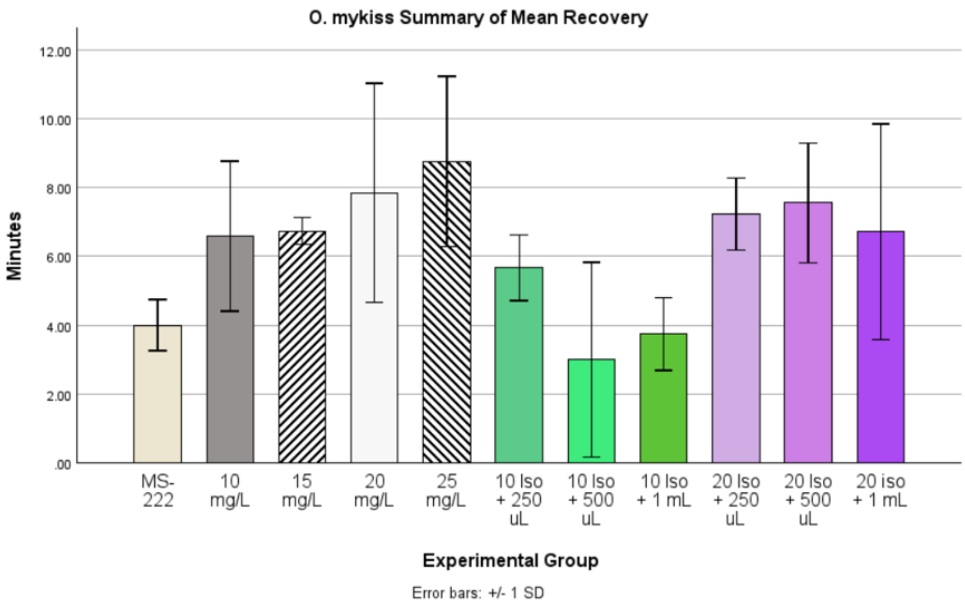

Figure 3-8 Mean recovery times for different doses of isoeugenol and MS-222 across experimental trials, illustrating variations in recovery duration based on dose and combination with nutraceuticals

3.1.2 Impact of the Anaesthetics on the Behavioural Observations and Anaesthesia Depth: Preliminary trial under Lab conditions

We employed a monitoring system to record the behaviour and to further verify the anaesthesia and recovery times of the fingerlings in response to the exposure to the anaesthetics under study (Figure 3-9). Rainbow trout fingerlings sedated at exposed to a lower dose of isoeugenol (10 mg/L) exhibited behaviours typical of light sedation, including schooling (swimming in groups) and resting at the bottom of the tank with mild equilibrium disturbances. This suggests that while they retain enough consciousness to display natural schooling behaviour, they experience some loss of balance.

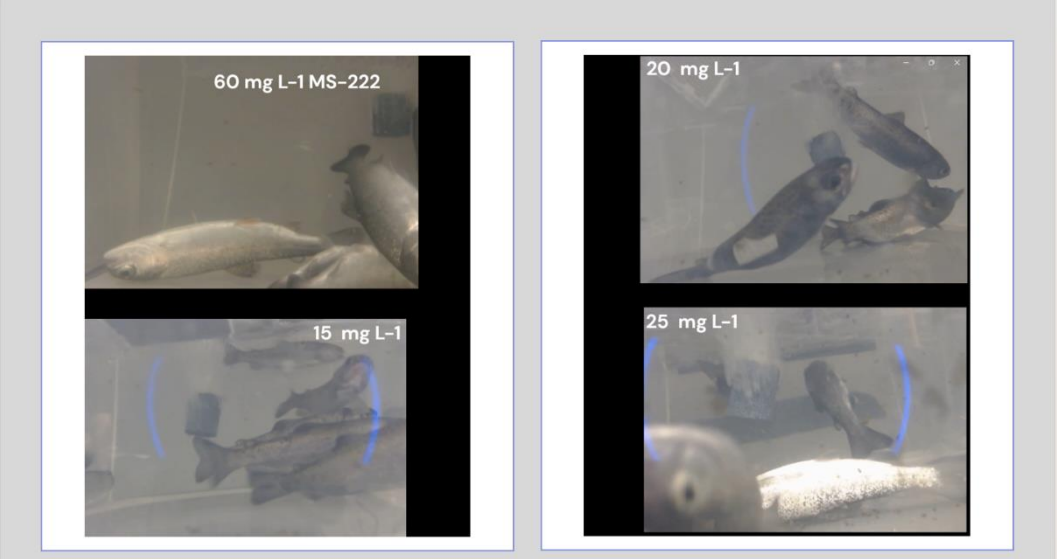

Figure 3-9 Video snapshot of different variations of behaviour and sedation depth at 3 minutes, 42 seconds. Isoeugenol 15 mg/L swimming and schooling present. MS-222 60 mg/L, Sedation level III, surgical to deep. Isoeugenol 20 and 25 mg/L equilibrium challenges with 25 mg/L lateral and nearing depth of MS-222 group.

In treatments involving higher doses, such as 60 mg/L of MS-222 or isoeugenol at 20 mg/L or 25 mg/L, fish showed more pronounced behaviours like no-swimming or lateral positioning (lying on their side) with minimal fin or tail movement, indicating deeper sedation or anaesthesia and significantly reduced mobility (Figure 3-9). The fingerlings that were exposed to combinations of isoeugenol and nutraceutical formulations displayed signs of excitement, such as increase swimming or circling, and react to external stimuli like tank tapping or lid opening. These reactions suggest that the fish remain somewhat responsive and are in a state of light to moderate sedation. The most notable behaviours at higher doses of isoeugenol (20 mg/L and 25 mg/L) include deep sedation or anaesthesia, where fish exhibit minimal movement, typically describe as “slight fin movement” or “no to minimal fin movement.”, indicating successful anaesthesia with minimal reactivity to external stimuli. Isoeugenol (20 mg/L) compares to MS-222 (60 mg/L) in terms of inducing deep sedation or surgical-level anaesthesia in the fish, highlighting similar sedation behaviours. The Sneddon (2012) ethogram (see Table 1) was used to measure the anaesthesia depth and stages in fish, behavioural responses and depth (Appendix 1, Figure 3-10).

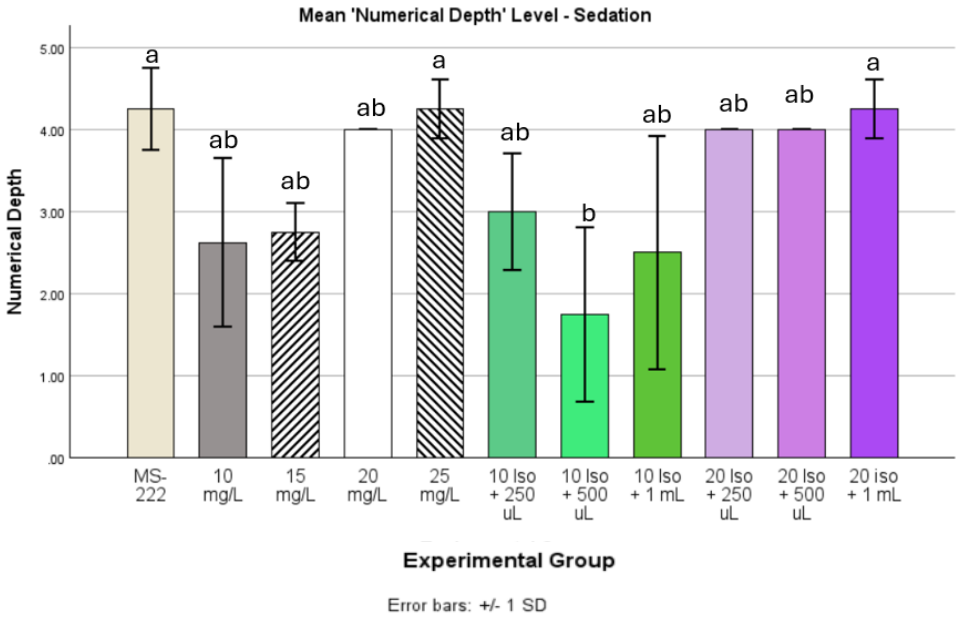

Figure 3-10 Overview of the mean sedation 'Numerical Depth Level' across all experiment 1 trials. The Sneddon (2012) ethogram (see Table 1) was used to assess anaesthesia depth, stages, and behavioural responses in fish (Appendix 1)

During the preliminary experimental trials 1-3, MS-222 achieved "III Surgical to Deep" anaesthesia with a numerical 'Numerical Depth Level' of 4.25 (See Appendix 1; Table 1). MS-222 induction response is indicative of successful deep sedation and anaesthesia with minimal to no level of tail movement. Similarly, isoeugenol at 20 mg/L reached a "III Surgical" depth with a Numerical Depth Level of 4, demonstrating comparable deep anaesthesia effects to MS-222.

A One-Way ANOVA was employed on the data of the "Numerical Depth Level" to evaluate the effects of different anaesthetic dosages on sedation and anaesthesia depth. There was a significant effect of the anaesthetics on the anaesthetic depth of the fingerlings ($p = 0.004$), with MS-222 at 60 mg/L showing the deepest and most consistent anaesthesia (average depth level = 4.25), while the group that received isoeugenol at 10 mg/L exhibited a shallower effect with an average depth level of 2.625, indicative of lighter sedation, and the difference between the two groups was significant ($p < 0.05$). Post hoc analysis using Tukey HSD indicated that lower doses of isoeugenol, including 10 mg/L alone (average = 2.625) and its combinations with nutraceutical doses of 500 µL (average = 1.75), 1 mL (average = 2.5), and 250 µL (average = 3.0), as well as the 15 mg/L isoeugenol treatment (average = 2.75), produced significantly shallower sedation depths. These results indicate that lower doses of isoeugenol alone or in combination primarily result in light to moderate sedation.

In contrast, higher doses of isoeugenol, such as 20 mg/L and 25 mg/L, as well as MS-222 at 60 mg/L, achieved deeper levels of anaesthesia. The second subset in the post hoc analysis showed that 20 mg/L (average = 4.0), 25 mg/L (average = 4.25), and MS-222 (average = 4.25) were not significantly different from one another, nor were combinations of 20 mg/L isoeugenol with 250 µL (average = 4.0), 500 µL (average = 4.0), or 1 mL (average = 4.5) of the nutraceutical formulation. Notably, the combination of 20 mg/L isoeugenol with 1 mL nutraceutical achieved the highest average depth level (average = 4.5), suggesting it may be even more effective than MS-222 in reaching surgical-level anaesthesia.

Standard deviation values reflected variability in depth responses among individual fish, indicating that some fish responded differently within the same treatment. In the future, individualized sedation and evaluation might simplify assessment and provide more precise depth data across groups. These findings suggest that while lower doses of isoeugenol (10 mg/L) and its combinations are sufficient for light to moderate sedation, a higher dose of 20 mg/L, particularly with the addition of nutraceuticals, closely approximates or exceeds the anaesthetic depth achieved by MS-222, making it a viable alternative for achieving deep anaesthesia.

3.1.3 Experiment 2: Brown Trout Anaesthesia Trials under Semi-Industrial Conditions

This experiment aims to validate the findings obtained from the rainbow trout trials conducted under laboratory conditions. We used brown trout, another salmonid species, as the test species. This species is from natural fisheries, and the rationale for using it is to test the broader applicability of the findings. Validating the results in a fish species of importance to fisheries ensures that the results are relevant across both farmed and wild populations.

Impact of anaesthetic formulation on anaesthesia and recovery times

No significant difference in anaesthesia time was observed between the groups exposed to the newly developed anaesthetic formulation, which combined 20 mg/L of isoeugenol with nutraceuticals, and the groups exposed to either isoeugenol alone or MS222. The mean anaesthesia times in the fish exposed to isoeugenol alone or MS222 ranged from 7.67 to 8.33 minutes (Figure 3-11a). Whereas the groups that received the natural anaesthetic formulation had an average anaesthesia time between 10.33 and 11.00 minutes.

While the recovery times ranged from 11.67 minutes in Treatment 2 to about 14.33 minutes in both control 1 and Treatment 3, there was no significant difference in the recovery times (p=0.861) between the groups (Figure 3-11b). Mortality remained low across all groups, though some "Did Not Recover" (DNR) cases were noted, particularly in Treatment 3. Gill bleeding was observed in several groups, indicating possible stress or physiological effects from the Isoeugenol anaesthetic process.

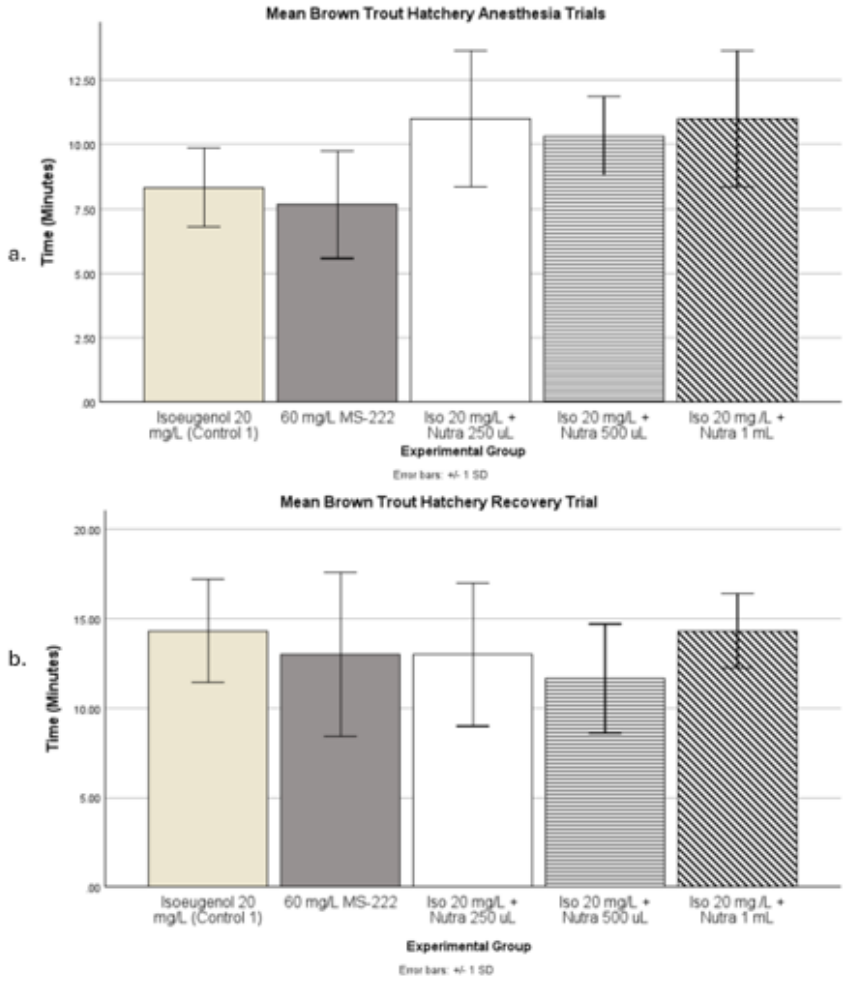

Figure 3-11 Effect of isoeugenol alone or in combination with a nutraceutical formulation on the anaesthesia (a) and recovery times(b) of brown trout fingerlings. Fingerlings exposed to 60 mg/L of MS-222 served as a positive control. Error bars represent mean ± SD

Impact of anaesthetic formulation on Mortality During Experimental Groups

To examine the impact of different anaesthetic treatments on mortality, a one-way ANOVA was conducted comparing the five experimental groups (Figure 3-12).

Mortality was defined as the inability to recover or perish during the experimental process. The analysis revealed no statistically significant differences in mortality.

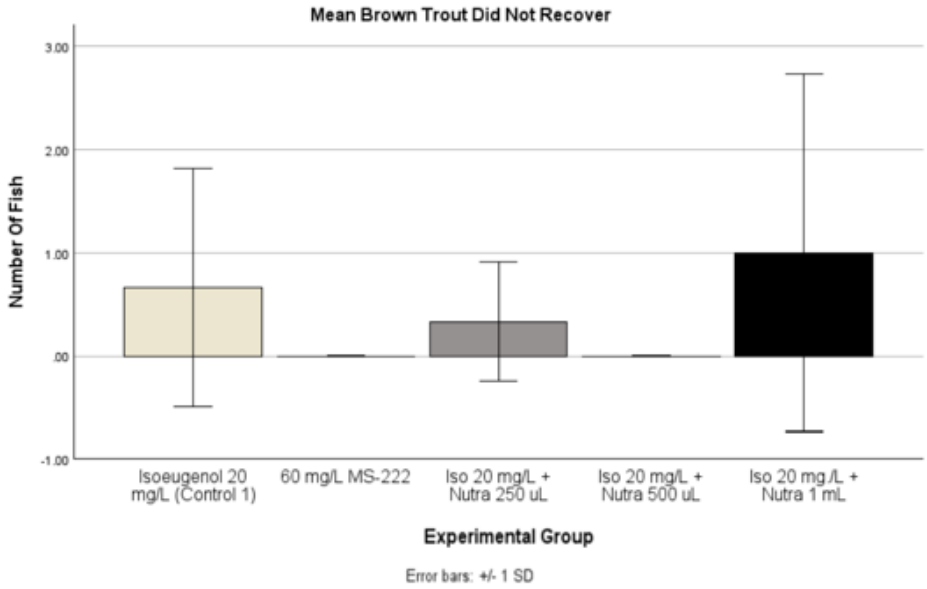

Figure 3-12 Mean number of brown trout that did not recover under different experimental treatment groups. Error bars represent ± standard deviation. Variability in non-recovery is observed especially in Isoeugenol 20 mg/L + Nutra 1 mL, but no significant difference between them.

Gill Bleeding Observations

Gill bleeding was observed in the groups exposed to isoeugenol either alone or in combination with the nutraceutical formulation during experimental trials. Bleeding frequency varied across replicates for each group. However, the group exposed to MS222 showed no bleeding in any replicates (Figure 3-13).

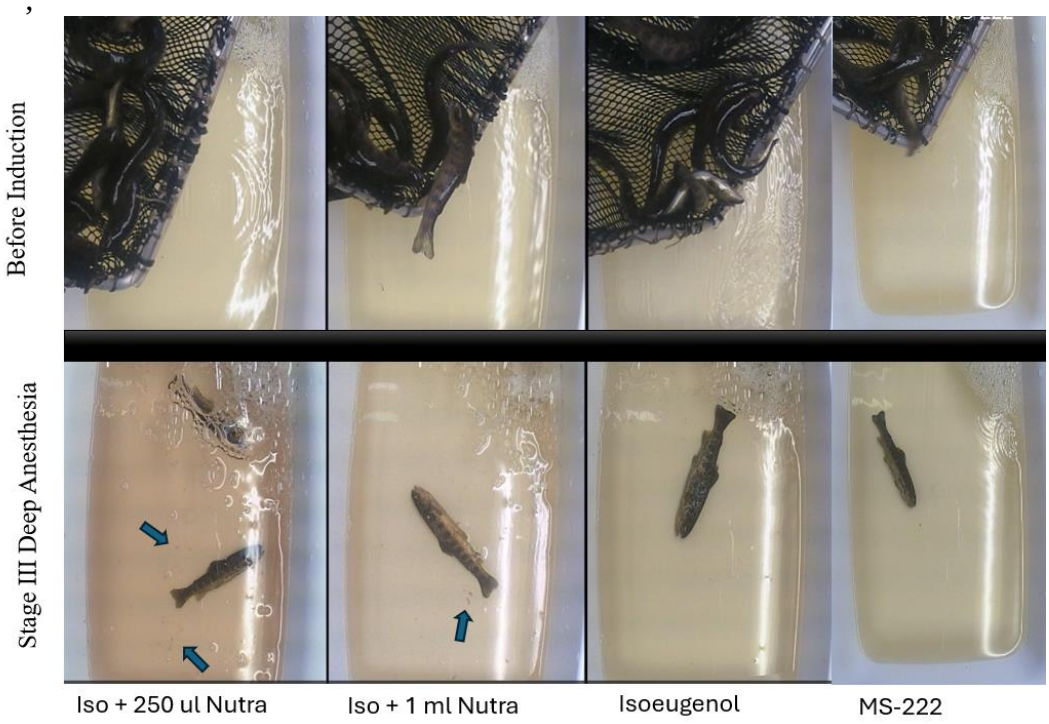

Figure 3-13: Occurrence of gill bleeding in brown trout. Colour of rearing water before (top three pictures) and after induction (bottom three pictures), showcasing change in colour or 'red water'. Additional artifacts, possibly coagulated blood can be seen as indicated by arrows. Snapshots taken from Time Lapse Camera (Brinno TLC200Pro HDR).

3.1.4 *Salmo Tuttra* Gill Histology Preliminary Results

The histological analysis of the left second-gill arch sections highlighted various structural changes between the control and treatment groups. Various pathological features including bleeding, excessive erythrocytes, leukocyte infiltration, epithelial lifting and alterations in the secondary lamellae were recorded.

Gill Histology: Control Groups

The experimental groups consisted of fish exposed to 20 mg/L of isoeugenol and 60 mg/L MS-222. Trials were conducted on May 22, 2024 (replicate 1), May 28, 2024 (replicate 2), and May 29, 2024 (replicate 3) (Figures 3-14 and 3-15) One replicate (Isoeugenol R2; Figure 3-14) displayed largely intact gill morphology, with well-preserved lamellae and only minor abnormalities. In contrast, other isoeugenol replicates showed clear pathological changes, including bleeding and epithelial lifting. The MS-222 group showed mainly normal lamellae, with some increase in erythrocyte presence. However, some fish in the MS-222 replicates (Figures3-15) displayed more pronounced abnormalities, such as excess

erythrocytes, swollen cells possibly indicating chloride cell hypertrophy, and leukocyte infiltration, suggesting an inflammatory response.

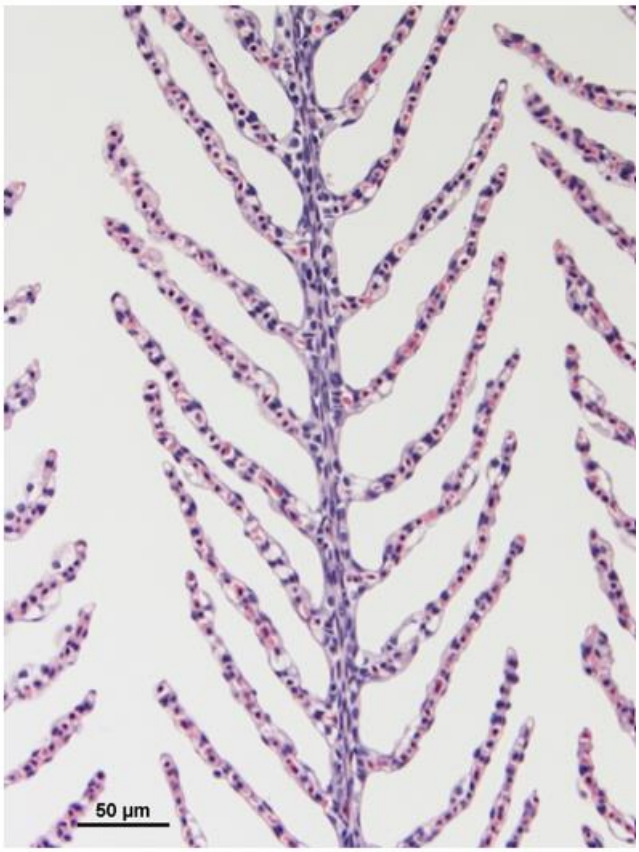

Figure 3-14 Single gill section from isoeugenol replicate 2 (R2), showing relatively intact lamellae with minimal abnormalities compared to other isoeugenol replicates. The lamellae are well-structured, with erythrocytes visible in pink to red and nuclei in purple-blue, showing no obvious signs of inflammation or epithelial lifting. Scale bar = 50 µm

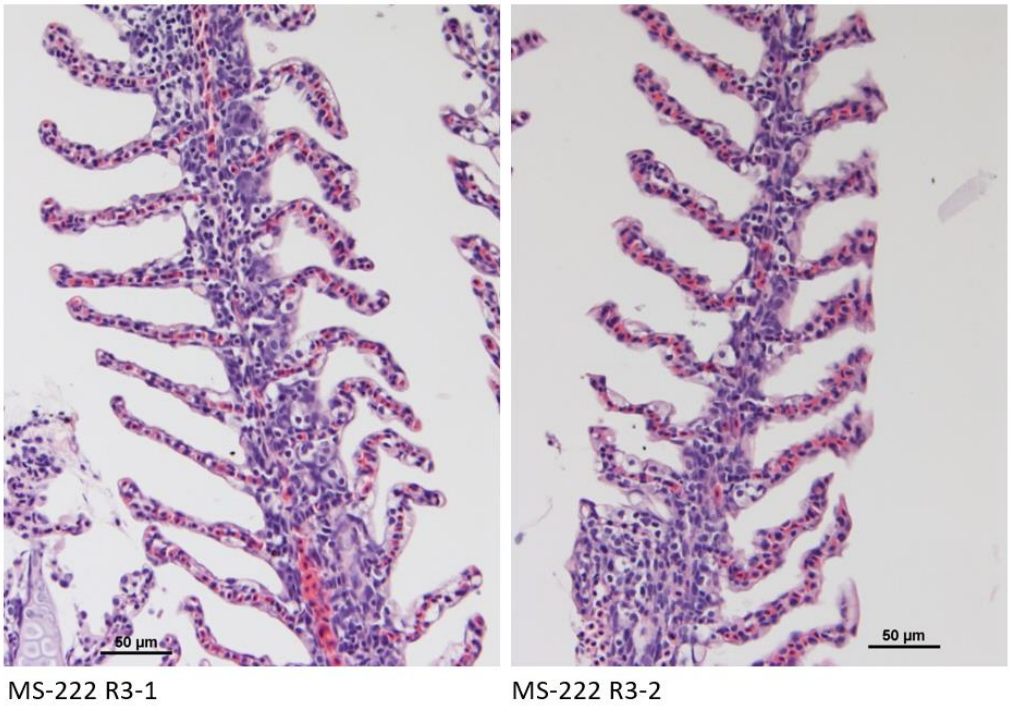

Figure 3-15 Gill sections from MS-222 replicate 3 (R3), showing two images from the same section. The secondary lamellae look irregular, with some shorter, blunted structures. Notable leukocyte infiltration is present, with leukocytes appearing pale pink or light purple, and their nuclei in darker purple-blue. Erythrocytes are visible in pink to red. Overall, these sections show more pronounced morphological changes compared to the other MS-222 replicates. Scale bar = 50 µm.

Gill Histology: Treatment Groups

In treatment groups were defined as the following: Experimental group 1; Isoeugenol 20 mg/L + 250 µL Nutra, Experimental group 2; Isoeugenol 20 mg/L + 500 µL Nutra, and Experimental group 3; Isoeugenol 20 mg/L + 1 mL Nutra. Trials were conducted on replicate 1, May 22nd, 2024, replicate 2, May 28th, 2024, and replicate 3, May 29th, 2024 (Figure 3-16, Figure 3-17, and Figure 3-18). Brown trout fingerlings exposed to the anaesthetic formulation of isoeugenol (20 mg/L) and 250 µL nutraceuticals displayed compression with architectural changes and excess erythrocytes in secondary lamellae. The same experimental group showed architectural changes, with thicker secondary lamellae containing erythrocytes (Figure 3-16). Fingerlings from the group that received a combination of isoeugenol and 500 µL nutraceuticals displayed swollen cells or potential epithelial uplifting, and symptoms of edema (Figure 3-17). Some fish in this experimental group had erythrocytes in secondary lamellae, mixed with areas appearing relatively normal. Fingerlings that received an anaesthetic formulation combining isoeugenol and 1 mL of nutraceutical formulation showed two images from the same section, with

normal lamellae on the left and shorter secondary lamellae on the right. The same experimental group included three images showing architectural changes, with excess blood and shortened secondary lamellae in some areas (Figure 3-18).

Overall, controls maintained normal gill structure, while nutraceutical-treated groups, especially at higher concentrations, showed increasing signs of circulatory or inflammatory responses.

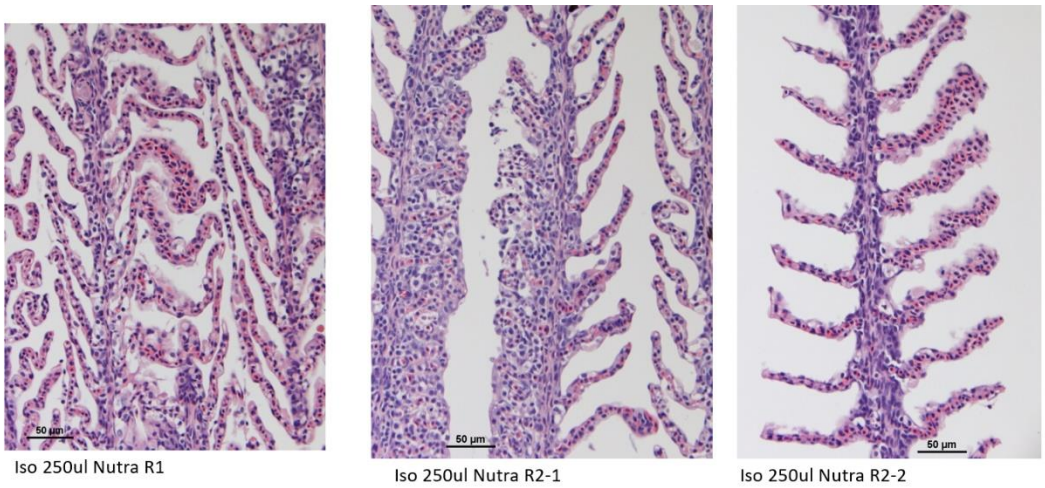

Figure 3-16 Gill sections from Isoeugenol and 250 µL Nutraceutical replicates 1 (R1) and 2 (R2), with two sections from the same gill in R2 (R2-1 and R2-2). R1 displays compressed gill structures with architectural changes, including excess erythrocytes visible in red in some secondary lamellae. In R2, architectural changes continue, with some secondary lamellae appearing thicker and containing erythrocytes, particularly visible in the second section (R2-2), indicating potential circulatory changes.

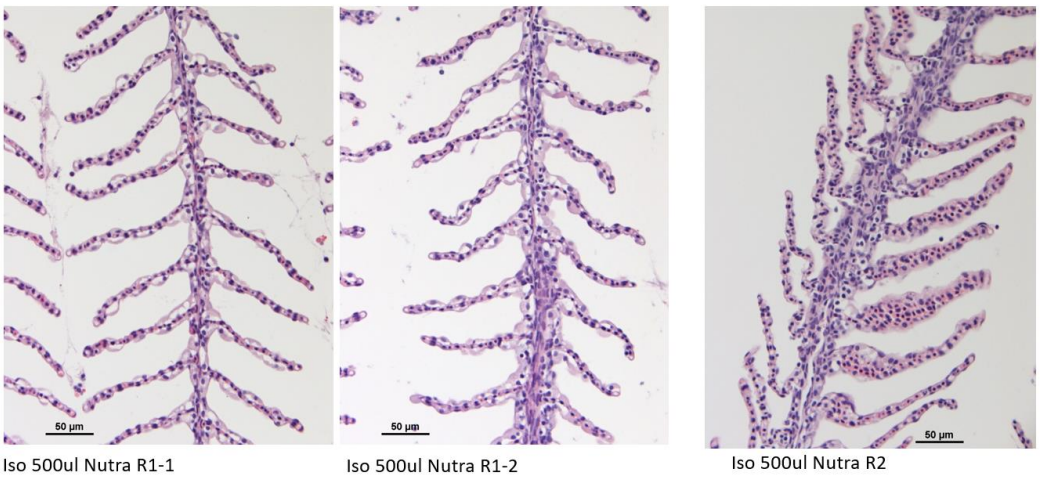

Figure 3-17 Gill sections from Isoeugenol combined with 500 µL Nutraceuticals showcasing replicate 1 (R1), with two sections (R1-1 and R1-2), and replicate 2 (R2). R1 sections (R1-1 and R1-2) display mostly intact lamellae with minor structural changes and low levels of erythrocytes visible in red within the secondary lamellae. In R2, there are more prominent architectural changes, with some secondary lamellae showing increased erythrocyte presence, indicating potential circulatory alterations.

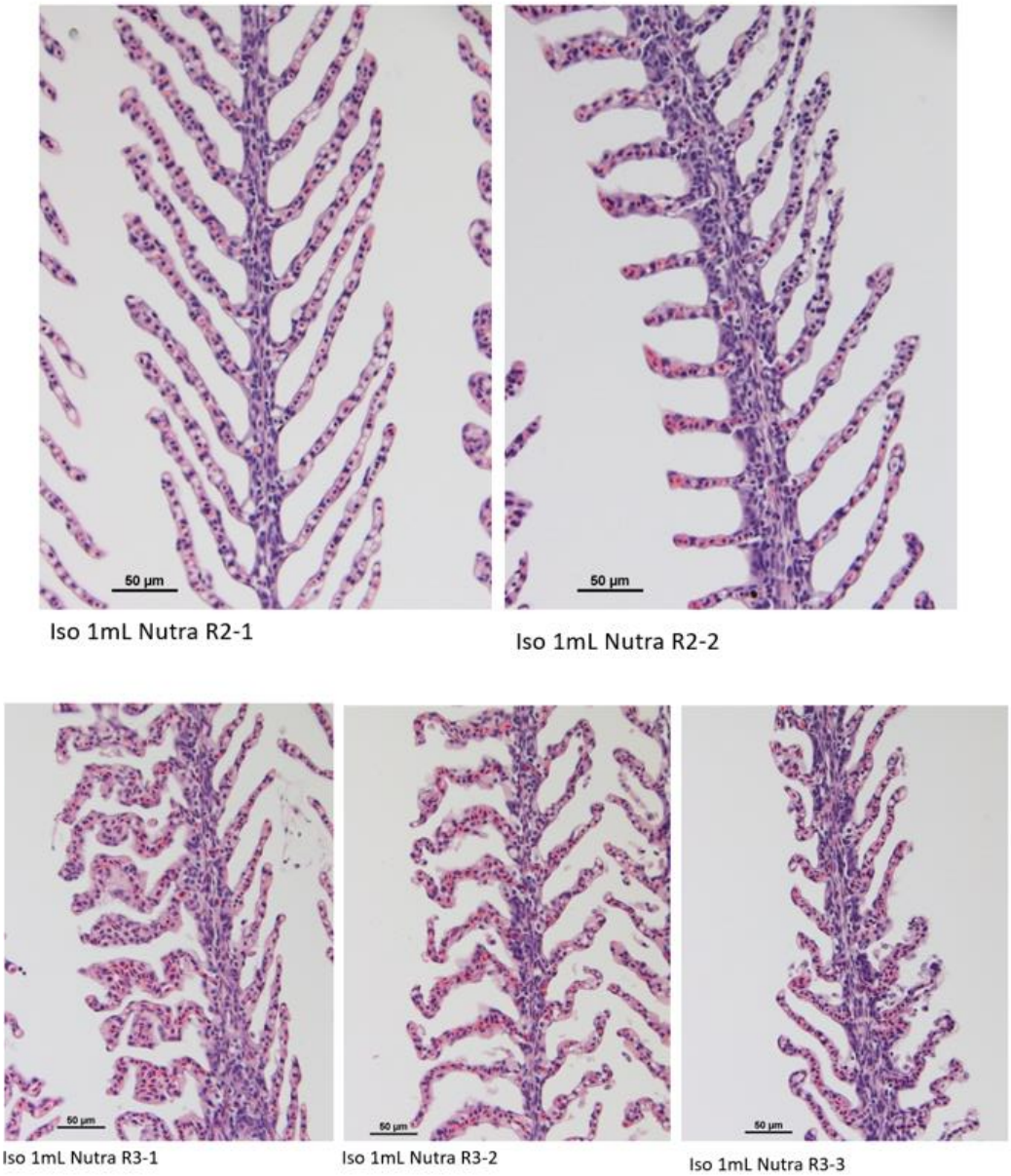

Figure 3-18 Gill sections from Iso 1 mL Nutra, with replicate 2 (R2) on top and replicate 3 (R3) on the bottom. The top row shows R2 with two sections, R2-1 (left) and R2-2 (right); R2-1 appears mostly intact, red erythrocytes within the secondary lamellae, while R2-2 has shorter secondary lamellae with areas of erythrocytes. The bottom row displays three sections from R3 (R3-1, R3-2, and R3-3), showing architectural changes with excess blood (red erythrocytes) in some secondary lamellae, shorter lamellae, and purple-stained nuclei contrasting with surrounding tissue, indicating moderate structural modifications.

3.1.5 ELISA Plasma Cortisol Results

Plasma cortisol level in the brown trout fingerlings exposed to different anaesthetic formulations was measured by ELISA (Figure 3-19). The ANOVA results showed no significant difference in the cortisol level between the different groups. Yet, the highest cortisol concentration was recorded in the group exposed to isoeugenol 20 mg/L combined with 1 mL of Nutra, indicating a heightened stress response. In contacts, the 60 mg/L MS-222 positive control group showed the lowest cortisol levels, suggesting a lower stress response under this treatment condition. This response was followed by the group exposed to isoeugenol 20 mg/L and 250 µL of nutraceutical formulation. The results suggest that at a lower concentration, the nutraceutical formulation could potentially mitigate the stress induced by the anaesthesia effects caused by isoeugenol. However, at a higher concentration, the nutraceutical formulation might cause additional physiological stress.

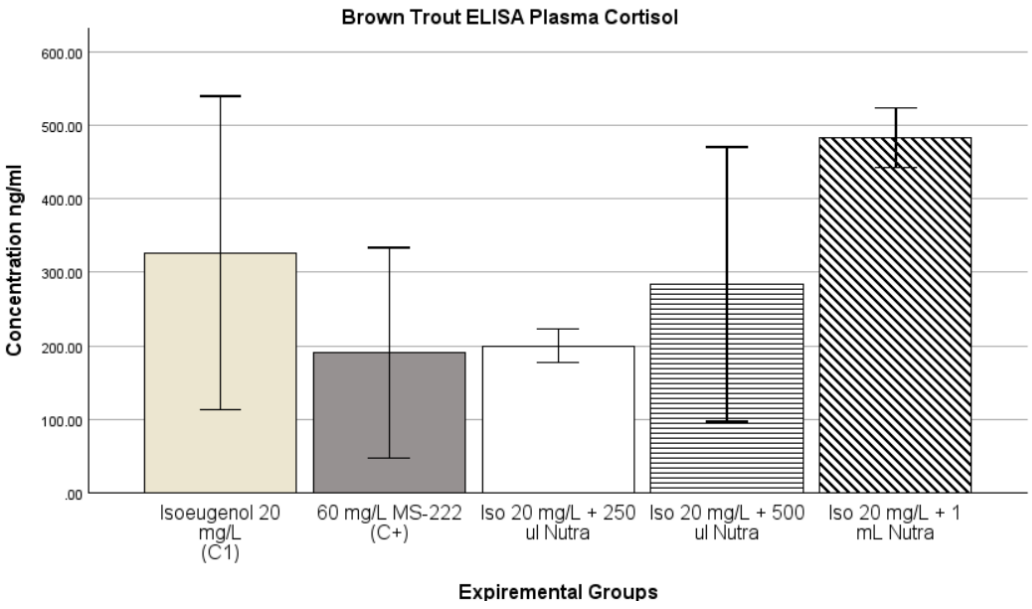

Figure 3-19 Plasma cortisol concentrations in brown trout across experimental groups showing variability. Error bars represent standard deviation.

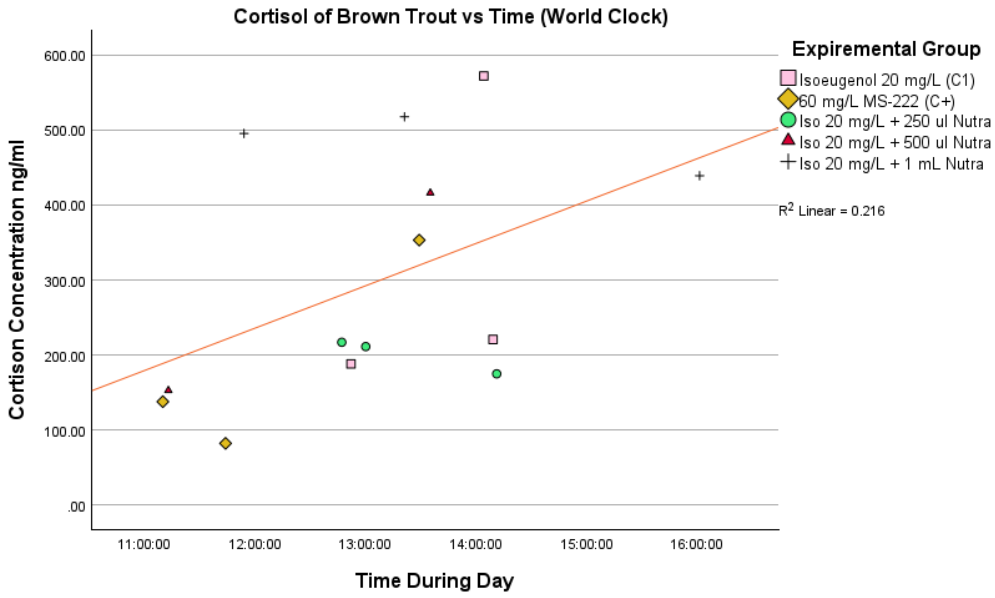

Figure 3-20 Cortisol of Brown Trout vs Time scatter plot displaying cortisol concentrations (ng/mL) over time for various experimental groups. Trendline suggests a weak positive trendline (R^2=0.216)

Additionally, a scatter plot (Figure 3-20) illustrates the relationship between cortisol concentrations and time of day for each treatment. Although, there is no strong correlation (R^2=0.216), a light positive trend suggests a potential increase in cortisol concentrations later in the day. The line indicates only 21.6% of variation in cortisol levels is due to the time of day. This weak correlation suggests that fluctuations in cortisol levels are more likely influenced by experimental conditions, external factors or individual biological responses rather than a diurnal rhythm. The distribution of data points across time and treatment groups shows a complex interaction between stress response and treatment type. These observations hint at potential time-dependent component to cortisol responses in brown trout, but further investigation is required to determine a consistent circadian or treatment-related pattern exists.

3.1.6 High-Performance Liquid Chromatography Analysis

Isoeugenol residue concentrations in skin-on fillet samples were analysed 48 hours post-exposure using HPLC following a standardized extraction protocol. This analysis aimed to assess residual isoeugenol levels to understand the retention characteristics of the compound in fish tissues and to develop a methodology and protocol for future studies. Isoeugenol was detectable at 7.92 to 8.10 minutes (Figure 3-21).

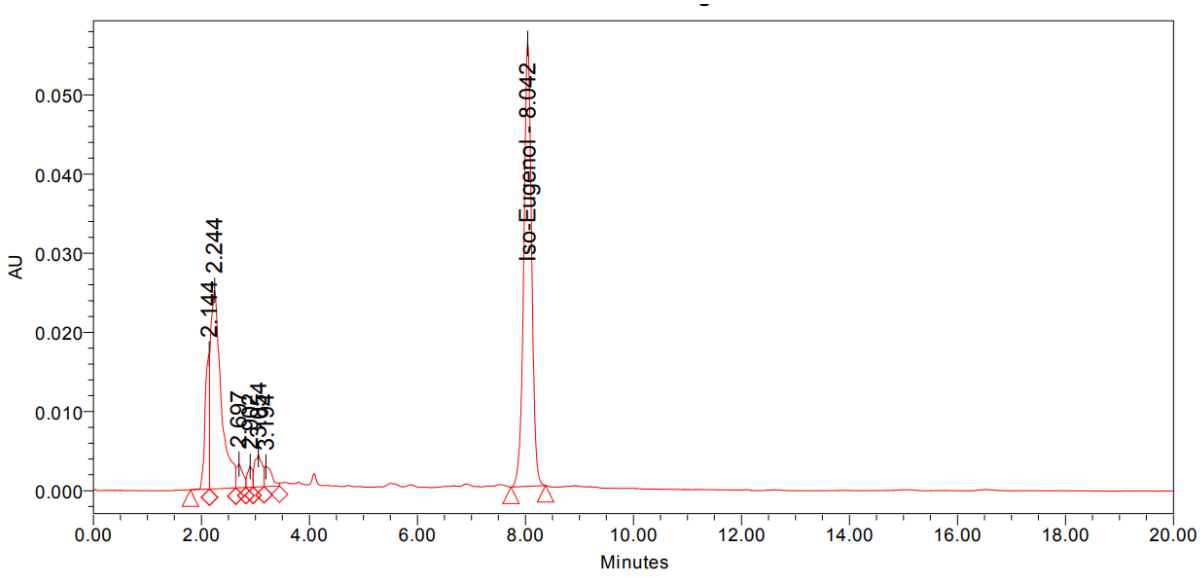

Figure 3-21 Example of a chromatographic profile of Isoeugenol from Run 1. The chromatogram was obtained at 261 nm. The primary peak for Isoeugenol retention time, 8.042 minutes, with an area of 566,587 and a height of 55,986, measured in Absorbance Units (AU). Minor peaks observed between 2 and 3 minutes likely represent residual components from the extraction process, such as acetonitrile, matrix compounds from the fillet tissues.

The HPLC isoeugenol concentrations (µg/mL) for each run were as follows: Run 1 -15, and apart from Run 8, which was spiked with 136 µg/mL of isoeugenol, all samples showed residual isoeugenol levels at 48 hours (Table 3). These samples were pooled from 20 mg/L isoeugenol treatments in hatchery brown trout, with or without nutraceuticals, validating the experimental extraction and detection methodology.

Detected concentrations were low, ranging from 0.02 µg/g to 0.08 µg/g (µg of extracted isoeugenol per g of tissue). Among unspiked samples, runs 1 and 7 had the highest concentrations at 2.88 µg/g and 10.30 µg/g, respectively. Samples showing concentrations of isoeugenol at or above 0.006 µg/g can be confidently reported as quantifiable (PQL). Run 15, though only 2.5 g of tissue was used during the extraction process (< 5.0g ± 2.0), was detectable (PQL > 0.006 µg/g). This variability suggests that isoeugenol retention may be affected by fish metabolism, tissue characteristics, or nutraceuticals, warranting further investigation.

Table 3 HPLC Isoeugenol concentrations showing samples runs 1 through 15. All samples had some sort of minimal concentrations, ranging from 0.02 µg/g to 0.08 µg/g. Sample 8, Spiked with 136 µg/mL of Isoeugenol and acted as a control indicator.

Sample Run	Concentration Isoeugenol µg/ml	Tissue Weight (g)	Concentration Per Weight of Fish (µg/g)
Run 1	2.82	4.89	2.88
Run 2	0.078	4.92	0.08
Run 3	0.052	5.2	0.05
Run 4	0.049	4.92	0.05
Run 5	0.045	4.88	0.05
Run 6	0.057	4.82	0.06
Run 7	9.845	4.78	10.30
Run 8 **	141.6	5.22	135.63
Run 9	0.19	5	0.19
Run 10	0.059	5.18	0.06
Run 11	0.052	5.049	0.05
Run 12	0.057	4.915	0.06
Run 13	0.036	5.032	0.04
Run 14	0.035	5.097	0.03
Run 15	0.01	2.5	0.02
	** *Spiked with 136 µg/mL of Isoeugenol*		

4. Discussion

4.1 Overview of Findings and Contextualization in Fish Welfare Research

This study aimed at evaluating the effects of combining isoeugenol with an anti-stress nutraceutical formulation on the anaesthesia depth, recovery times, cortisol levels, gill health, and post-anaesthesia isoeugenol concentrations in fish tissues. The goal was to develop a natural anaesthetic formulation as an alternative to the chemical-based anaesthesia for farmed and restocking fishes, which are crucial for sustainable practices. Synthetic anaesthetics commonly used in farmed fishes often pose risks, such as prolonged residual effects in the animal´s tissues. In addition, they may also cause negative physiological responses in fish, potentially affecting growth, reproduction, and overall health as well as welfare. A natural anaesthetic formulation, derived from plant-based or other bioactive natural compounds, would not only reduce risks but also align with consumer demand for natural and sustainable food products. In this thesis, we chose to study the effects of a natural anaesthetic formulation on two fish species: i) farmed rainbow trout *Oncorhynchus mykiss*) under lab conditions (Experiment 1) and ii) fisheries species brown trout *Salmo trutta* under semi-industrial conditions (Experiment 2). The rationale for such a dual-setting experimental approach was to assess whether the natural anaesthetic formulation could enhance fish welfare and operational efficiency in aquaculture and fisheries production, ensuring the findings are both scientifically robust and practically relevant. The results from our studies suggested that combining isoeugenol with an anti-stress nutraceutical formulation, at a specific dose influenced anaesthesia depth, had variable impacts on gill health, and reduced cortisol levels under certain conditions.

In both experiments, clear differences in anaesthesia times were observed across treatments. Control groups receiving MS-222 (60 mg/L) showed the shortest anaesthesia times for both species, with *O. mykiss* averaging around 3.58 minutes and *S. trutta* at 7.67 ± 2.08 minutes, consistent with its rapid onset as documented in previous studies (Barton and Iwama, 1991; Schreck and Tort, 2016). Conversely, isoeugenol treatments in *O. mykiss* had longer induction times, particularly at lower

concentrations. For instance, the 10 mg/L isoeugenol group with 250 µL/L nutraceuticals averaged 15.34 minutes, suggesting that while isoeugenol may not act as quickly as MS-222, combining it with nutraceuticals at lower doses helps improve its efficacy and tolerance (Small, 2003; Iversen et al., 2003). In contrast, 10 mg/L of isoeugenol alone had significantly longer induction times (~19.96 minutes), indicating a clear dose-dependent relationship in its anaesthetic efficacy.

In *O. mykiss* treated with 20 mg/L of isoeugenol combined with nutraceuticals, induction times shortened further. With averages of 5.97 minutes for 250 µL, 5.52 minutes for 500 µL, and 4.78 minutes for 1 mL nutraceutical addition, the higher isoeugenol concentration combined with nutraceuticals achieved more rapid sedation, approaching the efficacy of MS-222. This points to a clear dose-dependent enhancement in anaesthetic efficacy of isoeugenol, particularly as nutraceutical concentrations increase alongside 20 mg/L isoeugenol. This suggests a synergistic or additive effect, where the combined action of isoeugenol at 20 mg/L with increasing nutraceutical concentrations seems to further improve anaesthetic potency, approaching the sedation efficiency with MS-222. Such a dose-dependent relationship underscores the potential for nutraceuticals to enhance the efficacy of isoeugenol, potentially offering an alternative to MS-222. However, the practical implications require careful consideration. While increased nutraceutical doses tend to enhance sedation speed, it is vital to evaluate any long-term physiological impacts on fish and ensure consistent safety and recovery profiles across various environmental and operational conditions. Further studies should be carried out to explore these aspects.

In Experiment 2, we aimed to address a few of the aspects described above. Our results showed no significant difference in the induction of anaesthesia and recovery times between the different experimental groups. It is noteworthy to mention that an interesting observation was made in the isoeugenol exposed group that were also simultaneously exposed to higher nutraceutical concentrations. In particular, the group treated with 20 mg/L isoeugenol, and 1 mL/L nutraceutical had prolonged anaesthesia times ($P > 0.05$), along with increased bleeding and gill damage. Bleeding was observed across all isoeugenol-treated groups, suggesting that higher nutraceutical concentrations or isoeugenol may affect mucous membrane integrity. Mortality rates (DNR cases) were also highest in this group, indicating that certain isoeugenol-nutraceutical combinations may elevate physiological stress or toxicity at higher doses.

Histological examination showed nutraceutical-treated groups had increased erythrocytes, swollen cells, and occasional leukocyte infiltration, indicating mild to moderate inflammation. Although no gill-related mortality occurred, “red water”

and red artifacts were noted in anaesthetic baths. Eugenol exposure can lead to temporary gill damage, including capillary ectasia and epithelial sloughing, with recovery generally within 24 hours (Velisek et al., 2005a; Velisek et al., 2005b; Singh, 2021; Martins et al., 2024). PAS staining may enhance visualization of mucous cells in secondary lamellae, improving clarity between normal and pathological states. Future research should examine lower nutraceutical concentrations with isoeugenol and assess mucosal protectants to protect gills without reducing anaesthesia effectiveness. Minor inflammatory cell and erythrocyte variation can fall within normal ranges (Smith et al., 2018). Diagnostic challenges in fish gill histology are due to high morphological variability, making it difficult for non-specialists to distinguish normal from pathological features (Wolf et al., 2015; Smith et al., 2018). No completely "normal" sections further illustrate these complexities, with artifacts and orientation affecting interpretation. Control groups exhibited typical gill morphology, highlighting potential stress from isoeugenol treatments, especially at higher nutraceutical doses.

The pharmacokinetic properties of isoeugenol offer therapeutic benefits in aquaculture, however, has slower recovery due to gradual elimination. Kiessling et al. (2009), suggested that a slower elimination phase could be mitigated quicker by increasing gill ventilation and air flow. Establishing correct dosage regimes, and optimal use requires pharmacokinetic data, with elimination kinetics studies essential for determining withdrawal times of drugs used in food production. This highlights the broader challenge of using herbal compounds in aquaculture: the limited toxicological data demands thorough testing due to the complex and sometimes unpredictable effects of these compounds (Bona et al., 2024; Martins et al., 2024; Tchobanov et al., 2024). This challenge is also highlighted by Alagoz et al. (2021), who observed unexpected toxic responses in a study on spurge, emphasizing the importance of pre-application studies to ensure aquatic welfare and manage secondary effects.

The findings from Experiment 2 provide important insights into the stress responses of brown trout under different anaesthetic treatments, as indicated by plasma cortisol levels. In salmonids, such as rainbow trout, basal plasma cortisol levels are 0-5 ng/mL, but during acute stress they can spike to 40-200 ng/mL (Pickering and Pottinger, 1989). In experiment 2, the lack of significant differences in cortisol across experimental groups suggests that the treatments may not induce markedly distinct stress responses. However, the trends observed are noteworthy and merit closer examination The group exposed to 20 mg/L isoeugenol with 1 mL Nutra had a maximum level of cortisol, indicating a heightened stress response at higher nutraceutical doses. This dose-response trend aligns with Bona et al. (2024) and Alagöz et al. (2021), who reported that higher concentrations of clove oil and

Euphorbia rigida induced adverse effects in fish, requiring refined dosing to avoid stress responses like oxidative damage or asphyxia. Such a response suggests an upper limit to the beneficial effects of nutraceuticals in combination with isoeugenol, beyond which the treatment may become counterproductive and potentially stressful for the fish. Such a dose-response relationship could reflect physiological thresholds where higher nutraceutical concentrations induce discomfort or other metabolic stressors. Prolonged cortisol elevation, as demonstrated by Pfalzgraff et al. (2021), increases basal energy expenditure and tissue-specific oxygen demands, particularly in metabolically active tissues such as the gills and heart. This aligns with my findings of gill morphological changes and varying cortisol trends across treatment groups, suggesting that higher isoeugenol doses may exacerbate stress-related metabolic costs while influencing recovery dynamics.

While nutraceuticals are often incorporated for their beneficial properties, including potential stress reduction and complementary anaesthetic effects (Saydmohammed and Pal, 2009; Varghese et al., 2021), evidence suggests that surpassing a certain dosage may cause adverse effects or lead to stress (Sladky et al., 2001; Neuwinger, 2004; Dimitrijević et al., 2007; Alagöz et al., 2021; Zhang et al., 2022; da Paz et al., 2024; Sintuprom et al., 2024). This dose-sensitive response could be attributed to several factors, such as the generation of excessive level of free radicals by higher nutraceutical concentrations, which may disrupt homeostasis and activate stress-related pathways (Baruah et al., 2015).

Conversely, the MS-222 control group had the lowest cortisol levels, confirming its role in stress reduction during anaesthesia. Interestingly, the lower nutraceutical concentration (250 µL) appeared to slightly reduce stress responses, indicating at a potential stress-mitigating effect as lower dose. This finding points toward a dose-dependent relationship where moderate nutraceutical additions may complement isoeugenol's anaesthetic effects without triggering heightened stress responses. Overall, these results emphasize the importance of dose optimization when combining isoeugenol with nutraceuticals. Additional research exploring a broader range of doses and various physiological stress markers (such as glucose, stress proteins, and antioxidants) would help clarify the relationship between nutraceutical concentrations, isoeugenol, and stress response. This would enhance our understanding of how to effectively balance the anaesthetic efficacy of a natural formulation for optimal welfare of fish.

Anaesthesia residues in fish tissue are a major concern in aquaculture, especially for species consumed by humans. Persistent anaesthetic compounds or their metabolites can impact food safety, and regulatory standards often enforce strict

residue limits to protect public health. This underscores the importance of understanding anaesthetic pharmacokinetics and clearance rates in fish (Kiessling et al., 2009; Meinertz et al., 2006; Meinertz et al., 2008).

Lastly, we performed HPLC analysis of skin-on fillet samples to detect isoeugenol residues in brown trout 48 hours post-exposure. Our results showed concentrations ranging from 0.02 µg/g to 0.08 µg/g, apart from a spiked control. Notably, certain samples (Run 7, 10.30 µg/g) had higher concentrations, likely reflecting individual differences or retentional influence caused by nutraceuticals. These results underscore the need for further investigation into isoeugenol's retention characteristics, as it has promising anaesthetic properties but may have cumulative effects. Additionally, the HPLC methodology developed within the lab accurately quantifies isoeugenol residues on skin-on-fillet samples of 1-year-old salmonid smolts, enhancing specificity and sensitivity.

4.2 Conclusion and Future Perspectives

This study explores the synergistic combination of isoeugenol and anti-stress nutraceuticals as a natural anaesthetic alternative in aquaculture, supporting fish welfare while minimizing the need for synthetic chemicals. Additionally, this study lays a foundation for refining aquaculture anaesthetic protocols, demonstrating that herbal products can support stress management and welfare. These findings resonate with FAO's (2024) emphasis on sustainable aquaculture practices that prioritize animal welfare and food safety, addressing concerns related to traditional anaesthetics like MS-222, which have longer withdrawal times and potential environmental impacts (Liu et al., 2018; Bavumiragira and Yin, 2022).

However, challenges remain that highlight the need for further refinement. Higher nutraceutical doses combined with isoeugenol led to gill damage and mild inflammation, underscoring the importance of dose optimization to balance efficacy with fish health. Post-exposure fillet samples also showed detectable isoeugenol residues, raising questions about withdrawal periods and food safety compliance. Additionally, cortisol results need further refinement due to the variability across treatments and replicates, which may reflect inconsistencies in stress response or the need for more precise sampling protocols. Addressing this will help clarify the impact of isoeugenol and nutraceuticals on stress response.

Future studies should optimize dosing strategies to balance anaesthesia depth with reduced stress. Increasing sample replicates will improve statistical power, while

incorporating stress biomarkers, such as heat-shock proteins (e.g., Hsp70), oxidative markers, and blood glucose, will provide deeper insight into physiological responses. Testing lower nutraceutical concentrations alongside isoeugenol could help identify thresholds that prevent gill damage while maintaining efficacy. Histopathological analysis, including PAS staining and expert pathology, will allow detection of subtle gill changes. Further refinement of HPLC methodologies should focus on accurately measuring isoeugenol residues under varied treatment conditions and assessing the impact of nutraceuticals on pharmacokinetics.

While improvements are needed, these findings highlight the potential for isoeugenol and nutraceuticals to support sustainable aquaculture practices. This combination provides a natural alternative that addresses fish welfare and food safety concerns while reducing reliance on synthetic chemicals.

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Popular science summary

Aquaculture plays a crucial role in global food supply, yet maintaining fish health in farming, laboratory, or captive settings presents unique challenges. Fish in these environments often face stress from handling, crowding, and environmental changes, all of which can impact their health and growth. This research explores the potential of herbal-based treatments combined with isoeugenol—a component of clove oil—to enhance fish welfare by reducing stress responses in commonly farmed species like trout.

In a preliminary trial, a specific herbal mix combined with isoeugenol was tested on fish health. Key metrics included stress indicators, such as cortisol levels, and tissue concentrations of residual isoeugenol to assess both welfare impacts and safety for human consumption. The study introduced new methods to measure these effects, focusing on tissue analysis, stress response over time, anaesthesia induction and recovery, and using statistical methods to observe trends across treatment groups.

Early results suggest that herbal supplements combined with anaesthetics may improve induction and recovery times and bolster resilience in farmed fish, laying the groundwork for future studies. This approach not only supports fish welfare but aligns with sustainable practices by reducing reliance on synthetic additives.
Looking forward, increasing sample sizes for plasma and tissue analysis will help to validate these findings, along with developing more advanced protocols for aquaculture welfare.

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Appendix 1

Table 4 Anaesthetic-Sedation Levels and Behavioural Observations for O. mykiss: Experiment 1, adapted from Sneddon (2012); (Table 1).

Anaesthetic Combination	***Sedation Level***	***Numeric Sedation Level***	***Behavioural Observations***
Isoeugenol 10 mg/L	II to III Light	2.5	Lateral swimming, handling, light sedation
MS-222 60 mg/L	III Surgical to Deep	4.5	Lateral, non-swimming
Isoeugenol 10 mg/L + 250 µL/L Nutra	III Light to Surgical	3.5	Lateral or swimming, handling, light sedation
Isoeugenol 10 mg/L + 500 µL/L Nutra	II to III Light	2.5	Lateral or swimming, handling, light sedation
Isoeugenol 10 mg/L + 1 mL/L Nutra	III Light to III Surgical	3.5	Lateral, minimal fin movement, light sedation
Isoeugenol 10 mg/L	I to II	1.5	Lightly sedated, schooling, slight excitement
MS-222 60 mg/L (Control 2)	III Light to Surgical	3.5	Lateral, tail movement
Isoeugenol 10 mg/L + 250 µL/L Nutra	II to III Light	2.5	Lightly sedated, schooling, slight excitement
Isoeugenol 10 mg/L + 500 µL/L Nutra	I	1	Lightly sedated, no reaction, non-lateral
Isoeugenol 10 mg/L + 1 mL/L Nutra	I to II	1.5	Lightly sedated, circling, no reaction to stimuli
Isoeugenol 10 mg/L	I to II	1.5	Very light sedation, schooling, equilibrium difficulties
Isoeugenol 15 mg/L	I to III Light	2.5	Slight swimming sedation, lateral, reduced movement
Isoeugenol 20 mg/L	III Surgical	4	Deep sedation, slight fin movement
Isoeugenol 25 mg/L	Surgical III Deep	4.5	Rapid sedation, minimal movement, lateral
Isoeugenol 20 mg/L	III Surgical	4	Tail movement present, lateral
Isoeugenol 20 mg/L + 250 µL/L Nutra	III Surgical	4	Tail movement present, lateral
Isoeugenol 20 mg/L + 500 µL/L Nutra	III Surgical	4	Tail movement present, lateral

Isoeugenol 20 mg/L + 1 mL/L Nutra	III Surgical	4	Tail movement present, lateral
MS-222 60 mg/L	III Surgical to Deep	4.5	No to minimal fin movement, lateral

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