# THE PHENOTYPIC AND PHYSIOLOGICAL RESPONSE OF ZEBRAFISH (*Danio rerio*) TO THREE COMMONLY USED ANAESTHETICS: CLOVE OIL, 2-PHENOXYETHANOL (2-PE) AND ETHYL 3-AMINOBENZOATE METHANE SULPHONIC ACID (MS-222)

**by**

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**Submitted in fulfilment of the academic requirements of**

**Master of Science**

in Biological Sciences

School of Life Sciences

College of Agriculture, Engineering and Science

University of KwaZulu-Natal

Westville

South Africa

February 2025

# COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

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# PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Biological Sciences, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, South Africa. The research was financially supported by the National Research Foundation.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

_____

Signed: Prof A Vosloo

Date: 5 February 2025

## Acknowledgements

I would like to acknowledge my academic supervisors, Prof. Andre Vosloo and Dr Dalene Vosloo for their guidance throughout the years. I would like to acknowledge Dr Zander Lindeque and Mr Marcel Burger for their quantification of the metabolomics from fish issue. I would like to acknowledge my parents for their support throughout everything. Finally, I dedicate this dissertation to my sister, Dr Mbalenhle Thabile Sosibo. I wish you could have been here to read it. Lala kahle Gema. Lala kahle Ncobeni. Lala kahle Mshingila…

# ABSTRACT

The use of chemical anaesthesia in fish reduces the risk of mechanical injuries or stress in any husbandry practices or handling procedures in aquaculture, research, transport, surgery, or other veterinary practices. However, the type of the anaesthetic agent needed, the dosage required to induce a desired level of anaesthesia, and the physiological effect incurred can differ between species of fish. Since zebrafish are the most held lab fish and widely used in the aquarium trade, it is essential to determine protocols to anaesthetise zebrafish to reduce the risk of injury and stress due to their high demand and importance in these fields. It is also key to understand the physiological consequence of the anaesthetics for the benefit of studies centred around the use of physiological endpoints. This study focussed on assessing the efficacy and the metabolomic consequence of the three most used anaesthetics for fish: clove oil, ethyl 3-aminobenzoate methane sulphonic acid (MS-222), and 2-phenoxyethanol (2-PE). The study exposed adult zebrafish to various concentrations of the three anaesthetic agents and recorded the anaesthetics induction times and made observations of the phenotypic responses to each concentration. The time it took to recover from each exposure was recorded along with the phenotypic changes that occurred. The exposures were repeated using only dosages that induced the fish to stage IV (surgical) anaesthesia in 60-90 s and samples were taken for metabolomics analyses. Clove oil at 60 mg/L and 100 mg/L was the recommended anaesthetic for fast induction of surgical anaesthesia. For maintaining fish in surgical anaesthesia for an extended duration without artificial respiration, 2-PE at 500 mg/L was most effective, and for fish transport, 2-PE at 100 mg/L was suitable. Induction time was faster at higher concentrations across anaesthetics. Anaesthesia with clove oil yielded the shortest induction time at effective concentrations but required longer for full recovery at lower doses. However, recovery was comparably rapid at higher concentrations. Fish anaesthetised with MS-222 recovered quickly, as did those with 2-PE. The metabolic consequence of clove oil anaesthesia was less widespread and severe than that of 2-PE and MS-222-anaesthised zebrafish, while MS-222 affected a wider range of metabolic pathways and metabolites in comparison to 2-PE and clove oil. While all three anaesthetics elicited a stress-related metabolic response, 2-PE and clove oil did not induce a response indicative of acute or life-threatening physiological disruption. These findings serve as a basis for selecting suitable anaesthetics and dosages for zebrafish, striking a balance between physiological safety and procedural requirements to promote both animal welfare and the integrity of scientific findings.

**Key words:** Zebrafish; Anaesthesia; Anaesthetics; Metabolomics, Phenotypic response, Physiological response.

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# CHAPTER 1: INTRODUCTION

# 1. INTRODUCTION

Veterinarians, researchers and fish aquaculturists often require the use of anaesthetics to alleviate the stress that fish experience during handling procedures, transport, and even surgery (Zahl *et al*., 2012). Anaesthetics are meant to reduce the stress and pain experienced by the animals; however, several issues arise when using anaesthetics (Ross and Ross, 2009; Zahl *et al*., 2012). Anaesthetic agents are often used in fish studies to keep the animals controllable for experiments and to ensure their welfare. However, information on the optimal dosage is not always freely accessible (Rairat *et al*., 2021). Firstly, the correct dosage required to induce a specific level of anaesthesia must be established. Should the dosage be insufficient, analgesia (the absence of pain) is not achieved, and the animal will not achieve pain relief and will continue to experience pain during invasive procedures. Should the dosage be too high, the animal could die from anaesthetic overdose (Ross and Ross, 2009). Research suggests that the correct anaesthetic dosages and sensitivity to anaesthetics may differ quite substantially between species due to body size and metabolic rates (Sneddon, 2012). For example, zebrafish generally require lower dosages of clove oil to reach a similar plane of anaesthesia in comparison to larger fish such as salmon and catfish (Sneddon, 2012). However, there are contradictory results to this, where compared to rainbow trout and black sea bass, zebrafish, which are much smaller in size, can require a greater concentration of clove oil to achieve surgical anaesthesia (Priborsky and Velisek, 2018). Therefore, researchers must refine dosage ranges of anaesthetics to determine their dosage ranges which are often not published in scientific literature, making it difficult to find for others needing the information.

Secondly, there is a paucity of information regarding the link between the phenotypic and the physiological response of a fish to the anaesthetic (Ross and Ross, 2009). When determining if anaesthesia is achieved, physical or behavioural cues (the phenotypic response) are used (Bell, 1964; Ross and Ross, 2009). However, it is usually not known if all fish achieve analgesia when displaying anaesthesia symptoms, or whether they are just immobilised but still feel the full extent of pain that they would normally experience (Ross and Ross, 2009). A method to quantify the stress animals experience is to record stress biomarker levels (the physiological response) apparent at the exact stage of anaesthesia (Priborsky *et al*., 2015). Should an animal under anaesthesia have stress biomarker levels above baseline levels, then the anaesthetic was possibly not successful in its purpose. From an ethical perspective, it is in the interest of veterinarians, aquaculturists, and researchers to understand the link between the phenotypic and physiological response to anaesthetics to ensure that the anaesthetic agents used achieve the desired effect.

A first step in the process to fill these gaps in the literature that this study explores was to investigate the issues starting with the three most used anaesthetics: clove oil; ethyl 3-aminobenzoate methane sulphonic acid (MS-222); 2-phenoxyethanol (2-PE), and the most common lab kept fish *Danio rerio* (zebrafish). Of the stages of anaesthesia, stage IV and stage V (light, surgical and deep anaesthesia – see Table 3-1 in Chapter 3) are the most relevant to veterinary and aquaculture facilities since fish are

induced to those stages for handling and surgical purposes (McFarland, 1959; Bell, 1964; Iwama *et al*., 1989; Martins *et al*., 2016). Therefore, this study focused primarily on inducing the zebrafish to these two stages of anaesthesia and comparing the response to each of these stages.

This study aimed to evaluate and compare the suitability of MS-222, clove oil, and 2-phenoxyethanol (2-PE) as anaesthetics in adult zebrafish by linking phenotypic responses to physiological changes at the metabolomic level, in accordance with the criteria for a good anaesthetic outlined by Priborsky and Velisek (2018). . The objectives of this study were as follows:

- To confirm safe dosages to induce the five levels of anaesthesia through trials for MS-222 and clove oil based on what is in the literature, and to establish safe dosages for 2-PE on zebrafish based on what was found for MS222 and clove oil, and what is known in the literature.
- To record the induction and recovery time of each anaesthetic dose and compare their statistical differences.
- To determine the metabolomic consequence that the zebrafish experience from each anaesthetic using Nuclear Magnetic Resonance (NMR), Gas Chromatography–Time-of-Flight Mass Spectrometry (GC-TOFMS), and Liquid chromatography–mass spectrometry (LC–MS) on the whole-body tissue of fish exposed to each treatment.
- To relate the phenotypic response to the metabolomic response of each anaesthetic and use the Priborsky and Velisek (2018) standard of a good anaesthetic to determine which of the three should be recommended, as well as the optimal dose for specific stages and durations of anaesthesia in zebrafish.

## Potential impact

Zebrafish are the most common lab-held fish; they have great importance to scientific research since they commonly serve as a model species (Readman *et al*., 2013). Therefore, this study will have a positive impact in giving scientists, veterinarians, and aquaculturists the information needed to successfully anaesthetise the fish, for as long as required and at the desired level of anaesthesia. This study will be the first to give insight in an anaesthesia protocol to anaesthetise zebrafish using various concentrations of 2-PE and additionally provide insight to the metabolomic consequence of 2-PE, MS-222 and clove oil. By elucidating the metabolic consequences of anaesthetics, more accurate translational deductions can be drawn from zebrafish-based studies on human diseases. The goal in research (not the goal in this dissertation) is often to use zebrafish-based data in human translational medicine applications, but if the zebrafish response is attributed to the anaesthetic rather than the drug or treatment, false results may be produced, affecting the translational application of these studies. Therefore, the current study is in relation to the process of Refinement of the 3Rs: Replacement, Reduction, and Refinement (Russell and Burch, 1959). It aims to identify and address accompanying physiological changes which may increase the variability of scientific results and to consequently

improve data quality and contribute to/affect the process of Refinement in the 3Rs. This study will also pave the way for future research in this field through eventually expanding to include several other species of fish and several other anaesthetic agents.

# CHAPTER 2: RATIONALE AND LITERATURE REVIEW

# 2. RATIONALE AND LITERATURE REVIEW

## 2.1 Aquaculture and the use of fish in research

The utilisation of aquaculture has become vital to meet the expanding food demands of the ever-growing human population and maintaining the global fish supply due to wild fish stocks becoming increasingly under pressure (Food and Aquaculture Department Fao, 2014). In addition to supplying fish for consumption, aquaculture facilities also conduct research and contribute to our understanding of fish physiology. Examples of this include a study conducted on the influence of light intensity and photoperiod on the physiology and stress pattern observed in juvenile great sturgeon (*Huso huso*) (Bani *et al*., 2009). Another example is an investigation of the daily rhythms in the hypothalamus-pituitary-interrenal axis in the flatfish (*Solea senegalensis*) (López-Olmeda *et al*., 2013). The study investigated the daily rhythms of the expression of corticotropin-releasing hormone (CRH), CRH-binding protein, proopiomelanocortin a and b and circadian rhythm of stress metabolites glucose, lactate, and cortisol. Such knowledge may be useful for the advancement of fish farming and the understanding of the stress response, because routine procedures on farms that involve handling fish and, thus, cause stress to the animals, such as grading, vaccinations, or sampling, could be scheduled for a time of day when the animals exhibit a reduced stress response (López-Olmeda *et al*., 2013). Land-based aquaculture may enable the growth and advancement of aquatic resources outside the ocean (Brummett *et al*., 2008). Science has significantly developed thanks in large part to animal experimentation.

Animal models are still required in scientific study for development, reliability, and credibility of science, even though certain models have been replaced by other methodologies, such as *in vitro* cell cultures*, in silico* computer imaging and simulations (Doke and Dhawale, 2015; Simonetti *et al*., 2015). However, in aquaculture, laboratory enclosures, or any scenario where fish are outside their natural environment, would involve handling or moving the fish from place to place (Wendelaar Bonga, 1997; Barton, 2002). However, since the cultured or research fish are no longer in their accustomed environment; the new environment subjects them to various degrees of stress and elicits a stress response. (Wendelaar Bonga, 1997; Barton, 2002).

While research and knowledge regarding the effects of scientific methods on aquatic animals, particularly fish and amphibians, continues to develop, the expertise in those fields and the amount of evidence required to support their improvement is relatively sparse in comparison to that of mammalian models (Sneddon, 2009; Prescott and Lidster, 2017). There are varying degrees of awareness of potential areas for improvement, and in some cases, there is a reluctance to challenge and question long-standing norms and cultures (Prescott and Lidster, 2017). According to the 3Rs refinement principle, the wellbeing of research animals should be maximised, unpleasant effects including distress, fear, and pain must be averted or reduced, in addition the animals must be kept in environments that support their health and welfare (Russell and Burch, 1959; Prescott and Lidster, 2017). Optimal conditions that ensure

fish welfare are species specific. For example, factors such as stocking density may have differential effects on fish depending on the species, age, and individual responses as seen in rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus cajtrpio*) (Huntingford *et al*., 2006) Additionally, factors affecting fish welfare often interact, so a given condition may lead to poor welfare under some circumstances but not others. Therefore, it is not possible to guarantee fish welfare with a single set of conditions; instead, sensitive, on-the-spot welfare indicators are necessary (Huntingford *et al*., 2006). Such indicators that relate to fear, pain, discomfort, and freedom from hunger can be assessed through physiological, behavioural, anatomical, performance, and metabolic indicators (Toni *et al*., 2019). Assessing the level of animal welfare based on tissue and blood parameters essentially necessitates invasive sampling procedure and capture which may then elicit a stress response in the animal (Barton, 2002; Toni *et al*., 2019). Understanding the mechanisms involved in fish's stress response is therefore critical for preserving their health and well-being.

## 2.2 Stress in fish

In fish, stimulation of the Hypothalamic-Pituitary-Interrenal (HPI) axis initiates and regulates the response to various stressors, both acute and chronic, governed by a feedback mechanism that stops the response procedures initiated along the axis after they have finished their function to mitigate adversity from ongoing stress response processes (Wendelaar Bonga, 1997; Barton, 2002). The HPI axis stimulates the secretion and circulation of numerous stress hormones depending on the type of stress encountered by the organism. However, it typically initiates the circulation of the steroid hormones catecholamine, in chromaffin tissue, and cortisol, the major glucocorticoid in fish (Barton, 2002; Cao *et al*., 2017). The process stimulates the synthesis and release of corticosteroids into the bloodstream for delivery to the appropriate tissues (Barton, 2002; Cao *et al*., 2017). Plasma catecholamines primarily adjust cardiovascular and respiratory function to maintain sufficient blood oxygen levels and, consequently, an ample supply to the tissues (Reid *et al*., 1998). In addition, these biogenic amines aid to release energy reserves to satisfy elevated energy needs that are frequently associated with stress (Reid *et al*., 1998).

The circulation of cortisol initiates the relocation of metabolic energy from processes such as immune response, reproduction or growth via glucose metabolism through glycogenolysis to assist in combatting apparent stressors (Barton, 2002). The stress response may benefit the organism in that moment, the chronic cortisol elevation often follows tertiary effects that can eventually reduce fecundity reduce growth rates, and reduce immune system functioning consequently reducing the average population size of housed organisms (Wendelaar Bonga, 1997; Barton, 2002). Thus, stress management in aquaculture is critical to ensuring long-term organismal growth. Given that cortisol persists in the body longer than catecholamine, elevated blood cortisol levels and the subsequent metabolism of

glucose due to cortisol release are widely used as indicators for elevated stress levels in fish (Wendelaar Bonga, 1997; Barton, 2002; Barcellos *et al*., 2012).

## 2.3 Anaesthesia

The continuing and rapid expansion of aquaculture and biomedical research, technological advances have prompted the use of chemicals to alleviate the strain incurred during handling interventions relating to aquaculture management and research (Priborsky and Velisek, 2018). The struggling of the fish occurring during handling interventions elicits a response that affects their physiology and behaviour, hence the necessity to immobilise or sedate the fish before performing simple handling procedures (Priborsky and Velisek, 2018). The use of anaesthetics during transit and husbandry in fish aquaculture consequently reduces mechanical injuries and increases fattening and survival rates in the long term (Wosnick *et al*., 2018). Anaesthesia is commonly defined as "a reversible state resulting in unconsciousness and loss of sensation against external and internal stimulation, through the depression of the central nervous system." (Kim and Nam, 2018). This state may be succeeded by varying degrees of analgesia and muscle relaxation (Owen and Kelsh, 2021). External stimuli typically refer to external physical inputs or environmental factors that an organism perceives through sensory organs such as eyes, ears, nose, skin, and other tissues. In contrast, internal stimuli are stimuli originating from within the organism's body, such as visceral perturbations or nociceptive signals generated by internal organs or tissues, and psychological perturbations (Montupil *et al*., 2023). Records of the use of anaesthetic agents for fish date to the middle of the 20$^{th}$ century where the classification system for stages of anaesthesia in fish became foundational and experimentation of agents such as tricaine methansulfonate (MS-222) became standard for fish anaesthesia (McFarland, 1959; Sehdev *et al*., 1963; Bell, 1964; Schoettger and Julin, 1967; McFarland and Klontz, 1969).

The use of anaesthetic agents on any animal aims to induce analgesia, the relief or loss of pain without retention of other sensory abilities (Ross and Ross, 2009). However, determining whether many non-mammals, such as fish, have achieved analgesia is difficult since they do not express pain in the same manner as humans and other mammals do (Ross and Ross, 2009). Furthermore, analgesia does not necessarily result in the loss of equilibrium, making analgesia in fish difficult to judge by mere observation (Ross and Ross, 2009). Alternatively, stress biomarkers give an indication of the degree of deviation from homeostasis experienced by the organism (Wendelaar Bonga, 1997; Barton, 2002). Therefore, observing the physiological changes instead of behavioural responses through analysing the various metabolites produced from response to the anaesthetic agent could indicate as to whether the anaesthetic induced analgesia.

Anaesthetics can be administered via immersion or parenteral routes (Neiffer and Stamper, 2009). Induction of anaesthesia follows the flow of the anaesthetic agent transported from the gills through to the central nervous system via the arterial blood (Priborsky and Velisek, 2018). In turn, the placement

of fish in anaesthetic-free water to recover post-anaesthesia allows the excretion of the anaesthetic agent or its metabolites via the skin and the gills (Priborsky and Velisek, 2018). Good anaesthetics should: induce anaesthesia rapidly with minimal hyperactivity or stress; be easily administered and maintain the animal at the desired anaesthetized state; allow rapid recovery when removing the animal from anaesthetic; be effective at low doses while the toxic dose should significantly exceed effective dose to guarantee a wide margin of safety; have high solubility in fresh and salt water; be easily accessible, cost effective, and non-toxic to humans; and finally, produce no long lasting physiological effects after clearance from the body (Priborsky and Velisek, 2018).

The most used anaesthetics in aquaculture include ethyl 3-aminobenzoate methane sulphonic acid (MS-222), quinaldine, etomidate, quinaldine sulphate, clove oil, and 2-phenoxyethanol (2-PE) (Ackerman *et al*., 2005; Neiffer and Stamper, 2009; Ross and Ross, 2009; Carter *et al*., 2011; Priborsky and Velisek, 2018), with anaesthesia commonly induced via immersion (Priborsky and Velisek, 2018).

## 2.4 Stages of anaesthesia

There are five main stages of anaesthesia described induced by certain anaesthetic dosages, depending on the species of fish. The first stage, known as light sedation, induces a loss of equilibrium in the fish, a slight decrease in respiratory rate, and a slight loss of response to stimuli (McFarland, 1959; Martins *et al*., 2016). The second stage (deep sedation) results in the total loss of reactivity to external stimuli (apart from strong pressure) and a slight decrease in respiratory rate (McFarland, 1959; Martins *et al*., 2016). The third stage results in a loss of equilibrium and muscle tone. Erratic swimming and increased respiratory rate will occur (McFarland, 1959; Martins *et al*., 2016). The fish will only react to strong stimuli. This stage is known as the excitation phase, where the fish still displays spinal reflexes. In the fourth stage of anaesthesia, if providing pressure to the tail, no reaction to the unpleasant stimuli will occur (McFarland, 1959; Martins *et al*., 2016). This stage is known as the surgery stage of anaesthesia. The surgical stage of anaesthesia is advised for invasive procedures such as surgery, drug injection, and blood sampling, where tissue damages will occur (Rairat *et al*., 2021). In the fifth stage, the respiratory rate drops to near cessation. Surgery is typically undergone between stages 4 and 5; however, not done too deep into stage 5 (McFarland, 1959; Martins *et al*., 2016).

**Table 2-1: The stages of anaesthesia, the parameters used to monitor anaesthesia in fish and examples of procedures that can be done to the fish under each level (McFarland, 1959; Bell, 1964; Iwama *et al*., 1989; Martins *et al*., 2016).**

| Stage | Level of Anaesthesia | General Demeanour | Activity | Equilibrium | Gill ventilation rate | Reactivity | Heart rate | Muscle tone | Description | Example of procedures |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | Regular | Regular | Regular | Regular | Regular | Regular | Regular | Regular | | |
| I | Lightly sedated | Disorientated | Reduced | Regular | Regular | | Regular | Regular | Loss of equilibrium | Minimise stress and injuries during transportation |
| II | Deeply sedated | Disorientated | Reduced | Reduced | Low reduction | No reaction to visual and light tactile stimulation | Regular | Low reduction | | |
| III | Excitation | Agitated | Increased | Reduced | Increased | Increased | Increased | Reduced | Increased chance of physical harm or escape from the container or tank | |
| IV | Light | Anaesthetised | Ceased | Lost | Reduced | Reflex responses | Regular | Reduced | Regular opercular movements with the lack of gross bodily motion | Weight; close visual inspection; external non-invasive tags; gill scrape |
| V | Surgical | Anaesthetised | Ceased | Lost | Shallow | Ceased | Decreased | Reduced | As with stage IV with the loss of opercula motion | Recovery surgery; tissue removal; invasive tags; lesion dressing; gill biopsy; blood sampling |
| VI | Deep | Anaesthetised | Ceased | Lost | Sparse movements | Ceased | Decreased | Relaxed | | Non-recovery surgery |
| VII | Overdose | Deceased | Ceased | Lost | Ceased | Ceased | Cardiac failure | Ceased | | Euthanasia |

## 2.5 The three most used anaesthetics in fish

This section provides information of the anaesthetics clove oil, ethyl 3-aminobenzoate methane sulphonic acid, and 2-phenoxyethanol with regards to their use, dosage, and effects on the physiology of fish and other mentioned organisms. Table 2-2 below provides a summary of the aspects of each anaesthetic discussed in this section.

**Table 2-2: Comparative Summary of Common Fish Anaesthetics: Clove Oil, Ethyl 3-aminobenzoate methane sulphonic acid (MS-222), and Phenoxyethanol**

| Aspect | Clove Oil | Ethyl 3-aminobenzoate methane sulphonic acid (MS-222) | Phenoxyethanol (2-PE) |
|---|---|---|---|
| **Chemical Nature** | Amber liquid from S. aromaticum; 90–95% eugenol and iso-eugenol | White, crystalline powder; very water-soluble | Colourless, oily aromatic liquid |
| **Administration Route** | Immersion – absorbed via gills and skin | Immersion – absorbed via gills and skin | Immersion – absorbed via gills and skin |
| **Mechanism of Action** | $GABA_A$ receptor modulation in the brain (similar to benzodiazepines) | Sodium channel blocker (also minor $K^+$ channel inhibition) | NMDA receptor inhibition and neuronal membrane expansion |
| **Common Dosage Range** | 40–175 mg/L (species-specific); up to 1500 mg/L for trout surgery | 20–150 mg/L for anaesthesia; ≤100 mg/L for salmonids; 400–500 mg/L for euthanasia | 100–600 mg/L |
| **Induction Time** | Varies with species & dose: <1–10 min, e.g. 1 min at 100 mg/L in tilapia | As short as 15 seconds | Rapid, species- and dose-dependent |
| **Recovery Time** | Varies: 3–10 min generally; longer at higher doses | Short if optimal dose; >10 min suggests overdose | Smooth and fast |

**Table 2-2 Continued: Summary of Common Fish Anaesthetics: Clove Oil, Ethyl 3-aminobenzoate methane sulphonic acid (MS-222), and Phenoxyethanol**

| Aspect | Clove Oil | Ethyl 3-aminobenzoate methane sulphonic acid (MS-222) | Phenoxyethanol (2-PE) |
|---|---|---|---|
| **Use Cases** | Husbandry, tagging, breeding, surgery, research | Sampling, transport, surgery, selection, euthanasia | Short-term handling, minor procedures, out-of-water work |
| **Metabolism & Excretion** | Lipophilic – accumulates in fat/brain; metabolised slowly | Rapid liver metabolism; polar/non-polar metabolites; excreted via gills and kidneys | Not well described; fast CNS penetration |
| **Physiological Effects** | Affects haematology and biochemistry; reduced feeding with repeated use | Oxidative stress, antioxidant enzyme inhibition, no genotoxicity in some species | CNS depression; reduced HR, BP, $O_2$; increased $CO_2$, glucose, adrenaline |
| **Species Examples** | Zebrafish, Nile tilapia, meagre, turbot, rainbow trout | Salmonids, Nile tilapia, barbel, many warm/cold-water fish | Various fish species; general use in aquaculture |
| **Advantages** | Inexpensive, effective, natural, and easy to obtain | Rapid onset/recovery, widely used, effective at low doses | Low cost, fast action, antibacterial & antifungal properties |
| **Disadvantages / Limitations** | Variable effects; possible stress or metabolic changes with high doses | Narrow safety margin; overdose risk; regulatory limits | CNS and cardiovascular side effects; handling risk for humans |

### *2.5.1 Clove oil*

Clove oil is an amber coloured liquid, attained through the distillation of leaves, stems and flowers of *Syzgium aromaticum* (Priborsky and Velisek, 2018). The active ingredients of clove oil include iso-eugenol (4-propenyl-2-methoxyphenol) and eugenol (4-allyl-2-methoxyphenol, C10H12O2, molecular weight 164.2 g/L), make up 90-95% of clove oil's total weight. As a fish anaesthetic, Clove oil enters

the body via the gills and skin, where it enters the bloodstream and circulated throughout (Priborsky and Velisek, 2018). Clove oil's highly lipophilic nature allows it to rapidly penetrate gill epithelium, and once it enters the bloodstream, body tissues such as fat and brain rapidly absorb the substance (Jorge *et al*., 2021)

Clove oil is a potential candidate for the anaesthesia of ornamental fish and several artificially reared species (Priborsky and Velisek, 2018). It has been proposed as an appropriate, inexpensive, and readily obtainable anaesthetic for fish. Many hatcheries and research studies use clove oil to immobilise fish during husbandry, breeding, tagging, and surgery, as well as a sensory system suppressor during invasive procedures (Javahery *et al*., 2012). A variety of doses are used to induce anaesthesia in various species of fish. Dosages range from 60-100 mg/L in zebrafish (*Danio rerio*) (Grush *et al*., 2004), 40-50 and 85 mg/L in meagre (*Argyrosomus regius*) (Barata *et al*., 2016; Cárdenas *et al*., 2016), 40 and 150-175 mg/L in Nile tilapia (*Oreochromis niloticus*) (Ribeiro *et al*., 2015; Panthukumphol, 2017).

Clove oils' anaesthetic action is thought to be mediated by γ-Aminobutyric acid type A ($GABA_A$) receptor modulation, which is found in rat brain tissue. This function is thought to be conserved in the zebrafish brain as well. Clove oil has also been shown to have analgesic effects on frog (*Xenopus laevis*) oocyte ionotropic α1β2-$GABA_A$ receptors. Clove oil can have various effects on fish physiology, and reports show that changes in haematology and biochemistry occurred in clove oil anaesthetised fish (Priborsky and Velisek, 2018). Overuse of any anaesthetic can be stressful, causing uncharacteristic metabolic rates, blood pressure, oxygen consumption, and abnormal blood physiological response (Priborsky and Velisek, 2018). The use of optimal anaesthetic concentrations can reduce the adverse impacts of the anaesthetic and reduce stress in fish (Ross and Ross, 2009). Reports show that the use of clove oil could result in reduced feed intake after repeated use in anaesthesia in comparison to unanaesthetised controls (Hikasa *et al*., 1986; Javahery *et al*., 2012). Clove oil induced light surgical anaesthesia in rainbow trout in 1.05-3.36 min within a dosage range of 500-1500 mg/L, where recovery time was between 3.23-5.54 min (Yildiz *et al*., 2013). Clove oil induction time in tilapia (*Oreochromis mossambicus*) was 10 minutes in 50 mg/L and 1 minute in 100 mg/L clove oil, where the recovery was 3 minutes and 10 minutes, respectively (Nambiar *et al*., 2024). Clove oil induction time in turbot (*Psetta maxima*) was 4.45 minutes using 150 mg/L and 2 minutes using 600 mg/L, where the recovery times were 4.32 minutes and 8.29 minutes, respectively (Aydın *et al*., 2015). Therefore, the general induction and recovery times of clove oil may vary depending on species, size of the fish, and concentrations used.

### *2.5.2 Ethyl 3-aminobenzoate methane sulphonic acid*

Ethyl 3-aminobenzoate methane sulphonic acid, commonly abbreviated as MS-222, is also known as TMS, tricaine methanesulfonate, tricaine mesilate, metacaine, tricaine, and methane sulfonate (Priborsky and Velisek, 2018). MS-222 is widely used worldwide for various poikilotherm organisms and is known for its rapid induction and recovery times (Priborsky and Velisek, 2018). In humans, it is

used as an analgesic and was introduced as a replacement to cocaine during the 1920s (Ross and Ross, 2009; Priborsky and Velisek, 2018). MS-222 appears as a white, crystalline powder that is odourless and very soluble in water (1 g/L at 20°C) and often administered to fish via immersion (Priborsky and Velisek, 2018). In fish, MS-222 is absorbed by the skin and gills, then circulates through the bloodstream and functions as a muscle relaxant by blocking sodium channels in muscles and, to a lesser extent, potassium pathways in neuron membranes (Matthews and Varga, 2012; Jorge *et al*., 2021).

The anaesthetic undergoes rapid metabolism after absorption, primarily in the liver, but also in muscle, blood, and the kidneys (Priborsky and Velisek, 2018). The primary metabolites of MS-222 are acetyl-conjugates of ethyl m-aminobenzoate (non-polar) and m-benzoic acid (polar), with conjugation and hydrolysis functioning as the primary catabolic pathways (Kolanczyk *et al*., 2003; Rombough, 2007; Priborsky and Velisek, 2018). The gills eliminate MS-222 and its non-polar metabolites, while the kidneys excrete polar metabolites. (Priborsky and Velisek, 2018).

In aquaculture, MS-222 is commonly used to chemically restrain fish for a variety of interventions, including fish selection, blood sampling, transportation, and sorting (Ross and Ross, 2009; Priborsky and Velisek, 2018). Like other anaesthetics, the efficacy of MS-222 depends on both biological and environmental factors (Ross and Ross, 2009; Chambel *et al*., 2015; Priborsky and Velisek, 2018). Exposure of MS-222 exceeding 10 minutes and dosage of 50 mg/L may induce mortality (Priborsky and Velisek, 2018). The effective dosage to anaesthetise fish with MS-222 ranges between 20-150 mg/L depending on species, where it is recommended not to administer dosages greater than 100 mg/L for salmonids and not more than 250 mg/L for warm water fish . A dosage of 400-500 mg/L is suitable for salmonids euthanasia (Ackerman *et al*., 2005).

The anaesthetics induction time can be as short as 15 seconds (Coyle *et al*., 2004). The optimal concentration depends on the size of the fish and the required plane of anaesthesia or sedation, which is the same for several other anaesthetics (Priborsky and Velisek, 2018). The time of exposure varies from a few minutes for high doses to 48 hours for low doses (Topic Popovic *et al*., 2012). Fish mass is inversely related to induction and recovery times, where the effects are more pronounced in smaller fish (Priborsky and Velisek, 2018) . Recovery times greater than 10 minutes suggest that a dosage of MS-222 was too great for the exposure time (Priborsky and Velisek, 2018).

With regards to the reported effects that MS-222 has on the physiology of fish, a dosage of 600 mg/L showed no genotoxic effect on both *in vitro* and *in vivo* exposed erythrocytes of Nile tilapia, from the Cichlidae family, which suggests that MS-222 induces no significant DNA damage (Barreto *et al*., 2007). In rainbow trout, 100 mg/L of MS-222 elevated reactive oxygen species production, oxidative damage to proteins and lipids, and antioxidant capacity suppression (Velisek *et al*., 2011). A report observed an induction of the normal oxidation processes barbel (*Barbus barbus*), indicating a malfunction of antioxidant defence mechanisms exhibited by superoxide dismutase (SOD) and catalase (CAT) (Priborsky *et al*., 2015).

### *2.5.3 2-Phenoxyethanol*

2-Phenoxyethanol (or 2-PE) is a colourless aromatic oily liquid often used for short-term immobilisation of fish and for handling fish out of the water (Priborsky and Velisek, 2018). It enters the body through the skin and gills, then passes to the central nervous system via arterial blood. (Priborsky and Velisek, 2018). 2-phenoxyethanol's antibacterial qualities permit its use as a preservative in fragrances, dermatological products, and vaccines (Zahl *et al*., 2012). A report relates the anaesthetic effects of 2-PE to the expansion of neuronal cell membranes (Zahl *et al*., 2012) , which inhibits the excitatory N-methyl-D-aspartate receptor activity (NMDA) seen in *Xenopus lavis* oocytes. This implies that its anaesthetic effect is related to inhibiting high-region neuronal activity in the nervous system (Zahl *et al*., 2012). Furthermore, 2-PE's anaesthetic action relates to NMDA receptor activation, which lowers the threshold for action potential firing by allowing charged ions to enter through ion channels, increasing neuron excitability (Zahl *et al*., 2012). The dosage for anaesthesia and light sedation generally range from 100 to 600 mg/L (Neiffer and Stamper, 2009).

Some of the adverse side effects of 2-PE in fish include reduced heart rate and blood pressure, slowed breathing, decreased blood $O_2$ partial pressure followed by increase in $CO_2$, decreased blood pH, and elevated amounts of adrenalin and glucose in the plasma (Zahl *et al*., 2012). Despite the adverse secondary effects that could occur in the central nervous system of both the treated fish and the handler, 2-PE is deemed appropriate for aquaculture due to its low cost, fast action, smooth recovery, and ease of preparation, and further beneficiated by its anti-bacterial and fungicidal properties (Priborsky and Velisek, 2018).

## 2.6 Anaesthetic phenotypic vs physiological response

The importance of measuring the physiological response to the anaesthetic is to ensure that analgesia is achieved. Merely observing the phenotypic response to the anaesthetic may not be sufficient since the fish may only be immobilised and lose recumbency, however, may still experience pain in its immobilised state (Ross and Ross, 2009). Studies show some anaesthetics can be aversive based on the phenotypic or behavioural response of the fish (Readman *et al*., 2013). However, there is a lack of information based on the physiological response (i.e., the stress biomarkers/metabolites). The brief literature review presented shows that most information relating to the link between the phenotypic and physiological response to anaesthetics is lacking in some areas and the effects of 2-PE have not been as thoroughly investigated as that of clove oil and MS-222.

Readman *et al*. (2017) studied the behavioural response of carp (*Cyprinus carpio*); fathead minnows (*Pimephales promelas*); Medaka (*Oryzias latipes*); and rainbow trout (*Oncorhynchus mykiss*) to three commonly used anaesthetics, MS-222, etomidate and benzocaine. Of the four species, only medaka exhibited aversion to benzocaine and MS-222, and only carp showed aversion to etomidate. Rainbow trout and minnow showed no significant aversion to either anaesthetic. However, the lack of a

behavioural response due to the anaesthetics MS-222 and benzocaine may be due to these anaesthetics' mode of action to reduce movement by blocking voltage-sensitive sodium channels, i.e., the anaesthesia left them too immobile to evade the anaesthetic. The study concluded that only an investigation of the physiological changes in response to the anaesthetic would indicate whether the lack of avoidance of the anaesthetic was due to the inhibition of movement due to anaesthesia or if the anaesthetics were not aversive (Readman *et al*., 2017). These authors also point out that the observed aversion may have been due to the anaesthetic irritating peripheral taste or olfactory receptors, rather than the anaesthetic itself being aversive through a central neurological effect.(Readman *et al*., 2017).

## 2.7 Metabolomics

Many studies employ blood plasma cortisol or glucose levels as markers of stress levels in fish. However, there is always a chance that this kind of analysis may not accurately reflect the stressed organism's total reaction (Mushtaq *et al*., 2014). Therefore, as the ultimate objective of metabolomics is to evaluate all of an organism's metabolites qualitatively and quantitatively, it should be able to reflect the total physiological response of an organism more correctly (Mushtaq *et al*., 2014). Metabolomics describes the all-inclusive study of the assemblage of small molecule metabolites related with a cell, tissue, organ or organism in a context-reliant manner, being influenced by the environment, pathological stimuli or genetic modification (Ong *et al*., 2009). By definition, the metabolic profile of a biological system refers to its output that reflects its biochemical makeup or response to external stimuli. (Ong *et al*., 2009). Metabolic profiling not only shows changes in the levels of each metabolite, but it also provides a complete picture of the metabolic processes triggered by harmful xenobiotics.

Targeted and untargeted metabolic analysis are often characterised as two complementary techniques. A targeted approach aims to detect and quantify specific metabolites (or metabolite classes), such as metabolic pathway components, protein direct products, enzyme substrates, or a compound class. A targeted strategy allows for sample preparation to be modified to minimize matrix effects and companion compound interference, since the chemical properties of the compounds of interest are known. In contrast to targeted analysis, which is normally hypothesis driven, untargeted analysis can produce new hypotheses for additional testing by determining (ideally) the entire metabolome of a biological system. A common goal of several metabolomics analysis platforms is to compare different biological groups to identify metabolites that have undergone significant changes. Starting with an untargeted analysis, prospective and putatively interesting metabolites are screened. Following that, a targeted examination of these metabolites is performed for metabolite identity confirmation, quantification, functional interpretation, and pathway analysis (Zhou *et al*., 2012).

There is a lack of information with regards to the baseline metabolomic profiles of zebrafish and a lack of investigations involving the zebrafish metabolome (Ong *et al*., 2009). While Ong *et al.* (2009) established a baseline metabolomic profile of female and male zebrafish livers, metabolomic research

in zebrafish has progressed considerably since then. More recent studies, such as Aguilar *et al*. (2022), have provided comprehensive tissue-specific metabolomic profiles, demonstrating how metabolic responses vary across the liver, intestine, and brain under thermal stress. The report highlights the sensitivity of the zebrafish metabolome to external stimuli and reaffirms the need for controlled conditions during sample collection. Despite these advances, there remains a lack of targeted studies assessing how different anaesthetic agents and euthanasia protocols influence baseline metabolomic profiles in adult zebrafish (Aguilar *et al*., 2022). In Ong *et al*. (2009), the study did not disclose how the zebrafish were euthanised, nor whether anaesthetic was used. Therefore, it is difficult to assess the reliability of this baseline metabolomic profile. Possible confounding factors include not knowing if the profile is a baseline or the result of the process of euthanasia. The type of anaesthetic may have influenced the metabolome, or even a lack of using an anaesthetic would affect the profile by reflecting the handling stress. Taking too long to euthanise the zebrafish may have also initiated a stress response, consequently raising the concentrations of metabolites involved in the stress response. Furthermore, the study does not indicate what measures are taken to extract the livers to ensure that the profile remains at baseline levels. Therefore, although Ong *et al*. provided an early foundation, more rigorous and standardised metabolomic studies, such as those demonstrated by Aguilar *et al*. (2022) are needed to establish reliable baselines for zebrafish, particularly in the context of anaesthesia related research. A more targeted study by Burger (2022) conducted an LC-MS, GC-TOFMS, and NMR-based metabolomic analysis of zebrafish exposed to MS-222, clove oil, and 2-PE, using samples and an experimental protocol developed for the current study. While Burger (2022) focused solely on the metabolomic outcome of three concentrations, the present research integrates a broader range of exposures with phenotypic observations, aiming to recommend appropriate anaesthetics for zebrafish research based on both physiological and behavioural data.

An experiment tested if the anaesthetic benzocaine was able to induce light sedation in the euryhaline teleost *Centropomus parallelus* at different salinities, using stress biomarker (cortisol and glucose) levels in response to the anaesthetic and salinity (Wosnick *et al*., 2018). It was found that biomarker levels were unchanged within 3-6 minutes of exposure to the anaesthetic but significantly increased during prolonged exposure. Salinity caused no changes to the biomarkers. Despite the increase in response to the anaesthetic, no mortalities or adversities were recorded. The authors did not test biomarker levels during recovery and noted that it would be worth investigating. Thus, the study concluded that the exposure period for benzocaine should be between 3-6 minutes and not exceed that (Wosnick *et al*., 2018).

More recent studies show the effects of the three anaesthetics that the current study (clove oil, MS-222, and 2-PE) have on the metabolomes of various fish. A study on the combined transcriptomics and metabolomics for the brain tissue of grass carp (*Ctenopharyngodon idella*), a freshwater teleost, via Light Chromatography-Mass Spectrometry (LC-MS) identified 27 differential metabolites in 80 mg/L MS-222 anaesthetised carp, 36 in 200 mg/L MS-222 anaesthetised carp, 15 differential metabolites in

0.6 mL/L 2-PE anaesthetised carp, and 14 differential metabolites in 1.2 mL/L 2-PE anaesthetised carp (Wang *et al*., 2022). Both concentrations of MS-222 significantly altered 20-hydroxyeicosatetraenoic acid and 12-hydroxyeicosatetraenoic acid. Both concentrations of 2-PE significantly altered salidroside. The combined metabolomics and transcriptomics analyses results showed that MS-222 inhibited arachidonic acid metabolism, and 2-PE influenced the upstream and downstream arachidonic acid metabolism pathways, consequently affecting arachidonic acid metabolism. The study's metabolomics results showed that the two anaesthetics' effect on sedation altered metabolites related to stress response and autoimmunity. However, neither anaesthetic had a significant effect on cortisol release (Wang *et al*., 2022). A study that aimed to investigate the effect clove oil and MS-222 had on the HPI axis to reduce the stress incurred from the transport of juvenile gilthead seabream (*Sparus aurata*) quantified the effect through several parameters, such as targeted transcriptomics, plasma cortisol analysis, and targeted liver metabolite quantification (Jerez-Cepa *et al*., 2019). The outcome of the metabolite results showed that 6 h exposure to 2.5 mg/L clove oil stimulated amino acid catabolism, while 5mg/L MS-222 reduced liver glycogen and mobilised triglycerides, showing that differential alterations in the energy management of amino acids, carbohydrates, and lipids depending on the anaesthetic used. Additionally, cortisol showed an increase in clove oil anaesthetised fish, while cortisol levels remained constant on MS-222 anaesthetised fish (Jerez-Cepa *et al*., 2019).

## 2.8 Zebrafish

The zebrafish (*Danio rerio*) is native to South Asia's Himalayan region, which includes India, Nepal, Bhutan, Pakistan, Bangladesh, and Myanmar. It is a tropical freshwater teleost fish that dwells in rivers, mostly the Ganges River and is a member of the Cyprinidae family and the class Actinopterygii. George Streisinger began to utilise zebrafish as a biological model at the University of Oregon in the 1960's, laying the foundation for vertebrate genetic research (Khan and Alhewairini, 2018; Mullins *et al*., 2021).

The main reasons zebrafish have become an excellent model organism in various research fields and laboratories include the transparency of its embryos and larvae, ease of culture, high fecundity, ease of access to all stages of its body development, small size, short life cycles, high genetic similarity, and comparable physiology with humans. (Readman *et al*., 2013; Bozkurt, 2020). Zebrafish share many physiological and genetic characteristics with humans, particularly in terms of the central nervous system, including the innate immune system, musculature, and digestive tract (Khan and Alhewairini, 2018). Zebrafish disease genes share functional similarities with 70% of genes related to human diseases (Santoriello and Zon, 2012; Khan and Alhewairini, 2018) Zebrafish have liver functions and immune responses that closely resemble those of mammals; therefore, researchers see them as a useful model for studying how drugs can harm the liver (Jiang *et al*., 2024). Additionally, zebrafish are the most commonly held lab fish (Potter *et al*., 2020) and are more genetically tractable (Tavares and Lopes,

2013; Khan and Alhewairini, 2018). The commercial applications of the zebrafish as an animal model include the discovery of genes involved in the development of behavioural traits, the metabolism of nutrition, disease, and stress pathways, and the formation of lipids, bones, and muscles. In particular, in pharmacology a medication's impact on a variety of alleles can be evaluated in zebrafish to determine how they alter the fish's physiology and determine the alleles genetic makeup (Dahm and Geisler, 2006; Khan and Alhewairini, 2018).

The advantage of eggs laid and fertilised outside the mother, as well as fertilisation being induced by light succeeding a dark period, allows both observations and manipulation of the timing of fertilisation. The embryo's relative transparency, as well as the development of the internal organs, can be examined under dissection or research microscopes. The fertilised egg is around 650 µm in diameter, although much of the initial volume comprises the yolk, which is gradually digested as food (Figure 2-1). As a result, the organism's narrowest length is less than 650 µm after seven days of development. This allows the use of live embryos in various microscopic procedures (Linney *et al*., 2004). Unlike the mouse embryo, which is approximately 90 µm, the zebrafish embryo's comparatively large size of 650 µm makes it simple to introduce chemicals into the developing cell. Potential transgenes can be injected into the zebrafish embryo cytoplasm, whereas one of the mouse embryo's must be injected into the pronuclei. Therefore, zebrafish require less expensive and specialised equipment for this type of gene transfer than mouse embryos do (Linney *et al*., 1999; Linney *et al*., 2004).

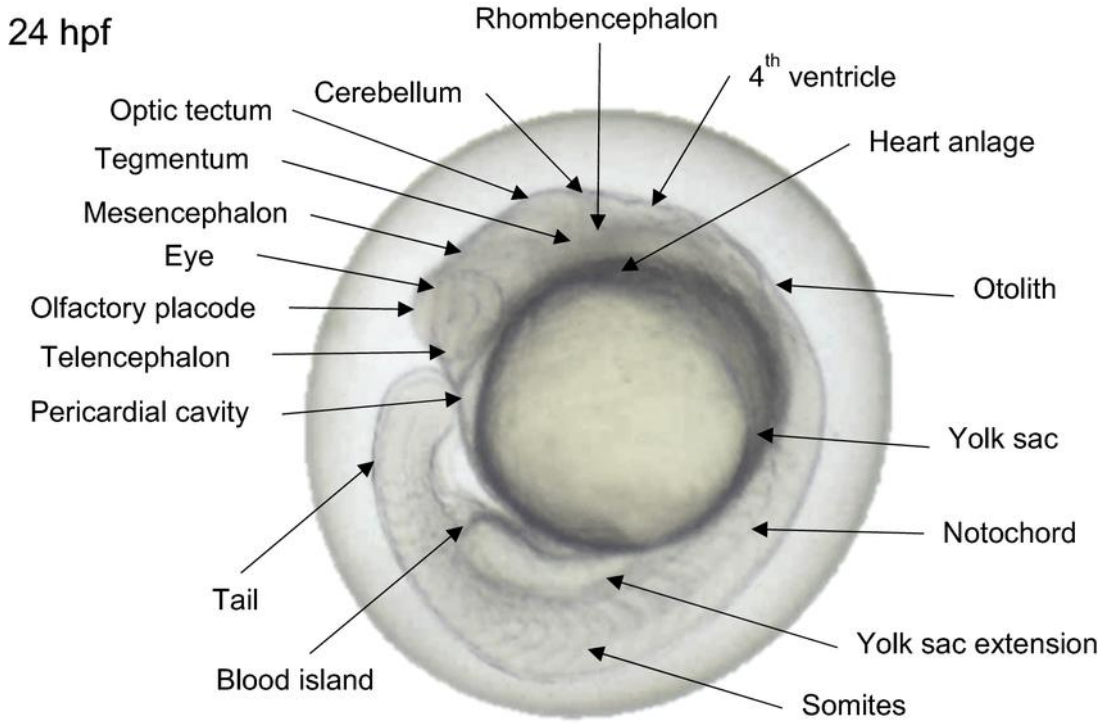


**Figure 2-1: The morphology of a normal zebrafish (*Danio rerio*) embryo at 24 hours post-fertilisation. Figure adapted from (von Hellfeld *et al*., 2020)**

# CHAPTER 3: METHODS AND MATERIALS

# 3. METHODS AND MATERIALS

## 3.1 Anaesthetic trials

### *3.1.1 Ethics statement*

All experimental work on live animals was approved by the Animal Research Ethics Committee (AREC) of UKZN under reference AREC/086/018M and the Department of Forestry, Fisheries, and the Environment in terms of section 20 of the Animal Diseases Act, 1984 (ACT NO 35 of 1984), reference: 12/11/1/5 (1054). All experiments were performed while the fish were under anaesthesia, except for the control sampling. All efforts were made to minimise suffering.

### *3.1.2 Anaesthetic preparation*

*Clove oil*: 500 µL of Clove oil (Sigma-Aldrich, CAS-No. 8000-34-8) stock solution (1.04 g/mL in 98% Ethanol at 1:10 ratio) (Anderson *et al*., 1997) was made up to 1 litre using the system water (final stock concentration of 520 mg/L of clove oil) and stored at room temperature (~19°C). *MS-222*: 450 mg of Ethyl 3-aminobenzoate methane sulfonate (Sigma-Aldrich, CAS-No. 886-86-2) was dissolved in 1 L of system water to make a 450 mg/L stock solution, then buffered to pH 7.11 with sodium bicarbonate (Sigma-Aldrich, CAS-No. 144-55-8). The solution was stored at ~17°C and used the day after the stock solutions preparation. *2-PE*: 4516.712 µL of 2-Phenoxyethanol (Sigma-Aldrich, CAS-No. 122-99-6) was topped up to 1 litre with system water making a 5000 mg/L stock solution and stored at room temperature (~19°C).

### *3.1.3 Anaesthetic protocol*

Before any experimentation, locally sourced adult zebrafish were held in quarantine and housed at UKZN Westville campus (Durban, South Africa) for at least seven days in water made up to a concentration of 60 mg Instant Ocean® sea salt (Instant Ocean, USA) per litre $dH_2O$ (Westerfield, 2007). The anaesthetic trials took place between 24 June 2019 and 29 July 2019. The initial five stages of anaesthesia based on the works of McFarland (1959); Bell (1964); Iwama *et al*. (1989); Martins *et al*. (2016) (Table 2-1) were determined on separate groups of zebrafish (N=9 per concentration) starting with the lowest concentration to avoid negative outcomes and increasing concentrations until no further reductions in induction times were observed. The final concentrations that were tested for clove oil were 60, 80, 100 mg/L (Grush *et al*., 2004; Chambel *et al*., 2015). The final concentrations tested of MS-222 were 50, 75, 125 and, 180 mg/L (Grush *et al*., 2004; Chambel *et al*., 2015; Martins *et al*., 2016). The purpose of using the concentrations listed for each anaesthetic was to confirm observations reported in the literature. This important baselining experiment was used to develop and validate an experimental

approach that can be used for anaesthetics, such as 2-PE, for limited data is available. Based on the reported concentration range for the family Cyprinidae to which *D. rerio* belongs and recent studies which evaluate 2-PE in adult zebrafish, the final concentrations tested for 2-PE were 100, 300, 500, 600, and 700 mg/L (Neiffer and Stamper, 2009; von Krogh *et al*., 2021).

To prevent regurgitation and to reduce nitrogen waste production, fish were not fed for 12-24 hours before the experiments (Harms and Bakal, 1995; Stetter, 2001). Zebrafish were placed individually in separate beakers that contained anaesthetic water of a specific dosage (Figure 3-1) and kept until they reached stage V of anaesthesia (The fish became completely immobile. The opercular movements were observed to slow down and become irregular (i.e. brief pauses between movements), strong pressure was applied to the tail to show no response to stimuli), or until they were anaesthetised for 15 minutes if they did not reach stage V (Table 3-1). Anaesthesia induction time and duration of anaesthesia to reach stage V were recorded. For the recovery, the fish were then transferred to beakers containing anaesthetic-free water at the same temperature (~26-28°C), where the time for recovery of equilibrium and fear response (full recovery) was recorded, and the fish were monitored for any post-experimental adversities or changes to their behavioural patterns. Water was gently swirled towards the gills until the fish began to swim (Zang *et al*., 2015). Since exposure to clove oil exceeding 15 min results in an increased recovery time, all fish were moved into a recovery tank once reaching stage V of anaesthesia or once 15 min had elapsed (Waterstrat, 1999). Because the fish used in this part of the study were only used for observations of the depth of anaesthesia, they were then put into quarantine and left to recover. Had any fish shown any complications during recovery, those fish were euthanised with MS-222 under the discretion of SAVC authorised staff. Had the fish made a full recovery, they were moved to a recovery tank but were noted as being previously exposed to anaesthetic treatments and were not reused in other experiments.

### *3.1.4 Statistical analyses*

A non-parametric ranked two-way MANOVA was used to A) determine if there was a difference in the time it took for fish to reach full recovery in the different concentrations of the three anaesthetics. B) To determine if there was a difference in the time it took for fish to reach stage IV and C) stage V anaesthesia in the different concentrations of the three anaesthetics. The statistical tests were performed using the Companion to Applied Regression (CAR) package (Fox and Weisberg, 2018) in R Studio (version 2022.07.2.). The residuals of the data were not normalised despite Log10 transformations ($p < 0.05$); hence, the non-parametric test was performed for each. The dependent variables were the total time it took to reach full recovery, induction time to achieve stage IV, and stage V anaesthesia, respectively. The independent variables were the anaesthetic agents and the concentrations of the anaesthetics to which the fish were exposed. A Tukey's HSD (honestly significant difference) multiple comparisons post hoc test was used to determine the statistical differences between the dependent

variables within each treatment. A critical *p*-value of 0.05 was used to test statistical significance for both the non-parametric ranked two-way MANOVA and post hoc tests, where a *p*-value less than 0.05 was regarded as a significant result. A Levene's test for homogeneity of variance showed that the residuals of the ranked data of all three non-parametric ranked two-way MANOVA's were not significantly different ($p > 0.05$); therefore, the non-parametric ranked two-way MANOVA's assumption of covariance of the residuals was satisfied. Line graphs of the anaesthetic induction times of each stage of anaesthesia were constructed with GraphPad Prism (version 8) for a visualisation of the induction times of the concentrations of each anaesthetic.

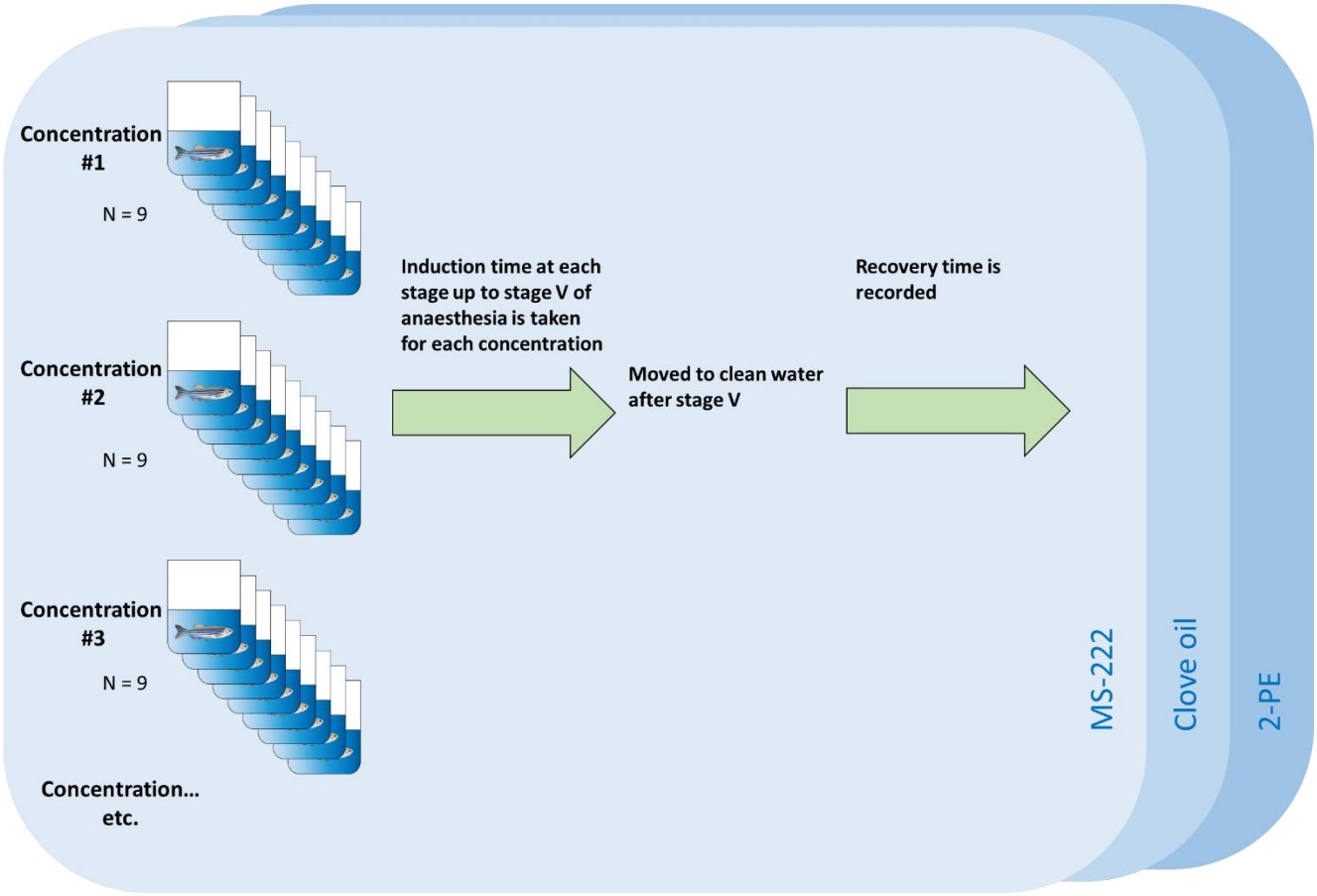


**Figure 3-1: Experimental layout to assess the efficacy of various concentrations of three anaesthetics (MS-222, clove oil, and 2-phenoxyethanol [2-PE]) in adult zebrafish. Anaesthesia was induced to stage V (defined by loss of response to tactile stimulation and slowed, irregular opercular movements), after which fish were transferred to recovery. Each concentration of each anaesthetic was tested with 9 individual fish (n = 9 per concentration).**

**Table 3-1: Indicators used to determine the level of anaesthesia in zebrafish**

| Stage of anaesthesia | Level of anaesthesia | The indicator used to determine the stage of anaesthesia | Action used to identify the stage of anaesthesia |
|---|---|---|---|
| **I** | Lightly sedated | Slight loss of equilibrium | Observation of swimming slowing down and body equilibrium beginning to be lost |
| **II** | Deeply sedated | Further loss of equilibrium, slight loss of response to external stimulation, and a decrease in opercular movement. | Observation of body equilibrium being further lost (fish struggling to stay upright). Response to external stimuli was tested by adding slight pressure to the tail with a glass rod. Opercular movements of the gills were visually observed to determine any change. |
| **III** | Excitation | Greater loss of equilibrium, an increase in respiratory rate, a slight loss in muscle tone, and sometimes an increase in erratic movement | The slight loss of muscle tone was observed, which was also observed through a change in the fish's colouration. The increase in opercular movements of the gills was visually observed. Erratic or hyperactive movements and/or increased swimming around the beaker were observed. |
| **IV** | Light | Complete loss in equilibrium, only responsive to strong external stimulation, and regular opercular movement. | The fish sinks to the bottom of the beaker. Opercular movements were observed to be normal. Only adding strong pressure to the tail with a glass rod would elicit a response from the fish. |
| **V** | Surgical | Irregular opercular movement or a greatly reduced respiratory rate, no response to external stimuli, and no movement | The Fish becomes immobile. Observation of the opercular movements slowing down and becoming irregular (i.e. brief pauses between movements). Strong pressure was applied to the tail to show no response to stimuli. |

## 3.2 Metabolomics

### *3.2.1 Anaesthetic exposure and sampling*

Once the induction/recovery times and dosages for each desired stage of anaesthesia had been determined and confirmed, zebrafish were placed individually in their own beakers that contained anaesthetic water of specific dosage (Figure 3-2) as previously described. The final concentrations of each anaesthetic used were: 180 mg/L MS-222, 100 mg/L clove oil, and 600 mg/L 2-PE. The chosen concentrations reflect the dosage that induced stage IV anaesthesia at approximately one minute of exposure (N = 7 x 3 anaesthetics + 7 control = 28) to standardise the phenotypic effect of the anaesthetics. Henceforth, all metabolomics observations were based on this level of anaesthesia for this duration. Once a fish displayed the desired stage of anaesthesia (stage IV), the fish was removed and decapitated; gently rinsed then inserted into a labelled and perforated 2.5 mL centrifuge tube, snap-frozen in liquid $N_2$ and stored whole at -80°C pending metabolomics analyses. This study used whole body tissue samples instead of blood given the fish's small size of making it difficult to extract large enough volumes of blood.

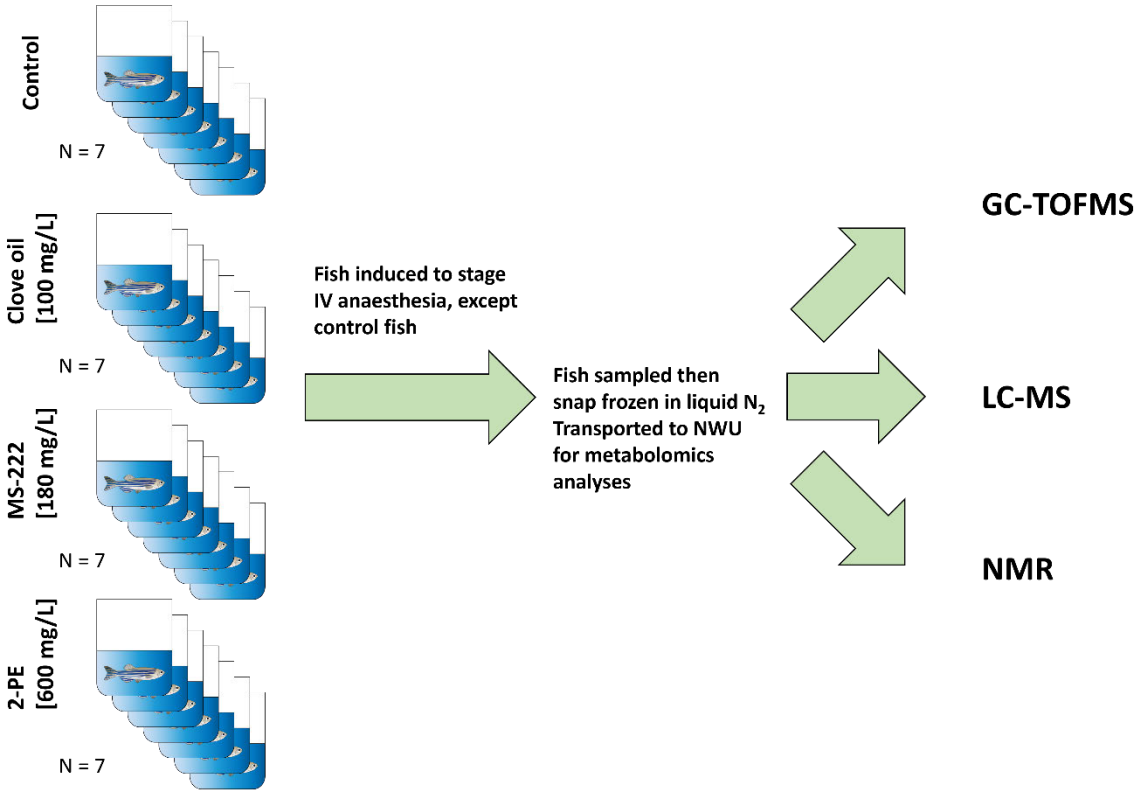


**Figure 3-2: Experimental layout to assess the metabolic consequence of the depth of anaesthesia using three anaesthetics (MS-222, clove oil and 2-phenoxyethanol (2-PE)) in zebrafish. GC-TOFMS = Gas Chromatography – Time-of-Flight Mass Spectrometry. LC-MS = Liquid Chromatography – Mass Spectrometry. NMR = Nuclear Magnetic Resonance**

### *3.2.2 Transportation of samples*

Samples were packaged with 20 kg of dry ice and transported overnight to the North-West University Mitochondrial Laboratory, where metabolomics analyses took place. The following protocol for sample extraction, quantification, and analysis was developed and carried out by Prof. Zander Lindeque, Mr Peet Jansen Van Rensburg and Mr Marcel Burger of North-West University.

### *3.2.3 GC-TOFMS, LC-MS, and NMR approach*

NMR

Nuclear Magnetic Resonance (NMR) spectroscopy is a method that makes use of the magnetic properties of the nucleus to detect the chemical environment of a nucleus in a molecular structure (Kaliva and Vamvakaki, 2020). Sensitivity of NMR can be a problem because it has a lower detection limit for metabolites than other detection methods like gas chromatography or mass spectrometry. Other benefits of NMR, such as simple sample preparation, minimal technical variability, non-destructive sample

analysis, and simple quantification of identified metabolites, readily offset this disadvantage (Mushtaq *et al*., 2014).

GC-TOFMS

Gas Chromatography–Time-of-Flight Mass Spectrometry (GC-TOFMS) can be used to quantify/confirm target analytes as well as identify non-target sample components, even in extremely complex mixtures, because spectrum information is available for a given molecule even at very low levels (high mass analyser efficiency) (Cajka and Hajšlová, 2007).

LC-MS

Unlike GC-MS, Liquid chromatography–mass spectrometry (LC-MS) can detect non-volatile, thermally labile, and polar compounds using electrospray ionisation (ESI) and provides a means if detecting a broad assortment of metabolites and drugs without the need of extensive sample preparations (Lynch, 2017). Due to its high throughput, gentle ionisation, and adequate coverage of metabolites, LC–MS has grown in favour as a platform for metabolomic investigations (Zhou *et al*., 2012).

### *3.2.4 Reagents and chemicals used*

The metabolite extraction used chloroform (Honeywell, cat # 67663), water (Honeywell, cat # 7732185), and methanol (Honeywell, cat # 67561).

### *3.2.5 Metabolite extraction*

Pre-analysis normalisation involved the weighing of experimental samples. Using a razor blade, the tissue (whole fish) was coarsely diced. For each mg of tissue, 2 µL of water and 4 µL of methanol were then added. Each tube received a 7 mm and a 5 mm stainless steel bead. After that, the mixture was homogenized using a Retch M400 vibrating mill at 30 Hz using for 10 min. The beads and homogenate were separated, and the contents were then dispensed in a sterile tube and centrifuged at 4°C for 10 min at 3000 g. For GC-TOFMS, LC-MS, and NMR, the supernatant from each homogenate was split into three microcentrifuge tubes. Metabolites were extracted from the samples using a modified two-phase extraction procedure that was previously published (Lindeque *et al*., 2018). Each tube received additional internal standard (nonadecanoic acid and 3-phenylbutyric acid, at 50 mg/L each) chloroform, methanol, and water during the extraction procedure. The tubes were vortexed for 30 seconds, preceded by a 10-minute incubation period on ice. To achieve phase separation, the samples were centrifuged at 4°C at 2000 g for 5 min. Following extraction, 150 µL of the nonpolar phase and 300 µL of the polar phase was dispensed into screw-top vials for the LC-MS and GC-TOFMS analyses and the same aliquots were dispensed into 2 mL microcentrifuge tubes for the NMR analysis.

### *3.2.6 GC-TOFMS, LC-MS, and NMR analyses*

**GC-TOFMS**

Agilent's 7890A GC and LECO's Pegasus HT mass spectrometers were used to perform the gas chromatography time-of-flight mass spectrometry (GC-TOFMS) analysis. For chromatographic separation, an Agilent DB1 column (20 m; 0.180 mm internal diameter) was used. Each run's autosampler approach involved injecting a 1 µL volume of sample with a split ratio of 1:5. The temperature at the front inlet was maintained at 250°C. Following an initial hold at 50°C for one minute, the oven temperature was raised as follows, maintaining each temperature for two minutes: raised to100°C at 5°C/min, 160°C at 10°C/min, 230°C at 13°C/min, and 300°C at 20°C/min. This resulted in an average run time per sample of about 30 minutes. At a constant flow rate of 1.40 mL/min, helium, was utilised as a carrier gas. Throughout the entire run, the ion source temperature was kept at 200°C, while the transfer line was kept at 225°C (Venter *et al*., 2016). For the first 350 seconds, an acquisition delay was used as a solvent delay. Twenty spectra (40-950 m/z) per second of data were acquired at a detector voltage of 50 V over the daily tuning voltage and an electron energy of about -70 V.

**LC-MS**

The samples were prepared, and then using an Agilent 6410 LC system, they were submitted to reverse phase liquid chromatography. The sample injection volume was 1 L, and the autosampler temperature was maintained at 4°C. The separating column utilised was an Agilent C18 SB-Aq column (2.1 x 100 mm, 1.8 µm) with a guard pre-column. The temperature of the column was maintained at 45°C. Acetonitrile and water, both containing 0.1% formic acid, were used as mobile phase solvents. (Venter *et al*., 2017).

The separation was carried out using the following gradient: 0 min 5% acetonitrile, 1 min 5% acetonitrile, 6 min 25% acetonitrile, 10 min 25% acetonitrile, 15 min 100% acetonitrile, 20 min 100% acetonitrile, and 21 min 5% acetonitrile. Apart from the 15–20-minute interval when a 0.3 mL/min flow rate was utilised, the entire run was conducted at a 0.2 mL/min flow rate to sustain the residual gradient for 4 minutes. The post-run was utilised to verify complete analyte elution and column equilibration. Due to the post-run and time, the gradients used, the runtime per sample was 28 minutes. Multiple reaction monitoring (MRM) identified the target metabolites, particularly amino acids and acylcarnitine’s.

**NMR**

A Bruker Advance III HD NMR spectrometer set to a 500 MHz frequency was utilised to perform the 1-H NMR analysis. The x, y, and z gradient coils and triple-resonance inverse (TXI) (1H, 15N, 13C) probe head were included in this instrument. The NMR instrument was automatically tuned, matched, locked, and shimmed each day using the Bruker standard algorithms: TopShim, LOCK, and ATMA. The NMR spectra contained 32,000 data points, including 256 transients and a spectral width of 6000 Hz. The water resonance was pre-saturated with single-frequency irradiation during a 4-second relaxation delay, with an excitation pulse of 8 µs at 90°C and a constant sample temperature of 27°C, obtaining spectra from 256 scans/sample at a runtime of 32 min/sample. The sample was shimmed automatically using the deuterium signal. For trimethylsilylpropionic acid (internal standard) and

metabolites, the resonance line widths were 1 Hz at half the peak height. Automatic phase correction, baseline correction, and Fourier transformation were carried out. Bruker Topspin (Version 3.1) software analysed the spectral data, and Bruker AMIX (Version 3.9.12) distinguished and identified the metabolites (Burger, 2022).

### *3.2.7 Peak identification*

Agilent's MassHunter Qualitative software (Version B.06) initially checked retention time drifts, the presence of metabolites, and isotopes in the LC-MS data. Following a qualitative analysis of the data, supervised peak integration was undergone with Agilent's MassHunter Quantitative software to transform the raw data into a useable data matrix. For data pre-processing and normalisation, the data matrix encompassing the retention periods and integrated peak regions was then retrieved electronically.

Leco-ChromaTOF GC software was used in this investigation to retrieve the GC-TOFMS data. In this investigation, peak identification, baseline subtraction, and deconvolution were carried out using ChromaTOF as described in Venter *et al*. (2018b) and Burger (2022). The offset for the baseline tracking mode was configured to 1. The application chose the smoothing parameters automatically. An expected peak width of 3 s and a signal-to-noise ratio of > 20 were used to detect peaks. In addition, any true peak required to have five apexed masses, and the detector was programmed to exclusively use model ions with masses between 100 and 950 m/z. To detect metabolites, two spectral libraries, the mass spectral library from the National Institute of Standards and Technology (NIST) 2011 and a custom library (Reinecke *et al*., 2012; Burger, 2022), were utilised. Before a feature could be assigned a preliminary identity, a spectral match similarity of 80% had to be achieved. Only identities with not less than a single silicon (Si) element in their composition were permitted the classification of a metabolite to prevent spectrum matching of inaccurate derivatives or non-derivatised compounds (Venter *et al*., 2018b). By comparing the retention times or retention indices of the significant compounds to those of the libraries and previously examined standards, the identity (spectral match) of the compounds was established. The metabolite identities were assigned to features that could be determined with a great deal of certainty (level one identification) and discussed further in the discussion. The features that were not level one identities were left out of further discussion and remained as the analyte number.

Bruker Topspin (V3.1) software was used to automatically shim the sample depending on the deuterium signal during NMR analysis. The resonance line width of trimethylsilylpropionic acid was found to be less than 1Hz, demonstrating that the shimming was effective (Burger, 2022). Additionally, phase and baseline correction, as well as automatic Fourier processing, were carried out using the Bruker Topspin software. The NMR spectra were further processed using Bruker AMIX (V3.9.12) software. Apart from the area around the water peak, the spectra of the NMR were segmented into 0.02 mg/L-sized bins of spectra for the range of 0.5–10 mg/L (Burger, 2022).

### *3.2.8 Data clean-up*

Microsoft Excel 365 was used for data clean-up. Features that could not be measured reliably were removed using the following quality control procedure: the relative standard deviation (RSD) was calculated by dividing the standard deviation by the mean for all quality control samples in both the experimental and the non-experimental datasets. This allowed for assessment ofthe reliability of each feature. Features with an RSD greater than 50% were excluded, indicating low reliability. All zero values were changed to half of the dataset's minimum observed value, which served as an estimate of the detection limit (Venter *et al*., 2018a). Where the data were not normally distributed, they were scaled, and log transformed. The estimated concentrations of the determined metabolites underwent a generalised logarithm transformation for normalisation.

### *3.2.9 Statistical analyses*

MetaboAnalyst version 6.0 was used to perform statistical analysis on the data produced from GC-TOFMS, LC-MS, and NMR from the samples obtained from zebrafish. After a general logarithmic transformation, a supervised univariate (students' t-test) statistical analysis was carried out to determine the metabolic markers that best described the distinction between the anaesthetised fish and control fish. The assumption that the treatments were independent were satisfied. A Levene's Test for Homogeneity was performed, and the assumption was satisfied ($p > 0.05$, $F > 1$). A One-Sample Kolmogorov-Smirnov Test was performed to determine if the data had a Gaussian distribution, and the assumption was satisfied ($p > 0.05$). Effect size using glass' delta value was determined as an additional indicator of practical importance. Additionally, the partial least square discriminant analysis (PLS-DA), a multivariate test, was performed. The PLS-DA was applied because it highlights the substances that best capture sample variation. A Principal Component Analysis (PCA) was used to ascertain whether there was a natural separation between the anaesthetised and unanaesthetised zebrafish.

A metabolite needed to have both a significant *p*-value (<0,05), calculated from the students' t-test in MetaboAnalyst, and glass' delta value (≥|0,8|) ($\Delta = \text{Mean}_{\text{Experimental group}} - \text{Mean}_{\text{control}} / \text{Standard deviation}_{\text{control}}$) for at least one of the anaesthetics to be considered a significant metabolite. Additionally, a high level of confidence in the identification was required by comparing the retention periods to the retention indices present in the NIST retention index database. Once significant metabolites of interest were identified, Venn diagrams were generated using Microsoft Excel to manually identify common significant metabolites between the anaesthetics and metabolites that were uniquely significantly altered by each anaesthetic treatment.

A metabolite set enrichment analysis (MSEA) and a metabolic pathway analysis (MetPA) were performed for the significant metabolites of each anaesthetic group using the corresponding control as a reference group on MetaboAnalyst version 6.0. The metabolic pathway analysis module integrates findings from robust pathway enrichment analysis and pathway topology analysis. to assist researchers

in locating the routes that are most important to the circumstances being studied. Numerous tried-and-true techniques, such as univariate analysis and over-representation analysis, were combined with cutting-edge concepts and algorithms in this module to perform pathway analysis, including GlobalTest, GlobalAncova, and network topology analysis (Pang *et al*., 2021). The uploaded concentration table of significant metabolites was subjected to quantitative enrichment analysis (QEA) for the MSEA, using the package global test 3. The Q statistic, which expresses the association between compound concentration profiles, X, and clinical outcomes, Y, was estimated for each metabolite set using a generalised linear model. The average of the Q statistics for each of the metabolites in a set of metabolites is the set's Q statistic (Pang *et al*., 2021).

# CHAPTER 4: RESULTS

# 4. RESULTS

## 4.1 Descriptive phenotypic observations

Table 4-1 below summarises the descriptive phenotypic observations of each dosage of each anaesthetic detailed in section 4.1.

**Table 4-1: Summary of phenotypic effects of the anaesthetics, clove oil; MS-222; and 2-phenoxyethanol (2-PE) by dose, stage reached, and recovery onset in zebrafish.**

| Anaesthetic | Dose (mg/L) | Highest Stage Reached | Time to Recovery Onset | Recovery Notes |
|---|---|---|---|---|
| **MS-222** | 50 | Stage III | Immediate | Slow initial swim, full recovery soon after |
| | 75 | Stage IV (few reached V) | Immediate | Like 50 mg/L, full recovery followed |
| | 125 | Stage V | ~43 sec | Struggled initially, then full recovery |
| | 150 | Stage V | Not specified | Very fast acting, immobile at deep stages |
| **Clove Oil** | 30 | Stage V | Immediate | Regained equilibrium gradually |
| | 60 | Stage V | 3–8 min | Gulping during and after recovery |
| | 100 | Stage V | ~5 min | Slower recovery, gulping noted |
| **2-PE** | 100 | Stage I (rarely Stage III) | Slightly delayed | Mostly quick full recovery |
| | 300 | Stage IV (few) | Shortly after immobility | Gradual upright swimming to full recovery |
| | 500 | Stage IV (rarely V) | 2–3 min | Initial stillness, full recovery shortly after |
| | 600 | Stage V | ~1 min | Full recovery in 2.5–4.5 min |
| | 700 | Stage V | Slightly >600 mg/L | Like 600 mg/L but slower onset of stage V |

### 4.1.1 MS-222

#### *4.1.1.1 MS-222 50 mg/L*

Fish in Stage I anaesthesia showed disorientation, for instance, bumping into the walls of the beaker. In stage II, the disorientation was either the same or progressed. In stage III, the fish began swimming rapidly in circles with the body pointing upwards with the aboral end pointing upwards, then swam erratically and sometimes in circles. The movement of the tail and body seemed rapid (hyperactive), struggling to maintain equilibrium, but mostly maintaining it, instead of the regular, smooth movement. While still in stage III, if the fish sank lower to the bottom of the water column, one end of the fish pointed down, and the other end pointed up, while the fish continued to swim in circles. In recovery, the fish started swimming as it entered the fresh water of the recovery tank. The swimming was initially slow but improved quickly to full recovery.

#### *4.1.1.2 MS-222 75 mg/L*

The fish showed slow movement in stages I and II. As for 50 mg/L, the fish entered stage III, showing erratic movements and swam in circles. The swimming eventually slowed down, and after 6-8 mins, but the fish continued to swim in circles in the beaker. At this point, some fish completely lost equilibrium, entering stage IV. Very few individuals progressed into stage V anaesthesia. While in recovery, at 50 mg/L, fish began swimming upon entry to anaesthetic-free water. Swimming started slowly then improved rapidly to full recovery.

#### *4.1.1.3 MS-222 125 mg/L*

The concentration immobilised the fish rapidly. When the fish entered stage I, they were disoriented like the previous concentrations. In stage II, the fish remained still, then in stage III, the fish began swimming in a similar manner described for the previous concentrations, showing rapid tail movements. However, the fish wholly and quickly lost equilibrium after swimming slowly in circles with the ventral side upwards; eventually ending up at the bottom of the beaker, mostly immobile, maintaining some response to tactile stimulation. The depth was identified as stage IV. The fish soon entered stage V through losing response to any stimulus and showed irregular opercular movements.

During recovery, the fish did not move immediately upon entry into the anaesthetic-free water, but the movement occurred within an average of 43 s. After initial movement, the fish swam to the surface of the water, struggling to maintain equilibrium, then quickly regained it and made a full recovery.

#### *4.1.1.4 MS-222 150 mg/L*

The initial stages were as for 125 mg/L. However, the fish appeared more distressed in the first two stages, flailing around more. The fish's movement slowed down much more rapidly. In stage III, it lost equilibrium much more rapidly (the fish swam a hyperactive yet slow manner with the body inverted). The fish then eventually entered stage IV with some response to tactile stimulation and then stage V losing response to stimuli with irregular opercular movements. Most fish were not as active in stage III but very immobile. Overall, the anaesthetic was very fast acting.

### 4.1.2 Clove Oil

#### *4.1.2.1 Clove oil 30 mg/L*

The initial exposure and entry into stage I at this concentration showed the fish appearing immobile before entering stage II showing a slight loss of equilibrium and swimming slowly relative to the control group. Once in stage III, the fish further struggled to keep equilibrium eventually wholly losing it while swimming in circles upside down in the beaker. The fish eventually sank to the bottom (still showing some tail and body movement and response to external stimuli). The fish were presumed to have entered stage IV yet appeared to be very mobile and responsive compared to fish exposed to other concentrations and other anaesthetics that had entered stage IV. The fish that managed to enter stage V did so shortly after entering stage IV. The fish in stage V showed no/little response to tactile stimuli and very irregular opercular movement. Some that entered stage V showed some increase in movement before entering stage V. Recovery began by swimming to the surface of the anaesthetic free water with little equilibrium, then eventually regaining equilibrium and responses until fully recovered.

#### *4.1.2.2 Clove oil 60 mg/L*

The fish entered the initial stages (I-III) rapidly, and they lost mobility very quickly. In the excitation phase, the fish completely lost equilibrium but still showed some movement. It is possible that stage IV may have been conflated with a more extended stage III. Still, some fish went straight into stage V (irregular opercular movements and little response to external stimuli). It is suspected that the mobile phase observed was stage IV i.e., shows that there are signs of variation in the reaction particularly at stages III and IV. Some fish in stage IV showed some response to external stimuli. When exposed to this concentration, the fish that could swim while exposed, equilibrium would have been lost (fish remaining at the bottom of the beaker in this case) or the fish would briefly swim while upside down (early stage II) (one fish was disorientated).

The fish's movement in stage IV seemed peculiar with this dosage. Just before entering stage 5, the motion ceased as for the other anaesthetics and concentrations but was much briefer before rapidly

entering stage V. Recovery was longer than fish exposed to 30 mg/L of clove oil, taking between 3-8 min to start moving then fully recovering a few minutes after moving. However, during and after recovering, fish exhibit a gulping behaviour which only ended a few minutes after reaching full recovery.

#### *4.1.2.3 Clove Oil 100 mg/L*

Exposure showed a speedy induction time with little to no movement in the three early stages, as if the excitation phase (stage III) didn't occur. This concentration of the anaesthetic made it clear to observe the point in stage IV, where the response to tactile stimulation ceases but regular opercular movement continues before entering stage V. Recovery was slow. It took the fish approximately 5 min to begin swimming after entering anaesthetic free water then fully recovered shortly after but still exhibiting gulping behaviour.

### 4.1.3 2-Phenoxyethanol

#### *4.1.3.1 2-PE 100 mg/L*

The fish moved slightly slower than the control with a slight change in equilibrium and continuous, yet slower, swimming in relation to the control group. It is difficult to determine if any fish entered stage III, most did not make it past stage I. One of the only indicators that the anaesthetic had any effect was that recovery was initially slower than that of the control group. However, the full recovery was very rapid, unnoticeably different.

#### *4.1.3.2 2-PE 300 mg/L*

The first stage, like the other concentrations/anaesthetics, showed disorientation in the fish's movement then a lack of movement in stage II. In stage III, few of the fish appeared to be immobile and showed a loss of muscle tone and an increase in respiration. But most of the fish displayed similar swimming patterns to those exposed to 75 mg/L MS-222, i.e., swimming in circles with rapid tail movements but slower locomotion than the MS-222 group. Very few (three) fish made it to stage IV, one showed the plane of loss of response to tactile stimulation but a regular opercular movement.

Some fish entering stage III showed further loss equilibrium, swimming upside down in circles at the top of the water column then swam at the bottom of the beaker. When recovering in anaesthetic free water, the fish were first lying on their sides immobile at the bottom of the tank then positioned themselves upright and began to swim slowly. Swimming improved then the fish reached full recovery.

### *4.1.3.3 2-PE 500p mg/L*

Initial exposure to the anaesthetic at this concentration appeared to quickly elicit a response where the fish swam around disoriented with the tail racing, losing their equilibrium (stage I), eventually completely losing equilibrium and sank to the bottom of the beaker (stage II). The opercular movements elevated, and the fish lost muscle tone (stage III). At this point, the fish were mostly immobile though reacting to noxious stimuli—all of which occurring one minute into exposure. Approximately 1 minute after entering stage III, the fish entered light anaesthesia (stage IV). Between 4-7 minutes after reaching light anaesthesia, the fish no longer responded to noxious stimuli, however, maintained regular opercular movement. It is suspected that this may be a deeper plane of light anaesthesia that occurs before stage V. Most of the fish did not enter stage V when exposed to this concentration. Two fish ceased opercular movements approximately 9 min into exposure. Noticeably, these fish took longer to reach the deeper plane of stage IV and were a bit more mobile in stage III in comparison to the other fish exposed to this concentration. Recovery initially took the fish 2-3 min to start moving. Full recovery followed shortly after initial movement.

### *4.1.3.4 2-PE 600 mg/L*

Exposure to this concentration elicited a similar response as 500 mg/L, but a more rapid induction. Once the fish entered the anaesthetic water, they became disoriented, swimming erratically, like stage III of other treatments, but a bit slower. The fish then quickly lost its equilibrium and sank to the bottom. The respiratory rate increased, suggesting it had entered stage III, though very immobile. Some fish showed some aggressive movement but quickly entered stage IV, after which it took between 2-3 min to enter stage V, where opercular movements were either irregular or extremely slow. The induction in response to this concentration was very rapid across all stages.

Upon entering the anaesthetic free water, recovery began with the fish mostly lying still on their side for the first minute, then quickly positioned themselves upright, remaining still, then began to swim shortly after. The swimming was regular and gradually increased in speed till full recovery. Full recovery was rapid compared to the other treatments, reaching full recovery between 2 ½ and 4 ½ min.

### *4.1.3.5 2-PE 700 mg/L*

The phenotypic response to this concentration was like that of 600 mg/L. In this case, the fish initially showed some escaping behaviour but were quickly immobilised (stage I-II). Stage III showed an increase in opercular movement, loss in muscle tone, and loss in mobility. Stage IV was like the previous concentrations but achieved more rapidly (approximately 10 sec earlier than 600 mg/L). However, induction into stage V took slightly longer than that of fish exposed to 600 mg/L. Stage V showed prolonged opercular movement if any. Overall, induction was extremely rapid.

Recovery of fish in this treatment took slightly longer than that of fish in 600 mg/L but was very different. The response was like fish in 600 mg/L, but some fish swam closer to the surface, then stayed still at the bottom of the tank, then continued to swim again till gradually reaching full recovery.

## 4.2.1 Onset of anaesthesia

### *4.2.1.1 Stage I anaesthesia*

The time for induction of stage I anaesthesia for clove oil treated fish averaged between 9.4-22s in 30, 60, and 100 mg/L clove oil; 12-28s in 50-180 mg/L MS-222; and averaged between 12-68s in 100-700 mg/L 2-PE (Figure 4-1). Fish exposed to 100 mg/L achieved the fastest average induction for of 9.4s. Overall, the time to reach stage I anaesthesia induction in each anaesthetic was more rapid with increase in concentration of the anaesthetic. The pattern that 2-PE exposure shows is that the induction time of exposure to 500, 600 and 700 mg/L did not decrease as drastically as the previous concentrations, suggesting a plateau effect at higher concentrations. Within each anaesthetic group, the lower concentrations tested, i.e. 30 mg/L Clove oil; 50-125 mg/L; and 100-300 mg/L 2-PE, showed greater variability indicated by the larger standard deviations in the error bars.

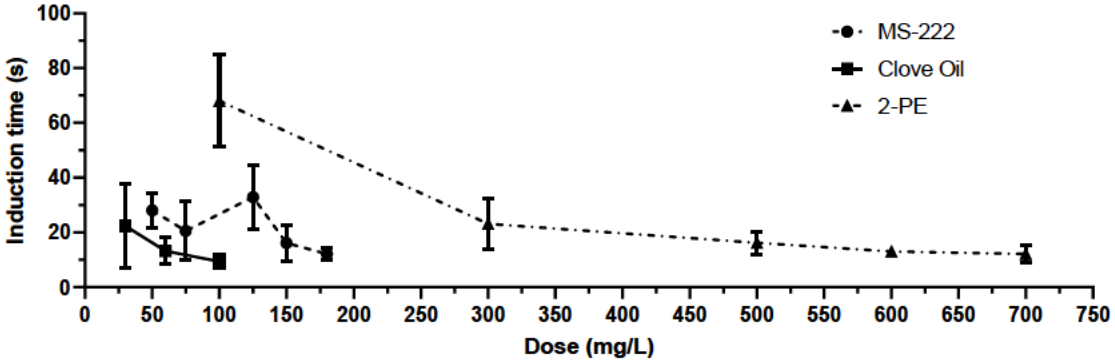


**Figure 4-1: Time required for zebrafish to achieve stage I anaesthesia at various concentrations of 2-Phenoxyethanol (2-PE), MS-222 and clove oil. Data points represent the mean ± SD, n = 9.**

### *4.2.1.2 Stage II anaesthesia*

The time for induction of stage II anaesthesia averaged between 18-58s in 30-100 mg/L clove oil; 24-88s in 50-180 mg/L MS-222; and averaged between 19.89-175s in 2-PE anaesthetised fish (Figure 4-2). The time to reach stage II was the fastest in fish exposed to 100 mg/L clove oil and the overall pattern of the three anaesthetics induction time showed that and increase in anaesthetic concentration led to faster induction times. Notably, variability is highest in 100, 300 mg/L 2-PE and 75 mg/L MS-222 indicated by the large standard deviations. A similar plateau effect can be seen in the higher

concentrations of 2-PE (500, 600, and 700 mg/L) as with that which occurred in stage I anaesthesia in Figure 4-1.

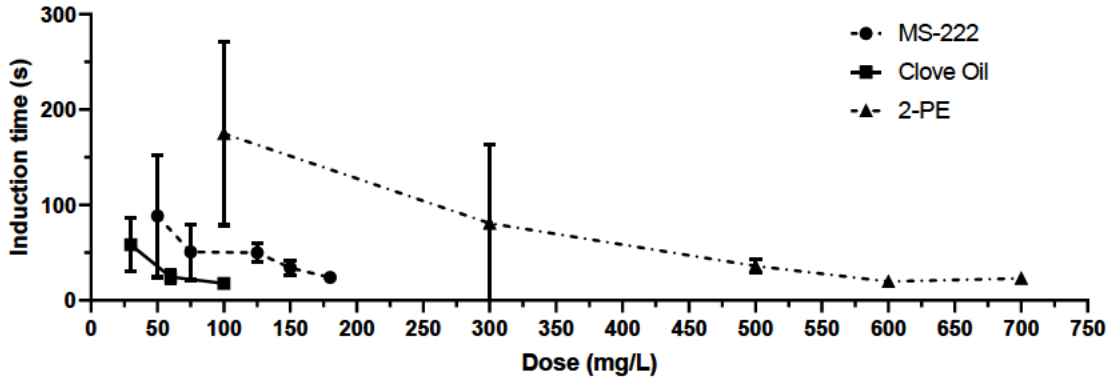


**Figure 4-2: Time required for zebrafish to achieve stage II anaesthesia at various concentrations of 2-Phenoxyethanol (2-PE), MS-222 and clove oil. Data points represent the mean ± SD, n = 9.**

### *4.2.1.3 Stage III anaesthesia*

The time of stage III anaesthesia induction averaged around 28-192.5s in 30-100 mg/L clove oil anaesthetised fish; 39-230.6s in 50-180 mg/L MS-222; and around 32-477.5s in 100-700 mg/L 2-PE anaesthetised fish (Figure 4-3). Like other stages of anaesthesia, an increase in the anaesthetic concentration led to faster induction of stage III anaesthesia. Zebrafish anaesthetised with clove oil 100 mg/L achieved stage III anaesthesia faster than other treatments at 28.11s. Notable variation was observed in the lower concentrations tested (100 and 300 mg/L 2-PE; 50 and 75 mg/L MS-222; and 30 mg/L clove oil). The other treatments showed less variability in the standard deviation.

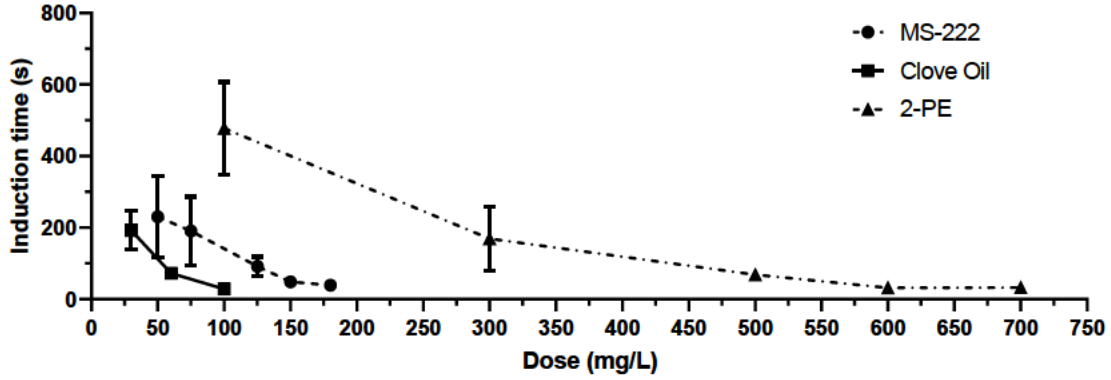


**Figure 4-3: Time required for zebrafish to achieve stage III anaesthesia at various concentrations of 2-Phenoxyethanol (2-PE), MS-222 and clove oil. Data points represent the mean ± SD, n = 9.**

### *4.2.1.4 Stage IV anaesthesia*

The time to reach stage IV anaesthesia induction averaged around 59.3-192.5s in 30-100 mg/L clove oil anaesthetised fish; 69.7-515.14s in 75-180 mg/L MS-222; and around 55.56s-134.5s in 500-700 mg/L 2-PE anaesthetised fish (Figure 4-4). Since only one fish anaesthetised with 50 mg/L MS-222 reached stage IV anaesthesia, that concentration was left out of further discussion of stage IV anaesthesia. Only three zebrafish anaesthetised with 30 mg/L 2-PE achieved stage IV anaesthesia, where the average time of induction was 410.33s with a range of 698s. The large standard deviation and the few fish that achieved stage IV anaesthesia indicate that 300 mg/L did not reliably anaesthetise the fish. The treatments 30 mg/L clove oil and 75 mg/L MS-222 also showed great variability, indicated by the standard deviation. Anaesthesia induction time became more rapid with an increase in concentration of each anaesthetic. As concentrations increased beyond 150 mg/L in MS-222 and 600 mg/L in 2-PE, the induction time did not significantly decrease further observed in a Tukey HSD test ($p$ adj. = 0.92 for 600 vs 700 mg/L 2-PE; $p$ adj. = 0.77 for 150 vs 180 mg/L MS-222), suggesting a plateau effect at higher concentrations.

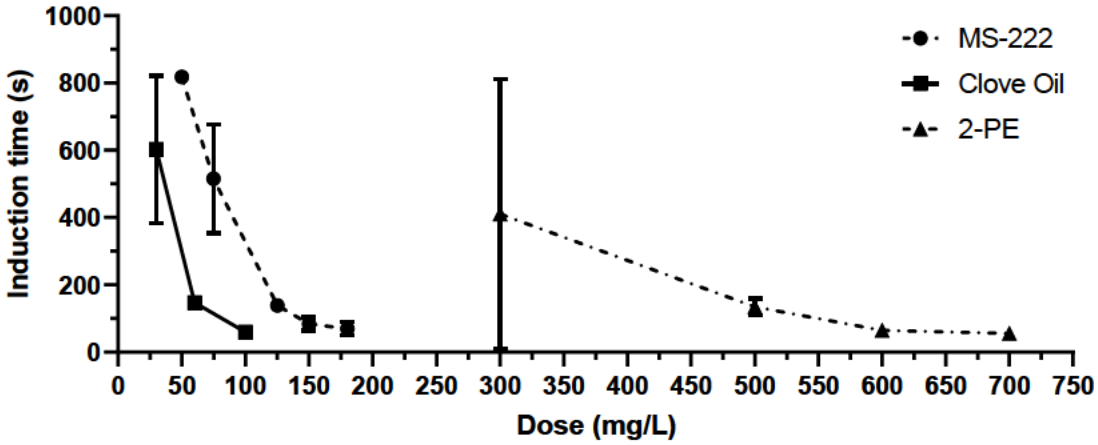


**Figure 4-4: Time required for zebrafish to achieve stage IV anaesthesia at various concentrations of 2-Phenoxyethanol (2-PE), MS-222 and clove oil. Data points represent the mean ± SD, n = 9.**

### *4.2.1.5 Stage V anaesthesia*

The time to reach stage V anaesthesia induction averaged around 59.3-192.5s in 30-100 mg/L clove oil anaesthetised fish; 69.7-515.14s in 75-180 mg/L MS-222; and around 55.56s-134.5s in 500-700 mg/L 2-PE anaesthetised fish (Figure 4-5). Only three out of the nine fish anaesthetised with 30 mg/L clove oil reached stage V anaesthesia during the 15 min exposure period at an average induction time of 797s. Though the standard deviation was low, only a third of the animals in the treatment were effectively induced to stage V; therefore, the treatment did not reliably induce stage V anaesthesia. Similarly, only

two of the nine fish anaesthetised with 500 mg/L 2-PE reached stage V and three of the nine fish anaesthetised with 75 mg/L MS-222 reached stage V. Therefore, these two treatments also did not reliably induce stage V anaesthesia in zebrafish in the 15-minute exposure period. As with the other stages of anaesthesia, induction to stage V was faster at higher concentrations, where 180 and 150 MS-222 induced stage V the fastest among the other anaesthetics at an average induction time of 147.6 and 147.2s, respectively. However, the standard deviation for the induction time from 180 mg/L MS-222 was greater than the other higher concentrations of the anaesthetics tested.

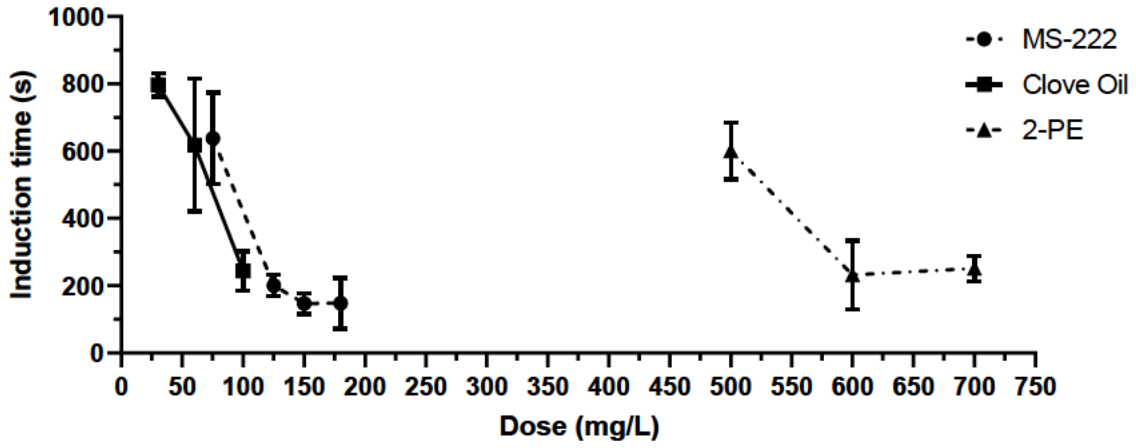


**Figure 4-5: Time required for zebrafish to achieve stage V anaesthesia at various concentrations of 2-Phenoxyethanol (2-PE), MS-222 and clove oil. Data points represent the mean ± SD, n = 9.**

## 4.2.2 Anaesthesia recovery

The outcome of the ranked two-way MANOVA found significant differences between each anaesthetic and concentrations for full recovery after anaesthesia ($p = 4.7\text{x}10\text{-}13$, $F = 12.71$, $df = 9$). Fish exposed to clove oil took longer to reach recovery of equilibrium and fear response (full recovery) than those that were exposed to MS-222 and 2-PE (Figure 4-6). Fish exposed to 500 mg/L 2-PE had a significantly higher mean full recovery time than those exposed to the other concentrations of 2-PE in this study (Figure 4-6), and this group's standard deviation showed that there was greater variation in the recovery. Fish that were exposed to the tested concentrations of MS-222 were not significantly different to one another (Figure 4-6; $p > 0.05$). A Tukey-HSD post-hoc test after a non-parametric ranked two-way MANOVA revealed that fish exposed to 300, 600 and 700 mg/L 2-PE were not significantly different to fish exposed to the tested concentrations of MS-222.

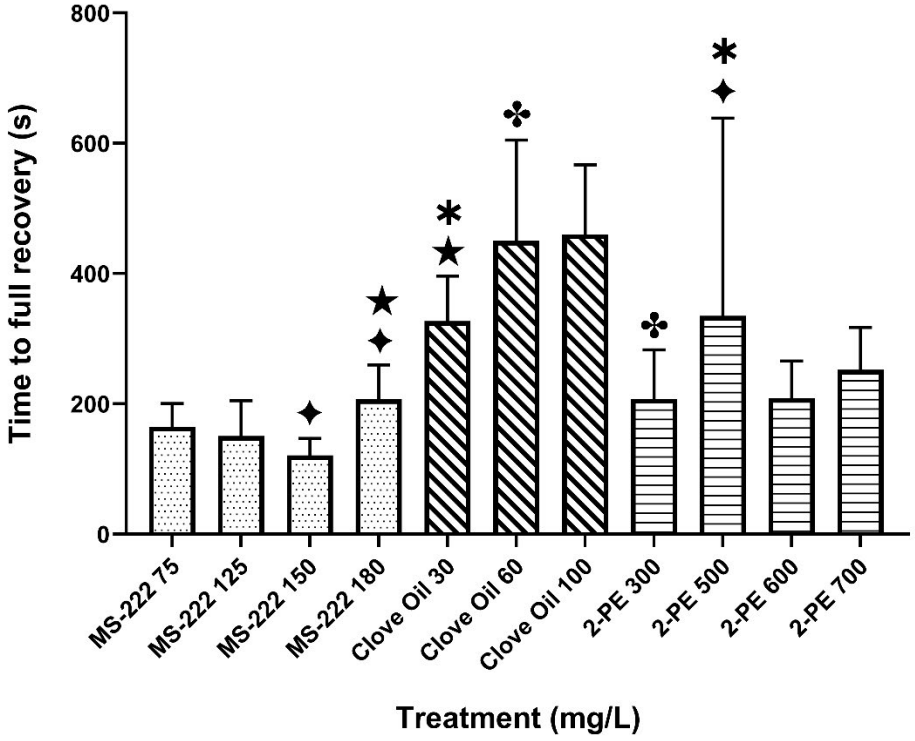


**Figure 4-6: Time required for zebrafish to achieve full recovery after anaesthesia at various concentrations of 2-Phenoxyethanol, MS-222 and clove oil. Data points represent the mean ± SD. The outcome of the Tukey HSD multiple comparisons post hoc test is shown above each column. The special characters above the columns indicate that the groups with matching special characters are significantly different.**

## 4.2.3 Onset of stage IV and V anaesthesia: Analysis of Variance

The outcome of each ranked two-way MANOVA is shown in Figure 4-7. Significant differences were found between each anaesthetic and concentrations for A) stage IV anaesthesia ($p = 2 \times 10^{-16}$, F = 72.048, $df = 8$), and B) stage V anaesthesia ($p = 4.45 \times 10^{-12}$, F = 14.79, $df = 8$). The post-hoc test revealed no significant difference between the induction time for 100 mg/L clove oil, 180 mg/L MS-222, and 600 mg/L 2-PE ($p > 0.05$) for stage IV anaesthesia. These three concentrations allowed the zebrafish to achieve anaesthesia within 59-70 s, and there was no significant difference between the time to reach full recovery from anaesthesia of the three concentrations ($p > 0.05$; Figure 4-6). Therefore, the phenotypic response to anaesthesia and recovery could be compared; henceforth, the three concentrations became the final treatments used for the metabolomics analyses.

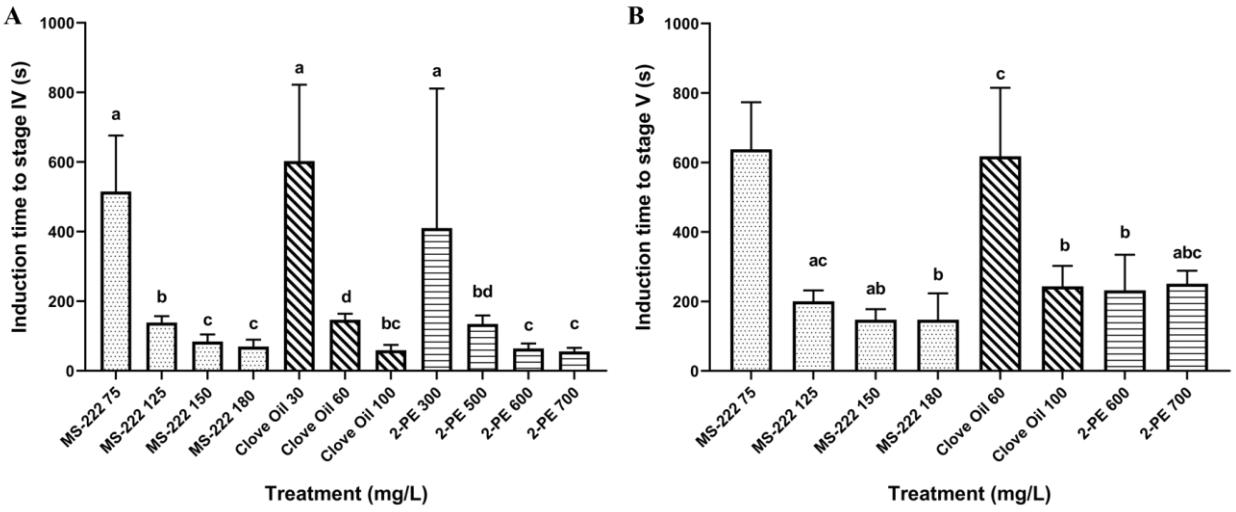


**Figure 4-7: Time required for zebrafish to achieve A) stage IV anaesthesia and B) stage V anaesthesia at various concentrations of 2-Phenoxyethanol, MS-222 and clove oil. Data points represent the mean ± SD. The outcome of the Tukey HSD multiple comparisons post hoc test is shown above each column. The letters above the columns indicate that the group of that column is not significantly different to a group with a matching letter.**

## 4.3 Metabolomics

### *4.3.1 Principal Component Analysis (PCA)*

Multivariate statistical modelling using PCA was performed on the combined platform spectra of the whole zebrafish tissue samples from the control and anaesthetic groups, using the entire metabolomic profiles of the control and anaesthetic groups. The score plot (Figure 4-8.) showed little discrimination between the metabolome of the three anaesthetic groups or separation from the control group

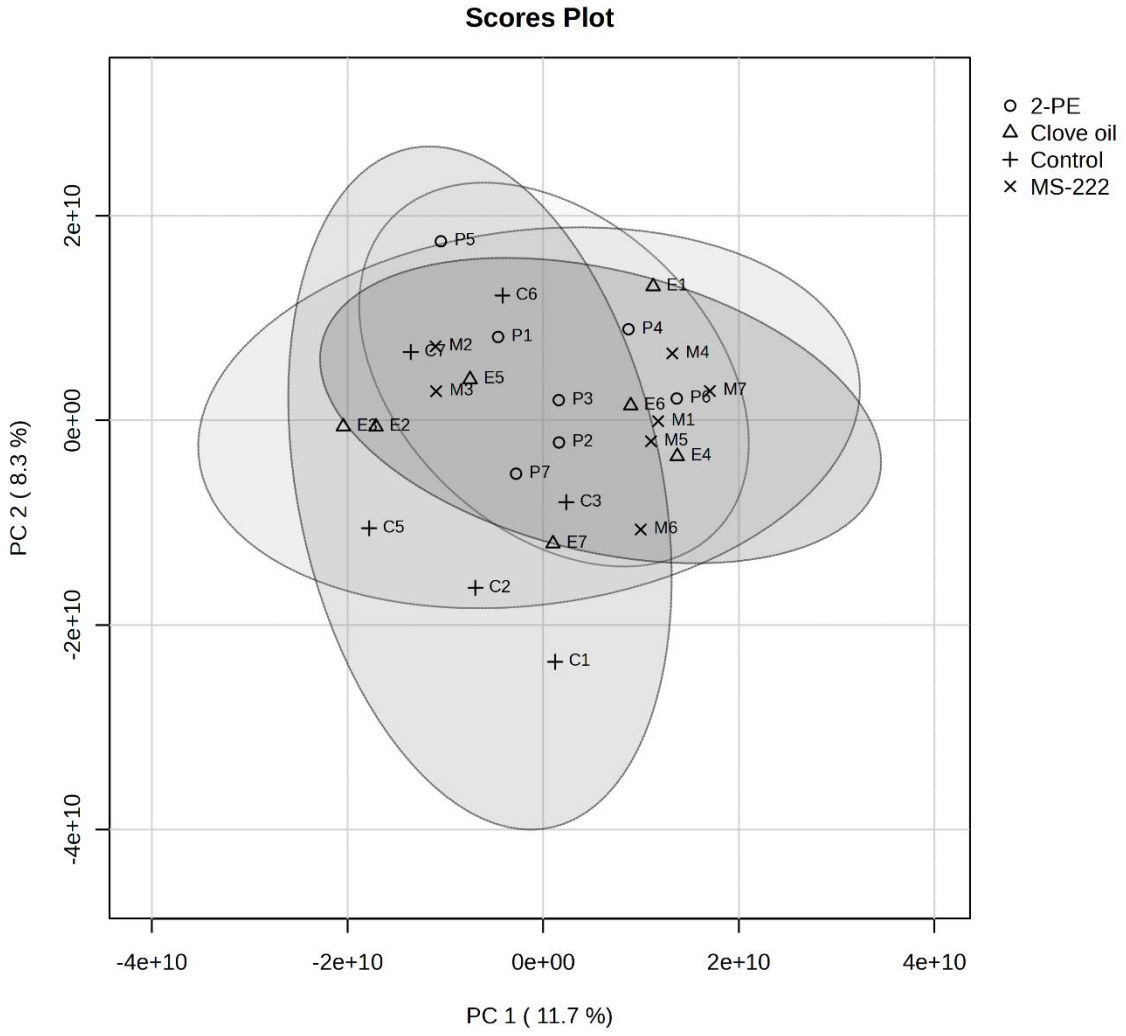


**Figure 4-8: Principal Component Analysis Score plot between the variance of the zebrafish metabolite concentrations from the control group and the three anaesthetic groups (600 mg/L 2-phenoxyethanol (2-PE); 100 mg/L clove oil; and 180 mg/L MS-222). The explained variances are shown in brackets. The data points represent each individual fish sampled, while ellipses describe 95% confidence regions for each group.**

### *4.3.2 Partial Least Squares - Discriminant Analysis (PLS-DA)*

Figure 4-8 shows a two-dimensional PLS-DA scores plot of Component 1 (6.5% of variance) and Component 2 (7.2% of variance), used for discrimination visualisation between the zebrafish metabolome of experimental groups: 2-PE, Clove oil, Control, and MS-222. The MS-222 group (×) shows wide separation from the 2-PE (○) and Clove oil (△) groups along Component 1, suggesting distinct metabolic profiles. The Control group (+) shows some overlap with MS-222 but is mostly separate from the 2-PE and Clove oil treatments. Clove oil and 2-PE have a partial overlap, indicating some degree of similarity in their profiles, but also produce distinguishable clusters. The low explained variance percentages (6.5% and 7.2%) indicate that even though discrimination has been shown between the groups, additional components may be required to explain more variance and effectively separate the groups. The PLS-DA analysis provides evidence that MS-222 elicits a response that is more distinguishable from the remaining anaesthetic treatments as well as the control.

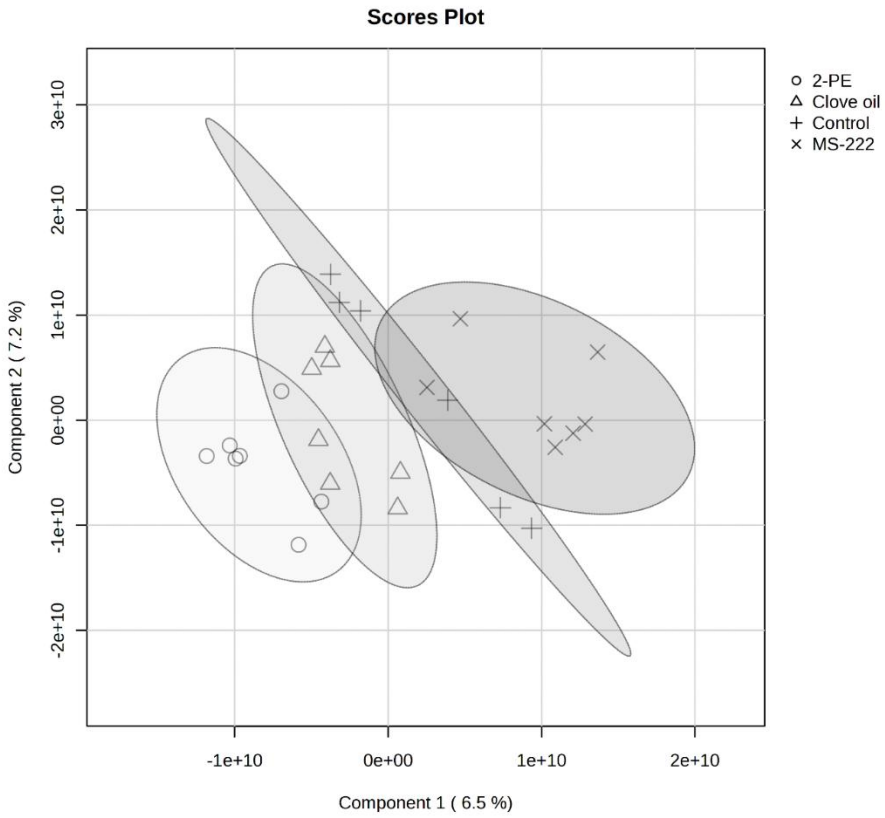


**Figure 4-9: Partial Least Squares-Discriminant Analysis scores plot between the principal components 1 and 2 (PCs) of zebrafish metabolite concentrations from the control group and the three anaesthetic groups (600 mg/L 2-phenoxyethanol (2-PE); 100 mg/L clove oil; and 180 mg/L MS-222). The explained variances are shown in brackets. The data points represent each individual fish sampled, while ellipses describe 95% confidence regions for each group.**

### *4.3.3 Independent samples t-test and effect size*

**Table 4-2: Compounds significantly different to the control compounds determined by independent samples t-test and effect size (Glass's *delta*) in clove oil treated zebrafish. The arrows indicate that the concentrations of compounds in the anaesthetic treatment were greater (↑) or lower (↓) in relation to the control.**

| **Compounds** | ***t*-statistic** | ***p*-value** | **Glass's *delta*** |
|---|---|---|---|
| Gluconic acid | -3.1342 | 0.0095 | -1.242 ↓ |
| Gluconic acid, lactone | 2.4174 | 0.0342 | 1.519 ↑ |
| Urea | 2.302 | 0.0419 | 6.197 ↑ |
| Analyte 269 | -2.2578 | 0.0453 | -0.962 ↓ |

Table 4-2 shows that four metabolites identified from the clove oil treated zebrafish group were found to be statistically significantly different from the control group. The metabolites that were both statistically significant and had an effect size ≥ |0.8| were: Gluconic acid; Gluconic acid, lactone; Urea; and Analyte 269.

**Table 4-3: Compounds significantly different to the control compounds determined by independent samples t-test and effect size (Glass's *delta*) in MS-222 treated zebrafish. The arrows indicate that the concentrations of compounds in the anaesthetic treatment were greater (↑) or lower (↓) in relation to the control.**

| Compounds | *t*-statistic | *p*-value | Glass's *delta* |
|---|---|---|---|
| L-Threonine | -3.5512 | 0.0045 | 3.523 ↑ |
| D-Galactose | -3.4566 | 0.0054 | 6.186 ↑ |
| Gluconic acid | 3.2955 | 0.0071 | -1.279 ↓ |
| 1,2-Propanediol | 3.2433 | 0.0078 | -1.375 ↓ |
| L-Isoleucine | 3.2382 | 0.0079 | -1.699 ↓ |
| Urea | -3.2336 | 0.0080 | 6.377 ↑ |
| N-Acetyl-Lysine | 3.161 | 0.0091 | -1.411 ↓ |
| Citrulline | 2.8181 | 0.0167 | -1.063 ↓ |
| Analyte 295 | -2.8171 | 0.0168 | 7.542 ↑ |
| 3-Hydroxyisobutyric acid | 2.5679 | 0.0261 | -1.332 ↓ |
| Pyrimidine | 2.5524 | 0.0269 | -1.142 ↓ |
| Glutamine | 2.5376 | 0.0276 | -0.963 ↓ |
| Mannonic acid, lactone | -2.4622 | 0.0316 | 1.191 ↑ |
| Creatinine | -2.4455 | 0.0325 | 6.156 ↑ |
| Analyte 312 | -2.4159 | 0.0342 | -6.483 ↓ |
| Uridine | 2.3973 | 0.0354 | -1.866 ↓ |
| Glutamic acid | 2.3779 | 0.0366 | -1.005 ↓ |
| Methionine | -2.3756 | 0.0368 | 13.104 ↑ |
| Analyte 33 | 2.3547 | 0.0382 | -1.227 ↓ |
| 2-Aminobutyric acid | 2.2722 | 0.0441 | -0.960 ↓ |
| Pantothenic acid | -2.2433 | 0.0464 | 6.751 ↑ |
| Glycoside | -2.2381 | 0.0469 | 4.823 ↑ |
| Myristic acid | -2.2185 | 0.0485 | 0.913 ↑ |

Table 4-3 shows that 23 metabolites identified from the MS-222 treated group were found to be statistically significantly different from the control group. The metabolites that were both statistically significant and had an effect size ≥ |0.8| were: L-threonine; D-galactose; gluconic acid; 1,2-propanediol; L-isoleucine; urea; N-acetyl-lysine; citrulline; analyte 295; 3-hydroxyisobutyric acid; pyrimidine;

glutamine; mannonic acid, lactone; creatinine; analyte 312; uridine; glutamic acid; methionine; analyte 33; 2-aminobutyric acid; pantothenic acid; glycoside; and myristic acid. The most metabolites found to be significant were identified in the MS-222 treated group.

**Table 4-4: Compounds significantly different to the control compounds determined by independent samples t-test and effect size (Glass's *delta*) in 2-PE treated zebrafish. The arrows indicate that the concentrations of compounds in the anaesthetic treatment were greater (↑) or lower (↓) in relation to the control.**

| Compounds | *t*-statistic | *p*.value | Glass's *delta* |
|---|---|---|---|
| Analyte 45 | -3.096 | 0.0102 | -1.1687 ↓ |
| Oleic acid | 2.9766 | 0.0126 | 1.1956 ↑ |
| Mannonic acid, lactone | 2.9485 | 0.0133 | 1.2940 ↑ |
| L-Aspartic acid | -2.9439 | 0.0134 | -1.1645 ↓ |
| Glutamic acid | 2.8521 | 0.0158 | -1.1209 ↓ |
| Pantothenic acid | 2.812 | 0.0169 | 7.2287 ↑ |
| Gluconic acid | -2.6848 | 0.0212 | -1.0922 ↓ |
| Analyte 312 | 2.6497 | 0.0226 | 1.0292 ↑ |
| Gluconic acid, lactone | 2.4922 | 0.0300 | 1.2273 ↑ |
| Eicosapentaenoic acid | -2.4209 | 0.0340 | -1.6034 ↓ |
| L-Alanine | -2.4185 | 0.0341 | -0.9599 ↓ |
| Glycoside | 2.3975 | 0.0354 | 1.7729 ↑ |
| D-(+)-Xylose | -2.3382 | 0.0393 | -0.9144 ↓ |
| N-Acetyl-Lysine | -2.313 | 0.0411 | -0.9258 ↓ |
| Phenylalanine | 2.3008 | 0.0420 | 1.3537 ↑ |
| Phosphate | -2.2409 | 0.0467 | -0.8588 ↓ |
| Octanoylcarnitine | 2.2132 | 0.0490 | 3.2583 ↑ |

Table 4-4 shows that 17 metabolites identified from the 2-PE treated group were found to be statistically significantly different from the control group. The metabolites that were both statistically significant and had an effect ≥ |0.8| were: Analyte 45; Oleic acid; Mannonic acid, lactone; L-Aspartic acid; Glutamic acid; Pantothenic acid; Gluconic acid; Analyte 312; Gluconic acid, lactone; Eicosapentaenoic acid; L-Alanine; Glycoside; D-(+)-Xylose; N-Acetyl-Lysine; Phenylalanine; Phosphate; and Octanoylcarnitine.

### *4.3.4 Venn diagram*

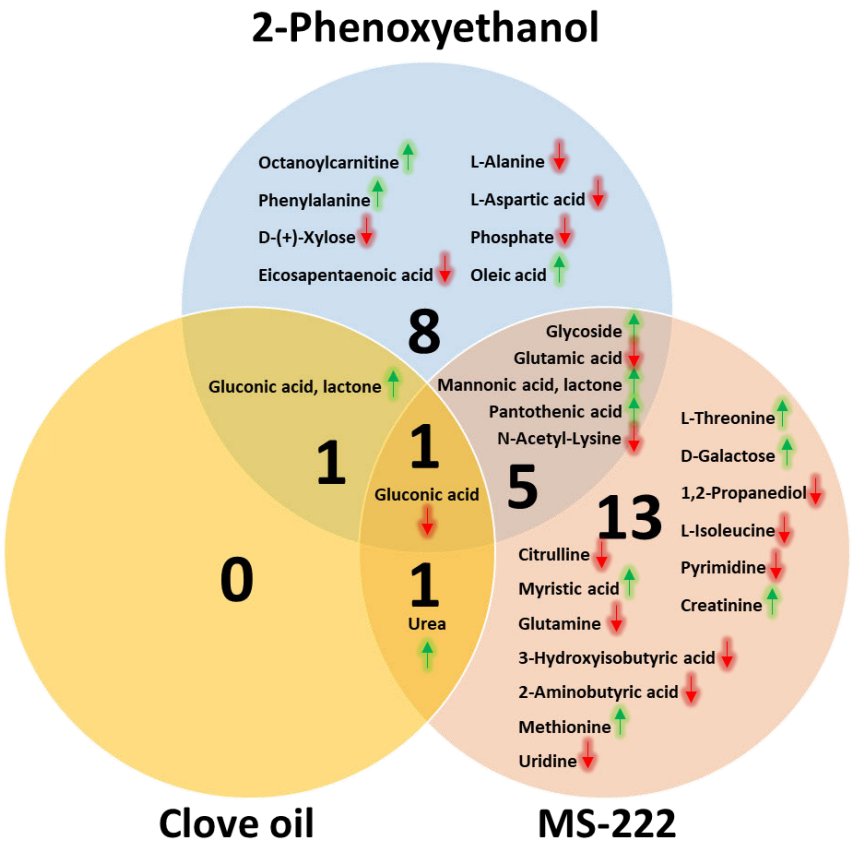


**Figure 4-9: Venn diagram of significant metabolites unique and common to each anaesthetic. The arrows indicate that the concentrations of compounds in the anaesthetic treatment were greater (↑) or lower (↓) in relation to the control.**

The Venn diagram (Figure 4-9) reveals that gluconic acid was the only metabolite significantly altered by all three anaesthetics. Gluconic acid lactone was the only metabolite significantly altered by both clove oil and 2-PE. Urea was the only metabolite significantly altered by both clove oil and MS-222. The metabolites glutamic acid, N-acetyl-lysine, pantothenic acid, mannonic acid lactone, and glycoside were significantly altered by 2-PE and MS-222. Metabolites uniquely altered by MS-222 include creatinine, 2-aminobutyric acid, methionine, glutamine, L-threonine, citrulline, 3-hydroxyisobutyric acid, L-isoleucine, d-galactose, 1,2-propanediol, myristic acid, pyrimidine, and uridine. Metabolites uniquely altered by 2-PE include, octanoylcarnitine, L-alanine, L-aspartic acid, phenylalanine, D-(+)-xylose, phosphate, eicosapentaenoic acid, and oleic acid.

### *4.3.5 Metabolite pathway analysis*

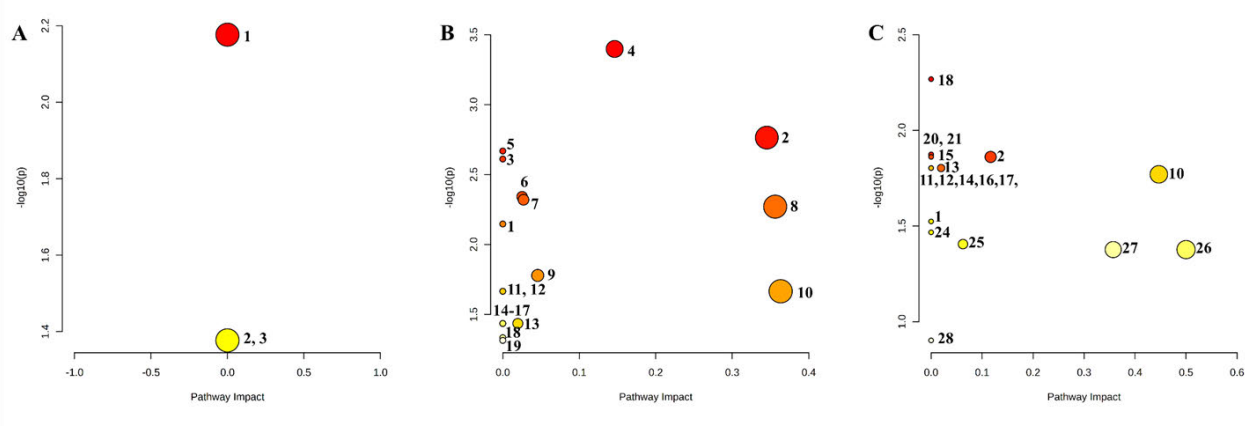


**Figure 4-10: Metabolome view of the outcome of the quantitative metabolite pathway analyses for A) clove oil, B) MS-222, and C) 2-PE exposed zebrafish. The size of each circle represents the pathway impact value, and the colour represents -log (p) value. The deeper the red colour of the node, the greater its l-log (p) value. Each pathway is labelled with a number as follows: 1) Pentose phosphate pathway; 2) Arginine biosynthesis; 3) Purine metabolism; 4) Cysteine and methionine metabolism; 5) Valine, leucine and isoleucine biosynthesis; 6) Glycine, serine and threonine metabolism; 7) Valine, leucine and isoleucine degradation; 8) Galactose metabolism; 9) Pyrimidine metabolism; 10) Alanine, aspartate and glutamate metabolism; 11) Glyoxylate and dicarboxylate metabolism; 12) Nitrogen metabolism; 13) Glutathione metabolism; 14) Arginine and proline metabolism; 15) Histidine metabolism; 16) Butanoate metabolism; 17) Porphyrin metabolism; 18) Pantothenate and CoA biosynthesis; 19) Fatty acid biosynthesis; 20) beta-Alanine metabolism; 21) Nicotinate and nicotinamide metabolism; 22) Arginine and proline metabolism; 23) Porphyrin metabolism; 24) Selenocompound metabolism; 25) Pentose and glucuronate interconversions; 26) Phenylalanine, tyrosine and tryptophan biosynthesis; 27) Phenylalanine metabolism; 28) Biosynthesis of unsaturated fatty acids.**

The quantitative metabolite pathway analysis linked the statistically significant metabolites from the clove oil exposed group to three metabolic pathways (Figure 4-10A) . Overall, the clove oil exposure showed to affect the smallest number of metabolic pathways in the zebrafish.

The outcome of the quantitative metabolite pathway analysis linked the statistically significant metabolites from the MS-222 exposed group to 19 metabolic pathways (Figure 4-10B). The most impacted pathways were cysteine and methionine metabolism; arginine biosynthesis; glycine, serine and threonine metabolism; valine, leucine and isoleucine degradation; galactose metabolism; pyrimidine metabolism; alanine, aspartate and glutamate metabolism; arginine and proline metabolism; and glutathione metabolism. Overall, the MS-222 exposure affected more metabolic pathways than the other anaesthetics.

The outcome of the quantitative metabolite pathway analysis linked the statistically significant metabolites from the 2-PE exposed group to 18 metabolic pathways (Figure 4-10C). The most impacted pathways were the phenylalanine, tyrosine and tryptophan biosynthesis; alanine, aspartate and glutamate metabolism; phenylalanine metabolism; arginine biosynthesis; pentose phosphate pathway; and glutathione metabolism.

# CHAPTER 5: DISCUSSION

# 5. DISCUSSION

## 5.1 Phenotypic observations

### *5.1.1 Onset of anaesthesia*

In contrast to MS-222 and clove oil, very little data exists on the use of 2-PE on zebrafish. While MS-222 is currently the only chemical anaesthetic permitted by the United States of America's Food and Drug Administration (FDA) for the use in fish intended for human consumption (Grush *et al*., 2004; Priborsky and Velisek, 2018), clove oil is readily available to veterinarians and commercially available for consumers of the pet trade, research, and dental use (Grush *et al*., 2004; Neiffer and Stamper, 2009) However, eugenol, the main constituent of clove oil, is approved for use as a food fish anaesthetic in South Africa, New Zealand, Japan and some south (Ke *et al*., 2021; MPI, 2022). Given their frequent use and availability of research data, both MS-222 and clove oil serve as suitable reference compounds for comparison with 2-PE in this study. The results demonstrate that appropriate concentrations of clove oil, MS-222, and 2-PE can successfully anesthetise zebrafish.

The observed progressions through the various stages of anaesthesia with MS-222 and clove oil were consistent with the descriptions for onset and recovery states from chemical anaesthesia (Grush *et al*., 2004; Martins *et al*., 2016). In accordance with the findings of Grush *et al*. (2004), anaesthesia was achieved more quickly at lower concentrations of clove oil than MS-222 in zebrafish. Similarly, a study showed that zebrafish exposed to clove oil lost equilibrium faster than propofol-lidocaine and MS-222 exposed zebrafish, indicative of clove oil's faster induction (Jorge *et al*., 2021). The current study also found that anaesthesia was achieved more rapidly at a lower dosage of clove oil and MS-222 than for 2-PE in zebrafish. The tested concentration range required to induce all fish to stage V anaesthesia reliably/consistently was 30-100 mg/L and 75-180 mg/L for clove oil and MS-222, respectively. However, the range for fish to consistently achieve stage V anaesthesia was 600-700 mg/L for 2-PE.

Like the findings of Grush *et al*. (2004), the time to reach stage IV and stage V anaesthesia decreased with increasing concentration for all anaesthetics. However, for clove oil and MS-222, reports indicate that beyond a certain threshold concentration, further increases did not significantly enhance anaesthetic effects and suggested that clove oil, which showed a significantly higher dose-response, had a wider safety margin than MS-222 in zebrafish (Hikasa *et al*., 1986; Keene *et al*., 1998; Grush *et al*., 2004). This plateau effect was not observed for 2-PE, which continued to show a concentration-dependent response at higher doses, particularly for achieving stage V anaesthesia; however, this may be due to fewer concentrations tested that induced stage IV and V anaesthesia. Additionally, the response of all three anaesthetics in the current study was consistent with the response seen in several other studies using the same anaesthetic agents on *Oreochromis niloticus*, *Carassius auratus*, wild *Alosa mediocris*, *Siganus rivulatus*, and *Solea senegalensis* where anaesthesia induction time decreases as the anaesthetic

dose increases because the drug reaches the threshold concentration, i.e. minimum effective concentration, sooner (Tengjaroenkul *et al*., 2004; Charoendat *et al*., 2009; Weber *et al*., 2009; Ghanawi *et al*., 2013; Misawa *et al*., 2014; Matsche, 2017; Rairat *et al*., 2021). The current study did not determine the effective dose / lethal dose of zebrafish response to each anaesthetic: therefore, it cannot comment on the safety margin of each anaesthetic used in this study. However, a study comparing the anaesthesia overdose and rapid cooling for euthanasia of zebrafish euthanised zebrafish using 250 mg/L MS-222 and 50mg/L clove oil. After cessation of opercular movements of the fish needing 11 min 19s and 13 min 15s for MS-222 and clove oil, respectively, the fish were left immersed in the anaesthetic solution for 2 min till death (Ferreira *et al*., 2022). Therefore, it is possible for the safety margin for these anaesthetics to be deduced to lie within a range of the effective doses determined from the current study to euthanasia doses of the mentioned study. However, the current study used greater concentrations of clove oil (60 mg/L and 100 mg/L) without any mortalities. Although the duration of exposure to the anaesthetic was less, and fish moved to recovery after opercular movements slowed/ became irregular, it is possible that either of the two concentrations used may have euthanised the zebrafish from asphyxiation through cessation of opercular movements due to lack of artificial respiration. Therefore, clove oil may have a narrower safety margin than MS-222.

### *5.1.2 Anaesthesia recovery*

The findings from this study indicate that the time to recover fear response and equilibrium (full recovery) did not differ significantly among several anaesthetic treatments, particularly among the three concentrations of interest: 100 mg/L clove oil, 180 mg/L MS-222, and 600 mg/L 2-PE. Despite lower concentrations of these agents not producing the same effect, the comparable recovery times observed with high concentrations of clove oil (100 mg/L) and 2-PE (600 mg/L) compared to MS-222 may be due to shorter exposure durations resulting from faster induction at higher anaesthetic levels, which likely leads to reduced drug uptake and faster elimination (Rairat *et al*., 2021). These results contrast with those of Grush *et al*. (2004), who reported that recovery following MS-222 was consistently faster than that following clove oil. Reports may suggest that the eugenol in clove oil has more significant effects on the cardiac and respiratory system than does MS-222, which would result in reduced respiratory rate and heart rate (McFarland, 1959). Furthermore, Grush *et al*. (2004) suggest that these widespread effects may result in prolonged retention of clove oil in the blood circulation. It is not clear that the result of the current study supports that clove oil also has a more significant impact in reducing heart rate and respiratory rate and is retained in the bloodstream longer than 2-PE and MS-222. However, an effect of the respiratory rate may explain the gulping behaviour described in sections 4.1.2.2 and 4.1.2.3 exhibited by many of the fish recovering from clove oil, suggesting that they were gasping for air during recovery like the results of Ehrlich *et al*. (2019), whose results similarly showed no significant difference with the recovery of zebrafish from anaesthesia to clove oil, AQUI-S, and MS-222. However,

a study on the efficacy and behavioural recovery of different anaesthetics in adult zebrafish found that the high opercular rate observed in animals treated with 175 mg/L MS-222 may have promoted a more rapid recovery rate. The study suggested that the high opercular rate allowed for a faster metabolisation and subsequent elimination of the anaesthetic via the gills. Therefore, the rapid recovery from MS-222 anaesthesia was likely due to its action as a local anaesthetic and ability to stimulate the respiratory and cardiovascular system (Jorge *et al*., 2021). The study's reasoning for why a faster opercular rate did not aid in reducing the recovery for 45 mg/L clove oil anaesthetised zebrafish was due to clove oil being described to coat gill epithelium making anaesthesia elimination difficult, therefore prolonging recovery (Sladky *et al*., 2001; Jorge *et al*., 2021).

This study found that the recovery of all the tested concentrations (50-180 mg/L) of MS-222 were not significantly different from one another. Reports confirm this and found that the recovery from MS-222 in the lower dosages tested (100-150 mg/L) was not significantly different; however, they were significantly different from recovery when fish are anaesthetised with 200 mg/L (Grush *et al*., 2004; Chen *et al*., 2014). Like the duration for reaching full recovery from MS-222, the total time for the full recovery of the tested 2-PE concentrations were not significantly different from one another, except for the recovery from 100 and 500 mg/L. However, the onset of anaesthesia of fish exposed to 100 mg/L only induced light sedation in all the fish and the excitation phase in half of the fish. Two of the nine fish exposed to 500 mg/L entered stage V anaesthesia and had much longer recovery times to the rest, resulting in a large standard deviation.

## 5.2 Metabolomics

The goal of this dissertation was to investigate the metabolic response of zebrafish in response to three anaesthetics: clove oil, MS-222, and 2-PE. It also aimed to link the metabolic response (physiological) to the phenotypic response of the zebrafish to the anaesthetic. It presents evidence of the anaesthetics impacts on metabolic pathways by identifying differences in the metabolite concentrations of the anaesthetised fish in comparison to the un-anaesthetised fish. The anaesthetic MS-222 affected more metabolic pathways than clove oil and 2-PE. The largest group of metabolites significantly different from the control were from amino acid metabolism. The three anaesthetics affected two of the same metabolic pathways, the arginine biosynthesis, and the pentose phosphate pathway. However, the anaesthetics affected different metabolites linked to those two pathways.

To determine if the anaesthetics had any adverse physiological effect, this study used the metabolomics analyses to identify metabolites linked to stress response metabolic pathways. To recap, the HPI axis stimulates the secretion and circulation of numerous stress hormones depending on the type of stress encountered by the organism. However, it typically initiates the circulation of the steroid hormones catecholamine in chromaffin tissue and cortisol, the major glucocorticoid in fish (Barton, 2002; Cao *et al*., 2017). Catecholamines function to adjust respiratory and cardiovascular function to

maintain sufficient blood oxygen levels and, consequently, an adequate supply to the tissues and additionally to help release energy reserves to meet the higher energy needs that occur frequently with stress (Reid *et al*., 1998). Circulating cortisol initiates the relocation of energy from metabolic activities through the metabolism of glucose via glycogenolysis (Barton, 2002). Therefore, the identification of metabolites linked to energy metabolism may indicate the activation of a stress response due to the anaesthetics.

### *5.2.1 Metabolic consequence of Clove oil*

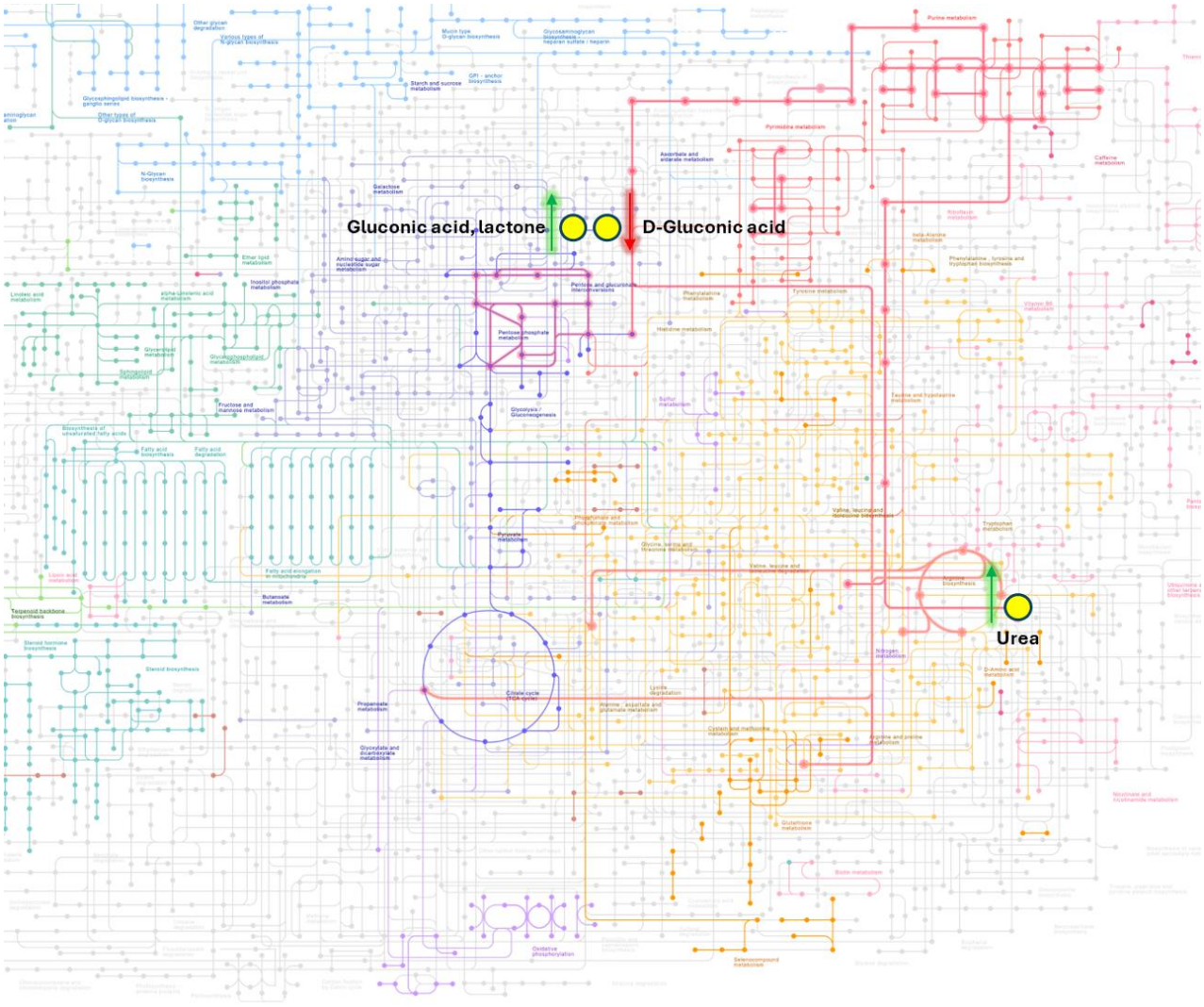


**Figure 5-1: A KEGG metabolic pathway map showing significant metabolites quantified from whole body tissue from clove oil treated zebrafish. Each metabolite is shown with their respective fold change regulation (Red arrow: down-regulation and Green: up-regulation) in response to the anaesthetic in zebrafish. Pathways that were identified by the metabolite set and metabolic pathway analyses that were significantly represented were highlighted in pink.**

An alteration common to the three anaesthetics was the decrease in gluconic acid. The production of D-gluconic acid occurs from the oxidation of the carbonyl (aldehyde) carbon of glucose to the carboxyl level (Nelson and Cox, 2021). Gluconic acid is produced through the process of oxidation of α-D-glucose. Alpha-D-glucose is oxidised to β-D-glucose through spontaneous reaction or catalysed by mutarotase (Ramachandran *et al*., 2006). Glucose oxidase or glucose dehydrogenase catalyses the

oxidation of β-D-glucose to form D-glucano-δ-lactone (gluconic acid lactone), which can then be further catalysed by D-glucano-δ-lactonase or by spontaneous reaction to form D-gluconic acid (Ramachandran *et al*., 2006). D-Gluconic acid can then be further catalysed to form the other glucose derivatives in the gluconate pathways (Ramachandran *et al*., 2006). The results revealed that gluconic acid was found in lower concentrations in the experimental group than in the control group, while gluconic acid lactone was found to be greater in the experimental group than it was in the control group (Figure 5-1). This may suggest that the induction of the anaesthetics disrupted the conversion of gluconic acid lactone to D-gluconic acid or, instead, gluconic acid lactone was diverted to form β-D-glucose, a process catalysed by glucose dehydrogenase (Kanehisa and Goto, 2000; Kanehisa *et al*., 2021). Glucose is a major metabolic fuel for vertebrate heart functioning (Driedzic, 1992) and a secondary stress response metabolite (Barton, 2002); however, it was not identified as a significant metabolite by the metabolomics analyses. Urea, a by-product of the breakdown of amino acids, was significantly altered by clove oil. However, no other amino acids were significantly altered by clove oil in zebrafish.

In contrast, after achieving surgical anaesthesia, the use of 150 mg/L clove oil in rainbow trout revealed an increase in branched chain amino acids linked to pathways upregulated to meet increased energy demands, as well as an increase in glucose and galactose, in rainbow trout due to the circulation of cortisol and catecholamines in the HPI axis (Rahimi *et al*., 2020). However, a comparison of the response of black sea bass (*Centropristis striata*) to clove oil, metomidate (5 mg/L), MS-222 (125 mg/L), and 2-PE (300 mg/L) revealed that the use of clove oil, metomidate, and 2PE anaesthesia did not significantly increase cortisol levels after 30 minutes of exposure (King *et al*., 2005). This finding is consistent with the findings of the current study, which found that clove oil had the least metabolomic impact on zebrafish when compared to MS-222 and 2-PE, as well as fewer metabolites associated with a stress response. The responses of rainbow trout and black sea bass to clove oil suggest that the physiological effect of clove oil varies by species, with zebrafish responding very differently than rainbow trout, though the concentration used on rainbow was greater than what was used on zebrafish in this study, the plane of anaesthesia and the time it took to reach that stage (60-90s) was however similar (Keene *et al*., 1998). Induction time for black sea bass anaesthetised with clove oil 40 mg/L was not reported (King *et al*., 2005). However, such interspecies differences may also be influenced by other physiological and environmental factors, including water temperature and fish size. (Zahl *et al*., 2009) demonstrated that anaesthesia induction and recovery times were significantly influenced by water temperature and body weight, with smaller fish reaching anaesthesia more quickly. These findings support the likelihood that the variability in response times across species may not solely reflect pharmacological differences but also differences in experimental conditions and animal physiology.

### *5.2.2 Metabolic consequences of MS-222*

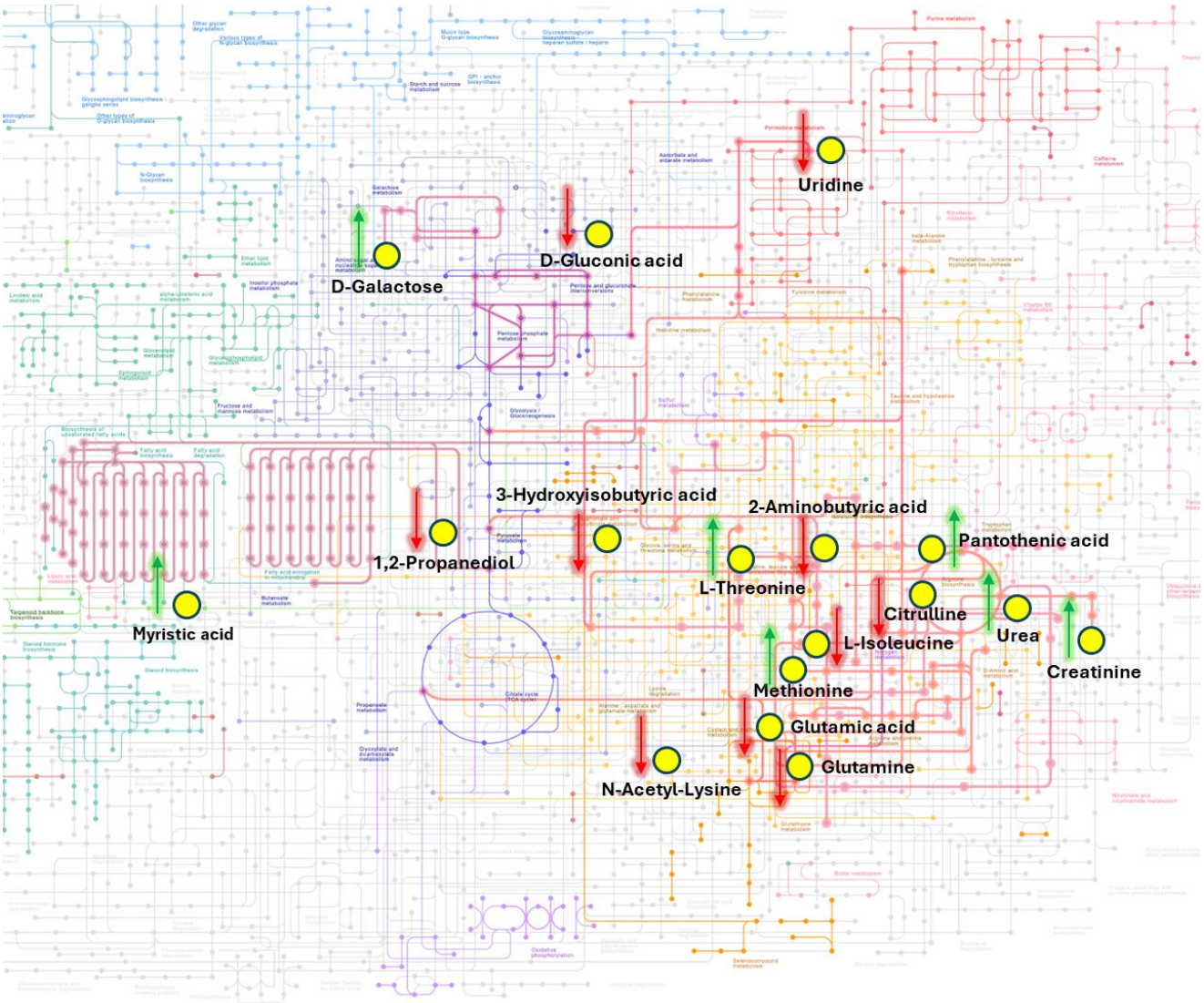


**Figure 5-2: AKEGG metabolic pathway map showing significant metabolites quantified from whole body tissue from MS-222 treated zebrafish. Each metabolite is shown with their respective fold change regulation (Red arrow: down-regulation and Green: up-regulation) in response to the anaesthetic in zebrafish. Pathways that were identified by the metabolite set and metabolic pathway analyses that were significantly represented were highlighted in pink.**

The primary function of intermediate metabolism is to sustain adenosine triphosphate (ATP)for cell growth, reproduction, and stress responses (Salway, 2017). Basic metabolism is dominated by anabolic activity, which synthesises macromolecules and stimulates cell division, repair, and growth

(Venter *et al*., 2018a). However, during a response to stress, catabolic processes can be favoured to guarantee that energy is available for processes involved in the maintenance of cellular homeostasis, recovery, and survival (Venter *et al*., 2018a). In response to MS-222, 13 out of the 21 significant metabolites determined by the metabolomics analyses have at least one or more functional role in energy metabolism, the redistribution of metabolic energy, or were altered in response to the processes involved in energy metabolism or the redistribution of metabolic energy.

The metabolite set enrichment analysis (Figure 5-2) illustrates the significant perturbation of 22 metabolic networks including but not limited to the aminoacyl-tRNA biosynthesis; cysteine and methionine metabolism; arginine biosynthesis; valine, leucine, and isoleucine biosynthesis; purine metabolism; glycine, serine, and threonine metabolism; and valine, leucine, and isoleucine degradation. The above-mentioned pathways involve glucogenic amino acids (methionine and glutamine) and amino acids that can be both glucogenic and ketogenic (isoleucine and threonine) were significantly altered. Additionally, amino acids that are intermediates to glucogenic amino acids were also significantly altered due to the anaesthetic. Acetoacetate or acetyl CoA are produced by ketogenic amino acids. Oxaloacetate, succinyl CoA, α-ketoglutarate, fumarate, or pyruvate are products of glucogenic amino acids (Litwack, 2017). The following paragraphs explains in greater detail of the response of the glucogenic amino acids resultant from response to MS-222.

The elevation of glucogenic/ketogenic and essential amino acids threonine and methionine indicates that a catabolic process occurred to cause the elevation since essential amino acids cannot be synthesised in the body (Nelson and Cox, 2021). Both L-methionine and L-threonine enter the tricarboxylic acid (TCA) cycle and can be degraded to succinyl CoA, which can be further metabolised to pyruvate for energy metabolism (Salway, 2017). Methionine is a glucogenic and essential amino acid needed for growth and tissue repair in humans and an antioxidant that aids in fat decomposition and the removal of hazardous substances such as heavy metals and serves as a lipotropic agent, preventing the accumulation of excess fat in the liver (Junjie and Shengjie, 2019). L-Threonine, an essential amino acid used in protein synthesis, is a precursor of glycine and functions as fuel for energy metabolism. Most is used for energy production and a large proportion is metabolised to form glycine and acetyl-CoA via the threonine cleavage complex (Kohlmeier, 2015). Another glucogenic amino acid, glutamine, decreased in response to MS-222. In the TCA cycle, it can be metabolised to form α-ketoglutarate, which is then converted to succinyl CoA. Glutamine, like glucose, supplies energy to immune cells by acting as a carrier of $NH_3$ through glutamine degradation that leads to the formation of $NH_3$ and aspartate leading to the synthesis of purines and pyrimidines of the DNA and RNA (Cruzat *et al*., 2018).

Isoleucine was the only essential amino acid that decreased in response to MS-222. L-isoleucine is important energy fuel especially in skeletal muscle (main oxidation site) and other areas responsible for a smaller percentage of utilising it as energy are liver intestines and other organs (Kohlmeier, 2015). In response to glycogen or fatty acid exhaustion, L-isoleucine and L-valine are catabolised to produce succinyl CoA, a TCA' cycle intermediate that maintains anaplerotic reactions to supplementary

oxaloacetate for acetyl CoA production necessary for ATP generation via oxidative phosphorylation in the primary respiratory chain (Salway, 2017). The metabolomics analyses did not identify L-valine as a significant metabolite; however, it identified 3-hydroxyisobutyric acid, an intermediate of the metabolism of L-valine, which was decreased in response to MS-222. In summary, the decrease in L-glutamine, L-isoleucine, and 3-hydroxyisobutyric acid suggests catabolic reactions occurred in response to MS-222 to release metabolic energy.

Even though the results show an upregulation of metabolites entering the TCA cycle, no TCA cycle intermediates were significantly altered. Neither was glucose significantly altered. However, α-D-Galactose, a glucose intermediate increased in response to MS-222. α-D-Galactose is used for the synthesis of glycoproteins and glycolipids and is used as energy fuel where it is metabolised to form glucose-1-phosphate and then glucose-6-phosphate in the liver (Kohlmeier, 2015). Several vitamins such as thiamine, lipoic acid, riboflavin, niacin, and pantothenic acid provide cofactors for the enzymes involved in pathways where glucose is conserved to ATP (Salway, 2017). Pantothenic acid increased in response to MS-222, further adding to the notion of the anaesthetics impact on metabolic energy redistribution, in this instance the upregulation of glycolysis.

The alteration of several other metabolites can also be explained due to catabolic processes triggered by the anaesthetic. Myristic acid, a beta-oxidation product was increased in the lipid metabolism in response to MS-222. Myristic acid is a saturated fatty acid containing a straight chain of 14 carbons, is a remarkably high energy containing metabolite in comparison to other energy nutrients (Kohlmeier, 2015). Oxidation supports the generation of approximately 92 ATP. It also functions as membrane anchor for some proteins especially those with signalling function by acylating them as a substrate (Kohlmeier, 2015).

Creatinine and urea increased in response to MS-222, both of which are breakdown products of pathways involved in energy production. Creatinine is the breakdown product of creatine phosphate, a phosphagen used to replenish ATP to ensure the energy needs for cell tissues and physiological processes within cells in zebrafish (Mushtaq *et al*., 2014; Yin *et al*., 2014). A study on *O. mykiss* found that creatinine levels in anaesthetised fish were elevated due to the anaesthetic MS-222, and intermediate metabolites dimethylglycine and creatine were the main metabolites that demonstrated higher plasma levels (Rahimi *et al*., 2020). Urea is the by-product of amino acid degradation where most animals will excrete ammonia as urea or uric acid, but some teleost fish will excrete ammonia directly from the gills to the environment (Nelson and Cox, 2021). Adult zebrafish, as most teleosts, are mostly ammoniotelic but can be partially or fully ammoniotelic depending on their habitat (Mathai, 2005). Teleosts such as eels have shown to increase plasma osmolality and urea concentration when experiencing severe osmotic stress (Mathai, 2005). Urea transporter mRNA expression in the gills is widely spread amongst teleosts (Mathai, 2005). Following a brief anaesthesia with essential oils from two different chemotypes of *Lippia alba*, an increase in plasma urea and plasma creatinine was observed in silver catfish, *Rhamdia quelen* (Souza *et al*., 2017). The increase in urea was first thought to be due to kidney lesions caused by

the anaesthetic clove oil after long exposure, as observed in goldfish (Gholipourkanani *et al*., 2015). The study discovered, however, that the increase in plasma urea concentrations was most likely due to shifts in thee excretion of creatinine and ammonia from the gills rather than renal lesions because the exposure to anaesthesia was brief (Souza *et al*., 2017). Thus, the upregulation of the urea cycle in silver catfish because of anaesthesia can be seen as a parallel to the upregulation of the urea cycle in zebrafish exposed to MS-222 and clove oil, as urea was also increased by clove oil exposure in zebrafish.

Citrulline is a key intermediate in biosynthesis of arginine, and in the urea cycle (Nelson and Cox, 2021). Citrulline is formed in the urea cycle when ornithine interacts with ammonia in the form of carbamoyl phosphate. Citrulline receives a second amino group from aspartate, resulting in arginine, the immediate precursor to urea. Arginase catalyses the hydrolysis of arginine into ornithine and urea; ornithine is replenished in each turn of the cycle. (Nelson and Cox, 2021). Therefore, the decrease of citrulline in the MS-222 group may suggest that it was converted into urea since urea increased in the pathway.

2-Aminobutyric acid is an alpha-amino acid derived from the catabolism of, serine, threonine and methionine (Haschke-Becher *et al*., 2016). L-Threonine and L-methionine had increased in response to MS-222, thus, suggesting the decrease of 2-aminobutyric acid in response to MS-222. This could suggest that the stage of the TCA cycle did not go past the formation of succinyl CoA. The changes in glucogenic/ketogenic amino acids indicate that the TCA cycle were being activated. However, intermediates of the TCA cycle were not significantly altered by the anaesthetic, implying that the anaesthetic had little effect on the TCA cycle. Alternatively, the increase in methionine levels in response to certain chemicals might reflect an attempt by the fish to boost its antioxidant defences or modulate its immune response by producing taurine and other methionine-derived metabolites, as shown by juvenile Nile tilapia fed a DL-methionine diet (Teodósio *et al*., 2022). Taurine aids fish in retaining amino acids and is essential for osmoregulation (Takagi *et al*., 2006), lipid digestion (Richard *et al*., 2017), antioxidant defence (Li *et al*., 2016; Martins *et al*., 2019), and bile salt synthesis (Kim *et al*., 2007; Kim *et al*., 2008). It has been demonstrated that feeding taurine supplements to Nile tilapia improves growth performance (Al-Feky *et al*., 2016; Teodósio *et al*., 2022). Similarly, the elevation of threonine may be indicative of an antioxidant response to stress from chemical exposure. Studies have shown that the metabolism in Zebrafish exposed to 10% and 100% sediment-derived water-soluble fraction of diluted bitumen (Fujita *et al*., 2021) and to PAHs benz[a]anthracene (BAA) or benz[a]anthracene-712-dione (BAQ) (Elie *et al*., 2015; Fujita *et al*., 2021) showed a significant increase in threonine, which may have disrupted the metabolism of glycine, serine, and threonine. The studies suggests that this alteration was related to the metabolism of glutathione which plays a role as an antioxidant that detoxifies reactive oxygen species and functions in phase II biotransformation of xenobiotic chemicals, as seen in zebrafish exposed to BAA or BAQ (Elie *et al*., 2015; Fujita *et al*., 2021). Additionally, zebrafish exposed to diluted bitumen and PAHs showed greater expression of genes involved in phase II biotransformation and responses to oxidative stress (Madison *et al*., 2015; Santana

*et al*., 2018; Fujita *et al*., 2021). Furthermore, threonine is essential for various biological functions such as cell signalling mediated by serine-threonine kinases (Miyazono *et al*., 1994). Therefore, an increase in threonine may impact signalling processes independently of protein-bound threonine (Fujita *et al*., 2021).

Alterations of metabolites that were not the result of catabolic processes due to anaesthesia are discussed in the following paragraphs. In the pyrimidine metabolism pathway pyrimidine and uridine decreased in response to MS-222. The pyrimidine ring forms through deriving bicarbonate, glutamine, and aspartate through a series of reactions catalysed by carbamoyl phosphate synthetase II (CPS II) (Salway, 2017) . The degradation of nucleic acids and nucleotides form the pyrimidine bases (Salway, 2017). With regards to uridine decrease, protein acetylation rises because of the production of acetyl-CoA and alanine by the catabolism of uridine. Uridine phosphorylase (UPase), which is produced by the UPP gene (EC=2.4.2.3), converts uridine to uracil. In vertebrates, UPase exists in two homologous forms: UPase1 and UPase2. Uridine homeostasis is regulated by UPase1, which is ubiquitously expressed and encoded by the UPP1 gene. Uridine levels in plasma and tissues are increased when UPase1's enzymatic activity is inhibited or UPase1 gene is knocked out. UPase2, a protein only found in the liver and is required for pyrimidine salvage processes, is encoded by the UPP2 gene. The liver is safeguarded against xenobiotic-induced lipid build-up by elevated endogenous uridine as a result of UPase2's enzymatic activity inhibition (Zhang *et al*., 2020).

Overall, the results may suggest that ATP was depleted to correct abolished mitochondrial membrane potential caused by anaesthesia, which could explain the upregulation of catabolism of lipids and proteins and the formation of glucose derivatives in response to MS-222 (Kishikawa *et al*., 2018).

### *5.2.3 MS-222 metabolic consequence has a phenotypic link*

Unlike clove oil and 2-PE, anaesthesia with MS-222 was the only instance in the study where fish displayed a prominent excitation phase in stage III anaesthesia. A stress response is likely to have occurred prior to the anaesthesia effect, particularly at stage III anaesthesia. The hyperactivity of the zebrafish exposed to MS-222 could indicate an adverse effect caused by the anaesthetic. Furthermore, an avoidance behaviour has been observed where a report suggests that the avoidance behaviour may have occurred not because of an anaesthetic effect but simply because the anaesthetic itself may have been unpleasant in smell or taste (Readman *et al*., 2013). Similar behaviour was also observed in juvenile tambaqui, *Colossoma macropomum* anaesthetised with *Acmella oleracea* (Barbas *et al*., 2016) and tambaqui anaesthetised with benzocaine (Gomes *et al*., 2001). The results of the current study suggest that the avoidance behaviour and hyperactivity exhibited by fish exposed to MS-222 may be more severe due to the presence of a stress response and not an unpleasant smell or taste, henceforth the upregulation of energy-generating metabolic pathways.

To support this, a study on *Oncorhynchus tshawytscha* found that glucose and triglycerides were elevated in anaesthetised fish due to an initial phase before achieving anaesthesia that exhibits excitement (hypertaxia & tachyventilation) and agitation in response to the depression of inhibitory neurons (Young *et al*., 2019). The metabolomic profile determined from the current study did not reveal glucose or triglycerides, however, glucose derivatives were present. However, the current study cannot determine how much of the metabolic profile was due to the stage III anaesthesia, or stage IV anaesthesia since sampling was only done at stage IV therefore a direct comparison could not be made. This raises the question as to how much of the stress response was due to the agitation phase in stage III in response to depression of inhibitory neurons before achieving anaesthesia, or stage IV and deeper anaesthesia and if or how soon after would the stress response dissipate with longer exposure to anaesthesia.

More evidence demonstrates how the adverse phenotypic response of MS-222 anaesthesia is linked to adverse physiological consequences in fish. Studies show that MS-222 itself results in a stress response of increasing cortisol levels in rainbow trout even in the absence of physical stimuli for the fish (Strange and Schreck, 1978; Small, 2003; Wagner *et al*., 2003; Davies and Buckley, 2011; Topic Popovic *et al*., 2012), and a stress response similar in fish that were subject to handling exposure without an anaesthetic (Small, 2003; Palić *et al*., 2006; Topic Popovic *et al*., 2012). Because MS-222 has extremely distinct chemical characteristics, the delayed induction of stage I anaesthesia upon exposure to MS-222 may allow the fish enough time to recognise the anaesthetic agent (Topic Popovic *et al*., 2012). It can irritate the skin and be detected through taste and smell (Topic Popovic *et al*., 2012; Readman *et al*., 2017). Additionally, once the anaesthetic begins to act, a stress response might also be triggered by a loss of balance. The amount of time required to begin inducing anaesthesia is crucial. For mammals, to calm a patient and lessen any tension that the anaesthetic or the anaesthetic treatment may cause, anaesthesia is frequently preceded in both human and veterinary medicine by the administration of a sedative (EFSA, 2009; Zahl *et al*., 2009; Zahl *et al*., 2010; Topic Popovic *et al*., 2012). For fish, to lessen the stress response, several fish species, including salmon, have successfully undergone pre-anaesthesia sedation using low dosages of metomidate or 2-phenoxyethanol as pre-anaesthetics (EFSA, 2009; Zahl *et al*., 2009; Zahl *et al*., 2010; Topic Popovic *et al*., 2012). As a result, the anaesthetic MS-222 may not be a suitable candidate for use as an anaesthetic agent on its own. An example of a pre-anaesthetic method used in zebrafish involves administering low doses of isoflurane combined with MS-222, which has been shown to prolong the duration of safe anaesthesia (i.e., without causing mortality) and shorten recovery time in adult zebrafish, with minimal impact on heart rate (Huang *et al*., 2010; Owen and Kelsh, 2021).

### *5.2.4 Metabolic consequence of 2-PE*

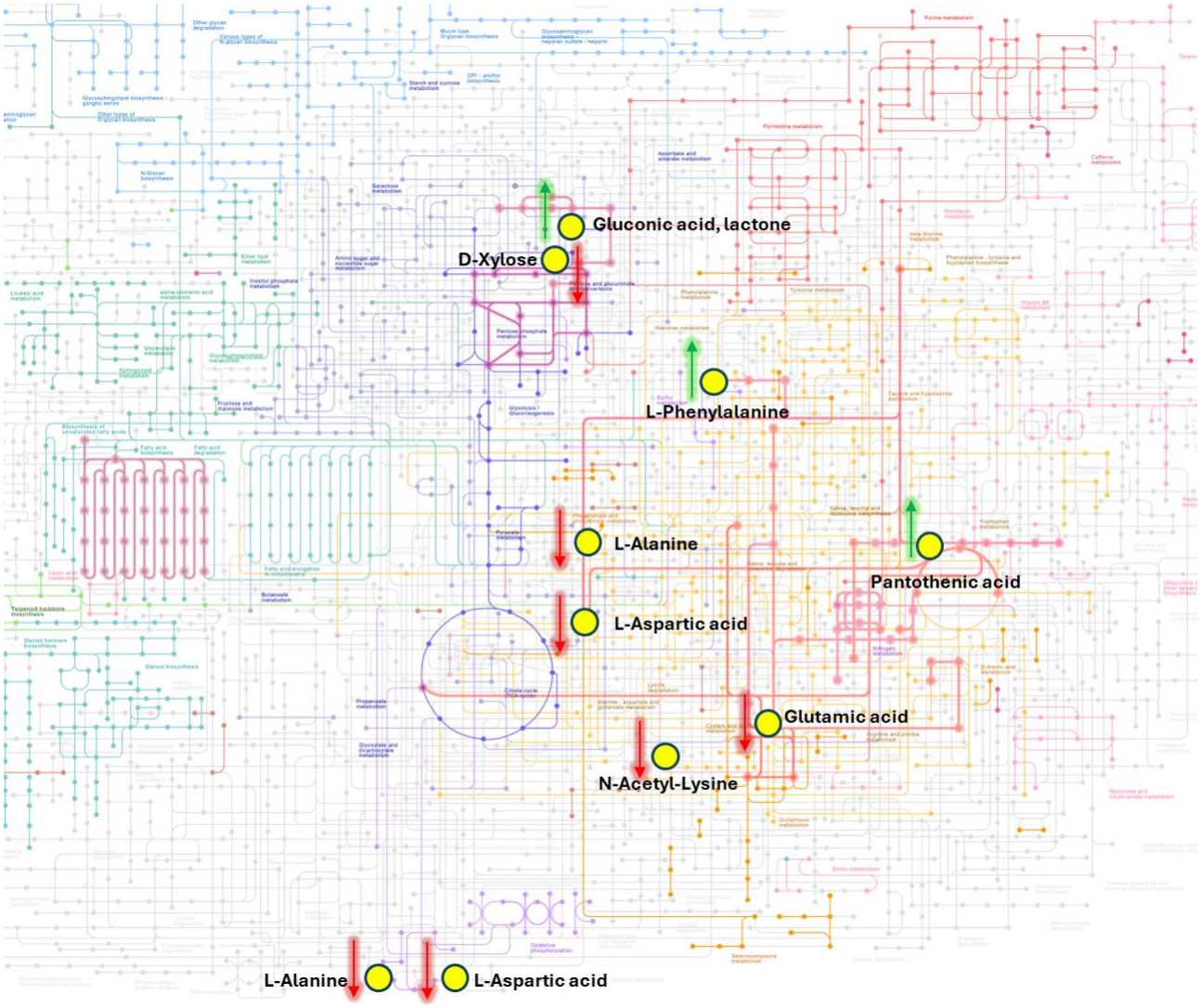


**Figure 5-3: A KEGG metabolic pathway map showing significant metabolites quantified from whole body tissue from 2-PE treated zebrafish. Each metabolite is shown with their respective fold change regulation (Red arrow: down-regulation and Green: up-regulation) in response to the anaesthetic in zebrafish. Pathways that were identified by the metabolite set and metabolic pathway analyses that were significantly represented were highlighted in pink.**

The anaesthetic 2-PE affected two metabolites directly linked to catecholamine release by upregulating phenylalanine and downregulating alanine. Phenylalanine is an essential amino acid used in the synthesis of proteins, melanin, and catecholamines and an important precursor to the amino acid L-tyrosine (Kohlmeier, 2015). It is used as fuel in energy metabolism where it is eventually broken down and oxidised to carbon dioxide, water, and urea (Kohlmeier, 2015). An increase in phenylalanine

levels indicates the dysfunction of the phenylalanine, tyrosine, and tryptophan biosynthesis pathway, as shown in the metabolite set enrichment analysis (Figure 5-3). As a precursor to the catecholamine neurotransmitters dopamine, norepinephrine, and adrenaline, phenylalanine may account for the reported neurobehavioral effects of the anaesthetic and its link to suppressing neuro-signalling (Elie *et al*., 2015). The reaction of the heart and peripheral nervous system to catecholamines in the blood have both been shown to be modulated by alanine (Butta and Adler-Graschinsky, 1987). Skeletal muscles and erythrocytes both synthesise alanine, an essential for the process of gluconeogenesis (Mushtaq *et al*., 2014). Any variation in the body's free alanine concentrations may be a sign of its involvement in the gluconeogenesis pathway when under stress (Mushtaq *et al*., 2014). Unlike in great sturgeon, *Huso huso* (Shaluei *et al*., 2012) and Senegalese sole (Weber *et al*., 2009), 2-PE did not significantly elevate glucose in zebrafish despite showing signs of catecholamine release.

β-oxidation processes were upregulated in fish exposed to 2-PE. This is indicated by the elevation of β-oxidation products oleic acid and octanoyl carnitine. Oleic acid, which was upregulated in the biosynthesis of unsaturated fatty acids pathway, is a monounsaturated omega 9 fatty acid and a major fatty acid in the membrane of nerves. It is abundant in freshwater fish and can be broken down for energy (Brody, 1998). Octanoyl carnitine increased and is a carnitine conjugate excreted in the urine in medium-chain acyl-CoA dehydrogenase MCAD deficiency in humans (Salway, 2017). It is formed when octanoyl-CoA reacts with L-Carnitine resulting in CoA and L-octanoylcarnitine catalysed by carnitine O-octanoyltransferase (Kanehisa and Goto, 2000; Kanehisa *et al*., 2021). In this process, octanoyl-CoA is oxidised for the formation of acetyl CoA hence, the octanoyl carnitine is the by-product of the formation of acetly-CoA (Grevengoed *et al*., 2014). Eicosapentaenoic acid, a substrate of β-oxidation and an omega 3 fatty acid which function as the body’s main energy fuel and can be extracted from freshwater fish (Ashfaq *et al*., 2020), had decreased in response to 2-PE possibly due to the metabolism of the β-oxidation to release metabolic energy during stress (Bai *et al*., 2021).

The metabolic response to the anaesthetic 2-PE altered metabolites linked to energy metabolism and energy redistribution but not to the extent of the administration of MS-222. In the amino acid metabolism, pantothenic acid increased in response to 2-PE. Like in MS-222, pantothenic acid may have increased to provide cofactors for the enzymes involved in pathways where glucose is conserved to ATP (Salway, 2017). Other metabolites not involved in catabolic processes that were significantly altered by 2-PE included N-acetyl-lysine, D-(+) xylose, and glycoside.

### *5.2.5 The mode of action of 2-PE*

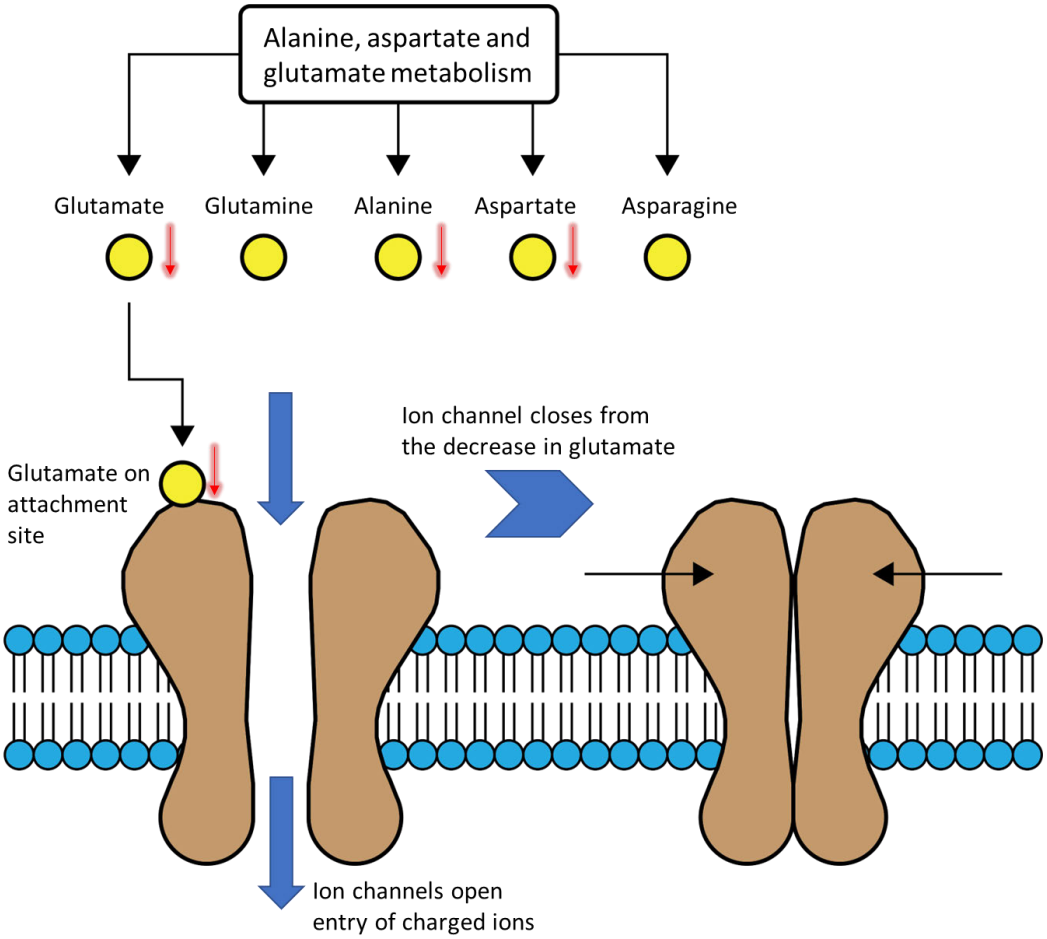


**Figure 5-4: The hypothesised inhibition of excitatory N-methyl-D-aspartate receptor activity in response to zebrafish treated with 2-PE based on results from the current study and supporting literature on the function of glutamate on ion channels (Garattini, 2000; Grasshoff *et al*., 2006; Kohlmeier, 2015)**

The metabolomics analyses revealed metabolites linked to 2-PE's expansion of neuronal cell membranes, which inhibits the excitatory N-methyl-D-aspartate receptor activity (NMDA) seen in *X. lavis* oocytes (Zahl *et al*., 2012). N-methyl-D-aspartate receptor activity is part of the L-glutamate receptor family (Grasshoff *et al*., 2006). Depression of the glutamate receptor function contributes to the immobilisation action of volatile anaesthetics (Grasshoff *et al*., 2006).

Glutamic acid (glutamate) is a non-essential amino acid that naturally occurs in the L configuration and is the most common excitatory neurotransmitter in the central nervous system (Garattini, 2000). It is released via several stimuli and functions at several subtypes of presynaptic and postsynaptic receptors (Garattini, 2000). There are two major groups of glutamate receptors: the ionotropic receptors that include the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and NMDA receptors (Garattini, 2000). Since glutamic acid, a metabolite that acts as a neurotransmitter

in but not limited to NMDA receptor activity, was downregulated in the experimental group, this could suggest a direct link to the anaesthetic's mode of action of suppressing the firing potential of NMDA receptors, as illustrated in Figure 5-4.

It has been demonstrated that aspartic acid (aspartate) is a functional agonist in NMDA and interestingly showing greater potency at four recombinantly expressed NMDA receptors than D-glutamate (Johnson, 2017). Potentially a neurotransmitter, however it is not certain since it has not been released in adequate quantities to activate postsynaptic NMDA receptors (Johnson, 2017). Evidence and studies are lacking to define it as a neurotransmitter (Johnson, 2017). L-Alanine is an amino acid used in the form of proteins broken down for energy metabolism such as glucose through glutamine and glutamate formation (Kohlmeier, 2015). Glutamate metabolism and the three metabolites linked to glutamate metabolism and NMDA were downregulated. Therefore, this suggests that the metabolomics analyses can provide evidence for the anaesthetic's mode of action in suppressing excitatory NMDA receptor activity.

## 5.3 Suitability as an anaesthetic

As described in section 2.3, a good anaesthetic should: induce anaesthesia rapidly with minimal hyperactivity or stress; be easily administered and maintain the animal at the desired anaesthetized state; allow rapid recovery when removing the animal from anaesthetic; produce no long lasting physiological effects after clearance from the body; have high solubility in fresh and salt water; be easily accessible, cost effective, and non-toxic to humans; and finally, be effective at low doses while the toxic dose should significantly exceed effective dose to guarantee a wide margin of safety (Priborsky and Velisek, 2018). Based on the induction/recovery times and phenotypic response shown by the fish of this study, we can summarise how well each anaesthetic in Tabe 5-1.

**Table 5-1: The criteria according to Priborsky and Velisek (2018) of a good anaesthetic met by clove oil MS-222 and 2-Phenoxyethanol.**

| Criterion | Clove oil | MS-222 | 2-PE |
|---|---|---|---|
| **Rapid induction with minimal hyperactivity/stress.** | ✓ All doses | ✓ 125-180 mg/L | ✓ 500-700 mg/L |
| **Easily administered and maintaining the animal at the desired state.** | ✓ | ✓ | ✓ |
| **Rapid recovery when removing the animal from anaesthetic.** | × | ✓ | ✓ |
| **Effective at low doses while toxic doses should significantly exceed effective dose to guarantee a wide margin of safety.** | ✓ | ✓ | × |
| **No long-lasting physiological effects after clearance from the body.** | Could not be determined in this study | | |
| **High solubility in fresh and saltwater** | × | ✓ | ✓ |
| **Readily available, cost-effective.** | ✓ | × | ✓ |
| **Nontoxic to humans.** | ✓ | × | × |
| **Positive attributes.** | 5 | 5 (from 125-180 mg/L) | 5 |

The anaesthetics that met most of the criteria were clove oil and 2-PE (Table 5-1) with five positive attributes each. At the higher concentrations, MS-222 met the same number of criteria. The lasting physiological effects of each anaesthetic after clearance from the body were not determined as the fell outside of the scope of this study. The metabolomics analyses only determined the physiological effect of acute exposure. Therefore, a metabolomics analysis from further experimentation by sampling the fish kept under anaesthesia for a longer duration, as well as sampling fish recovered from more prolonged exposure, may address this point since the long-term effects of anaesthesia are still not well understood (Jorge *et al*., 2021). Research is being done to address this, such as a study of the long-term exposure of 0.2 mg/L propofol in zebrafish, which found an increase in levels of malondialdehyde and reactive oxygen species that contributed to the oxidative damage to zebrafish liver (Jiang *et al*., 2024). Additionally, the study found: increased zebrafish liver lipid accumulation; increased triglyceride; and

high-density lipoprotein cholesterol levels after exposure to 0.2 mg/L propofol, which affected lipid metabolism and was potentially related to fatty acid synthesis and degradation pathways, and caused mitochondrial dysfunction (Jiang *et al*., 2024).

Looking at both the phenotypic observations and the metabolomic consequences of surgical anaesthesia, the results of this study suggest that the concentration of 100 mg/L clove oil is better suited for surgical anaesthesia because anaesthesia was achieved rapidly with minimal hyperactivity and at the lowest effective dose that yields a rapid recovery time after exposure and revealed a metabolomic consequence. However, the metabolomic response suggests that the acute exposure to MS-222 results in a stress response due to the evidence of energy mobilisation to a greater extent compared to clove oil and 2-PE.

Fish induced with 100 mg/L clove oil achieved anaesthesia more rapidly at lower concentrations; the recovery time was not significantly different from that of both 180 mg/L MS-222 and 600 mg/L 2-PE's recovery time. Though the recovery from 2-PE was not significantly different from that of MS-222, the effective dose to achieve stage IV and V anaesthesia was greater than the dose required to achieve the same level of anaesthesia at a similar induction time in MS-222 and clove oil; however, fewer metabolites were altered consequently to 2-PE compared to MS-222. A study tested the effect of clove oil and MS-222 on the swimming performance of rainbow trout. The study found that the time to full recovery from clove oil was significantly longer than the time to full recovery from MS-222. However, the recovery time was not excessively long enough to exclude it as a candidate for aquaculture use (Anderson *et al*., 1997). The study also commented that the low cost of clove oil has an economic benefit to aquaculture over MS-222 (Anderson *et al*., 1997). Additionally, the lower cost and lower effective dosage needed for clove oil, in comparison to 2-PE and MS-222, make clove oil not only the more economical choice but also the one that generates the least pollution by weight for the environment, making it a favourable option from a practical standpoint (Rairat *et al*., 2021)

This study can confirm prior studies induction and recovery times of the tested doses of clove oil; MS-222; and 600 mg/L of 2-PE (Grush *et al*., 2004; Zahl *et al*., 2012; Chambel *et al*., 2015) and that 2-PE is a viable candidate as an anaesthetic for zebrafish. Reports show 2-PE used for short term immobilisation and transportation (Priborsky and Velisek, 2018). The lowest dosage of 100 mg/L showed a reduction in the movement of the fish but did not affect opercular movement. Full recovery was achieved under 30 s suggesting that low doses of 2-PE are suitable for reducing stress experience during transportation of zebrafish. The dose of 500 mg/L 2-PE showed to reliably keep the fish in stage 4 for an extended period greater than any concentration of any of the three anaesthetics investigated in this study. This could prove useful to those wanting to keep zebrafish in an immobile state for an extended period without the need for an artificial respirator. This study confirms that clove oil is a suitable anaesthetic for short term exposure in every criterion regarding assessing an anaesthetic's viability since induction time was rapid at a low dosage with minimal hyperactivity and stress. It is easily administered, cost effective and had the least physiological impact for this exposure period. The

low solubility with water is easily addressed with the usage of a small amount of ethanol when preparing stock concentrations (Anderson *et al*., 1997).

# CHAPTER 6: CONCLUSION

# 6. CONCLUSION

This dissertation is the first to establish and compare both the phenotypic response of various concentrations and the metabolic consequence of rapid acute induction of the anaesthetics 2-PE, clove oil and MS-222 on zebrafish (*D. rerio*). This study found that the phenotypic and metabolomic consequence of MS-222 were more pronounced than those of clove oil and 2-PE on zebrafish. The metabolic consequence of the anaesthetics showed metabolite concentrations altered in a way that illustrates the redistribution and mobilisation of metabolic energy characteristic of a stress response, more so with MS-222. In the case of 2-PE, the study revealed that the metabolomics analyses were able to detect metabolites linked specifically to catecholamine release and the anaesthetic's mode of action on the NMDA receptor. The very limited metabolomic consequence of clove oil as an anaesthetic suggests that it may be more suitable for scientific investigations where metabolic endpoints are being measured. Based on the findings, clove oil at 60 mg/L and 100 mg/L is recommended for fast induction of surgical anaesthesia. For extended procedures requiring stable surgical anaesthesia without artificial respiration, 2-PE at 500 mg/L was most effective. For light sedation, such as during transport, 2-PE at 100 mg/L is advised. This study does not establish the consequence of longer-term exposure to the anaesthetics on zebrafish, nor the consequence of the other ranges of dosages used in this study. A repeat of the exposure protocol to sample and investigate the response to the various other dosages, exposure periods, and recovery will aid in closing these gaps in the understanding of the full extent of the three anaesthetics. Furthermore, an investigation on a 2-step anaesthetic protocol involving either clove oil or 2-PE before the use of MS-222 is warranted to see if the high number of metabolites altered due to MS-222 anaesthesia is a result of phenotypic stress during the earlier stages of anaesthesia (particularly stage III) or the anaesthetic itself. This will also elucidate whether the metabolic consequence of MS-222 can be avoided to benefit from its speed of anaesthesia and speed of recovery.

# CHAPTER 7: REFERENCES

# 7. REFERENCES

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