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A Protocol for Anaesthesia of the Oviparous Fish Model Organism *Betta splendens*

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Dissertation for applying to the degree of Master in Marine Sciences – Marine Resources, Specialization in Marine Biology and Ecology as submitted to the ICBAS – School of Medicine and Biomedical Sciences of the University of Porto

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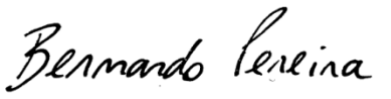

Bernardo Pereira

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“Moonlight drowns out all but the brightest stars.”

— **J.R.R. Tolkien, in “*The Lord of the Rings*”**

Resumo

Os peixes-modelo desempenham um papel fulcral na investigação científica, sendo o peixe betta (*Betta splendens)* um importante organismo modelo, amplamente utilizado em diversas áreas de investigação como a neurobiologia e comportamento, muito devido à agressividade dos machos, e até áreas como a genética e endocrinologia.

Apesar da sua vasta utilização como peixe modelo, há ainda pouca informação e protocolos específicos de anestesia para o peixe betta que permitam um maneio adequado e que assegurem os critérios de bem-estar animal, numa abordagem espécie-específica.

Desta forma, o objetivo deste trabalho passou por tentar estabelecer um protocolo de anestesia adequado e específico para o peixe betta, testando dois anestésicos diferentes, o 2-fenoxietanol e o óleo de cravinho. Os dois anestésicos têm sido usados para diversas espécies, mas existem lacunas acerca da eficácia na indução do estado de anestesia ou sedação nesta espécie, o peixe betta.

Assim, os objetivos visaram a obtenção da concentração ideal de 2-fenoxietanol e de óleo de cravinho para o peixe betta, considerando o sexo do animal, e comparar os efeitos dos dois anestésicos nesta espécie, monitorizando todas as condições de bem-estar animal durante os procedimentos.

Os ensaios experimentais consistiram, em primeiro, submeter os peixes a indução com 2-fenoxietanol, machos e fêmeas, individualmente, às concentrações de 0,20, 0,30, 0,45, 0,60, 0,75, 0,90 e 1,05 mL/L. A mesma dose de 1,05 mL/L foi replicada com uma placa de esferovite (para bloquear o acesso à superfície da água). Posteriormente, induções de anestesia com óleo de cravinho nas doses de 0,20, 0,30 e 0,35 mL/L. Os tempos de indução foram registados para subsequente interpretação.

Após a indução, os peixes foram transferidos para um tanque de recuperação e o respetivo tempo de recuperação foi registado para posterior análise de dados.

As induções com o 2-fenoxietanol revelaram variabilidade para os níveis de anestesia que os peixes atingiram, sendo que nenhuma das concentrações teve uma taxa de 100% de indução no nível 4 de anestesia (anestesia cirúrgica), exceto na concentração de 0,90 mL/L nas fêmeas (todas atingiram o nível 4).

O tempo de recuperação dos bettas anestesiados com a concentração de 1,05 mL/L de 2-fenoxietanol, usando a placa de esferovite, foi superior, revelando um efeito causado pela concentração mais o bloqueio do acesso à superfície da água no tempo de recuperação.

Os tempos de indução e recuperação do óleo de cravinho foram afetados pelo sexo do animal, sendo que as fêmeas apresentaram um maior tempo de indução e recuperação que os machos. O tempo de recuperação também foi afetado pela concentração de óleo de cravinho, sendo que o tempo de recuperação após indução com a dose de 0,35 mL/L foi significativamente superior.

Os resultados indicaram que todos os machos e todas as fêmeas (n = 5 machos e n = 5 fêmeas) atingiram o nível 4 de anestesia para a concentração de 0,30 e 0,35 mL/L de óleo de cravinho, apresentando-se este anestésico como adequado para um protocolo de anestesia para o peixe betta.

Após a análise de resultados concluiu-se que, comparando com o 2-fenoxietanol, o óleo de cravinho revelou ser um anestésico mais adequado para o peixe betta para se atingir o nível 4 de anestesia.

Investigar mais acerca de protocolos de anestesia e também para outras espécies de peixes é muito aconselhável, uma vez que a abordagem espécie-específica é útil para assegurar as melhores condições de bem-estar do peixe e para obter um protocolo melhor, mais preciso e mais adaptado ao peixe-modelo utilizado.

Palavras-chave: 2-fenoxietanol; Anestesia; Bem-estar animal; *Betta splendens*; Óleo de cravinho.

Abstract

Fish model organisms have an essential role in scientific research, and betta fish (*Betta splendens*) is a relevant fish model used in several research fields, such as genetics or endocrinology, but particularly in neurobiology and behaviour, mainly due to the male's aggressiveness.

Despite its wide use as a fish model, there is little information and standard anaesthesia protocols for safe handling and to ensure the welfare standards on a species-specific approach for betta fish.

Then, this work aimed to establish a suitable anaesthesia protocol for betta fish, using two different anaesthetics, 2-phenoxyethanol and clove oil. Both anaesthetics have been used for several fish species, but there needs to be more certainty on the efficacy to induce the state of sedation and anaesthesia in betta fish.

Hence, the objectives were to obtain the ideal concentration and the exposure period of 2-phenoxyethanol and clove oil for this species to reach an anaesthesia state, taking into account the animals' sex, and to compare the effects of both chemical agents while monitoring welfare conditions through the procedures.

The experimental trials consisted first of anaesthesia inductions with 2-phenoxyethanol on males and females at the concentrations of 0.20, 0.30, 0.45, 0.60, 0.75, 0.90 and 1.05 mL/L. The concentration of 1.05 mL/L was replicated with a board of polystyrene (to block the access to the water surface). Then, anaesthesia inductions with clove oil at a concentration range of 0.20, 0.30 and 0.35 mL/L were performed. The times of induction were registered for further interpretation. After the induction, the fish went to the recovery tank and the respective recovery time was recorded for data analysis.

The inductions with 2-phenoxyethanol revealed variability in the levels of anaesthesia reached by the fishes, and it was not achieved at any concentration a rate of 100% induction on level 4 (surgical anaesthesia), except for the concentration of 0.90 mL/L in females (all reached stage 4).

The recovery time of bettas anaesthetised with a 2-phenoxyethanol concentration of 1.05 mL/L with the polystyrene board was the highest, revealing an effect caused by this concentration and the blocked access to the water surface on the recovery time.

The induction and recovery times of clove oil were affected by the sex of the animal, where females showed longer induction and recovery times than males. Also, the recovery time was influenced by the clove oil concentration since the recovery time after the induction with the concentration of 0.35 mL/L was significantly higher.

The results indicated that all males and females (n = 5 males and n = 5 females) reached level 4 of anaesthesia for the concentration of 0.30 mL/L and 0.35 mL/L of clove oil, presenting this anaesthetic agent as suited for a protocol of anaesthesia for betta fish.

After analysing the results, it was concluded that to reach stage 4 of anaesthesia, clove oil was a more suitable anaesthetic agent for betta fish than 2-phenoxyethanol.

Further studies regarding anaesthesia protocols for betta fish and other fish species are highly advisable since a species-specific approach is useful to ensure the best welfare conditions for the fish and a better and more precise protocol adapted to the fish model organism at use.

Keywords: 2-phenoxyethanol; Anaesthesia; Animal welfare; *Betta splendens*; Clove oil.

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Chapter 1. Introduction

1. Fish model organisms in science

Among the numerous fish species used as research model organisms, zebrafish (*Danio rerio*) is the most used in life science research (Potter et al., 2020), but there are some other relevant fish species, such as betta fish (*Betta splendens*), who plays a central role in science fields like animal behaviour (Braddock and Braddock, 1955; Robins et al., 1991).

Anaesthetic agents have an important role in fish research, and their application has been customary since the beginning of the 20th century. However, their utilization began by trial and error in the first anaesthesia protocols (McFarland, 1959; McFarland and Klontz, 1969; Schoettger and Julin, 1967; Zahl et al., 2012). Anyway, the use of anaesthetics is essential to work with any fish model, either for manipulation, surgical procedures or humane killing.

2. The oviparous fish *Betta splendens*

The betta fish (Figure 1), commonly known as Siamese fighting fish, named this way primarily because of the male's typical aggressive behaviour towards other fish (Monvises et al., 2009), is an oviparous freshwater anabantoid fish (Order Perciformes, Osphronemidae Family), capable of breathing atmospheric air due to the labyrinth apparatus, a modified organ that is distinctive of this group. Betta fish are obligatory freshwater fish, composing the Anabantoidei Suborder who comprises three families (Anabantoidae, Helostomatidae and Osphronemidae) that appeared at about 60 million years ago, being naturally found in the southern Asia and the African continent (Berra, 2001; Goldstein, 2001; Rüber, 2009; Tate et al., 2017).

Figure 1 – Male (left) and female (right) specimens of *Betta splendens*. Male: authorship Bernardo Pereira. Female: royalty-free image from Shutterstock.

Betta fish are native to Thailand, ranging from Mae Khlong to Chao Phraya basins, as well as the eastern region of the Cardamom mountains or even in the Isthmus of Kra zone (Figure 2) (Vidthayanon, 2011). It is also considered by some authors to be naturally distributed in other adjacent countries and regions of Southeast Asia, e.g., the Mekong Basin (FishBase, 2022; Monvises et al., 2009; Nico and Neilson, 2022; Rainboth, 1996; Taki, 1978; Witte and Schmidt, 1992). However, it is dispersed throughout the globe due to the trade on ornamental fish business as an aquarium species (Smith, 1945). This fish inhabits intact standing waters (slow-moving streams or even with no currents at all) like those found in swamps of superficial areas or ponds, ditches and drain sites, being also able to inhabit flooded fields of arable crops such as paddy fields (Smith, 1945; Vidthayanon, 2011), as well as rivers of intermediate or big dimension (FishBase, 2022; Taki, 1978).

B. splendens shares the genus *Betta* and a similar ecological distribution with other betta fish species like *Betta smaragdina* Ladiges, 1972, which inhabits eastern and northern Thailand (while *Betta splendens* is originally found in central Thailand) or *Betta imbellis* Ladiges, 1975, and *Betta stiktos* that can be found in southern Thailand, certain parts of Malaysia, Singapore and north Sumatra, in the case of *Betta imbellis*, and in Cambodia for *Betta stiktos* fish (Tan and Ng, 2005).

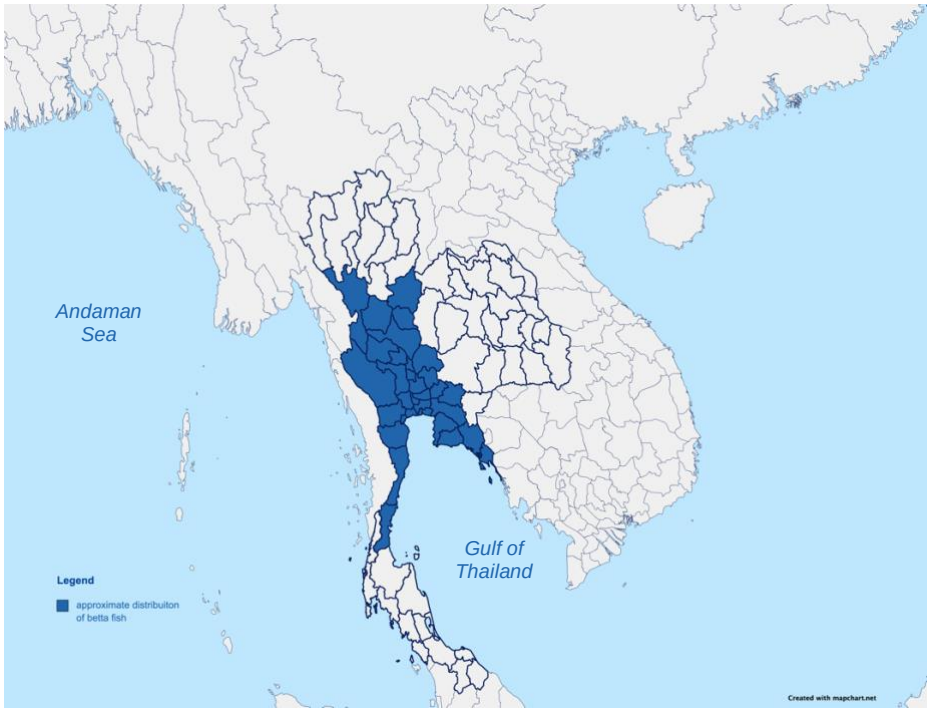


Figure 2 – Approximate native geographic range of *Betta splendens*. Data obtained from Vidthayanon, (2011). Generated in mapchart.net.

2.1. The labyrinth organ

The labyrinth organ is an intricate ossified structure, characteristic of the anabantoid fish, linked to a slim, exceptionally vascularised respiratory epithelium. The labyrinth apparatus covers most part of the suprabranchial chambers (which come two by two in Anabantoidae, Helostomatidae and Osphronemidae families), and the spatial arrangement consists of its extension of each side of the first epibranchial gill arch directly into the opercular cavity and where the several pleats that shape the labyrinth surface explain its complex display (Figure 3) (Huang et al., 2011; Kang and Lee, 2010; Rüber et al., 2004; Tate et al., 2017).

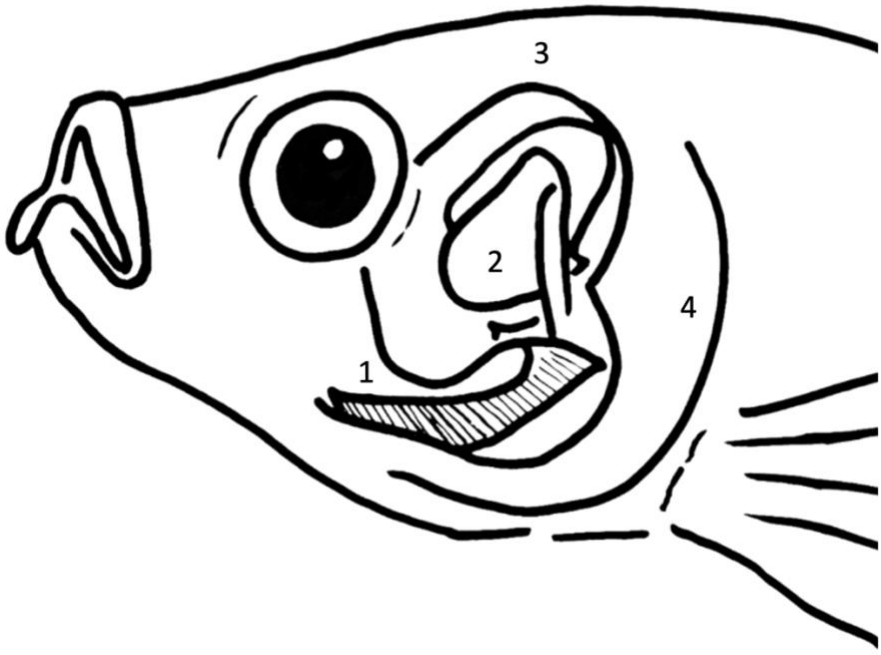


Figure 3 - Example of an anabantoid from a lateral perspective: gill (1), labyrinth apparatus (2), suprabranchial chamber (3), operculum (4). Adapted from Graham (1997) and Tate et al., (2017).

Hence, the oxygen uptake is higher due to this morphological adaptation, and the dorsal position of the labyrinth organ and annex structures permit the stream of the inhaled air into the chamber effortlessly (Shadwick and Lauder, 2006; Tate et al., 2017).

These air-breathing fish often swim to the surface to take air that flows to the suprabranchial chamber, the labyrinth, and associated organs. This differentiated mechanism allows a more efficient oxygen intake than the typical water gaseous trade by other fish, considerably due to the different diffusion distance on the capillaries, which are short on the air-breathing fish when compared to other fish and the fact that the labyrinth has a relatively large surface area (Hughes and Singh, 1970; Tate et al., 2017). However, these air-breathing fishes have

developed smaller gills than those that do not breathe air since the labyrinth occupies a considerable part of the inner operculum (Shadwick and Lauder, 2006; Tate et al., 2017).

2.2. Betta fish – commercial value and conservation threats

A highly commercialised species in the international market of ornamental fish trade, having numerous domestic variants, the betta fish is also used in some countries for game fighting, mainly in Asia (Vidthayanon, 2011). The trend of betta as an ornamental fish in the fishkeeping market is high and lucrative (with an increasing tendency), especially in male bettas that are of great interest for exportation or local trade due to several characteristics such as body colour and types of fins (Monvises et al., 2009).

There is an emergent change in the export market concept of betta fish, focusing more on breeding for ornamental purposes instead of aiming for the fish fight business, not only because of ethical reasons but also for commercial purposes since that ornamental fish trade is more profitable economically (Monvises et al., 2009).

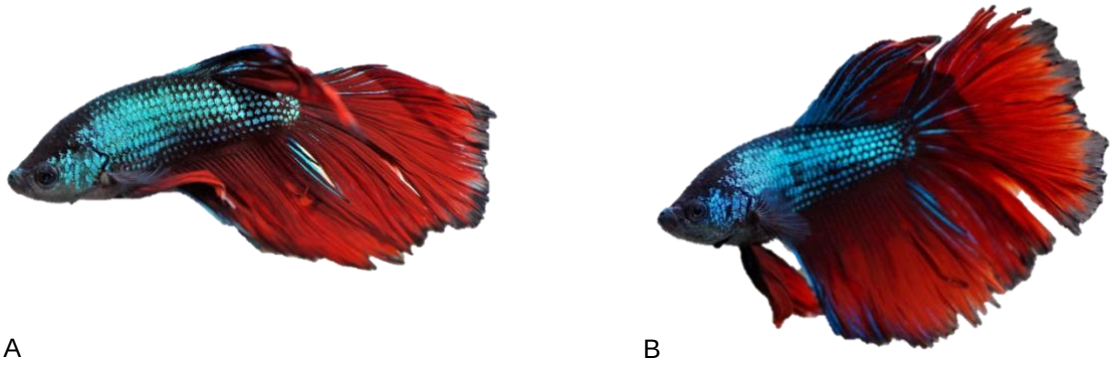
Betta splendens is established as Vulnerable (probable regression of about 30% of the population) by the IUCN Red List (Vidthayanon, 2011), with habitat dilapidation as the main threat, typically for transformation into agricultural fields as well as pollution from urbanisation aside with genetic depletion due to the escape of domestic variations from farms into the natural habitats (Vidthayanon, 2011). Therefore, some conservation actions have been recommended, e.g., managing known habitats combined with betta fish breeding using the wild populations in captivity (Vidthayanon, 2011).

According to the International Betta Congress (2020) the origin of the ornamental varieties of betta fish comes from the innumerous crossings of this fish wild types and even other species wild types like *Betta imbellis*, *Betta mahachaiensis* or *Betta samaragdina* (International Betta Congress, 2020; Matthew and MacLean, 2012).

Bettas are interesting fish for research fields such as behavioural studies, or neurobiology and evolution, and have been part of research works for about a century. However, there is still little information and established protocols for this species' adequate and effective husbandry, delaying its higher and broader use as a fish model organism (Lichak et al., 2022).

2.3. Betta fish anaesthesia

Fish transportation is a critical process for the animal, causing distress whether it is due, e.g., to the transport device vibration or the noise, shocks or abrupt movements throughout the travel, a circumstance that may provoke injuries in the fish, such as fin collapse (Figure 4A), which not only compromises the fish welfare but can also reduce the fish price and value when compared to a healthy fish (Figure 4B) (Pattanasiri et al., 2017).



A B

Figure 4 – *Betta splendens* in distress exhibiting caudal fin collapse (A) and with normal appearance showing a total display of dorsal, caudal and anal fins (B). Figures (royalty-free) by ivabalk from pixabay.com.

Therefore, anaesthetic agents like clove oil (CO), or its active ingredient, eugenol, have been used in betta fish to reduce possible stress reactions, although these two anaesthetic agents have few studies, and there is a lack of literature in their appliance for betta fish anaesthesia (Fabregat et al., 2015; Pattanasiri et al., 2017).

The correct use of anaesthesia dose and time of induction is essential to avoid a disproportionate appliance of the anaesthetic agent, which is a stressful factor to fish if it is overly administered, causing collateral effects such as higher oxygen intake, deregulated metabolic rates and blood pressure and blood physiological responses with a possibility of lasting for increased periods after the fish recovers from the anaesthesia (Pattanasiri et al., 2017; Summerfelt and Smith, 1990). Eventually, an overdose could lead to the fish's death.

The usual optimal induction and recovery times in fish anaesthesia are approximately of 3 minutes to induce sedation and a full recovery until a maximum of 10 minutes, a time range that can reduce possible stress reactions in fish (Gilderhus and Marking, 1987; Pattanasiri et al., 2017; Son et al., 2001).

The induction of anaesthesia can cause hypoventilation due to the anaesthetic agents properties and its effects on fish physiology, observable by the lower buccal movements of the fish, being this anaesthesia induction a main cause of hypoxemia and in the case of air-breathing fish this hypoxemia event can be intensified by making more difficult their contact with the water's surface (Neiffer and Stamper, 2009; Rantin et al., 1998; Rantin et al., 1993).

3. Welfare in fish research

Several factors in the fish life cycle (particularly in aquaculture) can lead to stress, reducing general welfare conditions (Sneddon, 2007). Among the factors that contribute to fish stress are handling and transport, which may decay the immunological capacity of the fish, lead to injuries or, ultimately, death (Coyle et al., 2004).

Therefore, some actions can ensure the fish's welfare, such as adaptations in aquaculture procedures, like a species-specific approach (each species' particular requirements), that can improve their growth and development, since fishes present high diversity compared to other vertebrate groups (Sneddon, 2007).

Among the various welfare parameters that can serve as displays of the fish welfare status, the physiological and biochemical data, as well as the fish behaviour, play a central role in a perceptive analysis of the welfare condition (Huntingford et al., 2006; Wang et al., 2017).

In research, ensuring higher fish welfare conditions improves the probability of getting more trustworthy results on the research work (Sneddon, 2007). Also, better animal use can be enhanced by guaranteeing the finest welfare procedure (Lidster et al., 2017).

To ensure optimal welfare conditions, some methods can be applied, such as the monitoring and analysis of the animal's plasma, specifically the cortisol levels, since this is the primary hormone in fish that is linked to stress (Ellis et al., 2007; Wang et al., 2017).

This measurement of the cortisol levels in fish can be an efficient method to verify the possible effects of a certain stress agent (Sadoul and Geffroy, 2019), and the plasma levels quantification of this hormone is a reliable way to assess the fish welfare conditions (Barcellos et al., 1999; Wang et al., 2017).

Regarding the implementation of welfare conditions in research protocols, there is a pattern in most laboratories of using their own guidelines, including the anaesthetic used (Figure 5), making these protocols heterogeneous between each institution, adding to the fact that most local regulations are inconsistent (Lidster et al., 2017).

Animal welfare is a subject of great interest and eminence in research due to the importance of ensuring the minimum stress reactions of the animal to a given laboratory protocol. Therefore studies regarding animals' stress responses are of topmost significance since stress levels reveal if the fish's well-being is balanced or in suffering conditions (Wang et al., 2017).

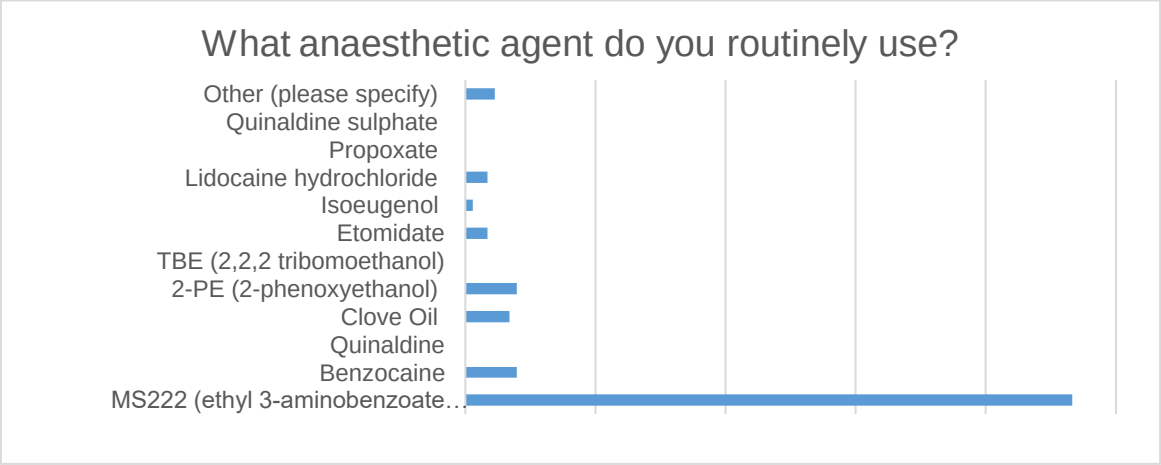


Figure 5 - Most used anaesthetics. Inquiry adapted from Lidster et al., (2017).

The concept of animal welfare in laboratory fish and the use of anaesthetic agents in these animals is correlated with the humane (a notion hard to define) manipulation of fish; this is the guarantee of good practice behaviour by the researcher or the person in charge of the handling (Readman et al., 2017).

Besides anaesthesia and the subsequent recovery process, welfare is also essential in euthanasia — many times defined as "good death" (APC Secretariat, 2009; Readman et al., 2017) — as the exposure to the anaesthetic agent should not incite avoidance behaviour so that the protocol can match with the humane use concept (Readman et al., 2017).

3.1. Stress and pain

A way to avoid or minimise cortisol release as a mechanism derived from stress response becomes challenging due to the difficulty in choosing the most appropriate anaesthesia protocol for each procedure, considering that anaesthesia has been described by some authors as a practice that negatively affects cognitive functions and causes neurotoxicity (Martins et al., 2016). However, these unwanted effects depend on the animal age, type of administration, dose of anaesthesia and its duration and also the type of anaesthesia, adding the fact that there is still little consistent information and detailed mechanistic knowledge about fish anaesthesia, which turns the fittest anaesthesia protocol choice even more complicated (Martins et al., 2016).

Anaesthesia and sedation are used customarily in clinical procedures, research studies and general husbandry. Although the pain perception issue in fish is still a debated theme among the scientific community, the importance of fish welfare and the attempts to minimise pain and distress are in agreement among researchers (Martins et al., 2016). Consequently, using anaesthetic, sedative or analgesic agents is vital to reduce stress or pain in fish, since they can be considered sentient animals capable of pain perception (Martins et al., 2016).

Formulating adequate anaesthesia protocols or refining already established and standard procedures is essential to minimise possible collateral effects of the used anaesthetics. As seen in Martins et al. (2016), the most commonly used laboratory anaesthetic and analgesic agents and protocols for each species (in this case, zebrafish) as well as the depth of anaesthesia and adult recovery were analysed and compared, recommending the use of a score sheet as a way of monitoring distress and pain to supervise zebrafish welfare during the experimental procedure (Martins et al., 2016).

4. Analgesia, anaesthesia and euthanasia

Anaesthesia and sedation as a way of chemical restraint in fish are topics of great interest in science with a high information demand, due to their importance for several activities even beyond research (it is increasing the use of fish instead of other classes of vertebrates and it requires the use of anaesthetic agents) as aquaculture. This activity needs the accurate usage of anaesthesia and sedation in many fish for handling or transport, or even for veterinary treatments and technical care of fish at industries such as public aquariums or zoos (Neiffer and Stamper, 2009).

Anaesthetic agents are widely used as the first choice to reduce stress on fish (Berka, 1986; Coyle et al., 2004; McFarland, 1959; Pattanasiri et al., 2017). Not only numerous procedures become possible due to anaesthetic agents such as transport, restraint, application of assisted reproduction methods and tagging, handling or even blood or tissue sampling and some medical practises, but also some issues provoked by stress such as lower immunity and feeding loss are weakened since the possible stress caused by those practices was reduced or attenuated due to the anaesthesia administration (Brown, 1993; Carmichael and Tomasso, 1988; Ross and Ross, 2008).

However, there is still a lack of concern about the real effect of the anaesthetic agent since these drugs are primarily used to obtain different stages of the anaesthesia process, despite the potential anaesthetic's negative influences on the fish (Zahl et al., 2012).

The range of effects of the anaesthetic agents can be a simple loss of balance in the lower levels of anaesthesia or total loss of movement and respiratory arrest in the higher phases of anaesthesia (McFarland, 1959; Pattanasiri et al., 2017).

Anaesthetic agents were initially applied in fish to enable safe handling, but the rising knowledge of fish physiology and the importance of treatment with anaesthesia to improve the welfare conditions led to an increase in the use of anaesthesia procedures (Zahl et al., 2012).

In the case of human and veterinary medicine, the anaesthesia protocols encompass a combination of several drugs, which individually act towards the desired effects on the anaesthesia procedure; thus, they are used collectively to obtain all those effects in one operation (Zahl et al., 2012). For instance, many drugs are applied for anaesthesia induction and maintenance, while the analgesic procedure is commonly utilized during surgery and after it (Zahl et al., 2012).

Fish anaesthesia is also important for situations that demand handling like transport, which requires sedation levels for the animal handling, measurement, weighing or vaccination, as well as blood sampling procedures or biopsies (Iversen et al., 2003; Mitjana et al., 2014; Mylonas et al., 2005).

The anaesthesia effects in fish may vary as well by influence of variables such as route of administration, pH, salinity or even temperature, oxygenation and nitrogen compounds, beyond the fact that anaesthesia depth and recovery depend on its extent, anaesthetic

concentration, animal's body weight, metabolism, gill surface, fish health status, strain, age and also different particularities of each fish species (Martins et al., 2016).

Performing pilot tests on a reduced number of fish to determine the ideal time and concentration of exposure is fundamental for establishing and enhancing anaesthesia procedures (Martins et al., 2016). Beyond that, to avoid complications that may lead to fish death, it is crucial that appropriate training and supervision of the fish anaesthesia procedures exists, a situation still neglected in literature (Martins et al., 2016).

Fish anaesthesia protocols include several anaesthetic agents which have been (or are still) applied, being ones more regularly utilized than others, as seen in Martins et al. (2016) review, where different anaesthetic agents used in zebrafish are summarized. The study also suggested the permanent monitoring throughout the entire anaesthesia protocol so that stress, discomfort or pain are screened, even at the maintenance stage, as well as the use of analgesics in fish during and after painful experimental procedures, thus ensuring that welfare concept is respected and maintained not only before the anaesthesia but also during and after the testing procedure (Martins et al., 2016).

Anaesthesia by immersion is the most common anaesthesia method, and for small fish like zebrafish (*Danio rerio*), it is the main process of anaesthesia induction, whereas other types of more invasive anaesthesia are almost unviable (Martins et al., 2016).

4.1. Anaesthesia stages

Along the anaesthesia process, several stages can be displayed, such as sedation, immobilization/restriction, narcosis (which comprises unconsciousness), amnesia and analgesia (pain relief) (Zahl et al., 2012). Each stage has intrinsic characteristics. For example, sedation reduces the animal sensitivity, leading to a calm state. In contrast, in narcosis (general anaesthesia), the animal attains not only an unconsciousness and amnesia state but also reaches immobilization and pain relief (analgesia) (Zahl et al., 2012). The characterization of each anaesthesia stage can be described as proposed by Zahl et al. (2012) (Table 1).

Table 1 - Stages of anaesthesia in fish. Adapted from Zahl et al., (2012).

Stage	Description	Appearance	Swimming	Equilibrium	Responsiveness*	Respiration
0	Normal	Normal	Normal	Normal	Normal	Normal
1	Light Sedation	Disoriented	Reduced	Normal	Slightly reduced	Normal
2	Excitatory stage	Excited	Increased	Struggles to maintain balance	Normal or exaggerated	Irregular or increased
3	Light anaesthesia	Anaesthetised	Stopped	Lost	Reacts to strong tactile stimuli	Normal or decreased
4	Surgical Anaesthesia	Anaesthetised	Stopped	Lost	None	Shallow
5	Deep narcosis	Anaesthetised	Stopped	Lost	None	Nearly absent

*Responsiveness refers to reaction to external stimuli. The stimulus may be visual or tactile.

4.2. Anaesthetic agents

The applied routine for anaesthesia induction is usually based on fish immersion in an anaesthetic solution with a given concentration (Mitjana et al., 2014; Topic Popovic et al., 2012; Weber et al., 2009). There are several anaesthetic agents used in fish anaesthesia procedures, such as MS-222 (tricaine methanesulfonate), benzocaine and 2-phenoxyethanol (2-PE), or even metomidate hydrochloride, isoeugenol and quinaldine (Zahl et al., 2012). The anaesthetics MS-222, 2-PE and CO are the most used in aquaculture, according to Mitjana et al., (2014).

In both human and veterinary medicine, numerous anaesthetics with an attested analgesic effect (used as local analgesics), e.g., benzocaine, are given to fish to obtain a state of sedation or for immobilization, analgesia or even for general anaesthesia (Zahl et al., 2012).

There are many factors to consider when determining a good anaesthetic, such as quick effect, fast recovery and low toxicity not only for the fish but also for the staff and the environment, as well as low tissue residues, low cost, availability, type of technique and simplicity of use (Mitjana et al., 2014; Soto and Burhanuddin, 1995).

Furthermore, since not all anaesthetics available for fish have demonstrated analgesic properties, it is important to consider administering analgesics beyond the anaesthetic agent. Additionally, combining anaesthetics instead of a single anaesthetic agent may diminish individual concentrations and reduce the risk of unwanted secondary effects (Martins et al., 2016).

4.2.1. 2-phenoxyethanol

Considering all the above-explained aspects, anaesthetic agents like 2-PE (Figure 6), also known simply as phenoxyethanol and ethylene glycol monophenyl ether, among other synonyms, present advantageous characteristics, e.g., rapid induction time of anaesthesia, fast recovery and low price (Mitjana et al., 2014; Weyl et al., 1996), being, for instance, suggested as a short-term immobilization anaesthetic in fish (Mitjana et al., 2014; Ortuño et al., 2002; Tsantilas et al., 2006).

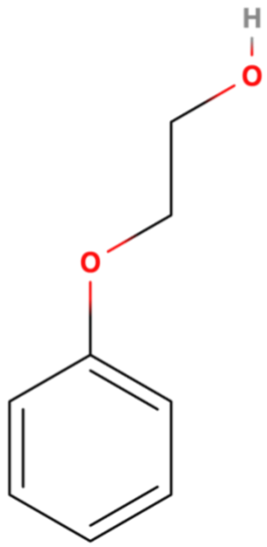


Figure 6 - Molecular structure of 2-phenoxyethanol. Image generated in molview.org.

2-PE is a clear or pale yellow-coloured oily liquid with moderate water solubility and is vastly used as an anaesthetic agent and sedative, mainly for fish transportation purposes (Neiffer and Stamper, 2009; Ross, 2001). However, 2-PE also shows adverse characteristics already described in fish, such as decreased heart rate, blood pressure, or hypoventilation (Mitjana et al., 2014; Ortuño et al., 2002). Moreover, there is a probability of 2-PE bioaccumulation in fish (Abreu et al., 2019).

Its advantages are highlighted by the fact that it does not provoke pH alterations when added to seawater, and it is a low-cost anaesthetic (Neiffer and Stamper, 2009; Oswald, 1978). However, the same works point to disadvantages in the use of 2-PE, such as low analgesic effect, causes hypoventilation and induces a neuropsychological syndrome in some handlers when they are subjected to prolonged exposure to 2-PE solutions.

The range of favourable concentrations for 2-PE in black sea bass (*Centropristis striata*) anaesthesia was between 200 and 600 mg/L with an induction time of 3 to 4 minutes and a

recovery time below 10 minutes in King et al. (2005). Still, research works in species such as rainbow trout (*Oncorhynchus mykiss*) and juveniles of atlantic cod (*Gadus morhua*) have reported failures in reaching a level of anaesthesia capable of immobilizing the fish in these species with moderate doses of 2-PE (Gilderhus and Marking, 1987; Mattson and Riple, 1989; Tort et al., 2002). These inconsistencies reveal the response specificity of each organism to the same anaesthetic agent and, eventually, the possible indication of variances between different life stages development in the same species (e.g., juvenile or adult) in the reaction to the same anaesthetic, which regularly requires the establishment of screening dosages to different anaesthetics (King et al., 2005).

4.2.2. Clove oil

Another frequently applied anaesthetic agent is CO, an oil that has eugenol as its active principle (Figure 7), extracted from plants of the genus *Eugenia* (*E. aromatica* and *E. caryophylata* by distillation of stem, leaves and flowers (Mitjana et al., 2014; Soto and Burhanuddin, 1995). CO stands out for its low cost, soft environmental impact, and few adverse reactions to fish and staff safety (Cho and Heath, 2000; Mitjana et al., 2014).

Ascertaining the rightest anaesthetic agent for each species is of great research interest, and there is still a need for data in this field since there are species from which there is a lack of knowledge on the best anaesthesia protocol to use specifically and simultaneously including welfare measures and humane use. The potential advantages of CO can be illustrated in the case of angelfish (*Pterophyllum scalare*). After the establishment of the lowest effective dose (LED) of three anaesthetic agents on juvenile angelfish — CO, 2-PE and MS-222 — along with the effect of multiple exposures of the juveniles to the anaesthetic at induction and recovery times, CO was affirmed as apparently the most advantageous, possibly being the less noxious and the cheapest for large scale administrations in angelfish juveniles (Mitjana et al., 2014).

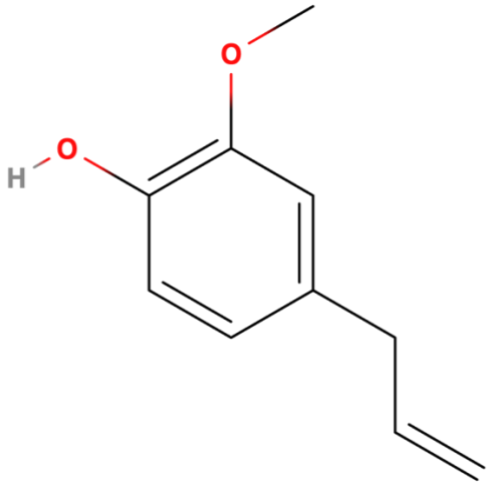


Figure 7 - Molecular structure of Eugenol, the active principle of clove oil. Image generated in molview.org.

CO, a commonly used anaesthetic agent, has revealed some inconsistencies regarding anaesthesia in juveniles and adults of black sea bass. King et al. (2005) conducted an experiment to evaluate the anaesthetic effect of CO on juvenile black sea bass, where all juveniles reached stage II of anaesthesia (characterized by “loss of gross body movements but continued opercular movement”) at a concentration of 20 mg/L. However, the juveniles still reacted to needle puncture even in stage II of anaesthesia at the CO induction phase. In contrast, adults showed no reaction to invasive procedures like ovarian biopsy or tag insertion at a concentration of 30 mg/L of CO.

Another discrepancy regarding CO resides in its eventual analgesic properties in fish, implied, e.g., in Keene et al. (1998) for juvenile rainbow trout using its derived compound, eugenol. However, the results obtained for black sea bass in King et al. (2005) and for red pacu (*Piaractus brachypomus*) in Sladky et al. (2001) contest that possibility.

CO also prevented the increase of cortisol levels of black sea bass in King et al. (2005) since fish reached stage II of anaesthesia. This CO “attribute” was already reported in previous works like Iversen et al. (2003) and Small (2003).

Additionally, it is verified that higher doses of CO are associated with longer recovery times as well as an increase in the mortality risk, being this anaesthetic agent more useful at low concentrations (<20 mg/L) for sedation (the stage where equilibrium is not lost yet) during transport or sorting procedures (King et al., 2005).

4.2.3. Other anaesthetics

MS-222 is a water-soluble anaesthetic agent that is intensely used in fish and other cold-blooded animals (Mitjana et al., 2014), being, additionally, the only legal anaesthetic for use in a restricted number of fish for human consumption in the United States of America, along with Canada, where MS-222 is the only approved drug that can be applied in fish used for human consumption (Carter et al., 2011). Also, MS-222 is the most used anaesthetic agent in zebrafish, according to the international survey of Lidster et al. (2017) that revealed this drug as the top choice among the enquired participants, standing out as the foremost applied local anaesthetic for inhalation anaesthesia protocols in fish, being highly absorbed by the gills inducing general anaesthesia (Martins et al., 2016).

Despite MS-222 being considered a safe and effective anaesthetic agent for many fish species (Topic Popovic et al., 2012), it has some negative characteristics that incite undesirable effects as an anaesthetic in zebrafish, like bradycardia (Mitjana et al., 2014) together with some other adverse points of MS-222, e.g., its high cost and noxious effects to the handler, chronically exposed without gloves, such as reversible retinal toxicity (Bernstein et al., 1997; Mitjana et al., 2014). Most researchers use a concentration of 164 mg/L of MS-222 in zebrafish studies when a state of surgical anaesthesia is required (Collymore et al., 2014).

Beyond the stress caused by the handling of fish (King et al., 2005), the anaesthetic agent itself can cause an increase in fish cortisol levels, depending on the anaesthetic and the species used in the anaesthesia protocol as in King et al. (2005) where the anaesthetic agent MS-222 raised the cortisol levels of adult sea bass as a stress response to the anaesthetic. There is evidence of substantial increases in cortisol levels in the plasma caused by MS-222, and this effect is detached from other stressor effects, acting independently (King et al., 2005; Small, 2003).

A premise that lower doses of MS-222 in salmonids (<50 mg/L) show cortisol reactions, whereas higher doses (>100 mg/L) may not incite cortisol levels to rise has been considered in research which reveals that the loss of discernment by the fish is often necessary so that a cortisol response to an acute stressor can be avoided (King et al., 2005).

In King et al. (2005), cortisol levels of black sea bass did not increase after induction with anaesthetic agents like CO, metomidate and 2-PE, showing only cortisol responses before anaesthesia induction at capture (with net) phase because after induction changes in

cortisol levels were not verified for at least 30 minutes. Hence, it was concluded that 2-PE and metomidate have several characteristics, like a safe dose range and a plane of anaesthesia suitable for procedures such as tag implementation on adult sea bass, that make them promising as anaesthetic agents for black sea bass. However, 2-PE is advantageous compared to metomidate due to its low cost (King et al., 2005).

The anaesthetic agent metomidate can prevent fish cortisol rises after stress through a mechanism that consists in blocking the adrenocorticotrophic hormone (ACTH) activity at the interrenal gland level (King et al., 2005; Olsen et al., 1995), and it works apparently through an inhibition mechanism of the 11β-hydroxylase activity, a phenomenon that was already observed in mammals for etomidate (King et al., 2005).

In King et al. (2005), differences in reactions to the same anaesthetic agent between juveniles and adults of black sea bass were observed. A comparison of four anaesthetic agents was made – CO, metomidate, MS-222 and 2-PE – to define the lowest effective dose (LED) of each one of these anaesthetics in both juveniles and adults of black sea bass, including induction and recovery times as a way to establish the ideal doses for usual procedures such as blood sampling or measuring and weighing (King et al., 2005). In this work, researchers also managed to study the effect of these anaesthetic agents on the cortisol stress reaction (King et al., 2005).

The effects of MS-222 and benzocaine were tested as possible solutions for decreasing stress in betta fish transport (Sommani et al., 1999).

4.3. Relevance in scientific research

Anaesthesia procedures have an important role in fish model organisms used in research, which may require immobilization to allow the handling and ensure the welfare and stress mitigation for safe laboratory protocols (Martins et al., 2018). The urgency and need for valuable data and information that may allow a better insight into chemical restraints in fish are driven by an interest in promoting fish welfare in research (Neiffer and Stamper, 2009).

According to Neiffer and Stamper (2009), beyond basic studies, research should be directed towards standardising fish anaesthesia practices similar to what already exists in other vertebrate classes. Anaesthesia and sedation protocols have been usually effective for both veterinaries and researchers for minor procedures. However, this subjective efficacy is not sufficient in these professional areas of work. Therefore, a need for a more precise and

objective appliance of anaesthesia protocols to turn fish medical activities into a routine (especially surgery) is imperative, assuring, that way, a high level of confidence in fish survival rates when subjected to medium to long-term procedures. Although many fish survive the surgery or long technically advanced procedures afterwards, metabolic crisis is frequently observed during the recovery period or immediately after the procedure (Neiffer and Stamper, 2009).

4.4. Species-specific anaesthesia in fish

The species-specific concept is essential since the ideal dose of anaesthesia varies among different fish species, so generalising one perfect anaesthetic agent concentration and exposure mode and duration for one species to another is not recommended since it is scientifically erroneous (Chambel et al., 2013; Pattanasiri et al., 2017).

The anaesthetic agents are, in their majority, administered indiscriminately, regardless of the fish species (Zahl et al., 2012). In the case of new species added to scientific research or aquaculture, the protocols of anaesthesia are mostly based on established anaesthetic agents, quantities given and immersion times for the currently most used species, as seen in the case of the Atlantic cod and the Atlantic halibut (*Hippoglossus hippoglossus*), two species first introduced in the aquaculture activity in the 1980s whose anaesthesia protocols were the same used for salmonids – the leading fish species with economic value in Norway, used in aquaculture since the 1960s (Zahl et al., 2012).

In the case of human and veterinary medicine (regarding mammals in this example), the anaesthetic agents are selected by their properties and appropriateness to that specific surgery and also to the human or animal type of patients (as well as their physiological condition) (Zahl et al., 2012).

The difficulty in defining the humane use of anaesthesia in fish can be avoided by conceiving anaesthesia protocols based on a species-specific perspective, already established in mammal anaesthesia procedures, bringing the improvement of more effective anaesthesia and the decrease of negative behavioural responses of the animal to the anaesthetic agent induction (Readman et al., 2017)

In 2009, there was almost no data about proper anaesthetic or analgesic drugs that suited the requirements for each fish species, but euthanasia techniques were shown to be satisfactory in their majority (Neiffer and Stamper, 2009). The scenario improved with time.

Research works on the advantageous method of species-specific anaesthesia through the use of the most fitting anaesthetic drug for each fish species, instigating the possibility of applying each anaesthetic to each species, are growing and the concept has been reviewed in some works (Neiffer and Stamper, 2009; Ross and Ross, 2008; Sneddon, 2012; Valentim et al., 2016; Zahl et al., 2010) however, it has been given more focus on the possible qualities of the anaesthetic agent (e.g. anaesthetic stability or induction time), so that, apparently, there is a generalized lack of the variable welfare in fish anaesthesia protocols used in research in a species-specific basis (Readman et al., 2017).

For instance, expressions of avoidance behaviours are present in zebrafish for most anaesthetics since the most commonly used agents seem to promote negative reaction behaviours in this species that appear to retain memories of the anaesthetic events (Readman et al., 2017; Readman et al., 2013; Wong et al., 2014). Hence, a good and adequate anaesthesia protocol should be grounded in a species-specific approach, and it is advantageous to use several behavioural models to consider all the demonstrated behaviours possible by the animal, understanding, in that way, the anaesthesia protocol appropriateness to the fish and turning it a humane anaesthetic protocol, evading the unwelcome aversion and avoidance reactions by the fish (Readman et al., 2017).

Beyond the concept of a species-specific approach in fish anaesthesia, the aim of the research project is also a relevant factor in defining the best protocol possible, and some parameters like pH, pCO_2 or osmolarity need to be minded closely since changes in these parameters may induce avoidance and aversion behaviours that can arise by the addition of the anaesthetic agent to the fish tank water (Readman et al., 2017).

Readman et al. (2017) attested the need for an approach in anaesthesia protocols that includes a species-specific basis because, in this work, in which a chemotactic choice chamber was used, it was substantiated that four species of closely related fish — carp (*Cyprinus carpio*), fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and rainbow trout — vastly used in laboratory, revealed behavioural differences when exposed to three anaesthetic agents (benzocaine, MS-222 and etomidate). Medaka and zebrafish showed no avoidance towards etomidate but exhibited it to benzocaine and MS-222 exposure, while, for instance, carp indicated the opposite behaviour (evasion towards benzocaine and MS-222 but no avoidance for etomidate) (Readman et al., 2017).

5. Euthanasia

Euthanasia is a practice that attempts to end a life to mitigate pain and distress/suffering. The word euthanasia originates from the junction of the Greek terms "*eu*" which means "good" and "*thanatos*" (meaning "death") (AVMA, 2020). For instance, in fish slaughtering (for human consumption, e.g., in aquacultures), the use of euthanasia methods reduces the state of stress before slaughtering (Wang et al., 2017).

The use of CO as an euthanising drug for fish can be applied, according to the work of Fernandes et al. (2017), with a concentration of 0.20 mL (or 200 mg) per 500 mL of water to provide an accelerated death inducted by anaesthesia with no distress, for studies and situations that demand the fish death for laboratory sampling or analyses (e.g., dietary or reproductive). In the study of (Fernandes et al., 2017), fish showed high mortality rates at CO concentrations over 0.05 mL per 500 mL of water, so the concentration of 0.20 mL of CO was recommended to ensure total mortality at the lowest induction time.

Despite the growing research studies that investigate the effects of induction and recovery times of the anaesthesia procedures, the results have been contradictory, with a high variability within fish species for a given anaesthesia time of both induction and recovery despite their weight and size (e.g., in some cases larger sized fish had a lower induction time than the smaller fishes, while in other species the opposite occurred), turning it hard to establish a protocol pattern (Fernandes et al., 2017; Hoskonen and Pirhonen, 2004; Perdikaris et al., 2010; Prince and Powell, 2000; Walsh and Pease, 2002; Woody, 2002).

An international survey carried out by Lidster et al. (2017) concerning the zebrafish welfare conditions in research gathered information on the most adopted methods by the laboratories on death confirmation (Figure 8).

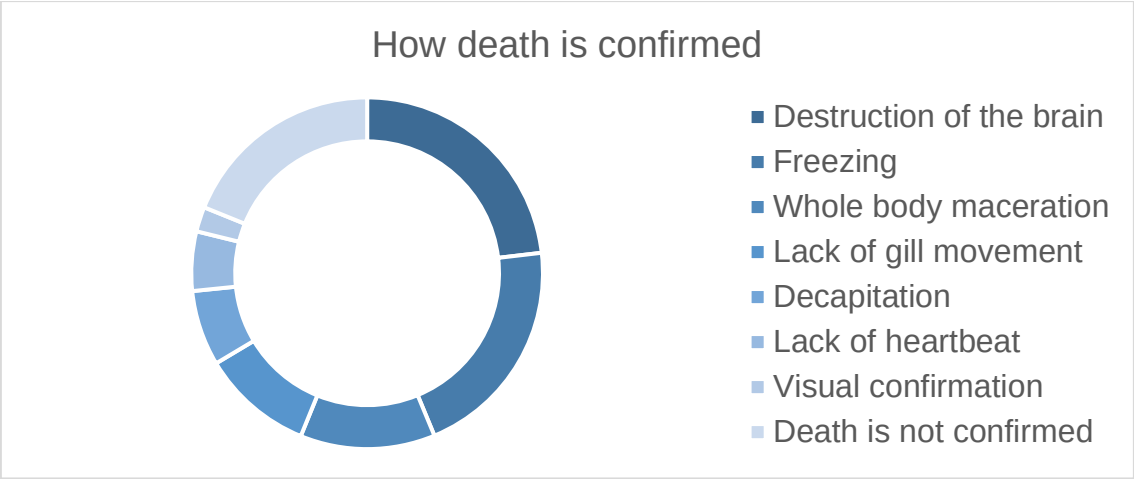


Figure 8 - Methods of death confirmation in fish. Adapted from (Lidster et al., 2017).

6. Dissertation objectives

There is a lack of information regarding anaesthesia protocols specifically standardized for betta fish. The vast majority of these protocols are based on previous works with zebrafish. Therefore, this master's dissertation aims to:

1. Determine the ideal concentration and exposure time of two anaesthetics (2-PE and CO) for inducing anaesthesia and recovering in male and female of betta fish;
2. Compare the effects of both anaesthetics in minimizing distress during the procedure.

Chapter 2. Materials and Methods

1. Fish husbandry and routines

This research was performed at the Aquatic Animal facility of the ICBAS – U.Porto. The legal processes were followed, including the personnel accreditation to perform animal experimentation.

A rack system with 24 inert plastic tanks of approximately 10 litres each was placed to accommodate the animals (betta fish) (Figure 9). Each tank had a mechanical filter with aeration, a heater and a thermometer.

Figure 9 - Rack system from the aquatic animal facility of ICBAS – U.Porto, used for the *Betta splendens*.

Adult males and females of betta fish were obtained from a local pet shop (Patinhas Pet Shop, Porto, Portugal) and were acclimated in the laboratory for an hour by slowly adding the chlorine-free water already present in each tank of the rack system to each fish, in its own transportation water bag. Then, each fish was released into tanks, and the bag with the transportation water was discarded. The fish were separated by males and females. The females were gathered in groups of 3 to 4 in each tank, and the males were placed individually (1 male per tank).

For the betta fish males used, the mean weight was 2.23 ± 0.55 g and the mean maximum standard length (caudal fin not considered) was 3.80 ± 0.30 cm. For the betta fish females used, the mean weight was 1.64 ± 0.40 g and the mean maximum standard length (caudal fin not considered) was 3.29 ± 0.27 cm.

The quarantine period corresponded to 7 days, and only after this period could the fish be submitted to the experimental trials since there was no evidence of pathology signs or stress. Also, this stabilisation period is important since some mortality may occur depending on the transport conditions, acclimation procedures (Table 2), and fish health status.

All the tanks of the rack system were maintained with aerated water at a temperature of 27 ± 1ºC, and the water quality parameters (pH, NO_2, NH_3/NH_4^+, GH and O_2) were monitored

once a week to guarantee the specific requirements of the betta fish, using Prodac® test kits.

The initial routine involved daily monitoring of the water temperature to make any necessary adjustments, and later on, it transitioned to weekly monitoring. A partial water change of about 30% was performed weekly, as well as the cleaning of the tanks. However, when the water quality parameters were not within the expected range, a partial water change of 50-65% was immediately performed.

Table 2 - Fish reception procedures and acclimation step by step.

STEP	DESCRIPTION
1	Begin by unboxing and do an initial check for any fish casualties while assessing the overall condition of the fish;
2	Confirm the fish species and divide them by male and female;
3	Place the sealed plastic bags into one of the tanks, allowing them to gradually equilibrate with the water temperature in the respective tank;
4	After 20 minutes, when the temperature levels are harmonised, unseal the bags;
5	Gradually introduce water from the destination tank into each bag (approximately 25% of the bag's volume) – repeat this step at 15-minute intervals and at least three times;
6	Using a fishnet, carefully transfer the fish from the bags to their designated tanks;
7	Monitor the fish closely for signs of stress, such as rapid or heavy breathing;
8	The day following their arrival, offer the fish their first meal. A positive response, with active feeding behaviour, indicates a successful acclimatisation process.

The fish were fed with Tropical® Supervit Granulat once per day throughout the workweek (except for the day before the trial) until they reached a state of satiation (the fish loses interest in the feed), making sure not to overfeed, which would compromise water quality.

Before any trial, the animals underwent a fasting period of at least 24 hours to minimise eventual digestive implications and ensure water quality stability during the procedures. Following each trial, the animals were fed to monitor for any behavioural changes, such as a reduced or lack of appetite, which might indicate potential recovery complications.

The anaesthesia induction started after verifying the temperature of the selected fish tank and a check-up of the fish's behaviour. If normal behaviour was observed and all the fish species' well-being standards (i.e., regular breathing, typical swimming status and normal equilibrium) were in accordance, the anaesthesia procedures could begin.

2. Materials, equipment and chemical substances

The list of materials and equipment used are summarised as follows:

- 1 anaesthesia tank (5 L)
- 1 recovery tank (5 L)
- 2 heaters
- 1 aeration pump
- 1 glass rod
- 1 ruled glass tub
- 1 precision scale
- 1 fishnet
- 1 Gilson pipette P1000
- Pipette tips for P1000

The anaesthesia induction tank and the recovery tank (both of plastic material) had 5 L capacity, but they were filled with up to 4 L, the total volume used for both the anaesthesia and the recovery. The tanks were equally provided with heaters with thermostats to maintain the 27ºC, and the recovery tank had an aeration pump. The induction tank did not have aeration pump during the anaesthesia procedure (only before the introduction of the fish in the tank) to exclude possible interferences in the anaesthesia induction.

After the fish was moved from its origin tank with a fish net to the anaesthesia induction tank, a glass rod was used to mix the water with the anaesthetic agent to prevent it from settling on the bottom of the tank. The glass rod was also used to touch the fish gently to check its reaction to that stimulus (a behavioural parameter for anaesthesia stage monitoring).

Once or if the fish reached surgical anaesthesia, it would be handled over to a ruled glass jar filled with anaesthesia tank water for precise weighing and measuring.

Any quantity of anaesthetic introduced in the induction tank would serve a maximum of 3 inductions (3 different individuals). After the third induction, the tank would be washed and refilled with 4 L, adding the anaesthetic agent to induce the following (up to 3) individuals. This was performed in order to guarantee the same anaesthetic concentration and water quality parameters for every fish.

The anaesthetic agents used were 2-PE (Sigma-Aldrich, ≥ 99%) and CO (Biover, Clove Essential Oil, 100% pure) (Figures 10A and 10B). Stock solutions were prepared using ethanol as solvent to minimise the hydrophobic trait of CO. Ethanol has no known anaesthetic properties on fish at low concentrations (Anderson et al. 1997; Munday and Wilson 1997). Ethylic alcohol 99.9% was acquired from Manuel Vieira & Ca (Irmão), Sucrs., Lda.

A B

Figure 10 - Original flasks of A - 2-phenoxyethanol (Sigma-Aldrich) and B - clove essential oil (Biover).

3. Experimental design

The experiments were designed to obtain and compare adequate anaesthesia protocols for 2-PE and CO in betta fish. The 1st experiment (A) used the anaesthetic 2-PE, and the 2nd experiment (B) had the same logic but tested CO.

For that, sequential trials were conducted to select concentrations, starting with a lower one and gradually raising it depending on the number of surgically anaesthetised fish, taking into account the time of induction and recovery, which should be the lowest possible in both.

The fish were anaesthetised alone in a total of 5 males and 5 females per concentration, except for the lower concentrations since, in these cases, the fish did not even achieve the state of sedation in 10 min, the trials ended to avoid fish stress.

To perform the anaesthesia (Figure 11), the anaesthetic agent was added to the induction tank water and mixed with the glass rod to avoid deposition in the bottom of the tank. The induction and recovery tanks were both at 27°C, like the tanks of the rack system.

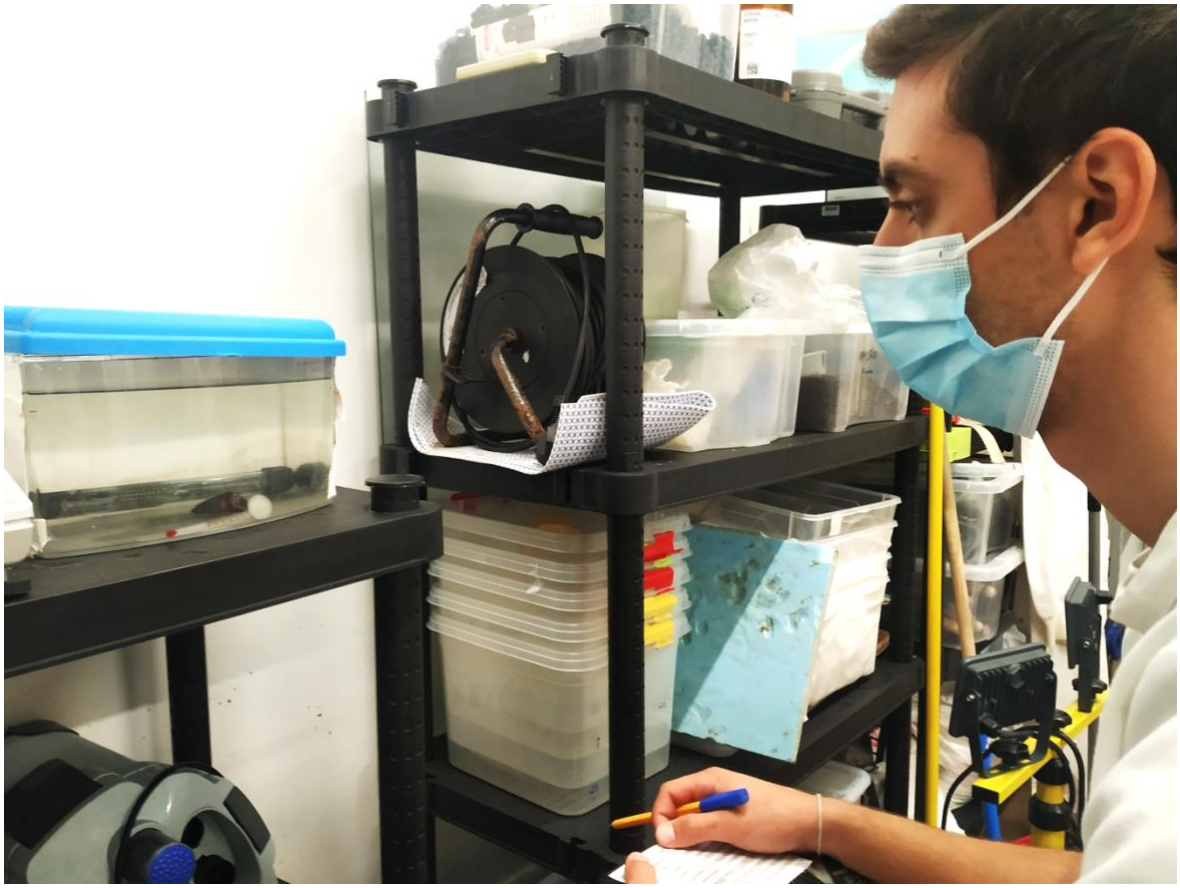


Figure 11 - Anaesthesia procedure. The tank with the fish being anesthetised is at the left, over the bench.

3.1. Experiment A: 2-phenoxyethanol

Before starting, it was confirmed that the temperature of the origin tank was equal to that of the anaesthesia induction tank and the recovery tank (all three needed to be at 27°C). The fish to test was randomly selected from the acclimated stock. The anaesthesia trial induction would start by moving the fish with a net from its tank of origin to the induction tank with the 2-PE mixed with 4 L of dechlorinated freshwater (by evaporation) at the selected concentration.

A registration sheet (Table 4) was used to annotate the timing of occurrence of all the behaviours exhibited by the fish during the induction process. The sheet was divided into 5 categories, namely Behaviour, Swimming, Respiration, Equilibrium, and Responsiveness, based on Zahl et al. (2012). These categories were used to determine the stage of anaesthesia at the end of the induction by observation.

Table 3 details the stages/levels of anaesthesia considered in this work.

Table 3 - Anaesthesia stages, from the normal status to death.

Level	Status
0	Normal
1	Light sedation
2	Excitatory stage
3	Light anaesthesia
4	Surgical anaesthesia
5	Deep narcosis
6	Death

Table 4 – Registration table adapted from Zahl et al., (2012) - see table 1 – used to record the behaviours observed during the anaesthesia procedures. “Muscle tone” and “Heart rate” parameters were both analysed on the original table, but in this case were not used.

Factor	Appearance	Induction (min)	Recovery (min)
Behaviour	normal		
	disoriented		
	anaesthetised		
	dying		
Swimming	normal		
	reduced		
	increased		
	stopped		
Equilibrium	normal		
	struggles to maintain balance		
	loss of equilibrium		
Responsiveness	normal		
	slightly reduced		
	normal or exaggerated		
	reacts only to strong tactile stimuli		
	none		
Respiration	normal		
	irregular or increased		
	normal or reduced		
	shallow		
	nearly absent		
	none		

The criteria to determine the stage of anaesthesia were the following (adapted from Zahl et al., (2012)):

Level 0 (Normal) – normal behaviour, swimming, equilibrium, responsiveness and respiration;

Level 1 (Light sedation) – disoriented behaviour, reduced swimming activity (not always present), normal equilibrium and respiration and slightly reduced responsiveness;

Level 2 (Excitatory stage) – excited behaviour, increased swimming activity, struggle to maintain balance, normal or exaggerated responsiveness and irregular or increased respiration;

Level 3 (Light anaesthesia) – anaesthetised appearance, no swimming activity, equilibrium loss, reaction only to strong tactile stimuli and normal or decreased respiration;

Level 4 (Surgical anaesthesia) – similar to level 3 but with no response to strong tactile stimuli and shallow respiration;

Levels 5 was not achieved since the anaesthesia procedures stopped when the animal reached level 4.

The induction time had a limit of 10 minutes – if the fish did not reach the stage of surgical anaesthesia (stage 4), then it would be considered the higher stage achieved, i.e., light sedation, light anaesthesia, and so on. The recovery time was not limited – it only stopped when the fish was fully recovered by observational parameters.

The induction concentration started on 0.20 mL/L of 2-PE, then 0.30 mL/L and after were gradually raised in 0.15 mL/L intervals, finishing in the maximum amount of 1.05 mL/L of 2-PE. Therefore, the concentrations used were: 0.20 mL/L, 0.30 mL/L, 0.45 mL/L, 0.60 mL/L, 0.75 mL/L, 0.90 mL/L, 1.05 mL/L, and 1.05 mL/L with a polystyrene board blocking the water surface. The later variant was not initially planned, but it was introduced in the end because betta fish is an Anabantoid, so it has the particularity of air-breathing, which happened several times during the anaesthesia induction. Therefore, to see if it would be an influencing factor, the anaesthesia inductions with the concentration of 1.05 mL/L were first applied normally, and this concentration was repeated but with a polystyrene board over the water surface to see if the induction time would reduce by impairing the fish to directly contact air. This polystyrene board was only used in the higher concentration (1.05 mL/L of 2-PE) since, by observation, the animals came more to the surface in the higher concentrations. This test was not repeated since it had no apparent observational effects on the induction time but seemed to negatively influence the fish recovery.

During induction, the 2-PE was periodically mixed with the glass rod to avoid deposition. After the induction period, the fish was moved with a net to a ruled glass tub filled with water from the induction tank (containing the anaesthetic to maintain the state of anaesthesia) to be weighed and measured, taking the least time possible in this procedure. The water in the rulled glass tub was returned to the induction tank after the fish weighing and measurement. The procedure mimics what is required in many laboratory experiences: fish weighing and measurement (Figure 12).

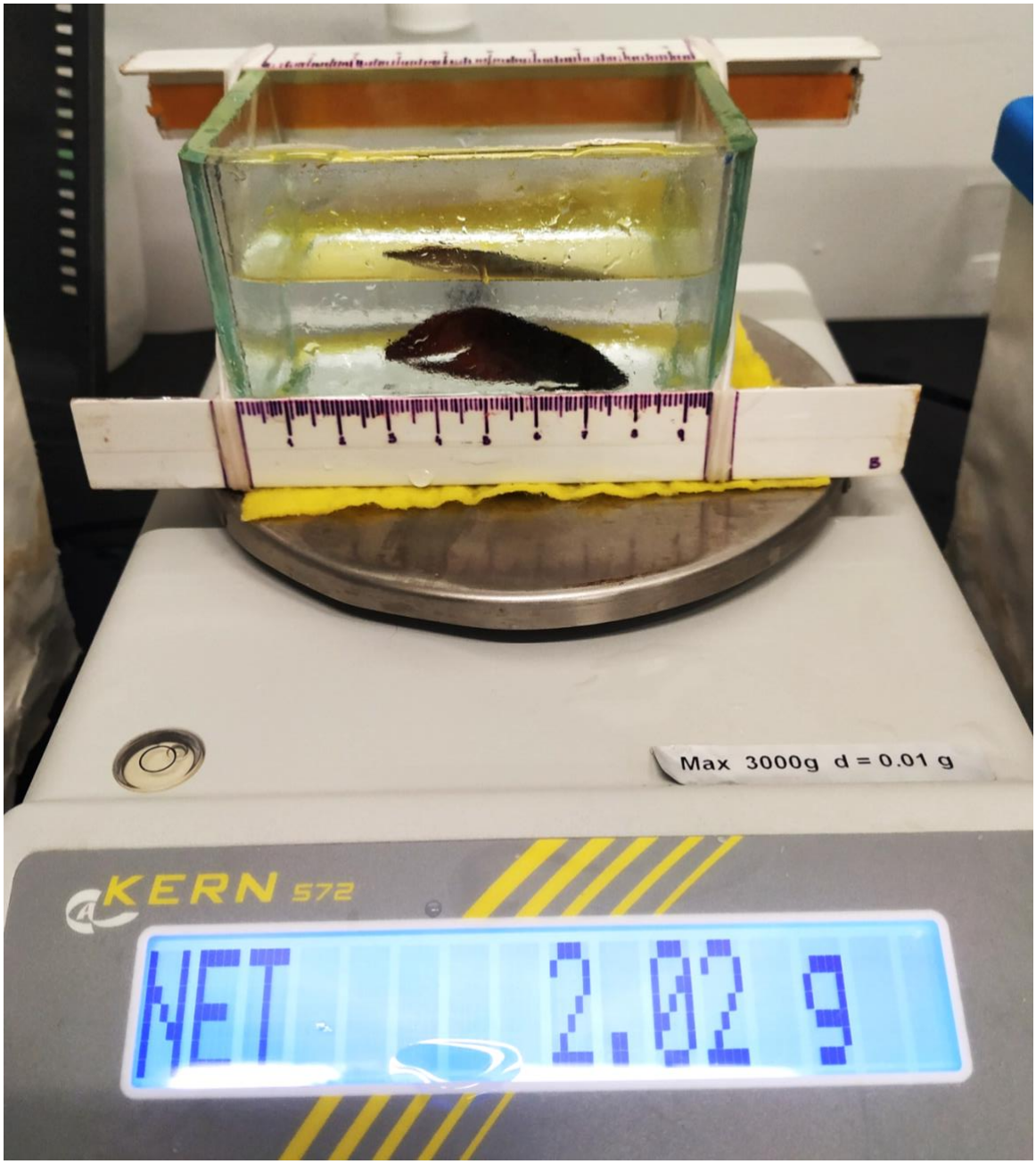


Figure 12 - Anesthetised *Betta splendens* being weighed and measured within a jar placed over a scale.

Next, the fish passed to the recovery tank (which contained an aeration stone to help the recovery through more oxygenation), and the time would start counting with a chronometer immediately after the fish entered the tank. Once the fish was fully recovered, it would return to its tank of origin in the rack. The fish was then observed for 1 to 2 minutes to check for any sign of stress. Typically, the fish behaved as usual soon after inside its tank of origin.

The anaesthetised animals would be fed only half an hour after the induction and only if they showed receptiveness to the feed. Fishes could only be anaesthetised again after 48h.

3.2. Experiment B: Clove Oil

The assay was done as described for Experiment A but recurring to CO. As mentioned, a stock ethanolic solution was prepared to facilitate the preparation of the final anaesthetic solution in the aquaria. For making the stock solution, 2 ml of pure CO was mixed with 8 ml of pure ethanol. Discounting the stock dilution factor, three final concentrations of CO in the water tank were tested: 0.20, 0.30 and 0.35 mL/L. No further dilutions were necessary.

For comparison with other authors that used CO concentrations expressed as mg/L, a conversion was made considering the supplier's information (Biover, Figure 10B). According to the supplier there is 68 mg of CO in 3 drops of the original flask (CO essential oil). Assuming that 20 drops corresponds approximately to 1 mL it was calculated that in 1 mL of the stock solution used (CO + ethanol) there is 90.67 mg of CO.

4. Statistical analysis

The descriptive and inferential analysis used Excel (Microsoft 365), Jamovi (v.2.3.) and the Social Science Statistics (https://www.socscistatistics.com/). The descriptive statistics displayed are for data related only to the aimed anaesthesia level: surgical anaesthesia (stage 4). Nevertheless, Z-score tests for two proportions were performed regarding the number of males and females that reached each anaesthesia stage (from stage 0 to 4).

A two-way ANOVA assessed if the variables sex and anaesthetic concentration (mL/L) might have influenced the induction time (seconds - s) and for the possible effects in the recovery time (s). The post-hoc Tuckey test was performed when the ANOVA unveiled a statistically significant factor to further identify which specific pairs of means differed.

The ANOVA assumptions of normality and the homogeneity of variances were tested with the Shapiro-Wilk normality test and Levene test, respectively. Virtually all data sets fulfilled the assumptions, except for one situation when studying 2-PE, where the homogeneity of variances was not verified despite the normal distribution. Anyway, the parametric ANOVA was performed since this test is very efficient and sufficiently robust to ensure a precise statistical estimate if the sample number is coherent for each tested group (here n = 5 for males and females), even with the lack of a normal distribution or variance homogeneity (Miranda-Fontaiña and Fernández López, 2009; Sabin and Stafford, 1990; Scheffe, 1999).

Chapter 3. Results

1. Proportions of fish reaching each anaesthesia level

The proportions of males and females at each concentration of the anaesthetic agent and the level of anaesthesia (0 to 4) they reached are shown in Tables 5 to 8. The increasing greyscale density reveals the relative number of individuals coming to each anaesthesia stage on a scale of 0 to 1, with 0 for no individuals (0%) and 1 for all individuals (100%).

For 2-PE in males (Table 5), the concentrations 0.20 and 0.30 mL/L were not considered in the analysis of proportions since no individuals went beyond level 0 (normal stage). For the lowest 2-PE concentration triggering effects (0.45 mL/L), most males reached level 3 (light anaesthesia). For the higher concentration (1.05 mL/L), there was more incidence in level 3, and the rest of the fish reached level 4, displaying no cases in stages 0, 1 and 2. For the highest concentration (1.05 mL/L), using the polystyrene board seemed to raise the proportion of fish reaching level 4 of anaesthesia compared to not using that air blocker.

Table 5 - Proportions (0 to 1) of male *Betta splendens* reaching each anaesthesia level in 2-phenoxyethanol (2-PE) displayed concentrations. A greyscale gradient was used to convey an easier perception of where the greater proportions are (lighter grey for lower numbers and darker grey for greater proportions).

	2-PE (mL/L)	Anaesthesia Levels				
		0	1	2	3	4
MALES	0.45		0.2		0.6	0.2
	0.60				0.8	0.2
	0.75		0.2	0.2	0.4	0.2
	0.90			0.2	0.4	0.4
	1.05				0.6	0.4
	1.05*				0.4	0.6

*Direct access of the fish to the water surface was blocked with a polystyrene board.

For 2-PE in females, Table 6 does not include concentrations of 0.20 and 0.30 mL/L for the reasons mentioned for males. Table 6 reveals that for 0.45 mL/L, all fish reached level 3. At a concentration of 0.90 mL/L, all individuals reached level 4. On the highest tested amount (1.05 mL/L), with and without polystyrene on the water surface, most fish reached level 4, and the others got level 3. Overall, scenarios were similar, from 0.75 to 1.05 mL/L.

Table 6 - Proportions (0 to 1) of female *Betta splendens* reaching each anaesthesia level in 2-phenoxyethanol (2-PE) displayed concentrations. A greyscale gradient was used to convey an easier perception of where the greater proportions are (lighter grey for lower numbers and darker grey for greater proportions).

	2-PE (mL/L)	Anaesthesia Levels				
		0	1	2	3	4
FEMALES	0.45				1	
	0.60			0.2	0.4	0.4
	0.75				0.4	0.6
	0.90					1
	1.05				0.4	0.6
	1.05*				0.4	0.6

*Direct access of the fish to the water surface was blocked with a polystyrene board.

Table 7 shows the proportion of males exposed to different concentrations of CO and the respective stages of anaesthesia they reached. At the lowest concentration (0.20 mL/L), 80% of the animals reached stage 3, and the remaining attained stage 2 only. For the concentrations 0.30 and 0.35 mL/L, all males reached stage 4.

Table 7 - Proportions (0 to 1) of male *Betta splendens* reaching each anaesthesia level using clove oil at the three tested concentrations. A greyscale gradient was used to convey an easier perception of where the lowest and highest proportions are (lighter grey for lower numbers and darker grey for greater proportions).

	Clove Oil (mL/L)	Anaesthesia Levels				
		0	1	2	3	4
MALES	0.20			0.2	0.8	
	0.30					1
	0.35					1

Table 8 exhibits the data from females exposed to CO. Overall, the pattern is similar to that of males, with 0.20 mL/L able to induce stages 2 and 3, with most reaching the latest level. The 0.30 and 0.35 mL/L concentrations offered the same outcome for males, with all females entering level 4.

Table 8 - Proportions (0 to 1) of female *Betta splendens* reaching each anaesthesia level using clove oil at the three tested concentrations. A greyscale gradient was used to convey an easier perception of where the lowest and highest proportions are (lighter grey for lower numbers and darker grey for greater proportions).

	Clove Oil (mL/L)	Anaesthesia Levels				
		0	1	2	3	4
FEMALES	0.20			0.4	0.6	
	0.30					1
	0.35					1

Table 9 indicates the possible differences between the proportion of males and females at the lower and higher concentrations of 2-PE in the intervals of 0.45 to 0.75 mL/L and 0.90 to 1.05 mL/L, respectively, for each anaesthesia level reached. Stage 0 was not considered in table 9 since no fish stayed on this level. Stage 1 only considers males as no females reached level 1. No significant differences were observed between the lower and higher concentrations of 2-PE and the proportion of males and females for each stage of anaesthesia, except for the block of males and females for the stage 4 (p=0.016).

Table 9 – Z-tests for the proportions of groups of males and females (individually and grouped) for two blocks of 2-PE concentrations - 0.45 mL/L to 0.75 mL/L and 0.90 mL/L to 1.05 with the polystyrene board and the stage of anaesthesia. The proportion is in number of fish that reached the given stage of anaesthesia divided by the total number of fish anaesthetised in that block of concentrations. P-values are given and considered significant when < 0.05.

Stage of anaesthesia	Parameter	2-PE concentration (mL/L)		p-value
		0.45 - 0.75	0.90 - 1.05+poly	
4	♂+♀	8/28	18/30	**0.016**
4	♂	3/15	7/15	0.121
4	♀	5/13	11/15	0.063
3	♂+♀	16/28	11/30	0.119
3	♂	9/15	7/15	0.465
3	♀	7/13	4/15	0.142
2	♂+♀	2/28	1/30	0.516
2	♂	1/15	1/15	1
2	♀	1/13	0/15	0.276
1	♂+♀	2/28	0/30	0.136
1	♂	2/15	0/15	0.144
1	♀	N.a	N.a	N.a

*N.a - Not applicable

In Table 10 are the possible differences between the proportion of males and females at the lower and higher concentrations of CO in the concentration of 0.20 mL/L and the concentration range of 0.30 to 0.35 mL/L, respectively, at each stage of anaesthesia. Stage 0 and 1 are not represented on the table since no fish stood in those levels. Data revealed

significant differences in CO concentration of 0.20 mL/L and the interval of CO concentration of 0.30 to 0.35 mL/L in almost all stages of anaesthesia for the proportions of males and females, individually and grouped, except for the males in stage 2, where there were no significant differences (p=0.144). The lower concentration of CO (0.20 mL/L) differed significatively from the higher concentrations (0.30 and 0.35 mL/L) for the stage 4 of anaesthesia, for both males and females (p=0,0001). At the concentration of 0.20 mL/L no fish reached stage 4 of anaesthesia while on the concentrations of 0.30 and 0.35 mL/L all fish reached stage 4.

Table 10 - Z-tests for the proportions of groups of males and females (individually and grouped) for the CO concentration of 0.20 mL/L and the concentration range of 0.30 to 0.35 mL/L and the stage of anaesthesia. The proportion is in number of fish that reached the given stage of anaesthesia divided by the total number of fish anaesthetised in that block of concentrations. P-values are given and considered significant when < 0.05.

Stage of anaesthesia	Parameter	CO concentration (mL/L)		p-value
		0.20	0.30 + 0.35	
4	♂+♀	0/10	20/20	**< 0.00001**
4	♂	0/5	10/10	**0.0001**
4	♀	0/5	10/10	**0.0001**
3	♂+♀	7/10	0/20	**< .00001**
3	♂	4/5	0/10	**0.001**
3	♀	3/5	0/10	**0.006**
2	♂+♀	3/10	0/20	**0.010**
2	♂	1/5	0/10	0.144
2	♀	2/5	0/10	**0.032**

2. Induction and recovery times

The induction and recovery times for each considered concentration of 2-PE and CO are described in Tables 11 and 12. Only the animals that reached the surgical anaesthesia (level 4) were evaluated. Data in the Tables grouped males and females, but the statistics tested the effects of concentration and sex in the speed of induction and recovery.

For statistics, the lowest 2-PE concentrations were grouped and data are given in Table 9 as follows: group 1 (G1), from 0.45 mL/L to 0.75 mL/L; group 2 (G2) for 0.90 mL/L; group 3 (G3) for 1.05 mL/L and group 4 (G4) for 1.05 mL/L with polystyrene board (Table 9). The relevant CO concentrations were 0.30 mL/L (G1) and 0.35 mL/L (2) (Table 10).

As to the induction time, the mean values were below the maximum defined target here, 10 minutes. For 2-PE, the mean was from 7 to 8 min up to the 0.90 mL/L concentration and

from 6.7 to 7.2 min in two conditions for 1.05 mL/L (Table 9). Regarding CO, the induction time was approximately 5 min, irrespective of the concentration (Table 10).

Regarding recovery, for 2-PE, the mean ranged from 8.4 min for the lowest concentrations to 9.3 min under 1.05 mL/L without covering the water surface, but climbing to almost 15 min with 1.05 mL/L but impairing the fish to capture air by directly gulping at the surface.

Table 11 - Induction and Recovery times for the different concentration groups of 2-phenoxyethanol (2-PE). – group 1 (G1): 0.45 - 0.75 mL/L 2-PE; group 2 (G2): 0.90 mL/L 2-PE, group 3 (G3): 1.05 mL/L 2-PE and group 4 (G4): 1.05 mL/L 2-PE + polystyrene board (poly). Time in minutes (min).

2-PE	Induction Time (min)	Recovery Time (min)
G1	7.47 ± 1.45	8.38 ± 3.13
G2	7.72 ± 2.40	10.53 ± 3.45
G3	7.18 ± 2.22	9.30 ± 1.97
G4	6.65 ± 2.83	14.70 ± 6.02

*All values are presented as mean ± standard deviation (n = 26).

Table 12 - Induction and Recovery times for the different concentration groups of clove oil. – group 1 (G1): 0.30 mL/L; group 2 (G2): 0.35 mL/L. Time in minutes (min).

Clove Oil	Induction Time (min)	Recovery Time (min)
G1	5.12 ± 1.92	14.33 ± 4.20
G2	5.18 ± 1.55	22.97 ± 6.78

*All values are presented as mean ± standard deviation (n = 20).

The resulting significance (p-values) from the two-way ANOVA when testing the 2-PE are exposed in Table 13. There were no significant effects of concentration, sex or interaction for the induction time, but 2-PE levels significantly shaped the recovery time (p = 0.041).

Table 13 - Two-way ANOVA evaluation of the effects of 2-phenoxyethanol (2-PE) concentration and sex on the induction and recovery times (seconds - s). P-values are given and considered significant when < 0.05.

Time (s)	Two-way ANOVA (P-values)		
	2-PE	2-PE * Sex	Sex
Induction	0.702	0.574	0.670
Recovery	**0.041**	0.441	0.228

After observing the significative influence of the 2-PE concentration in the recovery time, a post-hoc Tuckey test evidenced a significant difference between groups G1 and G4 (p = 0.030), which shows that the use of 1.05 mL/L with the lid of polystyrene board (Poly) increased the recovery time when compared to lowest concentrations (0.45 to 0.75 mL/L).

The G2 and G3 are intermediate groups, not statistically differing from G1 and G4. Recovery times are reproduced as a box blot to better visualize variability and group distances (Figure 13).

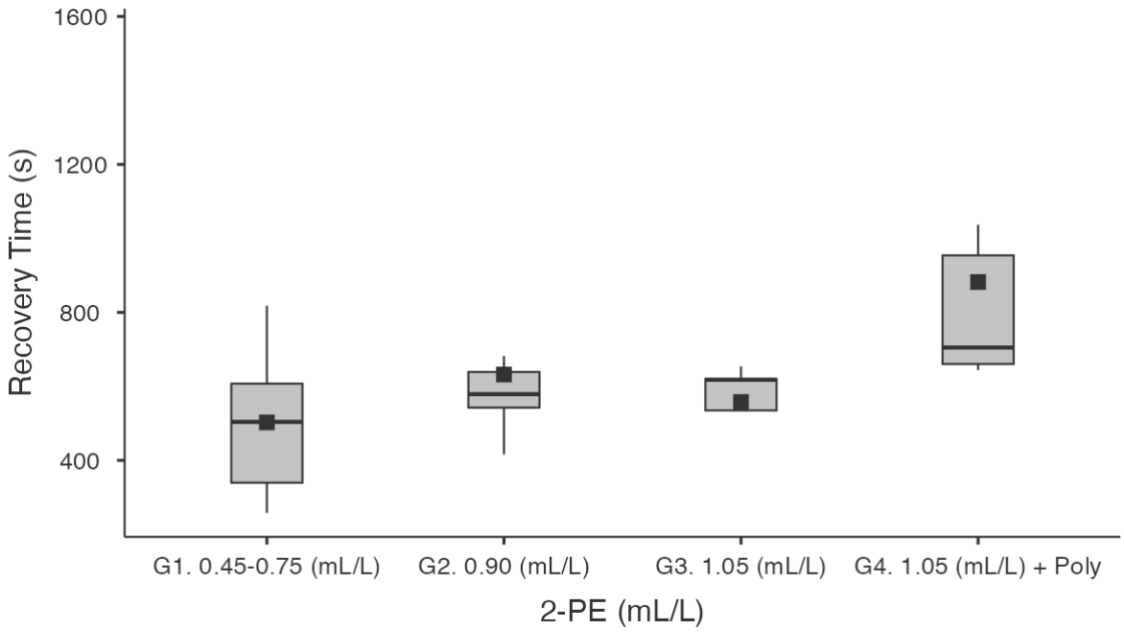


Figure 13 - Box-plot of recovery time (s) under the diverse 2-phenoxyethanol (2-PE) concentrations. In addition to the median, interquartile range, maximum and minimum, the mean is represented by black squares.

The possible influence of CO concentration and sex on the induction and recovery times was analysed, and a significant effect of sex on the induction time (p = 0.036) was observed (Table 14). Significant results were also observed for the CO concentration on recovery time (p=0.002) as well as sex on the recovery time (p=0.022) (Table 14). There were no interactions between the drug used and sex on induction or recovery time.

Table 14 - Two-way ANOVA evaluation of the effects of clove oil concentration and sex on the induction and recovery times (seconds - s). P-values are given and considered significant when < 0.05.

Time (s)	Two-way ANOVA (P-values)		
	Clove Oil	Clove Oil * Sex	Sex
Induction	0.923	0.120	**0.036**
Recovery	**0.002**	0.912	**0.022**

A Tuckey post-hoc analysis was performed to check the significant differences in each CO induction and recovery group. A distinction was confirmed between males and females for induction time, with females taking more time to reach surgical anaesthesia (p = 0.036).

Induction times are reproduced as a box blot to better visualize variability and group distances (Figure 14).

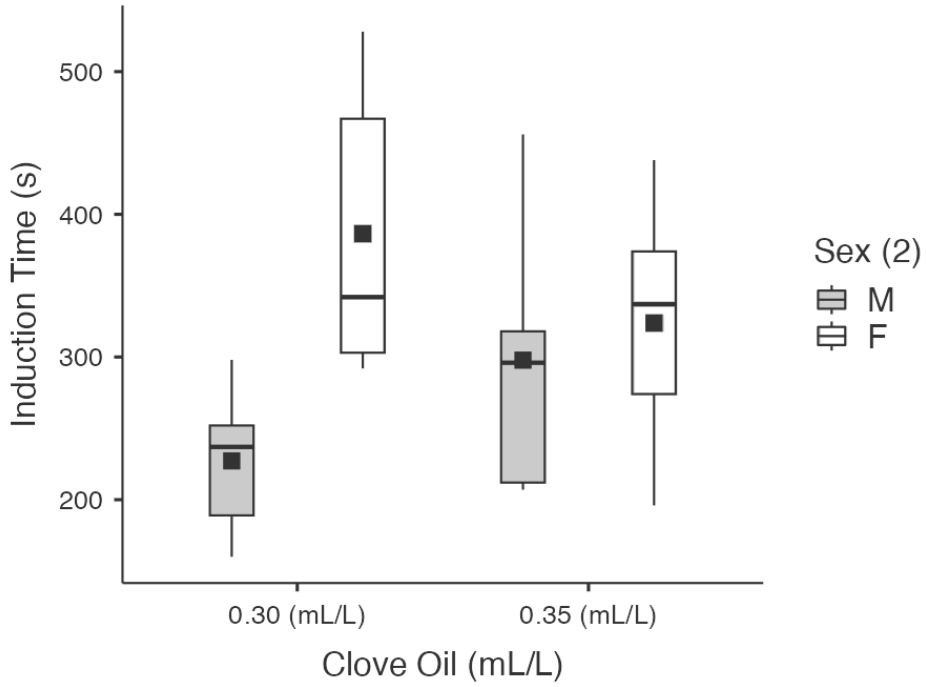


Figure 14 - Box-plot of induction time with clove oil by concentration and sex. In addition to the median, interquartile range, maximum and minimum, the mean is represented by black squares.

Furthermore, a post-hoc analysis confirmed the significant differences in the ANOVA. It unveiled a significant sex difference ($p = 0.022$) and also in the CO concentration ($p = 0.002$), so both factors influenced the recovery time of CO anaesthesia (Figure 15).

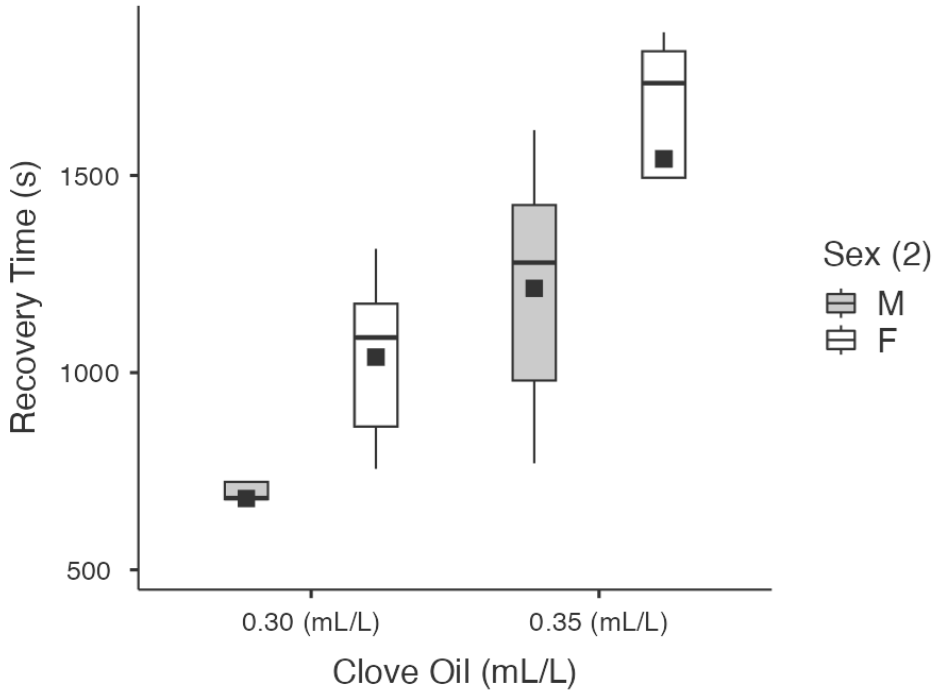


Figure 15 - Box-plot of recovery time with clove oil by concentration and sex. In addition to the median, interquartile range, maximum and minimum, the mean is represented by black squares.

3. Supplementary data

Supplementary tables (Tables S1 to S6) containing the raw, detailed data sets collected in the assays and used for the statistical analyses are provided for optional consultation. The tables are provided at the end of the manuscript in the section Supplementary Material.

Chapter 4. Discussion and Conclusions

Scientific research has a vast range of study fields. Still, many of them require the use of fish model organisms, such as the betta fish (also known as Siamese fighting fish or simply betta), a valuable model for fields like neurobiology, genetics, endocrinology, behaviour and even developmental biology and evolution (Lichak et al., 2022). Moreover, works regarding genes responsible for colour, studies on fin regeneration and analyses of the neural and behavioural correlations of social competition have used betta as a fish model organism (Dupeyron and Wallace, 2023; Zhang et al., 2023).

The use of fish in scientific research needs handling practises, and therefore, to measure parameters like length and weight with precision, immobilization while ensuring welfare

conditions is necessary. Consequently, it is essential to use sedatives or anaesthetics to reduce fish stress responses and for euthanasia (Neiffer and Stamper, 2009).

However, despite the increased use of betta fish in research, there is little information regarding anaesthesia protocols suited for the species, where most of the guidelines are generalistic and not based on sound and reproducible species-specific basis, mainly keeping in mind that concentrations of each anaesthetic and length of administration may differ between fish species (Neiffer and Stamper, 2009; Readman et al., 2017).

This work pretended to evaluate and improve protocols for anaesthesia and recovery in both betta males and females for two different anaesthetic agents commonly used in research, 2-PE and CO (Kheawfu et al., 2022; López-Cánovas et al., 2020; Martins et al., 2018; Robertson and Smith-Vaniz, 2010; Tsantilas et al., 2006). A comparison between the effects of both anaesthetics in reducing distress during procedures was aimed to establish the most appropriate agent for the betta fish, in accordance with a species-specific approach, as one anaesthetic agent may be adequate for a given fish species but may not work or cause distress in another (Neiffer and Stamper, 2009; Ross and Ross, 2008; Sneddon, 2012; Valentim et al., 2016; Zahl et al., 2010). An innovative aspect of this study was considering the animal's sex as one variable, which is seldom tackled matter.

In this work, the anaesthesia with 2-PE showed no differences between the induction time and 2-PE concentration (that is, a higher concentration did not necessarily mean a lower induction time and vice-versa), which means that there was not a dose-response relation with the induction time, but rather a marked individual variability (Table 13, Chapter 3). These postulations are reinforced in the proportion analysis in Chapter 3 (see Tables 5, 6 and 9). This inter-individual variability effect in the induction time of anaesthesia has already been suggested in Rairat et al. (2021) for Nile tilapia (*Oreochromis niloticus*), where for several concentrations of both eugenol and 2-PE, no differences were seen in the induction times.

However, the recovery time after 2-PE anaesthesia revealed differences regarding 2-PE concentrations. The highest tested one, with a board of polystyrene blocking the water surface, increased the time of recovery (Table 13 and Figure 13, Chapter 3). A possible explanation for this effect can be because the fish was barred from accessing the water surface for atmospheric air intake (since betta is an Anabantoid, Chapter 1), which may have caused hypoventilation and lower oxygen availability and therefore increased carbon dioxide accumulation, as reported in some studies which indicate the noxious effect of

blocking the water surface in air-breathing fish, intensifying the risk of hypoxemia (Neiffer and Stamper, 2009; Rantin et al., 1998; Rantin et al., 1993).

Hence, using the polystyrene board with the hypothesis that it would help decrease the induction time (and, consequently, reduce distress) did not influence the induction time and caused a longer recovery time, possibly triggering more distress, which is undesirable for ensuring fish welfare. In the author's view, it is inadvisable for an anaesthesia protocol in the target species to fulfil correct anaesthesia procedures according to the guidelines in the literature (Brønstad, 2022; Neiffer and Stamper, 2009).

Along the inductions with the several concentrations of 2-PE in this work, the results showed low effectiveness of this anaesthetic agent to induce the surgical stage of anaesthesia in betta fish, and a recurrent behaviour demonstrated by the fish during the anaesthesia procedures was a pattern of swimming to the surface to obtain atmospheric air. This behaviour was intensified in the higher concentrations of 2-PE. This may have worked as a compensation mechanism to delay the anaesthetic absorption as the anaesthetic agents cause initial distress and provoke an increase in metabolic rate and oxygen consumption, as reported in previous studies (Brønstad, 2022; Martins et al., 2018). Such air-breathing behaviour has been observed in betta male-male aggressive interactions where the males display a typical opercular flaring behaviour, causing amplified oxygen uptake and higher metabolic rate, which leads to increase the air-breathing behaviour in this stressful situation (Alton et al., 2013; Castro et al., 2006), causing possible parallelism with the distress due to the higher anaesthetic concentrations of 2-PE which also causes this higher metabolic rate and oxygen uptake, directing the fish to raise its air-breathing behaviour.

The induction and recovery with CO, however, revealed a different scenario since there were disparities between the lower and the higher concentrations of CO for stage 4 (surgical anaesthesia), in which the two higher concentrations lead all the individuals up to that stage (Tables 7, 8, 10, Chapter 3), revealing the efficacy of those concentrations in successfully induce anaesthesia in bettas and allow their recover. In previous work, concentrations of 10 and 15 mg/L of CO showed effectiveness for bettas sedation (Pattanasiri et al., 2017), while in the present study, it was established that the stage of surgical anaesthesia was achieved with both 0.30 and 0.35 mL/L (27.20 mg/L and 31.73 mg/L, respectively), delineating a range through various levels of anaesthesia for this fish species.

According to results from CO anaesthesia, it seems that although there are no differences between the higher concentrations (0.30 and 0.35 mL/L) in the induction time (despite the

differences between males and females – Table 14 and Figure 14) the ideal concentration is 0.30 mL/L because the higher dose of 0.35 mL/L leads to a higher recovery time, which agrees with the literature (Coyle et al., 2004; Martins et al., 2018).

At a concentration of 0.20 mL/L of CO, the animals presented more instability on the level of anaesthesia reached. However, more males and females reached level 3 of anaesthesia (Tables 7 and 8), and all the other individuals reached stage 2 (excitatory stage). Hence, an intermediate dose, between 0.20 and 0.30 mL/L (a concentration of 0.25 mL/L of CO), may be proposed to induce the fish until level 3 of anaesthesia (light anaesthesia) for less invasive or stressful procedures. This level of anaesthesia or even sedation stage is helpful for numerous purposes, e.g., distribution and transport of smolt or brood stock sorting in aquaculture (Schroeder et al., 2021).

In summary, a concentration of 0.30 mL/L of CO can be suggested to induce stage 4 of betta anaesthesia safely in both male and female fish. This will enable several procedures, such as weighing, transporting, and handling the animal most likely without distress.

This work also showed that the induction and recovery times were higher in females for CO anaesthesia, revealing the influence of sex in this case. The specific impact of sex in anaesthesia induction and recovery is poorly documented or has not yet described any significant impact in fish anaesthesia, despite some works considering the analysis for both males and females (Musk et al., 2020). Nevertheless, in three spotted tilapia (*Oreochromis andersonii*), there were differences in males and females for anaesthesia induction and recovery time (Siavwapa et al., 2022). Most works do not contemplate the animal's sex as an influencing factor, whether because only juveniles of the target species were used or there was the assumption that the sex did not influence the anaesthesia, neglecting sex as a parameter of analysis (Sladky et al., 2001; Tsantilas et al., 2006; Weber et al., 2009; Weyl et al., 1996). Even works that used betta fish as a model did not discriminate if they used males or females or only males, disregarding the possible sex influence as a determining factor in anaesthesia (Fabregat et al., 2015; Pattanasiri et al., 2017).

The dose range of both 2-PE and CO for bettas still lacks information in the literature. The majority of the studies about anaesthetic concentration criteria are not species-specific or do not have data specifically for bettas, focusing more, e.g., in aquaculture fish species (Coyle et al., 2004; Neiffer and Stamper, 2009) and making it more challenging to replicate into an anaesthesia protocol suited for this species. This work emphasises the significance of a suitable and specific anaesthesia protocol for bettas to standardise and make these

procedures more well-known and reproducible to the scientific community studying this species. This study supports the idea that CO is more effective as an anaesthetic agent for bettas than 2-PE, and the recommended dose to achieve level 4 of anaesthesia is 0.30 mL/L of CO. The study warned that each sex may be differentially impacted on occasion.

This study contributed insights on a proper anaesthetic agent for bettas like CO. It can provide a background for further studies to improve the knowledge in bettas anaesthesia and to better understand the individual variability and behaviours demonstrated by this species with these anaesthetic agents. Here were highlighted the findings in line with the objectives of this work. It defined the ideal concentration of the CO for bettas anaesthesia induction and recovery and suggested a range of 2-PE concentrations when compared to both of the anaesthetic agents, in which CO was evinced as the most suited to this species.

As a suggestion, research studies should be addressed to compare more anaesthetic agents for bettas anaesthesia protocols, also considering some extra parameters of analysis such as metabolic heart rate or cortisol levels determination to better infer the distress levels of the fish to a given anaesthetic, as seen in other works, that could provide a more detailed analysis on the anaesthesia procedure and its effects (Ferreira et al., 2022; Føre et al., 2021; Le et al., 2019; Lemos et al., 2023; Yousaf et al., 2022).

This work validates the importance of a species-specific approach to ensure the optimal welfare conditions depending on the species used since a specific anaesthetic agent may suit a given species but not others and, also, even for the same anaesthetic, the ideal concentrations of anaesthesia may differ between species, making further studies essential to obtain species-specific protocols of anaesthesia for each fish models used in research.

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Supplementary material

Table S1 - Times of induction and recovery and doses of 2-phenoxyethanol, for the fishes that reached the stage 4 of anaesthesia (surgical anaesthesia), in seconds (s). Polystyrene – a board of polystyrene was used to block the betta fish access to the tank water surface.

	Dose (mL/L)	Sex	Induction (s)	Recovery (s)
2-phenoxyethanol	0.45	♂	420	258
	0.60	♂	490	340
		♀	429	818
		♀	419	512
	0.75	♂	358	672
		♀	547	338
		♀	586	496
		♀	336	586
	0.90	♂	582	1066
		♂	564	579
		♀	433	416
		♀	522	528
		♀	412	557
		♀	553	596
		♀	172	682
	1.05	♂	358	654
		♂	546	620
		♀	456	535
		♀	241	362
		♀	556	618
	1.05 + Polystyrene	♂	326	704
		♂	246	1556
		♂	552	706
		♀	177	646
		♀	564	644
		♀	527	1037

Table S2 - Mean, median and standard deviation of induction and recovery time, separately for male and female and with both male and female, for the concentrations of 0.45 mL/L and 0.60 mL/L of 2-phenoxyethanol. Time in seconds (s).

	Dose (mL/L)	Sex	Category	Induction (s)	Recovery (s)
2-phenoxyethanol	0.45 mL/L	♂	time (s)	420	258
			mean	420	258
			median	420	258
			standard deviation	0	0
	0.60 mL/L	♂	time (s)	490	340
			mean	490	340
			median	490	340
			standard deviation	0	0
		♀	time (s)	429	818
				419	512

			mean	424	665
			median	424	665
			standard deviation	5	153
		TOTAL ♂ + ♀	mean	446	556.67
			median	429	512
			standard deviation	31.38	197.68

Table S3 - Mean, median and standard deviation of induction and recovery time, separately for male and female and with both male and female, for the concentrations of 0.75 mL/L and 0.90 mL/L of 2-phenoxyethanol. Time in seconds (s).

	Dose (mL/L)	Sex	Category	Induction (s)	Recovery (s)
2-phenoxyethanol	0.75 mL/L	♂	time (s)	358	672
			mean	358	672
			median	358	672
			standard deviation	0	0
		♀	time (s)	547	338
				586	496
				336	586
			mean	489.67	473.33
			median	547	496
			standard deviation	109.82	102.51
		TOTAL ♂ + ♀	mean	456.75	523
			median	452.50	541
			standard deviation	110.89	123.62
	0.90 mL/L	♂	time (s)	582	1066
				564	579
			mean	573	822.50
			median	573	822.50
			standard deviation	9	243.50
		♀	time (s)	433	416
				522	528
				412	557
				553	596
				172	682
			mean	418.40	555.80
			median	433	557
			standard deviation	134.03	87.02
		TOTAL ♂ + ♀	mean	462.57	632
			median	522	579
			standard deviation	133.16	192.00

Table S4 - Mean, median and standard deviation of induction and recovery time, separately for male and female and with both male and female, for the concentrations of 1.05 mL/L and 1.05 mL/L of 2-phenoxyethanol with the use of a polystyrene board to avoid atmospheric air intake by the betta fish (water surface area blocked) . Time in seconds (s).

	Dose (mL/L)	Sex	Category	Induction (s)	Recovery (s)
2-phenoxyethanol	1.05 mL/L	♂	time (s)	358	654
				546	620
			mean	452	637
			median	452	637
			standard deviation	94	17
		♀	time (s)	456	535
				241	362
				556	618
			mean	417.67	505
			median	456	535
			standard deviation	131.42	106.64
		TOTAL ♂ + ♀	mean	431.40	557.80
			median	456	618
			standard deviation	119.08	105.46
	1.05 mL/L + Polystyrene	♂	time (s)	326	704
				246	1556
				552	706
			mean	374.67	988.67
			median	326	706
			standard deviation	129.58	401.17
		♀	time (s)	177	646
				564	644
				527	1037
			mean	422.67	775.67
			median	527	646
			standard deviation	174.37	184.79
		TOTAL ♂ + ♀	mean	398.67	882.17
			median	426.50	705
			standard deviation	155.48	329.97

Table S5 - Mean, median and standard deviation of induction and recovery time, separately for male and female and with both male and female, for the concentration of 0.30 mL/L of clove oil. Time in seconds (s).

	Dose (mL/L)	Sex	Category	Induction (s)	Recovery (s)
Clove oil	0.30 mL/L	♂	time (s)	189	723
				237	803
				252	517
				298	679
				160	682
			mean	227.20	680.80
			median	237	682
			standard deviation	48.37	93.33
		♀	time (s)	467	756
				303	1314
				292	1089
				528	863
				342	1175
			mean	386.40	1039.40
			median	342	1089
			standard deviation	94.22	203.81
		TOTAL ♂ + ♀	mean	306.80	860.10
			median	295	779.50
			standard deviation	109.29	239.32

Table S6 - Mean, median and standard deviation of induction and recovery time, separately for male and female and with both male and female, for the concentration of 0.35 mL/L of clove oil. Time in seconds (s).

	Dose (mL/L)	Sex	Category	Induction (s)	Recovery (s)
Clove oil	0.35 mL/L	♂	time (s)	318	770
				212	1425
				207	980
				456	1615
				296	1279
			mean	297.80	1213.80
			median	296	1279
			standard deviation	90.60	303.79
		♀	time (s)	374	1815
				274	1734
				196	1494
				438	1863
				337	804
			mean	323.80	1542
			median	337	1734
			standard deviation	83.12	390.23
		TOTAL ♂ + ♀	mean	310.80	1377.90
			median	307	1459.50
			standard deviation	87.91	386.28

Table S7 - Mean water quality parameters values during the experimental period.

Parameters	Mean	Standard deviation (SD)
pH	6.660	0.176
O2 (mg/L)	6.000	0
NO3 (mg/L)	3.333	4.714
NO2 (mg/L)	0.063	0.093
$NH3/NH_4^+$ (mg/L)	0.003	0.011
GH (ºD)	6.438	0.998