POLITÉCNICO DE LEIRIA
ESCOLA SUPERIOR DE TURISMO E TECNOLOGIA DO MAR

Anaesthesia with MS-222, 2-Phenoxyethanol and clove oil in doctor fish *Garra rufa* (Heckel, 1843)

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Dissertation Report for obtaining the Master's Degree in Aquaculture

Masters project carried out under the guidance of Doctor Susana Margarida de Freitas Ferreira and co-supervision of Doctor Sílvia Correia Gonçalves Fernandes

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Anaesthesia with MS-222, 2-Phenoxyethanol and clove oil in doctor fish *Garra rufa* (Heckel, 1843)

Escola Superior de Turismo e Tecnologia do Mar – Peniche
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Resumo

Considerando a importância dos peixes *G. rufa* para a aquacultura, comércio de aquários ornamentais, indústria dos *spa* e o turismo termal, sendo o seu bem-estar uma questão ética importante. A anestesia pode ser necessária para minimizar o sofrimento dos animais e sem comprometer o bem-estar dos mesmos, durante os procedimentos de rotina ou mesmo na administração de um tratamento em aquacultura. O presente trabalho verificou a viabilidade do uso do MS-222, 2-fenoxietanol e óleo de cravinho em *Garra rufa* (Heckel, 1843) para esse fim, determinou-se também a concentração efetiva mínima de acordo com o tamanho do corpo do peixe. Portanto, três classes de tamanho de *Garra rufa* (peixes pequenos, médios e grandes) foram submetidas a oito concentrações diferentes de MS-222 (225 a 400 mg L^{-1}, com incrementos de 25 mg L^{-1}), seis concentrações de 2 -fenoxietanol (525 a 900 mg L^{-1}, com incrementos de 75 mg L^{-1}) e quatro concentrações de óleo de cravinho (110 a 170 mg L^{-1}, com incrementos de 20 mg L^{-1}). Os tempos de indução, monitorização e recuperação da anestesia (além de seus respectivos estádios) foram registrados para cada peixe. Durante a fase de monitorização, *G. rufa* foi medido quanto ao comprimento total, pesado, observado para determinação do sexo e também se verificou a ausência de movimentos operculares / contrações musculares, para avaliar a anestesia. Posteriormente, foi avaliado o apetite imediato por alimento e os peixes foram monitorados durante uma semana quanto à ocorrência de mortalidade ou lesão. Todos os peixes recuperaram rápido e bem, sem sequelas visíveis ou mortalidade. Todos os peixes eram do sexo masculino e apresentaram apetite pela comida após anestesia, ingerindo a dose diária total de ração. Quanto aos tempos de indução, monitorização e recuperação, variaram de acordo com o anestésico, concentração e tamanho do peixe. O óleo de cravinho foi o que apresentou os maiores tempos de indução e recuperação, enquanto o MS-222 e o 2-fenoxietanol apresentaram resultados semelhantes. Recomenda-se que, para 29 °C, uma concentração de 300 mg L^{-1} de MS-222 seja usada em peixes pequenos (4,31 ± 0,42 cm e 0,86 ± 0,70 g), enquanto em peixes médios (6,46 ± 0,85 cm e 3,20 ± 1,62 g) deve ser de 350 mg L^{-1} e 375 mg L^{-1} para peixes de tamanho grande (9,42 ± 0,70 cm e 9,74 ± 1,97 g). Em relação ao 2-fenoxietanol, 750 mg L^{-1} para peixes de tamanho grande (9,30 ± 0,67 cm e 10,0 ± 2,06 g) e 825 mg L^{-1} para peixes pequenos (4,53 ± 0,32 cm e 0,75 ± 0,26 g) e peixes médios (6,44 ± 0,90 cm e 3,29 ± 1,62 g).

Da mesma forma, a dose recomendada de óleo de cravinho para *G. rufa* a 29 ° C é de 130 mg L^{-1} para todas as classes de tamanho (4,41 ± 0,26 cm e 0,69 ± 0,19 g para os pequenos, 6,48 ± 0,89 cm e 3,15 ± 1,36 g para os médios e 9,43 ± 0,54 cm e 9,86 ± 1,84 g para os grandes), embora 150 mg L^{-1} também funcionasse bem.

Estas concentrações eram geralmente mais altas do que as descritas para outras espécies de peixes, mesmo aquelas com temperatura da água, tamanho corporal e filogenia semelhantes.

Palavras-chave: Anestesico, Tempo de Indução, Tempo de Monitorização, Tempo de Recuperação, Aquicultura, Ictoterapia, Bem-Estar, Ciprinídeo, Dose, Concentração.

Abstract

Considering the importance of *G. rufa* fish for aquaculture, ornamental aquarium trade, spa industry and thermal tourism, its welfare is an important ethical issue. Anaesthesia may be required to minimize animal suffering and reinforce its well-being, during aquaculture routine procedures or even, treatment administration. The present work verified the viability of using MS-222, 2-phenoxyethanol and clove oil in *Garra rufa* (Heckel, 1843) for that purpose, determined also their minimum effective concentration according to fish's body size. Therefore, three size classes of *G. rufa* (small, medium and large fish) were subjected to eight different concentrations of MS-222 (225 to 400 mg L^{-1}, with increments of 25 mg L^{-1}), six concentrations of 2-phenoxyethanol (525 to 900 mg L^{-1}, with increments of 75 mg L^{-1}) and four concentrations of clove oil (110 to 170 mg L^{-1}, with increments of 20 mg L^{-1}). The times for anaesthesia induction, monitoring and recovery phases (plus of their respective stages) were recorded for each fish. During the monitoring phase, *G. rufa* were measured for total length, weighted, observed to determine the gender and verify the absence of opercular movements/muscle contractions, as to assess anaesthesia. Afterwards, the immediate appetence for food was evaluated and the fish were monitored during a week for the occurrence of mortality or injury. All fish recovered fast and well, without visible sequels or mortality. All fish were males and presented appetence for food after anaesthesia, ingesting the total daily dose of feed. Regarding the induction, monitoring and recovery times, they varied according to the anaesthetic, concentration and fish size class. Clove oil was the one with the longest induction and recovery times, while MS-222 and 2-phenoxyethanol presented similar results. It is recommended that for 29 °C, a MS-222 concentration of 300 mg L^{-1} should be used in small fish (4.31 ± 0.42 cm and 0.86 ± 0.70 g) whilst for the medium fish (6.46 ± 0.85 cm and 3.20 ± 1.62 g) should be 350 mg L^{-1} and 375 mg L^{-1} for the large size fish (9.42 ± 0.70 cm and 9.74 ± 1.97 g). In regard to 2-phenoxyethanol, it is 750 mg L^{-1} for the large fish (9.30 ± 0.67 cm and 10.0 ± 2.06 g) and 825 mg L^{-1} for the small (4.53 ± 0.32 cm and 0.75 ± 0.26 g) and medium fish (6.44 ± 0.90 cm and 3.29 ± 1.62 g). Likewise, the recommended dose of clove oil for *G. rufa* at 29 °C is 130 mg L^{-1} for all size classes (4.41 ± 0.26 cm and 0.69 ± 0.19 g for the small, 6.48 ± 0.89 cm and 3.15 ± 1.36 g for the medium and 9.43 ± 0.54 cm and 9.86 ± 1.84 g for the large) even though 150 mg L^{-1} worked good as well. These concentrations were usually higher than the ones described for other fish species, even those with similar water temperature, body size and phylogeny.

Keywords: Anaesthetic, Induction Time, Monitoring Time, Recovery Time, Aquaculture, Ichthyotherapy, Welfare, Cyprinid, Dose, Concentration

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1. Introduction

1.1 - Anaesthesia

The word anaesthesia comes from a Greek derivation, of the words *an* – “without” and *aisthesis* – “sensation” which combined mean loss of sensitivity (Ross & Ross, 2008; Zahl *et al*., 2012). This theme covers several components such as sedation, immobilisation, unconsciousness (narcosis), amnesia (loss of memory) and analgesia (pain relief) (Zahl *et al*., 2011). According to Zahl *et al.* (2012) sedation is a reduction in sensitivity, which results in tranquillity and calmness. Narcosis (general anaesthesia) causes a state of unconsciousness and amnesia including also immobilisation and pain relief (analgesia).

Generally speaking, anaesthesia is characterized by a specific acting on the peripheral and/or central nervous system in which occurs a suppression of the ation potencial of the nerve cells, through the release of inhibitory neurotransmitters or by the change in plasma membrane permeability, or even a combination of these two elements (Ross & Ross, 2008).

Fish have a basic neural system that confers them the perception of painful stimuli, namely nociception. This has been verified and demonstrated in studies with rainbow trout (*Oncorhynchus mykiss*) (Walbaum, 1792) and goldfish (*Carassius auratus*) (Linnaeus, 1758) (Sneddon 2002, 2003; Sneddon *et al.,* 2003; Dunlop & Laming 2005). When applying procedures that can inflict harm or pain, minimising animal suffering is essential, which is why it is necessary to use anaesthetics with the ability to block nociceptive pathways. Benzocaine, isoeugenol and MS-222 are substances known to have this ability (Zahl *et al*., 2012).

In many parts of the world and for various species of fish, anaesthesia has been used in many processes and procedures, involving their maintenance in aquaculture (Wagner *et al.,* 2002; Coyle *et al.,* 2004; Purbosari *et al.,* 2019). These processes (such as handling, vaccination, transportation, surgery, assessment of health and physical conditions, plus others) can cause long periods of stress, creating negative impacts on the growth, reproduction, health, and survival of fish (Wagner *et al.,* 2002; Iveresen *et al.,* 2003; Mylonas *et al.,* 2005; Ross & Ross 2008; Weber *et al.,* 2009; Perdikaris *et al.,* 2010; Heo & Shin, 2010; Javahery *et al.,* 2012).

Using anaesthesia makes these processes easier, minimizing the risks of handling and alleviating the stress (Ross & Ross 2008; Perdikaris *et al.,* 2010).

The selection of anaesthetics to be used in fish must be made to taking into account the following factors: fast effect on the immobilisation of fish good recovery rates, no toxicity or low levels of hazardousness for the fish, staff and the environment, easiness to administer, low cost, availability and legislation (Marking & Meyer, 1985; Gilderhus & Marking, 1987; Soto & Burhanuddin, 1995; Burka *et al.,* 1997; Cho & Heath, 2000; TrevesBrown, 2000). According to Burka *et al.* (1997) and Ross & Ross, (1999) the efficacy of anaesthetics is conditioned by environmental (*e.g.:* pH, temperature, salinity) and biological factors, such as fish species, individual weight, size, gender, sexual maturity, body condition and composition (*e.g:* lipid content), health status, and stress.

1.2 - Application of anaesthesia

Anaesthetic agents used for general anaesthesia are usually combined with analgesic agents that target nociception (Zahl *et al*., 2012). Resistance and tolerance to anaesthetic action are known to vary between closer phylogenetic species, but also from species to species and even in between individuals of the same species (Hikasa *et al.,* 1986). Given this situation, each fish species reacts differenty to various concentrations from the same anaesthetic. So determining the most effective minimum anaesthetic concentrations is very important issue (Pawar *et al.,* 2011).

The effect of anaesthesia is usually assessed by the time of induction and recovery, reflex reactions to external stimuli and the responsiveness to handling (Zahl *et al*., 2012). Frequently, the higher the concentration used, the shorter will be the anaesthetia induction time. Conversely, there is also an inverse relationship between the induction and the recovery times (Park *et al.,* 2008). Anaesthesia can also be affected by water temperature (Walsh & Pease, 2002), fish size and gender (Woody *et al.,* 2002). Moreover, an anaesthetic should not produce any lasting physiological effects, for which it should be rapidly excreted from the body and must show high water solubility, regardless of its salinity (Ross & Ross, 1999, 2008; Coyle *et al.,* 2004).

1.3 - States of anaesthesia

Exposure of fish to different anaesthetics causes different responses. These responses are influenced according to the species under study, the concentrations used, environmental factors (*e. g.*: temperature and pH) and biological factors (Summerfelt & Smith 1990; Coyle *et al.,* 2004). Anaesthesia ranges from light sedation - to reduce stress during handling and non-invasive procedures to general anaesthesia - used to avoid inflicting pain during surgeries and other invasive procedures (Summerfelt & Smith 1990; Ross & Ross, 2008; Neiffer & Stamper, 2009; Zahl *et al*., 2012). To assess the progress of induction and depth of anaesthesia, different stages are evaluted. To determine these stages during anaesthesia of animals, clinical indicators such as behaviour, activity, corneal reflexes and pupil size, muscle tone, reflexes, respiratory rate, heart rate and blood pressure are chosen. Some of these indicators are difficult to assess in fish, so others may be used used instead, which are mostly based on swimming activity changes, buoyancy balance, respiratory rate and reactions to external stimuli (Zahl *et al*., 2012), as the ones demonstrated in Table I.

Table I – Stages of anaesthesia in fish by Akinrotimi *et al.* (2015).

Stage of	Anaesthesia	Description
Induction	I	Slow swimming
	II	Slight increase in opercula beat frequency
	III	Loss of equilibrium
	IV	Loss of reflexes and movement
	V	Deep anaesthesia, fish lies on one side
Recovery	I	Reappearance of opercula movement
	II	Partial recovery of equilibrium
	III	Irregular balance
	IV	Total recovery of equilibrium
	V	Normal swimming

1.4 - Anaesthesia methods

Immersion anaesthesia is the most commonly used methodology for the administration of anaesthetics in aquaculture and consists of dissolving an amount of the anaesthetic agent in water to obtain the desired concentration (Coyle *et al.,* 2004; Ross & Ross, 2008; Neiffer & Stamper, 2009). Immersion anaesthetics may be water-soluble or insoluble. The insoluble ones are first dissolved in an organic solvent and then diluted in water (Neiffer & Stamper, 2009).

For simple and short-term procedures, the anaesthetic solution is prepared in an aerated container, filled with water from the fish rearing system (Coyle *et al.,* 2004; Ross & Ross, 2008; Neiffer & Stamper, 2009). This water should be changed frequently to ensure the desired anaesthetic concentration (Ross & Ross, 2008; Neiffer & Stamper, 2009). The fish is immersed in the anaesthetic solution until reaching the pretended anaesthesia stage. Afterwards, the fish can be handled or intervened and then transferred to a clean water container, where it will be let to recover. The water used in the recovery container should also be originally from the animals rearing system as well (Ross & Ross, 2008; Neiffer & Stamper, 2009).

During the recovery phase, water should also be renewed frequently to avoid reabsorption of metabolites excreted by anesthetized fish (Ross & Ross, 2008; Neiffer & Stamper, 2009).

The immersion anaesthetics are generally absorbed through the gills, via inhalation as route of administration. While the fish ventilates in the anaesthetic solution, the anaesthetic agent contacts with gills and diffuses into the bloodstream, rapidly reaching the nervous system (Summerfelt & Smith 1990; Ross & Ross, 2008; Neiffer & Stamper, 2009). Nevertheless, many anaesthetics may also be absorbed through the skin of fish (scalless or with few or thin scales). The efficiency of absorption by immersion in gill tissue and skin is directly related to the lipid solubility of the anaesthetic used, as the composition of these organs surfaces contain large amounts of lipids (Neiffer & Stamper, 2009). Afterwards, when the fish is put back into water without anaesthetic, it excretes the agent (or its metabolites) through the gills, kidneys and skin (Ross & Ross, 1999, 2008; Walsh & Pease, 2002). Futhermore, there are also anaesthetics that can be administered orally, intravenously and intramuscularly (Ross & Ross, 2008; Neiffer & Stamper, 2009). Oral anaesthesia is used less frequently, because there are few studies on oral anaesthetics. Even more, the anaesthetic needs to be incorporated into the fish diet, which makes it difficult to apply the exact dose needed to be administered. The rate and degree of absorption are uncertain, as there are no guarantees that the anaesthetic is evenly distributed in the diet (Ross & Ross, 2008; Neiffer & Stamper, 2009), if part of the anesthetic is dissolved and lost in the water, or even if the fish eat the all dose of anaesthetic. This technique is not practical and is not commonly used in aquaculture routines, but can be used in medical or research laboratories (Neiffer & Stamper, 2009).

1.5 - Anaesthetics

There are two types of anaesthetics: non-chemical and chemical (Ross & Ross, 2008; Neiffer & Stamper, 2009). Non-chemical anaesthetics are not used as often but are still a possibility in aquaculture, such as electro-anaesthesia and hypothermia (Coyle *et al.,* 2004; Ross & Ross, 2008).The chemical anaesthesics are the most commonly used anaesthetics in aquaculture, namely MS-222 (tricaine methane sulphonate), 2-phenoxyethanol (ethylene glycol monophenyl ether), clove oil, benzocaine (ethyl-paminobenzoate), etomidate, quinaldine, and quinaldine sulphate (Neiffer & Stamper, 2009; Ross & Ross 2008; Mylonas *et al.,* 2005; Perdikaris *et al.,* 2010; Mercy *et al.,* 2013; Carter *et al.,* 2011).

1.5.1 – Tricaine methane sulphonate

The anaesthetic tricaine methane sulphonate ($C_9H_{11}O_2N + CH_3SO_3H$) is also referred to as MS-222 and is one of the most widely used anaesthetics in poikilotherm organisms worldwide with fast induction and recovery times (Hunn & Allen, 1974; Ross & Ross, 2008; Popovic *et al.,* 2012; Priborsky & Velisek, 2018).

MS-222 is the only one to be approved in the United States by the Food and Drug Administration (FDA) (Coyle *et al.,* 2004; Carter *et al.,* 2011). In Europe, this anaesthetic is licensed by the European Medicines Agency (EMA) to be used on fish but under specific circumstances, depending on each country legislation (Popovic *et al.,* 2012; Priborsky & Velisek, 2018).

Tricaine methane sulphonate is an odourless white crystalline powder with high water solubility (1 g L^{-1} at 20 °C). Is generally used in the immersion anaesthesia method (Priborsky & Velisek, 2018). MS-222 anaesthesia becomes more effective and at the same time, safer for fish when it is neutralized (Neiffer & Stamper, 2009; Priborsky & Velisek, 2018). MS-222 causes drastic changes in pH, acidifying the water, especially in freshwater, whose buffering capacity is lower than the one of saltwater (Ross & Ross, 2008; Neiffer & Stamper, 2009; Priborsky & Velisek, 2018). Therefore, sodium bicarbonate is generally added to buffer the anaesthetic solution, ajusting pH in accordance with fish welfare. Usually, the amount of sodium bicarbonate should be the same as MS-222, as to neutralize the solution (Neiffer & Stamper, 2009; Priborsky & Velisek, 2018). Buffers such as sodium hydrogen phosphate, sodium hydroxide, imidazole, and calcium carbonate may also be used (Stetter, 2001; Küçük, 2010).

MS-222 is absorbed by the gills and skin of the fish, enters the bloodstream and thenis distributed throughout the body (Hunn & Allen, 1974; Summerfelt & Smith 1990; Carter *et al.,* 2011). Afterwards, MS-222 is rapidly metabolized in the liver by acetylation reactions, where primary metabolites are obtained: acetyl conjugates of ethyl m-aminobenzoate (nonpolar) and m-benzoic acid (polar). Its nonpolar metabolites are excreted through the gills, whereas polar metabolites are excreted through the kidneys according to Hunn & Allen, (1974) and Burka *et al.* (1997).

1.5.2 - 2-Phenoxyethanol

The 2-phenoxyethanol (ethylene glycol monophenyl ether, $C_8H_{10}O_2$) is an oily aromatic liquid, lipophilic and colourless (Coyle *et al.,* 2004; Ross & Ross, 2008; Neiffer & Stamper, 2009; Misawa *et al.,* 2014). This anaesthetic is moderately soluble in water but dissolves better in ethanol, and remaining active in the diluted state for at least 3 days (Coyle *et al.,* 2004).

According to the study of Ghanawi *et al.* (2013) the 2-PE is used as an anaesthetic in veterinary medicine and surgery as well as aquaculture. In this area, 2-phenoxyethanol is a popular anaesthetic because of its safety, efficacy, fast induction time, short recovery time (Ghanawi *et al.,* 2013), easy preparation, low price, rapid action and also presents bactericidal and fungicidal properties (Priborsky & Velisek, 2018). In other studies, the suitability and effects of using 2-PE as an analgesic are reported Velíšek & Svobodova, (2004a); Mylonas *et al.* (2005); Weber *et al.* (2009); Yildiz *et al.* (2013), as well as its effectiveness in inducing both light and deep anaesthesia in aquaculture (Misawa *et al.,* 2014; Priborsky & Velisek, 2018). The study of Priborsky & Velisek, (2018) demonstrate the 2-phenoxyethanol is absorbed by the gills and skin being transported by the arterial blood until the central nervous system, being rapidly excreted, primarily via gills, aftwerwards. As reported by Waterstrat, (1999) temperature is a factor that might interfer with affec the effects of this anaesthetic in fish. It was found that when water temperature increases, the efficiency of 2-phenoxyethanol also increases.

1.5.3 - Clove oil

Despite the recent interest in clove oil as a fish anaesthetic (Soto & Burhanuddin, 1995; King *et al*., 2005; Neiffer & Stamper, 2009; Javahery *et al*., 2012; Priborsky & Velisek, 2018), There are restrictions on its use in fish intended for human consumption in some countries. Clove oil is currently not approved by the FDA in the United States of America as a fish anaesthetic (Melton, 2007; Adel *et al.,* 2016).

The European Union has approved maximum residue limits for iso-eugenol (European Commission, 2011). Eugenol is the active compound of Aqui-STM (AQUI-S New Zealand, Lower Hutt, New Zealand), which has been approved as an anaesthetic with no withdrawal period in Australia, Chile, Finland, New Zealand, and the Faroe Islands, but not in the EU or the USA (Hoskonen & Pirhonen, 2004). Clove oil ($C_{10}H_{12}O_2$) is distilled from the flowers, stalks, and leaves of *Syzygium aromaticum* (i.e. *Eugenia aromaticum*) or *Eugenia* caryophyllata. It is a dark brown liquid (Coyle *et al.,* 2004; Ross & Ross, 2008; Priborsky & Velisek, 2018).

Eugenol and Iso-eugenol are active components of clove oil with a representation of 90–95% of its weight (Priborsky & Velisek, 2018). According to Ross & Ross, (2008) the anaesthetic clove oil is easily dispersible in the water by vigorous shaking. But at lower temperatures, it can be prepared as a 10% solution in ethanol. A 10 cm^3 L^{-1} (≈ 10 g L^{-1}) stock solution is still effective after 3 months of storage at room temperature.

In an anaesthetic immersion, the clove oil is absorbed through the gills and skin of fish entering the bloodstream and being distributed through the body (Hunn & Allen, 1974; Summerfelt & Smith 1990). Clove oil is a highly lipophilic substance. So, it adheres and penetrates quickly into the branchial epithelium and, once in the bloodstream, is absorbed into body tissues, such as adipose tissue and central nervous system (Hunn & Allen, 1974; Summerfelt & Smith 1990). This anaesthetic is considered as a good alternative to other fish anaesthetics because it is inexpensive and poses no risk to human health (Perdikaris *et al.,* 2010), however, it also requires a relatively long recovery time compared to MS-222 (Coyle *et al.,* 2004; Neiffer & Stamper, 2009).

This anaesthetic and the others previously mentioned are being used only in non-food fish and in research (Priborsky & Velisek, 2018).

1.6 - Characteristics of *Garra rufa* (Heckel, 1843)

1.6.1 - Morphology of *Garra rufa* (Heckel, 1843)

Garra rufa (Heckel, 1843) belongs to the family Cyprinidae and is one of 73 species that represent the genus *Garra* (Fig. 1, Esmaeili *et al.,* 2009). *G. rufa* is a freshwater fish who has two pairs of barbels, a flat nose tip, a scaleless head, a developed adhesive disc, and has a specific crescent-shaped ventral mouth and is toothless (Jarvis, 2011; Cicek *et al.,* 2016). Its body form is fusiform, slightly flattened in the abdominal part. It has a visible lateral line system, with 31 to 38 scales, from the dorsal to the pelvic fin and the dorsal fin is anterior to the pelvic fin.

The number of rays is different between fins, 12 to 14 rays the pectoral fin, 8 rays in the dorsal fin, 5 on the ventral fin, 7 to 8 rays in pelvic fin and 17 on the caudal fin (Jarvis, 2011). The body of this fish also contains medium and large size cycloid scales (Coad, 2019).

The colour of the *G. rufa*´s body is variable between platinum and sliver grey, but sometimes a few individuals are almost black (Fig. 1). This can be explained by the fact that they can adapt to the brightness and the colours of their surrounding environment. Despite the variation of colour, their body is considered typically grey, except for their abdomen, which is white (Jarvis, 2011; Coad, 2019).

Figure 1 - *Garra rufa* (Heckel, 1843). Source: Aqua Orinoco

The total length of *Garra rufa* has been recorded by some researchers in different countries and water basins where this fish can be found as native species. In Iran, Esmaeili & Ebrahimi, (2006) collected and measured fish with 13 cm, as well as Yalçin-Özdilek & Ekmekçi, (2006) at the Asi Riser (Orontes), Turkey.

1.6.2 - Biogeography and ecology

Garra rufa is geographically distributed across southern Asia, northern and central middle east (Jarvis, 2011; Jayasree *et al.,* 2016; Froese, 2019). It can be found in countries like Israel, Jordan, Iran, Syria, Iraq and the regions around the Tigris and Euphrates River systems (Fig. 2) (Cicek *et al.,* 2016).

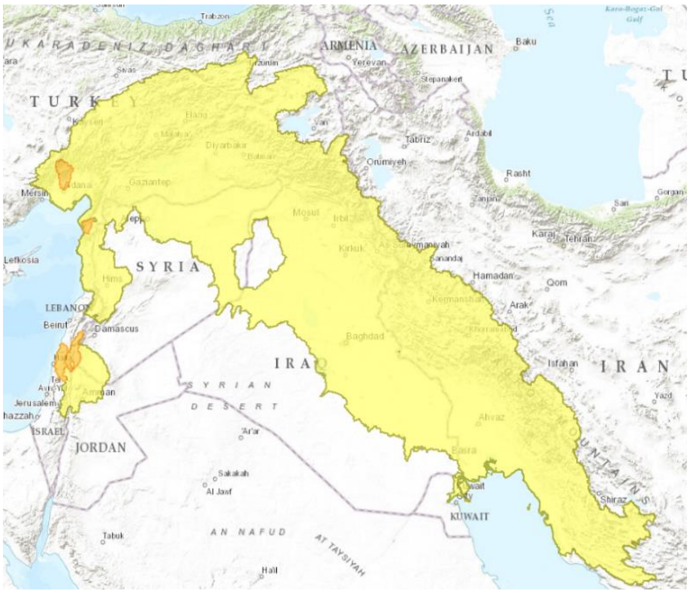

Figure 2 – Geographic distribution of the species *Garra rufa* (Heckel, 1843). Source: IUCN

The doctor fish is a sub-tropical, benthopelagic and a non-migratory species,who lives in rivers, lakes, and small lagoons (Ruane *et al.,* 2013). They can withstand fast waters and live in places with strong currents, such as rivers running from mountains. This ability is due to their adhesive disc, present near the mouth, which allows them to grab to a hard substrate (Keivany *et al.,* 2016).

Some studies refer that *Garra rufa* lives in aquatic ecosystems whose environmental parameters can be described as the following: temperature between 15 and 31 °C, water depth from 30 to 50 cm, oxygen dissolved among 6.1 and 14.8 mg L^{-1}, salinity ranging from 0.10-0.80, water velocity until 4.5 m s^{-1} and pH between 7.0 and 9.0 (Jarvis, 2011; Cicek *et al.*, 2016). This fish is also refered as capable of surviving in environments contaminated with trace metals, demonstrating to be resilient to dry periods and destruction of their habitat, being able to withstand more easily variations of temperature than oxygen (Gümgüm *et al.,* 1994; Yazdanpanah, 2005; Jarvis, 2011; Özçelik & Akyol, 2011).

1.6.3 - Feeding habits

Garra rufa is considered an omnivore species. Once in their natural environment, it feeds on macroalgae, phytoplankton, zooplankton and detritus (Teimori *et al.,* 2011; Froese, 2019). According to the study of Yalçin-Özdilek & Ekmekçi, (2006) gut content analysis on fish caught in Turkey showed the presence of bacteria and algae like Cyanobacteria, Chrysophyta and Chlorophyta (*Navicula sp*. and *Gomphonema sp.*), Rotifers and Protozoa. *Garra rufa* uses its adhesive disc for feeding, as the mouth pads stick to the substrate and the disc scratches the algae from a hard surface (Zhang, 2005; Teimori *et al.,* 2011; Ruane *et al.,* 2013). This species has as recognised predators, the European eel *Anguilla anguilla* (Linnaeus, 1758), the African catfish *Clarias gariepinus* (Burchell, 1822 and other cyprinids, like *Carasobarbus canis* (Valenciennes, 1842) (Jarvis, 2011; Froese, 2019).

1.6.4 - Reproduction

Garra rufa individuals are gonochoric, with no records of simultaneous or sequential hermaphroditism (Jarvis, 2011). Spawning occurs once a year during spring, between March to June in Iraq and between May to September in Iran (Ünlü, 2006; Patimar *et al.,* 2010; Abedi *et al.,* 2011). Males and females of *G. rufa* are very similar to each other which makes gender separation difficult. Nevertheless, this species has sexual dimorphism, due to the presence of white tubercles on the males, displayed temporarily uring the breeding season (Fig. 3) (Fowler & Steinitz, 1956; Jarvis, 2011). These tubercles are rounded dermal formations between the eyes and the nostrils (Coad, 2019).

Figure 3 – Male of *Garra rufa* (Heckel, 1843) in the breeding season. Source: Pro Aquarium

Males may acquire a brighter colour during the breeding season, being slightly larger than females. Females may have pectoral, pelvic and anal fin bases different from males during the spawning period (Coad, 2019). *G. rufa* can be considered a broadcast spawner, as breeding individuals execute postures on open substrate and do not protect their eggs after spawning being completed (Ünlü, 2006; Jarvis, 2011).

Sexual maturity of *G. rufa* varies according to different populations. In Iraq, its lifespan is 2 or 3 years for individuals with 10 cm total length and a weight of 50 g (Al-Rudainy, 2008), while in Iran, *G. rufa* has a lifespan of 4 years, in which the older individulas reach 15 cm of total length and weight of 9 g (Abedi *et al.,* 2011).

1.6.5 - Ichthyotherapy

This species is also known under the trade name doctor fish, due to the use of this fish in therapeutic treatments that go by the designation of Ichthyotherapy (Sayili *et al.,* 2007; Jarvis, 2011; Jayasree *et al.,* 2016). Ichthyotherapy (Fig. 4) is a treatment in which *G. rufa* uses its developed adhesive disc and mouth to remove dead skin cells from a human individual, around a specific part of the body in which a skin problem needs to be cured (Wildgoose, 2012). There are evidences that at the end of an ichthyotherapy treatment with *G. rufa*, skin becomes smooth and clean (Church, 2013).

Figure 4 - Ichthyotherapy session with *Garra rufa* (Heckel, 1843). Author: Adriana Gaveta.

This therapy has been used in diseases like psoriasis, eczema, acne, and other skin disorders (Grassberger & Hoch, 2006; Sayili *et al.,* 2007; Church, 2013).

Ichthyotherapy started in central Anatolia, Turkey, more specifically in the Kangal hot springs, which have been world-renowned since 1989 (Özçelik & Akyol, 2011; Majtán *et al.,* 2012; Church, 2013). Two species of fish coexist in this hot springs: the Tigris kingfish

Cyprinion macrostomus Heckel, 1843 and *G. rufa.* Both fish are members of the carp family Cyprinidae (Jarvis, 2011; Özçelik & Akyol, 2011; Church, 2013; Majtán *et al.,* 2012).
The temperature of Kangal hot springs varies between 35 °C to 37 °C (Özçelik & Akyol, 2011; Church, 2013). Few aquatic primary producers flourish in this system at these high temperatures, for which a food web problem arises. *C. macrostomus* and *G. rufa* fish feed on phyto and zooplankton scarcing in these waters. So, they may adapt their diet and feed on the skin of other vertebrates that come in contact with them in the water (Özçelik & Akyol, 2011; Church, 2013). Both fish are used to clean, soften and help treat people's skin, while they feed on epithelial cells. However, *G. rufa* is considered the principal therapeutic species, once it bares no teeth that could inflict harm to others subjecting themselves to their nibbling. Due to its recognised success in the Kangal hot springs, *G. rufa* has been propelled to be used in this type of treatments, not only in Kangal, but in *spas* worldwide. Nowadays, *G. rufa* is considered the key species for Ichthyotherapy (Özçelik & Akyol, 2011; Church, 2013).

In *spas* or thermal pools, some caretakers proved small amounts of food to *G. rufa*, in order to keep them interested in nibbling and feed of epithelial cells and thus, in interacting with their human patients. Deprived of a normal balanced diet and, sometimes of a proper quality aquatic environment, these animals evidence slow growth rates, low physical and health conditions, that might even revert to a more aggressive and predatory behaviour (Sayili *et al.,* 2007; Wildgoose, 2012). By adopting this type of procedure, animal welfare is undermined. Besides affecting its growth, fish can die without a proper diet (Wildgoose, 2012). The concepts of animal welfare incorporate physical, physiological and mental states of each animal (Segner *et al.,* 2019). The definition of animal welfare is based on nature, functions and feelings.
According to Segner *et al.* (2019), the nature-based definition considers animal welfare in good order if the animal can engage in natural behaviour. With this concept, it will be difficult, if not impossible, to obtain a good welfare status for farmed fish. The function-based concept considers well-being satisfied if the animal is in good health and shows normal biological functioning and growth. Factors that affect fish welfare are water quality, high stock densities in tanks, handling, stress, transport and diseases (Segner *et al.,* 2019). Water quality is one of the most critical factors for fish welfare and needs to be monitored more often. Poor water quality, or sudden changes in water parameters, can lead to acute and chronic health and well-being problems. The parameter values for water quality are specific to each species. Water quality parameters include temperature, conductivity, pH, oxygen concentration, nitrogenous compounds concentration assessment (ammonia, nitrite and nitrate) and much more (Segner *et al.,* 2019).

Optimal stocking densities endorsing fish welfare depend on biological factors, such as species or life stage, and also on technical factors, such as water flow rates. Stocking density is limited by water quality and thus, has to be adjusted to maintain optimal water quality (Segner *et al.,* 2019).

Handling is necessary throughout fish production cycle. As fish are very sensitive to handling, this should be done as fast as possible, minimizing animal discomfort. All equipment used for handling must be in good hygiene and operational conditions (Segner *et al.,* 2019).

Prophylaxis of the disease is also an important aspect of fish welfare. Any introduction of disease agents should be avoided, as it can occur through the transfer of infected fish, the use of contaminated equipment or personnel. This also includes separating infected fish during a disease outbreak and removing any dead animals from the rearing system (Segner *et al.,* 2019). But, an effective measure of disease prophylaxis is to use vaccination of fish against bacterial and viral diseases, in order to prevent an outbreak of disease (Segner *et al.,* 2019).

1.6.6 - Ecological Status

Garra rufa fish are considered as a least concern species by the International Union for Conservation of Nature (IUCN, 2019). Nevertheless, it is referred that there is a decreasing population trend, mostly of mature individuals, due to many ongoing threats. Moreover, it is not evaluated by Convention on International Trade in Endangered Species of Wild Fauna and Flora (also known as the Washington convention; CITES, 2017) and Convention on the Conservation of Migratory Species of Wild Animals (CMS, 2015).

In Turkey, *G. rufa* was listed as a vulnerable species, due to significant regional decline caused by overexploitation of the species for therapeutic purposes and exportation, plus the destruction of its natural habitats, being recently protected in legal terms (Baeck *et al.,* 2009; Jarvis, 2011).

1.7 - Objectives

Accordingly, this study pretended to verify the viability of using the anaesthetics MS-222, 2-phenoxyethanol and clove oil in *Garra rufa* fish. Moreover, it was intended to determine the minimum effective concentration to be used with each anaesthetic for this species, as well as to verify if it differed according to *G. rufa*'s body size. Therefore, tree size classes of *G. rufa* (small, medium and large fish) were subjected to eight different concentrations of MS-222 (225 to 400 mg L^{-1}, with increments of 25 mg L^{-1}), six concentrations of 2-phenoxyethanol (525 to 900 mg L^{-1}, with increments of 75 mg L^{-1}) and four concentrations of clove oil (110 to 170 mg L^{-1}, with increments of 20 mg L^{-1}). The times for anaesthesia induction, monitoring and recovery phases were recorded for each fish, as well as the time spent in the entire procedure. During the monitoring phase, *G. rufa* were measured for total length, weighted, observed to determine the gender and verify the absence of opercular movements/muscle contractions. The monitoring phase was carried out for 90 seconds, to assess anaesthesia success for aquaculture routine procedures. The anaesthesia stages observed in *G. rufa*, according to its behaviour, were described and timed within anaesthesia induction and recovery phases. Statistical analyses were performed to search possible correlations between induction and recovery times, according to each size class, or even between opposite stages within each of those phases. Also, linear regressions were assessed to find a possible relation between the induction and recovery times towards fish size (total length and individual wet weight), for each anaesthetic agent. After the anaesthesia procedures, the fish were evaluated also in what concerns their immediate appetence for food and were monitored during a week for the occurrence of mortality or injury.

2. Materials and Methods

2.1 - Acclimatisation and maintenance of *Garra rufa* (Heckel, 1843)

A total of 330 *G. rufa* specimens from 3 size classes (small 4-5 cm, medium 6-7 cm and large 8-11 cm) were selected among the stock kept at the CETEMARES Bioterium (Polytechnic Institute of Leiria, Peniche, Portugal). Their adaptation period took place over 2 weeks and all the individuals were kept in a freshwater recirculation system (RAS), in a controlled temperature room at 25 °C, where they remained until the end of the experimental activity. The systems consisted of three PVC shelves (EA, EB and EC), each with twelve aquariums distributed by three racks and one *sump* (Fig. 5). Each rack was illuminated by an 11W LED track light over four rectangular aerated aquariums of 18 L capacity. Each aquarium was covered with nets fixed with cloth clip springs, in order to avoid the animals from escaping the rearing systems. The water was directed to a 90 L *sump* that contained: aeration, a mechanical filter consisting of two glass wool sponges; a biological filter of Pure Water Bio-ring ceramic rings; an EHEIM reflex UV 350 ultraviolet filter of 14 W (EHEIM GmbH & Co KG, Stuttgart, Germany); a TMC Vecton2 400 skimmer (Tropical Marine Centre, London, UK) with a SICCE Syncra Silent 1.5 pump (700 - 1350 L h^{-1}; Pozzoleone, Italy); an EHEIM Compact + 5000 circulation pump (2500 - 5000 L h^{-1}; EHEIM GmbH & Co KG, Stuttgart, Germany) and an EHEIM JÄGGER heater of 300 W set to 28 ºC (EHEIM GmbH & Co KG, Stuttgart, Germany).

The fish were randomly distributed by rearing systems, with 10 fish of the same size class per aquarium (Fig. 6). In fact only 240 fish were subjected to the experimental procedures. The remaining fish were maintained only as a precaution, to be used in the case that some fish could die or become injured during the experimental procedures and/or eventual attempts to escape from the aquaria. Each rearing system maintained also an empty aquarium to isolate weaken or injured fish, as to protect them from being disturbed by their peers.

Figure 5 - *Garra rufa* (Heckel, 1843) rearing systems.

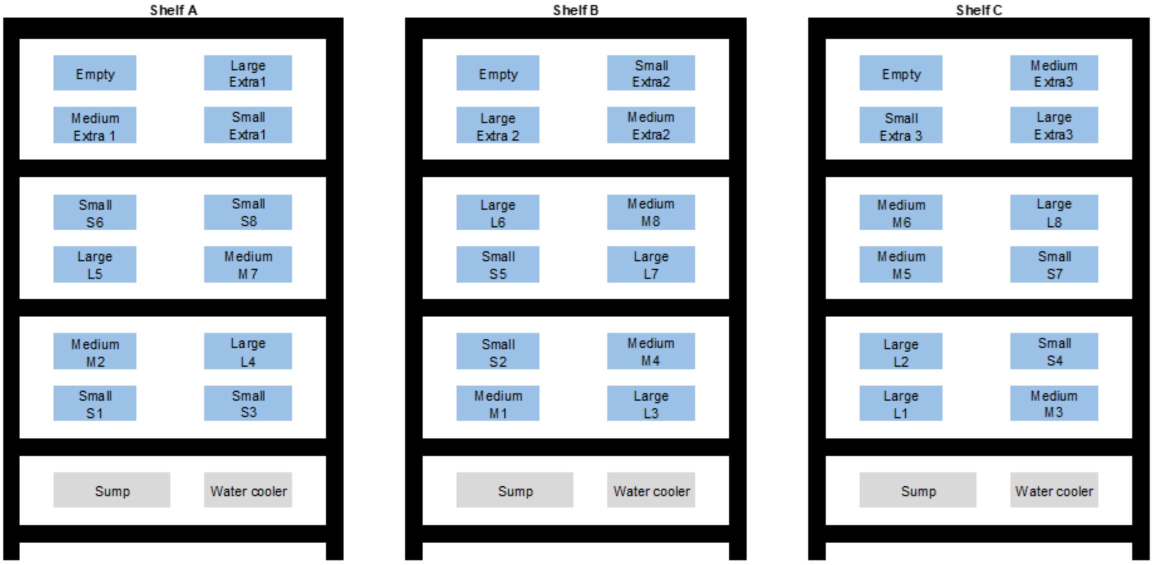

Figure 6 - Schematic distribution of *Garra rufa* (Heckel, 1843) fish in the rearing systems. There were 8 replicate aquaria for each size class and every single aquarium contained 10 fish. Plus, each shelf contained one empty aquarium (to isolate fish that might present small injuries) and three others with 10 fish each, to substitute those that could die or become ill during the procedures.

2.2 - Water quality monitoring

Partial water changes of the rearing systems were made once a week, at about 75 % of their volume. Water quality monitoring was performed three times a week. Environmental parameters temperature (28.90 °C), pH (8.09) and dissolved oxygen (6.94 mg L^{-1}) were measured every day with a YSI Professional Plus handheld multiparameter meter (YSI Incorporated, Yellow Springs, United States of America).

If the water quality parameters were outside the ranges suitable for animal welfare, a partial water exchange was made and environmental parameters were measured again. Additonally, ammonia (< 0.25 mg L^{-1}), nitrite (< 0.25 mg L^{-1}) and nitrate (< 5.0 mg L^{-1})

concentrations were assessed qualitatively with API - Aquarium Pharmaceuticals rapid tests (Mars Fishcare North America, Inc., Chalfont, Pennsylvania, United States of America).

2.3 - Feeding

During the acclimatisation and maintenance, *G. rufa* fish were fed *ad libitum* twice a day with a granulated commercial feed Dr. Bassleer Biofish Food Regular M (Aquarium Münster, Telgte, Germany; with 54 % crude protein, 16 % crude oils and fats, 10 % crude ash, 4 % crude fibre, 2 % calcium, 1.5 % phosphorus, 4,230 kcal kg^{-1}).

2.4 - Experimental design

This experimental work was carried out entirely at the CETEMARES bioterium (Polytechnic Institute of Leiria, Peniche, Portugal), following the European legislation (Directive No. 2010/63 of the European Parliament, Portuguese Decree-Law No.113/2013). This work studied the use of three substances (MS-222, 2-phenoxyethanol and clove oil) to anaesthetise *G. rufa* from 3 different size classes at their mean rearing temperature (29 °C). Ten fish from a random aquarium within the three rearing systems were chosen to induce anaesthesia, using one concentration of an anaesthetic. The three sizes classes were subjected to the same combination of anaesthetic × concentration in the same day. Previous studies showed that the concentrations 425 mg L^{-1} of MS-222 (Ferreira *et al.,* 2015a), 825 mg L^{-1} of 2-phenoxyethanol (Ferreira *et al.,* 2015b) and 130 mg L^{-1} clove oil (Ferreira *et al.,* 2016), were the most suitable to anaesthetise small *G. rufa* (4-5 cm of total length). However, those studies did not use controlled temperature of the water, for which it dropped from 29 °C (recommended temperature) to a room temperature of approximately 20 °C, during the anaesthetic procedures. As higher temperatures usually potentiate the effects of anaesthetics (Prince & Powell, 2000; Walsh & Pease, 2002; Hoskonen & Pirhonen, 2004), a concentration immediately lower to the recommended ones was firstly used to anaesthetise *G. rufa*. Its results were evaluated, so as to decide which concentration should be used afterwards. The concentrations of the anaesthetics were increased or reduced, accordingly to the induction and recovery times observed, in order to comply with those recommended (3 minutes for induction and and less 10 minutes, respectively; Gilderhus & Marking, (1987) and Ross & Ross, (2008). In an effort to abide by the concept of animal welfare (Directive 2010/63/Eu, Portuguese Decree-Law No. 113/2013), whenever a certain anaesthetic concentration became unsuited for *Garra rufa* size class, the following increment/reduction dose was not assessed anymore (Table II). Accordingly, the fish were subjected to eight different concentrations of MS-222 (225 to 400 mg L^{-1}, with increments of 25 mg L^{-1}), six concentrations of 2-phenoxyethanol (525 to 900 mg L^{-1}, with increments of 75 mg L^{-1}) and four concentrations of clove oil (110 to 170 mg L^{-1}, with increments of 20 mg L^{-1}). The first anaesthetic to be used was MS-222, then 2-phenoxyethanol and clove oil for last. The fish were left to rest for 1 month in between anaesthetics, in order to eliminate its residues from the organism and avoid synergistic effects (as the withdraw period of 21 days when using MS-222 in fish destined for human consumption (Hoskonen & Pirhonen, 2004; FDA, 2007; Popovic *et al.,* 2012; Priborsky & Velisek, 2018).

Table II - Concentrations of MS-222, 2-phenoxyethanol and clove oil used to anaesthetise three size classes of *Garra rufa* (Heckel, 1843).

	Size class		
Anaestesics	Small	Medium	Large
MS-222			
225 mg L^{-1}	X	X	
250 mg L^{-1}	X	X	X
275 mg L^{-1}	X	X	X
300 mg L^{-1}	X	X	X
325 mg L^{-1}	X	X	X
350 mg L^{-1}	X	X	X
375 mg L^{-1}	X	X	X
400 mg L^{-1}	X	X	X
2-phenoxyethanol			
525 mg L^{-1}	X	X	
600 mg L^{-1}	X	X	
675 mg L^{-1}	X	X	X
750 mg L^{-1}	X	X	X
825 mg L^{-1}	X	X	X
900 mg L^{-1}	X	X	X
clove oil			
110 mg L^{-1}	X	X	X
130 mg L^{-1}	X	X	X
150 mg L^{-1}	X	X	X
170 mg L^{-1}	X	X	X

2.5 – Anaesthesia

The fish were fasted for 24 hours prior to the anaesthetic procedures.

Anaesthesia was carried out under controlled temperature - the same as the one in the rearing systems (29 °C), by using water heaters and aeration within the recipients in which the fish were anaesthetised and recovered.

Plastic recipients with a 5 L capacity were filled with 3 L of rearing water and placed on a work station (Fig. 7), by the following order: 1) one for receiving the fish from the rearing system and holding them until being anaesthetised (blue lid); 2) one for inducing anaesthesia (pink lid); 3) two for recovering the animals (green lid), in order to divide a batch of fish and avoid the contamination of the recovering water with anaesthetic residues; and 4) one container for holding the fish until the last one has been recovered and transfer them all back into their respective aquarium (blue lid). The anaesthetic solution was prepared in advanced and let to dissolve for 30 minutes, with strong aeration. A new solution was prepared for each 10 fish from a *G. rufa* size class. The MS-222 (Sigma-Aldrich, St. Louis, United States of America) was weighted in an analytical balance (AE ADAM PGL 3002, Milton Keynes, England, ± 0.01 g accuracy) and added to the water in the induction recipient, along with the same amount of sodium bicarbonate (Sigma-Aldrich, St. Louis, United States of America) as to buffer the solution. The clove oil (Bio clove essential oil, Biover Nv, Nazareth, Belgium) was firstly dissolved in ethanol at 1:10 ratio and then added to the water. The 2-phenoxyethanol (Sigma-Aldrich, St. Louis, United States of America) was added directly into the water. Before the anaesthetic procedures, the water quality in the recipients was assessed with a YSI Professional Plus handheld multiparameter meter, in order to verify if they were suitable to the fish welfare (Table III).

Table III - Physico-chemical parameters of the water quality during the anaesthesia for the three anaesthetics: MS-222, 2-phenoxyethanol and clove oil. Data are expressed as mean ± SD.

Anaesthetics	Temperature (°C)	pH	Dissolved oxygen (mg L^{-1})
MS-222	29.3 ± 0.41	8.40 ± 0.19	6.65 ± 0.20
2-phenoxyethanol	29.2 ± 0.81	8.09 ± 0.05	7.04 ± 0.30
clove oil	29.2 ± 0.67	7.95 ± 0.06	6.69 ± 0.13

The anaesthetic procedures comprised three different phases: induction, monitoring and recovery. In each of these phases, fish behaviour was surveyed and timed according to several stages adapted from Coyle *et al.* (2004), but adjusted to the specific external signs exhibited by *G. rufa.* Ten *G. rufa* were taken from a random aquarium, placed in the first recipient and let to rest for 10 minutes.

To induce anaesthesia by inhalation, a single *G. rufa* was removed with a fish net, let to drip the excessive water and placed in the second recipient, which had a certain amount of anaesthetic agent (as to obtain the desired concentration).

The anaesthesia induction phase (I) corresponded to the time that run since immersion in the anaesthetic solution until cessation of the opercular movements (Summerfelt & Smith, 1990).

In the case of *G. rufa*, the following stages of anaesthesia induction were observed: 1) impaired motion (IM), which was the time that took for the fish to lose swimming coordination, after being inserted in the anaesthetic solution; 2) loss of buoyancy loss (BL) that was the time running between the moment in which the fish lost swimming coordination and fell sideways on the bottom of the container; and 3) cease of opercular movements (COM), corresponding to the time it took for the fish to stop moving the opercula, after losing buoyancy.

Afterwards, the fish were removed from the anaesthetic solution to measure their total length (TL) with the aid of an ichthyometer, weighted with a portable digital scale Pesola MS500 (± 0.1 g; PESOLA Präzisionswaagen AG, Schindellegi, Switzerland) and observed through the Stereo Microscope Zeiss Stemi DV4 LED (Carl Zeiss Microscopy GmbH, Göttingen, Germany) to determine the gender. Then, the fish were be held to detect muscular contractions or opercular movements until being 90 seconds out of the water. If the fish demonstrated any of these signs, they were immediately inserted in the recovery container. All these procedures were carried out during the monitoring phase (M).

After that, the fish was placed in another recipient, containing just water from the rearing system, near the aeration stone. The anaesthesia recovery phase (R) was evaluated as the time running since the immersion of the fish in clear water until it showed voluntary and coordinated swimming movements. This phase comprised the following stages: 1) initiation of opercular movements (IOM), as the time it took for the fish to restart breathing by moving the opercula; 2) initiation of body movements (IBM), which corresponded to the time it took for the fish to present muscular contractions afterwards, in order to straighten up and swim; 3) buoyancy control (BC) that was the time for the fish to recover its buoyancy control after its first attempts to swim; and 4) normal swimming (NS), as the time it took for the fish to swim voluntarily and co-ordinately after regaining its buoyancy control.

For every 5 anaesthetised fish, the recovery container was changed so that there was were no anaesthetic residues in the water. Then, the fish was removed from the recovery recipient with a net, let to drip the excessive water and transferred to a last container, where it was left to rest. When all the 10 fish went through these steps, they were returned to their respective aquarium in the rearing system.

Ten minutes later, they were given a daily dose of feed corresponding to the amount ad libitum (0.04 g fish^{-1}; Catarino *et al.,* 2019), previously weighted on an analytical balance Sartorius TE124S (Sartorius AG, Göettingen, Germany). Their feeding behaviour was observed in what concerns their appetence for food and how long it took them to ingest the whole daily dose of feed. The amount of food placed on the bottom of the aquarium was verified at each 15 minutes. If the food was not all ingested in 45 min, then it was considered that the fish´s appetite was impaired by the anaesthesia

During the following week, the fish were monitored for their survival, health state and behaviour. This monitoring was done by observing the fish in their respective aquariums at a visual level.

Figure 7 - Photographs of the work station for the anaesthetic procedures in *Garra rufa* (Heckel, 1843), with six recipients containing 3 L of water from *G. rufa's* rearing system, a water heater set for 29 °C and strong aeration (**a**). The recipients had lids with different colour to indicate their purpose (**b**): the blue lid corresponded to recipients in the beginning and in the end of the work station, used to receive the fish bound to be anaesthetised and those ready to be returned to the rearing system, respectively (the last one was always covered with the lid to avoid *G. rufa* from jumping out of the water); the pink lid signalled the recipient with the anaesthetic solution; the green lid identified the containers used to recover the fish from anaesthesia,(being the first replaced by the second after 5 fish, to avoid contamination by anaesthetic residues)

2.6. – Data analyses

All calculations and statistical analyses were performed with IBM SPSS™ Statistics for Windows, version 26 (IBM Corporation, Armonk, New York, U.S.A), with a significance level set as α = 0.05. The results were presented as mean ± standard error (S.E.).

All data were tested for normality with the Kolmogorov Smirnov normality test and homogeneity of variances with Levene's test. After that, two-way ANOVA ($F_{\text{(degrees of freedom between groups, degrees of freedom within groups)}}$ = value; *p-value*) were made, having as factors the anaesthetic concentration and the fish size class (independent variables). These analyses were applied to the induction, monitoring and recovery phases, plus the several stages comprised in each of them, for the three anaesthetics: MS-222, 2-phenoxyethanol and clove oil. In the case of statiscally significant differences, the multiple comparison post-hoc Bonferroni tests were performed and the respective *p*-values were presented.

Linear regression analyses were performed to relate the total length (TL) and individual wet weight (IWW) of the fish with the induction and recovery phases, with fish from all size classes for each concentration of the three anaesthetics: MS-222, 2-phenoxyethanol and clove oil. The equation, Pearson's correlation coefficient (r), coefficient of determination (R^2); sample size (N) and *p*-value were presented.

Pearson product-moment correlations were used to measure the strength and direction of association between the time it took for the fish to be anaesthetised and to recover from it, regardless of *G. rufa's* size class and the anaesthetic concentration, or also for each fish size class regardless the anaesthetic concentration. The results were expressed as correlation coefficient (r), sample size (N) and *p*-value. The strength of correlation was interpreted according to Evans (1996).

3. Results

All *Garra rufa* fish were male and presented appetence for food immediately after being reintroduced in their rearing systems, subsequent to the anaesthetic procedures.

In what regards the anaesthetic MS-222 (Fig. 8 a), the feeding time established for the 30 minutes was verified for the lowest concentrations 225, and 250 and 300 mg L^{-1} and it exceeded 45 minutes for all the other concentrations. After this time, there was still food left in the aquariums. The medium class, fed in between 5 min to 30 min, being faster within the highest MS-222 concentrations. The larger fish were even faster to consume the daily feed dose, within 2 to 8 min. However, their feeding frenzy was faster for 300 mg L^{-1}, increasing up and downwards. For the 2-PE anaesthetic (Fig. 8 b), the small class usually fed within 30 min, but they took 45 minutes when anaesthetised with 675 and 750 mg L^{-1}. In the medium size class, the feeding ranged from 9 to 30 min, decreasing progressively with the increasing anaesthetic doses until 825 mg L^{-1} . The same was verified for the large size class. The large fish fed faster, ranging from 4 to 8 min.

The feeding time for the clove oil anaesthetic (Fig. 8 c) were similar to the other two anaesthetics for the small size class, taking at least 30 minutes to ingest the daily dose of feed or exceeding 45 min .

The medium and large fish fed faster, ranging from 10 to 17 min and from 3 to 7 min, respectively. All size classes of fish fed faster at the intermediate clove oil concentrations (130 and 150 mg L^{-1}) than in the smaller and larger anaesthetic doses.

Regarding survival, there were no mortalities registered during the anaesthetic procedures and within one week time afterwards. The only exception was a single individual from the medium size class, 24 hours after being anaesthetised with 170 mg L^{-1} of clove oil.

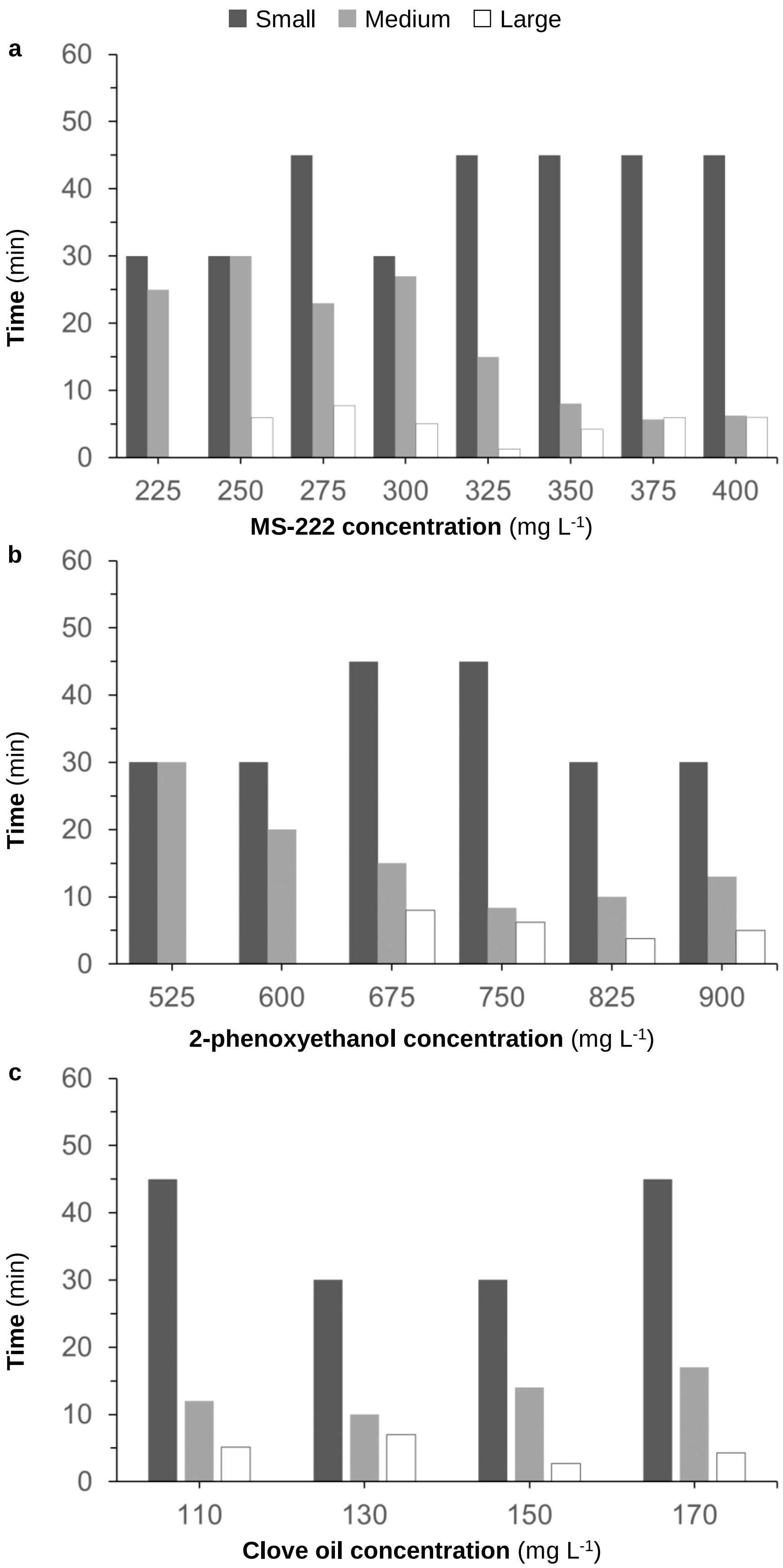

Figure 8 - Absolute time that three size classes (small, medium and large) of *Garra rufa* (Heckel, 1843) took to ingest the daily ad libitum dose of feed after anaesthesia with a) MS-222, b) 2-phenoxyethanol and c) clove oil.

3.1 – Anaesthesia with MS – 222

3.1.1 Total length and individual wet weight

The fish *Garra rufa* measured a total length of 4.31 ± 0.42 cm within the small size class. For the medium size class, they measured 6.46 ± 0.85 cm and those from the large size class were 9.42 ± 0.70 cm (Fig. 9 a).

There were statistically significant differences in the total length amongst all the size classes (Two-way ANOVA: $F_{2,207}$ =11075.458, *p*-value = 0.000 < 0.05; Bonferroni tests with *p*-value = 0.000 < 0.05).

There were differences between the various concentrations of MS-222, in what concerns the total lengths of *G. rufa* (Two-way ANOVA: $F_{7,207}$ = 2.591; *p*-value = 0.014 < 0.05), but only for concentration 225 mg L^{-1} of MS-222 towards all the other concentrations (Bonferroni tests with *p*-value < 0.05).

Statistical analyses confirmed that there were no significant total length differences amongst the several concentrations for the medium fish (Two-way ANOVA: $F_{7,72}$ = 0.898, *p*-value = 0.830 > 0.05) and large fish size classes (ANOVA: $F_{6,63}$ = 0.259, *p*-value = 0.954 > 0.05).

There was no interaction between size classes and the various MS-222 concentrations tested in what concerns the total length of *G. rufa* (Two-way ANOVA: $F_{13,207}$ = 1.635; *p*-value = 0.078 > 0.05).

G. rufa presented an individual wet weight of 0.86 ± 0.70 g for the small size class, 3.20 ± 1.62 g for the medium size class and those from the large size class weighted 9.74 ± 1.97 g (Fig. 9 b).

The individual wet weight of the fish from the three size classes were all significantly different from each other (Two-way ANOVA: $F_{2,207}$ = 682.900, *p-value* = 0.000 < 0.05; Bonferroni tests with *p*-value < 0.05).

Contrarily to the total length, statistically significant differences in the individual wet weight of the fish in relation to the various concentrations of MS-222 were not obtained (Two-way ANOVA: $F_{7,207}$ = 0.546, *p*-value = 0.857 > 0.05).
Also, there was no interaction between size classes and the various concentrations of Ms-222 tested for the individual wet weight (Two-way ANOVA: $F_{13,207}$ = 3.569, *p-value* = 0.083 > 0.05).

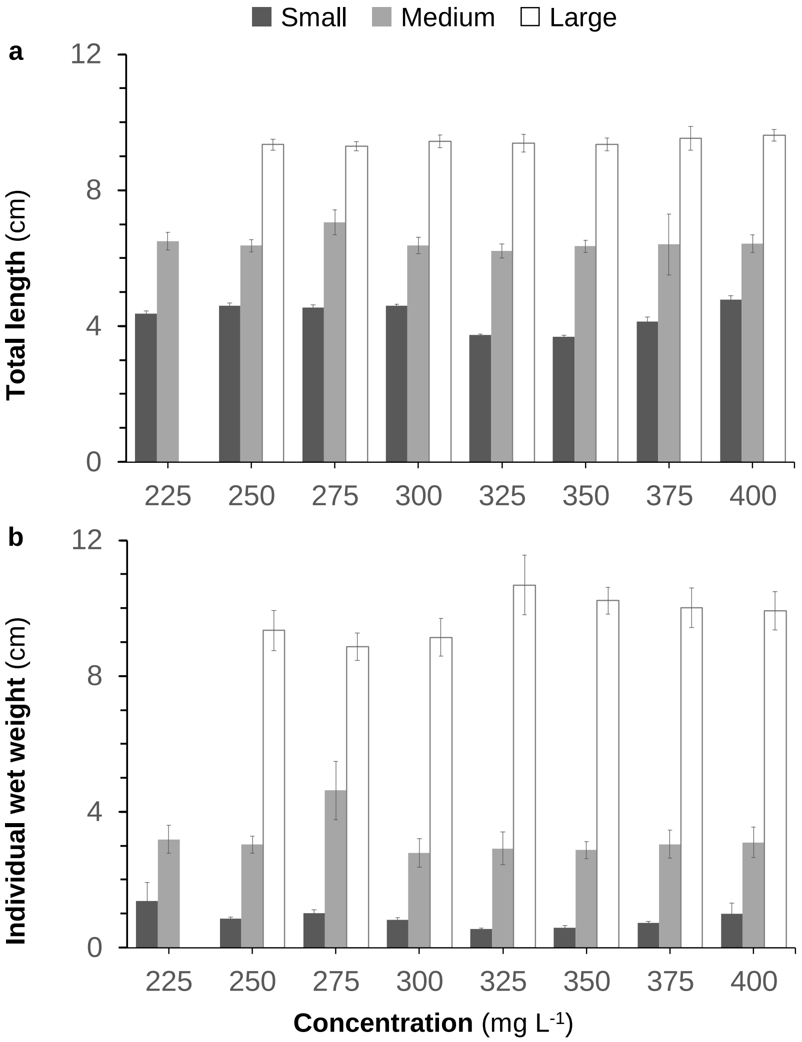

Figure 9 - *Garra rufa* (Heckel, 1843) **a)** total length and **b)** individual wet weight (mean ± S.E.) obtained in three size classes (small, medium and large fish) and eight concentrations of MS-222 (225, 250, 275, 300, 325, 350, 375 and 400 mg L^{-1}) assessed during the experimental trial.

3.1.2 -Timing of the induction, monitoring and recovery phases

3.1.2.1 Induction phase

The induction phase (Fig. 10 a) showed statistically significant differences in length between all three *G. rufa* size classes (Two-way ANOVA: $F_{2,207}$ = 43.313, *p*-value = 0.000 < 0.05; Bonferroni tests with *p*-value < 0.05), MS-222 concentrations (Two-way ANOVA: $F_{7,207}$ = 46.207, *p*-value = 0.000 < 0.05), with an interaction between the 2 factors (Two-way ANOVA: $F_{13,207}$ = 5.110, *p*-value = 0.000 < 0.05).

Generally, *G. rufa* from the largest size class presented a longer induction phase than those from the medium size class, regardless of MS-222 concentrations, while the smallest size class showed shorter induction times. The only exception was registered in the concentration 325 mg L^{-1}, in which the smaller *G. rufa* took more time to be anaesthetised than the medium and even the larger fish.

The induction phase lasted longer in the concentrations from 225 to 300 mg L^{-1} of MS-222 in relation to those from 325 to 400 mg L^{-1} (Bonferroni tests with *p*-value < 0.05). Also, the induction phase in this last concentration of MS-222 was significantly higher than the ones measured for 325 and 375 mg L^{-1} (Bonferroni tests with *p*-value < 0.05).

In sum, the MS-222 concentrations that induced a faster anaesthesia in *G. rufa* were: 375 mg L^{-1} for both the small (65.80 ± 11.183 s) and large (78.70 ± 21.69 s) size classes, plus 325 mg L^{-1} (64.50 ± 11, 97 s) for the medium size class. In contrast, the concentrations that took more time to produce an anaesthesia state were: 225 mg L^{-1} (118.50 ± 22.36 s) for the small size class, 250 mg L^{-1} (166.70 ± 33.50 s) for the medium size class and 300 mg L^{-1} (169.90 ± 27.97) for the large size class.

Significant linear regressions, with strong correlation and 62 to 77 % determination coefficients, were established between the time to induce MS-222 anaesthesia and *G. rufa* total length, plus with individual wet weight, at 275 and 300 mg L^{-1} (Table IV). Also, significant linear regressions, with moderate correlation and 18 to 42 % determination coefficients, were observed between the anaesthesia induction time and fish total length at the concentrations 225 and 350 mg L^{-1}, plus individual wet weight at the concentrations 250 and 350 mg L^{-1} (Table IV).

3.1.2.2 - Monitoring phase

The monitoring phase (Fig. 10 b) presented statistically significant differences between various size classes (Two-way ANOVA: $F_{2,207}$ = 6.109, *p*-value = 0.003 < 0.05), MS-222 concentrations (Two-way ANOVA: $F_{7,207}$ = 3.219, *p*-value = 0.003 < 0.05), but with significant interaction between the two factors (Two-way ANOVA: $F_{13,207}$ = 1.7881, *p*-value = 0.043 < 0.05).

Bonferroni test showed that small class behaved differently from the medium and large size classes, enduring the full 90 s of the monitoring phase (Bonferroni tests with *p*-value = 0.008 < 0.05 and *p*-value = 0.025 < 0.05, respectively), contrarily to these last two groups (Bonferroni test with *p*-value = 1.000 > 0.05).

The monitoring phase at MS-222 concentration 250 mg L^{-1} was significantly shorter than at 325 and 350 mg L^{-1} (Bonferroni tests with *p*-value < 0.05).

Almost all fish remained totally immobilised during the monitoring phase, during which they were measured, weighed and observed for gender identification.

In the medium size class, some fish exhibited opercular movements and/or muscular contractions when anaesthetised with concentrations lower than 325 mg L^{-1} of MS-222, with the shortest monitoring phase registered at 225 mg L^{-1} (80.90 ± 16,86 s). Within the highest concentrations, a single fish endured only 40 s of anaesthesia at 375 mg L^{-1}, reducing the monitoring time to 85.00 ± 4.74 s.

Likewise, fish from the large size class were maintained immobilised for 90 ± 0.00 s for concentrations above 325 mg L^{-1}, with exception of two individuals at 400 mg L^{-1}, reducing the monitoring time to 87.50 ± 1.99 s. The shortest monitoring phase was observed at 250 mg L^{-1} (75.50 ± 17.22 s), with only 30 % of the fish enduring anaesthesia for 90 s. For this reason, the larger *G. rufa* were not submitted to the downward concentration 225 mg L^{-1}.

3.1.2.3 - Recovery phase

The anaesthesia recovery phase for MS-222 (Fig. 10 c) presented statistically significant differences amongst size classes (Two-way ANOVA: $F_{2,207}$ = 21.867, *p*-value = 0.000 < 0.05) and anaesthetic agent concentration (Two-way ANOVA: $F_{7,207}$ =8.258, *p*-value = 0.000 < 0.05), with a significant interaction between those two factors (Two-way ANOVA: $F_{13,207}$ = 5.561, *p*-value = 0.000 < 0.05).

Generally, *G. rufa* from the small size class took longer to recover from MS-222 anaesthesia than those from the medium and large size classes (Bonferroni tests with *p*-value = 0.000 < 0.05), with these two showing similar responses (Bonferroni test with *p*-value = 0.277 > 0.05).

Predominantly, *G. rufa* took more time to recover from MS-222 anaesthesia within concentrations from 325 mg L^{-1} upwards than downwards. The recovery time measured for this concentration was significantly higher than that from all the other smaller anaesthetic doses. In the concentrations 225 and 300 mg L^{-1}, it was also significantly lower than in all the other doses above 325 mg L^{-1} (Bonferroni tests with *p*-values < 0.05).

In sum, the MS-222 concentrations in which *G. rufa* recovered faster from anaesthesia were: 225 mg L^{-1} for both the small (94.10 ± 37.11 s) and medium (99.40 ± 41.54 s) size classes, plus 300 mg L^{-1} (99.40 ± 41.54 s) for the large size class. In contrast, the concentrations that took more time to recover from an anaesthesia state were: 350 mg L^{-1} (426.70 ± 203.34 s) for the small, 375 mg L^{-1} (225.60 ± 138.32 s) for the medium and 325 mg L^{-1} (236.90 ± 153.59) for the large size classes.

No significant linear regressions, with weak correlation and determination coefficients, were observed between the anaesthesia recovery time and the individual wet weight of *G. rufa* at 275 mg L^{-1} of MS-222 (Table IV).

A weak, but significant, negative correlation was found between the time it took for the fish to be anaesthetised and to recover from it, regardless of *G. rufa's* size class and MS-222 anaesthetic (r = -0.251, N = 230, *p*-value = 0.001 < 0.05). All sizes classes of *G. rufa* evidenced this trend: small (r = -0.307, N = 80, *p*-value = 0.006 < 0.05), medium (r = -0.229, N = 80, *p*-value = 0.0.41 < 0.05) and large fish (r = -0.259, N = 70, *p*-value = 0.030 < 0.05).

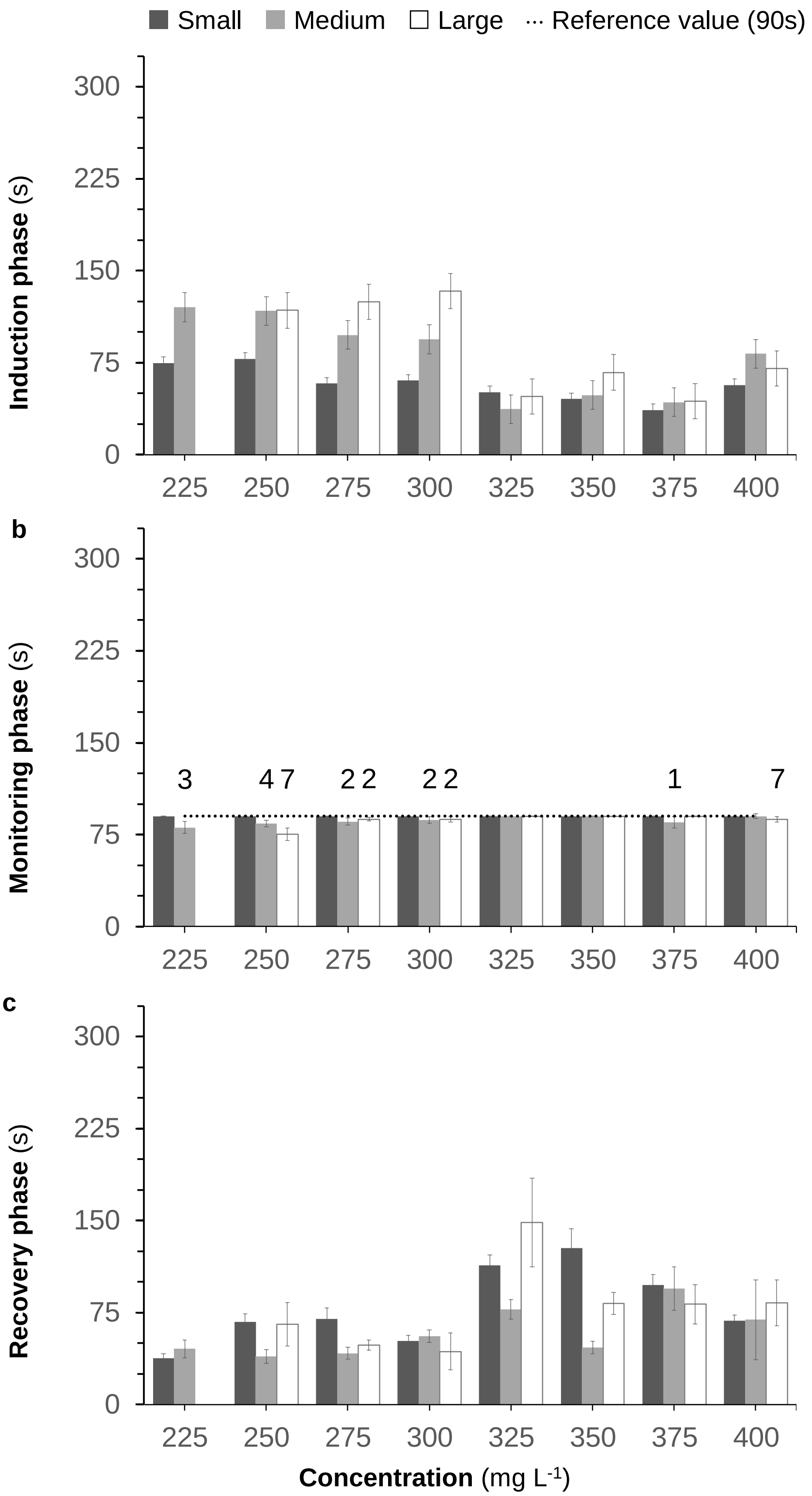

Figure 10 – Timing of the **a)** induction , **b)** monitoring and **c)** recovery phases (mean ± S.E.) for the three size classes of *Garra rufa* (Heckel, 1843) (small, medium and large) and for the eight concentrations of MS-222 (225, 250, 275, 300, 325, 350, 375 and 400 mg L^{-1}) assessed during the experimental trial. Note: The numbers above the columns in the monitoring phase indicate the number of fish that not endure 90 seconds of anaesthesia without presenting body contractions or opercular movements.

Table IV - Results of linear regression analyses performed to assess the relationship between the independent variables total length (TL) or individual wet weight (IWW) of *Garra rufa* (Heckel, 1843) with the dependent variables time anaesthesia induction (I) or recovery (R). Fish from all size classes were used in for each concentration of concentration of MS-222. Pearson's correlation coefficient (r), coefficient of determination (R^2), *p*-value, regression equation and classification of the regression analysis are indicated.

Concentrations	r	R^2	*p-value*	Regression equation	Classification
225 mg L^{-1}	0.64	0.42	0.00	I = - 19.02 + 21.42 TL	Significant, moderately correlated
	0.39	0.15	0.09	I = 76.92 + 8.94 IWW	Non-significant, weakly correlated
	0.19	0.04	0.42	R = 26.06 + 2.84 TL	Non-significant, weakly correlated
	0.00	0.00	0.99	R = - 0.008 + 41.52 IWW	Non-significant, very weakly correlated
250 mg L^{-1}	0.45	0.21	0.12	I = 52.33 + 7.67 TL	Non-significant, moderately correlated
	0.43	0.18	0.02	I = 87.41 + 3.82 IWW	Significant, moderately correlated
	0.05	0.03	0.79	R = 63.96 - 0.99 TL	Non-significant, very weakly correlated
	0.05	0.03	0.78	R = 59.6 - 0.53 IWW	Non significant very weakly correlated
275 mg L^{-1}	0.88	0.77	0.00	I = - 6.26 + 14.3 TL	Significant, strongly correlated
	0.84	0.70	0.00	I = 55.97 + 7.73 IWW	Significant, strongly correlated
	0.37	0.13	0.04	R = 82.42 - 4.19 TL	Significant, weakly correlated
	0.35	0.13	0.05	R = 64.37 - 2.03 IWW	Non-significant, weakly correlated
300 mg L^{-1}	0.84	0.71	0.00	I = - 0.84 + 14.21 TL	Significant strongly correlated
	0.79	0.62	0.00	I = 64.78 + 7.31 IWW	Significant strongly correlated
	0.23	0.05	0.22	R = 62.51 - 1.81 TL	Non-significant weakly correlated
	0.25	0.06	0.19	R = 54.72 - 1.06 IWW	Non-significant weakly correlated
325 mg L^{-1}	0.04	0.00	0.84	I = 46.54 - 0.23 TL	Non-significant very weakly correlated
	0.10	0.11	0.58	I = 43.56 + 0.31 IWW	Non-significant very weakly correlated
	0.22	0.05	0.23	R = 67.7 + 7.06 TL	Non-significant weakly correlated
	0.27	0.07	0.15	R = 92.94 + 4.28 IWW	Non-significant weakly correlated
350 mg L^{-1}	0.46	0.21	0.01	I = 30.61 + 3.55 TL	Significant moderately correlated
	0.49	0.24	0.00	I = 43.77 + 2.15 IWW	Significant moderately correlated
	0.39	0.16	0.03	R = 1.37 - 7.98 TL	Significant weak correlated
	0.23	0.05	0.23	R = 97.16 - 2.6 IWW	Non-significant very weakly correlated
375 mg L^{-1}	0.19	0.03	0.32	I = 34.52 - 0.92 TL	Non-significant very weakly correlated
	0.17	0.03	0.38	I = 38.58 + 0.46 IWW	Non-significant very weakly correlated
	0.04	0.00	0.83	R = 96.85 - 0.81 TL	Non-significant very weakly correlated
	0.10	0.01	0.59	R = 96.72 -1.16 IWW	Non-significant very weak correlated
400 mg L^{-1}	0.27	0.07	0.15	I = 49.56 + 2.88 TL	Non-significant weakly correlated
	0.20	0.04	0.29	I = 64.48 + 1.09 IWW	Non-significant weakly correlated
	0.05	0.00	0.78	R = 64.55 + 0.78 TL	Non-significant very weakly correlated
	0.06	0.00	0.73	R= 67.7 + 0.49 IWW	Non-significant very weakly correlated

3.1.3 -Timing of the stages observed within the induction phase

During the anaesthesia induction with MS-222, *G. rufa* firstly lost control of their swimming coordination, followed by the loss of buoyancy control, in which the fish laid sideways on the bottom of the container, and finally stopped breathing, which was visible by the ceasing of opercular movements. No exceptions were observed. Generally, the cease of opercular movements was the longest induction stage, followed almost equally by the other two stages (Fig. 11).

3.1.3.1 Impaired motion

G. rufa showed no statistically significant differences in the time it took them to demonstrate impaired motion (Fig. 11 a) when submerged in a MS-222 solution to what concerns size classes (Two-way ANOVA: $F_{2,207}$ = 1.285, *p*-value = 0.279 > 0.05. Nevertheless, there were differences amongst the MS-222 concentrations (Two-way ANOVA: $F_{7,207}$ = 10.400, *p*-value = 0.000 < 0.05) and a significant interaction between the two factors (Two-way ANOVA: $F_{13,207}$ = 6.437, *p*-value = 0.000 < 0.05).

The time *G. rufa* took to display impaired motion was significantly higher in the smallest MS-222 concentration (225 mg L^{-1}) in relation to all others. The same was observed for the following concentration 250 mg L^{-1} and the highest ones that were used: 275 and 400 mg L^{-1} (Bonferroni tests with *p*-value < 0.05).

The time for observing *G. rufa* impaired motion was longer for the concentration of 225 mg L^{-1} in smaller fish (19.40 ± 7.01 s), 250 mg L^{-1} in the medium size class (20.30 ± 6.53 s) and 350 mg L^{-1} in the larger fish (13.20 ± 3.90 s). Then again, the fastest impairment of swimming was observed at 400 mg L^{-1} both for the small and medium size fish (7.60 ± 2.37 s and 7.90 ± 3.04 s, respectively) and 350 mg L^{-1} for the large fish (8.60 ± 3.90 s).

3.1.3.2 – Buoyancy loss

The time spent to lose Buoyancy (Fig. 11 b) differed between *G rufa* size classes (Two-way ANOVA: $F_{2,207}$ = 16.472, *p*-value = 0.000 < 0.05)., MS-222 concentrations (Two-way ANOVA: $F_{7,207}$ = 6.277, *p*-value = 0.000 < 0.05 in which there was an interaction between the two factors (Two-way ANOVA: $F_{13,207}$ = 3.486, *p*-value = 0.000 < 0.05).

Fish from the large class presented statistically significant differences in comparison with the small and medium size ones, being less prone to lose buoyancy control (Bonferroni tests with *p*-value = 0.000 < 0.05). But, no differences were found between the other two size classes (Bonferroni test with *p*-value = 0.534 > 0.05)

In terms of concentration, the buoyancy loss timings recorded at 250 mg L^{-1} were significantly different from those at 250 mg L^{-1}, whilst these and those observed at 300 mg L^{-1} were also significantly higher than the ones from concentrations 325 and 350 mg L^{-1} (Bonferroni tests with *p*-value = 0.000 < 0.05).

The loss of buoyancy took longer time at 250 mg L^{-1} for the small (14.80 ± 4.96 s) and large (20.00 ± 5.83 s) size classes, while for the medium fish it was at 300 mg L^{-1} with 16.9 ± 5.30 s. In the contrary, it was faster between 325 and 375 mg L^{-1} for the small size class (6,10 ± 3.31 to 6.70 ± 3.83 s) and 325 mg L^{-1} for the medium (7.70 ± 4.81 s) and large fish (8.50 ± 2.95 s).

3.1.3.3 – Cease of opercular movements

The time it took for *G. rufa* to cease the opercular movements (Fig. 11 c) varied significantly between size classes (Two-way ANOVA: $F_{2,207}$ = 37.204, *p*-value = 0.000 < 0.05), MS-222 concentrations (Two-way ANOVA: $F_{7,207}$ = 36.371, *p*-value = 0.000 < 0.05), with interaction between both factors (Two-way ANOVA: $F_{13,207}$ = 6.353, *p*-value = 0.000 < 0.05).

The period observed for the fish to cease the opercular movements was statistically shortest for the small size class than for the medium and large size fish (Bonferroni tests with *p*-value = 0.000 < 0.05), but these two were not statistically different between each other (Bonferroni test with *p*-value = 0.716 > 0.05)

The lower MS-222 concentrations (from 225 to 300 mg L^{-1}) presented significantly higher times for the cessation of opercular movements in *G. rufa* than those from the higher concentrations (from 325 to 400 mg L^{-1}).

For the small size class of *G. rufa*, the period to cease opercular movements decreased progressively from 250 mg L^{-1} (53.00 ± 18.44 s) until 375 mg L^{-1} (18.20 ± 8.78 s), to increase again at 400 mg L^{-1} (36.90 ± 9.76 s). The medium and large size classes followed the same trend, in which the opercular movements ceased briefly at 375 mg L^{-1} (22.90 ± 9.97 and 16.50 ± 10.19 s, respectively), whilst the longer periods were recorded at 225 (91.70 ± 43.91 s) and 300 mg L^{-1} (66.80 ± 19.81 s), respectively.

3.1.4 -Timing of the stages observed within the recovery phase

During the recovery from anaesthesia with MS-222, *G. rufa* generally began by initiating the opercular movements, followed by presenting body movements to try swimming or stand up straight on the bottom of the container, regaining buoyancy control. For last, the fish were only considered fit to be transferred to another container without supervision when they swam normal and voluntarily in the recovery recipient. Few exceptions were observed, in which *G. rufa* first recovered buoyancy control and then tried to swim. Generally, the initiation of opercular movements and the normal swimming behaviour were the longest recovery stages, followed by the initiation of body movements. The buoyancy control was the shortest one, being frequently simultaneous with the initiation of body movements (Fig. 11).

3.1.4.1 Initiation of opercular movements

G. rufa presented statistically significant difference within the times recorded for initiating their opercular movements (Fig. 11 c'), in what concerns size classes (Two-way ANOVA: $F_{2,207}$ = 58.324, *p*-value = 0.000 < 0.05), MS-222 concentrations (Two-way ANOVA:

$F_{7,207}$ = 9.887, *p*-value = 0.000 < 0.05), with interaction between the two factors (Two-way ANOVA: $F_{13,207}$ = 5.207, *p*-value = 0.00 < 0.05).

The initiation of opercular movements took significantly longer to occur within the small size than for the medium and large fish (Bonferroni tests with *p*-value = 0.000 < 0.05), while no statistical differences were observed between these two last size classes (Bonferroni test with *p*-value = 1.000 > 0.05).

Generally, the lower MS-222 concentrations (from 225 to 300 mg L^{-1}) took significantly more time than the higher ones (from 325 to 400 mg L^{-1}) to initiate the opercular movements, although 275 mg L^{-1} was only statistically different from 350 mg L^{-1} (Bonferroni test with *p*-value < 0.05).

The smaller *G. rufa* presented an increasing period to initiate their opercular movements after being anesthetised until the MS-222 concentration of 350 mg L^{-1} (81.20 ± 14.05 s), decreasing slightly until 400 mg L^{-1}. The medium and large fish took more time to initiate opercular movements gradually until the higher concentrations that were tested (24.91 ± 7.39 s and 24.10 ± 10.20 s, respectively). For the smaller fish, this stage of the recovery phase was one of the longest ones, but for the other two size classes, it was comparable to the stage in which they start to move their body, being the second longest ones.

Apparently, it seemed that the longer *G. rufa* took to cease the opercular movements during the anaesthesia induction, the sooner they initiated breathing during recover. However, a very weak negative significant correlation was found between the ceasing and the beginning of the opercular movements, respectively during the anaesthesia induction and recovery phases, in the smaller (r = -0.293, N = 80, *p*-value = 0.008 < 0.05 and medium fish (r = -0.232, N = 80, *p*-value = 0.038 < 0.05),), but it was not significant for the large fish (r = -0.129, N = 70, *p*-value = 0.287 > 0.05) .

3.1.4.2 Initiation of body movements

The time taken to initiate body movements by *G. rufa* (Fig. 11 d), in order to swim and regain buoyancy, showed no statistical differences amongst size classes (Two-way ANOVA: $F_{2,207}$ = 0.178, *p*-value = 0.837> 0.05), or amongst the MS-222 concentrations (Two-way ANOVA: $F_{7,207}$ = 1.652, *p*-value = 0.122 > 0.05), although a significant interaction was found between the two factors (Two-way ANOVA: $F_{13,207}$ = 2.374, *p*-value = 0.006 < 0.05).

This parameter presented many value fluctuations between size classes and concentrations, without apparent trends. It varied between 9.20 ± 2 07 s at 225 mg L^{-1} and

29.50 ± 4.76 s at 325 mg L^{-1} for the small fish, 9.90 ± 1.92 s at 350 mg L^{-1} and 32.40 ± 9.90 s at 375 mg L^{-1} for the medium fish, plus 13.60 ± 1.68 s at 300 mg L^{-1} and 35.00 ± 11.11 s at 250 mg L^{-1} for the large fish.

3.1.4.3 Buoyancy control

In what concerns the buoyancy control (Fig. 11 b'), there were no statistically significant differences between size classes (Two-way ANOVA: $F_{2,207} = 1.666$, *p*-value = 0.192 > 0.05), or amongst MS-222 concentrations (Two-way ANOVA: ($F_{7,207}= 1.305$, *p*-value = 0.249 > 0.05) neither was there an interaction between the two factors (Two-way ANOVA: $F_{13,207} = 1.135$, *p*-value = 0.331 > 0.05).

The stage in which *G. rufa* gained buoyancy control was generally short. It varied from 1.70 ± 0.20 s at 300 mg L^{-1} and 10.40 ± 3.12 s at 350 mg L^{-1} of MS-222. It seemed that the medium and large fish from higher concentrations of MS- 222 took more time to stand up straight in the bottom of the recovery recipient. Some of these fish also inverted the recovery stages sequence (gaining buoyancy first and then starting to swim), although few situations were observed.

The buoyancy loss in the anaesthesia phase was usually longer than its achievement during recovery. A moderate negative and significant correlation was observed between these two stages for small fish (r = -0.359, N = 80, *p*-value = 0.001 < 0.05), but no correlation was observed for medium fish (r = -0.150, N = 80, *p*-value = 0.185 > 0.05), nor large fish (r = -0.095, N = 70, *p*-value = 0.432 > 0.05).

3.1.4.4 Normal swimming

The time taken by *G. rufa* to exhibit a normal and voluntary swimming behaviour (Fig. 11 a') presented statistically significant differences between size classes (Two-way ANOVA: $F_{2,207} = 12.745$, *p*-value = 0.000 < 0.05) and MS-222 concentrations (Two-way ANOVA: $F_{7,207} = 8.361$, *p*-value = 0.000 < 0.05) with interaction between the two factors (Two-way ANOVA: $F_{13,207} = 3.651$, *p*-value = 0.000 < 0.05).

The fish from the large size class took longer to achieve this stage than the small and medium ones (Bonferroni tests with *p*-value = 0.000 < 0.05), but there were no significant difference amongst these to last size classes (Bonferroni test with *p*-value = 1.000 > 0.05).

In terms of concentrations, there were statistically significant differences regarding 325 mg L^{-1} and all the other MS-222 concentrations (Bonferroni tests with *p*-value < 0.05), in which the fish have taken a longer time to achieve a normal swimming behaviour. Generally, this parameter took a longer time from concentration 325 mg L^{-1} upwards. The maximum value was recorded for the large fish at 325 mg L^{-1} (109.50 ± 32.41 s) and the minimum for the small fish at 275 mg L^{-1} (10.40 ± 1.89 s).

The normal swimming behaviour in the recovery phase was usually longer than motion impairment during the anaesthesia induction phase. A moderate negative and significant correlation was observed between these two stages for medium fish ($r = -0.344$, $N = 80$, p-value $= 0.002 < 0.05$), but no correlation was observed for small fish ($r = -0.144$, $N = 80$, p-value $= 0.203 > 0.05$), nor large fish ($r = -0.025$, $N = 70$, p-value $= 0.840 > 0.05$).

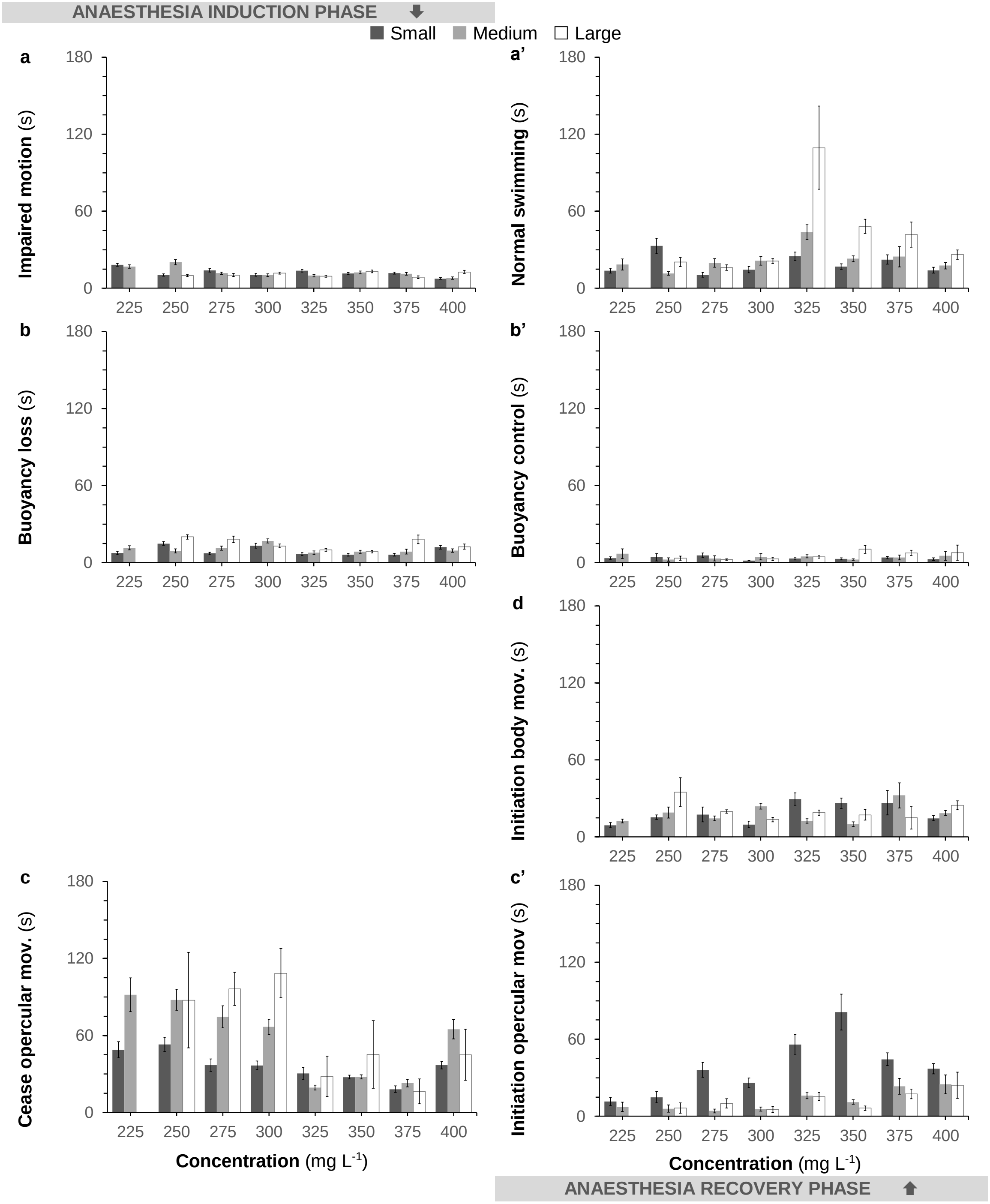

Figure 11 – Timing of the anaesthesia stages (mean ± S.E.) identified during the induction (**a** impaired motion, **b** buoyancy loss and **c** cease of opercular movements) and recovery phases (**c'** initiation of opercular movements, **d** initiation of body movements, **b'** buoyancy control and **a'** normal swimming), for three size classes of *Garra rufa* (Heckel, 1843) (small, medium and large) and eight concentrations of MS-222 (225, 250, 275, 300, 325, 350, 375 and 400 mg L^{-1}) assessed during the experimental trial.

3.1.4.5. Total anaesthesia procedure

The anaesthesia procedures with MS-222, which successfully immobilised the fish for 90 s during examination routines, varied from 273.50 ± 14.61 s for the medium fish and 591 ± 60.31 s for the small fish, both at 350 mg L^{-1} (Fig. 12).

The total time for the anaesthesia procedures presented statistically significant differences amongst fish size classes (Two-way ANOVA: $F_{2,207} = 9.862$, *p*-value = 0.000 < 0.05), but not between MS-222 concentrations (Two-way ANOVA: $F_{7,207} = 1.696$, *p*-value = 0.112 > 0.05), with a significant interaction between factors (Two-way ANOVA: $F_{13,207} = 5.699$, *p*-value = 0.000 < 0.05).

The small and medium fish presented statistically significant differences regarding the time lengths for the entire procedures, varying amongst concentrations (Bonferroni test with *p*-value = 0.000 < 0.05), whilst large fish behaved in between these two, not differing from them (Bonferroni tests with *p*-value > 0.05).

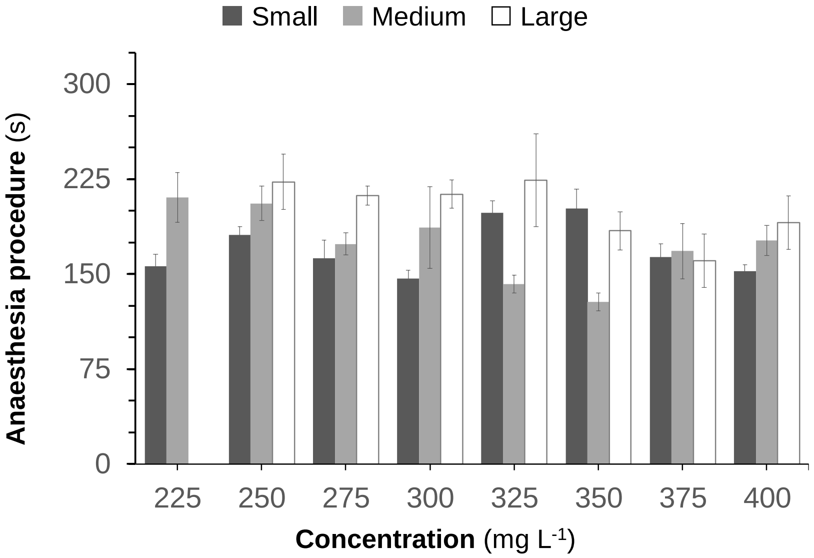

Figure 12– Timing of the anaesthesia procedure (mean ± S.E.) for the three size classes of *Garra rufa* (Heckel, 1843) (small, medium and large) and for the eight concentrations of MS-222 (225, 250, 275, 300, 325, 350, 375 and 400 mg L^{-1}) assessed during the experimental trial.

3.2. – Anaesthesia with 2- phenoxyethanol

3.2.1 Total length and individual wet weight

The fish *Garra rufa* measured a total length of 4.53 ± 0.32 cm within the small size class, 6.44 ± 0.90 cm within the medium size class and 9.30 ± 0.67 cm within the large size class (Fig. 13 a).

G. rufa also presented an individual wet weight of 0.75 ± 0.26 g within the small size class, 3.29 ± 1.62 g within the medium size class and 10.0 ± 2.06 g within the large size class (Fig. 13 b).

Both the total length and individual wet weight were statistically different between all size classes (Two-way ANOVA: $F_{2,144} = 533.756$, p-value = 0.000 < 0.05 and $F_{2,144} = 433.877$, p-value = 0.000 < 0.05, respectively; Bonferroni tests all with p-value < 0.05).

Likewise, there were no statistically significant differences of total length and individual wet weight amongst the various concentrations of 2-phenoxyethanol (Two-way ANOVA: $F_{5,144} = 0.906$, p-value = 0.479 > 0.05 and $F_{5,144} = 0.061$, p-value = 0.998 > 0.05, respectively).

Also, there were no interactions between fish size class and phenoxyethanol concentrations in relation to the total length and the individual wet weight of *G. rufa* (Two-way ANOVA: $F_{8,144} = 0.358$, p-value = 0.941 > 0.05 and $F_{8,144} = 0.427$, p-value = 0.903 > 0.05, respectively).

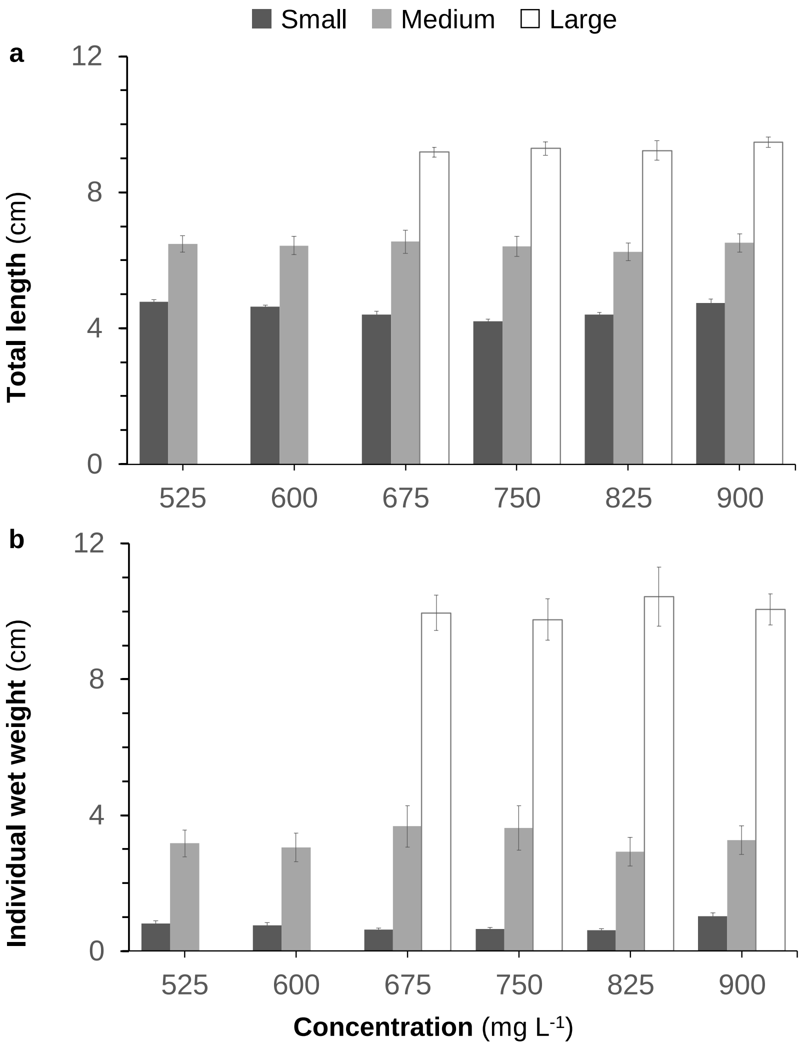

Figure 13– *Garra rufa* (Heckel, 1843) **a)** total length and **b)** individual wet weight (mean ± S.E.) obtained in three size classes (small, medium and large fish) and six concentrations of 2-phenoxyethanol (525, 600, 675, 750, 825 and 900 mg L^{-1}).

3.2.2 -Timing of the induction, monitoring and recovery phases

3.2.2.1 Induction phase

The induction phase (Fig. 14 a) was distinct for all size classes (Two-way ANOVA: $F_{2,144} = 28.260$, *p*-value = 0.000 < 0.05, Bonferroni tests with *p*-value < 0.05), 2-phenoxyethanol concentrations (Two-way ANOVA: $F_{5,144} = 5.227$, *p*-value = 0.000 < 0.05), with no interaction between the two factors (Two-way ANOVA: $F_{8,144} = 1.790$, *p*-value = 0.083 > 0.05).

Generally, *G. rufa* from the medium size class presented a longer induction phase than those from the large size class, regardless of 2-phenoxyethanol concentrations, while the smallest size class showed shorter induction times.

Also, the induction phase was only significantly shorter within the fish from 2-phenoxyethanol concentration 825 mg L^{-1} than in those from 525 mg L^{-1} (Bonferroni test with p-values < 0.05).

In short, anaesthesia induction of *G. rufa* with 2-phenoxyethanol was faster at 825 mg L^{-1} for the smaller (38.50 ± 3.13 s) and larger fish (57.90 ± 1.49 s), plus 900 mg L^{-1} for the medium ones (81.40 ± 5.46 s). On the other hand, it was longer at 525 mg L^{-1} within fish from the small (77.70 ± 5.03 s) and medium size classes (110.60 ± 9.03 s), while for the larger fish was at 675 mg L^{-1} (105.00 ± 5.01 s).

A significant linear regression, with strong correlation and 34 % determination coefficients, was observed between the anaesthesia induction time and individual wet weight of *G. rufa* within 525 mg L^{-1} of 2-phenoxyethanol (Table V). Those with moderate correlation and 20 to 25 % determination coefficients were obtained for the total length at 600 and 675 mg L^{-1}, while for the individual wet weight was at 600 mg L^{-1} (Table V).

3.2.2.2 Monitoring phase

The monitoring phase (Fig. 14 b) had statistically significant differences of time between size classes (Two-way ANOVA: $F_{2,144} = 4.904$, p-value $= 0.009 < 0.05$), 2-phenoxyethanol concentrations (Two-way ANOVA: $F_{5,144} = 20.986$, p-value $= 0.000 < 0.05$) , plus an interaction between the two factors (Two-way ANOVA: $F_{8,144} = 4.590$, p-value $= 0.000 < 0.05$).

The fish from the small size class endured significantly less the monitoring period than those from the medium and large size classes (Bonferroni tests with p-value < 0.05), with no statistically significant differences amongst these two last size classes (Bonferroni test with p-value $=0.134 > 0.05$)..

Also, the monitoring phase was less endured by *G. rufa* subjected to the 2-phenoxyethanol doses lower than 675 mg L^{-1}, in which 525 and 600 mg L^{-1} were statistically different between each other and all the other concentrations (Bonferroni tests with p-value < 0.05). In fact, *G. rufa* from the small and large size classes stood anaesthetised for all 90 s from 675 mg L^{-1} upwards, while those from the medium size class behaved similarly above 750 mg L^{-1}. As 10 % of the medium size fish recovered from anaesthesia before the end of the monitoring period at 675 mg L^{-1}, it was advisable not to use lower doses of 2-phenoxyethanol with larger fish.

3.2.2.3 Recovery phase

The recovery phase (Fig. 14 c) demonstrated significantly differences within all size classes (Two-way ANOVA: $F_{2,144} = 12.250$, p-value = 0.000 < 0.05, Bonferroni tests with p-value < 0.05), 2-phenoxyethanol concentrations (Two-way ANOVA: $F_{5,144} = 25.085$, p-value = 0.000 < 0.05) and an interaction between the two factors (Two-way ANOVA: $F_{8,144} = 3.673$, p-value = 0.001 < 0.05).

In most cases, *G. rufa* from the large size class presented a significantly longer recovery phase than those from the medium size class (except for concentration 750 mg L^{-1}, in which the medium fish took more time), while those from the small size class were the fastest to recover (with exception of 900 mg L^{-1}, in which the medium fish were the fastest). Also, the fish showed a significantly longer recovery phase in 900 mg L^{-1} of 2-phenoxyethanol regarding all the other concentrations (Bonferroni tests with p-value = 0.000 < 0.05). Also, the recovery phase was shortest in 525 mg L^{-1}, which was also significantly different from the ones recorded at 825 mg L^{-1}.

Summing up, *G. rufa* took longer to recover from 2-phenoxyethanol anaesthesia at 900 mg L^{-1} for all size classes (small = 102.90 ± 7.86 s; medium = 99.90 ± 12.12 s; large = 114.70 ± 7.87 s). Notwithstanding, the smaller and medium fish recovered faster at 525 mg L^{-1} (36.00 ± 2.17 s and 39.90 ± 39.99 s, respectively), while for the larger ones was at 750 mg L^{-1} (53.50 ± 5.35 s).

Significant linear regressions, with strong correlation and 39 to 47 % determination coefficients were observed between the anaesthesia recovery time and *G. rufa* total length, plus individual wet weight, at 675 and 825 mg L^{-1} of 2-phenoxyethanol (Table V).

The time it took for the fish to be anaesthetised and to recover from it, regardless of *G. rufa's* size class and 2-phenoxyethanol anaesthetic, was not correlated (r = 0.129, N = 160, p-value = 0.103 > 0.05). Neither was there, when analysing the fish size classes separately: the small *G. rufa* evidenced a week positive significant correlation between the two phases (r = 0.262, N = 60, p-value = 0.043 < 0.05), but not the medium (r = 0.001, N = 60, p-value = 0.995 > 0.05), or the large size class fish (r = 0.047, N = 40, p-value = 0.771 > 0.05).

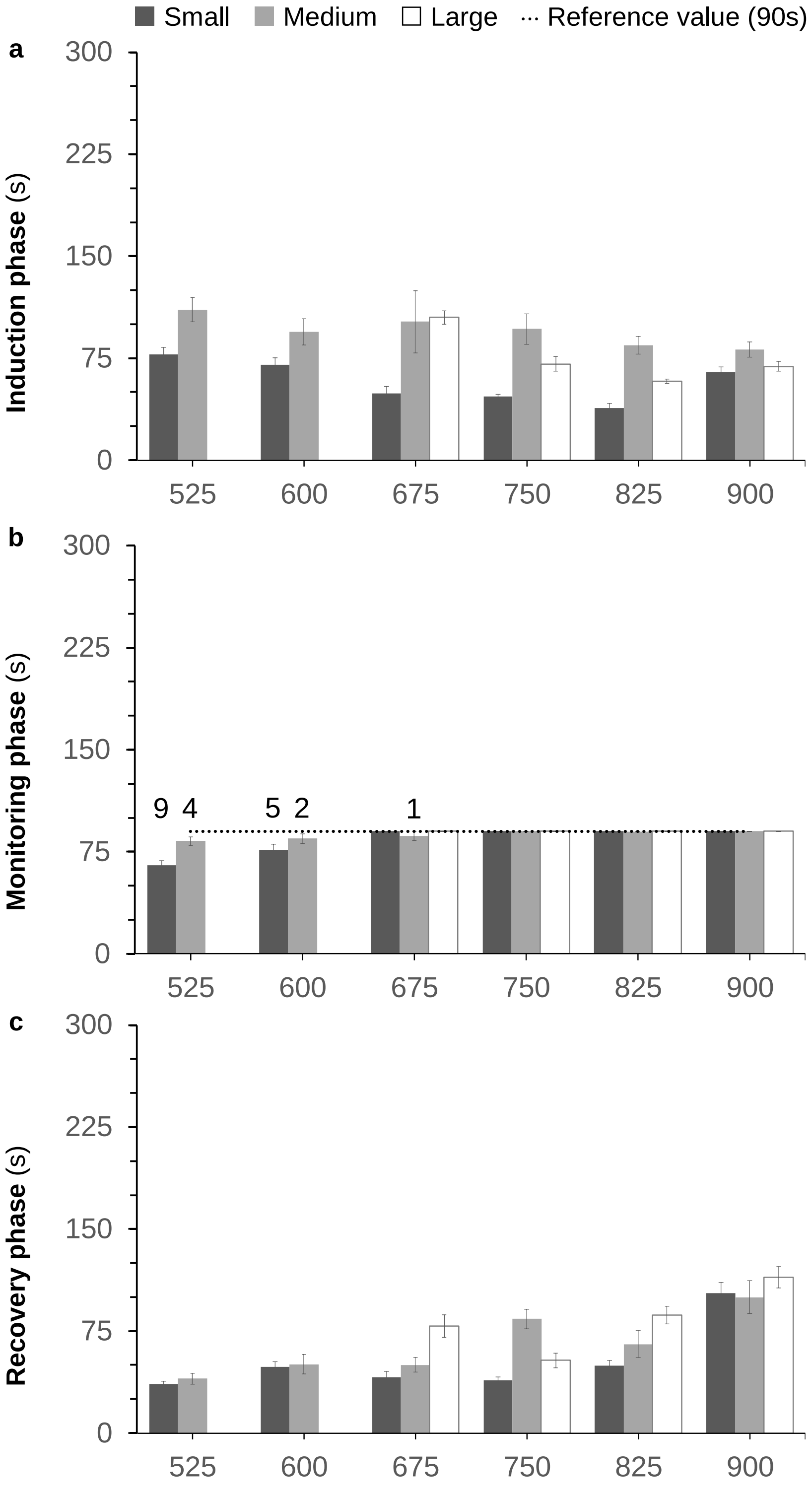

Figure 14 – Timing of the **a)** induction, **b)** monitoring and **c)** recovery phases (mean ± S.E.) for the three size classes of *Garra rufa* (Heckel, 1843) (small, medium and large) and for the six concentrations of 2-phenoxyethanol (525, 600, 675, 750, 825 and 900 mg L^{-1}) assessed during the experimental trial. Note: The numbers above the columns in the monitoring phase indicate the number of fish that not endure 90 seconds of anaesthesia without presenting body contractions or opercular movements.

Table V - Results of linear regression analyses performed to assess the relationship between the independent variables total length (TL) or individual wet weight (IWW) of *Garra rufa* (Heckel, 1843) with the dependent variables of time anaesthesia induction (I) or recovery (R). Fish from all size classes were used in each concentration of 2-phenoxyethanol. Indication of the simple correlation coefficient (r), coefficient of determination (R^2), *p*-value, regression equation and classification of the regression analysis.

Concentrations	r	R^2	*p*-value	Regression equation	Classification
525 mg L^{-1}	0.67	0.46	0.00	I = - 10.76 +18.65 TL	Significant, moderately correlated
	0.69	0.47	0.00	I = 68.27 + 13 IWW	Significant, moderately correlated
	0.31	0.09	0.19	R = 20.74 + 3.06 TL	Non-significant, weakly correlated
	0.34	0.11	0.14	R = 33.33 + 2.32 IWW	Non-significant, weakly correlated
600 mg L^{-1}	0.58	0.34	0.00	I = 2.05 + 14.5 TL	Significant, moderately correlated
	0.50	0.25	0.02	I = 64.79 + 9.17 IWW	Significant, moderately correlated
	0.22	0.05	0.34	R = 28.64 + 3.8 TL	Non-significant, weakly correlated
	0.19	0.04	0.41	R = 45.05 + 2.42 IWW	Non-significant ,very weakly correlated
675 mg L^{-1}	0.45	0.20	0.01	I = 12.2 + 10.89 TL	Significant, moderately correlated
	0.37	0.14	0.04	I = 63.8 + 4.53 IWW	Significant, weakly correlated
	0.65	0.42	0.00	R = 2.89 + 8.01 TL	Significant, moderately correlated
	0.63	0.40	0.00	R = 38.34 + 3.85 IWW	Significant, moderately correlated
750 mg L^{-1}	0.33	0.11	0.07	I = 41.03 + 4.58 TL	Non-significant, weakly correlated
	0.25	0.06	0.18	I = 62.87 + 1.83 IWW	Non-significant, weakly correlated
	0.19	0.03	0.33	R = 44.36 + 2.16 TL	Non-significant, very weakly correlated
	0.09	0.00	0.64	R = 56.14 + 0.55 IWW	Non-significant, very weakly correlated
825 mg L^{-1}	0.27	0.07	0.15	I = 40.62 + 2.96 TL	Non-significant, weakly correlated
	0.13	0.02	0.48	I = 92.17+ 1.80 IWW	Non significant, very weakly correlated
	0.65	0.42	0.00	R = 11.85 + 8.36 TL	Significant, moderately correlated
	0.62	0.39	0.00	R = 49.71 + 3.76 IWW	Significant, moderately correlated
900 mg L^{-1}	0.06	0.04	0.75	I = 68.46 + 0.46 TL	Non-significant, very weak correlated
	0.02	0.00	0.93	I = 71.97 - 0.06 IWW	Non-significant, very weakly correlated
	0.20	0.04	0.30	R = 85.54 + 2.94 TL	Non- significant, weakly correlated
	0.16	0.03	0.39	R = 99.86 - 1.25 IWW	Non-significant, very weakly correlated

3.2.3 -Timing of the stages observed within the induction phase

During the anaesthesia induction with 2-phenoxyethanol, *G. rufa* followed the same pattern observed for MS-222. The first stage observed was impaired motion (control loss of swimming coordination), followed by buoyancy loss (when fish laid sideways on the bottom of the container), and finally ceasing the opercular movements (when they stopped breathing). No exceptions to this sequence were observed. Also like reported for MS-222 anaesthesia, the cease of opercular movements was the longest induction stage, followed almost equally by the other two stages (Fig. 15).

3.2.3.1 Impaired motion

G. rufa showed statistically significant differences in the time it took them to demonstrate impaired motion (Fig. 15 a), when submerged in a 2-phenoxyethanol solution, to what concerns all size classes (Two-way ANOVA: $F_{2,144} = 21.887$, *p*-value = 0.000 < 0.05) and anaesthetic concentrations (Two-way ANOVA: $F_{5,144} = 4.083$, p-value = 0.002 > 0.05), but no interaction between the two factors (Two-way ANOVA: $F_{8,144} = 1.806$, p-value = 0.080 > 0.05).

Generally, the smaller fish were faster to show impaired motion than the other two size classes, followed by the larger fish and the medium ones for last, with statistically significant differences amongst all them (Bonferroni tests with p-value < 0.05).

The time *G. rufa* took to display impaired motion was significantly shorter in the 2-phenoxyethanol concentration 825 mg L^{-1} in relation to the lowest one (525 mg L^{-1}) (Bonferroni tests with p-value < 0.05).

The time for observing *G. rufa* impaired motion was longer for the concentration of 525 mg L^{-1} in smaller fish (15.25 ± 2.33 s), 825 mg L^{-1} in the medium size class (17.80 ± 1.32 s) and 900 mg L^{-1} in the larger fish (16.20 ± 1.77s). Then again, the fastest impairment of swimming was observed at 825 mg L^{-1} both for the small and large size fish (7.60 ± 1.06 s and 15.20 ± 1.27 s, respectively) and 900 mg L^{-1} for the medium fish (12.0 ± 1.52 s).

3.2.3.2 Buoyancy loss

The time spent to lose buoyancy (Fig. 15 b) did not differ between *G. rufa* size classes (Two-way ANOVA: $F_{2,144} = 1.556$, p-value = 0.215 > 0.05), 2-phenoxyethanol concentrations (Two-way ANOVA: $F_{5,144} = 0.950$, p-value = 0.451 > 0.05), not was an interaction between the two factors (Two-way ANOVA: $F_{8,144} = 0.479$, p-value = 0.869 > 0.05).

The loss of buoyancy took longer time at 525 mg L^{-1} for the small (16.1 ± 2.25 s) and medium (16.2 ± 2.01 s) size classes, while for the large fish it was at 825 mg L^{-1} with 15.7 ± 2.13 s. In the contrary, it was faster between 675 mg L^{-1} for the small size class (10.2 ± 1.74) and 750 mg L^{-1} for the medium (13.80 ± 1.60 s) and large fish (12.60 ± 2.49 s).

3.2.3.3 Cease of opercular movements

The time it took for *G. rufa* to cease the opercular movements (Fig. 15 c) varied significantly between all size classes (Two-way ANOVA: $F_{2,144} = 21.887$, p-value = 0.00 < 0.05), 2-phenoxyethanol concentrations (Two-way ANOVA: $F_{5,144} = 4.083$, p-value = 0.002 < 0.05), but there was no interaction between both factors (Two-way ANOVA: $F_{8,144} = 1.806$, p-value = 0.080 > 0.05).

The smaller fish tended to be faster to cease the opercular movements than the other two size classes, followed by the larger fish and the medium ones for last, with statistically significant differences amongst all them (Bonferroni tests with *p*-value < 0.05).
The time *G. rufa* took to cease the opercular movements was significantly shorter in the 2-phenoxyethanol concentration 825 mg L^{-1} in relation to the lowest one (525 mg L^{-1}; Bonferroni test with *p*-value < 0.05).

For the small and large size classes of *G. rufa,* the period to cease opercular movements decreased progressively from smallest concentrations (46.10 ± 4.79 s at 525 mg L^{-1} and 77.00 ± 5.06 at 675 mg L^{-1}) until 825 mg L^{-1} (18.10 ± 2.26 s and 27 ± 2.56 s), to increase again at 400 mg L^{-1} (36.90 ± 9.76 s). The medium size class showed more variance amongst concentrations, but also ceased the opercular movements faster and longer respectively at 825 mg L^{-1} (55.20 ± 5.45 s) and 525 mg L^{-1} (78.40 ± 8.69 s).

3.2.4 -Timing of the stages observed within the recovery phase

G. rufa recovered from anaesthesia with 2-phenoxyethanol in the same way was for MS-222. It began by initiating the opercular movements, followed by presenting body movements, regaining buoyancy control and swimming normally for last. Also, there were few exceptions in which *G. rufa* first recovered buoyancy control and then tried to swim. Generally, the initiation of opercular movements and the initiation of body movements were the longest recovery stages, followed by the normal swimming behaviour (contrarily to what was observed with MS-222). The buoyancy control was the shortest one, being frequently simultaneous with the initiation of body movements (Fig. 15).

3.2.4.1 Initiation of opercular movements

G. rufa presented statistically significant difference within the times recorded for initiating their opercular movements (Fig. 15 c’), in what concerns size classes (Two-way ANOVA: $F_{2,144}$ =3.320, *p*-value = 0.39 < 0.05), 2-phenoxyethanol concentrations (Two-way ANOVA: $F_{5,144}$ = 53.299, *p*-value = 0.00 < 0.05), with interaction between the two factors (Two-way ANOVA: $F_{8,144}$ = 3.902, *p*-value = 0.00 < 0.05).

The initiation of opercular movements took significantly longer to occur within the large size than for the small and medium fish (Bonferroni tests with *p*-value = 0.000 < 0.05), whilst no statistical differences were observed between these two last size classes (Bonferroni test with *p*-value = 0.169 > 0.05).

Most fish within the small and medium size classes started the opercular movements within the monitoring period, leading to an immediate recovery before 90s, at the lowest 2-phenoxyethanol concentrations (525 and 600 mg L^{-1}). From the concentration 675 mg L^{-1}, the time to initiate the opercular movements increased progressively. So much, that this parameter was significantly higher amongst all fish size classes within the 2-phenoxyethanol 900 mg L^{-1} in relation to all others (small fish: 69.20 ± 3.82 s; medium fish: 40.10 ± 7.88 s; large fish: 50.10 ± 7.29 s; Bonferroni tests with *p*-values < 0.05).

No correlation was found between the ceasing of the opercular movements during the anaesthesia induction and the breathing initiation during recovery (r = 0.029, N = 160, *p*-value = 0. 717), or even considering the size classes individually (small: = 162, N = 60, *p*-value = 0.215 > 0.05; medium: r = 0.075, N = 60, *p*-value = 0.567 > 0.05 and large fish: r = -0.057, N = 40, *p*-value = 0.729 > 0.05).

3.2.4.2 Initiation of body movements

The time taken to initiate body movements by *G. rufa* (Fig. 15 d), in order to swim and regain buoyancy, showed statistical differences amongst size classes (Two-way ANOVA: $F_{2,144}$ = 7.552, *p*-value = 0.001 < 0.05) and the 2-phenoxyethanol concentrations (Two-way ANOVA: $F_{5,144}$ = 4.602, *p*-value = 0.001 < 0.05), but no interaction was found between the two factors (Two-way ANOVA: $F_{8,144}$ = 0.937, *p*-value = 0.488 > 0.05).

The large fish took significantly more time to start moving than the small and medium fish (Bonferroni tests with *p*-values < 0.05), whilst these two groups did not differ between each other (Bonferroni test with *p*-values > 0.05). Also, *G. rufa* from the concentration 750 mg L^{-1} showed movements significantly sooner than those from 900 mg L^{-1}.

This parameter presented an apparent trend to decrease until 750 mg L^{-1}. The lower values for small and large size classes were observed at this concentration (9.10 ± 1.65 s and 19.50 ± 3.57 s, respectively), whilst for the medium fish was at 675 mg L^{-1} (11.80 ± 3.51 s). Afterwards, it tended to increase again until 900 mg L^{-1}, in which the medium and large fish recorded the longest times (28.80 ± 5.11 and 32.50 ± 5.24 s, respectively). On the other hand, the small size class recorded the longest times at 600 mg L^{-1} (25.10 ± 2.70 s).

3.2.4.3 Buoyancy control

In what concerns the buoyancy control (Fig. 15 b'), there were statistically significant differences between size classes (Two-way ANOVA: $F_{2,144}$ = 5.849, *p*-value = 0.004 < 0.05), but not amongst 2-phenoxyethanol concentrations (Two-way ANOVA: $F_{5,144}$ = 0.975, *p*-value = 0.435 > 0.05) , neither was there an interaction between the two factors (Two-way ANOVA: $F_{8,144}$ = 0.735, *p*-value = 0.661 < 0.05).

The stage in which *G. rufa* gained buoyancy control was generally short. The large fish from higher concentrations of 2-phenoxyethanol took significantly more time to stand up straight in the bottom of the recovery recipient than those from the other size classes (Bonferroni tests with *p*-values < 0.05), without significant differences amongst these two (Bonferroni test with *p*-value = 0.798 > 0.05).

Buoyancy control was faster at 525 and 600 mg L^{-1}, as most fish did not endure deep anaesthesia during the monitoring phase. In what concerns the remaining 2-phenoxyethanol concentrations, this parameter varied from 2.40 ± 0.51 s of the small fish and 13.40 ± 3.09 s of the large fish, both at 675 mg L^{-1}.

The buoyancy loss in the anaesthesia induction phase showed similar length to its gain during recovery for the higher concentrations, in which the fish remained successfully immobilised during the monitoring phase. No correlations were found between these two stages for small (r = -0.112, N = 60, *p*-value = 0.552 > 0.05), medium (r = -0.078, N = 60, *p*-value = 0.185 > 0.05) and large fish (r = -0.012, N = 40, *p*-value = 0.941 > 0.05).

3.2.4.4 Normal swimming

The time taken by *G. rufa* to exhibit a normal and voluntary swimming behaviour (Fig. 15 a') presented statistically significant differences between size classes (Two-way ANOVA: $F_{2,144}$ = 17.487, *p*-value = 0.000 < 0.05) and 2-phenoxyethanol concentrations (Two-way ANOVA: Two Way ANOVA: $F_{5,144}$ = 4.797, *p*-value = 0.000 < 0.05), with interaction between the two factors (Two-way ANOVA: $F_{8,144}$ = 6.560, *p*-value = 0.00 < 0.05).

In general, the fish from the small size class took a significantly longer time to achieve this stage than the medium and large ones (Bonferroni tests with *p*-value < 0.05), whilst there were no significant differences amongst these two last size classes (Bonferroni test with *p*-value = 0.112 > 0.05).

In terms of concentrations, there were statistically significant differences regarding 525 mg L^{-1} towards 750 and 825 mg L^{-1} (Bonferroni tests with *p*-value < 0.05). *G. rufa* from the medium size class presented longer time to achieve this stage in 750 mg L^{-1} (53.50 ± 5.58 s), while the larger fish presented the same trend in 825 mg L^{-1} (29.10 ± 4.78 s). The small fish took more time to achieve a normal swimming behaviour at 600 mg L^{-1} (16.20 ± 1.80 s).

The normal swimming behaviour in the recovery phase was longer than motion impairment during the anaesthesia induction phase, usually in the highest 2-phenoxyethanol concentrations. No correlations were found between these two stages for small (r = -0.061, N = 60, *p*-value = 0.646 > 0.05), medium (r = 0.108, N = 60, *p*-value = 0.413 > 0.05) and large fish (r = 0.001, N = 40, *p*-value = 0.993 > 0.05).

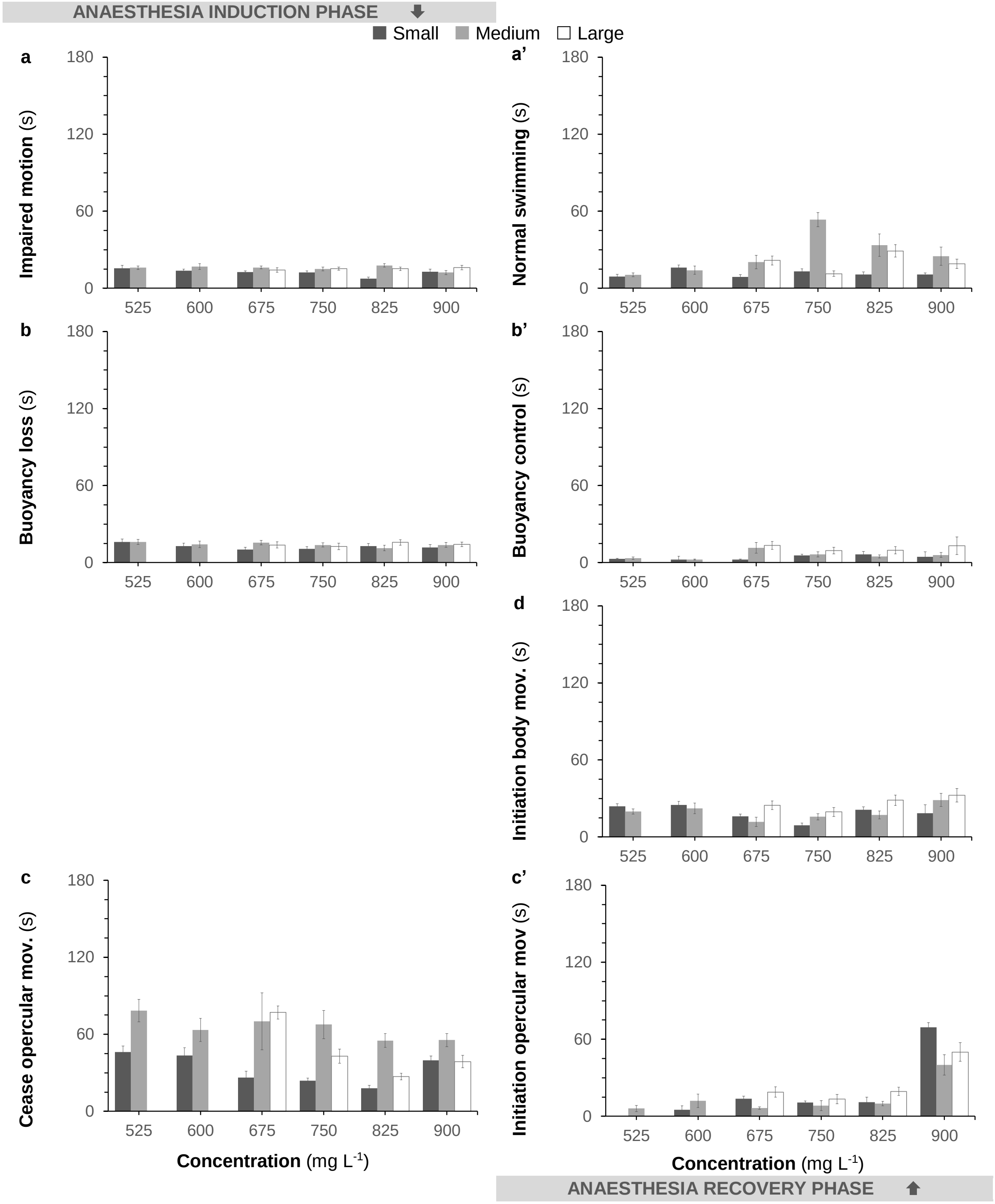

Figure 15 – Timing of the anaesthesia stages (mean ± S.E.) identified during the induction (**a** impaired motion, **b** buoyancy loss and **c** cease of opercular movements) and recovery phases (**c**’ initiation of opercular movements, **d** initiation of body movements, **b’** buoyancy control and **a’** normal swimming), for three size classes of *Garra rufa* (Heckel, 1843) (small, medium and large) and six concentrations of 2-phenoxyethanol (525, 600, 675,750, 825 and 900 mg L^{-1})

3.2.4.5 Total anaesthesia procedure

The anaesthesia procedures with 2-phenoxyethanol, which successfully immobilised the fish for 90 s during examination routines, varied from 128 ± 6.44 s for the medium fish at 350 mg L^{-1} and 224 ± 36.58 s for the large fish at 325 mg L^{-1} (Fig. 16).

The total time for the anaesthesia procedures presented statistically significant differences amongst fish size classes (Two-way ANOVA: $F_{2,144} = 35.818$, p-value = 0.000 < 0.05), 2-phenoxyethanol concentrations (Two-way ANOVA: $F_{5,144} = 5.789$, p-value = 0.000 < 0.05), with a significant interaction between factors (Two-way ANOVA: $F_{8,144} = 3.409$, p-value = 0.001 < 0.05).

Predominantly, the small fish presented a significant shorter time for the entire anaesthesia procedures with 2-phenoxyethanol than the medium and large fish (Bonferroni tests with p-value = 0.000 < 0.05), but not these two last size classes amongst each other (Bonferroni test with p-value = 1.000 > 0.05). Also, the fish took a significant longer time to be handled when anaesthetised with 900 mg L^{-1} than with 750 and 825 mg L^{-1}.

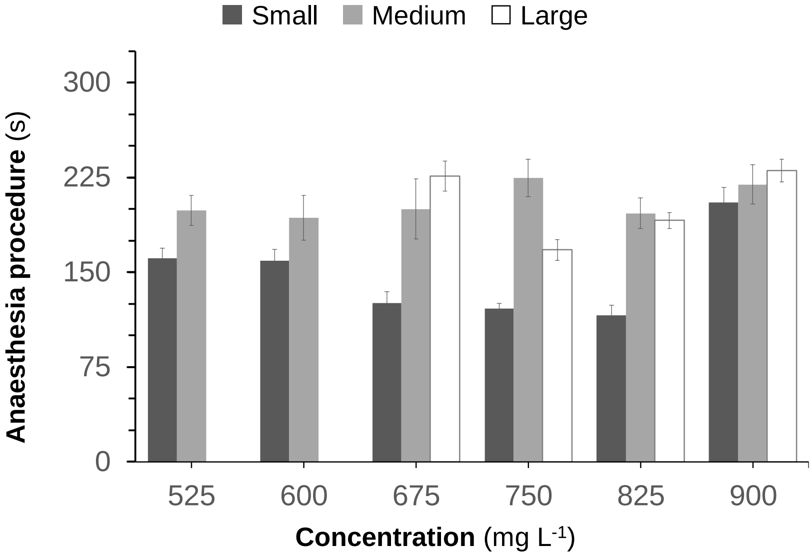

Figure 16 – Timing of the anaesthesia procedure (mean ± S.E.) for the three size classes of *Garra rufa* (Heckel, 1843) (small, medium and large) and for the six concentrations of 2-phenoxyethanol (525, 600, 675,750, 825 and 900 mg L^{-1}).

3.3. - Anaesthesia with clove oil

3.3.1 Total length and individual wet weight

The fish *Garra rufa* measured a total length of 4.41 ± 0.26 cm in the small size class, 6.48 ± 0.89 cm in the medium size class and 9.43 ± 0.54 cm in the large size classe (Fig. 17 a). Furthermore, *G. rufa* weighted 0.69 ± 0.19 g in the small size class, 3.15 ± 1.36 g in the medium size class and 9.86 ± 1.84 g in the large class (Fig. 17 b).

The total length and the individual wet weight presented statistically significant differences between all the three size classes (Two-way ANOVA: $F_{2,108} = 642.581$, p-value = 0.000 < 0.05 and $F_{2,108} = 528.574$, p-value = 0.000 < 0.05, respectively; Bonferroni tests with p-value < 0.05).

On the other hand, there were no statistically significant differences in the total length, nor in the individual wet weight of the fish, in relation to the four concentrations of clove oil (Two-way ANOVA: $F_{2,108} = 1.387$, p-value = 0.251 > 0.05 and $F_{3,108} = 2.550$, p-value = 0.059 > 0.05, respectively).

Moreover, there were no interactions between the three size classes and the four concentrations of clove oil in relation to the total length (Two-way ANOVA: $F_{6,108} = 0.211$, p-value = 0.973 > 0.05) and also to the individual wet weight ($F_{6,108} = 0.908$, p-value = 0.492 > 0.05).

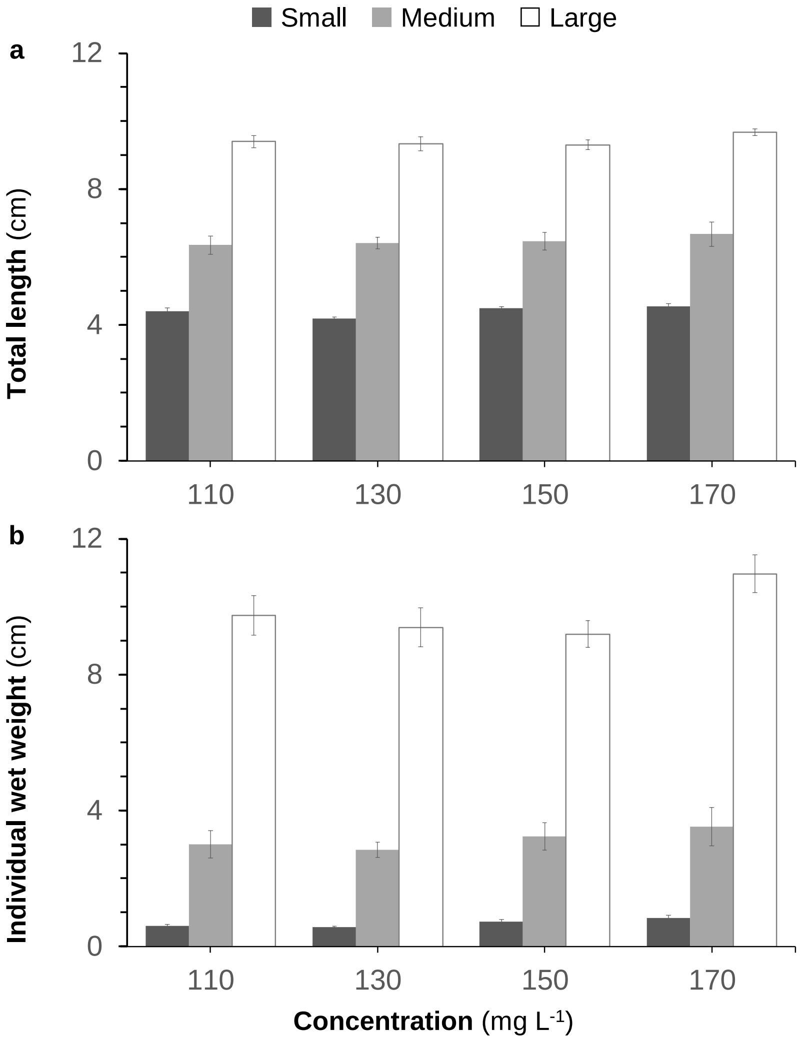

Figure 17 – *Garra rufa* (Heckel, 1843) a) total length and b) individual wet weight (mean ± S.E.) obtained in three size classes (small, medium and large fish) and four concentrations of clove oil (110, 130, 150 and 170 mg L^{-1}).

3.3.2 -Timing of the stages observed within the induction monitoring and recovery phase

3.3.2.1 Induction phase

The induction phase (Fig. 18 a) was distinct for size classes (Two-way ANOVA: $F_{2,108}$ = 5.013, *p*-value = 0.008 < 0.05, Bonferroni tests with *p*-value < 0.05), clove oil concentrations (Two-way ANOVA: $F_{3,108}$ = 2.737, *p*-value = 0.047 < 0.05), with no interaction between the two factors (Two-way ANOVA: $F_{6,108}$ = 0.756, *p*-value = 0.606 > 0.05).

Generally, *G. rufa* from the large size class presented a longer induction phase than those from the small one (Bonferroni test with *p*-value = 0.008 < 0.05), while the medium size class showed no differences regarding those two (Bonferroni tests with *p*-value > 0.05). Also, the induction phase was only significantly shorter within the fish from clove oil concentration 150 mg L^{-1} than in those from 110 mg L^{-1} (Bonferroni test with *p*-values < 0.05).

The clove oil concentrations that induced a faster anaesthesia induction in *G. rufa* were 150 mg L^{-1}, both for the small and large fish (60.90 ± 5.30 s and 101.60 ± 7.65 s, respectively), plus 130 mg L^{-1} (91.40 ± 11.90 s). On the other hand, those who took more time to induce anaesthesia were 110 mg L^{-1} for the small and large *G. rufa* (90.70 ± 8.80 s and 157.10 ± 48.24 s, respectively), plus 110 mg L^{-1} for the medium fish (130.10 ± 27.78 s). Apparently, larger G. rufa took more time to be anaesthetised than the smaller ones.

A significant linear regression, with strong correlation and 31 % determination coefficients, was observed between the anaesthesia induction time and fish's total length at 150 mg L^{-1} of clove oil. Similarly, significant moderate correlations were obtained at 130 mg L^{-1}, between the anaesthesia induction time and both fish's total length and individual wet weight (Table VI).

3.3.2.2 - Monitoring phase

The monitoring phase (Fig. 18 b) had no statistically significant differences of time between size classes (Two-way ANOVA: $F_{2,108}$ = 53.325, *p*-value = 0.535 > 0.05), clove oil concentrations (Two-way ANOVA: $F_{3,108}$ = 1.980, *p*-value = 0.121 > 0.05), or an interaction between the two factors (Two-way ANOVA: $F_{6,108}$ = 1.738, *p*-value = 0.119 > 0.05).

G. rufa from all size classes endured the all 90 s of the monitoring phase without presenting muscular contractions and opercular movements only at 130 mg L^{-1} of clove oil. The concentration 150 mg L^{-1} produced similar results, except for two large fish that initiated breathing before that time had ended. The clove oil concentrations below and above those values seem to be unsuited for routine procedures regarding fish physical assessments, as some fish recovered from anaesthesia short after ceasing their opercular movements.

3.3.2.3 - Recovery phase

The recovery phase (Fig. 18 c) demonstrated significantly differences within *G. rufa* size classes (Two-way ANOVA: $F_{2,108}$ = 4.550, *p*-value = 0.013 < 0.05, Bonferroni tests with *p*-value < 0.05), but not amongst clove oil concentrations (Two-way ANOVA: $F_{3,108}$ = 1.023, *p*-value = 0.386 > 0.05), neither was there an interaction between the two factors (Two-way ANOVA: $F_{6,108}$ = 1.231, *p*-value = 0.296 > 0.05).

Generally, *G. rufa* from the large size class recovered faster than those from the small one (Bonferroni test with *p*-value = 0.018 < 0.05). The medium size class showed no differences regarding those two (Bonferroni tests with *p*-value > 0.05), presenting intermediate values in the lowest concentrations (110 and 130 mg L^{-1}) and longest recovery in the highest concentrations (150 and 170 mg L^{-1}).

In fact, the recovery phase lasted similar times between concentrations. Considering just the concentrations in which clove oil anaesthesia was effective throughout the entire monitoring phase, *G. rufa* from the medium and large size classes recovered faster at 130 mg L^{-1} (106.60 ± 7.21 s and 83.50 ± 5.77 s, respectively), while those from the small size class were faster at 150 mg L^{-1} (105.10 ± 9.57 s).
Apparently, larger *G. rufa* recovered faster from anaesthesia than smaller ones.

Significant linear regressions, with moderate correlation and 26 % determination coefficients, were observed between the anaesthesia recovery time and G. rufa´s total length, as well as individual wet weight, at 130 mg L^{-1} of clove oil (Table VI).
The time it took for the fish to be anaesthetised and to recover from it, regardless of G*. rufa’s* size class and clove oil anaesthetic, were very weakly and positively correlated (r = 0.295, N = 120, *p*-value = 0.001 < 0.05). However, when analysing the fish size classes separately: the small *G. rufa* evidenced a strong positive significant correlation between the two phases (r = 0.737, N = 40, *p*-value = 0.000 < 0.05), while it was moderate for the medium fish (r = 0.413, N = 40, *p*-value = 0.008 < 0.05) and weak for the large fish (r = 0.346, N = 40, *p*-value = 0.029 < 0.05).

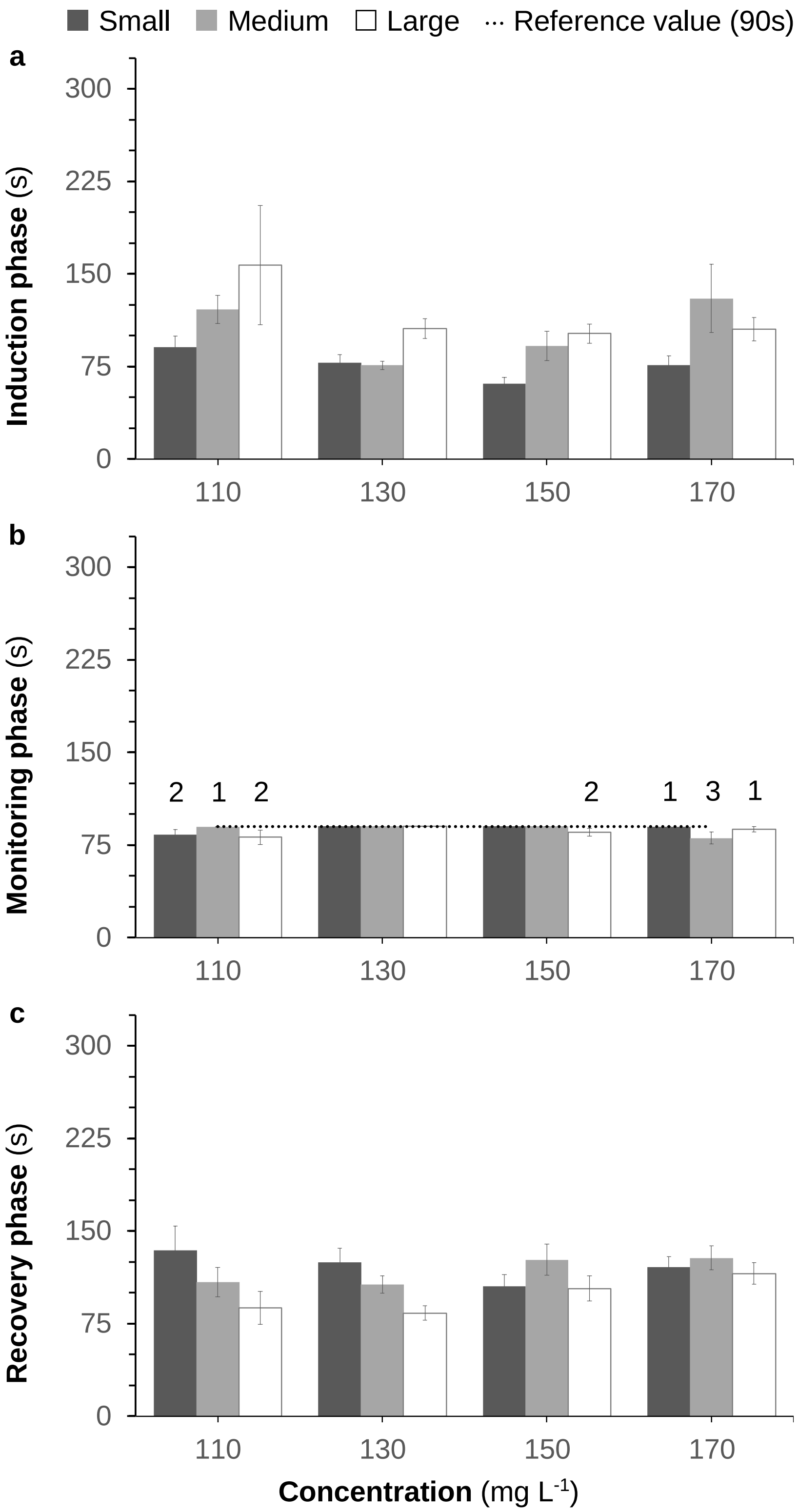

Figure 18 – Timing of the **a)** induction, **b)** monitoring and **c)** recovery phases (mean ± S.E.) obtained in three size classes of *Garra rufa* (Heckel, 1843) (small, medium and large fish) and four concentrations of clove oil (110, 130, 150 and 170 mg L^{-1}).

Note : The numbers above the columns in the monitoring phase indicate the number of fish that not endure 90 seconds of anaesthesia without presenting body contractions or opercular movements.

Table VI - Results of linear regression analyses performed to assess the relationship between the independent variables total length (TL) or individual wet weight (IWW) of *Garra rufa* (Heckel, 1843) with the dependent variables time anaesthesia induction (I) or recovery (R). Fish from all size classes were used in each concentration of clove oil. Pearson's correlation coefficient (r), coefficient of determination (R^2), *p*-value, regression equation and classification of the regression analysis are indicated.

Concentrations	r	R^2	*p-value*	Regression equation	Classification
110 mg L^{-1}	0.33	0.11	0.00	I = 35.49 + 9.45 TL	Significant, weakly correlated
	0.29	0.09	0.00	I = 79.12 + 4.46 IWW	Significant, weakly correlated
	0.22	0.05	0.02	R = 138.77 - 3.97 TL	Significant, weakly correlated
	0.25	0.06	0.06	R = 123 - 2.38 IWW	Significant, weakly correlated
130mg L^{-1}	0.51	0.26	0.00	I = 48.93 + 5.64 TL	Significant, moderately correlated
	0.51	0.31	0.00	I = 71.59 + 3.46 IWW	Significant, moderately correlated
	0.51	0.26	0.00	R = 154.45 - 7.47 TL	Significant, moderately correlated
	0.51	0.26	0.00	R = 122.47 - 4.14 IWW	Significant, moderately correlated
150 mg L^{-1}	0.60	0.31	0.00	I = 24.72 + 8.87 TL	Significant, moderately correlated
	0.49	0.24	0.00	I = 65.57 + 4.35 IWW	Significant, moderately correlated
	0.05	0.00	0.98	R = 111.11 + 0.09 TL	Significant, very weakly correlated
	0.07	0.00	0.69	R = 115 - 0.74 IWW	Non-significant, very weakly correlated
170 mg L^{-1}	0.33	0.11	0.08	I = 42.16 + 8.85 TL	Non-significant, weakly correlated
	0.24	0.06	0.20	I = 87.47 + 3.19 IWW	Non-significant, weakly correlated
	0.15	0.02	0.42	R = 3.57 - 6.18 TL	Non-significant, very weakly correlated
	0.05	0.02	0.80	R = 123 - 0.31 IWW	Non-significant, very weakly correlated

3.3.3 -Timing of the stages observed within the induction phase

During the anaesthesia induction with clove oil, G. rufa followed the same pattern observed for the other two anaesthetics (MS-222 and 2-phenoxyethanol). The stages of induction observed were impaired motion (control loss of swimming coordination), followed by buoyancy loss (when fish laid sideways on the bottom of the container) and cease of the opercular movements (stop breathing). No exceptions to this sequence were observed. The buoyancy loss was the shortest stage to attain, followed by motion impairment and cease of opercular movements was by far the longest one (Fig. 19).

3.3.3.1 Impaired motion

G. rufa showed statistically significant differences in the time it took them to demonstrate impaired motion (Fig. 19 a), when submerged in a clove oil solution amongst size classes (Two-way ANOVA: $F_{2,108}$ = 18.485, *p*-value = 0.000 < 0.05), but not in what concerns anaesthetic concentrations (Two-way ANOVA: $F_{3,108}$ = 1.649, *p*-value = 0.182 > 0.05). Although a significant interaction was found between the two factors (Two-way ANOVA: $F_{6,108}$ = 2.496, *p*-value = 0.027 < 0.05).

Generally, the large fish were slower to show impaired motion than those from the other two size classes (Bonferroni tests with *p*-value =0.000 < 0.05), with no statistically significant differences amongst small and medium fish (Bonferroni test with *p*-value = 1.000 > 0.05).

The fastest times for observing *G. rufa* impaired motion were: 12.10 ± 1.05 s for the small fish at 150 mg L^{-1}, 12.20 ± 0.77 s for the medium fish at 130 mg L^{-1} and 14.80 ± 1.72 s for the large fish at 170 mg L^{-1}.

3.3.3.2 – Loss of buoyancy

The time spent to lose buoyancy (Fig. 19 b) did present statistically significant differences between *G. rufa* size classes (Two-way ANOVA: $F_{2,108}$ = 3.737, *p*-value = 0.027 < 0.05), but not for clove oil concentrations (Two-way ANOVA: $_{F3,108}$ = 1.495, *p*-value = 0.220 > 0.05), nor was an interaction between the two factors (Two-way ANOVA: $F_{6,108}$ = 0.786, *p*-value = 0.583 > 0.05).

Generally, the smaller fish were faster to lose control of their buoyancy ability than those from the medium size classe (Bonferroni test with *p*-value =0.047 < 0.05), but they did not differ statistically from the large fish, neither did these last ones from the medium fish (Bonferroni tests with *p*-value > 0.05). Times varied from 7.00 ± 1.10 s for the small fish at 170 mg L^{-1} and the 18.20 ± 3.17 s for the large fish at 110 mg L^{-1} of clove oil.

3.3.3.3 – Cessation of opercular movements

The time it took for *G. rufa* to cease the opercular movements (Fig. 19 c) varied significantly between *G. rufa* size classes (Two-way ANOVA: $F_{2,108}$ = 3.245, *p*-value = 0.043 < 0.05), but not for clove oil concentrations (Two-way ANOVA: $F_{3,108}$ = 2.480, *p*-value = 0.065 > 0.05), nor was an interaction between the two factors (Two-way ANOVA: $F_{6,108}$ = 0.312, *p*-value = 0.720 > 0.05).

The smaller fish tended to cease the opercular movements faster than those from the large size class (Bonferroni test with *p*-value = 0.047 < 0.05), but not from the medium ones, neither did these two last group of fish differed one from the other (Bonferroni tests with *p*-value < 0.05).

The time *G. rufa* took to cease the opercular movements was apparently shorter in the lowest and highest clove oil concentrations (110 and 170 mg L^{-1}) than in the intermediate ones (130 and 150 mg L^{-1}). The times varied from 36.50 ± 5.75 s from the small fish at 130 mg L^{-1} and 118.60 ± 48.48 s from the small fish at 110 mg L^{-1}.

3.3.4 -Timing of the stages observed within the recovery phase

G. rufa recovered from clove oil anaesthesia in the same way as for the other two anaesthetics (MS-222 and 2-phenoxyethanol). The first recovery sign was seeing opercular movements. These occurred shortly after the insertion of the fish in clean water. It was followed by muscular contractions, in order to resume swimming, which was often a long stage to attain. Then, the fish were able to control their buoyancy ability, almost always immediately to the initiation of body movements. Finally, *G. rufa* ended up swimming normal and voluntarily, largely the longest stage to accomplish (Fig. 19). Rare exceptions were observed, in which *G. rufa* first recovered buoyancy control and then tried to swim.

3.3.4.1 Initiation of opercular movements

G. rufa presented statistically significant difference within the times recorded for initiating their opercular movements (Fig. 19 c') between *G. rufa* size classes (Two-way ANOVA: $F_{2,108}$ = 9.080, *p*-value = 0.000 < 0.05), but not for clove oil concentrations (Two-way ANOVA: $F_{3,108}$ = 0.950, *p*-value = 0.419 > 0.05), or was an interaction between the two factors (Two-way ANOVA: $F_{6,108}$ = 0.743, *p*-value = 0.118 > 0.05).

Generally, the initiation of opercular movements took significantly longer to occur within the small size class than for the medium and large fish (Bonferroni tests with *p*-value = < 0.05), whilst no statistical differences were observed between these two last size classes (Bonferroni test with *p*-value = 1.000 > 0.05).

Apparently, *G. rufa* regained opercular movements sooner within the lowest and highest clove oil concentrations (110 and 170 mg L^{-1}) than in the intermediate ones (130 and 150 mg L^{-1}). Considering just the concentrations in which clove oil anaesthesia was effective throughout the entire monitoring phase, *G. rufa* from the medium and large size classes recovered the opercular movements faster at 150 mg L^{-1} (16.00 ± 3.33 s and 10.80 ± 1.79 s, respectively), while those from the large size class were faster at 130 mg L^{-1} (6.90 ± 1.09 s).

No correlation was found between the ceasing of the opercular movements during the anaesthesia induction and the breathing initiation during recovery (r = 0.006, N = 120, *p*-value = 0. 945), nor even considering the size classes individually for medium (r = -0.188, N = 40, p-value = 0.244 > 0.05) and large fish (r = 0.006, N = 40, *p*-value = 0.970 > 0.05). Notwithstanding, the small fish presented a strong positive correlation, in which those fish that took longer to stop breathing also took longer to restart the opercular movements (r = 0.626, N = 40, *p*-value = 0.000 < 0.05).

3.3.4.2 Initiation of body movements

The time taken to initiate body movements by *G. rufa* (Fig. 19 d) showed statistical differences amongst size classes (Two-way ANOVA: $F_{2,108} = 3.270$, *p*-value = 0.042 < 0.05) and clove oil concentrations (Two-way ANOVA: $F_{3,108} = 4.083$, *p*-value = 0.009 < 0.05), with an interaction between the two factors (Two-way ANOVA: $F_{6,108} = 0.786$, *p*-value = 0.583 < 0.05).

The small fish took significantly less time to start moving than the large fish (Bonferroni test with *p*-values = 0.41 < 0.05), whilst the medium fish did not differ from those two fish size classes (Bonferroni tests with *p*-values > 0.05).

Apparently, *G. rufa* from the lowest clove oil concentrations moved sooner than those from the highest ones, but there were statistically significant differences only between the 130 mg L^{-1} and 170 mg L^{-1} (Bonferroni test with *p*-values < 0.05).

Within the clove oil concentrations that produced an effective anaesthesia during the monitoring phase, the fastest times to produce body movements were recorded for small fish at 150 mg L^{-1} (54.60 ± 7.50 s), while for the medium and large fish was at 130 mg L^{-1} (42.70 ± 4.49 s and 29.70 ± 5.30 s, respectively).

3.3.4.3 Buoyancy control

In what concerns the buoyancy control (Fig. 19 b'), there were no statistically significant differences between size classes (Two-way ANOVA: $F_{2,108} = 0.959$, *p*-value = 0.387 > 0.05), or amongst clove oil concentrations (Two-way ANOVA: $F_{3,108} = 0.656$, *p*-value = 0.581 > 0.05), but there was an interaction between the two factors (Two-way ANOVA: $F_{6,108} = 2.520$, *p*-value = 0.025 < 0.05).

The stage in which *G. rufa* gained buoyancy control was generally short. It varied from 2.70 ± 0.53 s of the medium fish at 170 mg L^{-1} and 17.60 ± 4.32 s of the large fish, both at 130 mg L^{-1}.

The buoyancy loss in the anaesthesia induction phase showed similar length to its gain during recovery for the higher concentrations, in which the fish remained successfully immobilised during the monitoring phase. No correlations were found between these two stages, regarding the fish from all size classes (r = -0.152, N = 120, *p*-value = 0.098 > 0.05), or for just small (r = -0.215, N = 40, *p*-value = 0.182 > 0.05), medium (r = 0.008, N = 40, *p*-value = 0.961 > 0.05) and large fish (r = -0.250, N = 40, p-value = 0.120 > 0.05).

3.3.4.4 - Normal swimming

The time taken by *G. rufa* to exhibit a normal and voluntary swimming behaviour (Fig. 19 a') presented statistically significant differences between size classes (Two-way ANOVA: $F_{2,108} = 5.869$, *p*-value = 0.004 < 0.05), but not amongst clove oil concentrations (Two-way ANOVA: $F_{3,108} = 1.702$, *p*-value = 0.171 > 0.05), neither an interaction between the two factors (Two-way ANOVA: $F_{6,108} = 1.965$, *p*-value = 0.077 < 0.05).

In general, the fish from the medium size class took a significantly longer time to achieve this stage than the small and large ones (Bonferroni tests with *p*-value < 0.05), whilst there were no significant differences amongst these two last size classes (Bonferroni test with *p*-value = 1.000 > 0.05). This stage lasted from 18.10 ± 2.81 s to of 47.20 ± 9.06 s, by small fish at 170 mg L^{-1} and 110 mg L^{-1}, respectively.

The normal swimming behaviour in the clove oil recovery phase took longer to achieve than the motion impairment during the anaesthesia induction phase. No correlations were found between these two stages for all fish (r = -0.133, N = 120, p-value = 0.149 > 0.05), neither for just small (r = -0.004, N = 40, *p*-value = 0.982 > 0.05), medium (r = -0.025, N = 40, *p*-value = 0.877 > 0.05), or large fish (r = -0.153, N = 40, *p*-value = 0.345 > 0.05).

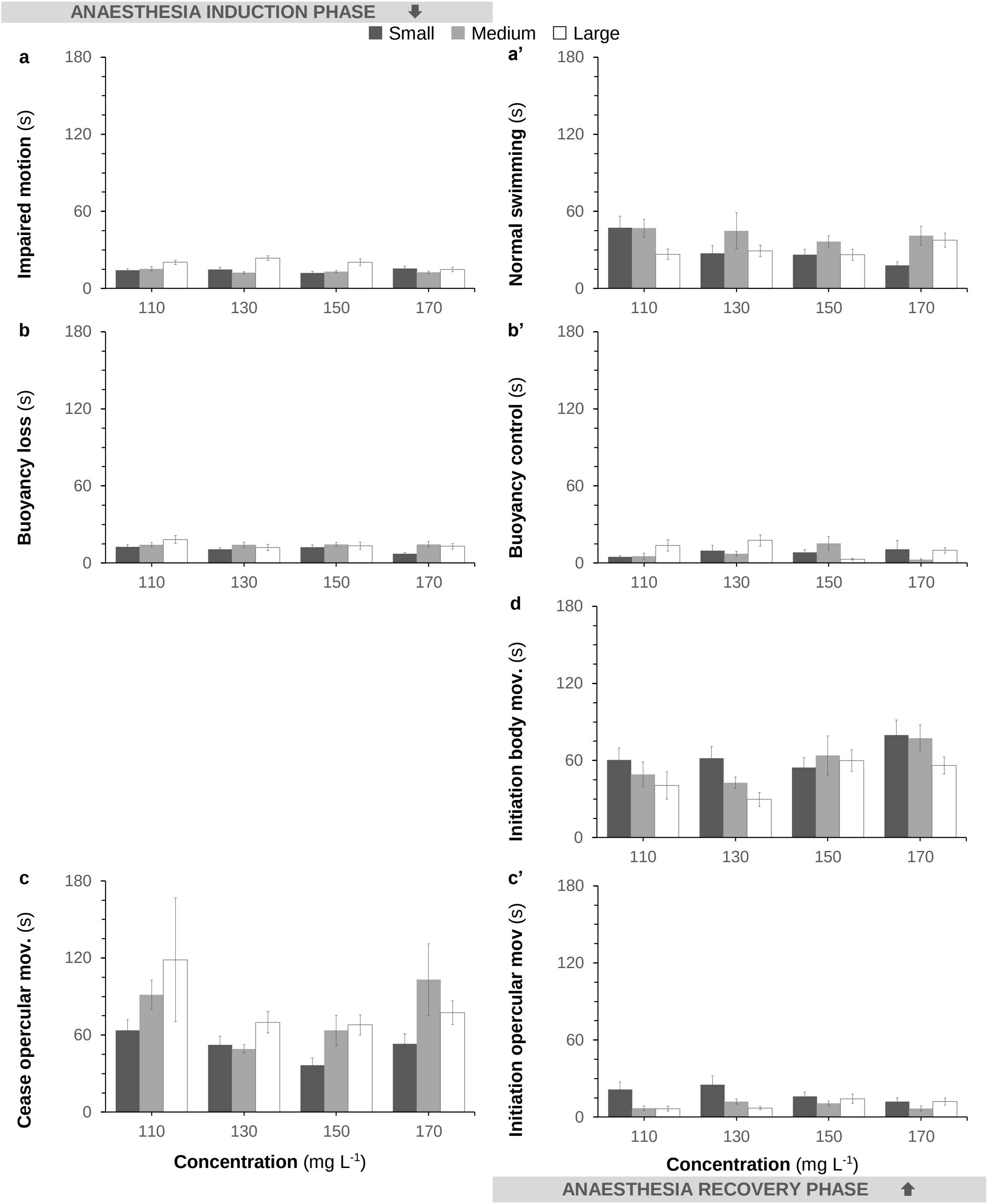

Figure 19 – Timing of the anaesthesia stages (mean ± S.E.) identified during the induction (**a** impaired motion, **b** buoyancy loss and **c** cease of opercular movements) and recovery phases (**c**' initiation of opercular movements, **d** initiation of body movements, **b'** buoyancy control and **a'** normal swimming), for three size classes of *Garra rufa* (Heckel, 1843) (small, medium and large) and four concentrations of Clove oil (110, 130, 150 and 170 mg L^{-1}).

3.3.4.5 - Total anaesthesia procedure

In the end, the total time for the anaesthesia procedures presented no statistically significant differences amongst fish size classes (Two-way ANOVA: $F_{2,108}$ = 0.944, *p*-value = 0.392 > 0.05), or among clove oil concentrations (Two-way ANOVA: $F_{3,108}$ = 1.899, *p*-value = 0.134 > 0.05), without a significant interaction between factors (Two-way ANOVA: $F_{6,108}$ = 0.613, *p*-value = 0.719 > 0.05).

The anaesthesia procedures with clove oil, which successfully immobilised the fish for 90 s during examination routines, varied from 166.00 ± 14.21 s for the small fish and 218.10 ± 23.74 s for the medium fish, both at 150 mg L^{-1} (Fig. 20).

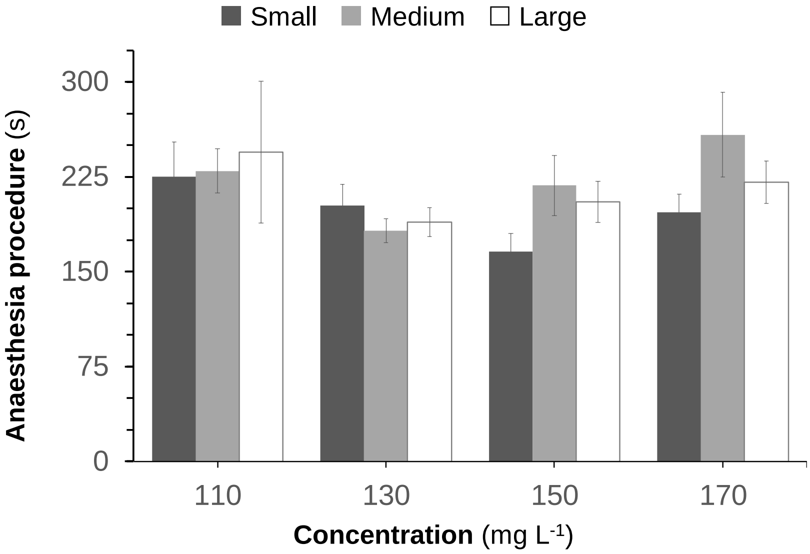

Figure 20 – Timing of the anaesthesia procedure (mean ± S.E.) for the three size classes of *Garra rufa* (Heckel, 1843) (small, medium and large) and for the four concentrations of Clove oil (110, 130, 150 and 170 mg L^{-1}).

4. Discussion

Garra rufa food intake after anaesthesia at 20 °C was previously described by Ferreira *et al.* (2015a, 2015b, 2016). The fish usually showed immediate appetence for food, after being subjected to all the three anaesthetics: MS-222, 2-phenoxyethanol and clove oil. Plus, they ingested their daily dose of food within a monitoring time of 30 min. In this study, *G. rufa* presented a similar behaviour, although the small fish took longer to ingest the same amount of feed. The dose administered was based on the work of Catarino *et al.* (2019), who determined that *G. rufa* with a total length of 4.80 ± 0.025 cm were satisfied every day with 0.04 g of feed per fish (0.17 kcal day^{-1} fish^{-1}; 3.2% of the body weight), correspondent to an *ad libitum* situation. Therefore, the present results sugest that the effects of the anaesthetics at 29 °C may bide for some time within the metabolism of this species, impairing its appetite, as the small fish had similar size and conditions to those studied by Ferreira *et al. (*2015a, 2015b, 2016) and Catarino *et al.* (2019). On the other hand, the medium and large *G. rufa* were very fast to ingest the food given to them. Afterwards, they still manifested some frenzy on the sight of their caretakers, for which they were given more food that was immediately consumed. Thus, it may be necessary to study in the future the dose of food required by larger *G. rufa* fish to be satiated, in order to satisfy their nutrional requirements and abide with animal welfare ethics.

The influence of anaesthesia has been approached in other studies, with different fish species. Anaesthesia with 80 mg L^{-1} of MS-222 and 40 mg L^{-1} of clove oil caused a reduction of feed consumption in juveniles of rainbow trout *Oncorhynchus mykiss* at a temperature of 12.6 °C (Pirhonen & Schreck, 2003). The fish were able to recover, presenting increased food intake 4 hours later.

Nevertheless, Pirhonen & Schreck, (2003) verified that the non-anaesthetised fish (control group) still ingested 15 to 20 % more food than the ones subjected to the anaesthetics within a period of 24 to 48 hours afterwards .

Also, Soto & Burhanuddin, (1995) observed that most rabbit fish *Siganus lineatus* (Valenciennes, 1835) had eaten the food given to them, within a few hours after being anesthetized with clove oil. Moreover, the siberian sturgeon *Acipenser baerii* (Brandt, 1869) was anaesthetised with 350 mg L^{-1} of clove oil by Akbulut *et al.* (2012). These authors found that the effects of this anaesthetic on feed consumption continued 4 hours after anaesthesia was performed, in which anaesthetised fish took longer to ingest the feed than non-anesthetized ones (control group). The longest feeding time was recorded in the 10 min after anaesthesia, being 4 minutes for anaesthetised fish and 2 minutes for control fish

(Akbulut *et al.,* 2012). Because of the probability of interfering with the digestive system, Fish are fasted for 12 to 24 hours prior to anaesthesia in order to reduce the risk of regurgitation of food that may lodge in the gills or foul the water (Ross & Ross, 1999). Fasting also decreases fecal contamination of water used for anaesthesia and recovery.

The exact mechanism by which MS-222, 2-phenoxyethanol and clove oil act to supress the nervous system in fish and reduce sensory perception are still cryptic (Popovic *et al.,* 2012; Zahl *et al.,* 2012). All of them are mostly absorbed by the gills and also through the skin, acting systemically to reach the body tissues (Carter *et al.,* 2011). According to Burka *et al.* (1997), Matthews & Varga, (2012), plus Balko *et al,* (2018), MS-222 operates at the level of the peripheral and central nervous system, decreasing neuronal activity. It acts on the sodium channels of neurons, blocking them. MS-222 inhibits the sodium entrance into the nerve cell and thus, limiting the depolarization of the plasma membrane, plus the consequent electric nerve impulse (Carter *et al.,* 2011) and/or muscle contraction (Priborsky & Velisek, 2018). This anaesthetic also acts, but to a lesser extent, on the potassium pathways of nerve membranes, being a muscle relaxant (Matthews & Varga, 2012).

The exact mechanism of 2-phenoxyethanol anaesthetic effect in fish has not been fully described (Priborsky & Velisek, 2018). For the anaesthetic 2-phenoxyethanol, it has been suggested by Burka *et al.* (1997) that the anaesthesia mechanism involves an expansion of neuronal cell membranes. The study of Zahl *et al.* (2012) demonstrated that 2-phenoxyethanol exerted some inhibitory activity on N-methyl-d-aspartate (NMDA) receptor, which is a glutamate receptor and ion channel protein found in nerve cells. Its activation results in the opening of an ion channel that is nonselective to cations, causing the depolarisation of the plasma membrane and originates the electric nerve impulse.

The anaesthetic clove oil is a highly lipophilic substance, which is rapidly distributed by the circulatory system and incorporated in the body tissues, especially the fat and nervous tissues (Priborsky & Velisek, 2018). It decreases neurosensory functions by affecting the nervous system, especially the cerebral cortex (Fernandes *et al.,* 2017). In addition to NMDA inhibition (Zahl *et al.,* 2012), clove oil potentiates gamma-amino butyric acid type A (GABAa) receptor (Zahl *et al.,* 2012). In vertebrates, GABA is the chief inhibitory neurotransmitter in the central nervous system. It binds to specific transmembrane receptors in both pre- and postsynaptic plasma membranes, at inhibitory synapses in the brain. GABA induces the opening of ion channels, allowing the flow of either negatively charged chloride ions into the cell or positively charged potassium ions out of the cell, causing a negative change in the transmembrane potential (usually resulting in hyperpolarization).

In what concerns the anaesthesia recovery, MS-222 is rapidly metabolised by acetylation reactions and, together with its non-polar metabolites, is mainly excreted through the gills (Wayson *et al.,* 1976 in Carter *et al.,* 2011). Unmetabolised MS-222 and its polar metabolites are mostly excreted by the kidneys (Burka *et al.,* 1997; Wayson *et al.,* 1976 in Carter *et al.,* 2011). MS-222 half-time was estimated at 1.5 to 4 hours (Hunn & Allen, 1974 in Carter *et al.,* 2011), being undetected in body fluids passed 8 to 24 hours after exposure (Burka *et al.,* 1997; Wayson *et al.,* 1976 in Carter *et al.,* 2011). This anaesthetic is generally accepted as a good agent for anaesthesia, euthanasia and sedation, reccomended for several procedures and routine operations (Popovic *et al.,* 2012). MS-222 is one of the few approved to be used in fish destined to human consumption, both by the United States of America (FDA, 2007) and European Union, as long as a withdrawal period of 21 days is applied. After all, MS-222 is regarded as a potential carcinogenic (Pirhonen & Schreck, 2003). Morover, it represents several disadvantages: 1) may have selective toxicity for poikilotherms due to their low metabolic rate of the liver (Wayson *et al.,* 1976); 2) its addition to the water causes pH changes, harmful for aquatic animals (Priborsky & Velisek, 2018); 3) it is quite expensive to acquire and 4) abides safety protocols for its use (Popovic *et al.,* 2012) . Therefore, the research for other alternative anaesthetics continues.

On the other hand, 2-phenoxyethanol is rapidly excreted, mainly by the gills, and its half-life was estimated in 30 min (Imamura-Kojima *et al.,*1987). But it is known to fail in supressing involuntary muscle reflexes. Thus, it may not be an effective anaesthetic and it is not recommended for surgical procedures. Also, fish subjected to this substance are known to recover often in an abrupt manner (Priborsky & Velisek, 2018). Moreover, 2-phenoxyethanol is an irritant substance, for which it should be handled with care.

Eugenol is the main component of the clove oil (83 – 95 %). It is rapidly and almost completely excreted through the kidneys within 24 hours (Fischer *et al.,* 1990 in Javahery *et al.,* 2012), having a 12.4 hours half-life in rainbow trout tissues (Guénette *et al.,* 2007). But the gills are also appointed as an excretion pathway for this anaesthetic in fish. Under repeated administration, eugenol may accumulate in the fish's tissues and become potentially toxic (Guénette *et al.,* 2007; Priborsky & Velisek, 2018). In high doses, eugenol may be a cytotoxic, namely for liver and kidney cells (Javahery *et al.,* 2012). Eugenol is known for its antioxidant, anti-inflammatory, antimicrobial, antitumor, antimutagenic, and antigenotoxic activities; however, it may present some toxicity depending on the type of histological structure exposed to this compound and the concentration used (Jaganathan & Supriyanto, 2012; Barboza *et al.,* 2018). Clove oil is considered generally regarded as safe (GRAS; FDA, 2007) and has different applications, such as food additive, flavouring and fragance agent, or even as a an attractant pheromone for insects (Moustafa *et al.,* 2012). Due to its market accessibility, price and rapid effects, it is a promising anaesthethic to be used for husbandry and transport of fish. Nonetheless, one other component of the clove oil is meythyleugenol, present in trace amounts, known to be carcinogenic (WHO, 2013). For this reason, the anaesthetic active compounds in clove oil have been selected to produce AQUI-S (AQUI-S New Zealand, Lower Hutt, New Zealand), an aquatic anaesthetic that has been approved in New Zealand, Australia, Chile, and Vietnam for animal husbandry, transportation and harvesting operations with a zero withholding period, plus Norway, Iceland and the Faroe Islands (but without clearance for harvesting) (AQUI-S, 2020).

For the above reasons, clove oil and 2-phenoxyethanol are not approved for use on fish intended for human consumption (Priborsky & Velisek, 2018). Almost all (if not all) anaesthetics have problematic issues associated, whose negative impacts can be reduced if optimal concentrations and operation conditions are employed to minimize and reduce stress in fish (Summerfelt & Smith, 1990; Priborsky & Velisek, 2018).

Studies on anaesthetics and concentrations to be used on *Garra rufa* are scarce (Ferreira *et al.,* 2015a, 2015b, 2016; Aydin *et al.,* 2019).

In this study, the fish *Garra rufa* were subjected to the influence of three anaesthetics: MS-222, 2-phenoxyethanol and clove oil: Induction and recovery times recorded were within the time limit recommended by Gilderhus & Marking, (1987) and Ross & Ross, (2008). According to these authors, fish should be anaesthetised within 3 minutes, in order to not compromise animal welfare. Also, a successful recovery should occur in less than 10 minutes and all anaesthetised fish must survive.

The three anaesthetics used in this experimental trial induced a deep anaesthesia in *G. rufa* for routine processes, but not all concentrations were effective. For the medium and large *G. rufa* size classes, MS-222 concentrations from 225 to 375 mg L^{-1} were ineffective, since the fish did not last the 90 s of the monitoring phase without opercular movements and/or body contractions. Concerning the 2-phenoxyethanol anaesthetic, the concentrations 525 and 600 mg L^{-1} shown to be ineffective for the small size class, while for the medium size class it were those bellow 675 mg L^{-1} and all the large fish endured anaesthetised the entire monitoring phase from this concentration upwards.

In what concerns clove oil, fish from the small and medium size classes did not hold 90 s of monitoring at 110 and 170 mg L^{-1} and for the large class the concentration 150 mg L^{-1} was also ineffective.

Balancing the faster induction and recovery phases, plus holding the full monitoring period anaesthetised, which resulted in a shorter total anaesthesia procedure, the recommended dose of MS-222 for *G. rufa* at 29 °C is 300 mg L^{-1} for the small *G. rufa* size class, whilst for the medium and large size fish should be 350 mg L^{-1}. The most effective doses to anaesthetise *G. rufa* at 29 °C resulted from a compromise between faster induction and recovery phases, which resulted in a shorter total anaesthesia procedure, plus holding the full monitoring period deeply anaesthetised. This balance for anaesthesia with MS-222 differed between size classes, with lower doses being effective for smaller fish and higher doses were required for larger size classes. A concentration of 300 mg L^{-1} worked better for these animals, being induced, monitored and recovered in 146 ± 6.44 s. For the medium fish it was 350 mg L^{-1}, being prossessed within 128 ± 6.88 s. And for last, the large fish were better anaesthetised using 375 mg L^{-1} being prossessed in 167 ± 21.13 s. It was not possible to establish a relationship between the induction and recovery times for MS-222.

On the other hand, the 2-phenoxyethanol concentration that worked better for *G. rufa* did not differ much between size classes. But the dose 750 mg L^{-1} produced better results for the large fish (total procedures in 168 ± 8.46 s) which was lower than the 825 mg L^{-1} endorsed for the small and medium fish (total procedures in 116 ± 7.80 s and 197 ± 12.27 s, respectively). Like MS-222, it was not possible to establish a relationship between induction and recovery times for 2-phenoxyethanol.

In contrast with the two other anaesthetics, the recommended dose of clove oil for *G. rufa* at 29 °C was 130 mg L^{-1} for all size classes (total procedures in 202 ± 17 s for the small size, 182 ± 9.39 s for the medium and 189 ± 11.43 s for the large size). Even though 150 mg L^{-1} worked good as well (except for one large fish that did not endure 90 s of monitoring without presenting opercular or body movements). There was no correlation between the induction and recovery times and it was not possible to establish a relationship between them from this anaesthetic as well. The anaesthesia procedures were all carried in average under 5 min per fish, regardless of the anaesthetic and concentrations that were used. Nevertheless, for the effective concentrations of clove oil and 2-phenoxyethanol lasted less than 3 min 50 s, while for MS-222 was slightly faster (less than 3 min).

The *G. rufa* fish were anaesthetised in warm water at 29 °C and the concentrations used were also high. This situation can be explained by the fact that this cyprinid fish is found in natural habitats with temperature ranging between 15 and 31 °C (Jarvis, 2011). However, the optimal rearing temperatures are between 28 and 30 °C (Catarino, 2015; Gomes, 2016). Bellow 24 ° C, *G. rufa* behave letargic, ignore food and are propense to diseases, namely skin and organ lesions caused by *Aeromonas* sp. (Ferreira SMF, personal observation).

Whereas in other studies using also warm water, higher temperatures seem to increase the effect of an anaesthetic by increasing the recovery time of anaesthetized fish (Weyl, *et al.,* 1996; Stehly & Gingerich, 1999; Prince & Powell, 2000; Walsh & Pease, 2002; Hoskonen & Pirhonen, 2004).

According to Coyle *et al.* (2004), cold water species respond to lower concentrations of anaesthetics than warm water species, as higher temperatures increase the diffusion rate of the anaesthetics dissolved in the water into the fish's gills. Moreover, when fish are exposed to higher ambient temperatures, their basal metabolism is (or becomes) more elevated and hence, they present a higher oxygen rate. With the increase in oxygen rate, breathing accelerates, as well as blood flow. Considering that the gills are the main pathway to absorb the anaesthetics, as well as to excrete it, the increasing ventilation and cardiac rates lead to the absorption of larger quantities of anaesthetics (Zahl *et al.,* 2011; Javahery *et al.,* 2012), thus reducing the induction time. Likewise, the excretion of the anaesthetic will be faster, resulting in faster recovey times (Javahery *et al.,* 2012). Notwithstanding, it is imperative to provide strong aeration, both in the anaesthetic solution and also in the recovery system, as the concentration of dissolved oxygen in the water decreases with temperature (Ross & Ross, 2008; Neiffer & Stamper, 2009).

In the study of Aydin *et al.* (2019), *Garra rufa* were exposed to two different temperatures (15 and 25 °C) and concentrations from 110.2 to 551.0 mg L^{-1} of 2-phenoxyethanol, plus 26.5 to 106.0 mg L^{-1} of clove oil. These authors observed that with the increasing concentration for both anaesthethics, at both temperatures, the induction time decrease, while the recovery time increased. A similar trend was observed for Senegalese sole *Solea senegalensis* Kaup 1858 (Weber *et al.,* 2009), kelp grouper *Epinephelus bruneus* Bloch, 1793 (Park *et al.,* 2008), rainbow trout *O. mykiss* (Yildiz *et al.,* 2013) and goldfish *C. auratus* (Küçük & Çoban, 2016). However Perdikaris *et al.* (2010) found that the induction time of goldfish *C. auratus* size class 20 to 25 cm at 18 °C increased with concentration, as well as the rainbow trout *O. mykiss* in all sizes class at 12 °C. The recovery time increased in both.

Aydin *et al.* (2019) recommended the minimum effective concentration of 330.6 mg L^{-1} of 2 phenoxyethanol for both temperatures (15 °C and 25 °C). For clove oil, these authors recommended the minimum of 79.5 mg L^{-1} at 15 °C and 53.0 mg L^{-1} at 25 °C. All these concentrations are bellow the ones endorsed in the present study, in spite of the similar size of the fish (*G. rufa* weighted 1.29 ± 0.24 g, which was in between the actual small and medium classes) and the extra higher 4 °C. Nevertheless, Aydin *et al.* (2019) did not refer any monitoring period between induction and recovery, that might be effective for routines procedures to evaluate the fish's behaviour, physical, physiological and health conditions.

In other studies using *Garra rufa* fish, Ferreira *et al.* (2015 a,b, 2016) used an ambient temperature of 20 °C to determine the most effective MS-222, phenoxyethanol and clove oil concentrations in fish measuring from 4 to 6 cm total length and approximately an individual wet weight of 0.86 to 1.28 g (correspondent to the small size class in the present work). These authors reached the conclusion that 425 mg L^{-1} of MS-222, 825 mg L^{-1} of 2-phenoxyethanol and 130 mg L^{-1} of clove oil were the advisable concentrations. In relation to the present work, those results were slightly superior, substantiating the trend theory that higher temperatures within the species tolerance amplitude do turn anaesthesia more effective. So, with higher temperatures, the same concentration of anaesthetic will, in theory, reduce the induction and recovery times, thus shortening the entire process. If these phases are already fast, then lower concentrations may be employed, as the results in this work may confirm towards the results of Ferreira *et al.* (2015a,b), but not those of 2-phenoxyethanol of Aydin *et al.,* (2019). Clove oil results were more ambiguous. Besides the fact that the most effective concentration of this anaesthetic was the same despite *G. rufa* size class, it was also the same regarless the water temperature (20 or 29 °C), when compared to the results of Ferreira *et al.* (2016). Still, the concentration 130 mg L $^{-1}$ was twice as higher than those recommended by Aydin *et al.* (2019), even though these authors did recommend a lower concentration for a higher temperature.

The reduction of the anaesthetic effective doses with higher temperatures might be beneficial or useful, as to minimise toxicity, tissue accumulation or residual metabolites in the fish. Thus, it will help assuring safer procedures both for animals and their caretakers. The anaesthesia procedures should be planned and carried out in manner to avoid or reduce stress, as stressed animals exhibit abnormal reactions to anaesthesia and may require higher doses for induction, manisfest undesirable behaviour during the monitoring phase and demand longer recovery periods (Zahl *et al.,* 2012).

During this experimental assay, no mortality was observed within 24 hours after the anaesthesia procedures, regardless of the anaesthetic that was used.

A single exception was observed, one fish belonging to the medium size class anaesthetised at a concentration of 170 mg L^{-1} of clove oil. One possible explanation for this mortality could have been its long-time exposure to the anaesthetic. This fish presented the longest induction time recorded during the assay - 412 seconds (6.87 min) and the longest recovery time of 660 seconds (11 min). Weak and sick animals are prone to make a difficulty recover from anaesthesia (Coyle *et al.,* 2004), which could have also been the case for that specific individual, although, no external signs of disease were observed. Clove oil is reported to induce faster efficient anaesthesia than other anaesthetics (Sladky *et al.,* 2001; Bressler & Ron 2004, Detar & Mattingly 2004 all in Neiffer & Stamper, 2009), but longer recoveries. According to Mylonas *et al.* (2005) and Misawa *et al.* (2014), clove oil was the anaesthetic agent with the longest recovery time, whilstand MS 222 had the shortest one, with 2-phenoxyethanol in between. Keene *et al.* (1998) explained that the shorter recovery times for MS-222 were based on different effects on the cardio-respiratory system of the fish, resulting in increased respiratory and heart rates, which in turn removed excess anaesthetic.

In this case, *G rufa* recovered faster with 2-phenoxyethanol (in average < 100 s) than MS-222 and clove oil (both in average < 125 s) for the most effective concentrations. Clove oil prolonged recovery times have been reported for several teleostei fish, such as: coral reef ambon damsel *Pomacentrus amboinensis* Bleeker, 1868 (Munday & Wilson 1997); in rainbow trout *O. mykiss* (Keene *et al.,* 1998), non-salmonids like lake sturgeon *Acipenser fulvescens* (Rafinesque, 1817), smallmouth bass *Micropterus dolomieu* Lacépède, 1802, walleye *Sander vitreus* (Mitchill, 1818) and northern pike *Esox lucius* Linnaeus, 1758 (Peake, 1998). Other authors have also mentioned a narrow safety margin, as for the red pacu *Piaractus brachypomus* (G. Cuvier, 1818) in comparison with MS-222 (Sladky *et al.,* 2001), and increased sensitivity to eugenol and AQUI-S in preliminary studies with southern stingrays *Dasyatis americana* Hildebrand & Schroeder, 1928 (DLN author in Neiffer & Stamper, 2009). Clove oil has also been reported to cause mortality in sockeye salmon *Oncorhynchus nerka* (Walbaum, 1792) (Woody *et al.,* 2002) and red pacu *P. brachypomus* (Sladky *et al.,* 2001).

Sladky *et al.* (2001) suggested that mortality could be due to clove oil toxicity, whose effects induced ventilatory failure and medullar collapse. Misawa *et al.,* (2014) demonstrated also serious effect on the goldfish *C. auratus* respiratory center in the medulla than that of 2-phenoxyethanol and MS-222. Moreover, clove oil is as a lipidic substance that might adhere to gills epithelial cells, forming an outer layer that will prevent gas exchanges (Sladky *et al.,* 2001). It might even cause mild gill necrosis, as observed for asian sea bass *Lates calcarifer* (Bloch, 1790) subjected to repeated exposure of low eugenol doses (Afifi *et al.,* 2001). Although, Misawa *et al.* (2014) have considered 2-phenoxyethanol to be safer than clove oil, Weyl *et al.* (1996) stated that the repeated use of 2-phenoxyethanol increases fish tolerance to this anaesthetic.

Also, clove oil might no be indicated for invasive or other deleterious procedures, once that it is a complex of several substances. Fish may produce physiological reactions when in contact with the anaesthetics. Sladky *et al.* (2001) also reported that red pacus *P. brachypomus* reacted more to a hypodermic needle puncture when using clove oil than MS-222. Additionally, clove oil is known to induce and increase of epidermal mucous, namely in rainbow trout *O. mykiss* (Velíšek *et al.,* 2005a), common carp *Cyprinus carpio* Linnaeus, 1758 (Velíšek *et al.,* 2005b) and sheatfish *Silurus glanis* Linnaeus, 1758 (Velíšek *et al.,* 2006). Fish produce this mucous to protect their skin against abrasion, which is also a barrier to pathogenic elements, besides turning them more slippery and difficult for predators to grab them. In this case, it seems that the epidermal globlet cells are stimulated to produce more mucous in response to the astringent action of the anaesthetic.

Furthermore, this reaction has also been reported for 2-phenoxyethanol for the same species rainbow trout *O. mykiss* (Velíšek & Svobodová, 2004a), common carp *C. carpio* (Velíšek & Svobodová, 2004b) and sheatfish *S. glanis* (Velíšek *et al.,* 2007b). Although it was not an intended aim, it was possible to notice that *G. rufa* subjected to clove oil produced more mucous during the monitoring phase, while it was being handled for biometrics and verification of their physical status.

Park *et al.* (2008) generalised that the higher the concentration of anaesthetic used, the shorter was the induction time and conversely, the longer was the recovery time. This trend was observed in the use of 2-phenoxyethanol and clove oil in *G. rufa*, by Aydin *et al.* (2019). However, taking into account other studies, other fish species revealed that anaesthesia with higher concentrations may have benefits, since it may result in both shorter induction and recovery times, namely the European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) and the gilthead sea bream *Sparus aurata* Linnaeus, 1758 (Mylonas *et al.,* 2005, Table VII). Therefore, higher concentrations of anaesthetic may be necessary for certain fish to be deeply anaesthetised, as is the case of *G. rufa*. The concentrations used in this study were higher compared to other species (Table VII).

Table VII - Comparison of results obtained for anaesthesia of several fish species with MS - 222, 2-phenoxyethanol (2-PE) and clove oil. Indication of anaesthetic, its effective concentrations, fish size (length and/or weight, when relevant), environmental conditions (salt, brackish and freshwater and respective temperatures) by different authors (Note : M - male; F – female; S - small and L – large).

Species	Anaesthetic	Concentrations	Type of Water	Temperature of Water	Induction Time	Recovery Time	Reference
Channel catfish ***Ictalurus punctatus*** **(Rafinesque, 1818)** **Ictaluridae**	Clove oil	75 and 100 mg L^{-1}	Freshwater	Warm 23 °C	75 – 5.50 min 100 – 5.17 min	75 – 6.53 min 100 – 3.77 min	Waterstrat, 1999
Nile tilapia ***Oreochromis niloticus*** **(Linnaeus, 1758)** **Cichlidae**	2-PE	600, 750 and 900 mg L^{-1}	Freshwater	Warm 28 °C	600 – 3.17 min 750 – 2.92 min 900 – 1.67 min	600 – 4.5 min 750 – 2.83 min 900 – 2.5 min	Mello *et al.*, 2012
Discus ***Symphysodon discus*** **(Heckel, 1840)** **Cichlidae**	MS-222	75 and 100 mg L^{-1} (3.6 g)	Freshwater	Warm 27 °C	75 – 1.10 min 100 – 1 min	75 – 1.20 min 100 – 2 min	Chambel *et al.*, 2013
Angelfish ***Pterophyllum scalare*** **(Lichtenstein, 1823)** **Cichlidae**	MS-222	160 mg L^{-1} (4.45 cm, 2.5 g)	Freshwater	Warm 24 - 25 °C	160 – 3.31 min	160 – 5.19 min	Mitjana *et al.*, 2014
	2-PE	800 mg L^{-1} (4.45 cm, 2.5 g)			800 – 2.36 min	800 – 4.67 min	
	Clove oil	100 mg L^{-1} (4.45 cm, 2.5 g)			100 – 2.31 min	100 – 3.31 min	
Southern platyfish ***Xiphophorus maculatus*** **(Günther, 1866)** **Poeciliidae**	Clove oil	100 and 200 mg L^{-1} (0.2 – 0.5 g)	Freshwater	Warm 27 °C	100 – 1.07 min 200 – 0.47 min	100 – 3.40 min 200 – 4.10 min	Hoshiba *et al.*, 2015

Green swordtail ***Xiphophorus helleri*** **Heckel, 1848** **Poeciliidae**	MS-222	125 and 150 mg L^{-1} (1.3 g)	Freshwater	Warm 27 °C	125 – 2.33 min 150 – 1.3min	125 – 2.07 min 150 – 2 min	Chambel *et al.*, 2013
Guppy ***Poecilia reticulata*** **Peters, 1859** **Poeciliidae**	MS-222	125, 150 and 200 mg L^{-1} (3.6 g)	Freshwater	Warm 27 °C	125 – 3.31 min 150 – 1.83 min 200 – 1.42 min	125 – 1 min 150 – 1.20 min 200 – 130 min	Chambel *et al.*, 2013
	Clove oil	125 and 150 mg L^{-1} (1.5 - 3.5 cm) (0.04 – 0.5 g)	Freshwater	Warm 30 °C	125 – 1.72/2.08 min (F/M) 150 – 1.32 min (F)	125 – 3.60/4.65 min (F/M) 150 – 3.62 min (F)	Cunha *et al.*, 2015
	MS-222	180 mg L^{-1}	Freshwater	Warm 25 °C	180 – 2.12 /2.02 min (M/F)	180 – 4.17 / 3.34 min (M/F)	Mitjana *et al.*, 2018
	2-PE	1000 and 1200 mg L^{-1}			1000 – 4.49 /4.14min (M/F) 1200 – 3.11 /3.87 min (M/F)	1000 – 3.93 /2.31min (M/F) 1200 – 3.88 /4.27min (M/F)	
	Clove oil	50 and 75 mg L			50 – 2.28 /4.41 min (M/F) 75 – 2.19 /2.40 min (M/F)	50 – 3.79 /3.32 min (M/F) 75 – 4.95 /3.96 min (M/F)	
Zebra fish ***Danio rerio*** **(Hamilton, 1822)** **Cyprinidae**	MS-222	75, 100 and 125 mg L^{-1} (0.5 g)	Freshwater	Warm 27 °C	75 – 1.66 min 100 – 1.33 min 125 – 1.20 min	75 – 0.68 min 100 – 0.75 min 125 – 0.80 min	Chambel *et al.*, 2013
Doctor fish ***Garra rufa*** **(Heckel, 1843** **Cyprinidae**	MS-222	425 mg L^{-1} – small fish (4 cm)	Freshwater	Cold 20 °C	425 – 0.77 min	425 – 1.04 min	Ferreira *et al.*, 2015a
	2-PE	825 mg L^{-1}– small fish (4 cm)			825 – 0.94 min	825 – 1.12 min	Ferreira *et al.*, 2015b
	Clove oil	130 mg L^{-1}– small fish (4 - 6 cm)			130 – 1.5min	130 – 4 min	Ferreira *et al.*, 2016

	2-PE	330.6 mg L^{-1} – 15 & 25 °C (1.3 g)	Freshwater	Cold 15 °C & Warm 25 °C	330.6 mg L^{-1} – 1.57 min 15 °C 330.6 mg L^{-1} – 1.33 min 25 °C	330.6 mg L^{-1} – 2.91 min 15 °C 330.6 mg L^{-1} –1.98 min 25 °C	Aydin *et al.*, 2019
	Clove oil	79.5 mg L^{-1} – 15 °C 53.0 mg L^{-1} – 25 °C (1.3 g)			79.5 mg L^{-1} – 2.71 min 15 °C 53.0 mg L^{-1} – 2.79 min 25 °C	79.5 mg L^{-1} – 5.37 min 15 °C 53.0 mg L^{-1} – 5.29 min 25 °C	
	MS-222	300 mg L^{-1} – small fish (4 cm) 350 mg L^{-1} – medium fish (6.5 cm) 375 mg L^{-1} – large fish (9 cm)	Freshwater	Warm 29 °C	300 mg L^{-1} – 1 min 350 mg L^{-1} – 0.80 min 375 mg L^{-1} – 0.72 min	300 mg L^{-1} – 0.87 min 350 mg L^{-1} – 0.77 min 375 mg L^{-1} – 1.36 min	Present work, 2020
	2-PE	825 mg L^{-1} – small fish (4 cm) 825 mg L^{-1} – medium fish (6.5 cm) 750 mg L^{-1} – large fish (9 cm)			825 mg L^{-1} – 0.82 min) 825 mg L^{-1} – 1.09 min 750 mg L^{-1} – 0.89 min	825 mg L^{-1} – 0.64 min 825 mg L^{-1} – 1.4 min 750 mg L^{-1} – 1.18 min	
	Clove oil	130 mg L^{-1} – small fish (4 cm) 130 mg L^{-1} – medium fish (6.5 cm) 130 mg L^{-1} – large fish (9 cm)			130 mg L^{-1} – 1.29 min 130 mg L^{-1} – 1.26 min 130 mg L^{-1} – 1.76 min	130 mg L^{-1} – 2.07 min 130 mg L^{-1} – 1.77 min 130 mg L^{-1} – 1.4min	
Bighead carp ***Hypophthalmichthys nobilis*** **(Richardson, 1845)** **Cyprinidae**	2-PE	771 mg L-1	Freshwater	Warm 26 °C	771 – 1.83 min	771 – 1.84 min	Akbary *et al.*, 2016
Koi carp ***Cyprinus carpio*)** **Linnaeus, 1758** **Cyprinidae**	MS-222	125 and 200 mg L^{-1}	Freshwater	Warm 24 – 25 °C	Not analysed	Not analysed	Bailey *et al.*, 2013
Common carp **(*Cyprinus carpio*)** **Linnaeus, 1758** **Cyprinidae**	Clove Oil	30, 40 and 50 mg L $^{-1}$	Freshwater	Temperate 20 °C	30 – 3.77 min 40 – 2.33 min 50 – 1.23 min	30 – 4.43 min 40 – 3.77 min 50 – 3.98 min	Hajek *et al.*, 2006
Goldfish ***Carassius auratus***	MS-222	200 mg L^{-1}	Freshwater	Warm 25.6 °C	200 – 3.86 min	200 – 5.13 min	Küçük & Çoban, 2016

(Linnaeus, 1758) **Cyprinidae**	2-PE	551 mg L^{-1}	Freshwater	Temperate 20 °C Hot 25 and 30 °C	551 – 1-1.9 min 551 – 1-1.5 min 551 – 1.9-2.8 min	551 – 4.8-5.6 min 551 – 3.7-5.5min 551 – 3.6-4.7 min	Weyl *et al.*, 1996
	Clove oil	150 mg L^{-1}	Freshwater	Temperate 18 °C	150 – 1.54 min	150 – 4.19 min	Perdikaris *et al.*, 2010
Silver carp ***Hypophthalmichthys molitrix*** **(Valenciennes, 1844)** **Cyprinidae**	2-PE	551, 771 and 992 mg L^{-1}	Fresh Water	Temperate 20.5 °C	551 – 4.67 min 771 – 2.67 min 992 – 2 min	551 – 1.33 min 771 – 2.5 min 992 – 5.5 min	Hedayati, 2018
Far eastern catfish ***Silurus asotus*** **Linnaeus, 1758** **Siluriformes**	MS-222	300 and 400 mg L^{-1}	Freshwater	Temperate 26-27 °C	300 – 1.28 /2.83 min (S/L) 400 – 0.93 /1.67 min (S/L)	300 – 1.8 /4.75 min (S/L) 400 – 1.9 /4.57 min (S/L)	Park, 2019
	Clove oil	300 and 400 mg L^{-1}			300 – 0.95 /1.72 min (S/L) 400 – 0.7 /1.10 min (S/L)	300 – 2.18 /3.05 min (S/L) 400 – 2.15 /2.98 min (S/L)	
Yellow perch ***Perca flavescens*** **(Mitchill, 1814*)*** **Perciformes**	MS-222	250 and 300 mg L^{-1}	Freshwater	Temperate 22 °C	250 – 1.58 min 300 – 1.41 min	250 – 1.51 min 300 –1.56 min	Zhai *et al.*, 2018
Hickory shad ***Alosa mediocris*** **(Mitchill, 1814)** **Clupeidae**	MS-222	75 and 100 mg L^{-1}	Freshwater	Cold 11.1 – 13 °C	75 – 1.5 min 100 – 1min	75 – 4 min 100 – 2.5 min	Matsche, 2017
	2-PE	100 and 400 mg L^{-1}			100 – 2.5 min 400 – 1.3 min	100 – 4.7 min 400 – 4.3 min	
	Eugenol	50 and 100 mg L^{-1}			50 – 1.8 min 100 – 1 min	50 – 4.5 min 100 – 3.3 min	
Rainbow trout	MS-222	60 mg L^{-1} at 150 mg L^{-1}	Freshwater	Cold 12 °C	1.7 – 3.3 min	5.2 – 6.2 min	Ross & Ross, 2008

Oncorhynchus mykiss **(Walbaum, 1792)** **Salmonidae**	2-PE	200 and 300 mg L^{-1}			Not analysed	Not analysed	Velíšek & Svobodová, 2004a)
	Clove Oil	50 mg L^{-1}			50 – 2.02 min	50 – 2.72 min	Perdikaris *et al.*, 2010
Persian sturgeon ***Acipenser persicus*** **Borodin, 1897** **Acipenseridae**	2-PE	330m g L^{-1}	Brackish water	Temperate 21.5 °C	330 – 5.5 min	330 – 4.75 min	Adel *et al.*, 2016
	Clove oil	25 and 50 mg L^{-1}			25 – 7.04 min 50 – 2.11 min	25 – 5.73 min 50 – 10.76 min	
Senegalese sole ***Solea senegalensis*** **Kaup, 1858** **Soleidae**	MS-222	100 mg L^{-1}	Saltwater	Warm 14 °C	100 – 2.25 min	100 – 2.99 min	Weber *et al.*, 2009
	2-PE	500 and 600 mg L^{-1}			500 – 4.37 min 600 – 1.50 min	500 – 5.47 min 600 – 1.94 min	
	Clove oil	30 and 40 mg L^{-1}			30 – 3.16 min 40 – 1.66 min	30 – 3.76 min 40 – 3.59 min	
Marbled spinefoot ***Siganus rivulatus*** **Forsskål & Niebuhr, 1775** **Siganidae**	MS – 222	100 and 125 mg L^{-1}	Saltwater	Warm 25.6 °C	100 – 3.07 min 125 – 2.14 min	100 – 1.71 min 125 –1.73 min	Ghanawi *et al.*, 2013
	2-PE	500 and 600 mg L^{-1}			500 – 1.53 min 600 – 1.12 min	500 – 2.18 min 600 – 2.10 min	
	Clove oil	70 and 100 mg L^{-1}			70 – 0.98 min 100 – 0.77 min	70 – 4.53 min 100 – 4.19 min	
Meagre ***Argyrosomus regius***	2-PE	400 and 550 mg L^{-1}	Saltwater	Temperate 18 °C	400 – 4.76 min 550 – 4.03 min	400 – 4.31 min 550 – 4.69 min	Barata *et al.*, 2016

(Asso, 1801) **Sciaenidae**	Clove oil	70 and 85 mg L^{-1}			70 – 3.06 min 85 – 2.47 min		70 – 6. 72 min 85 – 6.53 min		
Atlantic halibut ***Hippoglossus hippoglossus*** **(Linnaeus, 1758)** **Pleuronectidae**	MS – 222	80 mg L^{-1}	Saltwater	Cold 8 °C	80 – 1.9 min 80 – 3.22 min		80 – 9.78 min 80 – 12.43 min		Zahl *et al.*, 2011
	2-PE	600 mg L^{-1}			600 – 2.52 min 600 – 2.72 min		600 – 15.42 min 600 – 18.5 min		
European sea bass ***Dicentrarchus labrax*** **(Linnaeus, 1758)** **Moronidae**	2-PE	300 and 350 mg L^{-1}	Saltwater	Cold 15 °C & Warm 25 °C	At 15 °C 300 – 3 min	At 25 °C 350 – 1 min	At 15 °C 300 – 5 min	At 25 °C 350 – 3 min	Mylonas *et al.*, 2005
	Clove oil	30 mg L^{-1} and 40 mg L^{-1}			At 15 °C 30 – 2.5 min	At 25 °C 40 – 1 min	At 15 °C 30 – 7 min	At 25 °C 40 – 2.5 min	
Gilthead sea bream ***Sparus aurata*** **Linnaeus, 1758** **Sparidae**	2-PE	300 mg L^{-1} and 450 mg L^{-1}			At 15 °C 300 – 7 min	At 25 °C 450 – 1.5 min	At 15 °C 300 – 8 min	At 25 °C 450 – 4 min	
	Clove oil	40 mg L^{-1} and 55 mg L^{-1}			At 15 °C 55 – 3 min	At 5 °C 40 – 2 min	At 15 °C 55 – 6.5 min	At 25 °C 40 – 4.5 min	

The effective dose of MS-222 to achieve deep anaesthesia varies between 20 and 480 mg L^{-1} (Popovic *et al.,* 2012). This range of effective concentrations is large because it includes freshwater and saltwater species, as well as, different sizes of fish. For freshwater species such as grass carp *Ctenopharyngodon idella* (Valenciennes, 1844) this range is among 20 to 75 mg L^{-1}; in common carp *C. carpio* is from 25 to 100 mg L^{-1} and in tench *Tinca tinca* (Linnaeus, 1758) varies among 25 to 200 mg L^{-1}. For the saltwater species, concentrations among 60 to 75 mg L^{-1} may be used for the Atlantic cod *Gadus morhua* (Linnaeus, 1758), in the red seabream *Pagrus major* (Temminck & Schlegel, 1843) works between 50 to 100 mg L^{-1} and for the Atlantic halibut *Hippoglossus hippoglossus* (Linnaeus, 1758) it is among 250 to 480 mg L^{-1} (Popovic *et al.,* 2012).

For the 2-phenoxyethanol the effective dose to achieve deep anaesthesia varies between 60 and 900 mg L^{-1} (Priborsky & Velisek, 2018). This range of effective concentrations also includes freshwater and saltwater species and also different sizes of fish. For freshwater species such as common carp *C.carpio* this range is among 400 to 600 mg L^{-1}, for tench *T. tinca* it works between 100 to 500 mg L^{-1} and the concentration 900 mg L^{-1} was better for bighead carp *Hypophthalmichthys nobilis* (Richardson, 1845). For the saltwater species, it were referred the concentrations between 60 to 200 mg L^{-1} for gilt-head sea bream *S. auratus*, among 200 to 300 mg L^{-1} for the black sea bass *Centropristis striata* (Linnaeus, 1758) and 350 mg L^{-1} for the European sea bass *D. labrax* .

The study by Hoskonen & Pirhonen, (2004) demonstrated that amplitude of the ideal dose of clove oil was a result from different actions of eugenol, for each species of fish, for Atlantic salmon *Salmo salar* Linnaeus, 1758, brown trout *Salmo trutta* Linnaeus, 1758, rainbow trout (*O. mykiss*), whitefish *Coregonus lavaretus* Linnaeus, 1758, perch *Perca fluviatilis* Linnaeus, 1758, and roach *Rutilus rutilus* Linnaeus, 1758, so, the effective dose of to achieve deep anaesthesia varies between 20 and 120 mg L^{-1} (Ross & Ross, 2008) but this range can be amplified up to the 150 mg L^{-1} (Perdikaris *et al.,* 2010).

Perdikaris *et al.* (2010, Table VII) used three concentrations of clove oil (75, 100 and 150 mg L^{-1}) in three size classes of the goldfish *C. auratus* (1.5 - 2.5 cm; 5 - 7cm; 11 - 15 cm and 20 - 25 cm) at 18 °C. They found that the higher the concentration, the shorter the induction time for the size classes until 15 cm. The same situation happened for the recovery time, as mentioned by Mylonas *et al.* (2005; Table VII). For size class 20 to 25 cm, *C. auratus* presented a longer induction time inversely related to the anaesthetic concentration. These authors also assessed the same clove oil concentrations in two size classes of rainbow trout *O. mykiss* (20 - 23 cm and 30 - 33 cm, Table VII) at 12 °C. The both also took longer to be induced at higher anaesthetic doses. These three last observations contradicted those of Park *et al.* (2008), but all did in fact take longer to recover, in which the goldfish was faster than the rainbow trout.

On the other hand, Yildiz *et al.* (2013, Table VII) observed that the rainbow trout's (15.48 and 39.08 g) induction times for 2-phenoxyethanol (200 to 600 mg L^{-1}) and clove oil (500 to 1500 mg L^{-1}) decreased with increasing concentrations, while the recovery time for both anaesthetics increased. The results were according to Park *et al.,* (2008), despite the different temperatures used (7, 13 and 18 °C), but contradict the results obtained by Perdikaris *et al.,* (2010) for the same species.

Another study using goldfish *C. auratus* (2.4 cm and 23 g) assessed the influence of water salinity (0, 8, 12, 14 and 16) at 25.6 °C on the anaesthesia dose with MS-222 (150 to 500 mg L^{-1}). Küçük & Çoban, (2016) demonstrated that the induction time decreased with the MS-222 concentrations augmentation, which agrees with the studies of Park *et al.* (2008) and Yildiz *et al.* (2013). However, there was an inverse relationship between the recovering time and MS-222 concentration, contradicting those authors and supporting the findings of by Mylonas *et al.* (2005) and Perdikaris *et al.* (2010). Furthermore, as freshwater fish, *C. auratus* revealed increased anaesthesia induction and reduced recovery times with the incremetation of water salinity (Küçük & Çoban, (2016). Anaesthetics MS-222, 2-phenoxyethanol and clove oil were used for marine fish (Table VII), such as European sea bass *D. labrax* and the gilthead sea bream *S. aurata* (Mylonas *et al.,* 2005), Senegalese sole *S. senegalensis* (Weber *et al.,* 2009), Atlantic halibut *H.hippoglossus* (Zahl *et al.,* 2011), marbled spinefoot *Siganus rivulatus* Forsskål & Niebuhr, 1775 (Ghanawi *et al.,* 2013), Persian sturgeon *Acipenser persicus* Borodin, 1897 (Adel *et al.,* 2016) and meagre *Argyrosomus regius* (Asso, 1801) (Barata *et al.,* 2016). Generally, marine fish are anaesthetised with lower concentrations of the same anaesthetic agent than related fresh water species (Table VII). Such empirical observation may be related to the fact that marine fish need to copious drink seawater to avoid dehydration and excessive ion in their tissues This behaviour results from the fact that they live in a hyperosmotic environment and therefore, need to compensate the osmotic loss of water into the surroundings. So, they will absorb the ingested water into their circulatory system, along with other ions (Watanabe & Takei, 2012). Consequentely, they will also probably absorb the anaesthetic agent dissolved in the water through the intestine, turning the anaesthesia induction more effective than in freshwater fish. Moreover, the excessive ions (as sodium and chloride) are excreted by the gills (Watanabe & Takei, 2012), the common pathway for inhalation anaesthetics as MS-222, 2-phenoxyethanol and clove oil.

So, it can be concluded that the animals reaction to an anaesthetic agent can differ according to the environmental conditions, such as temperature, pH, nitrogenous compounds and salinity, but also on the species specific constraints (physiology, metabolism, integument, body composition, gill and body size), growth rate, sexual maturity and stage of the life cycle (Neiffer & Stamper, 2009; Zahl *et al.,* 2011). It was mentioned before that the variations in induction time may be due to the absorption rate of anaesthetics by inhalation, which is influenced by the ventilation frequency, blood flow and permeability of the gills. Increasing water temperatures may potentiate absorption by accelerating ventilation frequency, as a response to the increasing metabolic rate resulting from the induced stress situation. What is more, warm-water fish are known for having higher metabolic rates, plus physiological respiratory adaptations to compensate the lower dissolved oxygen in the water column, than cold-water fish. Once in the gills and blood stream, the fish will slow down opercular movements and metabolism, which will extend the time for clearing the anaesthetic fom the bloodstream. As more anaesthethic is absorbed during the induction period, the longer it will take for the fish to recover, after being placed in a tank with clean water (Stehly & Gingerich, 1999; Prince & Powell, 2000; Zahl *et al.,* 2011). This trend was apparent in the present work for *G. rufa* anaesthetised with MS-222 and 2-phenoxyethanol (although without a strong statistical support), but not for clove oil.

Furthermore, other factors may be taken into account. For instance, anaesthesia effectiveness may be related to the gill area – body mass ratio. Generally, large fish have a smaller gill area in relation to body mass than small fish and therefore, they have a smaller area for diffusion. So, large fish usually require a greater concentration of anaesthetic than small ones (Coyle *et al.,* 2004; Zahl *et al.,* 2011, 2012). This observation is also supported by the physiological evidences in which the basal metabolic rate is lower in large fish than in small ones, and hence their oxygen consumption and anaesthetic absorption rates as well (Zahl *et al.,* 2011). But there are reported cases in which large fish are easier to anaesthetise. As female towards males, large individuals have normally more adipose tissue than small ones. So, anaesthesia may occur faster and last longer in this individuals, as many anaesthetic agents are liposoluble (*e. g.* MS-222 and clove oil). The recovery may also be slower, as the agent needs to be removed from the fat tissues (Coyle *et al.,* 2004). In fact, the present work verified that *G. rufa* anaesthesia with MS-222 was more effective with lower concentrations in small individuals, thus larger concentrations should be used in larger fish. In the case of 2-phenoxyethanol, it was the other way around. The small and medium fish should be anaesthetised with a higher dose than the large ones, although within a small margin of 75 mg L^{-1}. Anaesthesia with clove oil produced similar results, regardless the body size of *G. rufa*.

In this work, it was possible to divide the anaesthesia phase in just three stages: impaired motion, buoyancy loss and cease of opercular movements. This sequence of events was consistent for every single fish, independently of the anaesthetic used, or even itds dosage. The description is usally similar in other authors (Coyle *et al.*, 2004), but there are those who described other steps that include a normal initial state to an excitatory phase, ending with the loss of reflexive movements, which in *G. rufa* was coincident with the cease of opercular movements (Summerfelt & Smith, 1990; Burka *et al.,* 1997; *Zahl et al*. 2011 Akinrotimi *et al.,* 2015, Table I). So, different fish species may present different behavioural signs and symptoms towards anaesthesia that should be described. Aydin *et al.* (2019) adopted for *G. rufa* a classification proposed for rainbow trout *O. mykiss* (Keene *et al.*, 1998) and guppy *P. reticulata* (Cunha *et al.*, 2015). They describe a first induction stage of relaxation and unresponsive to stimuli and a final stage in which the loss of buoyancy coincides with the absence of opercular movements. However, Aydin *et al.* (2019) do not describe the use of aeration in the immersion recipient, which could explain their incoherent classification regarding the behaviour observed during the experimental procedures of this work and the ones described by Ferreira *et al.* (2015a,b, 2016). In fact, *G. rufa* vigorously tried to escape from the recipient as soon as their were immersed in the anaesthetic solution. For this reason, it is advisable to use use barriers to prevent that situation. Also, the duration of each stage might bring some additional information to understand the anaesthetic effects and fish behavioural responses. However, these parameters are seldomly referred. Accordingly, the cease of opercular movements was the longest induction stage, followed almost equally by the other two stages, both for MS-222 and 2-phenoxyethanol. But for clove oil, the buoyancy loss was the shortest stage to attain, followed by motion impairment and cease of opercular movements was by far the longest one.

In what concerns the anaesthesia recovery phase for *G. rufa*, it was possible to time four stages: initiation of opercular movements, then body movements, buoyancy control and normal swimming behaviour. *G. rufa* generally followed this sequence, regardless of the anaesthetic agent that was used. But, in this case there few exceptions in each some of the intermediate stages were commuted. The first and last stages were consensual with others studies (Coyle *et al.,* 2004), but intermediate stages and their sequence may vary between species, individuals and anaesthetic agent. Cunha *et al*. (2015) considered just three stages for the anaesthesia recovery of guppy *P. reticulata*, combining both the initiation of opercular and body movements into the first stage. Aidyn *et al.* (2019) considered recovery phase as a whole moment, without differentiating stages in it. Generally, the initiation of *G. rufa*'s opercular movements and the normal swimming behaviour were the longest recovery stages for both MS-222 and 2-phenoxyethanol, followed by the initiation of body movements. The buoyancy control was the shortest one, being frequently simultaneous with the initiation of body movements. The clove oil anaesthesia recovery differed by *G. rufa* rapidly regaining opercular movements, shortly after being introduced in clear water, plus the much longer time needed to restart behaving normally. Similarly to the induction phase, a physical barrier (as a lid) should be used in the recovery container, after the fish starts to swim normally, as to prevent its escape.

Having this reservations in mind, it was notable that the most effective MS-222 concentrations in *G. rufa's* anaesthesia were usally much higher than the ones recommended for other warm-water species with similar size, most of them also used in the ornamental aquarium trade (Table VII). Among those other species are: the green swordtail *Xiphophorus helleri* Heckel, 1848 (Chambel *et al.,* 2013), the zebra fish *Danio rerio* (Hamilton, 1822) (Chambel *et al.,* 2013), the angelfish *Pterophyllum scalare* (Lichtenstein, 1823) (Mitjana *et al.,* 2014), the discus *Symphysodon discus* Heckel, 1840 (Chambel *et al.,* 2013) and the guppy *Poecilia reticulata* Peters, 1859 (Chambel *et al.,* 2013; Mitjana *et al.,* 2018). But also in relation to other cyprinid fish that can grow much larger than *G. rufa*, as: the Koi carp *C. carpio* (Bailey *et al.,* 2013) and the goldfish *C. auratus* (Küçük & Çoban, 2016). Notwithstanding, the results obtained were slightly lower than those appointed by Ferreira *et al.* (2015a) at 20 °C.

Likewise, the clove oil concentrations required to anesthetise *G. rufa* in this study were similar to those appointed before by Ferreira *et al.* (2016), plus the ones recommended for the guppy *P. reticulata* by Cunha *et al.* (2015) and the goldfish *C. auratus* (Perdikaris *et al.,* 2010). Also, they were much higher than those advised by Aydin *et al. (*2019) for *G. rufa* and others in similar conditions (Table VII), as: the Southern platyfish *Xiphophorus maculatus* (Günther, 1866) (Küçük & Çoban, 2016), the angelfish *P. scalare* (Mitjana *et al.,* 2014), the guppy *P. reticulata* by Mitjana *et al.* (2018). But also in relation to those cyprinids able to attain larger dimensions, as the common carp *C. carpio* (Hajek *et al.,* 2006).

A different trend was observed with 2-phenoxyethanol. The results obtained in this assay were indeed higher than others with those same species, namely of Aydin *et al.* (2019) also with *G. rufa*, as well as the goldfish *C. auratus* (Weyl *et al.,* 1996). Moreover, they were also bellow those brought forward by Ferreira *et al.* (2015b) at 20 °C. Yet, most other ornamental species were also effectively anaesthetised with similar doses of 2-phenoxyethanol, including: the he angelfish *P. scalare* (Mitjana *et al.,* 2014), the guppy *P. reticulata* (Mitjana *et al.,* 2018), the bighead carp *Hypophthalmichthys nobilis* (Richardson, 1845) (Akbary *et al.,* 2016) and the silver carp *Hypophthalmichthys molitrix* (Valenciennes, 1844) (Hedayati, 2018).

5. Conclusion

This trial showed that *Garra rufa* can successfully withstand anaesthesia with MS-222, 2-phenoxyethanol and clove oil at a temperature of 29 °C. The fish recovered fast and well, without visible sequels. All fish were males and presented appetence for food after anaesthesia, ingesting the total daily dose of feed recommended (corresponding to an *ad libitum* situation; Catarino *et al.,* 2019). Regarding the induction, monitoring and recovery times, they varied according to the anaesthetic, concentration and fish size class. In the induction and recovery times of the three anaesthesics it was not possible to establish a relationship between them in this concentrations. But in other studies, it is generally verified that the higher the concentration of anaesthetic used, the shorter was the induction time and conversely, the longer was the recovery time. Of the three anaesthetics used, clove oil was the one with the longest induction and recovery times. Afterwards, MS-222 and 2-phenoxyethanol presented similar times. Based on all results obtained during this study, it was also possible to find out which concentrations were most effective for each *G. rufa's* size class and each anaesthetic. For the anaesthetic MS-222 the concentration of 300 mg L^{-1} should be used in small fish (4.31 ± 0.42 cm and 0.86 ± 0.70 g) whilst for the medium fish (6.46 ± 0.85 cm and 3.20 ± 1.62 g) should be 350 mg L^{-1} and 375 mg L^{-1} for the large size fish (9.42 ± 0.70 cm and 9.74 ± 1.97 g). While in 2-phenoxyethanol the concentration should be used for the small (4.53 ± 0.32 cm and 0.75 ± 0.26 g) and medium fish (6.44 ± 0.90 cm and 3.29 ± 1.62 g) is 825 mg L^{-1} but on the other hand for the large fish (9.30 ± 0.67 cm and 10.0 ± 2.06 g) the recommended concentration is 750 mg L^{-1}. Likewise, the recommended dose of clove oil for *G. rufa* is 130 mg L^{-1} for all size classes (4.41 ± 0.26 cm and 0.69 ± 0.19 g for the small, 6.48 ± 0.89 cm and 3.15 ± 1.36 g for the medium and 9.43 ± 0.54 cm and 9.86 ± 1.84 g for the large). These concentrations are those recommended for the temperature of 29° C.

It can be concluded that the anaesthetics MS-222, 2-phenoxyethanol and clove oil are effective for *Garra rufa* routine handling procedures and physical assessment, regardless of the fish size class. They were able to produce deep anaesthesia, without causing noticible physical damages or affecting fish welfare.

6. Future perspectives

There is not much information/studies in the literature regarding *Garra rufa*. So, further studies on the life cycle, growth, feeding, reproduction and pathologies would be a good option and important for their maintenance in captivity, independently of its purpose (animal animal science, aquaculture, aquarium trade, spa and therapeutic industry). In the specific case of this study, namely at the level of anaesthesia, it would also be important to test different temperatures, other anaesthetics, concentration levels and size classes. Studies in embryos, larvae and juveniles would also be relevant as anaesthesia research is mainly performed in adult fish.

In addition, to animal behaviour when anaesthetised, physiological responses, hematological and histopathological surveys would be a very good complement to better understand health consequences of repeated anaesthesia procedures, in the scope of animal husbandry and welfare, as well as response to stress and disease situations. The resulting information would contribute to establish regulatory constraints on the use of anaesthetics, elaborate standard protocols and anticipate factor interdependencies impacting the specific efficacy of anaesthesia (Popovic *et al.,* 2012). All the information would be instrumental in ameliorating the rearing conditions of this species and the veterinary health support.

Ichthyotherapy studies are also necessary, since this *G. rufa* is widely used in spas around the world for skin treatments. These would help extending the knowledge on the interaction between humans and these fish, which is also important both for human health and *G. rufa's* ecological conservation status.

7. - References

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