POLITÉCNICO DE LEIRIA | ESCOLA SUPERIOR DE TURISMO E TECNOLOGIA DO MAR

Refinement of anaesthesia and analgesia protocol in gilthead seabream (*Sparus aurata*): 2-phenoxyethanol and clove-oil in synergy with lidocaine

Carolina Filipe Tchobanov

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Dissertação para obtenção do Grau de Mestre em Aquacultura

Dissertação realizada sob a orientação da Professora Especialista Teresa Maria Coelho Baptista e coorientação do Professor Doutor Luís Antunes

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Como muitos caminhos dão à ESTM, criou-se um grupo de amigos que irei levar para a vida. Numa fase mais tenra, a Beatriz e a Patrícia, a típica grupeta de miúdas - todas diferentes, mas que se encaixam que nem uma luva. A Beatriz, com a sua capacidade de desenrasque nunca antes visto, mas também poderá passar algumas coisas pacatas ao lado. E a Patrícia - fiel colega de casa e de estudo - com a coragem para tomar posse das rédeas, e onde a troca de um olhar comprometedor num dia cansativo poderá significar mais do que mil palavras de desabafo. E numa fase mais final, a chegada dos rapazes Diogo e Henrique, os forrageiros de outras universidades com habilidades de adorar carpas como quem adora chocolate, e encantar camarões ornamentais como cobras do circo. AHAH um brilharete para estes 5 maestros, em que a Malta é Fixe: 😊

Dagene går, og årene lages. Livet skapes, og fremtiden oppdages.

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Resumo

A redução do stress é um objetivo fundamental para promover o bem-estar e o sucesso na produção animal. É comum serem utilizados anestésicos para reduzir o stress dos animais durante alguns procedimentos. Este estudo visa refinar a concentração anestésica do 2-fenoxietanol (2PHE) e do óleo de cravo-eugenol (CO) e analgésica da lidocaína (L) em douradas (*Sparus aurata*).

O estudo consistiu na realização de dois ensaios de refinamento de concentrações, e de um ensaio de resposta fisiológica à exposição da anestesia/ analgesia. Neste último ensaio foi retirado sangue para determinação de parâmetros hematológicos e parâmetros de resposta imune e metabólica. Analisou-se o fígado avaliando a resposta metabólica e parâmetros de stress oxidativo. Avaliou-se a expressão de genes tanto no cérebro como nas brânquias. A análise histológica das brânquias permitiu estudar as alterações na estrutura e na morfologia.

Dos resultados obtidos para os parâmetros hematológicos analisados destaca-se o MCV onde se observou uma diminuição em 2PHE + L comparado com CO. No entanto, estes valores encontram-se dentro dos aceitáveis para a espécie. Nos parâmetros de stress oxidativo a CAT foi estatisticamente superior em 2PHE e 2PHE + L comparado com CO e CO + L. Para TG, existiu igualmente um aumento significativo em CO e CO + L quando comparado com 2PHE + L. A SOD mostrou um aumento significativo em 2PHE + L. Tendo em conta os resultados dos parâmetros de stress oxidativo, o melhor tratamento parece ser CO ou CO + L. Nos resultados dos parâmetros da resposta imune, na peroxidase, existiu um aumento significativo com a exposição de 2PHE + L comparado com o CO, indicando um melhor resultado quando é utilizado o óleo de cravo. Nos parâmetros metabólicos, o lactato apresentou valores acima dos valores normais para a espécie, tendo o 2PHE + L e CO + L apresentado valores significativamente superiores a CO. O ALT apresentou valores mais elevados em 2PHE + L e CO +L comparado com 2PHE e CO, e o AST apresentou valores superiores em CO + L comparando com CO. Podendo concluir-se que o uso da lidocaína apresenta um potencial efeito negativo. Na análise histológica, nas modificações progressivas, em CO observaram-se menos danos do que em 2PHE, e nas modificações regressivas o uso da lidocaína (2PHE + L e CO + L) causou mais danos do que o uso isolado da anestesia (2PHE e CO). Na análise de expressão de genes, há uma maior expressão do gene HSP70 nas brânquias havendo uma

maior expressão em CO e CO + L do que em 2PHE + L. Na expressão de genes no cérebro, destaca-se o gene GST3 com maior dano associado a 2PHE + L.

Em conclusão, este trabalho aponta para a utilização das doses ideais de 2-fenoxietanol (0,4 mL/L) com lidocaína (2,5 mg/L), e o óleo de cravo-eugenol (45 mg/L) em dourada. Embora o uso de lidocaína leve a uma melhoria nos tempos de indução e tempo de recuperação, sem alterações de outros parâmetros; houve também resultados que mostram um aumento de stress devido à exposição. Assim, dependendo dos objetivos do trabalho e do composto anestésico utilizado, a utilização da analgesia pode ser favorável. No entanto, são necessários mais estudos para compreender melhor a sinergia com a analgesia utilizando outros anestésicos sintéticos e naturais.

Palavra-chave: Veterinária; Aquacultura; Anestésico; Analgésico; *Sparus aurata*

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Abstract

Stress reduction is a key objective to promote welfare and success in animal production. Anaesthetics are commonly used to reduce animal stress during some procedures. This study aims to refine the anaesthetic concentration of 2-phenoxyethanol (2PHE) and clove-oil (CO) and analgesic concentration of lidocaine (L) in gilthead sea bream (*Sparus aurata*).

The study consisted of two concentration refinement experiments, and a experiments regarding the physiological response to anaesthesia/ analgesia exposure. In the third experiment, blood was drawn for the determination of haematological parameters and immune and metabolic response parameters. The liver was analysed to assess metabolic response and oxidative stress parameters. Gene expression was assessed in both brain and gills. Histological analysis of the gills allowed the study of changes in structure and morphology.

From the results obtained for the haematological parameters analysed, the MCV was highlighted where a decrease was observed with 2PHE + L compared to CO. However, these values are within the acceptable limits for the species. Regarding oxidative stress parameters, CAT was statistically superior in 2PHE and 2PHE + L compared with CO and CO + L. For TG, there was also a significant increase in CO and CO + L when compared with 2PHE + L. SOD showed a significant increase with 2PHE + L. Considering the results of the oxidative stress parameters, the best treatment seems to be CO or CO + L. In the results of the immune response parameters, in peroxidase, there was a significant increase with the exposure of 2PHE + L compared with CO, indicating a better result when clove oil is used. In the metabolic parameters, the lactate presented values above the normal values for the species, with 2PHE + L and CO + L presenting significantly higher values than CO. The ALT presented higher values in 2PHE + L and CO + L compared to 2PHE and CO, and the AST presented higher values in CO + L compared to CO. It may be concluded that the use of lidocaine presents a potential negative effect. Histologically, in progressive changes, less damage was observed in CO than in 2PHE, and in regressive changes, the use of lidocaine (2PHE + L and CO + L) caused more damage than the use of anaesthesia alone (2PHE and CO). In the gene expression analysis, there is a higher expression of the HSP70 gene in the gills with higher expression in CO and CO + L than in

2PHE + L. In the gene expression in the brain, the GST3 gene stands out with higher damage associated to 2PHE + L.

In conclusion, this work points to the use of the ideal doses of 2-phenoxyethanol (0.4 mL/L) with lidocaine (2.5 mg/L), and clove oil-eugenol (45 mg/L) in gilthead sea bream. Although the use of lidocaine leads to an improvement in induction times and recovery time, without changes in other parameters; there were also results showing an increase in stress due to exposure. Thus, depending on the objectives of the work and the anaesthetic compound used, the use of analgesia may be favourable. However, further studies are needed to better understand the synergy with analgesia using other synthetic and natural anaesthetics.

Keywords: Veterinary; Aquaculture; Anaesthetic; Analgesic; *Sparus aurata*

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Index

List of Figures

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List of Tables

List of Acronyms

2PHE	2-phenoxyethanol
ache	Acetylcholinesterase
act	β-actin
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
CAT	Catalase
CO	Clove oil-eugenol
crh	Corticotrophin-releasing Hormone
crhbp	CRHbinding Protein
GST	Glutathione S-transferase
gst3	Glutathione-S-transferase 3
Hb	Haemoglobin
hsp70	Heat Shock Protein 70
Ht	Haematocrit
il1β	Interleukin 1β
L	Lidocaine
LPO	Lipid Peroxidation
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
RBC	Red Blood Cells
SD	Standard deviation
SOD	Superoxide Dismutase
TG	Total Glutathione
trh	Thyrotropin-releasing Hormone
WBC	White Blood Cells

1. Introduction

In 2020, world fisheries and aquaculture production reached 177.8 million tonnes. World aquaculture accounted for 49% of production (88 million tonnes) being dominated by finfish 57.5 million tonnes (49.1 million tonnes from inland aquaculture and 8.3 million tonnes from marine and coastal aquaculture) (Figure 1) (FAO, 2022).

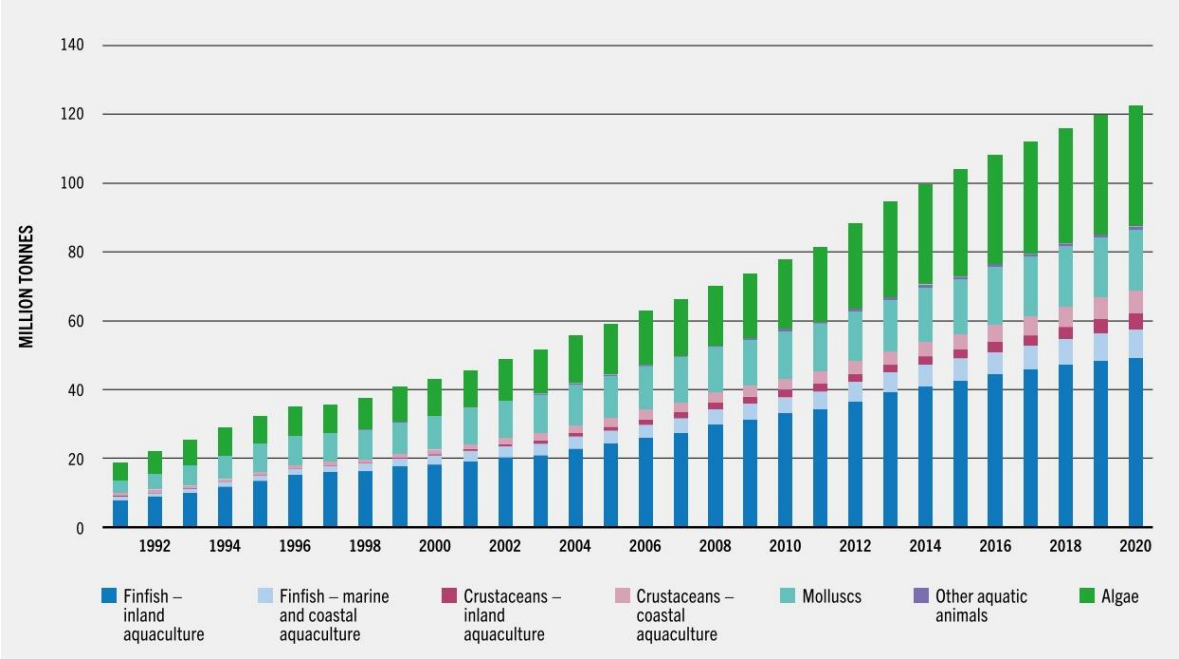

Figure 1 – World Aquaculture Production of Aquatic Animals and Algae, 1991-2020 (FAO, 2022).

Farming systems are very diverse in terms of culture methods, practices, and facilities (FAO, 2020). The main goals of this industry are to grow animals to market size in the shortest time, with minimum inputs and with reduced cost. Means that both growth rate and growth efficiency must be maximised and, their production environment must be optimized for minimal stress induction and to ensure the maximum 'comfort' possible (Ross & Ross, 2008).

Gilthead seabream (*Sparus aurata*) is a very important aquaculture fish from an economic perspective for many countries (Arechavala-Lopez et al., 2019; Grigorakis et al., 2002). The production of this species can be made, particularly, in intensive farming systems, which can cause welfare problems such as stress, health problems, and mortality (Conte, 2004). The majority of stressors in aquaculture are avoidable and reducing stress is a

fundamental goal for welfare and successful production (Ashley, 2007). The unavoidable stressors, such as handling and transport, can be reduced through the use of drugs (anaesthetics and analgesics).

The concept of animal welfare used for mammals has been extended to marine animals. Animal welfare has been under special observation recently by researchers and contributors to commercial practices (aquaculture and ornamental market) (Sloman et al., 2019). The definition of animal welfare depends on the position of the researcher and it is possible to have three methodologies in perspective: based on feelings (animals must feel good, without pain or fear, and have access to positive experiences); based on function (the animal has good health and an environment in which it expresses its normal function and capacity); and based on nature (the animal can live a natural life and express natural behaviour) (Sloman et al., 2019).

In veterinary sciences, pain minimization remains a fundamental principle (Chatigny et al., 2017). Also, in some areas of aquaculture and research, there may be an additional need to address the problem of pain (Ross & Ross, 2008). The perception and the need for debate about whether fish feel pain still exists. The argument is maintained by the fact that there are, or are not, pain receptors (Chatigny et al., 2018b; Neiffer & Stamper, 2009). Any procedure involving invasive methods, ranging from simple forms of needle punctures to surgery, causes a certain degree of pain (Ross & Ross, 2008). Studies based on brain responses and behavioural expression suggest that like mammals, fish experience pain, fear and stress (Terlouw et al., 2008).

The Directive 2010/63/EU emphasizes the improvement of animal welfare for animals which are exposed to procedures and research by implementing the principle of 3R: Reduce, Reuse and Refine (European Parliament and The Council of the European Union, 2010). Under Article 14, from the same law, procedures involving severe injuries that may cause severe pain cannot be performed without anaesthesia and appropriate methods to ensure that pain, suffering and distress are reduced to a minimum need to be proposed.

To improve animal welfare and meet ethics and law, anaesthetics and analgesics have been tested to prove their effectiveness in fish (Neiffer & Stamper, 2009). Anaesthetic agents are commonly used for surgical procedures in fish (such as fin clipping for genetic identification), but also as the first step towards euthanasia for most laboratory fish (Readman

et al., 2013). To date, only minimal investigations have been made in the field of fish analgesics (Chatigny et al., 2018b).

Anaesthesia consists of several moments, including sedation, immobilization, unconsciousness (narcosis), amnesia and analgesia (pain relief). Sedation is a reduction in sensitivity, which results in tranquillity and calmness. Narcosis creates a state of unconsciousness and amnesia which includes immobilization and pain relief (Zahl et al., 2012). Chemical anaesthetics are used crosswise between species. However, the anaesthesia protocol for new research and production species is based on compounds and concentrations established for more studied species (Zahl et al., 2012).

Sedation is the first stage of anaesthesia progression. The loss of the notion and the external receptors of animal stimuli are repressed whit excitation responses increasingly limited. Sedatives have a tranquillizer and anxiolytic function, and some chemicals have analgesic capabilities (Schroeder et al., 2021). Sedation is advised to be performed at times before handling, brief out-of-water examination, transport, and sorting of specimens (Aydın & Barbas, 2020; Schroeder et al., 2021).

General anaesthesia is a mandatory intervention previously performed when evasive procedures such as tagging, cardiac excision surgery, fin clipping or amputation, biopsy, curative interventions, vaccination, harvest, transport, clinical procedures, labelling, and blood collection were performed (Aydın & Barbas, 2020; Readman et al., 2013; Schroeder et al., 2021).

The anaesthetic is chosen due to the basis of its properties, adapting both to the surgical intervention and the physiological state of the animal. Pre-anaesthetic sedation is used to prevent anaesthesia-induced stress as part of some veterinary protocols. By combining drugs of different properties, more complete anaesthesia is obtained than with only a single substance. In addition, synergistic effects between different drugs may also allow a reduction in the dosage of each drug compared to individual administration. This may result in smoother induction and recovery and reduce the incidence of adverse effects (Zahl et al., 2012).

There are several methodologies for the administration of anaesthetics: immersion/inhalation, intravenous, intramuscular, and oral (Chatigny et al., 2018b; Neiffer &

Stamper, 2009). The most usual method is immersion. The drug is found in the water where the animal will be and is absorbed through the gills and excreted by the route of gills and urine (Schroeder et al., 2021; Zahl et al., 2012). These immersion drugs must be water-soluble or use a water-soluble solvent as a vehicle and are powder-shaped or liquid (Mylonas et al., 2005; Neiffer & Stamper, 2009; Schroeder et al., 2021).

The fish absorbs the anaesthetic in dissolved solution in the surrounding water, which enters the bloodstream through the gills and/or accessory respiratory organs and then quickly passes into the central nervous system. Both the tissue of the gills and the skin contain large amounts of lipids, so the efficiency of the absorption of immersion agents on these surfaces are directly related to the lipid solubility of the drug (Neiffer & Stamper, 2009).

Local anaesthetics produce analgesia since they block all nerve activity including nociceptive transmission. Local anaesthetics are mainly used in 4 ways: to induce skin or mucosal anaesthesia (topical anaesthesia), local tissue anaesthesia (local infiltration), regional anaesthesia, and intravenous anaesthesia (which has a regional action). These chemicals tend to have a low cost, minimal side effects and a brief recovery period when used properly (Chatigny et al., 2017).

For practical purposes, anaesthesia is reduced to three obvious phases: induction, maintenance, and recovery. Each of them varies in duration according to the drug or method used, species and conditions (Ross & Ross, 2008). Analgesia treatment is used in any phase to improve pain control and increase animal welfare during and after surgical procedures (Zahl et al., 2012). Pre-anaesthesia is also considered a period. This is the moment when a pre-anaesthesia evaluation is conducted to assess the risks and develop an anaesthesia plan. This evaluation specifically identifies the risks of the anaesthesia encounter and is the moment when preparation activities should be done (Zambouri, 2007). The ultimate goals of preoperative assessment are to reduce the surgical and anaesthetic perioperative morbidity or mortality, and to return animals to desirable functioning as quickly as possible.

Induction consists of the exposure of the animal to the anaesthetic agent, and it is possible to see several reactions in the animal characterizing an induction stage (Table I). When induction is done slowly, it is possible to observe the different stages, first the sedation stages and then the anaesthesia stages (Ross & Ross, 2008). Induction progress and deep anaesthesia are usually divided into different stages and 'planes'. Each stage is possible to be

distinguished by the behavioural activity of the animal (swimming and posture), muscle staining, response to reflexes, and respiratory rate (opercular ventilation). In fish, it may not be possible to be able to differentiate the stages if the induction is fast (Sneddon, 2012; Zahl et al., 2012).

Induction is usually accompanied by hyperactivity, a response of only a few seconds to the sensation or slightly irritating properties of the drug. In general, induction should be rapid (up to 3 minutes) and without marked hyperactivity, although some increase in activity is usually observed (Ross & Ross, 2008). Without considering the chemical compound itself, other factors can affect anaesthesia. These are biological factors like age, gender, body conditions, weight, stage of life, growth, physiological status, health, reproduction; and abiotic factors like water quality, temperature, available oxygen (Sneddon, 2012).

The level of anaesthesia depends on the drug itself, dose, water temperature, immersion time, biomass, fish species, size, and type of handling. The ideal anaesthetic will induce rapid anaesthesia with rapid excretion and recovery, a wide margin of safety and a short or absent withdrawal period (Schroeder et al., 2021).

Maintenance involves extending the stage achieved in a stable manner, without harming the health of the animal. It should run unquestioned and will most likely be effective with a reduced dose of the medicinal product (Ross & Ross, 2008).

The recovery phase involves the removal of the anaesthetic agent and the return to the normal state. Initial recovery can take from a few seconds to a few minutes (5-10 minutes), but in general, it should be fast and without change in behaviour or other side effects, although there is usually some muscle tremor (Ross & Ross, 2008; Schroeder et al., 2021).

Although an overdose of anaesthesia is often the recommended method for the euthanasia of ornamental and research fish, this is not a common practice for fish destined for the food chain. Drug-free slaughter is preferred to avoid chemical residues. For laboratory animals, euthanasia usually follows when an animal reaches the end of its procedure (Schroeder et al., 2021).

Table I - Description of each stage of anaesthesia and recovery. Based on Akgul & Can, 2020; Golomazou et al., 2016; Mylonas et al., 2005; Ross & Ross, 2008; Sneddon, 2012; Treves-Brown, 2000; and Zahl et al., 2012 - *some authors suggest that there is an intermediate stage between mild and surgical anaesthesia called *midplanium* (S - sedation; A - anaesthesia; R - recovery)

Stage	Plane	Code	Stage	Behavioural characteristics
I		S1/A1	Mild sedation	Disorientation, reduced activity, normal balance, normal branch ventilation, reduced reactivity
II		S2/A2	Commotion	Agitation, increased activity, difficult to maintain balance, increased opercular ventilation, increased reactivity, increased heartbeat
III	1	A3	Mild anaesthesia and Loss of balance	Anaesthetized, no activity, loss of balance, movement of pectoral fins, decreased regular opercular ventilation, touch reflexes, regular heartbeat
	2	A4	Surgical anaesthesia*	Anaesthetized, no activity, no balance, superficial opercular ventilation, reduced heartbeat
	3	A5	Deep anaesthesia	Anaesthetized, motionless, loss of response to tactile stimuli, slow and irregular opercular ventilation
IV		A6	Overdose	Appears dead, no opercular movement, heart failure
RECOVERY		R1	Partial opercular recovery	Opercular movement start
		R2	Partial balance recovery	Partial recovery of balance with partial recovery of swimming movements
		R3	Balance recovery	Complete and permanent balance recovery, regular opercular ventilation
		R4	Behavioural recovery	Reappearance of the escape and reaction swimming movement to external stimuli, but still the behavioural response is impassive
		R5	Full recovery	Response to visual stimuli, breakout swimming

The drugs used for humans have been tested for their use in fish (Zahl et al., 2012). The anaesthetic selection process is based on induction, maintenance, and recovery; cannot be

averse to the animal and should induce unconsciousness quickly, and reduce stress (Martins et al., 2019). There are several commonly used anaesthetic drugs, including MS222; benzocaine; AQUI-S; 2-phenoxyethanol; etomidate; and clove oil (Ross & Ross, 2008).

2-Phenoxyethanol (2PHE) is a clear, colourless, or straw-coloured oily liquid (Martins et al., 2019; Neiffer & Stamper, 2009; Ross & Ross, 2008) with a slight odour that easily passes into solution if diluted with a small quantity of water (Ross & Ross, 2008). It is slight water-soluble with easy preparation making this drug very suitable for aquacultural practices (Martins et al., 2019; Neiffer & Stamper, 2009; Ortuño et al., 2002). This drug is widely used as a sedative for transportation and anaesthetic which can provide light and deep stages of induction (Martins et al., 2019; Neiffer & Stamper, 2009).

Has an anaesthetic, 2-phenoxyethanol can be a low-price drug that acts rapidly creating a short induction time, if exposure is limited, the recovery tends to be positive and fast (Mitjana et al., 2014; Ortuño et al., 2002). The solution can be used also as bactericidal and fungicidal during laparotomy or abdominal surgery (Martins et al., 2019; Ross & Ross, 2008). Another advantage is the fact that there is no occurrence of pH change when added to seawater (Neiffer & Stamper, 2009).

The use of 2-phenoxyethanol can provoke several adverse reactions, such as heart-rate decrease, alterations in blood parameters, and respiratory depression (Martins et al., 2019; Mitjana et al., 2014). It has been reported that regular and continuous exposure can cause neuropsychological syndrome in some handlers (Neiffer & Stamper, 2009). Furthermore, studies showed that for 2-phenoxyethanol the lower the temperature, the longer the induction and recovery time (Mylonas et al., 2005). The amplitude used is 0.1 to 0.5 mL/L (Table II).

Clove oil (CO) is a natural product obtained by distillation of the flowers, stems and leaves of *Eugenia aromaticum* and/or *Eugenia caryophyllata* (Mylonas et al., 2005; Ross & Ross, 2008), and its major constituent is eugenol (70-90%) (Martins et al., 2019; Mitjana et al., 2014; Mylonas et al., 2005; Ross & Ross, 2008). The oil has a dark brown colour and has a rich, aromatic odour and flavour (Ross & Ross, 2008).

The advantages of clove oil include lower price, less environmental impact, being more potent than other anaesthetics used in fish, and being safe for staff (Mitjana et al., 2014; Mylonas et al., 2005). Clove may be dispersible in water at higher temperatures (Ross &

Ross, 2008) but since it is an oil insoluble in water at room temperature, it requires mixing with ethanol before its use (Martins et al., 2019).

In fish, clove oil exhibits fast induction times and provides consistent anaesthesia compared to other drugs. But it may be associated with longer recovery times and higher mortality (Martins et al., 2019). It has been reported also to change some physiological and biochemical parameters, with cardiovascular problems and lowering feed intake (Martins et al., 2019).

With clove oil anaesthesia the higher the temperature, the shorter the induction and recovery time. Higher concentrations reduce the induction time and prolong the recovery time (Hur et al., 2019; Mylonas et al., 2005). The range of clove oil used among species is vast, it varies from 20 to 150 mg/L. However, the concentrations are narrowed to gilthead seabream between 40 and 55 mg/L in a 15 to 25 ºC water temperature range (Table II).

Table II – 2-phenoxyethanol and clove oil anaesthetic concentrations for different fish species according to authors.

Chemical	Species	Concentration	Temperature (ºC)	Reference
2-Phenoxyethanol	*Gadus morhua*	0.25 mL/L		Zahl et al., 2009
	Sparus aurata	0.3 mL/L	25	Mylonas et al., 2005
		0.45 mL/L	15	
	Oncorhynchus mykiss	0.2 mL/L		Soltanian et al., 2018
	Dicentrarchus labrax	0.3 mL/L	25	Mylonas et al., 2005
		0.45 mL/L	15	

Clove Oil - Eugenol	General	20 to 150 mg/L		Neiffer & Stamper, 2009; Sneddon, 2012; Taheri Mirghaed et al., 2016
	Sparus aurata	40 mg/L	25	Mylonas et al., 2005
		55 ppm		Bahi et al., 2018
		55 mg/L	15	Bodur et al., 2018; Mylonas et al., 2005
	Paralichthys olivaceus	160 mg/L	21	Hur et al., 2019
	Oncorhynchus mykiss	25 ppm		Soltanian et al., 2018
		30 mg/L		Velisek et al., 2011
		30 mg/L	15	Mylonas et al., 2005
		40 mg/L	25	

Analgesics are used in veterinary practice to alleviate pain in animals, but in fish procedures generally, they are not administered because of the debate regarding their capacity to perceive pain (Mettam et al., 2011). Research on fish has shown that potentially painful event may change their normal behaviour and indicate a stressful event. The main classes of analgesic drugs are opioids, nonsteroidal anti-inflammatory (NSAIDs), local anaesthetics, and miscellaneous drugs (Sneddon, 2012).

The development of protocols regimes including anaesthesia and analgesia has been an important part of the drive towards refinement of methodology to minimize suffering and stress, and improve welfare in fish (Schroeder & Sneddon, 2017). The addition of the analgesic can create anaesthetic-sparing effects of the drug reducing the general anaesthetic

requirements during anaesthesia, which can reduce cardiovascular problems (Chatigny et al., 2018b). This is called balanced analgesia or anaesthesia, where in two or more drugs potentiate to produce the desired anaesthesia effects, while reducing the risk of side-effects, such as respiratory depression, hemodynamic instability, and mortality. This is possible when these drugs induce a synergetic and complementary effect, resulting in a rapid and recover anaesthesia, with decreased doses and safer analgesia and anaesthesia (Kehlet et al., 1999). The concept of 'balanced anaesthesia' and analgesia has a major impact on the development of protocols based on the combination of drugs.

Lidocaine hydrochloride (L) is an example of a local analgesic that is relatively inexpensive and is safe to handle (Martins et al., 2019). The hydrochloride salt is freely soluble in water (Ross & Ross, 2008). Low-dose lidocaine immersions can improve welfare in perioperative analgesia situations (Martins et al., 2019), which can allow the reduction of the anaesthesia dose at use.

In veterinary, lidocaine is often used in combination with other drugs but conjunctions for fish analgesia have not been tested (Chatigny et al., 2018b). The practice of this drug as infiltration in combination with general anaesthesia might be prudent (Chatigny et al., 2018b; Martins et al., 2019).

Lidocaine is a local anaesthetic and analgesic agent that inhibits the propagation of action potentials by blocking sodium channels and affecting membrane function. Thus, it prevents the sensation of pain due to the blockage of nociceptive transmission (Sneddon, 2012). There are few studies on the use of this drug, and it is still necessary to study the ideal concentration to be used in different species and what reaction causes them. In the case of gilthead seabream, there are no records of experimental lidocaine use.

Many authors refer the range between 1 and 5 mg/L for the use of lidocaine by immersion for zebrafish, with an effective dose between 2.5 to 5 mg/L. Lidocaine can be used as a compound for euthanasia with concentrations >100 mg/L (Collymore et al., 2016; de Abreu et al., 2019; Lopez-Luna et al., 2017).

Acute stress in fish is a topic still under study in fish. Most of these studies are related to the influence of stress on immunity. Through the analysis of blood, it is possible to observe cells that are related to an acute stress event (Guo & Dixon, 2021). Haematological

assessment of red blood cells, white blood cells, haematocrit and haemoglobin is recommended as a routine health check in aquaculture (Fazio, 2019).

Oxygen is a potent oxidant that can cause oxidative stress, being an essential compound for aerobic organisms for energy production. Oxidative stress occurs in the situation of destabilization in the production of oxidant and antioxidant components, as it refers to the destabilization of reactive oxygen species (ROS) and their antioxidants (Biller & Takahashi, 2018). Oxygen reduction leads to the production of reactive intermediates such as superoxide radical ($O_2^{\bullet -}$) by SOD, singular oxygen molecule ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO•) by CAT (Birnie-Gauvin et al., 2017; Yu et al., 2020). Antioxidant defences can account for damage such as enzyme inactivation and peroxidative damage (e.g., lipid peroxidation, LPO) (Almeida et al., 2010; Teles et al., 2019). In addition to antioxidant defences, there are also enzymatic response defences such as catalase (CAT), and superoxidase dismutase (SOD). Glutathione s-transferase (GST) is used as a biomarker being part of a set of biotransforming enzymes and total glutathione (TG) indicates oxidative damage (Almeida et al., 2010; Birnie-Gauvin et al., 2017; Teles et al., 2019).

The immune response can be analysed through different plasma biomarkers such as peroxidase, protease and antiproteases (Passos et al., 2021). These biomarkers are enzymatic and have been studied between species in situations of pathogenic infection, climate change, stress induction, transport, and exposure to contaminants. Peroxidase is known as one of the first lines of defence, in which different microbicides eliminate hydrogen peroxide, helping in the redox balance of the immune system (Espinosa et al., 2020; Guardiola et al., 2016). Protease and antiprotease are also related to stress situations in which it is possible to analyse the existence of inflammation and its resolution (Machado et al., 2015). These analyses are correlated, by the protease it is possible to observe the existence of inflammation and by the peroxidase it is possible to analyse the course of the inflammation, being possible to observe the end of this 'problem' through the antiprotease (Machado et al., 2015).

The effect of anaesthetic exposure on metabolic changes has been studied and may affect fish physiology and cause stress. Glucose and lactate are two parameters related to secondary stress effects, where their increase may be related to several factors (Bahi et al., 2018; Fazio et al., 2015). Alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are widely used to analyse the damage in fish and their

health status (Yousefi et al., 2022). These when found in high concentrations, translate as damage to the hepatobiliary cell system, which leads to a leakage of enzymes into the circulation (Yousefi et al., 2021).

The gills are an organ with a large surface of contact with the external environment, through the epithelium, which facilitates the monitorisation of the environment (Marinović et al., 2021). The use of this organ in histology also comes from its sensitivity to the environment based on its multifunction of respiration, osmoregulation and ion exchange (Teles et al., 2019).

The anaesthetic and analgesic are administered via the water, because of it they are inhaled through the gills where they are diffused through the lamellae, where they enter the arterial blood stream and are carried to the brain where they act (Martins et al., 2019). The gills are the first organ of contact and where it absorbs, and the brain is where it acts, this were the organs of choice for gene expression. Heat shock proteins (HSPs) are widely important proteins when studying cell stress response (Wang et al., 2021). The *hsp70* gene, plays an important role in regulating the restructuring of new synthetic proteins and denatured proteins (Yu et al., 2020). Fish neuroendocrine stress pathways presents the hypothalamus-pituitary-interrenal (HPI) axis. When stimulated at the hypothalamic level, CRH-binding protein (CRHBP) is considered an important player in the HPI axis, with antagonistic roles towards corticotropin-releasing hormone (CRH) in the control of stress pathways (Toni et al., 2015). CRHBP has a high affinity to CRH, which binds to decrease the actions from CRH (inhibitory activity), guaranteeing homeostasis (Jerez-Cepa et al., 2019; Toni et al., 2015) *crhbp* expression can be lower than CRH since stress induces *crh* expression, and CRHBP binds to its particles. The thyrotropin-releasing hormone (TRH) also regulates *crh* (Jerez-Cepa et al., 2019). If *crhbp* isn't enough expressed, *trh* gene helps to decrease the damage created by CRH binding to it reducing the receptors (Toni et al., 2015). The *il1β* gene is considered a marker for pro-inflammatory activity, with a cellular and humoral capacity of innate and adaptive immune responses (Balasch et al., 2021). It is a cytokine gene, a non-specific immunomarker often used in fish, since its expression levels are stimulated by toxic substances, pathogenic microorganisms, and stress (Wang et al., 2021). Acetylcholinesterase (AChE) is part of the Cholinesterase (ChE) family of enzymes, whose activity is among the most widely used biomarkers. AChE is responsible for the degradation of the neurotransmitter acetylcholine at cholinergic synapses, and pseudocholinesterase which are involved in the

detoxification of various xenobiotics (Almeida et al., 2010). Acetylcholinesterase acts on the central nervous system and neuro-muscular synapses via nicotinic and muscarinic receptors (de Abreu et al., 2019). Increased expression marks the regulation of inflammatory responses (Balasch et al., 2021). The decrease of *ache* in mice is correlated with an increase in anxiety behaviour. Theoretically, in fish the reduction of AChE activity may correlate with anxiolytic-like action (de Abreu et al., 2019). But it is a detail that still needs further study. Glutathione-S-transferase 3 is referred to as an antioxidant response and enzymatic gene, where its expression suggests the production of ROS species (Balasch et al., 2021; Teles et al., 2019).

This study aims to refine anaesthetic and analgesic concentration for gilthead seabream (*Sparus aurata*) comparing 2-phenoxyethanol and clove oil-eugenol anaesthetics, with and without the use of one analgesic (lidocaine). The behavioural, haematology, metabolic, immune, and stress parameters, gill histology and gene expression (brain and gills), will performed after anaesthesia. The potential beneficial effects of the local analgesic, lidocaine, as a synergistic agent to these anaesthetics will be compared when animals are subjected to a procedure.

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2. Materials and Methods

2.1 Ethics Statement

All procedures were conducted under personal and project licenses for this study approved by the Portuguese competent authority, Direcção Geral de Alimentação e Veterinária (DGAV, Lisboa, Portugal)., under the project authorization 0421/000/000/2019 and in agreement with the European Directive on the protection of animals used for scientific purposes (2010/63/EU) and its transposition to the Portuguese law (Decreto-lei 113/2013) ensuring minimal animal stress and discomfort.

2.2 Fish housing

Gilthead seabream (*Sparus aurata*) juveniles were maintained according to established procedures at the CETEMARES - Marine and Environmental Sciences Centre MARE-Polytechnic of Leiria, Peniche. Briefly, animals were kept at 18.59 ± 1.30°C in a photoperiod of 12:12 h light: dark cycle. The RAS systems had a mechanical filter to remove particles, like faeces and food waste, a biological filter, and a UV filter. The fish were fed daily with a commercial diet (SPAROS, Portugal), 1% of the total biomass. Fish fasted 24h before sampling, and the behaviour was monitored to register any difference post sampling or handling.

Daily cleaning of the aquaria and tanks to remove excess food and clean the faeces to maintain the quality of the water. Water quality parameters were measured and recorded daily with a handheld multiparameter meter (YSI, Professional Plus; Yellow Springs, United States of America) for pH, salinity, dissolved oxygen, and temperature. And kits for ammonia (Marine Test Kit Ammonia, ref. 21460, RedSea; Eilat, Israel), nitrites (Nitrite Test Kit, ref. #28, API; Chalfont, United States of America), and nitrates (Nitrate Test Kit, ref. #LR1800, API; Chalfont, United States of America).

All fish were maintained in a RAS system (system A) of 1 tank of 1000L. After their use they were transferred to another RAS system (system B) of 1000L tank, marked as ‘animals not to be used’ for having undergone manipulation. Fish used on experiment 3 (Physiological responses to anaesthesia and analgesia exposure), were transferred by treatment group to 60L aquaria, in 2 RAS systems of 3 aquariums of 60L each (system C –

Clove oil-eugenol and D – 2-phenoxyethanol), where they remained for 72H for behaviour and mortality observation.

The water of the induction and recovery aquaria was collected from the original tank.

2.3 Induction/ Recovery protocol

The fasted fishes were exposed individually to the anaesthetics and analgesic in the study in a 15L aquaria with a 10L of water volume. The recovery was made in a 15L aquaria with a flow-through system. All aquaria had aeration with an air stone.

The exposure solution was freshly prepared for each fish (new water and the necessary volume from the chemical to make the required concentration) in experiment 1 (Anaesthesia Refinement) and 2 (Analgesia Refinement).

The process of capture, transport, induction, and recovery was done singularly to reduce the stress associated with handling. The fish were caught with the aquarium net, inserted into the transport bucket, and transported to another laboratory. Then, individually animals were introduced in the induction aquarium with the anaesthetic. When the animal entered the solution, the inducing time was recorded at A3 and A5 (Table I). If the animal did not reach the induction stage A5 for up to 5 minutes, it was removed from the solution and set the time. To confirm the induction stage A5, it was verified the response to touching stimuli in the caudal fin and the regularity of the ventilation rates.

Upon reaching the desired stage or past the maximum induction time, the fish were transferred to recovery, and recorded the time to reach the recovery stage R3 and R5 (Table I). To check the arrival at stage R5, approximation movements were performed to the recovery aquarium. If the animal had an escape response, was transferred to a bucket, and transported to the aquaculture room. Fish were kept in 2 RAS systems of 3 aquaria each, to assess mortality and behaviour (to feed again) in the following days (72 hours) after being anaesthetised.

2.4 Anaesthetic and Analgesic Drugs

The anaesthetics in the study were: 2-phenoxyethanol (2PHE; VWR Chemicals; 99.4% purity) and Clove Oil-Eugenol (CO; Sigma Aldrich C8392-500ML; *Eugenia* spp., Oil of cloves; diluted in ethanol 95%, 1:9). The analgesic (local anaesthetic) in the study was lidocaine hydrochloride (L; Anestesin; Medinfar-Sorológico, Amadora, Portugal; concentration of 20 mg/mL). All solutions were freshly made with the water from the systems.

2.5 Experiment 1 - Anaesthesia refinement

The fish were exposed to three concentrations of 2-phenoxyethanol (0.2, 0.4, and 0.6 ml/L), and clove oil-eugenol (30, 45, and 60 mL/L) to record the induction time of anaesthesia (A3 and A5) and recovery time (R3 and R5). The anaesthetics chamber was a 15L aquarium with 10L of seawater, which was aerated continuously. Forty-eight (48) fish (eight per concentration) were caught from the 1000L tank and placed in the anaesthetic chamber, individually, and induction times were recorded (in the appendix, Figure 1). After the anaesthesia, the fish were weighed and placed in a 15L aquarium with aeration in an open system, and recovery times were recorded.

2.6 Experiment 2 - Analgesia refinement

Twenty-four fish (four animals per optimum concentration of each anaesthetic) were exposed to three concentrations (2.5, 5, 7.5 mg/L) of lidocaine in a 5-minute bath before anaesthesia. Then exposed to the chosen concentration of each anaesthetic and recorded the induction and recovery times (in the appendix, Figure 2). The analgesic chamber was a 15L aquarium with 10L water, with continuous aeration.

2.7 Experiment 3 – Physiological responses to anaesthesia and analgesia exposure

Based on the results of the previous experiments, four treatment groups were set up: 2-phenoxyethanol (2PHE); clove oil (CO); 2-phenoxyethanol + lidocaine (2PHE + L); clove oil + lidocaine (CO + L) (concentrations optimized in Experiment 1 and 2). A total of eighty (80) fish were randomly selected for sampling and, twenty (20) fish were used per treatment: 10

fish were sampled for blood and immediately sacrificed with an overdose of the anaesthetic in the experiment for organ collection (brain, gill, liver); the other 10 fish were sampled for blood and set to recuperate in the 60L aquaria for 72h observations. After the 72h observation, the fish were transferred to systems C and D (in the appendix, Figure 3).

2.7.1 Blood Parameters

The blood parameters analysed consisted of counts of red blood cells (RBC) and white blood cells (WBC), haematocrit (Ht), and haemoglobin (Hb). Consequently, was possible to calculate the mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) according to Machado et al. (2015).

The anaesthetized fish was placed in a tray to collect the blood using a heparinized syringe (Henke Sass Wolf Norm-Ject Insulin U-100 1ml; ref. 4010-A00V0; Tuttlingen, Germany) and needle (Henke Sass Wolf Henk-Ject 0.5X16mm 25GX5/8’’; ref. 4710005016; Tuttlingen, Germany). The blood was collected in the caudal vein and then placed in a microtube with 20 μL of heparin at 3000 units/ml. To analyze the concentration of cells, present in the blood, dilutions of 1:200 were performed for RBC and 1:20 for WBC, with PBS and heparin at 30 units/mL. For the concrete count, the Neubauer chamber was filled with the sample and counted 5 squares.

Microhaematic tubes were used to quantify hematocrit. These were filled by capillarity to their limit and one of the ends was filled with plasticine to seal. Consequently, the tubes were centrifuged (Haematokrit 200, ref. 1801, Hettich; Tuttlingen, Germany) for 10 minutes at 10 000 rpm. The results were read through a graph reader.

Hemoglobin concentration was determined using Drabkin's colorimetric method (SPINREACT Kit, ref. 1001230S, Spain). In a microtube, 500 μL of Drabkin reagent was placed with 1 μL of the blood sample to analyze, kept in the dark until placement in the microplate of 96 wells. Subsequently, 100 μL of the sample were placed, in triplicate, in the microplate and the absorbance was read at 540nm in a microplate reader (EPOCH 2 Microplate Spectrophotometer, Biotek, United States of America). To perform the calculations, the supplier's instructions were followed.

The microtubes containing the blood samples were centrifuged (VWR Micro Star 12, VWR; Leuven, Belgium) at 10 000 g for 10 minutes (4ºC) to collect the plasma into a new microtube. It was stored in a -80 freezer for the forward analysis.

2.7.2 Oxidative Stress Parameters

Oxidative stress analyzes different parameters. Initially, 0.1 g of the liver was homogenized with 1000 µL of ultrapure water in 2 mL microtube, using a pellet mixer. Then, was transferred 200 µL of the homogenized to another 2 mL microtube with 4 µL of 2.6-Di-tert-butyl-4-methylphenol (BHT) at 4% in methanol for the analysis of lipid peroxidation (LPO). In the microtube where the homogenized remains, 800 µL of k-phosphate buffer (0.2 M, pH 7.4) was added, which was centrifuged (Centrifuge 5415R, Eppendorf; Germany) for 20 minutes at 11 500 rpm (at 4ºC). After centrifugation, two aliquots were created with supernatant for protein measurement, and analyses of catalase (CAT), glutathione S-transferase (GST), total glutathione (TG), and superoxide dismutase (SOD) (Passos et al., 2021).

Protein measurement of liver samples was performed through the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific; ref. 23225; United States of America). Ten µL of the sample was diluted 1:10, placed in duplicated in 96-well microplate, together with 200 µL of the reaction solution. With the microplate cap, it was taken to incubate at 37ºC for 30 minutes. After incubation, the absorbance of the samples was read in two wavelengths, 550 nm and 630 nm in a microplate reader (EPOCH 2 Microplate Spectrophotometer, Biotek, United States of America). By quantifying the protein, it is possible to determine the amount of sample needed to be used in the following oxidative stress analyses, and it is necessary to dilute them.

The analysis of lipid peroxidation (LPO) followed Bird & Draper's (1984) protocol. Briefly, 100 µL of trichloroacetic acid (TCA) were added directly to the samples (microtubes prepared at homogenization) and to the blanks (200 µL of ultrapure water and 4 µL of BHT) and were mixed in a vortex. Then, 1 ml of thiobarbituric acid (TBA, Tris-HCl 60 mM, diethyleneriaminopentatic acid 0.1 mM; pH 7.4) at 0.73% solution was added to all microtubes and were incubated for 1 hour at 100 °C. Then, centrifuge (Centrifuge 5415R, Eppendorf; Germany) at 11 500 rpm for 5 minutes (room temperature), removed 200 µL of

the supernatant from each sample in triplicate for a microplate of 96-wells. The absorbance of the samples was read at a wavelength of 535 nm in a microplate reader (EPOCH 2 Microplate Spectrophotometer, Biotek, United States).

Catalase (CAT) determination used by Clairborne (1985) requires the use of an appropriate microplate for UV light. The samples were diluted with the buffer used for homogenization (k-phosphate buffer 0.2 M, pH 7.4), to obtain a protein value of 0.7 mg/ml in 10 μL in the microplate. Determinations were made in triplicates. Added 140 μL of k-phosphate buffer (0.05 M, pH 7.0) and 150 μL of buffer solution (H_2O_2 at 30% and k-phosphate buffer 0.05 M at pH 7.0). Absorbance was read at 240 nm for 2 minutes in a microplate reader (EPOCH 2 Microplate Spectrophotometer, Biotek, United States).

Glutathione S-Transferase (GST) was determined on a 96-well microplate according to Frasco & Guilhermino (2002). Samples were diluted in k-phosphate buffer (0.2 M, pH 7.4) to obtain a protein value of 0.7 mg/mL. Then, 250 μL of the reaction solution was added, consisting of k-phosphate buffer (0.05 M, pH 6.5), L-Glutathione reduced (GSH), and CDNB (1-Chloro-2,4-dinitrobenzene). The absorbance of the samples was read at 340 nm every 20 seconds for 5 minutes in a microplate reader (EPOCH 2 Microplate Spectrophotometer, Biotek, United States).

For the determination of total glutathione (TG) it was used the protocol used by Baker et al. (1990). The samples were diluted with k-phosphate buffer (0.2 M, pH 7.4) in order to obtain a protein value of 0.7 mg/mL in 50 μL. For the blanks, samples were replaced by a k-phosphate buffer (0.2 M, pH 7.4). A standard line has been made with an L-glutathione reduced (GSH). Then, 250 μL of the reaction solution was added, consisting of k-phosphate buffer (0.2 M, pH 8.0), NADPH (β-Nicotanimide adenine dinucleotide 2'-phosphate reduced tetrasodium salt), DTNB (5-5'- dithiobis (2-nitrobenzoic acid)) and Glutathione reductase (GR, 168 U mg/protein). Absorbance was measured at 340 nm for 3 minutes in a microplate reader (EPOCH 2 Microplate Spectrophotometer, Biotek, United States).

For the determination of superoxide dismutase (SOD) it was used the protocol described by Almeida et al. (2010). The samples were diluted in k-phosphate buffer (0.2 M, pH 7.4) to obtain a protein value of 0.3 mg/mL. The standard curve was made by consecutive dilutions of k-phosphate buffer (0.05M, pH 7.8 with 0.01M of Na-EDTA) with superoxide dismutase from bovine erythrocytes (300 U/mL of protein). The analysis was made in

triplicates with 50 μL of the samples and the standard curve. It was added 200 μL of the reaction solution to the microplate, to which it was made of Xanthine at 0.7 mM in NaOH, Cytochrome C at 0.03 mM in k-phosphate buffer (0.05 M, pH 7.8 with 0.01M of Na-EDTA). Shortly thereafter, Xanthine Oxidase at 0.03 U/ml in Na-EDTA (0.1 mM) was added. The absorbance of the samples was read at a wavelength of 550 nm every 20 seconds for 3 minutes in a microplate reader (EPOCH 2 Microplate Spectrophotometer, Biotek, United States).

2.7.3 Immune Parameters

The immune response parameters studied were: peroxidase, proteases and anti-proteases. Plasma samples were used for these parameters.

To analyze the activity of peroxidase, the procedure described by Quade & Roth (1997) was followed. Using 96-well microplates, 15 μL of plasma were diluted 1:10 and added 135 μL of PBS. Determinations were made in triplicates and 150 μL of PBS served as blanks. Then, 50 μL of 3.3′,5.5′-tetremethylbenzidine hydrochloride (TMB; 10mM) and 50 μL of H_2O_2 (5 mM) were added. After 2 minutes the reaction was stopped with 50 μL of H_2SO_4 (2 M). Absorbance was measured in a plate reader at 450 nm in a microplate reader (EPOCH 2 Microplate Spectrophotometer, Biotek, United States).

The protocol of Machado et al. (2015) was followed to analyze the anti-proteases and consequently the proteases. This analysis was made in duplicates. For anti-proteases, 10 μL of plasma was placed incubating with the same volume of trypsin solution (with $NaHCO_3$) in microtubes for 10 minutes at 22ºC. Then, another 100 μL of PBS and 125 μL of azocasein were added and taken back to incubate for 1 hour at 22ºC. After incubation, 250 μL of TCA was added and mixed, and taken after incubation for another 30 minutes at 22ºC. Finally, the samples were centrifuged (Centrifuge 5415R, Eppendorf; Germany) at 10 000 g for 5 minutes. It was transferred 100 μL of the supernatant to the microplate and added 100 μL of NaOH per well and read at 450 nm in a microplate reader (EPOCH 2 Microplate Spectrophotometer, Biotek, United States). A blank (PBS replacing plasma and trypsin) and a reference sample (PBS replacing plasma) were made. The percentage of inhibited trypsin activity is calculated using the reference sample.

To analyze the protease was placed 10 μL of plasma, 100 μL of PBS and 125 μL of 2% azocasein in sodium bicarbonate (100mM) in microtubes. Two controls were performed: positive control (100 μL of PBS with 10 μL trypsin; 100% protease activity) and negative control (110 μL of PBS; 0% protease activity). The controls and samples were incubated for 24 hours at room temperature with agitation. After incubation, 250 μL of 10% TCA was added, mixed and incubated for 30 minutes. Finally, the microtubes were centrifuged (Centrifuge 5415R, Eppendorf; Germany) at 10 000 g for 5 minutes. It was placed 100 μL of the supernatant in the microplate with 100 μL of NaOH and read at 450 nm in a microplate reader (EPOCH 2 Microplate Spectrophotometer, Biotek, United States). The percentage of trypsin activity is calculated using the reference sample.

2.7.4 Metabolic Parameters

To measure plasmatic metabolites, plasma samples were prepared in aliquots used for glucose (SPINREACT Kit, ref. 1001190, Spain), lactate (SPINREACT Kit, ref. 1001330, Spain), and alkaline phosphatase (ALP, SPINREACT Kit, ref. 41246, Spain).

Liver metabolites were measured in homogenized samples following the procedure described by Guerreiro et al. (2015). In brief, using 1:10 dilution (w/v) of the liver with ice-cold buffer (30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.25 mM saccharose, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 5 mM K_2HPO_4, 1mM dithiothreitol (DTT), pH 7.4), it was obtained a homogenization using a portable homogenizer (Peller Mixer, ref. 431-0100, VWR; Radnor, United States of America). After centrifugation for 10 minutes at 1 000 g (4ºC), supernatants were sonicated for 1 minute (pulse 1 second, amplitude 50), and centrifuged (Centrifuge 5415R, Eppendorf; Germany) again for 20 minutes at 15 000 g (4ºC). With the final supernatant was made aliquots to test alanine aminotransferase (ALT, SPINREACT Kit, ref. 41282, Spain), and aspartate aminotransferase (AST, SPINREACT Kit, ref. 41272, Spain).

Each parameter was measured following the manufacturer's kit instructions but adjusted the volumes for a microplate. All parameters were made in triplicates and read in a microplate reader (EPOCH 2 Microplate Spectrophotometer, Biotek, United States).

2.7.5 Histological Analysis

After sampling the gills, they were fixed in formaldehyde 4% for 48 hours. At the end of this time, they were passed on to ethanol 70%, where they were kept until processing.

The samples were transferred to histological cassettes duly identified to be placed in the tissue processor (Leica TP1020; Germany). In the processor, the samples were dehydrated by passing through various ethanol solutions (80, 96 and 100%), allowing gradual dehydration of tissues while maintaining tissue integrity. It was followed by successive immersions in xylene (ref. 28973.328, VWR Chemicals, France), allowing the ethanol present in the tissues to be replaced by it, so that in the following submersion with liquid paraffin (Histosec pastilles; ref. 1.11609.2504, Merk KGaA, France) is facilitated its impregnation. At the end of the processing, using a paraffin dispenser (Leica EG1120; Germany), liquid paraffin blocks were created where the samples were placed and then covered the block with the cassette. At the end of the assembly, the blocks were placed to cool down on a cooling plate (CP-4; Kunz Instruments; Sweden), to solidify.

In the microtome (ACCU-CUT SRM200, Sakura Finetek Europe; The Netherlands), the tissue was cut in the solid paraffin blocks. The desired cut was made in a thickness of 5 µm, passed to a 53ºC water bath. They were then placed on slides and dried in a heating chamber at 37ºC for at least 24 hours. The slides were stained using hematoxylin (Haematoxylin Harris; ref. 351945S, VWR Chemicals; France) and eosin (Eosin Y; ref. 341973R, VWR Chemicals; France) staining method. The slides initially went through xylene for deparaffination and were then hydrated with an ethanol gradient (100, 96, 70 and 50%). They were first colored with hematoxylin, and then to remove excess dye were passed by running water, acidic ethanol, and running water again and hydrated with ethanol (96%). The second dye was eosin, and they were taken again to wash with running water. Finally, the slides went through several ethanol solutions (96 and 100%) for dehydrates and were clarified with xylene. At the end of the xylene process, the definitive preparation of the tissues was carried out. On the slides, drops were placed with a mounting-base (DPX, ref. 1.00579.0500, Merk KGaA, Germany) and a lamella over the tissue. In the end, they were left to dry for 48 hours so that they could be observed.

The slides were observed under a microscope (Leica DM2000 Led; Germany) and photographed (Leica MC170 HD; Germany), with the program Leica Application Suite

(Version 4.4.0, Build 454; Switzerland). The gills were observed with the purpose to identify possible different structural and morphologic alterations (Table III). Posteriorly, classify the alterations with a histoscore adapted from Mitchell et al. (2012). Ten (10) histological slides were validated and classified with a score from 1 to 4 (1, 0-25%; 2, 25-50%; 3, 50-75%; 4, 75-100%). The score is given per observed slide after analyzing the tissue. Each score number characterizes the structure and morphology modifications: normal morphology is associated with a score of "1", mild morphology damage "2", moderate gill damage "3", while severe tissue damage is represented by "4".

Table III – Characterization of structural and morphologic alterations observed in gills from gilthead seabream *Sparus aurata* exposed to different anaesthetics, with and without analgesia (Adapted according to Mitchell et al. (2012), Ortiz-Delgado et al. (2007), Rodrigues et al. (2019)).

Circulatory	**Progressive**	**Regressive**	**Inflammatory**
Blood congestion	Lamellar hyperplasia	Epithelium lifting	Leukocyte infiltration (lymphocytes and mast cells)
Haemorrhage / Aneurism (Telangiectasis)	Hypertrophy from chloride cells	Epithelium rupture	
	Hyperplasia from chloride cells	Lamellar fusion	
		Rupture from pillar cells	
		Necrosis	

2.7.6 Gene Expression Analysis

The organs sampled were the brain and gills of six (6) fish. For sampling, sterile Eppendorf microtubes were prepared with 1 mL of RNAlater, where the collected organs were placed. After organ collection, storage gradually increased from 4ºC for 24 h to -20ºC for 24h (so that there is no thermal shock and degradation of the reagent) and then -80ºC, until its use.

Tissue RNA extraction and purification were performed with the use of a NZY Total RNA Isolation kit (NZYTech, Portugal). It was weighed between 30 to 50 mg of the organ for homogenization. For homogenization, 500 μL of NZYol (NZYTech) was added, and the samples were placed in the precellys (Precellys Evolution, Bertin Instruments, France) at 6 000 rpm for two cycles of 20 seconds. Chloroform is then added and centrifuged (Centrifuge 5415R, Eppendorf; Germany) for 15 minutes at 4ºC at 12 000 rpm. From this step, the protocol provided by the supplier was followed.

RNA quantification is observed in the Nanodrop 2000 spectrophotometer (Thermo Scientific, United States), where sample purity can be observed when the ratio is 1.9 to 2.4 in absorbance 260/280 and from 2.0 to 2.3 in absorbance of 260/230. The verification of the quality and integrity of the samples is done by performing a 2% agarose gel (1g pure agarose in 50 mL of 1x TAE, and 500 μL bleach, for an 8-sample gel) (Aranda et al., 2012), by adding 0.6 μL GreenSafe Premium (NZYTech, Portugal) and for each sample was added NZYTech DNA loading dye. The marker used was the NZYTech DNA Ladder V and was programmed 100V for 50 minutes in the Horizontal Electrophoresis System (Mini-sub cell GT, Bio-Rad, United States) connected to the Electrophoresis Power Supply. To verify the integrity, the gel was observed in the Gel Doc EZ Imager (Bio-Rad, United States) (in the appendix, Figures 4 and 5).

Depending on the RNA concentration obtained in Nanodrop (in the appendix, Table V and VI), the samples are diluted. Dilution is made based on the lowest concentration for each organ (brain 104.5 ng/μL, and gills 978.8 ng/μL). Thus, the cDNA was standardized. The cDNA synthesis is performed with the NZY First-Strand cDNA Synthesis kit for a total volume of 40 μL, was followed the instructions from the supplier. The thermocycler (T100 Thermal Cycler, Bio-Rad, United States), according to the supplier was programmed as follows in table IV.

Table IV – Thermocycler conditions for cDNA synthesis according to the supplier (Constant temperature on thermocycler lid at 105ºC).

Steps	Temperature (ºC)	Time (min)
Initial incubation	25	10 min
Incubation	50	30 min
Inactivation reaction	85	5 min
	4	Infinite time
Final incubation	37	20 min

The efficiency of primers of reference (*actb*), indicators of stress (*hsp70, crh, crhbp, trh*), immune response (*il1β*), neuro-muscular alterations (*ache*) and enzymatic response (*gst3*) has been tested (in appendix, Table IV), through a pool (2 μL) of cDNA of each of the brain and gill samples. A calibrator 0 is created from where successive dilutions are made. A Mix is prepared with 3.4 μL RNase-free water, 0.3 μL reverse do primer, 0.3 μL forward do primer, 5 μL SYBR (iTaq^{TM} Universal SYBR Green Supermix, Bio-Rad), e 1 μL cDNA from each dilution. After the preparation of the mix, the steps in table V were programmed in the CFX Connect Real-Time System (Bio-Rad, United States). Depending on the primer used, the annealing temperature is modified. The primers were considered as efficient if their slope of the standard curve presented acceptable values of -2.7 to -4, ideally being of -3.2 to -3.7; or have values of 90 to 120% efficiency.

For sample testing, the same procedure is used as in the efficiency of primers, using cDNA from each of the samples. This step is also carried out in the CFX Connect Real-Time System (Bio-Rad, United States). The calculations for the testing of samples were made according to the method of Pfaffl (2001), using the reference gene to uniformize the samples.

Table V – RT-qPCR protocol conditions. (*Annealing temperature variates with primer)

Steps	Temperature (ºC)	Time (min/seg)
A)	95 (initial denaturation)	3 min
B) 40 cycles of 2 stages	95	30 seg
	Annealing temperature*	20 seg
	72 (extension)	

2.8 Statistical Analyses

Statistical analysis was performed using the IBM SPSS program for Windows, version 28 (IBM Corporation, Armonk, New York, United States of America). The results were expressed as a mean ± standard deviation (SD), and the differences between experimental groups were considered statistically significant at the significance level p-value < 0,05. Data had an analytical procedure for significant differences using a one-way ANOVA, with each anaesthetic and analgesic as factors, followed by multiple comparison using Tukey's test, when normality and homogeneity of variance were met. In case of rejection of homogeneity, a Kruskal-Wallis test was performed. For experiment 1, times of induction and recovery are expressed as the means of eight fish per treatment ± SD (n = 8). As in experiment 2, times of induction and recovery are expressed as the means of four fish per treatment ± SD (n = 4). With experiment 3, times of induction and recovery, haematological parameters, oxidative stress, immune parameters, metabolic parameters, and histology are expressed as the means of ten fish per treatment ± SD (n = 10). Gene expression is expressed as the means of six fish per treatment ± SD (n = 6).

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3. Results

3.1 Experiment 1 - Anaesthesia refinement

Gilthead seabream used for testing different concentrations of 2PHE had 49.12 ± 12.52 grams, and the fish used for CO had 48.35 ± 11.59 grams. There was no mortality. The water parameters from maintenance tank were: temperature 23.00 ± 0.42 ºC, salinity 33.87 ± 0.31, pH 7.51 ± 0.31, and O_2 87.89 ± 1.24 %.

It was possible to observe different times of induction and recovery in 2-phenoxyethanol at different concentrations (Figure 2 and in the appendix, Table I). As in full induction time, there were statistical differences (Kruskal-Wallis; p-value<0.05) between 0.2 and 0.4 mL/L when compared with 0.6 mL/L. 2PHE 0.2 mL/L and 2PHE 0.4 mL/L overpassed the maximum induction time with 345.84 ± 39.16 and 295.25 ± 131.90 seconds in total. And reached the maximum time possible in the full induction study (180 ± 0 seconds), meaning fish were not anaesthetised. 2PHE 0.6 mL/L managed to reach induction, taking 106.89 ± 7.34 seconds. The anaesthetic 2-phenoxyethanol had statistical differences (Tukey HSD; p-value<0.05) in the recovery time. For the lowest concentration (2PHE 0.2 mL/L) 137.09 ± 27.29 seconds were needed to observe full recovered fish. This recovery time was not considered since fish were not anaesthetized. It took 2PHE 0.4 mL/L and 2PHE 0.6 mL/L, 210.63 ± 31.20 and 222.54 ± 56.66, respectively to complete recovery.

With different concentrations of clove oil-eugenol was possible to distinguish times of induction and recovery (Figure 2 and in the appendix, Table I). In the induction times, all concentrations were statistically different (Kruskal-Wallis; p-value<0.05). CO 30 mg/L (352.57 ± 57.63 seconds) took the longest time to induce anaesthesia, CO 45 mg/L (191.23 ± 25.86 seconds) took an average time and CO 60 mg/L (123.47 ± 18.37 seconds) the shortest. The recovery times for clove oil-eugenol didn’t demonstrate statistical differences. CO 30 mg/L took longer to recuperate (481.99 ± 100.92 seconds) in comparison with CO 45 mg/L (437.49 ± 87.50 seconds) and CO 60 mg/L (412.56 ± 41.85 seconds) (Tukey HSD; p-value>0.05).

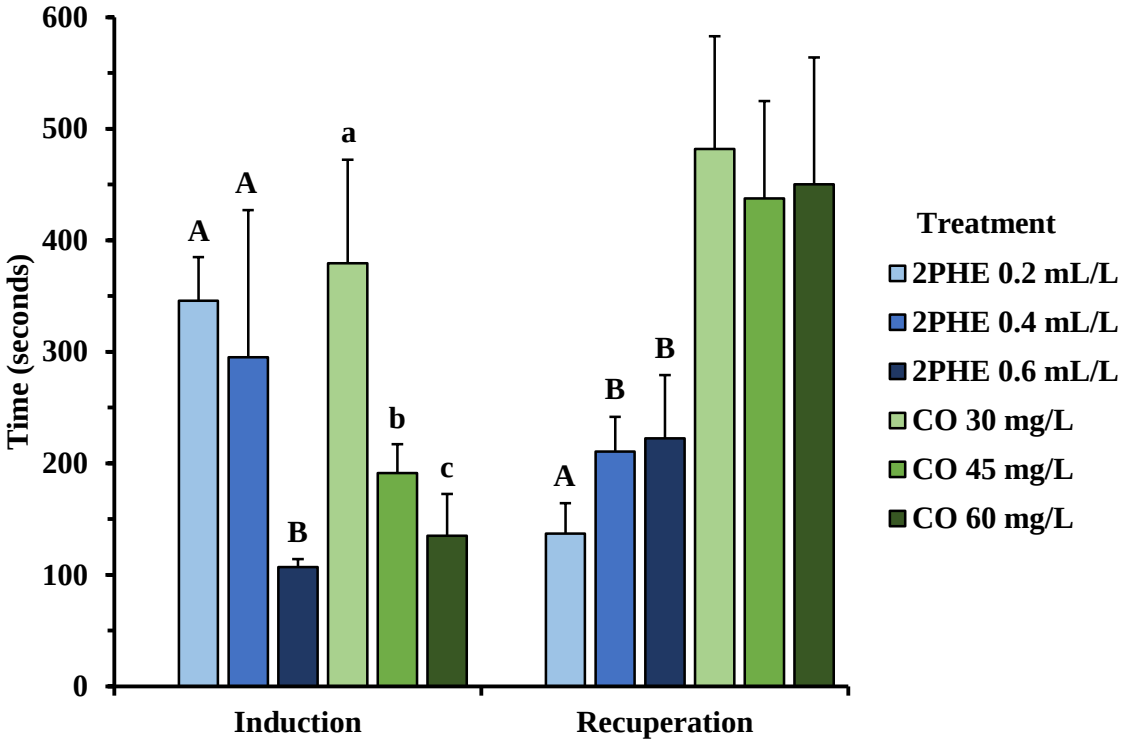

Figure 2 - Time of anaesthesia induction (stage A5) and recovery (stage R5) from 2-phenoxyethanol (2PHE) and clove oil-eugenol (CO) at different concentrations in experiment 1 (Anaesthesia refinement). Values expressed as means ± SD (n = 8), statistically significant differences identified with different upper (2PHE) or lowercase (CO) letters among times (p-value<0.05).

Based on the results obtained and attending to times of induction and recovery, the concentrations to be used in the further experiments were 2PHE 0.4 mL/L and CO 45 mg/L.

3.2 Experiment 2 - Analgesia refinement

Gilthead seabream used for testing different concentrations of 2PHE + L had 48.48 ± 6.57 grams, and the fish used for CO + L had 52.71 ± 14.36 grams. There was no mortality. The water parameters from tank maintenance were: temperature 18.91 ± 1.00 ºC, salinity 33.97 ± 0.58, pH 7.58 ± 0.43, and O_2 86.42 ± 2.39 %.

After choosing the most proper concentration of 2-phenoxyethanol (2PHE 0.4 mL/L), it was tested the addition of different concentrations of lidocaine and comparing their induction and recovery time (Figure 3 and in the appendix, Table II). It was observed that

among all exposed concentrations of analgesia, there was a euphoric behaviour on the part of the animals during exposure. The higher the concentration, more euphoric the fish were.

There were no statistical differences (Tukey HSD; p-value>0.05) for the induction times. The higher the lidocaine concentration, the longer it took to reach full anaesthesia (2PHE + L 2.5 mg/L 144.02 ± 68.26 seconds, 2PHE + L 5 mg/L 182.82 ± 70.10 seconds and 2PHE + L 7.5 mg/L 243.88 ± 121.04 seconds). Analysing the full recovery times, there were statistical differences (Kruskal-Wallis; p-value<0.05). 2PHE + L 2.5 mg/L (152.40 ± 32.12 seconds) recuperated faster than 2PHE + L 5 mg/L (248.88 ± 31.06 seconds) and between these two concentrations a statistically differences were observed. However, statistically differences were observed between these times and the recovery times observed with 2PHE + L 7.5 mg/L (232.43 ± 71.23 seconds).

After choosing the most proper concentration of clove oil-eugenol (CO 45 mg/L), it was tested the addition of different concentrations of lidocaine and compared their induction and recovery time (Figure 3 and in appendix, Table II). In the induction times, there were no statistical differences (Tukey HSD; p-value>0.05). The lowest concentration took less time to induce anaesthesia (CO + L 2.5 mg/L 129.00 ± 22.83 seconds), the other concentrations had similar inductions times (CO + L 5 mg/L 146.08 ± 13.40 seconds; CO + L 7.5 mg/L 147.10 ± 18.61 seconds). The recovery times for clove oil-eugenol with lidocaine didn’t demonstrate statistical differences (Tukey HSD; p-value>0.05). The treatment CO + L 2.5 mg/L took the longest time to reach full recovery (394.28 ± 43.93 seconds), CO + L 5 mg/L took the average time (379.08 ± 56.56 seconds), and CO + L 7.5 mg/L took the lower time (334.28 ± 41.24 seconds).

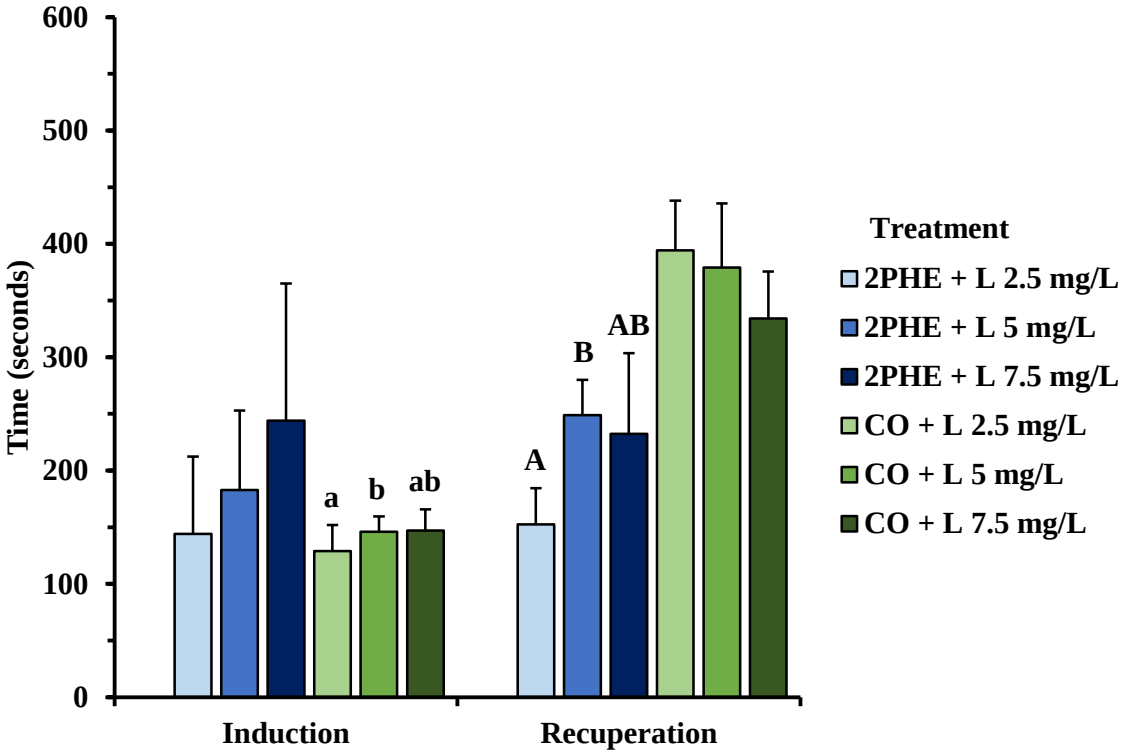

Figure 3 – Time of anaesthesia induction (stage A5) and recovery (stage R5) from 2-phenoxyethanol (2PHE) and clove oil-eugenol (CO), using different concentrations of lidocaine (L) in experiment 2 (Analgesia refinement). Values expressed as means ± SD (n = 4), statistically significant differences identified with different upper (2PHE + L) or lowercase (CO + L) letters among times (p-value<0.05).

The concentration to be used in the further experiment is 2.5 mg/L of lidocaine, attending its times of induction and recovery from both anaesthetics.

3.3 Experiment 3 – Physiological responses to anaesthesia and analgesia exposure

Gilthead seabream used for 2PHE had 58.17 ± 15.71 g, and the fish used for 2PHE + L had 68.05 ± 13.97 grams. The fish from CO had 61.98 ± 15.80 g and from CO + L 61.24 ± 13.63 g. There was no mortality. The water parameters from the maintenance system were: temperature 17.58 ± 0.67 ºC, salinity 33.20 ± 1.39, pH 8.02 ± 0.12, and O_2 87.60 ± 1.74 %.

The concentrations used for 2-phenoxyethanol, clove-oil and lidocaine were the ones determined in the previous sub-sections (2PHE 0.4 mL/L; CO 45 mg/L; L 2.5 mg/L). In general, from induction, there were some statistical differences (Kruskal-Wallis; p-

value<0.05). Animals from 2PHE (193.90 ± 74.94 seconds) showed a statistically longer time for induction of anaesthesia compared with 2PHE + L (130.65 ± 40.16 seconds) and CO (121.30 ± 16.30 seconds). All of these are statistically the same compared with CO + L (130.91 ± 25.02 seconds). Regarding anaesthesia recovery times, there were no statistical differences (Kruskal-Wallis; p-value>0.05) between groups. However, anaesthetics without analgesic took longer time to recuperate (2PHE 301.50 ± 86.99 seconds and CO 349.90 ± 123.69 seconds) than anaesthetics with analgesic (242.35 ± 46.41 and 250.11 ± 51.99 seconds, for 2PHE + L and CO + L, respectively) (Figure 4 and in the appendix, Table III).

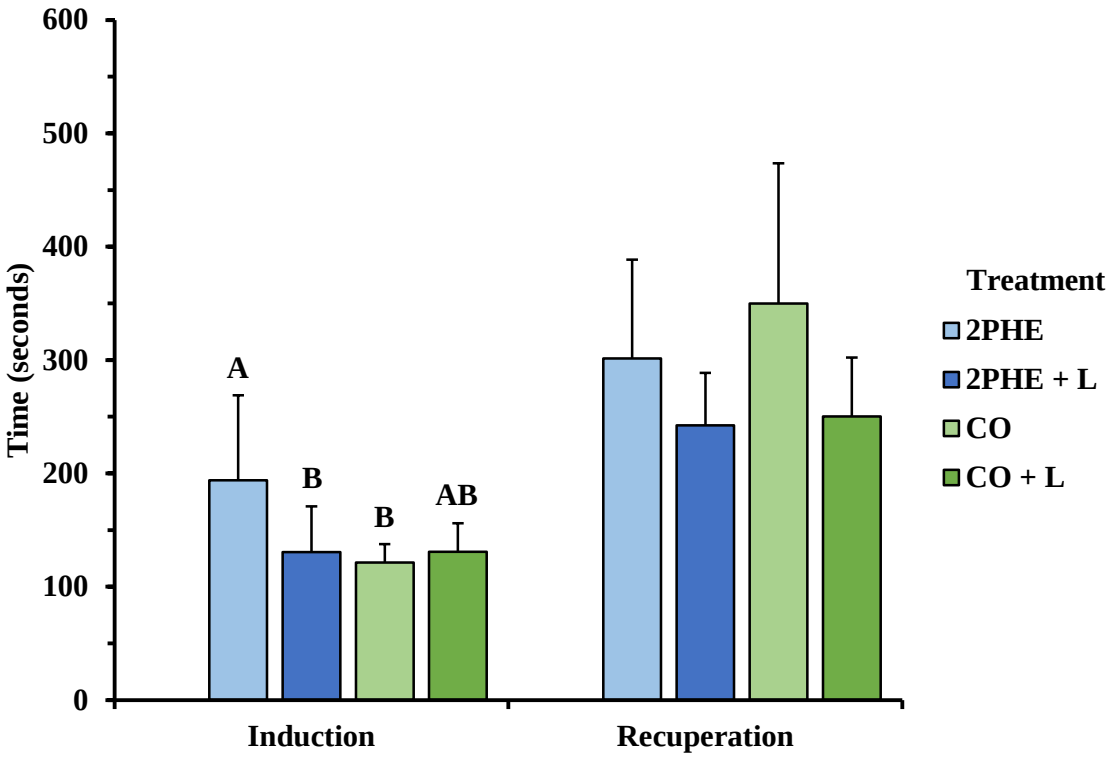

Figure 4 – Time of induction (stage A5) and recovery (stage R5) from 2-phenoxyethanol (2PHE 0.4 mL/L) and clove oil-eugenol (CO 45 mg/L), with and without lidocaine (L 2.5 mg/L) in experiment 3 (Physiological responses to anaesthesia and analgesia exposure). Values expressed as means ± SD (n = 10), statistically significant differences identified with different letters among times (p-value<0.05).

After recovery, the fish kept for observation for 72H in the 60L aquaria and had no mortality in any treatment group. The water parameters for the 2-phenoxyethanol and clove oil-eugenol groups were: temperature 16.85 ± 0.21 ºC, salinity 34.37 ± 0.27, pH 8.31 ± 0.05, and O_2 87.22 ± 0.33 %.

3.3.1 Blood Parameters

Table VI shows the mean values and standard deviation from the haematologic parameters to evaluate physiological responses to anaesthesia and analgesia exposure. There were no statistical differences (Tukey HSD; p-value>0.05), except in the mean corpuscular volume (Tukey HSD; p-value<0.05). In the MCV, 2PHE+L showed a statistically lower volume compared with CO.

Table VI – Haematological profile of gilthead seabream *Sparus aurata* exposed to different anaesthetics, 2-phenoxyethanol (2PHE 0.4 mL/L) and clove oil-eugenol (CO 45 mg/L) and combination with an analgesic, lidocaine (L 2.5 mg/L) for each treatment. Values expressed as means ± SD (n = 10), statistically significant differences are identified with different letters (p-value<0.05).

	2PHE	**2PHE + L**	**CO**	**CO + L**
RBC ($\times 10^6$ μL^{-1})	3.29 ± 0.95	3.21 ± 0.76	2.92 ± 0.30	3.28 ± 0.64
WBC ($\times 10^4$ μL^{-1})	6.27 ± 2.01	5.54 ± 1.86	5.75 ± 1.70	5.08 ± 1.01
Ht (%)	28.33 ± 3.57	28.56 ± 2.40	32.10 ± 3.76	30.44 ± 5.05
Hb (g dL^{-1})	2.36 ± 0.50	2.57 ± 0.63	2.58 ± 0.73	2.74 ± 0.69
MCV (μm^3)	92.71 ± 22.40^{AB}	84.93 ± 13.75^{A}	117.28 ± 23.22^{B}	96.48 ± 24.70^{AB}
MCH (g 100 ml^{-1})	8.03 ± 2.50	7.93 ± 1.70	9.18 ± 2.00	8.64 ± 2.70
MCHC (pg $cell^{-1}$)	8.42 ± 1.35	8.68 ± 3.60	8.10 ± 2.26	7.66 ± 0.73

3.3.2 Oxidative Stress Parameters

The lipid peroxidation (Figure 5A) had no statistical differences (Tukey HSD; p-value>0.05). 2PHE (24.55 ± 6.34 nmol/g wt) and CO + L (27.84 ± 12.95 nmol/g wt) had the lowest values, and 2PHE + L (35.17 ± 7.48 nmol/g wt) and CO (30.95 ± 11.31 nmol/g wt) had the highest values.

The catalase activity (Figure 5B) presented statistical differences (Tukey HSD; p-value<0.05). 2PHE (44.80 ± 10.27 U mg $prot^{-1}$) and 2PHE + L (50.09 ± 7.68 U mg $prot^{-1}$) are statistically the same. But both are higher and different from CO (29.43 ± 7.93 U mg $prot^{-1}$) and CO + L (29.40 ± 9.00 U mg $prot^{-1}$), which are statistically the same.

The glutathione s-transferase (Figure 5C) didn't present statistical differences (Tukey HSD; p-value>0.05). 2PHE (1793.09 ± 400.40 mU mg^{-1}) had a similar value as CO + L (1702.62 ± 263.30 mU mg^{-1}). 2PHE + L (1811.70 ± 97.04 mU mg^{-1}) and CO (2040.93 ± 182.80 mU mg^{-1}) presented the highest values.

The total glutathione (Figure 5D) showed statistical differences (Tukey HSD; p-value<0.05). 2PHE + L (141.30 ± 48.16 nmol mg $prot^{-1}$) is statistically different (lower) from CO (208.60 ± 28.00 nmol mg $prot^{-1}$) and CO + L (219.22 ± 27.32 nmol mg $prot^{-1}$). But all these are statistically the same as 2PHE (177.05 ± 58.18 nmol mg $prot^{-1}$).

The superoxide dismutase (Figure 5E) showed statistical differences (Kruskal-Wallis; p-value<0.05). 2PHE + L (73.41 ± 23.04 U mg $prot^{-1}$) is statistically higher compared with the other treatment groups (2PHE 43.70 ± 9.78 U mg $prot^{-1}$, CO 44.14 ± 14.45 U mg $prot^{-1}$, CO + L 43.75 ± 9.70 U mg $prot^{-1}$).

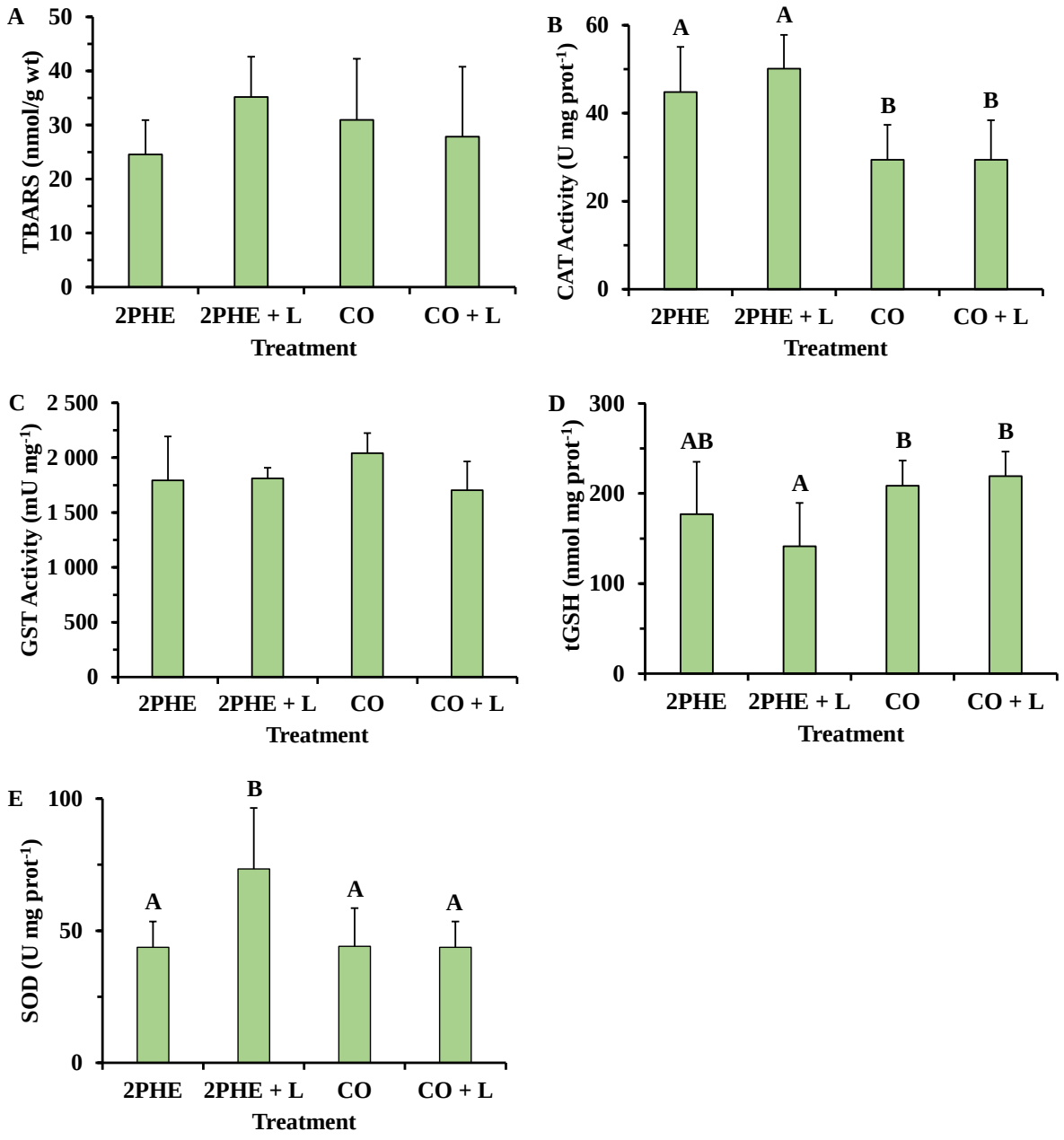

Figure 5 – Oxidative stress parameters in gilthead seabream *Sparus aurata* liver exposed to different anaesthetics, 2-phenoxyethanol (2PHE 0.4 mL/L) and clove oil-eugenol (CO 45 mg/L) and combination with an analgesic, lidocaine (L 2.5 mg/L), for each treatment (mean ± SD, n = 10). A) Lipid peroxidation; B) Catalase activity; C) Glutathione s-transferase; D) Total glutathione; E) Superoxide dismutase. Statistically significant differences are identified with different letters (p-value<0.05).

3.3.3 Immune Parameters

The peroxidase activity (Figure 6A) didn't present statistical differences between treatment groups (Kruskal-Wallis; p-value>0.05). 2PHE has the lowest value (1.41 ± 0.44 OD) and the highest value was from CO (1.87 ± 0.79 OD); 2PHE + L and CO + L have values of 1.54 ± 0.46 and 1.48 ± 0.30 OD, respectively.

The antiprotease activity (Figure 6B) didn't present statistical differences (Tukey HSD; p-value>0.05). All treatment groups had similar values: 2PHE 73.83 ± 6.68 %; 2PHE + L 76.57 ± 1.58 %; CO 78.45 ± 4.59 %; CO + L 75.16 ± 5.46 %.

The protease activity (Figure 6C) demonstrated statistical differences (Tukey HSD; p-value<0.05). CO (8.56 ± 0.46 %) showed the lowest value with statistically differences from 2PHE (9.48 ± 0.623 %) and 2PHE + L (9.99 ± 0.72 %). 2PHE + L has the highest value and is statistically different from CO + L (9.15 ± 0.78 %).

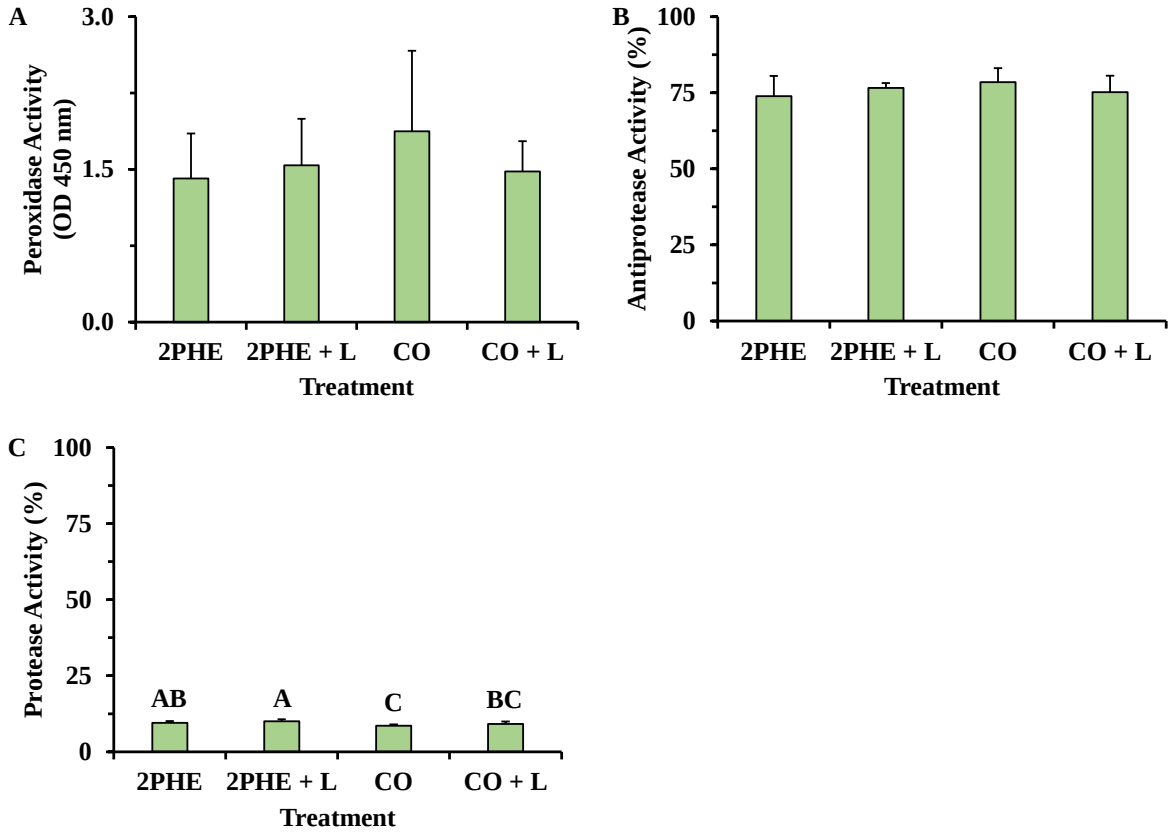

Figure 6 – Immune biomarkers in gilthead seabream *Sparus aurata* plasma exposed to different anaesthetics, 2-phenoxyethanol (2PHE 0.4 mL/L) and clove oil-eugenol (CO 45 mg/L) and combination with an analgesic, lidocaine (L 2.5 mg/L), for each treatment (mean ± SD, n = 10). A)

Peroxidase activity; B) Antiprotease activity; C) Protease Activity. Statistically significant differences are identified with different letters (p-value<0.05).

3.3.4 Metabolic Parameters

Glucose (Figure 7A) didn't present any statistical differences (Kruskal-Wallis; p-value>0.05) between treatment groups. 2PHE (134.51 ± 52.24 mg dL^{-1}) and CO + L (138.72 ± 68.92 mg dL^{-1}) had similar values. 2PHE + L (161.03 ± 63.20 mg dL^{-1}) and CO (194.33 ± 19.45 mg dL^{-1}) had the highest values.

Lactate (Figure 7B) demonstrated statistical differences (Tukey HSD; p-value<0.05). The use of lidocaine demonstrated to induce lower lactate (2PHE + L 68.76 ± 28.42 mg dL^{-1} and CO + L 50.74 ± 45.01 mg dL^{-1}). But are statistically the same as 2PHE (100.81 ± 46.26 mg dL^{-1}). CO (153.34 ± 61.44 mg dL^{-1}) had the highest lactate but statistically the same has 2PHE.

Alkaline phosphatase (ALP; Figure 7C) didn't show statistical differences (Kruskal-Wallis; p-value>0.05). All treatment groups had similar values (2PHE 41.95 ± 10.26 U L^{-1}; 2PHE + L 42.69 ± 13.02 U L^{-1}; CO 43.09 ± 12.29 U L^{-1}; CO + L 41.68 ± 1.35 U L^{-1}).

Alanine aminotransferase (ALT; Figure 7D) presents statistical differences (Kruskal-Wallis; p-value<0.05). 2PHE (3.85 ± 1.67 U L^{-1}) and CO (4.61 ± 3.12 U L^{-1}) had the lowest values, and they are statistically different from the treatments which used lidocaine. 2PHE + L (37.43 ± 23.34 U L^{-1}) and CO + L (74.32 ± 34.49 U L^{-1}) had the highest values.

Aspartate aminotransferase (AST; Figure 7E) demonstrated statistical differences (Kruskal-Wallis; p-value<0.05). CO (6.00 ± 3.71 U L^{-1}) had the lowest value but is statistically the same as 2PHE (19.99 ± 9.31 U L^{-1}). CO + L (162.44 ± 104.27 U L^{-1}) has the highest value and is statistically the same as 2PHE + L (135.56 ± 71.29 U L^{-1}). 2PHE and 2PHE + L are statistically the same.

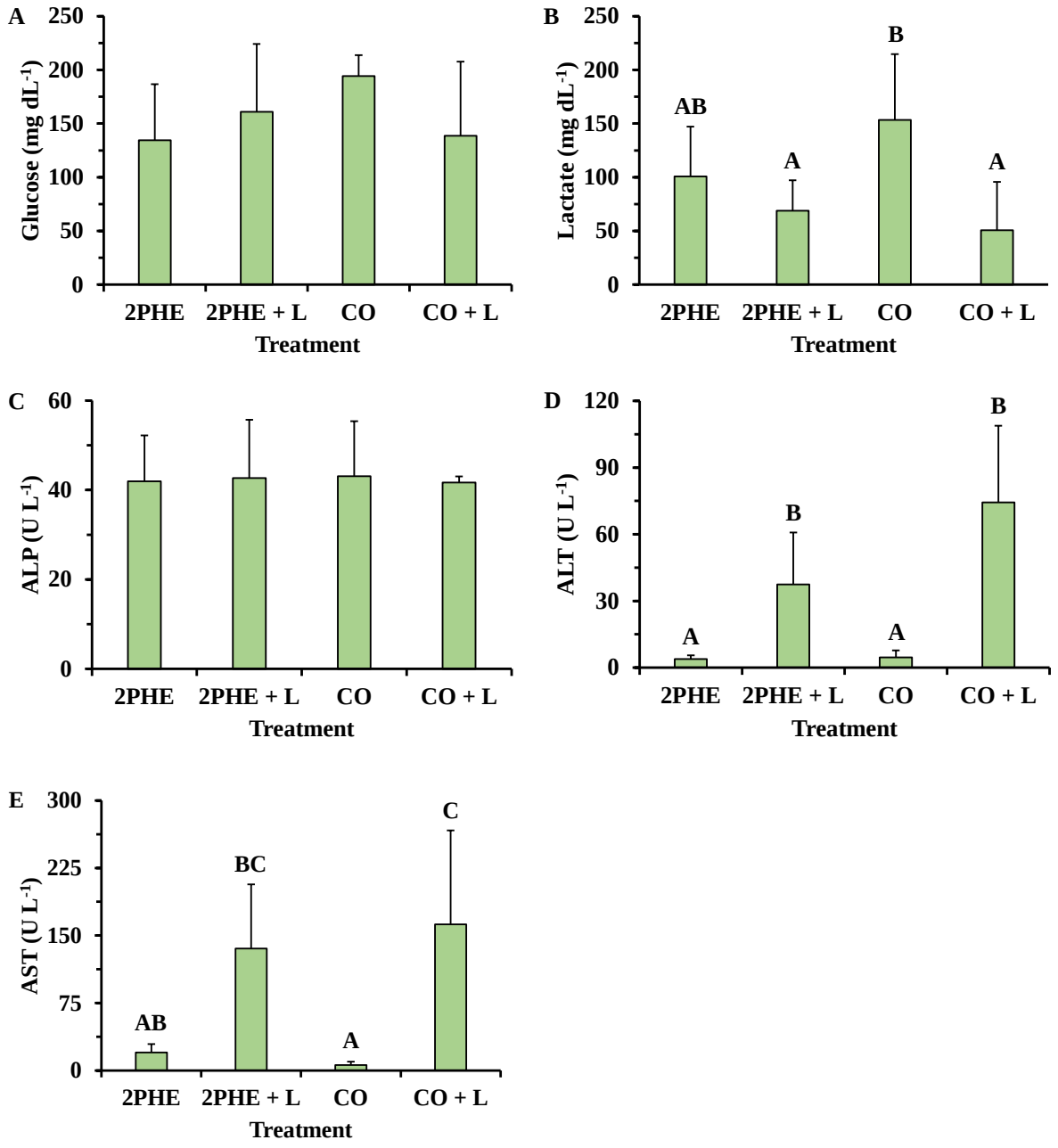

Figure 7 – Metabolic biomarkers in gilthead seabream *Sparus aurata* plasma and liver exposed to different anaesthetics, 2-phenoxyethanol (2PHE 0.4 mL/L) and clove oil-eugenol (CO 45 mg/L) and combination with an analgesic, lidocaine (L 2.5 mg/L), for each treatment (mean ± SD, n = 10). A) Glucose; B) Lactate; C) Alkaline phosphatase; D) Alanine aminotransferase; E) Aspartate aminotransferase. Statistically significant differences are identified with different letters (p-value<0.05).

3.3.5 Histological Analysis

The structural and morphologic alterations observed in histological slides from gilthead seabream gills exposed to different anaesthetics, with and without analgesic, was similar to all treatment groups (Figure 8 to 11). Exposure to anaesthetics and analgesic didn't present circulatory (blood congestion - Tukey HSD; p-value>0.05); haemorrhage/ aneurism (telangiectasis - Kruskal-Wallis; p-value>0.05) and inflammatory (leukocyte infiltration, lymphocytes and mast cells - Kruskal-Wallis; p-value>0.05) damage (Figure 8), being characterized by scores between 1 to 2.

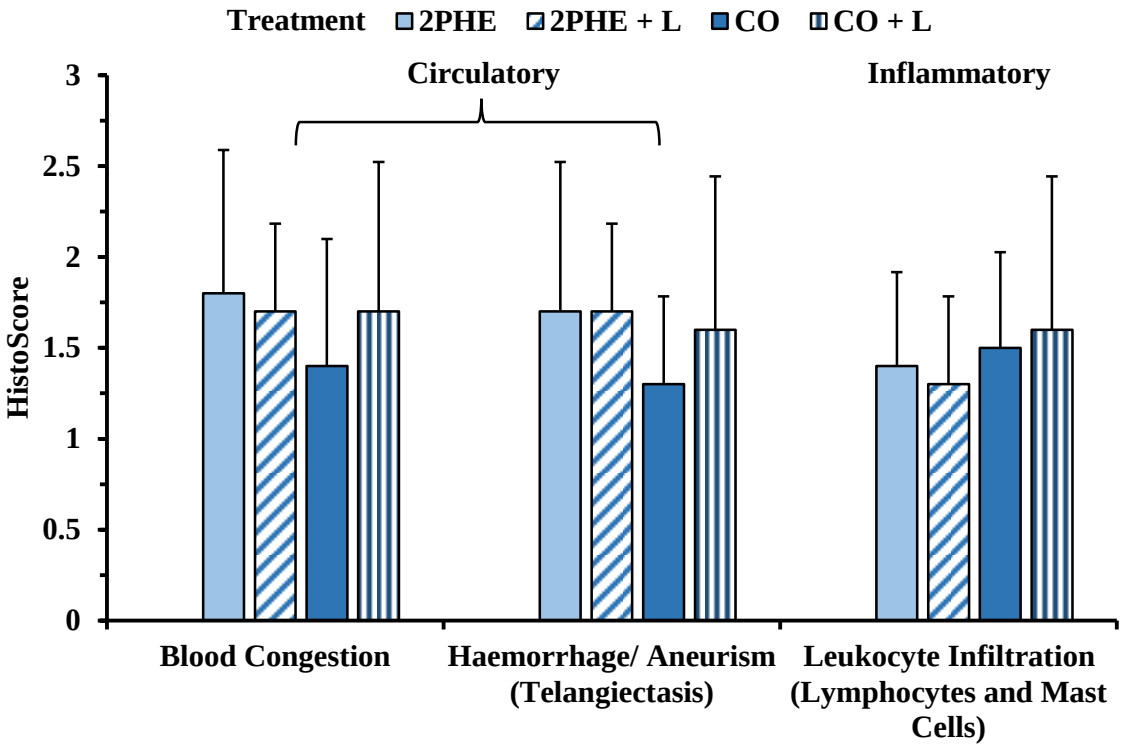

Figure 8 – Histology score of gill morphology on circulatory and inflammatory alterations of gilthead seabream *Sparus aurata* exposed to different anaesthetics, 2-phenoxyethanol (2PHE 0.4 mL/L) and clove oil-eugenol (CO 45 mg/L) and combination with an analgesic, lidocaine (L 2.5 mg/L), for each treatment. Normal morphology is associated with a score of "1", mild morphology damage "2", moderate gill damage "3", while severe tissue damage is represented by "4". Values expressed as means ± SD (n = 10), statistically significant differences identified with different letters (p-value<0.05).

Results showed a progressive alteration in the gills tissue (Figure 9) with the addition of lidocaine The use of lidocaine reduced the damage associated to the anaesthetics. The use of 2PHE showed the same damage has 2PHE + L, but more than CO and CO + L. The use of CO + L showed less damage than 2PHE and 2PHE + L, but the same has CO. Lamellar hyperplasia and hyperplasia from chloride cells demonstrated the same statistical differences between treatment groups (Kruskal-Wallis; p-value<0.05).

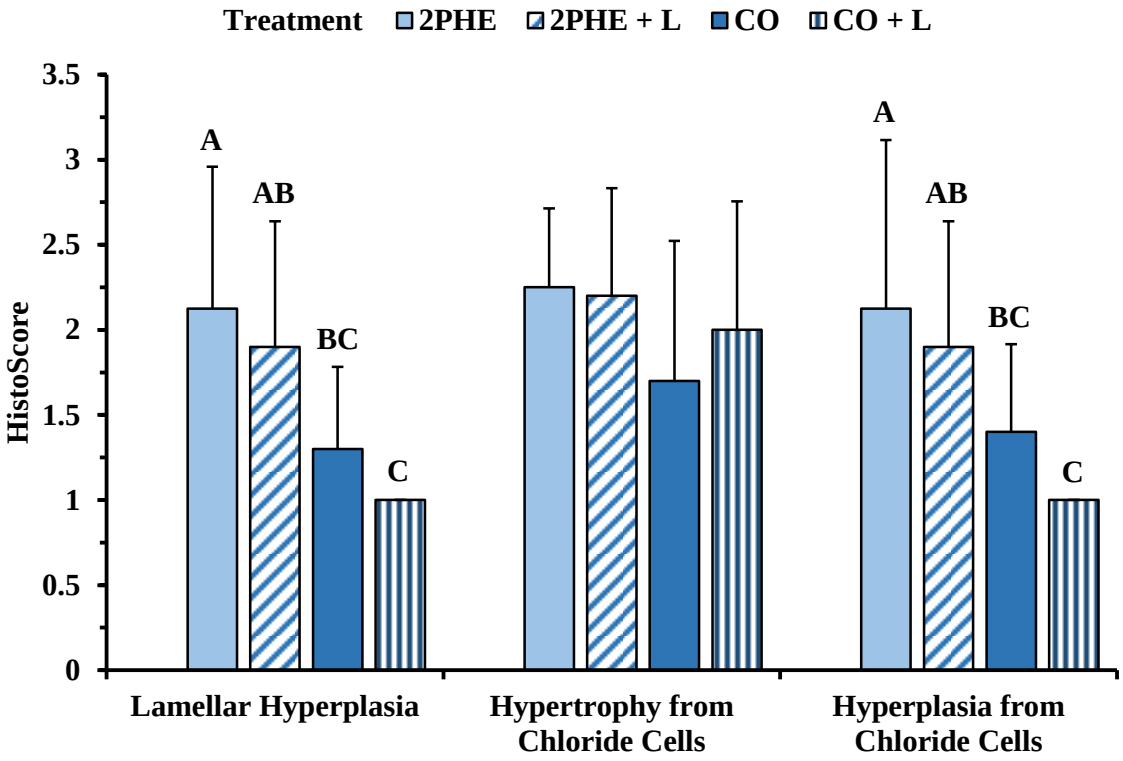

Figure 9 – Histology score of gill morphology on progressive alterations of gilthead seabream *Sparus aurata* exposed to different anaesthetics, 2-phenoxyethanol (2PHE 0.4 mL/L) and clove oil-eugenol (CO 45 mg/L) and combination with an analgesic, lidocaine (L 2.5 mg/L), for each treatment. Normal morphology is associated with a score of "1", mild morphology damage "2", moderate gill damage "3", while severe tissue damage is represented by "4". Values expressed as means ± SD (n = 10), statistically significant differences are identified with different letters (p-value<0.05).

Histological observations showed that it was possible to observe regressive alterations in the gills tissue (Figure 10). Epithelium rupture and rupture from pillar cells had the same statistical differences between treatment groups (Kruskal-Wallis; p-value<0.05). 2PHE and CO demonstrated less damage compared to the treatment group using analgesia with lidocaine (2PHE +L and CO + L).

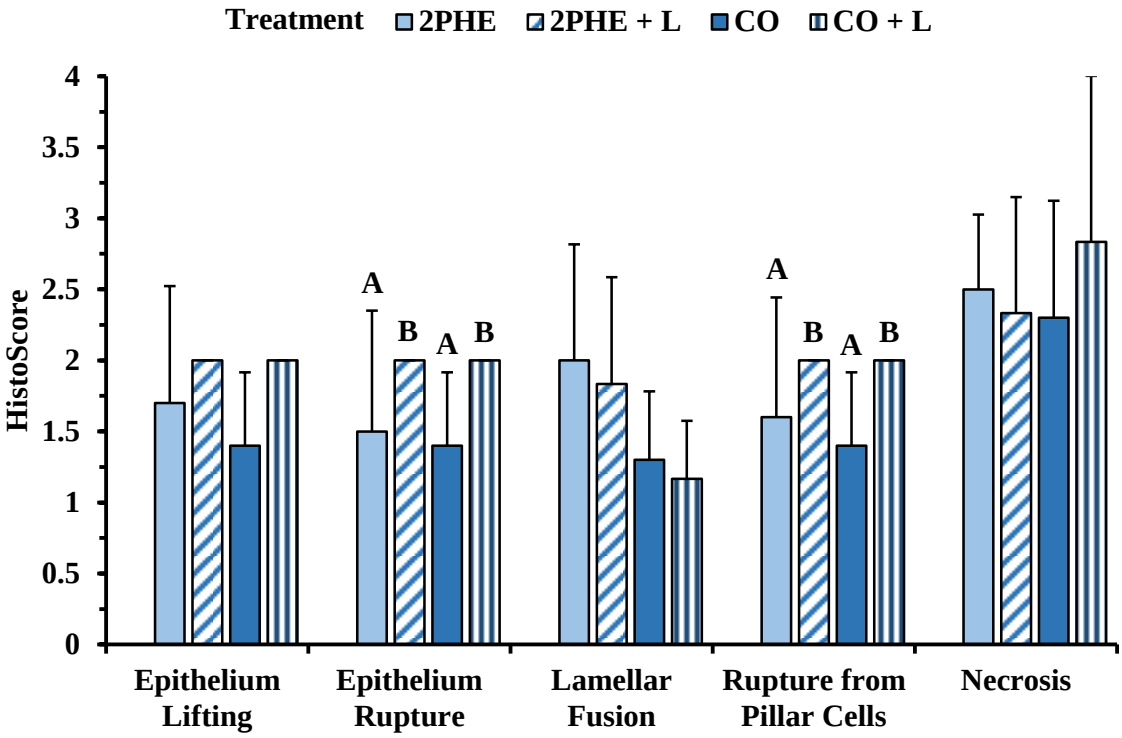

Figure 10 – Histology score of gill morphology on regressive alterations of gilthead seabream *Sparus aurata* exposed to different anaesthetics, 2-phenoxyethanol (2PHE 0.4 mL/L) and clove oil-eugenol (CO 45 mg/L) and combination with an analgesic, lidocaine (L 2.5 mg/L), for each treatment. Normal morphology is associated with a score of "1", mild morphology damage "2", moderate gill damage "3", while severe tissue damage is represented by "4". Values expressed as means ± SD (n = 10), statistically significant differences identified are with different letters (p-value<0.05).

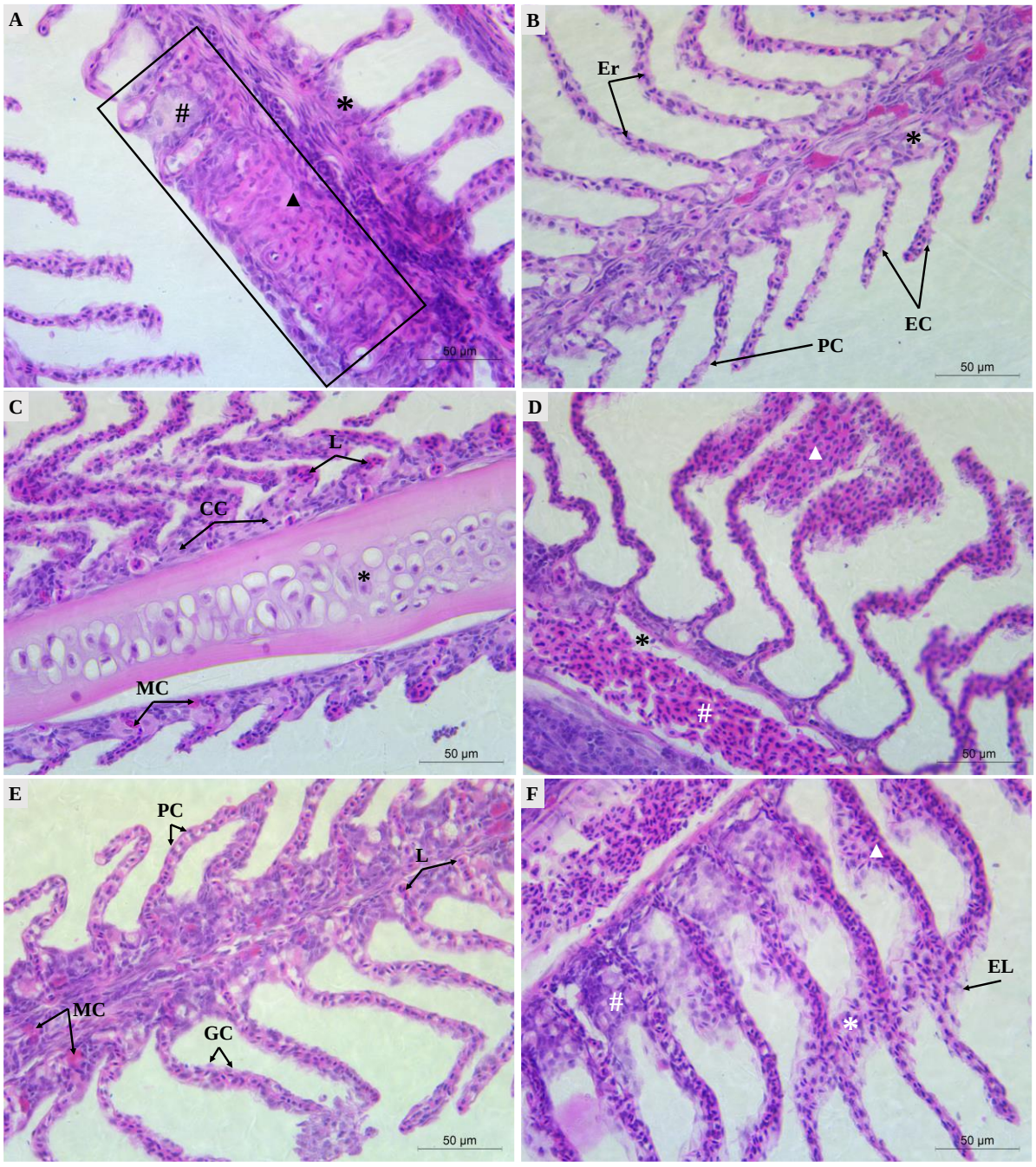

Figure 11 – Histology of gilthead seabream *Sparus aurata* gills, haematoxylin and eosin, 400×. A) exposed fish presenting lamellar hyperplasia (#) showing lamellar fusion (□) with an aneurism (▲), and hyperplasia from chloride cells (*); B) exposed fish showing hypertrophy from chloride cells (*) in the primary filament, possible to observe erythrocytes (Er), epithelium cells (EC) and pillar cells (PC); C) exposed fish showing various inflammatory cells, lymphocytes (L) and mast cells (MC), possible also to observe cartilage (*) and chloride cells (CC); D) exposed fish showing a haemorrhage (▲) in the second filaments, necrosis (*) in the primary filament and the possibility to observe the central venous sinus (#); E) exposed fish possible to observe lymphocytes (L), mast cells (MC), pillar

cells (PC) and goblet cells (GC); F) exposed fish showing hyperplasia from chloride cells (#) from primary filament, lamellar hyperplasia (*) and blood congestion (▲) in the secondary filament causing epithelium lifting (EL).

3.3.6 Gene Expression Analysis

The gene expression was analysed from different stress indicators, immune response, neuro-muscular alterations, and enzymatic genes, using β-actin (*actb*) as a reference gene to uniformize the data.

The indicators of stress genes are heat shock protein 70 (*hsp70*; Figure 12A), Corticotrophin-releasing hormone (*crh*; Figure 12B), CRHbinding protein (*crhbp*; Figure 12C) and Thyrotropin-releasing hormone (*trh*; Figure 12D) didn't present statistical differences between treatment groups in the brain (Tukey HSD; p-value>0.05).

The indicator of stress gene Heat shock protein 70 (*hsp70*; Figure 12E), presents statistical differences between treatment groups in the gills (Kruskal-Wallis; p-value<0.05). 2PHE + L had the lowest gene expression compared to CO and CO + L. The groups with clove oil-eugenol presented higher expression. Corticotrophin-releasing hormone (*crh*; Figure 12F) and CRHbinding protein (*crhbp*; Figure 12G), didn't present statistical differences between treatment groups in the gills (Tukey HSD; p-value>0.05).

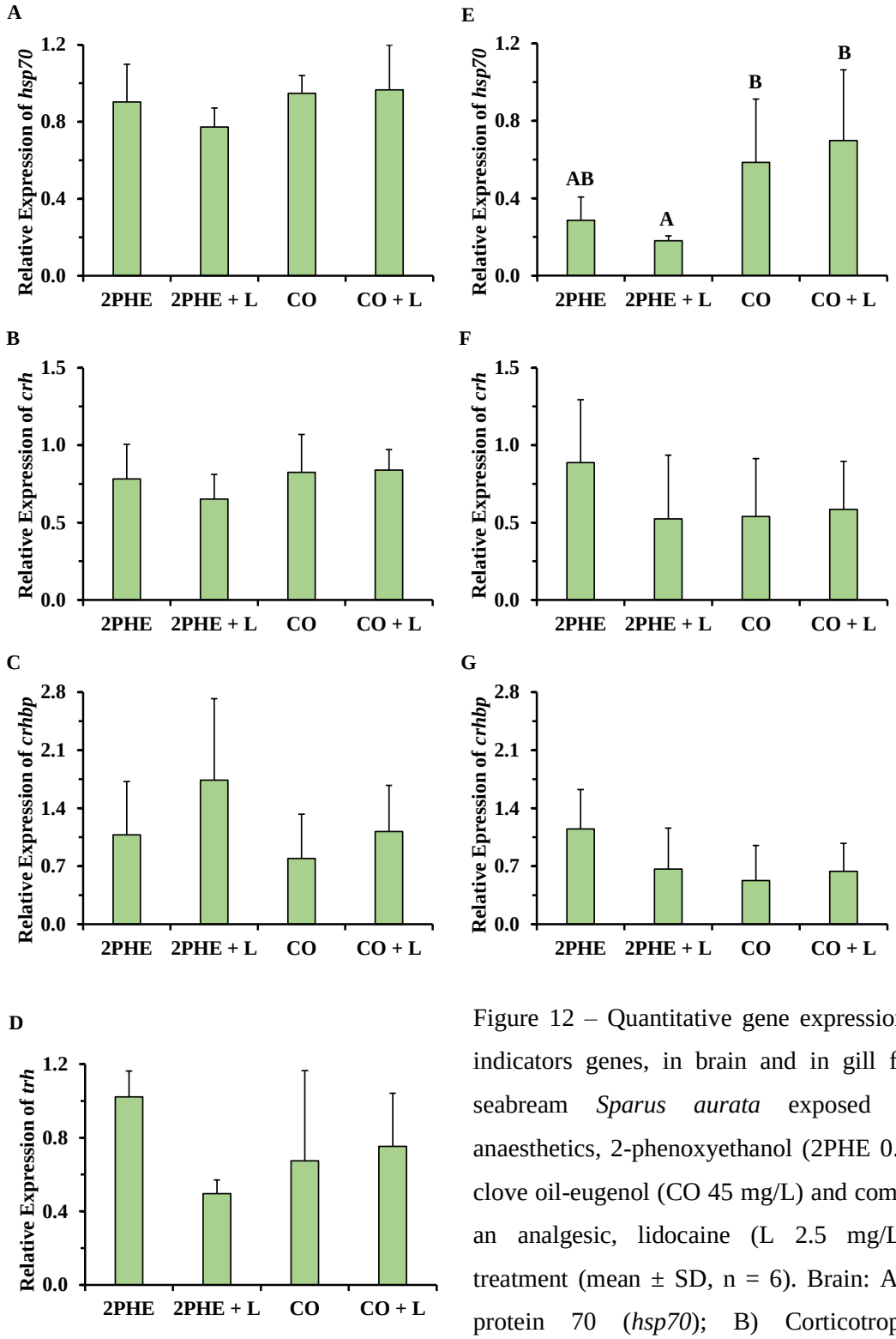

Figure 12 – Quantitative gene expression from stress indicators genes, in brain and in gill from gilthead seabream *Sparus aurata* exposed to different anaesthetics, 2-phenoxyethanol (2PHE 0.4 mL/L) and clove oil-eugenol (CO 45 mg/L) and combination with an analgesic, lidocaine (L 2.5 mg/L), for each treatment (mean ± SD, n = 6). Brain: A) Heat shock protein 70 (*hsp70*); B) Corticotrophin-releasing hormone (*crh*); C) CRHbinding protein (*crhbp*); D) Thyrotropin-releasing hormone (*trh*). Gill: E) Heat shock protein 70 (*hsp70*); F) Corticotrophin-releasing hormone (*crh*); G) CRHbinding protein (*crhbp*). Statistically significant differences are identified with different letters (p-value<0.05).

The immune response gene, Interleukin 1β (*il1β*), didn't present statistical differences in the brain (Figure 13A). and in the gills (Figure 13B) (Tukey HSD; p-value>0.05). The use of lidocaine tends to express more this gene in the gills.

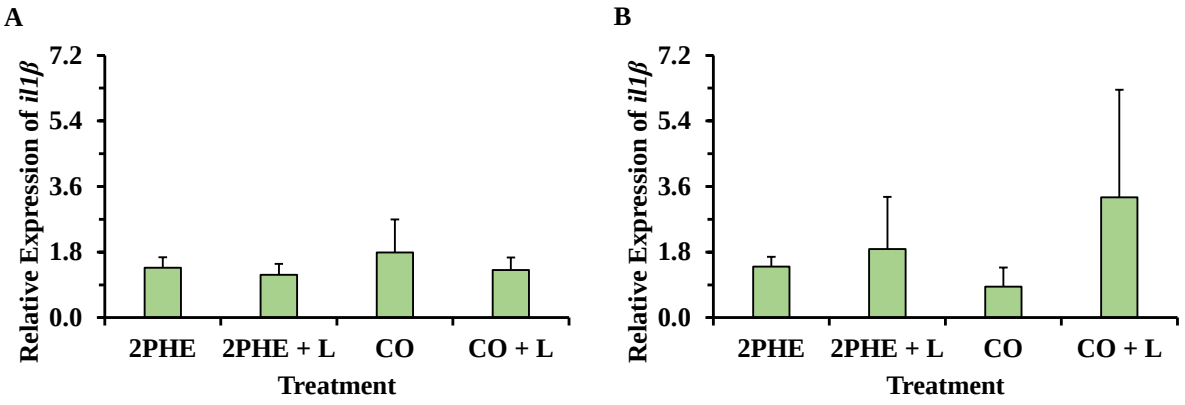

Figure 13 – Quantitative gene expression from immune response gene, Interleukin 1β (*il1β*), in brain (A) and in gill (B) from gilthead seabream *Sparus aurata* exposed to different anaesthetics, 2-phenoxyethanol (2PHE 0.4 mL/L) and clove oil-eugenol (CO 45 mg/L) and combination with an analgesic, lidocaine (L 2.5 mg/L), for each treatment (mean ± SD, n = 6). Statistically significant differences are identified with different letters (p-value<0.05).

The neuro-muscular alterations gene, Acetylcholinesterase (*ache*), didn't present statistical differences in the brain (Tukey HSD; p-value>0.05) (Figure 14A), and in the gills between treatment (Kruskal-Wallis; p-value>0.05) (Figure 14B). But the exclusive use of the anaesthetics tends to increase the expression in the gills.

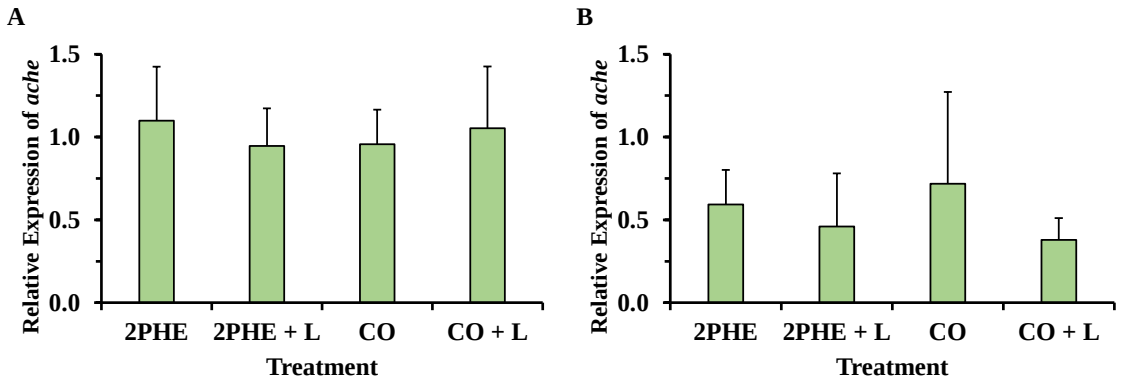

Figure 14 – Quantitative gene expression from neuro-muscular alterations gene, Acetylcholinesterase (*ache*), in brain (A) and in gill (B) from gilthead seabream *Sparus aurata* exposed to different anaesthetics, 2-phenoxyethanol (2PHE 0.4 mL/L) and clove oil-eugenol (CO 45 mg/L) and combination with an analgesic, lidocaine (L 2.5 mg/L), for each treatment (mean ± SD, n = 6). Statistically significant differences are identified with different letters (p-value<0.05).

The enzymatic response gene, Glutathione-S-transferase 3 (*gst3*), presents statistical differences in the brain (Tukey HSD; p-value<0.05) (Figure 15A). 2PHE + L had a higher gene expression than the rest treatment groups, being statistically different. But GST3 doesn't present statistical differences in the gills between treatments (Tukey HSD; p-value>0.05) (Figure 15B).

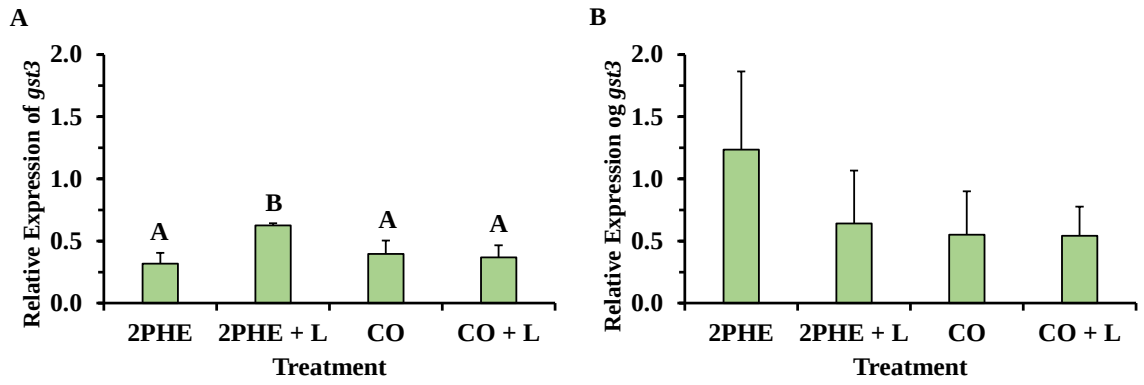

Figure 15 – Quantitative gene expression from enzymatic response gene, Glutathione-S-transferase 3 (*gst3*), in brain (A) and in gill (B) from gilthead seabream *Sparus aurata* exposed to different anaesthetics, 2-phenoxyethanol (2PHE 0.4 mL/L) and clove oil-eugenol (CO 45 mg/L) and combination with an analgesic, lidocaine (L 2.5 mg/L), for each treatment (mean ± SD, n = 6). Statistically significant differences are identified with different letters (p-value<0.05).

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4. Discussion

Gilthead seabream (*Sparus aurata*) is a species of great economic interest in the aquaculture industry (Toffan et al., 2017). During the production cycle, animals are subjected to various handling practices which may require the use of anaesthetic agents (Zahl et al., 2012). In these cases, research is looking for the best treatments to ensure animal welfare and takes into consideration the efficacy and costs (Mylonas et al., 2005; Zahl et al., 2012).

In experiment 1 (anaesthesia refinement), median concentration of clove oil-eugenol (45 mg/L) showed to meet the requirements of inducing anaesthesia below 3 minutes (Ross & Ross, 2008) and recovery between 5 to 10 minutes (Schroeder et al., 2021) at 23ºC. Mylonas et al. (2005) studied that for *S. aurata* anaesthesia, clove oil exposure at 25ºC had an optimal concentration with 40 mg/L, but since in this study fish had a higher weight, the median concentration (CO 45 mg/L) would meet his conclusions. In our study the median concentration of 2-phenoxyethanol (0.4 mL/L) only meet the requirement of recovery time at 23ºC, and yet was chosen to further use. Mylonas et al. (2005) discovered that for *S. aurata* with 41.9g, the 2-phenoxyethanol necessary anaesthetic concentration was lower at 25ºC (0.3 mL/L) than at 15ºC (0.45 mL/L). This is justified because the higher the water temperature the higher the anaesthetic effect, since there is a higher metabolism and opercular ventilation from fish when exposed to higher temperatures. In our study, the temperature was close to the highest temperature from Mylonas et al. (2005), but our fish had more weight (49.12g). Since bigger fish has higher metabolism, it was chosen the median concentration of 2-phenoxyethanol, as temperature and body weight weren't a controlled factor. The highest concentration also fitted the desirable induction and recovery times of anaesthesia, but the use of the median concentration allows for a smaller cost for the fish farmer and less polluting effect for the environment (Mylonas et al., 2005).

In experiment 2 (analgesia refinement), the lowest lidocaine concentration (2.5 mg/L) demonstrated the best effects on induction times (2PHE + L 2.5 mg/L 144.02 ± 68.26 seconds, and CO + L 2.5 mg/L 129.00 ± 22.83 seconds), and a better recovery time (2PHE + L 2.5 mg/L 152.40 ± 32.12 seconds, and CO + L 2.5 mg/L 394.28 ± 43.93 seconds) comparing to isolated use of the anaesthetic. Lidocaine demonstrated an effect in improving the times of anaesthesia. Lidocaine addition showed significatively the lowest times in the induction of CO and in the recovery of 2PHE. The chosen concentration is according to Zahl

et al. (2009), suggesting a synergistic effect produced by prior exposure to analgesia with some anaesthetics. Zahl et al. (2009) studied different combinations of anaesthetics has pre-sedatives in the way to create a synergetic effect with the anaesthesia to reduce concentrations. Atlantic cod (*Gadus morhua*) was the species studied with different weight (10, 100 and 1000 g) and at different temperatures (8 and 16ºC). Zahl et al. (2009) exposed the fish to diverse concentrations of single pre-sedative (metomidate) and double pre-sedation (metomidate with 2-phenoxyethanol), then anaesthetised with benzocaine or MS-222 at different concentrations. In our study, when fish were expose to higher concentrations of lidocaine, they tended to have a higher euphoric behaviour, which is correlated to anxiety-like behaviours as found by de Abreu et al. (2019). In de Abreu et al. (2019) study, the highest concentration (10 mg/L) showed hypolocomotor effect when applied systemically and peripherally in adult zebrafish during a 20-minute immersion exposure. When the concentration of 5 mg/L of lidocaine was used in stage 5 zebrafish larvae, the dose-effect was the same as the control groups (disturbed and undisturbed groups) used when studying the behaviour of the animals by Lopez-Luna et al. (2017).

In experiment 3 (physiological responses to anaesthesia and analgesia exposure), the combinations using analgesia (2PHE + L and CO + L) resulted in positive results to handling and blood sampling and also by reduction of recovery time as similarly found by Zahl et al. (2009). Although there were no statistical differences in the recovery, there was a synergetic effect leading to a decrease recovery time when compared to the use of the anaesthetic alone. Zahl et al. (2009) also observed a reduction of induction and recovery times, when there was a pre-sedative exposure prior to anaesthesia. Thus, the best treatment groups in this study are when lidocaine is used has analgesia, because of the reduction of the exposure time of the anaesthetic and a better recovery post-handling.

Witeska et al. (2022) states that the use of anaesthetic alters haematological parameters and states the need of determining the correct drug and dose to be used for each fish species and size, taking into consideration the water parameters of these animals.

Our study showed no statistical differences for RBC (2.92 to 6.39 $\times 10^6$ μL^{-1}), WBC (5.08 to 6.27 $\times 10^4$ μL^{-1}), Ht (28.33 to 32.10 %), Hb (2.36 to 2.74 g/dL), MCH (7.93 to 9.18 g 100 mL^{-1}) and MCHC (7.66 to 8.68 pg $cell^{-1}$). The values stated by Fazio et al. (2015) for RBC (3.0 to 4.2 $\times 10^6$ μL^{-1}), WBC (13.5 to 84.3 $\times 10^3$ μL^{-1}), Ht (17.80 to 53.33 %), Hb (4.8 to

16.3 g/dL), MCH (12.63 to 36.11 pg) and MCHC (13.33 to 28.90 pg $cell^{-1}$), for *S. aurata* with 62.43 to 243.10g. This demonstrates that in the present study the values are in range with Fazio et al. (2015). In this study, there was no visual damage directly to RBC, but a lower Hb affects MCH and MCHC. The MCV values varied between 84.93 to 117.28 μm^3 in the present study. According to Fazio et al. (2015), the MCV values are within the established range (92.00 to 168.00 μm^3), but despite statistical differences between 2PHE + L with CO, in general the values are shown to be lower when lidocaine is used.

There are no statistical differences in LPO (24.55 to 35.17 nmol/g wt) and in GST (1702.62 to 2040.93 mU mg^{-1}) values between the treatment groups. LPO values in this study are lower, and GST values are higher than Almeida et al. (2010) reported values for *Dicentrarchus labrax* (LPO – 100 to 150 nmol TBARS g wt^{-1}; GST – 20 to 60 nmol min^{-1} mg $protein^{-1}$). GST also presented higher values then in Teles et al. (2019) study (GST – 200 to 350 nmol/min/mg/prot), with *S. aurata* (42.7g). This increase corresponds to an antioxidant defence activity to eliminate unwanted xenobiotics that may create oxidative damage (Birnie-Gauvin et al., 2017). Boaventura et al. (2020) studied the use of essential oil *Ocimum gratissimum* as an anaesthetic for *Lophiosilurus alexandri*. Their results demonstrated that the use of 90 mg/L of *Ocimum gratissimum* induced changes to the antioxidant parameters, increasing the concentration of liver and brain ROS, and reducing the activity of brain GST, but did not cause tissue damage.

In the 2PHE + L treatment we found the highest amount of SOD (73 ± 23.04 U mg $prot^{-1}$) (statistically different from all treatments) defence enzymatic activity but nevertheless a high amount of CAT (50.09 ± 7.68 U mg prot-1), meaning that there will be a greater degradation of hydrogen peroxide and of the superoxide radical, when comparing to CO (SOD 44.14 ± 14.45 U mg $prot^{-1}$ and CAT 29.43 ± 7.93 U mg prot-1) and CO + L (SOD 43.75 ± 9.70 U mg $prot^{-1}$ and CAT 29.40 ± 9.00 U mg $prot^{-1}$). Regardless of the use of lidocaine in the clove oil-eugenol treatments, it implies inhibition of the defence enzymes of SOD and CAT. Despite the elevation of enzymatic defence, there is no significant lipid damage. However, in our study, SOD and CAT have lower values when compared to Hoseini et al. (2020) (CAT 40 to 100 U mg $prot^{-1}$) and Yu et al. (2020) (SOD 150 to 350 U mg $prot^{-1}$). Yu et al. (2020) studied MS222 as a stress reducer to juvenile silver pomfret (*Pampus argentus*).

Statistically, 2PHE + L (141.30 nmol mg prot^{-1}) is different from CO (208.60 nmol mg prot^{-1}) and CO + L (219.22 nmol mg prot^{-1}) when total glutathione (TG) is observed. Although they are a proper anaesthetic and concentration for sampling fish, they are considered minimum changes in the hepatic parameters when comparing to Zahran et al. (2021) values (4 to 10 mmol g^{-1} tissue).

The increase in protease in 2PHE + L demonstrates the existence of inflammation, when compared to CO. Looking at the antiprotease of 2PHE + L leads one to believe that the inflammation is still present and that it has not yet begun to resolve. In CO, as there are lower values of protease and higher values of peroxidase and antiprotease, it shows a decrease in inflammation. This could be because the use of two synthetic chemicals makes it more difficult for the body to recover from inflammation, while the single use of the anaesthetic being of natural composition, facilitates recovery. The values from protease variation between 8.56 to 9.99 %, and antiprotease variation between 73.83 to 78.45 %, observed in this study meets the values obtained by Guardiola et al. (2016) (protease 8.69 to 10.28 %; antiprotease 72.17 to 77.21 %), but Guardiola et al. (2016) reported higher peroxidase values (11.99 to 17.33 units ml^{-1}) compared to the observed values (1.41 to 1.87 OD 450 nm) in our study. Bahi et al. (2018) demonstrated that exposure to 55 ppm clove oil for 5 minutes for *S. aurata* doesn't compromise the immune status of the fish. Overall, the best treatment group is the unique use of clove oil-eugenol, because of the reduced existence of inflammation when comparing with the other combinations.

In the present study, the glucose parameters (134 to 194 mg dL^{-1}) are according to Fazio et al. (2015) reference values for glucose for *S. aurata* (glucose 89.00 - 327.00 mg dL^{-1}), being within normal range. There are statistically differences in lactate (50.74 to 153.34 mg dL^{-1}). When comparing to reference values from Fazio et al. (2015) to *S. aurata* (4.9 to 21.30 mg dL^{-1}), the fish from this study presents much higher values. In lactate, when analgesia is used, the stress that triggers this parameter is lower, namely in CO + L. However, among all treatments, the highest and lowest values of lactate have the anaesthetic clove oil-eugenol. Somehow 2-phenoxyethanol despite creating a stress reaction, isn't so aggressive its use when compared to CO, but coming to use lidocaine significantly decreases this negative reaction. Toni et al. (2015) showed the use of the 2-phenoxyethanol is more advisable than the essential oil *Lippia alba* for *S. aurata*, when comparing metabolic levels (glucose and lactate) with a control group, even with stressor exposure. Although different types of

anaesthetics and analgesia are used, numerous factors influence and make the interpretation of glucose difficult, such as exposure time, dose, species, and biotic and abiotic factors, before some kind of comparison (Bahi et al., 2018).

The increase in ALP, ALT and AST is seen as a negative reaction (Yousefi et al., 2018). There are no statistical differences between ALP treatments (41.68 to 43.09 U L^{-1}), regardless of the type of compounds exposed the secondary reaction is the same. Zahran et al. (2021) obtained values in the range of 90 to 140 U L^{-1} for Nile tilapia (*Oreochromis niloticus*) with 35g when exposed to propofol (2.5 mg/L) and eugenol (30 mg/L) and evaluated that didn't create health damage being considered safe levels of ALP. In ALT (3.85 to 74.32 U L^{-1}) and AST (6.00 to 162.44 U L^{-1}) from this study, the use of lidocaine creates a negative reaction due to the significant increase in these parameters. When observing Zahran et al. (2021) range values (ALT 3 to 12 U L^{-1} and AST 10 to 30 U L^{-1}).

Through histology, it was possible to observe histological changes in structures and morphological differences when the anaesthetic is administered, with or without analgesia. The circulatory alterations are transversal to all treatments, in which no damage is considered. Anaesthetics tested by Dong et al. (2022) on spotted seabass *Lateolabrax maculatus*, showed changes in the morphology of the gill tissue. The gills seem to have produced an inflammatory response.

Lamellar hyperplasia is related to hyperplasia from chloride cells. Chloride cells play a role in ionic transport with the possibility of being involved in detoxification (Mokhtar, 2017). The increase of these cells using 2-phenoxyethanol could mean the response of a defence mechanism against the anaesthetic, to try to maintain the homeostasis of the organism. The use of both anaesthetics presents a higher hyperplasia of chloride cells due to the exposure of a secondary chemical component, by which is often observed under conditions that challenge ionic regulation (Macirella & Brunelli, 2017). Lidocaine has a positive effect on this progressive damage, causing less damage than using anaesthetics alone. In the treatment groups using 2-phenoxyethanol (2PHE and 2PHE + L) can be observed mild damage in the chloride cell hypertrophy but not significant. This can be identified as an adaptive response due to their ionic function to balance with the external environment (Strzyżewska-Worotyńska et al., 2017).

The increase in epithelial cells may lead to disorganization and fusion of the lamellae, which can be seen as a defence mechanism by reducing the surface in contact with the external environment (Chance et al., 2018). The use of lidocaine, in regressive alterations, showed a higher damage related. The penetration of the chemicals used can cause a rupture of the epithelium which can be related to the pavement cell lifting not being able to adapt to the exposure creating a consequent damage (Reddy & Rawat, 2013). Epithelium lifting is considered a defence mechanism as well as necrosis, due to the increased space created between the external environment and the blood, serving as an entry barrier for chemicals (Reddy & Rawat, 2013). In Santos et al. (2020) study when exposing tambaqui (*Colossoma macropomum*) to 10.4 mg/L tea tree and clove essential oils, showed some gill alterations caused by exposure, may not cause harmful morphological alterations in the epithelium since it can be considered adjustment alterations and not damage. Jia et al. (2022) study exhibit different alterations during anaesthesia and recovery. Showing significantly more changes of gill histology using clove oil compared with MS222, inducing potentially more damage for spotted knifejaw (*Oplegnathus punctatus*). Which concluded that the gill histology of MS222 and clove oil exposure remains controversial.

Regarding gene expression, we observed a higher expression of *hsp70* in brain than in gill tissue. Expression of this gene was significantly lower in 2PHE + L treatment in the gills. When compared with Teles et al. (2019), the significant increase of *hsp70* in the brain and liver of *S. aurata* (42.7 g) by exposure to MS222 (5 mg/L) and clove oil (2.5 mg/L) during transport sedation, suggests the presence of stress after 6H and dissipation after 24H in the brain for both anaesthetics. However, in both the gene is not expressed enough to be characterised as the existence of cellular stress.

Neither treatment group (2PHE, 2PHE + L, CO, and CO + L) induced significant changes in the expression of neuroendocrine factors in brain tissue (*crh* 0.65 to 0.8; *crhbp* 0.79 to 1.13; and *trh* 0.49 to 1.02) and gills (*crh* 0.52 to 0.88; and *crhbp* 0.52 to 1.14). Thus, this gene expression being relatively similar with Toni et al. (2015) study. Toni et al. (2015) observed low expression of *crhbp* (0.5 to 1.5) in the stressed specimens of *S. aurata* exposed to essential oil *Lippia alba* (35 μL/L) possibly related to the consequent affinity to CRH receptors (expression 0.3 to 0.8), to guarantee homeostasis. Thus, as observed, they can regulate stress with hypothalamic factors depending on the type/source of induced stress. When compared with Jerez-Cepa et al. (2019) study, TRH control expression (1.11 ± 0.06) is

near or less expressed in the brain meaning the stress inducing CRH could modulate its particles.

The greater expression of *il1β* observed in gills characterises greater inflammation in the receptor organ, with special attention in the groups using lidocaine. Then, the opposite event is observed in the brain. This may be because local anaesthetics (lidocaine, as an analgesic) have the action of relieving pain by interrupting nerve conduction in the gills, thus temporarily preventing the sensation of the noxious stimulus to be conducted to the central nervous system (Chatigny et al., 2018a). In Hoseini et al. (2020), the anaesthesia with cineole with higher concentrations has shown an anti-inflammatory reaction, down-regulating *il1β*. The higher concentrations not only reduced exposure time (anaesthesia time/induction), but also lowers stress responses in common carp (*C. carpio*) with 1000 μL/L of cineole.

Although gills are the receptor organ of the drugs, there was a higher expression of *ache* in the brain, although there were no statistical differences. Looking at the gills, it is possible to detect that there is less expression when the lidocaine is used. It seems that lidocaine decreases the inflammation associated with drug absorption at the neuro-muscular level. With the fish behaviour observations in experiment 2 (analgesia refinement), we saw that increasing the concentration of lidocaine lead to a euphoric behaviour in fish, the same happening with mice (de Abreu et al., 2019). However, there was only a decrease in gill's *ache*, which explains the behaviour during the time of exposure and didn't show a decrease in expression in the brain as quickly as in the gills.

There is a special expression in 2PHE+L in brain *gst3* (0.62 ± 0.01) but overall insignificant when compared with Teles et al. (2019) coinciding with their control values (0 to 1). The expression in the gills was higher (0.54 to 1.23), where it is possible to see that there is a tendency to have a higher expression when lidocaine was used. This indicates that the presence of analgesia acts positively in inhibiting the production of ROS species in the gills.

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5. Conclusion

Currently, there are several anaesthetics widely used in research, however, the use of analgesics is rare. The analgesia factor is still a unknow effect, because there is still little knowledge in the fish scientific community.

This study used one of the most uses synthetic anaesthetics (2-phenoxyethanol) and a natural anaesthetic (clove oil-eugenol) in aquaculture, in combination with lidocaine to create a synergistic analgesia environment. This was to understand if it is possible to reduce the pain implied by handling.

In summary, according to the results presented, the ideal exposure to submit the gilthead seabream *Sparus aurata*, is the 2-phenoxyethanol (0.4 mL/L) with lidocaine (2.5 mg/L), and clove-eugenol oil (45 mg/L). This is because some results with the use of lidocaine are contradictory. Although the use of lidocaine proceeds to better induction and recovery times due to handling; there are also results showing an increase of stress in other parameters due to exposure. Therefore, in case of use of a synthetic anaesthetic, it is better to use the analgesia of lidocaine to decrease some of the side effects of stress induction. In the case of using a natural based anaesthetic, it is advised to use only the clove oil-eugenol, as the analgesia will create greater stress reactions in the fish. Thus, depending on the objectives of the study and the anaesthetic compound used, the use of analgesia may be favourable. However, further studies are needed to better understand the synergy with analgesia using other synthetic and natural anaesthetics.

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Appendix

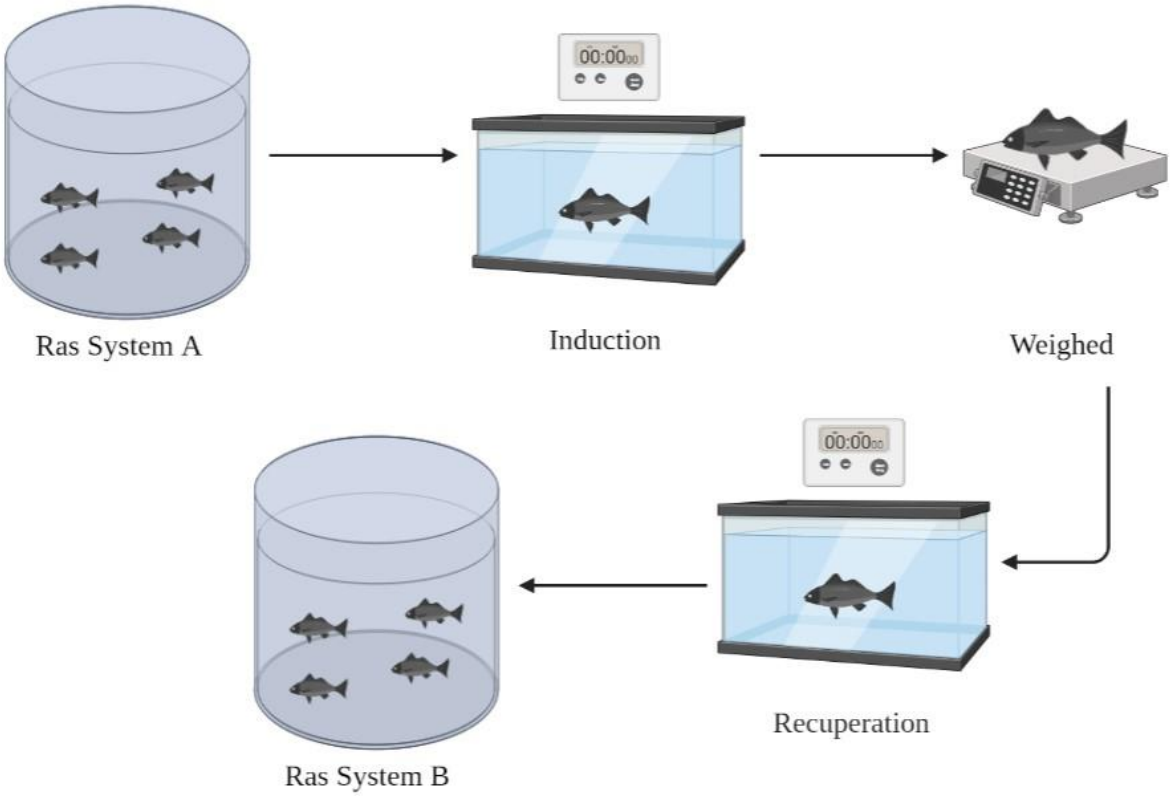

Figure 1 - Experiment 1 (Anaesthesia refinement) self-explaining experimental design.

Table I – Times of induction in seconds, at stage A3 and A5, and recovery at stage R3 and R5 in experiment 1 (Anaesthesia refinement) for 0.2; 0.4 and 0.6 mL/L 2-phenoxyethanol (2PHE) and 30, 45 and 60 mg/L clove oil-eugenol (CO) concentrations.

	A3	**A5**	**R3**	**R5**
2PHE 0.2 mL/L	45.94 ± 39.16	300 ± 0	36.93 ± 16.43	90.34 ± 13.62
2PHE 0.4 mL/L	63.33 ± 37.18	210.19 ± 68.35	78.20 ± 10.99	123.56 ± 17.54
2PHE 0.6 mL/L	35.25 ± 2.63	71.64 ± 8.66	89.38 ± 22.81	133.16 ± 35.34
CO 30 mg/L	94.66 ± 32.94	263.18 ± 38.53	170.68 ± 37.96	311.31 ± 68.45
CO 45 mg/L	54.33 ± 5.56	134.70 ± 21.80	167.33 ± 36.07	270.16 ± 61.44
CO 60 mg/L	43.46 ± 9.46	86.75 ± 22.74	173.37 ± 14.12	239.19 ± 36.00

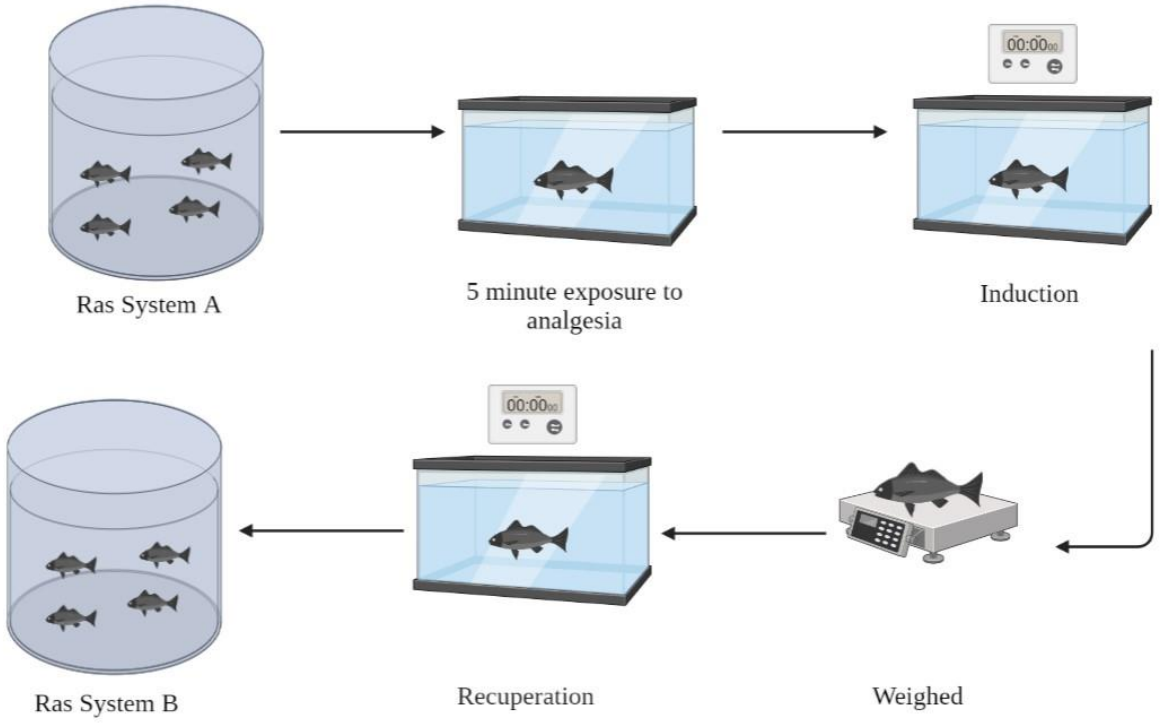

Figure 2 - Experiment 2 (Analgesia refinement) self-explaining experimental design.

Table II – Times of induction in seconds, at stage A3 and A5, and recovery stage R3 and R5 in experiment 2 (Analgesia refinement) for 2.5; 5 and 7.5 mg/L lidocaine (L) concentrations when using 2-phenoxyethanol 0.4 mL/L (2PHE) and clove oil-eugenol 45 mg/L (CO).

	A3	**A5**	**R3**	**R5**
2PHE 2.5 mg/L	61.18 ± 21.82	82.85 ± 47.25	98.58 ± 20.13	53.83 ± 25.39
2PHE 5 mg/L	57.33 ± 30.71	125.50 ± 53.05	130.88 ± 19.36	118.00 ± 18.00
2PHE 7.5 mg/L	67.48 ± 22.90	176.40 ± 102.40	141.88 ± 32.52	90.55 ± 44.37
CO 2.5 mg/L	49.80 ± 5.74	79.20 ± 20.57	129.00 ± 22.83	226.25 ± 35.77
CO 5 mg/L	57.20 ± 5.74	88.88 ± 15.37	146.08 ± 13.40	211.18 ± 18.04
CO 7.5 mg/L	75.05 ± 12.91	72.05 ± 11.73	147.10 ± 18.61	192.00 ± 18.30

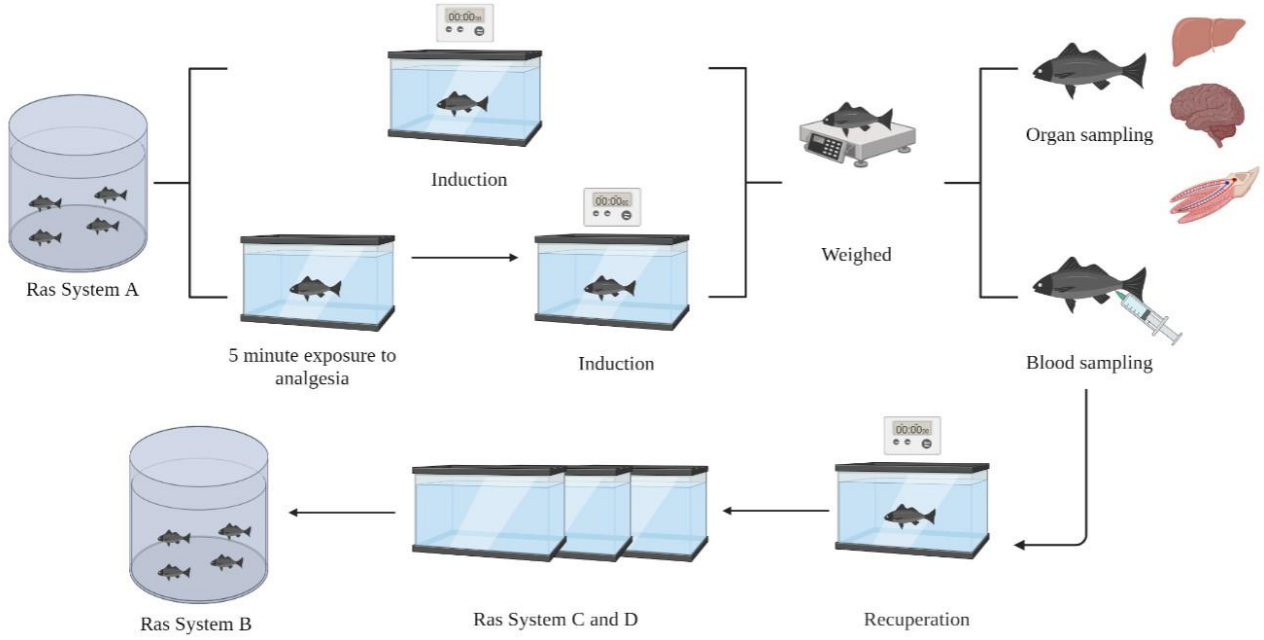

Figure 3 - Experiment 3 (Physiological responses to anaesthesia and analgesia exposure) self-explaining experimental design.

Table III – Times of induction in seconds, at stage A3 and A5, and recovery stage R3 and R5 in experiment 3 (Physiological responses to anaesthesia and analgesia exposure) for 2-phenoxyethanol (2PHE 0.4 mL/L), 2-phenoxyethanol with lidocaine (2PHE 0.4 mL/L + L 2.5 mg/L), clove oil-eugenol (CO 45 mg/L) and clove oil-eugenol with lidocaine (CO 45 mg/L + L 2.5 mg/L).

	A3	**A5**	**R3**	**R5**
2PHE	64.76 ± 25.84	122.49 ± 45.72	250.71 ± 10.26	107.60 ± 28.75
2PHE + L	51.46 ± 10.87	79.19 ± 39.41	223.90 ± 56.65	111.70 ± 29.39
CO	55.45 ± 10.52	65.85 ± 16.78	249.88 ± 41.93	228.60 ± 113.17
CO + L	54.91 ± 7.41	76.00 ± 19.57	303.10 ± 95.33	119.20 ± 53.97

Table IV – Genes used in the analysis from gene expression for brain and gills, optimized with annealing temperature and its efficiency. (*Accepted slope; **Not used)

Gene	**Acronym**	**Annealing temperature (ºC)**	**Efficyency (%)**		**Primer Sequence Foward Sequence (5´-3´) Reverce Sequence (5´-3´)**	**Reference**
			Brain	Gill		
β-actin	*actb*	62	104	115	F: TCTTCCAGCCATCCTTCCTCG R: TGTTGGCATACAGGTCCTTACGG	Jerez-Cepa et al., 2019
Heat shock protein 70	*hsp70*	60	93	100	F: AATGTTCTGCGCATCATCAA R: GCCTCCACCAAGATCAAAGA	Espinosa et al., 2020
Corticotrophin-releasing hormone	*crh*	60	120	91	F: GGAGCTTAAACGGGCTTGAC R: TCAAACATGAAGAACGGCTTAA	Jerez-Cepa et al., 2019
CRHbinding protein	*crhbp*	60	107	115	F: TTTGAAGAGCCACAGATCCA R: TCGAGCTTTATTCAGCGACA	Jerez-Cepa et al., 2019
Thyrotropin-releasing hormone	*trh*	60	95	215**	F: GAAACGCTTTTGGGATAACTCC R: CGGCGTGACTCTTGTTTATGTT	Jerez-Cepa et al., 2019
Interleukin 1β	*il1β*	60	82*	106	F: TCAGCACCGCAGAAGAAAAC R: TAACACTCTCCACCCTCCAC	Balasch et al., 2021
Acetylcholinesterase	*ache*	60	115	103	F: CGGAGTGGATGGGTGTGATC R: GTCGGCTCAGTTTCTCCTCC	Campos-Sánchez et al., 2021

Glutathione-S-transferase 3	*gst3*	60	98	95	F: CCAGATGATCAGTACGTGAAGACCGTC R: CTGCTGATGTGAGGAATGTACCGTAAC	Teles et al., 2019

Table V –*Sparus aurata* brain RNA concentration (ng/μL) with respective organ weight used for RNA extraction, ratio 260/280 nm and 260/230 nm for gene expression for different anaesthesia and analgesia combinations 2-phenoxyethanol (2PHE), 2-phenoxyethanol with lidocaine (2PHE + L), clove oil-eugenol (CO), and clove oil-eugenol with lidocaine (CO + L).

Treatment	Samples	Weight (mg)	RNA Concentration (ng/ul)	Ratio 260/280nm	Ratio 260/230nm
CO (Clove Oil)	**A1B**	39.8	310.6	2.11	2.31
	A2B	42.2	522.6	2.14	2.43
	A3B	47.6	533.0	2.13	2.43
	A4B	40.9	399.5	2.13	2.31
	A5B	43.2	463.0	2.09	2.13
	A6B	39.7	521.3	2.15	2.18
CO + L (Clove Oil + Lidocaine)	**B1B**	41.2	263.6	2.12	2.3
	B2B	41.4	104.5	2.14	2.17
	B3B	37.8	210.0	2.08	2.13
	B4B	35.1	239.8	2.09	2.15
	B5B	51.1	380.9	2.12	2.31
	B6B	54.8	888.6	2.11	2.36
2PHE (2-Phenoxyethanol)	**C1B**	45.4	479.7	2.09	2.13
	C2B	31.3	196.6	2.07	1.8
	C3B	30.9	265.0	2.1	1.99
	C4B	31.2	216.0	2.09	2.19
	C5B	46.4	245.8	2.09	2.32
	C6B	32.5	309.9	2.1	2.34

2PHE + L (2-Phenoxyethanol + Lidocaine)	**D1B**	31	241.3	2.1	2.16
	D2B	36.1	344.2	2.09	2.19
	D3B	38.7	472.3	2.09	2.3
	D4B	52	453.3	2.09	2.25
	D5B	40.8	342.6	2.1	2.1
	D6B	46.3	677.5	2.1	2.35

Table VI – *Sparus aurata* gill RNA concentration (ng/µL) with respective organ weight used for RNA extraction, ratio 260/280 nm and 260/230 nm for gene expression for different anaesthesia and analgesia combinations 2-phenoxyethanol (2PHE), 2-phenoxyethanol with lidocaine (2PHE + L), clove oil-eugenol (CO), and clove oil-eugenol with lidocaine (CO + L).

Treatment	**Samples**	**Weight (mg)**	**RNA Concentration (ng/ul)**	**Ratio 260/280nm**	**Ratio 260/230nm**
CO (Clove Oil)	**A1G**	39.8	1111.3	2.11	2.34
	A2G	42.2	1781.8	2.11	2.33
	A3G	47.6	1366.9	2.12	2.2
	A4G	40.9	1527.1	2.12	2.33
	A5G	43.2	1568.9	2.12	2.3
	A6G	39.7	2446.3	2.11	2.32
CO + L (Clove Oil + Lidocaine)	**B1G**	41.2	978.8	2.14	2.35
	B2G	41.4	2442.5	2.12	2.33
	B3G	37.8	1958.6	2.11	2.33
	B4G	33.1	2490.6	2.11	2.27
	B5G	51.13	1781.3	2.12	2.32

	B6G	54.8	2017.5	2.12	2.3
2PHE (2-Phenoxyethanol)	**C1G**	45.4	1105.1	2.12	2.35
	C2G	31.3	1004.4	2.11	2.33
	C3G	30.9	1665.7	2.14	2.35
	C4G	31.2	1458.6	2.13	2.31
	C5G	46.4	2328.3	2.12	2.31
	C6G	32.5	1508.1	2.14	2.27
2PHE + L (2-Phenoxyethanol + Lidocaine)	**D1G**	31	1626.9	2.11	2.36
	D2G	36.1	1562.1	2.12	2.35
	D3G	38.7	992.7	2.12	2.39
	D4G	52	2100.2	2.13	2.35
	D5G	40.8	1701.8	2.13	2.36
	D6G	46.3	1227.7	2.14	2.37

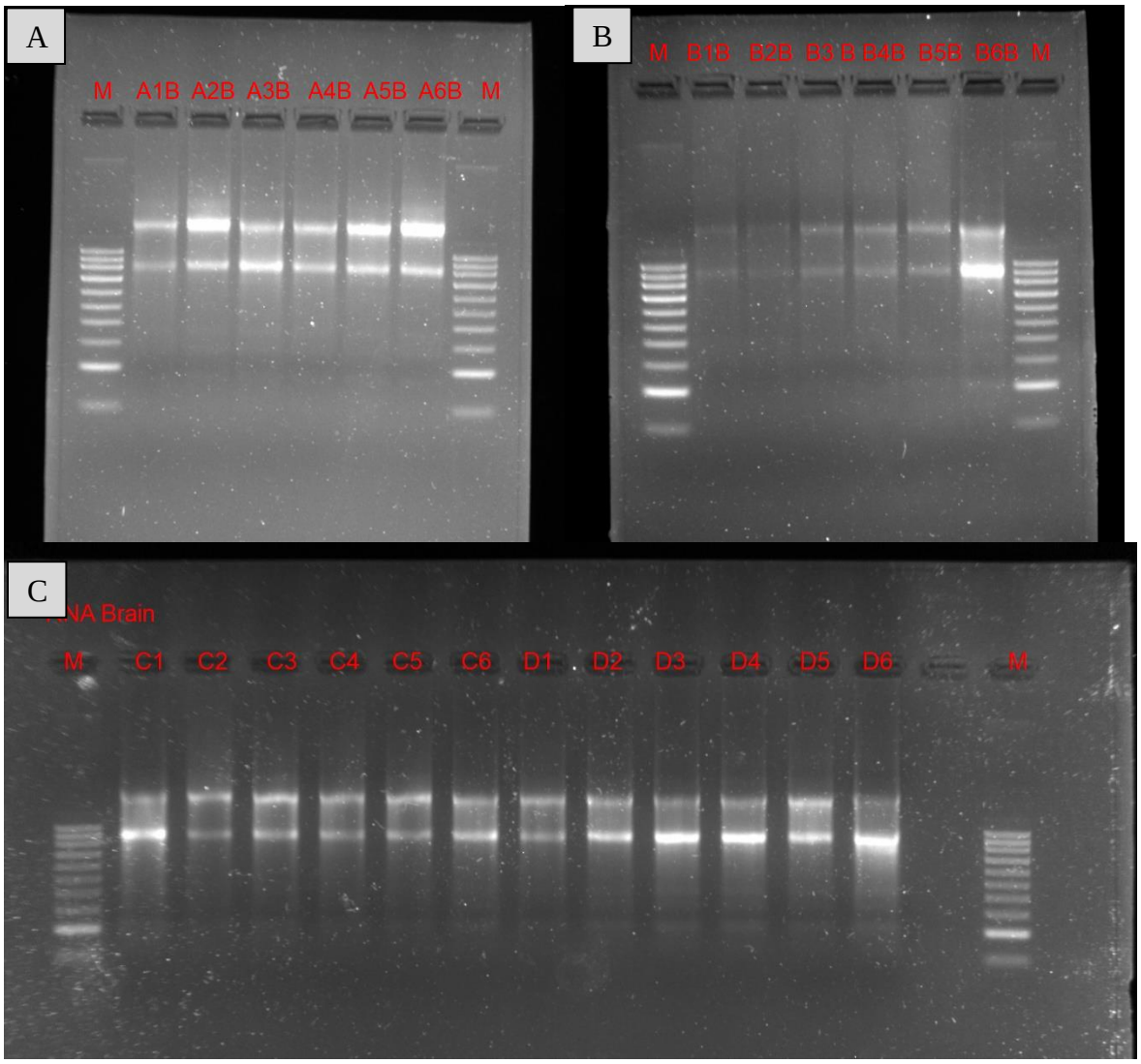

Figure 4 – Electrophorese gel for the verification of the quality and integrity of the RNA samples from brain. (A - M = marker, A = treatment clove oil; B - B = treatment clove oil + lidocaine; C - C = treatment 2-phenoxyethanol, D = treatment 2-phenoxyethanol + lidocaine)

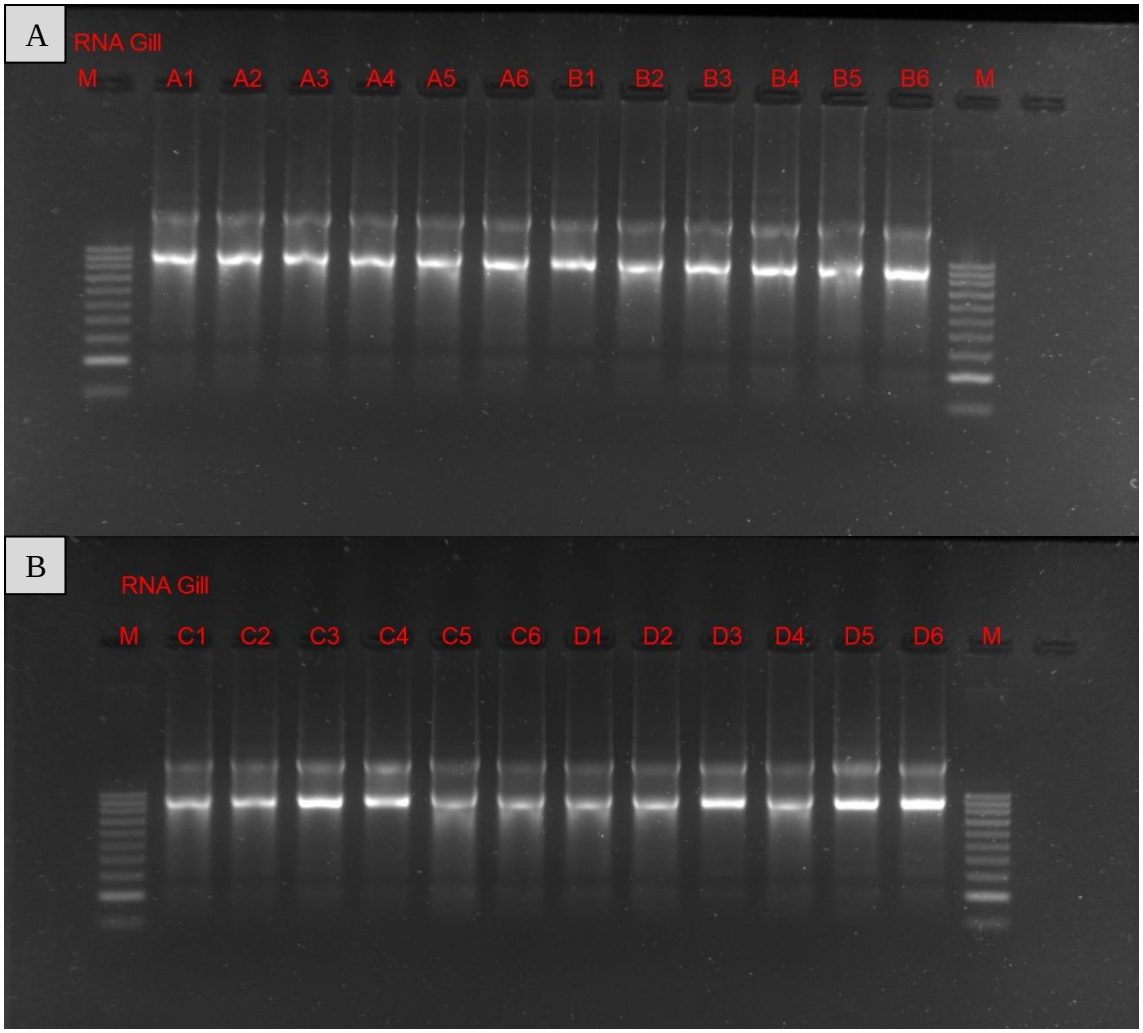

Figure 5 – Electrophorese gel for the verification of the quality and integrity of the RNA samples from gill (A - M = marker; A = treatment clove oil, B = treatment clove oil + lidocaine; C - C = treatment 2-phenoxyethanol, D = treatment 2-phenoxyethanol + lidocaine)