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Lidocaine supplementation in clove-oil and 2-phenoxyethanol anesthesia for gilthead seabream (*Sparus aurata*)

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ABSTRACT

Animal welfare and reducing stress during procedures are key objectives for success in animal production. Anesthesia has been used for procedures to reduce animal stress and its negative impact on welfare. This study aimed first to refine the concentrations of the anesthetic clove-oil (CO) and lidocaine (L) in gilthead seabream (*Sparus aurata*) juveniles (56.0 ± 15.09 g) and then combine clove-oil and 2-phenoxyethanol (2PHE) with the refined concentration of lidocaine. The concentrations of clove-oil (30, 45, and 60 mg L^{-1}), and the concentrations of lidocaine (2.5, 5, and 7.5 mg L^{-1}), were evaluated in the refinement trial. Based on these results, a second trial was performed with 45 mg L^{-1} CO or 0.4 mL L^{-1} 2PHE as anesthetics alone or combined with 2.5 mg L^{-1} of lidocaine. Results from this work showed an improvement in induction times for 2-phenoxyethanol when lidocaine was added (2PHE 179.53 ± 63.21 s; 2PHE + L 130.65 ± 40.16 s). Recovery time also showed a reduction for clove-oil when lidocaine was used (CO 349.90 ± 123.69 s; CO + L 250.11 ± 51.99 s). The use of lidocaine showed better results, reducing lactate and histological progressive alterations. Lidocaine showed stress-induced oxidative alterations when it was combined with 2-phenoxyethanol. Lidocaine exposure increased ALT, AST, histological regressive alterations for both anesthetics, and gene expression of *hsp70* in the gills when clove-oil was used. Further studies are necessary to comprehend the synergistic effects of lidocaine when combined with synthetic and natural anesthetics and to discern potential acute or chronic toxic responses in fish. These insights will be crucial for refining anesthesia protocols and ensuring the well-being of aquatic species in aquaculture practices and research settings.

1. Introduction

World fisheries and aquaculture procedures must be optimized for minimal stress induction and to ensure maximum animal welfare (Ross and Ross, 2008). Any procedure involving invasive methods may cause a certain degree of pain. Aquaculture and research with fish must ensure that pain, suffering, and distress are reduced to a minimum. Furthermore, the European Directive 2010/63/EU on the protection of animals used for scientific purposes, emphasizes that procedures that may cause severe pain cannot be performed without anesthesia and appropriate analgesia (European Parliament and The Council of the Europian Union, 2010).

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Anesthetic agents are commonly used for surgical procedures in fish (such as fin clipping for genetic identification) but also as the first step toward euthanasia for most laboratory fish (Readman et al., 2013). Rather than using a single substance, combining drugs with different properties results in a smoother induction, faster recovery, and a reduction in adverse effects during anesthesia (Zahl et al., 2012). In general, induction of anesthesia should be quick (up to 3 minutes) and without marked hyperactivity, although some increase in activity is usually observed (Ross and Ross, 2008). The recovery phase involves the elimination of the anesthetic agent and the return to a normal state. 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 behavior (which can be understood as stress behavior) or other side effects (Ross and Ross, 2008; Schroeder et al., 2021).

Clove-oil 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 and Ross, 2008), and its major constituent is eugenol (70–90 %) (Martins et al., 2019; Mitjana et al., 2014; Mylonas et al., 2005; Ross and Ross, 2008). The advantages of using clove-oil as an anesthetic for fish include its lower price, reduced environmental impact, greater potency compared to other anesthetics used in fish, and safety for staff (Mitjana et al., 2014; Mylonas et al., 2005). Clove-oil has a dark brown color and a rich, aromatic odor and flavor (Ross and Ross, 2008). It is insoluble in water at room temperature and requires mixing with ethanol before use (Martins et al., 2019). Clove-oil is frequently used as an anesthetic for gilthead seabream procedures. However, there is a lack of information regarding potential changes in markers such as hematological and biochemical parameters, histological alterations in target tissues and organs, cytotoxicity, and gene expression profiles when this anesthetic is used (Aydın and Barbas, 2020).

2-Phenoxyethanol is a more commonly used anesthetic. It is a clear, colorless, or straw-colored oily liquid (Martins et al., 2019; Neiffer and Stamper, 2009; Ross and Ross, 2008) with a slight odor that easily passes into the solution if diluted with a small quantity of water (Ross and Ross, 2008). This is a low-priced drug, widely used as a sedative for transportation and anesthesia which can provide light and deep anesthesia stages (Martins et al., 2019; Neiffer and Stamper, 2009). 2-Phenoxyethanol acts rapidly with a short induction time; if exposure is limited, the recovery tends to be good and fast (Mitjana et al., 2014; Ortuño et al., 2002), and there is no occurrence of pH change when added to seawater (Neiffer and Stamper, 2009).

Local anesthetics produce analgesia since they block nociceptive transmission. These chemicals tend to have a low cost, minimal side effects, and a brief recovery period when used properly (Chatigny et al., 2017). Analgesics are used in veterinary practice to alleviate pain in animals, but in fish procedures in general, they are not commonly administered. This is partially due to the debate regarding fish́s capacity to perceive pain (Mettam et al., 2011). However, several studies suggest a high possibility of fish experiencing pain (Braithwaite and Ebbesson, 2014; Rose, 2002; Sloman et al., 2019). These studies showed changes in pain-related behaviors after painful stimuli, indicating similarities between the nociceptive systems of fish and mammals. Therefore, the use of pain-relieving drugs is recommended to prevent these variations in fish (Sneddon, 2019).

Synergism between anesthetics occurs when a mixture of two or more drugs produces a greater response than expected (i.e., greater than the sum of their individual effects) (Muir, 2015). Generally, the combination of two anesthetics is likely to have a stronger effect compared to a single anesthetic because the drugs act through different mechanisms of action. Lidocaine hydrochloride is an example of a local analgesic that is relatively inexpensive and safe to handle (Martins et al., 2019). In humans and mammals, lidocaine infusion has been shown to reduce the requirements for other anesthetics used simultaneously (Altermatt et al., 2012). The hydrochloride salt is freely soluble in water (Ross and Ross, 2008). Low-dose lidocaine immersions can improve welfare in perioperative analgesia situations, which can allow the reduction of the anesthesia concentration at use (Martins et al., 2019). This analgesic agent 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 in fish, and it is still necessary to study the ideal concentration to be used in different species. In the case of gilthead seabream, there are no records of lidocaine use. The development of technical protocols that include anesthesia and analgesia has been an important part of efforts to refine methodology, reduce suffering and stress, and improve fish welfare (Schroeder and Sneddon, 2017).

This study aims to refine and evaluate the synergic effects of lidocaine combined with 2-phenoxyethanol and clove-oil anesthesia in gilthead seabream (*Sparus aurata*). Behavioral, hematology, metabolic, immune, and oxidative stress parameters, gill histology, and gene expression (brain and gills) studies were performed after anesthesia. After a preliminary research to determine the appropriate clove-oil concentrations, the potential beneficial effects of the local analgesic lidocaine as a synergistic agent with 2-phenoxyethanol and clove-oil anesthesia were compared when animals were subjected to a procedure, such as blood collection.

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, Direção 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) ensuring minimal animal stress and discomfort.

2.2. Animal maintenance

Juveniles of *S. aurata* ($n = 116$, 56.0 ± 15.09 g body mass) were maintained according to the procedures established at CETEMARES - Center for Marine and Environmental Sciences - MARE-Polytechnic of Leiria, Peniche. The fish were kept in a RAS system with a photoperiod of 12:12 h, a temperature of 20.78 ± 2.34 °C, a salinity of 33.93 ± 1.15, a pH of 7.81 ± 0.38, and a dissolved oxygen of 86.79 ± 2.79 %. Animals were fed daily (1 % of the total biomass per day) with a commercial diet (SPAROS, Portugal). Fish were fasted for 24 hours before experiment and their behavior was observed to detect any differences caused by sampling or handling. Following that, the fish were individually exposed to anesthetics and analgesic in a 15-liter aquarium. Recovery was conducted individually in a 15-liter aquarium with aeration. Fish were then housed per treatment group in a 60-liter aquarium for observation over the following 72 hours. During this period, appetite recovery was assessed through daily feed consumption according to the feeding tables.

2.3. Lidocaine refinement

The fish ($n = 8$ per concentration) were exposed to three concentrations (30, 45, and 60 mL L^{-1}) of clove-oil (Sigma Aldrich; St. Louis, Missouri, United States) dissolved in 95 % ethanol at a ratio of 1:9. The induction and recovery times for anesthesia were defined based on previous work by Mylonas et al. (2005). For the induction time assessment, the loss of equilibrium (A3) and the deep anesthesia (A5) stages were considered. For recovery time evaluation, the times for regaining equilibrium (R3) and total recovery (R5) were assessed and compared. The endpoints of induction and recovery were recorded individually. Based on the anesthesia results obtained with concentration clove-oil, the fish ($n = 4$ per concentration) were exposed to three concentrations (2.5, 5, and 7.5 mg L^{-1}) of lidocaine (Anestesin; Medinfar-Sorológico, Amadora, Portugal; concentration of 20 mg/mL) in a 5-minute bath before anesthesia. Induction and recovery times were recorded. These trials allowed for the selection of the combined clove-oil and lidocaine concentrations to be used in the following trials. The concentration of 0.4 mL L^{-1} of 2-phenoxyethanol (VWR Chemicals; Radnor, Pennsylvania, United States) was based on the literature (Mylonas et al., 2005; Velíšek et al., 2011). Four treatment groups were evaluated: clove-oil (CO); clove-oil with lidocaine (CO + L); 2-phenoxyethanol (2PHE); and 2-phenoxyethanol with lidocaine (2PHE + L). A total of 80 fish were randomly selected for analysis, with 20 fish used per treatment. From each treatment group, 20 fish were blood sampled after reaching deep anesthesia (A5). Ten fish were immediately sacrificed with twice the dose of the anesthetic used for organ collection (brain, gills, liver), while the other 10 fish were allowed to recover in the 60 L aquarium for 72-hour observation period. Fish anesthetized with 2-phenoxyethanol were given an overdose of 0.8 mL L^{-1}, while those anesthetised with clove-oil were given an overdose of 90 mg L^{-1}.

Blood was collected from the caudal vein with heparinized syringes (3000 U). Plasma was isolated by centrifugation of blood (VWR Micro Star 12, VWR; Belgium; 10 min, 10000 × g, 4 ºC) and then stored at −80 ºC. Liver samples were collected and immediately frozen in liquid nitrogen, then kept at −80 ºC until further processing. The first-gill arch from one side was fixed in 4 % buffered formaldehyde for 24 hours, then transferred to 70 % ethanol until processing. The whole brain and first-gill arch filaments from the other side were stored at −80 ºC in sterile microtubes with RNAlater (Sigma Aldrich, St. Louis, Missouri, USA).

2.4. *Hematological parameters*

The hematological profile of the fish consisted of counts of red blood cells (RBC) and white blood cells (WBC), hematocrit (Ht) (Haematokrit 200, Ref. 1801, Hettich; Germany; 10 min, 10000 × g), and hemoglobin (Hb, SPINREACT, Ref. 1001230, Spain). Consequently, it was possible to calculate the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) according to Machado et al. (2015).

2.5. *Oxidative stress parameters*

Liver tissue homogenates were obtained with 1:10 (m:v) ultrapure water, using a pellet mixer. For lipid peroxidation (LPO) testing, a 200 μL aliquot was placed in a microtube with 4 μL of 4 % BHT (2,6-Di-tert-butyl-4-methylphenol) in methanol. The remaining extract was mixed 1:1 (v:v) with potassium phosphate buffer (0.2 M, pH 7.4), centrifuged at 10000 x g at 4 °C for 20 min, and stored at −80 °C. Protein measurement of liver samples was performed through the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific; United States) for sample normalization. LPO was determined following the protocol described by Bird and Draper (1984). Catalase activity (CAT) was determined as described by Clairborne (1985) and modified for UV microplates. Superoxide dismutase activity (SOD) was obtained as described by Almeida et al. (2010). Glutathione-S-transferase activity (GST) was measured following the adapted method of Frasco and Guilhermino (2002). Total glutathione (tGSH) was determined according to Baker et al. (1990).

2.6. *Immune parameters*

Plasma peroxidase activity was determined following Quade and Roth (1997), where one unit of peroxidase represents an absorbance change of 1.0 OD at 450 nm. According to Machado et al. (2015), the antiprotease activity and, consequently, the proteases were determined. The percentage of inhibited trypsin activity is calculated using the reference sample.

For protease activity assessment, 10 μL of plasma were mixed with 100 μL of PBS (13.9 mg/mL, pH 7.0) and 125 μL of azocasein (20 mg/mL in $NaHCO_3$, pH 8.3). The samples were incubated for 24 hours at room temperature with agitation. After incubation, 250 μL of 10 % TCA (100 mg/mL) was added to stop the reaction, and the samples were read at 450 nm, as with the antiprotease activity. The percentage of trypsin activity is calculated using the reference sample.

2.7. *Metabolic parameters*

Thawed plasma samples were used to quantify glucose (Ref. 201001190), lactate (Ref. 1001330), and alkaline phosphatase (ALP, Ref. 41246), with SPINREACT (Spain) kits. Liver metabolites were measured in homogenized samples following the procedure described by Guerreiro et al. (2015) to quantify the alanine aminotransferase (ALT, Ref. 41282) and aspartate aminotransferase (AST, Ref. 41272), with SPINREACT (Spain) kits.

2.8. *Gene expression analysis*

Tissue RNA extraction and purification from the brain and gills were performed by using an NZY Total RNA Isolation Kit (NZYTech, Lisbon, Portugal), according to the supplier's instructions. RNA quantification and purification were evaluated by spectrophotometry (NanoDrop 2000, ThermoFisher Scientific, Waltham, MA, USA), and their quality and integrity were verified by electrophoresis (Wide Mini-Sub Cell GT, Bio-Rad, California, CA, USA) on 2 % agarose gel. Then cDNA synthesis was performed following the instructions of the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) on a T100TM Thermal Cycler (Bio-Rad, California, CA, USA).

Several genes (HSP70, CRH, and CRHBP) were studied as stress indicators, as well as immune response (IL1β), neuromuscular alterations (ACHE), enzymatic response (GST3), and β-actin (ACTB) was used as a reference gene (Supplement Table 1). The efficiency of the primers was evaluated through linear regression, considering the slope and the mean threshold cycle (Ct) obtained (Bio-Rad, Bio-Rad CFX Maestro 1.0, version: 4.0.2325.0418). For the gene quantitative PCR (qPCR), the CFX ConnectTM Real-Time System (Bio-Rad, California, CA, USA) was used. The qPCR reaction mixture, containing 1 μL cDNA (5-fold dilution factor), 0.3 μL of each primer reverse and forward (at 10 μM each), and 5 μL iTaqTM Universal SYBR® Green Supermix (Bio-Rad, California, CA, USA) at a final volume of 10 μL. The standard cycle conditions were 3 min at 95 ºC for initial denaturation, followed by 40 cycles of 30 s at 95 ºC denaturation and annealing/extension for 20 s with temperature-specific conditions for each set of primers (Supplement Table 1). All the samples were analysed in triplicate and normalized with the reference gene. The gene expression was determined according to the Pfaffl (2001).

2.9. *Histological analysis*

Gills were processed and embedded in paraffin. The Section (5 μm) were cut on a microtome (Sakura Accu-Cut® SRMTM Rotary) and stained with hematoxylin and eosin. An optical microscope (Leica DM2000LED) with a digital camera (Leica MC 170 HD) was used to observe and photograph the gill structures. The gills were examined for potential structural and morphological changes at the circulatory (blood congestion; hemorrhage and aneurism (telangiectasis)), inflammatory (leukocyte infiltration: lymphocytes and mast cells), progressive (lamellar hyperplasia; hypertrophy and hyperplasia from chloride cells); and regressive (epithelium lifting; epithelium rupture) levels (Mitchell et al., 2012; Ortiz-Delgado et al., 2007; Rodrigues et al., 2019) Posteriorly, the alterations were classified with a histoscore adapted from Mitchell et al. (2012).

2.10. *Statistical analysis*

To estimate the number of fish to use, a power calculation was done using the online tool available at https://www.stat.ubc.ca/~rollin/stat s/ssize/ from the University of British Columbia, Vancouver, BC Canada. Statistical analysis was performed using the IBM SPSS program for Windows, version 28 (IBM Corporation, Armonk, New York, USA). All anesthesia refinement data, when normality and homogeneity of variance were met, were subjected to a two-way ANOVA, with the anesthetics used (2-phenoxyethanol and clove-oil) and the use of analgesia (with or without lidocaine) as factors. If a significant interaction was detected, a one-way ANOVA followed by Tukey's test was conducted. A Kruskal-Wallis test was used in cases where homogeneity was rejected. The results were expressed as a mean ± standard deviation (SD), and the differences between experimental groups were considered statistically significant at the significance level of $P < 0.05$.

3. Results

3.1. Lidocaine refinement

The anesthesia refinement showed different induction times between clove-oil concentrations (Supplement Table 2). Concentrations of 45 mg L^{-1} and 60 mg L^{-1} were found to induce full anesthesia (deep anesthesia – A5) in less than 180 seconds (3 minutes) and allow for recovery in less than 600 seconds (10 minutes). Due to these results, a 45 mg L^{-1} concentration of clove-oil was used in the following trials.

The refinement of lidocaine did not result in variations between the three concentrations when combined with the anesthetics at any stage of anesthesia (see Supplement Table 3). Fish exposed to all combined lidocaine concentrations showed an agitation behavior. This response became stronger with increasing concentrations (observed data). The lowest lidocaine concentration (2.5 mg L^{-1}) was used for further trials.

The concentrations of clove-oil and lidocaine used were previously determined (CO 45 mg L^{-1} and 2.5 mg L^{-1} of lidocaine) and were compared with the reference concentration of 0.4 mL L^{-1} of 2-phenoxyethanol. Results showed a reduction in induction time when CO and 2PHE + L were used compared with 2-phenoxyethanol. Data showed a reduction in recovery time after the procedure (blood collection), when lidocaine was used (Fig. 1). The CO treatment showed statistically longer recovery times compared to the treatments that used lidocaine. 2PHE + L and CO + L showed the best recovery times, being significantly faster. Animals regained their appetite on the same day as the trial and exhibited normal behavior, with no observed mortality.

3.2. Hematological parameters

The hematological profile of gilthead seabream exposed to the anesthetics, with or without lidocaine (Table 1), did not show statistical differences between groups.

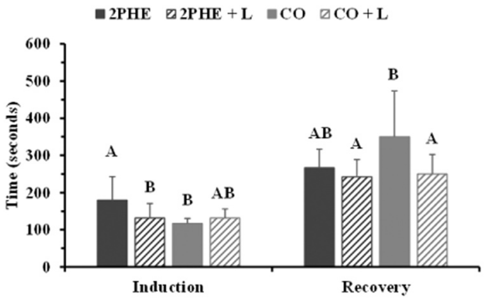

Fig. 1. – Time required to induce anesthesia and recover from 2-phenoxyethanol and clove-oil with or without lidocaine (mean ± SD, n = 10). Different letters stand for statistical differences between exposure treatments $P < 0.05$. 2PHE – 2-phenoxyethanol, 0.4 mL L^{-1}; 2PHE + L – 2-phenoxyethanol 0.4 mL L^{-1} with lidocaine 2.5 mg L^{-1}; CO – clove-oil 45 mg L^{-1}; CO + L – clove-oil 45 mg L^{-1} with lidocaine 2.5 mg L^{-1}.

Table 1
– Hematological profile of gilthead seabream *Sparus aurata* exposed to 2-phenoxyethanol and clove-oil, with or without lidocaine.

	2PHE	**2PHE + L**	**CO**	**CO + L**
RBC (x 10^6 μL^{-1})	2.82 ± 0.51	3.21 ± 0.76	2.92 ± 0.30	3.15 ± 0.54
WBC (x 10^4 μL^{-1})	6.27 ± 2.01	5.54 ± 1.86	5.42 ± 1.42	5.08 ± 1.01
Ht (%)	28.33 ± 3.37	28.56 ± 2.40	32.10 ± 3.75	29.13 ± 3.36
Hb (g dL^{-1})	2.36 ± 0.50	2.57 ± 0.63	2.58 ± 0.73	2.74 ± 0.69
MCV (μm^3)	97.94 ± 17.09	89.88 ± 20.33	114.31 ± 22.53	91.17 ± 20.17
MCH (pg cell^{-1})	8.46 ± 2.22	7.93 ± 1.69	8.41 ± 1.35	8.64 ± 2.70
MCHC (g 100 mL^{-1})	8.42 ± 1.35	9.55 ± 2.45	8.55 ± 1.86	7.66 ± 0.73

The values are given as the mean ± standard deviation (n = 10). 2PHE – 2-phenoxyethanol 0.4 mL L^{-1}; 2PHE + L – 2-phenoxyethanol 0.4 mL L^{-1} with lidocaine 2.5 mg L^{-1}; CO – clove-oil 45 mg L^{-1}; CO + L – clove-oil 45 mg L^{-1} with lidocaine 2.5 mg L^{-1}. RBC: red blood cells; WBC: white blood cells; Ht: hematocrit; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

3.3. Oxidative stress parameters

Fig. 2 shows the oxidative stress biomarkers results when fish were exposed to 2-phenoxyethanol or clove-oil, with or without lidocaine. The lipid peroxidation in seabream exposed to 2PHE + L was significantly higher compared to 2PHE. Catalase activity in seabream exposed to CO and CO + L was significantly lower than in 2PHE and 2PHE + L. Total glutathione was significantly lower in 2PHE + L, compared to CO and CO + L. In superoxide dismutase, 2PHE + L was significantly higher compared to 2PHE and CO + L. The glutathione s-transferase did not showed statistical differences.

3.4. Immune parameters

Plasmatic immune parameters only showed differences among exposures in protease activity (Table 2). The single exposure to CO (8.08 ± 1.57 %) was significantly lower than in the 2PHE (9.48 ± 0.62 %) and 2PHE + L (9.79 ± 0.92 %) treatment groups.

3.5. Metabolic parameters

Plasmatic and liver metabolites showed differences in all parameters (Fig. 3). Glucose levels were higher in the CO treatment compared to 2PHE and CO + L. Lactate levels were lower in the treatments with lidocaine, especially in the CO group. The alkaline phosphatase (ALP) for 2PHE + L values were significantly lower than for 2PHE and CO + L results. In the liver metabolites, the alanine aminotransferase (ALT) showed statistically higher values when lidocaine was used. The aspartate aminotransferase (AST) showed similar tendencies, but CO results were significantly lower than the other treatment groups.

3.6. Gene expression analysis

The analysis of gene expression in the gills revealed differences between treatment groups in only one gene (Table 3). Results showed upregulation of *hsp70* when CO was used, with or without lidocaine, compared to 2PHE + L.

3.7. Histological analysis

In the circulatory and inflammatory alterations, there were no differences between anesthetics (Fig. 4 A). In the progressive alterations, the lamellar hyperplasia showed higher damage when 2PHE was used compared to CO with and without lidocaine. Also, a higher damage in hyperplasia from chloride cells was observed for 2PHE compared to CO + L. Lidocaine reduced these changes for all anesthetics (Fig. 4B). On the

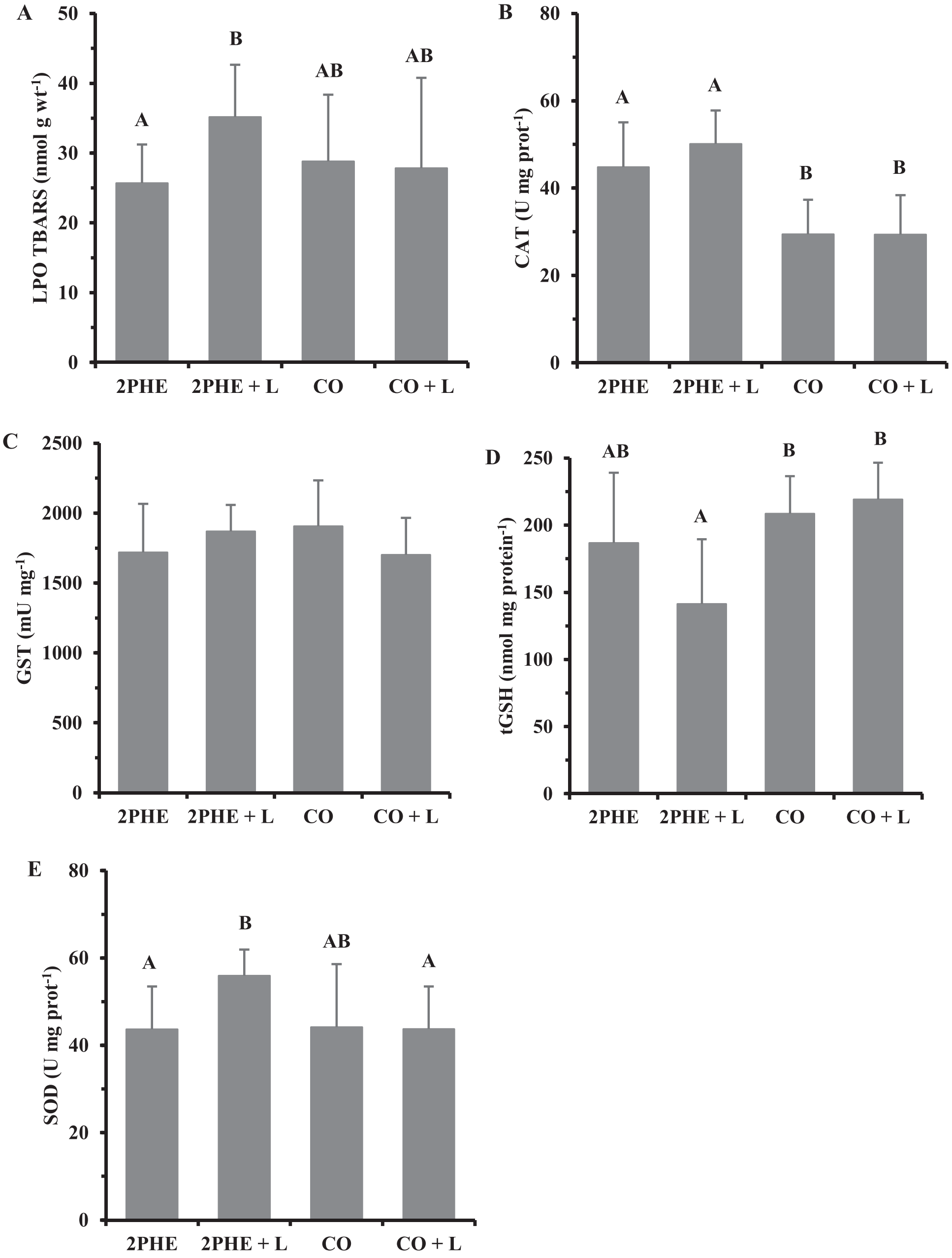

Fig. 2. – Oxidative stress parameters in gilthead seabream *Sparus aurata* liver exposed to 2-phenoxyethanol and clove-oil with or without lidocaine (mean ± SD, n = 10). Different letters stand for statistical differences between exposure treatments, $P < 0.05$. 2PHE – 2-phenoxyethanol 0.4 mL L^{-1}; 2PHE + L – 2-phenoxyethanol 0.4 mL L^{-1} with lidocaine 2.5 mg L^{-1}; CO – clove-oil 45 mg L^{-1}; CO + L – clove-oil 45 mg L^{-1} with lidocaine 2.5 mg L^{-1}. A) Lipid peroxidation (LPO); B) Catalase (CAT); C) Glutathione s-transferase (GST); D) Total glutathione (tGSH); E) Superoxide dismutase (SOD).

contrary, in the regressive alterations, the epithelium lifting and the rupture from pillar cells showed higher damage when lidocaine was used in the clove-oil treatment compared to the single use of anesthetic. In the epithelium rupture, 2PHE + L and CO + L had higher damage than CO (Fig. 4 C). Regressive alterations showed better results when CO was used alone. Fig. 5 illustrates the histomorphology alterations in the gill structures.

4. Discussion

In the aquaculture industry, gilthead seabream (*S. aurata*) is a species of great economic interest (Toffan et al., 2017). In this production, the animals may be subjected to several procedures that may require the use of anesthetics to minimize stress (Zahl et al., 2012). Research has been conducted to improve anesthesia protocols and ensure animal welfare while also considering cost and effectiveness (Mylonas et al., 2005; Zahl et al., 2012). In laboratory practices, a significant number of fish will undergo anesthesia, but for most of them, their initial and only experience with an anesthetic substance will occur during the process of humane euthanasia (Readman et al., 2017). General anesthetics are beneficial for immobilization, reducing stress and pain, collecting biological material, and providing pain relief when a human endpoint is reached. However, as anesthetic drugs actions are not limited to the nervous system, the methodology of anesthesia and euthanasia may interfere with the study results (Achtymichuk et al., 2022). To the best of our knowledge, no studies on gilthead seabream with lidocaine have been published.

In the refinement of clove-oil anesthesia using different

Table 2

– Immunology profile of gilthead seabream *Sparus aurata* exposed to 2-phenoxyethanol and clove-oil, with or without lidocaine.

	2PHE	2PHE + L	CO	CO + L
Peroxidase (OD 450 nm)	1.41 ± 0.44	1.54 ± 0.46	1.87 ± 0.79	1.48 ± 0.30
Protease (%)	9.48 ± 0.62^{A}	9.79 ± 0.92^{A}	8.08 ± 1.57^{B}	9.15 ± 0.78^{AB}
Antiprotease (%)	73.83 ± 6.68	76.57 ± 1.58	78.45 ± 4.59	76.42 ± 3.95

The values are given as the mean ± standard deviation (n = 10). Different letters stand for statistical differences between exposure treatments, $P < 0.05$. 2PHE – 2-phenoxyethanol 0.4 mL L^{-1}; 2PHE + L – 2-phenoxyethanol 0.4 mL L^{-1} with lidocaine 2.5 mg L^{-1}; CO – clove-oil 45 mg L^{-1}; CO + L – clove-oil 45 mg L^{-1} with lidocaine 2.5 mg L^{-1}.

concentrations (30, 45, and 60 mg L^{-1}), 45 mg L^{-1} met the requirements for inducing anesthesia in less than 3 minutes and recovering in 5–10 minutes (Ross and Ross, 2008; Schroeder et al., 2021). This aligns with Mylonas et al. (2005) studies for *S. aurata* anesthesia, which showed optimal results with 40 mg L^{-1} at 25ºC and 55 mg L^{-1} at 15ºC. The highest tested concentration, 60 mg L^{-1}; also met the desirable induction and recovery times of anesthesia. However, using a lower concentration reduces costs for the fish farmer, has a less polluting effect on the environment (Mylonas et al., 2005), and potentially results in fewer toxic effects of anesthetics on fish after techniques such as handling, sampling, transport, tagging and grading (Hoseini et al., 2019; Martins et al., 2019).

The lidocaine refinement trial compared 2.5, 5, and 7.5 mg L^{-1} concentrations, and results showed lower induction times when the 2.5 mg L^{-1} lidocaine concentration was used in combination with clove-oil. This effect is in accordance with Zahl et al. (2009) studies, which also suggest a synergistic effect between some anesthetics in Atlantic cod (*Gadus morhua*). In the mentioned study, the agents were tested individually or in combination, involving pre-anesthetic sedation with a low dosage of metomidate or 2-phenoxyethanol followed by anesthesia with benzocaine or MS-222.

In our study, when fish were exposed to elevated concentrations of lidocaine, they tended to exhibit agitation behavior. This is correlated with anxiety-like behaviors, as found by de Abreu et al. (2019). All the fish used in the trial, which had their blood drawn and were allowed to recover, showed a return to normal appetite. Responses to anesthetic exposure showed that the combinations using 2PHE + L and CO + L had positive results after handling and blood collection. A reduction in induction time was observed from 179.53 ± 63.21 s with 2PHE to 130.65 ± 40.16 s with 2PHE + L, and also a decrease in recovery time from 349.90 ± 123.69 s with CO to 250.11 ± 51.99 s with CO + L. These results are in accordance with Zahl et al. (2009) who tested various combinations of a pre-sedative (metomidate or 2-phenoxyethanol) with an anesthetic (benzocaine or MS-222) and found that induction and recovery times tended to be shorter when pre-anesthetic sedation was combined with anesthesia, compared to the use of anesthesia alone. This result by Zahl et al. (2009) aligns with our observations, where we also noted a reduction in induction and recovery times when lidocaine was used in combination. It is evident that there was a synergistic effect leading to a shorter recovery time when the combination was used, compared to using the anesthetic alone. The use of these drugs in combination helps reduce the stress caused by the action of anesthesia alone (Zahl et al., 2009). Nevertheless, potential lidocaine side effects during general anesthesia should not be dismissed. In humans, lidocaine infusion is generally safe, but cases of hypotension, cardiovascular block and arrhythmias, neuro-excitability, and hypersensitivity reactions have been reported (Chu et al., 2020).

Oxidative stress, characterized by an imbalance favoring oxidants over antioxidants, is a key factor in fish vulnerability to damage to cellular components (Birnie-Gauvin et al., 2017). In the context of fish health, the administration of anesthetics through the gills, significant sites for xenobiotic transfer, raises concerns about potential harmful effects on organs (Velíšek et al., 2011). In the 2PHE + L treatment, we found the highest amount of SOD defense enzymatic activity along with a high amount of CAT, indicating a greater degradation of hydrogen peroxide and superoxide radicals when compared to CO and CO + L. Mousavi et al. (2023) studied the use of myrcene which has anesthetic properties as water conditioner, to reduce stress and as an antioxidant agent in the transport of common carp (*Cyprinus carpio*). They observed an increase in the activity of the enzymes SOD and CAT that did not prevent oxidative stress, and there was also a decrease in reduced glutathione. Regardless of the use of lidocaine in the clove-oil treatment groups, there was an inhibition of the defense enzymes of SOD and CAT. Despite the elevation of enzymatic defense (SOD and CAT) during 2PHE + L anesthesia, there was a significant lipid damage, as indicated by the decline in total glutathione of 2PHE + L. These results are in line with the increase in antioxidant enzyme activity activated by the fish's antioxidant system observed by Hoseini et al. (2020) in study of common carp (*Cyprinus carpio*) anesthetized by cineole.

Protease activity plays a protective role against pathogens and stressors (Guardiola et al., 2016). The increase in protease values in 2PHE treatment groups, especially in 2PHE + L, demonstrates the presence of an immune response compared to CO. This could be because the use of two synthetic chemicals increases the organisḿs immune response, whereas the use of a single CO anesthetic is less aggressive since protease activity is affected by stressors (Soltanian et al., 2018). This immune response tendency is consistent with the oxidative stress parameters observed in the 2PHE + L treatment, as previously described. Bahi et al. (2018) demonstrated that exposure to 55 ppm clove-oil for 5 minutes for *S. aurata* does not compromise the immune status of the fish.

Glucose level is one of the most used indicators of stress in fish, which is related to a secondary effect of stress (Bahi et al., 2018). This study indicates that no stress-related increase in plasma glucose was observed after gilthead seabream specimens were exposed to the anesthetic clove-oil. However, in our study, there was an increase in glucose when using clove-oil which is in line with the increase in lactate levels. Both parameters showed lower values when lidocaine was used in combination with clove-oil. Lactate is known to be produced during anaerobic conditions caused by stress, leading to its excretion into the bloodstream (Small, 2004; Velíšek et al., 2011). In our study, when lidocaine is used, regardless of the anesthetic, the stress that triggers this parameter is significantly lower. When compared to the unique use of anesthetics, the addition of lidocaine led to a faster recovery to homeostasis (Dando, 1969). Similar results were found by Small (2004), who discovered that using isoeugenol as a sedative had a significant lowering effect on plasma lactate levels during stress caused by acute oxygen depletion. Toni et al. (2015) studied the sedative effect of 2-phenoxyethanol and *Lippia alba* essential oil on the stress response of gilthead seabream (*Sparus aurata*). They found that exposure to *Lippia alba* increased the stress response when a procedure was performed, increasing the plasma concentration of glucose and lactate. In contrast, exposure to low concentrations of 2-PHE for 4 hours did not alter the gilthead sea bream's plasma metabolites. These results are in line with our clove-oil results, since it is a natural compound and an essential oil (Aydın and Barbas, 2020; Jia et al., 2022) like *Lippia alba*. Our specimens anesthetized with clove-oil and exposed to a stressful procedure showed an increase in plasma glucose and lactate compared to fish anesthetised with clove-oil and lidocaine.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are widely used to analyze hepatic damage in fish and assess health status (Yousefi et al., 2022). Our study showed an increase in ALT and AST when lidocaine was used. The elevated activity from these enzymes can indicate stress, hepatic disease, toxicity, and muscle damage (Ozer et al., 2008). The increase in these enzymes may correspond to a potential short-acting toxicity response. However, we still

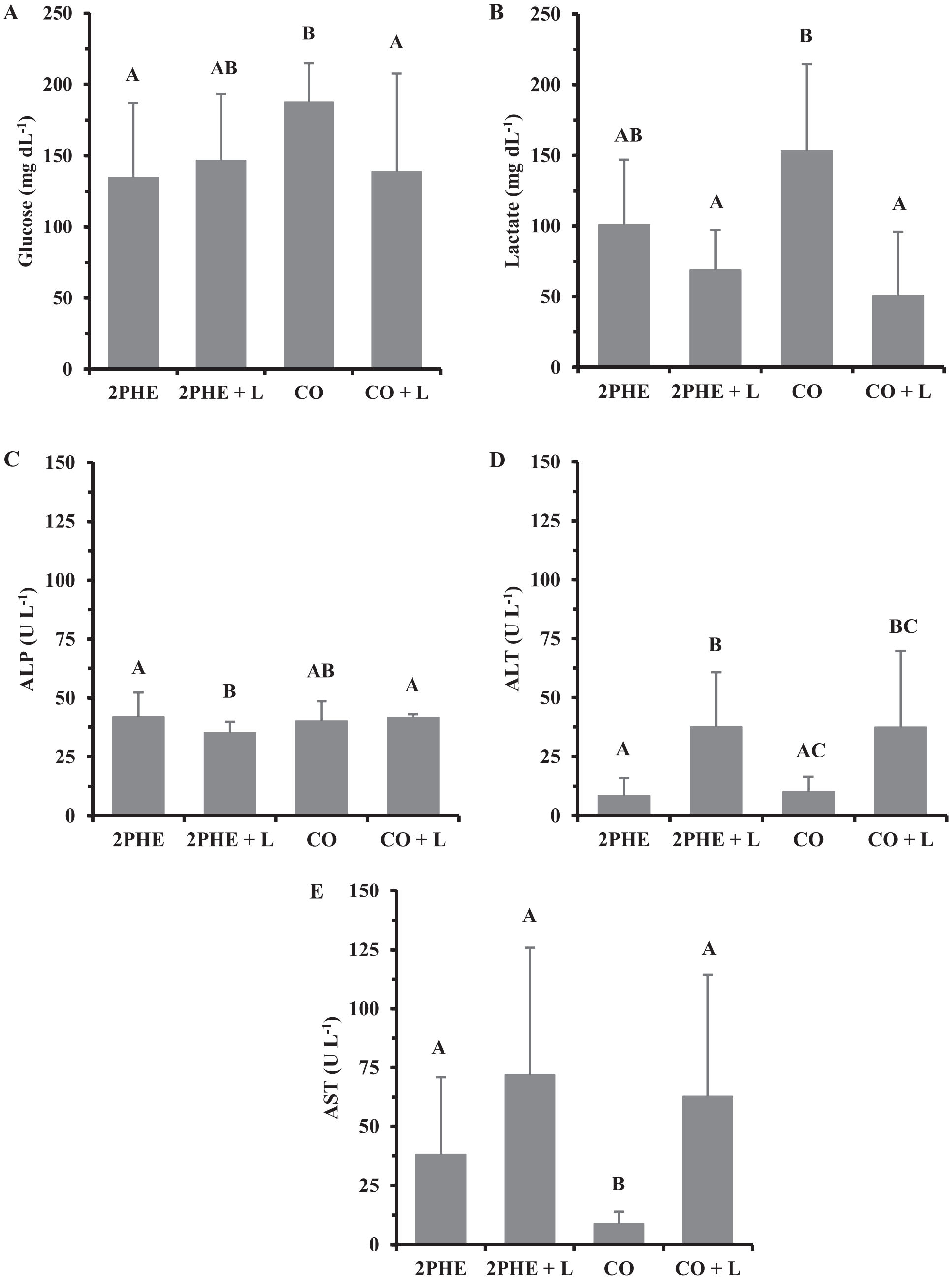

Fig. 3. – Metabolic biomarkers in gilthead seabream *Sparus aurata* plasma and liver exposed to 2-phenoxyethanol and clove-oil, with or without lidocaine (mean ± SD, n = 10). Different letters stand for statistical differences between exposure treatments, $P < 0.05$. 2PHE – 2-phenoxyethanol 0.4 mL L^{-1}; 2PHE + L – 2-phenoxyethanol 0.4 mL L^{-1} with lidocaine 2.5 mg L^{-1}; CO – clove-oil 45 mg L^{-1}; CO + L – clove-oil 45 mg L^{-1} with lidocaine 2.5 mg L^{-1}. A) Glucose; B) Lactate; C) Alkaline Phosphatase (ALP); D) Alanine Aminotransferase (ALT); E) Aspartate Aminotransferase (AST).

don't know if these changes will have prolonged health implications since our study evaluated acute exposure. The change in alkaline phosphatase activity can be considered a good indicator of stress. Its increase is seen as a protective action by immune and enzymatic molecules (Soltanian et al., 2018). In our study, the decrease of ALP in 2PHE + L group is in agreement with the observed ALT and AST levels.

Heat shock proteins (HSP) mediate protection against oxidative damage, allowing cross-protection from additional stressors (Eissa et al., 2017; Oksala et al., 2014). HSP synthesis is also regulated by a variety of stressors and plays a key role in maintaining homeostasis at the cellular level (Bodur et al., 2018; Yu et al., 2020). The previously discussed high hepatic levels of AST and ALT activities observed in CO + L, together with the upregulation of *hsp70* in fish gills, indicate a potential increase in stress and toxicity. In fish, an increased expression of *hsp70* not only reflects the body's response to oxidative stress but also mitigates the adverse effects of oxidative stress with its antioxidant function (Bodur et al., 2018; Yu et al., 2020). Teles et al. (2019) showed a significant increase in *hsp70* expression in the brain and liver of *S. aurata* (42.7 g) after exposure to sedation concentrations of MS222 (5 mg L^{-1}) and clove-oil (2.5 mg L^{-1}) during transport.

There is a link between lamellar and chloride cell hyperplasia and progressive histological changes, as these cells play a role in ionic transport and have the potential to be involved in detoxification (Mokhtar, 2017). The increase in chloride cells in 2PHE treatment could be a defense mechanism against the anesthetic in an attempt to maintain homeostasis (Macirella and Brunelli, 2017). The use of lidocaine

Table 3 –
Quantitative gene expression in the brain and gills of gilthead seabream *Sparus aurata* exposed to 2-phenoxyethanol and clove-oil, with or without lidocaine.

		2PHE	2PHE + L	CO	CO + L
hsp70	Brain	0.90 ± 0.20	0.77 ± 0.10	1.02 ± 0.19	0.97 ± 0.23
	Gills	0.29 ± 0.12^{AB}	0.22 ± 0.17^{A}	0.51 ± 0.31^{B}	0.70 ± 0.36^{B}
crh	Brain	0.78 ± 0.22	0.65 ± 0.16	0.75 ± 0.17	0.84 ± 0.13
	Gills	0.89 ± 0.41	0.52 ± 0.41	0.54 ± 0.37	0.59 ± 0.31
crhbp	Brain	1.08 ± 0.64	1.74 ± 0.98	1.12 ± 0.93	1.12 ± 0.56
	Gills	1.15 ± 0.48	0.66 ± 0.49	0.53 ± 0.42	0.64 ± 0.34
il1β	Brain	1.37 ± 0.29	1.18 ± 0.30	1.50 ± 0.65	1.30 ± 0.35
	Gills	1.40 ± 0.27	1.88 ± 1.44	0.85 ± 0.52	1.49 ± 0.78
ache	Brain	1.10 ± 0.33	0.95 ± 0.23	0.96 ± 0.21	1.05 ± 0.37
	Gills	0.59 ± 0.21	0.46 ± 0.32	0.72 ± 0.55	0.38 ± 0.13
gst3	Brain	0.32 ± 0.09	0.56 ± 0.14	0.50 ± 0.26	0.37 ± 0.10
	Gills	1.24 ± 0.63	0.64 ± 0.43	0.55 ± 0.35	0.54 ± 0.24

The values are given as the mean ± standard deviation (n = 6). Different letters stand for statistical differences between exposure treatments, $P < 0.05$. 2PHE – 2-phenoxyethanol 0.4 mL L^{-1}; 2PHE + L – 2-phenoxyethanol 0.4 mL L^{-1} with lidocaine 2.5 mg L^{-1}; CO – clove-oil 45 mg L^{-1}; CO + L – clove-oil 45 mg L^{-1} with lidocaine 2.5 mg L^{-1}.

provides a positive effect, with both used anesthetics, causing less damage in the progressive alterations than when the anesthetics were used alone. The hypertrophy of the chloride cells can be seen as an adaptive response due to their ionic function to balance with the external environment (Strzyżewska-Worotyńska et al., 2017).

In terms of regressive histological changes, the use of lidocaine showed higher damage, leading to the rupture of the epithelium and consequently the pillar cells. Subsequent exposure can cause lifting and rupture of the epithelium which may be related to the inability of pavement cells to adapt, resulting in consequential damage (Reddy and Rawat, 2013). Santos et al. (2020) demonstrated some gill alterations in tambaqui (*Colossoma macropomum*) caused by exposure to tea tree and clove essential oils. Since these changes did not induce harmful morphological alterations in the epithelium, they can be considered adaptative alterations rather than damage. Jia et al. (2022) observed significant changes in gill histology using clove-oil compared to MS 222, potentially causing damage to the spotted knifejaw (*Oplegnathus punctatus*).

In our study, the balance between damage and benefit is at stake, concerning whether lidocaine can be beneficial across different anesthetics. It has been shown that lidocaine does not appear to cause acute toxicity to zebrafish when exposed for 10 minutes at different concentrations (Collymore et al., 2016). However, any drug used in an animal (including humans) for a short time will have an effect on detoxification levels, involving changes in gene expression, enzymes, and tissues. After a few days, these changes may return to basal levels due to homeostasis.

5. Conclusions

In summary, this study on *S. aurata*, showed that the optimal concentration for clove-oil and 2-phenoxyethanol combined with lidocaine resulted in a synergistic effect, reducing both induction and recovery times. Lidocaine administration also led to decreased lactate levels and mitigated progressive histological alterations. However, it was associated with a significant increase in ALT and AST levels, indicating a potentially toxicological response. Regressive histological changes were more pronounced with lidocaine use. Oxidative stress biomarkers, such LPO, CAT, and SOD increased with 2-phenoxyethanol combined with lidocaine. Clove-oil as an anesthetic showed a trend of increasing parameters with negative impacts compared to 2-phenoxyethanol, which was supported by *hsp70* expression in the gills. Further studies are necessary to understand the synergistic effects of lidocaine when combined with synthetic and natural anesthetics, as well as to discern potential acute or chronic toxic responses in fish. These insights will be crucial for refining anesthesia protocols and ensuring the well-being of aquatic species in aquaculture practices and research settings.

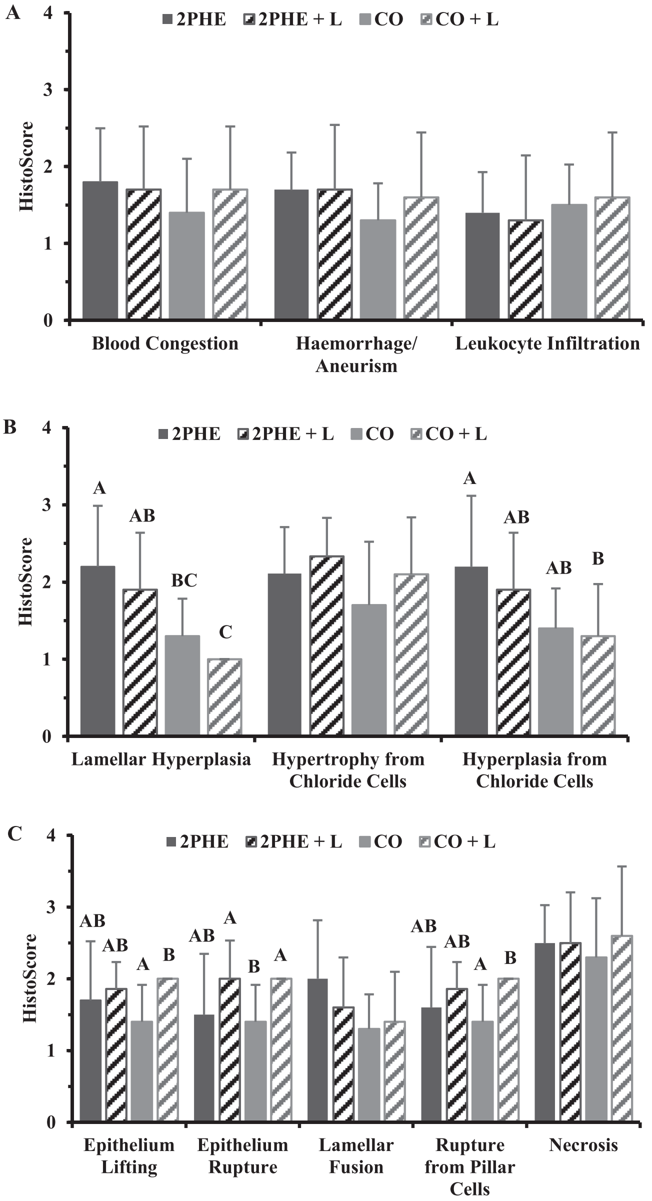

Fig. 4. – Histology score of gill morphology in gilthead seabream *Sparus aurata* exposed to 2-phenoxyethanol and clove-oil, with or without lidocaine (n = 10). A) Circulatory and inflammatory alterations; B) progressive alterations; C) regressive alterations. Normal morphology is associated with a score of "1", mild morphology damage is "2", moderate gill damage is "3", while severe tissue damage is represented by "4". Different letters stand for statistical differences between exposure treatments, $P < 0.05$. 2PHE – 2-phenoxyethanol 0.4 mL L^{-1}; 2PHE + L – 2-phenoxyethanol 0.4 mL L^{-1} with lidocaine 2.5 mg L^{-1}; CO – clove-oil 45 mg L^{-1}; CO + L – clove-oil 45 mg L^{-1} with lidocaine 2.5 mg L^{-1}.

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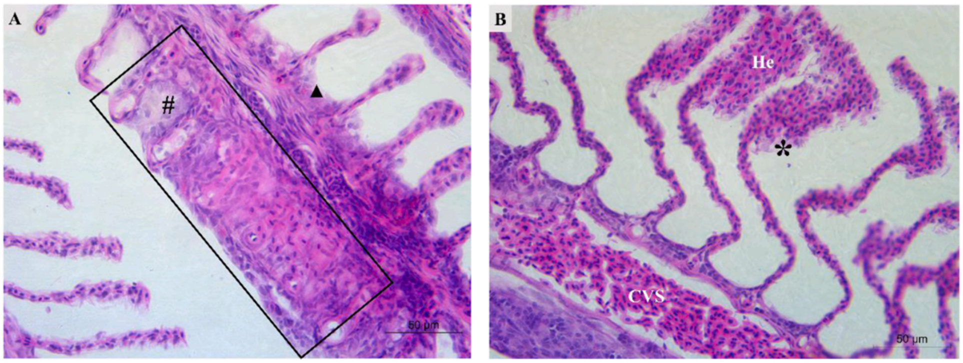

Fig. 5. – Histology of gilthead from exposed seabream *Sparus aurata* gills to 2-phenoxyethanol and clove-oil, with or without lidocaine, haematoxylin and eosin, 400×. A) Lamellar hyperplasia (#) lamellar fusion (▭), and chloride cells hyperplasia (▲); B) Hemorrhage (He) in the second lamella with epithelium rupture (*), and central venous sinus (CVS).

CRediT authorship contribution statement

Damiana Pires: Writing – review & editing, Investigation, Data curation. **Mariana Vaz:** Writing – review & editing, Investigation, Data curation. **Carolina F. Tchobanov:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Teresa Baptista:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Luís M. Antunes:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Conceptualization. **Ricardo Passos:** Writing – review & editing, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2024.102224.

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