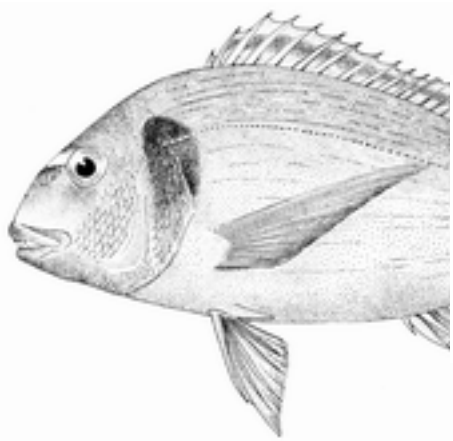

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Physiological effects of three different anesthetics: 2-phenoxyethanol, tricaine methanesulfonate and clove oil on *Sparus aurata*

Ricardo Manuel Pereira Pires
Recursos Biológicos Aquáticos
Departamento de Biologia
2017

Orientador
Doutora Helena Peres, Investigadora auxiliar, CIIMAR

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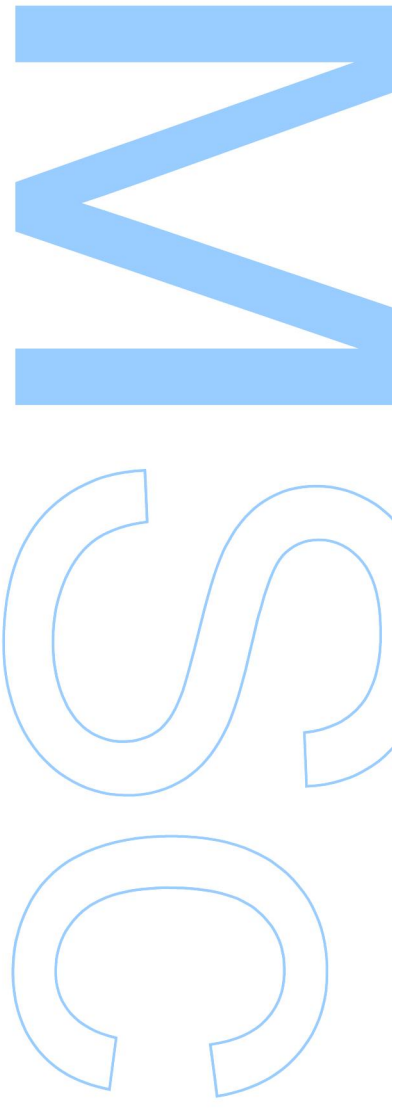

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Publications associated to this Master thesis

Part of this study was presented as a poster presentation with the title: ‘’Optimal dose of three anesthetics and their effect on plasma metabolites in gilthead seabream (*Sparus aurata*).’’ in 2nd AQUAIMPROVE Workshop, which took place at CIIMAR, in Oporto (Portugal), on 17th March 2017.

Aqualmprove
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5 **OPTIMAL DOSE OF THREE ANESTHETICS AND ITS EFFECTS ON PLASMA METABOLITES IN GILTHEAD SEABREAM (*Sparus aurata*) JUVENILES**

Pires, R.[1], Magalhães, R.[1,2], Diaz-Rosales, P.[2], Oliva-Teles, A.[1,2], Peres, H.[1,2]

[1]Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre, Edifício FC4, 4169-007 Porto, Portugal
[2]CIMAR/CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos, 4450-208 Matosinhos, Portugal
*Email: ricardomppires@gmail.com

Abstract

Blood sampling has been routinely used in fish nutrition research, and many of the commonly applied sampling techniques require anesthesia. Acute effects of sampling and anesthesia procedures may confound the results, and those potential effects are not well studied. The present work aimed to evaluate the effects on blood biochemical parameters, efficacy and optimal dose for induction of anesthesia of three anesthetics: tricaine methanesulfonate (MS-222), 2-phenoxyethanol (2-PE) and eugenol. For that purpose, seabream juveniles (IBW of 153,3g) were randomly distributed by 12 tanks and fed a commercial diet for 1 month. The optimal dose for each anesthetic was established using seabream juveniles (IBW of 153g) by dose-response tests, measuring the anesthesia induction and recovery times. Thereafter, 3 fish per tank (9 per treatment) were anesthetized with the optimal dose of each anesthetic established before, or not anesthetized, and blood samples were collected. Eugenol induced anesthesia faster at lower concentrations than 2-PE and MS-222, while its recovery time was 6 to 10 times higher than the other anesthetics when exposed to the same concentrations. 2-PE required 3 times higher anesthesia induction time at lower concentrations than the other anesthetics, while its recovery time was similar to that of MS-222. Irrespectively of the anesthetics used, plasma glucose levels were higher in nonanesthetized than in anesthetized fish. Lactate and triglyceride levels were lower in eugenol anesthetized fish than in fish non-anesthetized or anesthetized with MS-222 or 2-PE. No differences were observed in plasma protein, albumin, and cholesterol levels between non-anesthetized and anesthetized fish. Overall, results indicate that sampling procedures may affect plasma metabolites level, and this should be considered when designing experiments that require blood sampling in fish.

Keywords: Anesthesia; Anesthetics; Stress; Aquaculture; Metabolism.

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Abstract

The aquaculture industry is currently the fastest-growing food sector in the world, demonstrating to be an adequate solution to meet the world's fish demand. The intensification of aquaculture has increased fish susceptibility to several aquaculture practices stressors, which may have several harmful side effects. To mitigate such effects, anesthesia has been used in aquaculture. Also, at the research level, anesthesia has been used for invasive studies. Although it is widely used, for the majority of aquaculture species, its metabolic and physiological interactions are still unknown that, at the research level, it may confound the study results.

Under this context, the present work aimed to determine the optimal dose of three anesthetics (tricaine methanesulfonate (MS-222), 2-phenoxyethanol (2-PE) and clove oil) to induction of deep anesthesia in ongrowing gilthead seabream. The collateral effects of anesthesia on hematology, plasma metabolites profile, cortisol levels, and hepatic activity of key enzymes of intermediary metabolism and heat shock protein gene expression were evaluated and compared to non-anesthetized fish. For that purpose, ongrowing gilthead seabream (IBW of 153 g) were randomly distributed by 12 tanks and fed a commercial diet for 1 month. The optimal dose for each anesthetic was established by dose-response tests, measuring the anesthesia induction and recovery times after deep anesthesia with 5 different anesthetic doses (2-PE: 0.15; 0.3; 0.45; 0.6 and 0.75 mL/L; MS-222: 50, 100, 150, 200 and 250 mg/L, and clove oil: 0.2; 0.4; 0.6; 0.8 and 1 mL/L). Then, after a 14 days recovery period, 3 fish per tank (9 per treatment) were anesthetized, with the optimal dose of each anesthetic established before, or not anesthetized, and blood samples were collected. Then fish were euthanized with a sharp blow in the head and liver was sampled.

Clove oil induced anesthesia faster at lower concentrations than 2-PE and MS-222, while its recovery time was two-times higher than with other anesthetics, for concentrations higher than 0.2mL/l. 2-PE required 2 to 3 times higher anesthesia induction time, when used at concentrations between 0.15 and 0.45 mL/L, than the other anesthetics, while its recovery time was similar to that of MS-222.

Irrespective the anesthetic used, plasma glucose levels were higher in non-anesthetized than in anesthetized fish. Lactate and triglyceride levels were lower in clove oil anesthetized fish than in fish non-anesthetized. No differences were

observed in plasma protein, albumin, and cholesterol levels between non-anesthetized and anesthetized fish. Clove oil anesthetized group showed the lowest plasma LDH activity and the highest CK activity among the other treatments. Plasma cortisol levels were highest in fish anesthetized with 2-PE which were more than double of the control group, while the fish anesthetized with MS-222 and clove oil showed a decrease in plasma cortisol levels.

Regarding liver enzyme activity, there were differences in Alanine aminotransferase, aspartate aminotransferase, hexokinase, and glucose-6-phosphate dehydrogenase. Alanine aminotransferase and aspartate aminotransferase activity of control group was lower than that of 2-PE group, while glutamate-dehydrogenase was not affected by the anesthetic. Hexokinase activity was lower in the clove oil group than that of the other groups, while glucose-6-phosphate dehydrogenase was lower in the clove oil group that in the MS-222 group. Gene expression of heat shock protein 70 kDa was not affected by the anesthetic treatments.

Optimal doses for deep anesthesia for ongrowing gilthead seabream, providing the combined shortest induction and recovery times, was determined to be 0.45 mL/L for 2-PE, 150 mg/L for MS-222 and 0.6 mL/L for clove oil.

Results indicate that sampling procedures may affect plasma metabolites levels and liver enzyme activity, and this should be considered when designing experiments that require fish anesthesia.

The observed reduction of glycemia, plasma cortisol levels and RBC count in the fish anesthetized with clove oil, compared to the other anesthetics, suggest that clove oil is an effective anesthetic for ongrowing gilthead seabream. Clove oil not only sedates gilthead seabream but also inhibits the primary and secondary stress responses and its by-products. Moreover, clove oil is a natural and inexpensive product with an easy preparation and a safe handling.

Keywords: 2-phenoxyethanol; anesthesia; aquaculture; clove oil; gilthead seabream; metabolism; physiology; stress; tricaine methanesulfonate.

Resumo

A indústria da aquacultura eé actualmente o sector alimentar com maior crescimento, demonstrando ser uma solução adequada para satisfazer as necessidades do consumo mundial de peixe. A intensificação da aquaculture aumentou a susceptibilidade dos peixes aos vários stressores da prática da aquaculture, o que traz efeitos secundários nefastos. Para mitigar tais efeitos, são usados anestésicos. A anestesia também é usada em contexto de investigação, para estudos invasivos. Apesar de serem vastamente usados, as suas interações metabólicas e fisiológicas são desconhecidas e também se alteram o resultado de um estudo.

Neste contexto este trabalho visa avaliar os efeitos nos parâmetros bioquímicos do sangue, eficácia e a dose óptima para indução de anesthesia de três anestésicos: tricaína metanosulfonato (MS-222), 2-fenoxietanol (2-PE) e óleo de cravo.

Para esse propósito, juvenis de dourada (peso médio de 153,3g) foram aleatoriamente distribuídos por 12 tanques e alimentados com uma ração commercial durante um mês. A dose óptima foi estabelecida através de testes de dose-resposta, ao medir o tempo de indução e de recuperação de 5 concentrações diferentes (2-PE: 0.15; 0.3; 0.45; 0.6 e 0.75 mL/L; MS-222: 50, 100, 150, 200 e 250 mg/L, and clove oil: 0.2; 0.4; 0.6; 0.8 e 1 mL/L). Depois de um período de recuperação de 14 dias, 3 peixes por tanque (9 por tratamento) foram anestesiados com a dose óptima de cada anestésico estabelecida previamente, ou não anestesiados, e amostras de sangue foram colhidas. Seguidamente os peixes foram eutanasiados com um golpe na cabeça e o fígado foi colhido.

O óleo de cravo induziu a anestesia mais rapidamente, em concentrações mais baixas, do que o MS-222 e o 2-PE, enquanto que o seu tempo de recuperção foi duas vezes mais alto do que os outros dois anestésicos, para concentrações mais altas do que 0.2 mL/L. O 2-PE requiriu tempos de indução 2 a 3 vezes maiores, quando usado em concentrações entre 0.15 e 0.45 mL/L, do que os outros anestésicos, enquanto que o seu tempo de recuperação foi similar ao MS-222.

Independentemente do anestésico utilizado, os níveis de glucose no plasma foram mais altos nos peixes não anestesiados do que nos peixes anestesiados. Os níveis

de lactate e triglicerídeos foram mais baixos nos peixes anestesiados com óleo de cravo do que nos peixes não anestesiados. Não foram observadas diferenças nos níveis de plasma de proteína, albumina e colesterol entre os peixes não anestesiados e anestesiados. O grupo de peixes anestesiados com óleo de cravo mostrou os níveis de LDH no plasma mais baixos e os níveis de actividade da CK mais altos em relação aos outros tratamentos. Os níveis de cortisol no plasma foram mais altos no grupo anestesiado com 2-PE, que foram mais do dobro do grupo de control, enquanto que os peixes anestesiados com MS-222 e óleo de cravo mostraram um decréscimo nos níveis de cortisol no plasma.

Em relação às enzimas do fígado, houve diferenças na actividade da alanina aminotransferase, aspartato aminotransferase, hexokinase e glucose-6-fosfato desidrogenase. A actividade da alanina aminotransferase e aspartato aminotransferase do grupo control foi mais baixa do que o grupo 2-PE, enquanto que a actividade da glutamato desidrogenase não foi afectada pelo anestésico. A actividade da hexokinase foi mais baixa no grupo do óleo de cravo em relação a todos os outros grupos, enquanto que a da glucose-6-fosfato desidrogenase foi mais baixa no grupo do óleo de cravo do que no grupo do MS-222. A expressão génica de heat shock protein 70 kDa não foi afectada pelo tratamento dos anestésicos.
As doses óptimas para anestesia profunda de juvenis de dourada, dando os tempos de indução e recuperação mais curtos, foram determinadas ser as seguintes: 0.45 mL/L para 2-PE, 150 mg/L para MS-222 e 0.6 mL/L para óleo de cravo.

Os resultados indicam que o método de amostragem podem afectar os metabolitos do plasma e a actividade de enzimas do fígado, e que isso deve ser tomado em conta quando se realizam estudos que necessitem de anestesia em peixes.

A redução da glicémia, níveis de cortisol no plasma e contagem de glóbulos vermelhos no sangue observado nos peixes anestesiados com óleo de cravo, comparado com os outros anestésicos, sugerem que o óleo de cravo é um anestésico eficaz para juvenis de dourada. O óleo de cravo não só causa a sedação dos peixes mas também inibe as respostas primária e secundária ao stress e os seus produtos resultants. Adicionalmente, o óleo de cravo é um produto natural e de baixo custo, e de fácil preparação e seguro para manuseamento.

Palavras-chave: 2-phenoxyethanol; anestesia; aquacultura; dourada; fisiologia; metabolismo; óleo de cravo; stress; tricaina metanosulfonato

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Abbreviations

2-PE: 2-Phenoxyethanol

ALP: Alkaline phosphatase

ALT: Alanine transaminase

AST: Aspartate aminotransferase

CK: Creatine phosphokinase

CO: Clove oil

FBPase: Frutose 1,6-biphosphatase

G6PDH: Glucose-6-phosphate dehydrogenase

GDH: Glutamate dehydrogenase

GK: Glucokinase

Hb: Hemoglobin

HCT: Hematocrit

HK: Hexokinase

LDH: Lactate dehydrogenase

MCH: Mean corpuscular hemoglobin

MCHC: Mean corpuscular hemoglobin concentration

MCV: Mean corpuscular volume

MS-222: Tricaine methanesulfonate

PK: Pyruvate kinase

RBC: Red blood cell

WBC: White blood cell

Introduction

State of Aquaculture

The world's population demand for food is constantly increasing, with resources gradually decreasing. Human fish consumption has steadily grown over the past decades, from 9.9 kg in the 1960 to 19.72 kg in 2013 (FAO, 2016), which causes massive pressure on fish stocks to meet the high food demand. However, global fishery capture production has been relatively stable, at around 90 million tonnes, in the last decade, having reached its second highest value ever of 93.7 million tonnes in 2011 (Fig. 1), with the highest ever being 93.8 million tonnes in 1996 (FAO, 2014), being imperative to find an alternative and sustainable source of seafood, while attempting to mitigate the negative effects of fishing. To fit this role, Aquaculture is now recognized as a promise sustainable alternative to overfishing.

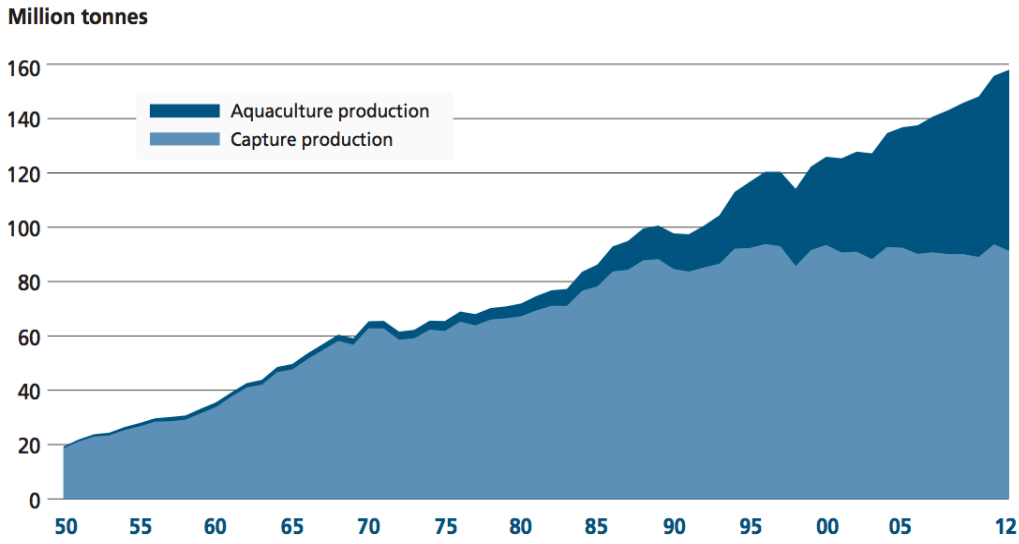

Figure 1. World capture fisheries and aquaculture production. Source: FAO (2014)

Aquaculture is, according to Haylor & Bland (2001), the ''farming of aquatic organisms in inland and coastal areas, involving intervention in the rearing process to enhance production and the individual or corporate ownership of the stock being cultivated''. It has proven to be a safe and sustainable source of food for human

consumption, with added benefits such as the creation of employment in both developed and underdeveloped countries as well as stimulating their economy. This practice was started in China in 2000 B.C. and has since then suffered a massive evolution, mainly due to the introduction of new technologies, better overall water quality and a higher understanding of the biology of the farmed species (Read and Fernandes, 2003), to the point where it is now one of the fastest growing industries in the world, with an average expansion rate of 5.8 percent between 2005 and 2014, despite it being lower than the 1980-1990 period, with a rate of 10.8 percent, and the 1995-2004 period, with a growth of 7.2 percent (FAO, 2016).

Aquaculture in Europe

European aquaculture production has been steadily growing since 2012. In 2014, the total production reached 1.28 million tonnes (Fig. 2), representing an increase of 8% over 2013, amounting to a total value of 3.96 billion Euros, 75 million more than in 2013 (EUMOFA, 2016).

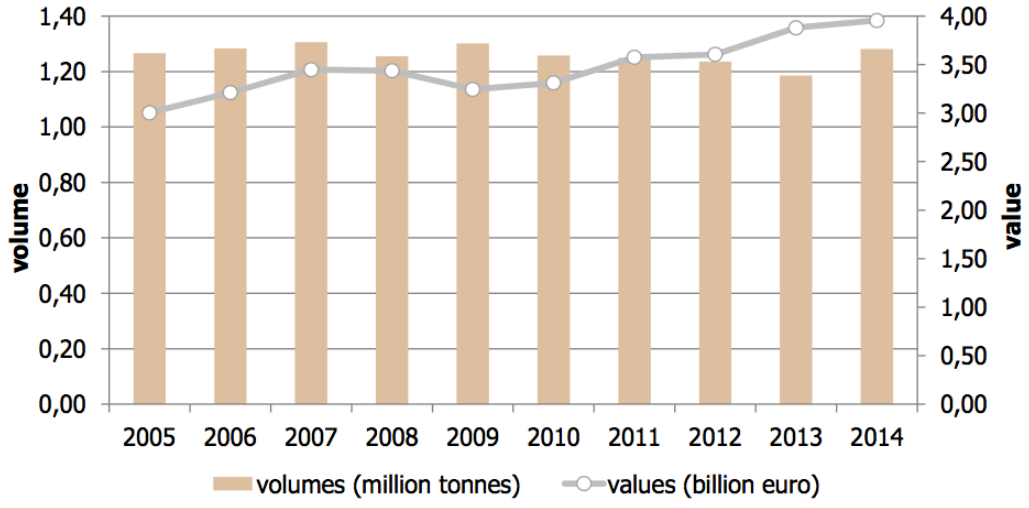

Figure 2. Total aquaculture production in the European Union. Source: EUMOFA (2016)

The main aquaculture producers in the European Union are Spain and the United Kingdom, both reaching peak productions in 2014, with Spain producing 285.000 tonnes, which amounted to a value of 472 million EUR, and the UK producing 214.000 tonnes and 953 million EUR. The other of European's top producers are France, Greece and Italy (EUMOFA, 2016).

The four most produced species are salmon, trout, gilthead seabream, and mussels. Mussels registered an increase in production between 2013 and 2014, while other species such as clam and Bluefin tuna suffered a sharp decrease in production (Fig. 3) (EUMOFA, 2016).

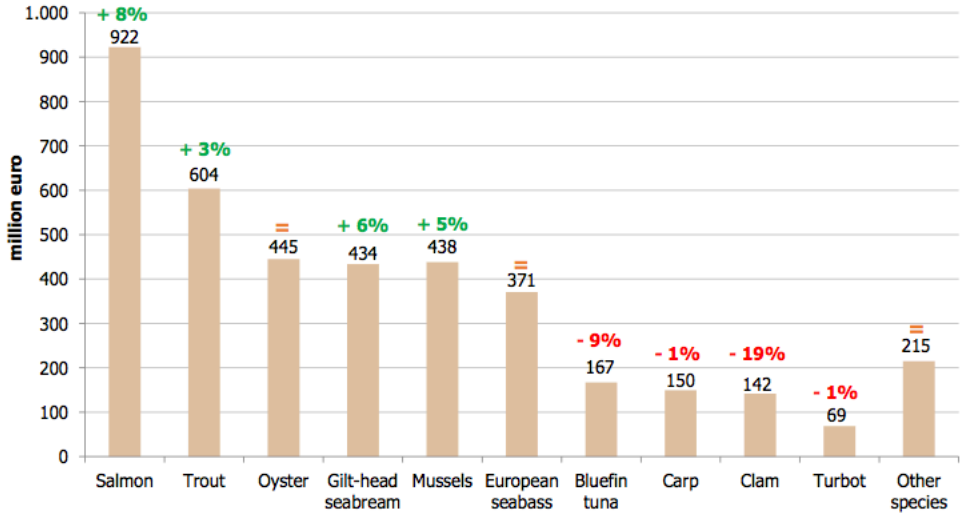

Figure 3. Values of main farmed products in the EU in 2014 and % variation 2013/2014. Source: EUMOFA (2016)

Gilthead Seabream

Biology

Gilthead seabream, *Sparus aurata* (Linnaeus, 1758) (Fig. 4), is a perciform fish that belongs to the family Sparidae. It has an oval and compressed body, with a head profile regularly curved with small eyes. The body is silver-grey with a large black blotch at the beginning of the lateral line, there is also a golden frontal band between the eyes and the fork and tips of the caudal fin are edged in black (Basurco et al, 2011).

Commonly found in the Mediterranean Sea and in the Eastern Atlantic Coast and rarely in the Black Sea, inhabits both marine and brackish waters, due to its euryhaline nature. It has a demersal behavior, usually found in seagrass (*Posidonia oceanica*) meadows, but also in sandy grounds, living in solitary or small groups. Younger fish remain in relatively shallow waters, up to 30 m, whereas adults can

reach deeper waters, generally no more than 150 m (FAO, 2015). It is, however, very sensitive to low temperatures, with 4ºC being the lethal minimum.

Being mainly a carnivorous species, gilthead seabream feeds mostly on crustaceans, molluscs, polychaeta and some teleost fish and echinoderms, but can be accessorily herbivorous (Wassef and Eisawy, 1985), adapting their diet according to temporal variations and food availability and accessibility (Gamito et al., 2003).

It is a protandrous hermaphrodite, where juveniles reach sexual maturity as males at around 2 years of age and around 30 cm, and as females when older than 2 years and over 30 cm (FAO, 2015). Spawning periods of this fish take place between October and December.

Figure 4. Gilthead seabream (*Sparus aurata* L.). Source: FAO

Gilthead Seabream Production

Seabream is one of the most important species in European aquaculture and it is already well established, with a production of over 150k tones per year, mainly produced by Greece and Spain (EUMOFA, 2016).

Historically, gilthead seabream aquaculture production began with extensive production in saltwater ponds and coastal lagoons, like the Italian ‘’vallicoltura’’ or the Egyptian ‘’hosha’’, that are rearing systems that act like natural fish traps (FAO, 2015).

During the 1980’s, intensive rearing systems were developed and in 1981-

1982 artificial breeding was successfully achieved in Italy and in Spain and, in 1988-1989, the large-scale production of gilthead seabream juveniles was achieved (FAO, 2015), allowing its mass production, being one of the most culture species nowadays. In the European Union, in 2014, gilthead seabream reached a ten-year peak at 43 million EUR, representing a 6% increase over 2013. However, in terms of volume of production it decreased 7%, relatively to 2013, to 86.400 tonnes (EUMOFA, 2016).

Aquaculture and Stress

In modern intensive aquaculture conditions, fish are involved in several practices, such as weighing, measuring, sorting, manual spawning, transportation, vaccination and tagging (Shaluei et al, 2012; Zahl et al., 2012), which will cause stress and consequently growth and immune response impairments and affect their reproduction and osmoregulation processes. All these effects make the fish more prone to disease or infections and can eventually lead to death. Indeed, the stress response diverts energy from normal metabolic processes including growth, to deliver energy to the physiological systems activated to adapt to the stressor (Tort et al, 2011). The several endocrine and physiological responses often result in changes in the fish's ability to survive, immune response decrease, increase in the incidence of diseases or growth limitations (Pickering, 1998; Tort et al., 2011). Although these changes may not be lethal, they can impair the health and appearance of the fish, which may result in environmental and/or aquaculture problems. Fish stress and mortality can cause significant losses of resources and productivity and so, fish welfare has become an increasing concern in commercial fish production and in scientific fish studies (Bystriansky et al, 2006; Jacquemond, 2004; Pramod et al, 2010; Welker et al, 2007; Zah et al, 2011).

The stress response is of vital importance for all living organisms. In response to a stressor, fish will undergo a series of biochemical and physiological changes in an attempt to compensate for the challenge imposed upon it and thereby cope with the stress (Bonga, 1997; Tort et al., 2011). The integrated stress response comprises the activation of the hypothalamus-pituitary-interrenal (HPI) and the hypothalamus-sympathetic-chromaffin (HSC) axis, with corticosteroids (namely cortisol) and catecholamines (adrenaline and noradrenaline) as endocrine factors, respectively (Bonga, 1997; Tort et al., 2011). When under stress, the first reaction is a neuroendocrine hormonal response resulting in the secretion of cortisol, which acts as an immunosuppressant, and epinephrine, followed by a second phase, defined by

the hormonal effects on blood and tissues, including changes in metabolism, hydromineral balance, cardio-respiratory and immune functions (Alves et al., 2010) causing an increase in plasma glucose (Davis and Griffin, 2004). Some authors claim that cortisol can have long term negative effects on the immune system and can compromise it, while others argue that a small increase in cortisol levels does not harm the immune system, but even enhances it, which is clearly beneficial for the fish (Neiffer and Stamper, 2009). Several indicators have been used to detect stress. For sparids, as well as for the majority of fish species, cortisol is known as a reliable and sensitive indicator of stress; heat shock proteins have been used as molecular indicators; metabolic changes have also been used, including plasma glucose and lactate levels, hepatic glycogenolysis and liver glycogen levels; and osmoregulatory parameters as plasma or serum osmolality and ion concentrations (potassium, sodium, calcium, chlorine) (Tort et al., 2011).

Anesthetics

Recent concerns with animal welfare and their ethical implications, both in aquaculture and scientific studies, demand a solution that reduces the stress of the fish while maintaining their normal physiological functions. In Europe, any experiment on animals has to abide to a very specific legislation- Directive 2010/63/UE, which aims to protect their welfare and rights, and undergo a very thorough ethical scrutiny. To achieve so, with animal welfare in mind, anesthetic agents have been used to mitigate stress in fish during several aquaculture routine handling operations, without causing any injuries or long-term negative effects (Neiffer & Stamper, 2009), by reducing fish energy use, enabling efficient transportation, increasing ease of handling and reducing handling stress (Park et al, 2009; Zahl et al., 2012).

Anesthesia is a biological reversible state, induced by an external agents which results in the partial or complete loss of sensation or loss of voluntary neuromotor control and reduced or even stopped respiration, through chemical or non-chemical means (Summerfelt and Smith, 1990). Anesthetics elicit or inhibit the corticosteroid response, being used to immobilize fish and to reduce metabolic rate and hence oxygen consumption. There are several anesthetics available, each one with pros and cons. For an anesthetic to be considered suitable it must present certain characteristics, such as low induction and recovery times, availability, low cost, not foam producing, stability under light and heat, rapid clearance from the body and most importantly nontoxic to humans, fish and environment (Schoettger

and Julin., 1967). Ideally it should induce anesthesia rapidly, with minimum hyperactivity or stress, and should be easy to administer and should maintain the animal in the chosen state (Coyle et al, 2004). The most commonly anesthetic agents used to induce inhalation anesthesia are tricaine methanesulfonate (MS-222), clove oil, 2-phenoxyethanol, benzocaine, and metomodiate (Coyle et al., 2004; Zahl et al, 2012). Among these, the most generally used for research purposes are tricaine methanesulfonate, clove oil and 2- phenoxyethanol.

For each anesthetic, the proper concentration varies according to water parameters such as pH, temperature, oxygen level, hardness and salinity and biological factors, i.e. fish size, weight, sex and age (Topic-Popovic et al., 2012), as well as branchia to body weight ratio and metabolic rates. It should be noted that despite their advantages, anesthetics may have unwanted and harmful side effects, especially with prolonged exposures and therefore should be used with caution (Cárdenas et al., 2016). Neiffer & Stamper (2009) compiled a series of data regarding the appropriate dose of several anesthetics, according the different fish taxa. Even thought, this knowledge may not be applied to all the situation, due to the complex and intricate interactions between the anesthetic, water parameters and fish metabolism as well as the desirable anesthesia stage light or deep sedation (Zahl et al., 2011).

The optimal dose and anesthesia protocols have been developed for several aquaculture species to reduce stress (Neiffer and Stamper, 2009). Nevertheless, only very few reports provide further information on its modulation effect on physiological status of fish during aquatic biomonitoring or laboratory studies. During these procedures, anesthesia would contribute to improve fish welfare, however, the anesthetic itself might induce and interfere with some physiological responses, and thus alter experimental results.

The most common route of administration of anesthesia is by inhalation. The anesthetic is added to the water tank or fish are placed in a separate tank with aerated water and the anesthetic, at the desired concentration. By fish immersion, the anesthetic will enter through the gills and quickly reach the bloodstream. Anesthesia can be divided in three different stages: light sedation, heavy sedation and complete (deep) anesthesia, each with different symptoms and characteristics. In light sedation, fish show reduced activity and breathing; in heavy sedation, fish suffer a loss of equilibrium and start to sink, while presenting minimal reactions to

visual and mechanical stimuli; in complete anesthesia, fish have a total loss of equilibrium and no reaction to stimuli at all, while laying still on the bottom of the tank. An overdose of anesthetic will be fatal and it is commonly used for fish euthanasia.

2- Phenoxyethanol

2-phenoxyethanol or ethylene glycol monophenyl ether (2-PE) is an oily and aromatic liquid with antimicrobial and fungicidal properties, making it very suitable for surgical procedures. 2-PE is the most commonly used anesthetic in aquaculture (Serezli et al, 2012). Its low cost and preparation effort, rapid induction and recovery time and the fact that it does not alter the water's pH when applied in seawater (Neiffer & Stamper, 2009) make it a suitable alternative for inducing anesthesia in fish. Depending on the concentration, it can induce different levels of sedation, ranging from 0.1 to 0.6 mL/L, inducing light sedation and surgical (deep) anesthesia, respectively (Coyle et al., 2004). It is still unclear how exactly this anesthetic acts but one possible explanation is that it causes an expansion on neuronal cell membranes, suppressing the central nervous system (Sneddon, 2012). The recommended anesthetic concentration of 0.3 mL/L can be considered safe for rainbow trout, Artic charr (*Salvelinus alpinus)*; European sea bass (*Dicentrarchus labrax*), meagre (*Argyrosomus regius*), but for gilthead sea bream (*Sparus aurata)* a higher dose is required (0.45 mL/L) (Husen and Sharma, 2014). However, for other species such as white sea bream (*Diplodus sargus* L.) and sharp snout sea bream (*Diplodus puntazzo*) 2-PE lower concentration are desirable (0.167mL/L) (Tsantilas et al, 2006), in fact, the fish's response to this anesthetic is species specific, its effects should tested experimentally beforehand (Moosavi et al, 2015). Handling this chemical should not be taken lightly as it has been shown that prolonged regular exposures can cause neuropsychological disorders on the handler (Neiffer & Stamper, 2009).

Tricaine Methanesulfonate (MS-222)

Tricaine methane-sulfonate (MS-222) is a benzocaine derivative and is the only anesthetic licensed for food fish in the European Union and the United States (Coyle et al, 2004), although fish treated with MS-222 must be held for a minimum of 21 days before human consumption (Maricchiolo, 2011; Zahl et al., 2011), and has been used since the 1970's. MS-222 is a powdered crystal and should be stored in darkness as it is unstable in light both in liquid and powder form (Neiffer & Stamper, 2009). It forms a colorless acid solution when dissolved in water and has a high

water solubility, making it suitable for both freshwater and saltwater (Topic Popovic et al., 2012). The main mode of action of MS-222 is by suppressing the nervous system, by inhibiting the entrance of sodium (Na^+) into the nerve, limiting its excitability (Carter et al., 2011). MS-222 is dissolved at a maximum of 11% and due to its acidity may cause unwanted side effects on fish (Coyle et al., 2004) as it lowers the water's pH, causing irritation on the gills. When using MS-222 to anesthetize fish the handler should be careful, as it can cause retinal irritation (Neiffer & Stamper, 2009). This anesthetic is more effective and safe in its neutralized form, which is achieved by buffering the solution to the pH of the fish's holding water, by adding sodium bicarbonate, the most commonly used, although there are several others like imidazole, sodium hydrogen phosphate, sodium hydroxide and calcium carbonate (Neiffer and Stamper, 2009). Optimum dose of MS-222 to induce deep anesthesia depend on the species, ranging from 50-125 mg/L (Neiffer & Stamper, 2009). The results indicated that MS-222 is effective as anesthetics for a great number of species. However, it was observed that it increases the cortisol levels, a stress indicator (Coyle et al., 2004).

Clove Oil

Clove oil (CO), a pale and yellow liquid extracted from the flowers, leaves and stems of the *Eugenia caryophyllata* or *Eugenia aromatica* trees, has been used as a mild topical anesthetic since antiquity (Javahery et al, 2012). Its main ingredient is eugenol (4-allyl-2-methoxyphenol), making up 90-95% of clove oil (Cunha et al., 2010). Because it is used as a food additive, it is considered safe for human consumption and is classified by the U.S. Food and Drug Administration (FDA) as ''generally considered as safe'' (GRAS) compound (Summerfelt and Smith, 1990). Clove oil has been used as a fish sedative in research and was approved to be used in food fish production in some countries with no withdrawal period. In Norway, Aqui-S (active ingredient - clove oil) was approved for sedation and anesthesia of Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*) prior and during handling events and during live fish transport (Zahl et al., 2012). Even thought, FDA did not approve its use in fish food production (Food and Drug Administration, 2007).

Clove oil has been proven to be an effective anesthetic for fish that presents advantages over other chemicals such as its low cost and natural origin and availability (Barata et al, 2016). Clove oil concentration varies greatly depending on the species and how strong the sedation is required, ranging from 2 to 60 mg/L.

Clove oil impedes the sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) channels (Sneddon, 2012), in a similar manner as MS-222. For marine fish, as gilthead sea bream, clove oil is a safe anesthetic and it does not induce immune depression in anesthetized fish (Bressler, 2004). Because it is an organic compound, fish anesthetized with clove oil intended for human consumption require no withdrawal period (Bressler, 2004). The optimal dose for European sea bass (*Dicentrarchus labrax*) was determined to be around 30 mg/L clove oil and for gilthead sea bream 55 mg/L clove oil (Mylonas et al, 2005). For blackspot seabream (*Pagellus bogaraveo*) and blackspot greater amberjack (*Seriola dumerilii)* clove oil is an effective anesthetic, at concentrations of 40 and 100 mg/L, respectively (Maricchiolo, 2011). Unlike MS-222 and 2-PE, clove oil is safe to handle but has a longer recovery time and is not completely appropriate to anesthetize fish for surgical procedures (Neiffer & Stamper, 2009).

Objectives of this study

This study aimed to determine the optimal dose of three anesthetics (tricaine methanesulfonate (MS-222), 2-phenoxyethanol (2-PE) and clove oil) to induction of deep anesthesia in ongrowing gilthead seabream. The physiological effects of anesthesia will be evaluated and compared to non-anesthetized fish, whit respect to:

- Hematology;
- plasma metabolites profile;
- cortisol levels;
- hepatic activity of key enzymes of intermediary metabolism;
- gene expression of hepatic heat shock protein.

The most suitable anesthetic for this species, causing the least impact on the fish normal physiological functions will also be determined. The use of the most adequate anesthetic and the correct dose will in turn allow a scientifically valid and ethically responsible aquaculture practices and fish research, promoting animal welfare.

Material and Methods

Fish trial and rearing conditions

Gilthead seabream juveniles were obtained from a commercial hatchery and maintained in the indoor facilities for 1 year. Prior to the experiment, fish were transferred to the experimental system and acclimatized to the new facilities and conditions for 1 month. Fish were determined to be healthy on the basis of history, general health, and appearance. For that, during this period, fish were visually controlled for signs of disease, lesions on the skin, tail or fins, feeding, territorial and aggression behavior, frantic swimming or increased opercular movements. During this period, fish were fed a commercial diet (diet Labrax with 46% protein and 18% lipids, Aquasoja, Soja de Portugal).

The experimental facilities consisted in a thermo-regulated recirculation water system equipped with 12 cylindrical fiberglass tanks of 100 L capacity, supplied with continuous flow of aerated filtered seawater. A thermostat installed in the system

heated the water.

At the beginning of the trial 12 homogenous groups of 9 seabream, with an initial body weight of 151 ± 4.3 g, were randomly distributed into 12 tanks. Fish were fed the same commercial diet used during the acclimatization period, to apparent visual satiety, twice a day, 6 days a week for 3 weeks. During this period water quality was maintained within acceptable levels for gilthead seabream. Temperature was maintained at 22 ± 1 °C, dissolved oxygen near to saturation, salinity at 35‰ and ammonia and nitrite near 0 ppm. A 12 h light – 12 h dark photoperiod was maintained.

Triplicate tanks were randomly assigned to a single anesthetic and another triplicate group did not receive anesthesia, being used as control group. Fish were fasted for 24h prior to anesthesia exposure experiment to minimize the risk of regurgitation.

Anesthesia induction and recovery time

The anesthesia induction and recovery time of the 3 target anesthetics: 2-phenoxyethanol (2-PE; VWR), tricaine methanesulfonate (MS-222; Sigma-Aldrich) and clove oil (CO; BioVer) extracted from the *Eugenia caryophyllata* tree, were measured.

MS-222 was mixed 1:2 with 95% sodium bicarbonate to make a stock solution of 1 mg/mL with a resulting pH of 7.3 in system water. The stock solution was added into to 10-L anesthetic tank containing system water to achieve different concentrations: 50, 100, 150, 200 and 250 mg/L. 2-PE was added to the anesthetic tank containing system water to achieve different concentrations: 0.15; 0.3; 0.45; 0.6 and 0.75 mL/L. Clove oil (100% pure extract) was mixed 1:10 with 95% ethanol and also added to the anesthetic tank to achieve different concentrations: 0.2; 0.4; 0.6; 0.8 and 1 mL/L. For each anesthetic, concentration 2 is the recommended concentration in the literature and the remaining concentrations correspond to a gradual increase or decrease of 50%.

To achieve the desired concentration, appropriate volumes of 2-PE and of stock solutions of MS-222 and clove oil were pre-solubilized into water from the experimental system to facilitate its administration and then added to the anesthetic glass tank of 10L. Another similar tank was prepared with water for the experimental time to be used for the recovery trial. Both tank water was strongly aerated prior and during the experiment and oxygen level and temperature were monitored during the

procedure.

For each anesthetic and each concentration, three fish from each tank (9 fish per treatment/anesthetic/concentration) were randomly selected and transferred, one at a time, to each anesthetic tank. The water of the anesthetic and recovery tanks was changed, every three fish, to ensure the maintenance of the desirable anesthetic concentrations and water quality.

A single designated person individually monitored the behavior of each fish, throughout anesthetic exposure and recovery phase using a stopwatch, following the recommendation of Shaluei et al. (2012). Fish were transferred to the anesthetic tank, observed for loss of equilibrium and slowing of opercular movement, and considered fully anesthetized (deep anesthesia) when showing total loss of reactivity, no reaction to handling and lying on the bottom of the tank (Shaluei et al., 2012). The time to reach deep anesthesia was recorded. Then fish were immediately transferred to the recovery tank and time to reach total recovery was recorded. Fish were considered fully recovered from the anesthesia when it regained its equilibrium, responded to mechanical stimuli and were actively swimming (Shaluei et al., 2012).

After the recovery, fish returned to a different tank, maintaining the initial groups, to avoid mixing anesthetized fish with non-anesthetized. For each anesthetic group 3 fish were anaesthetized twice, so a 14 days recovery period was set to ensure that the fish were fully recovered. No fish died after exposure.

The optimal concentration should induce anesthesia in under 3 minutes and with minimum hyperactivity or stress.

Effect of anesthetics on hematology, plasma metabolites, plasma cortisol, and hepatic enzymes activity

For each anesthetic, triplicate anesthesia tanks (10-L glass tank) were prepared, using the water of the system. Anesthetics were prepared as in previous trial. Another triplicate group of tanks (10-L glass tank), without anesthesia, were prepared and used as control.

On the sampling day, 24h after starvation, 3 fish per tank (9 fish per treatment/anesthetic) were randomly transferred to each of the 3 anesthesia tanks. The same procedure was applied to the control group, transferring the fish into the glass tank without anesthesia. Each tank was treated one at a time, to ensure that

biological samples will occur at the same time after anesthesia administration.

As soon as fish reached deep anesthesia, blood of the 3 fish were simultaneously collected, by a staff of three technicians, as described by Peres et al. (2013), in order to reduce the stress induced by handling. Blood was sampled from caudal vein by puncture with a heparinized syringe and divided into two aliquots. One blood aliquot (circa 1 mL) was immediately centrifuged at ambient temperature at 3000 g for 5 min, and five plasma aliquots were frozen at -80 C^{o} until analysis. One plasma aliquot was reserved for cortisol analysis. The other blood aliquot was placed in tubes with anticoagulant (ethylene diamine tetra acetic acid, EDTA; 1 mL/L) for hematological studies done immediately after blood sampling. Then fish were euthanized with a sharp blow in the head and liver was sampled and immediately frozen in liquid nitrogen until analysis.

Hematological and plasma analysis

Total erythrocytes (RBC) and leucocytes (WBC) counts were performed in a hemocytometer with fresh blood previously diluted in phenol-red free Hank's balanced salt solution (HBSS; Gibco, Paisley, UK). Hematocrit (HCT) was determined after microcentrifugation (10 000 g, for 10 min, at room temperature). Hemoglobin (Hb) concentration was determined with Drabkin's reagent using an analytical kit (Spinreact, ref.1.001.230). All counts were performed in duplicate for each fish. Mean cell volume (MCV) and cell hemoglobin concentration (MCHC) were computed from the HCT, Hb concentration and RBC.

Biochemical analysis of plasma was performed according to the protocol used by Peres et al. (2013), using commercial kits from Spinreact, S.A. (Gerona, Spain) to determine glucose (cod.1001191), triglycerides (cod. 1001312), cholesterol (cod.1001091), total protein (kit cod. 1001291), lactate (kit cod.1001330), and albumin (kit cod. 1001020). Other plasma parameters namely, total calcium, inorganic magnesium, sodium, potassium, chlorine, alkaline phosphatase, aspartate aminotransferase, lactic dehydrogenase and creatine phosphokinase were done by a veterinary clinical laboratory, NP EN ISO 9001–2000 certified by Bureau Veritas, using standard clinical methods, stringently monitored with appropriate quality control procedures, in an auto-analyzer (Architect ci8200; Abbot Diagnostics, Canada).

Plasma cortisol analysis

For cortisol analysis, plasma samples (50 mL) were diluted in phosphate buffer (450 mL) containing 0.5 g/l of gelatin (pH 7.6) and denatured at 80 °C for 60 min. After samples cooled, cortisol (11b,17,21-trihydroxy-pregn-4-ene-3,20-dione) was measured by radioimmunoassay (RIA). Details of the RIAs methodology have been published elsewhere (Scott et al., 1984). RIAs were performed using duplicate amounts (100 µL) of denatured samples. Cross reactions of antisera used in RIAs for cortisol was described previously in Rotllant et al. (2005). Average intraassay and interassay coefficient of variations for RIAs were 3.3% and 10.3% for cortisol, respectively.

Hepatic enzyme activity analysis

Prior to the analysis of the activity of key enzymes of intermediary metabolism liver was homogenized in six volumes of ice-cold 100 mM Tris–HCl buffer containing 0.1 mM EDTA and 0.1 % (v/v) Triton X-100, pH 7.8. The procedure was performed on ice. Homogenates were centrifuged at 30000 g for 30 min at 4ºC, and the resultant supernatants were kept in aliquots and stored at -80ºC until analysis. Then, the activity of glycolytic enzymes (hexoquinase (HK), glucokinase (GK), and pyruvate kinase (PK)); gluconeogenesis enzyme (fructose- 1,6-biphosphatase (FBPase); amino acid catabolic enzymes (glutamate-dehydrogenase (GDH), alanine transaminase (ALT), aspartate aminotransferase (AST) and lipogenic enzymes (glucose 6- phosphate dehydrogenase (G6PDH) were determined.

Hexokinase (HK; EC 2.7.1.1) and glucokinase (GK; EC 2.7.1.2) activities were measured as previously described by Vijayan et al. (1990). Reaction mixture contained 50 mM imidazole–HCl buffer (pH 7.4), 2.5mM ATP, 5mM $MgCl_2$, 0.4 mM NADP, 2 units/mL G6PDH, and 1 mM (HK) or 100 mM (GK) glucose.

Pyruvate kinase (PK; EC 2.7.1.40) activity was performed with a reaction mixture consisting of 50 mM imidazole–HCl buffer (pH 7.4), 5 mM MgCl2, 100 mM KCl, 0.15 mM NADH, 1 mM ADP, 2 units/mL LDH and 2 mM PEP (Morales et al., 1990).

Frutose-1,6-biphosphatase (FBPase; EC 3.1.3.11) activity was measured using a reaction mix containing 42,84 mM imidazole-HCl buffer (pH 7.4), 5 mM-$MgCl_2$, 0.5 mM NADP, 12 mM β-mercaptoethanol, 2 units/mL PGI, 2 units/mL G6PDH and 0.5 mM FBP (Morales et al., 1990).

Glutamate dehydrogenase (GDH; EC 1.4.1.2) activity was performed using a reaction mixture containing 50 mM imidazole–HCl buffer (pH 7.4), 0.2 mM NADH, 1

mM ADP, 100 mM ammonium acetate, 2 units/mL LDH and 10 mM α-ketoglutarate (Morales et al., 1990).

Alanine transaminase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1) were activities were measured using Spinreact commercial kits (Ref. 41272 and 41282).

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was assayed as previously described by Morales et al., using a reaction mixture containing 50 mM imidazole–HCl buffer (pH 7·4), 5 mM $MgCl_2$, 2 mM NADP and 1 mM glucose-6- phosphate.

The optimal sample dilution for each analysis was determined by preliminary assays, in order to ensure optimum ratio between enzyme and substrate. Enzyme activities were determined by monitoring the changes in NADH or NADP at 25ºC.

One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmol of substrate per minute under the assay conditions. Hepatic soluble protein concentration was determined according to Bradford (1976), using a commercial kit (Sigma-Aldrich protein Kit, ref. B6916) and bovine serum albumin as standard.

Gene Expression

Gene expression of the mitochondrial 70-kDa heat shock protein (mtHsp70) was determined by real-time PCR (qPCR, quantitative polymerase chain reaction) technique.

Firstly, tissue samples (liver) were lysed and homogenized in TRI-Reagent® (Invitrogen) using pellet pestles cordless motor (Sigma), previously treated with DEPC (diethyl pyrocarbonate) to eliminate any RNA-ases. Once the tissue was homogenized, it was centrifuged to remove any particulate debris. Then, total RNA was isolated and treated with Dnases, through columns, using the kit Direct-zol™ RNA MiniPrep (ZymoResearch), following manufacturer's instructions.

Quality of the RNA was checked through electrophoresis in a 1 % agarose gel. Total RNA was quantified by spectrophotometry, using a µDrop™ plate (ThermoScientific) and concentrations were adjusted to 1 µg. First-strand cDNA was obtained using the NZY First Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal), following the kit's instructions.

Real-time quantitative PCR was performed in a CFX Connect™ Real-Time System (Bio-Rad). Reactions were carried out in a final volume of 25 µL, using 12.5

µL of SsoAdvanced Universal SYBR® Green supermix (Bio-Rad), 10.5 µL of ultrapure water (Sigma-Aldrich), 0.5 µL of each specific primer (10 µM; Table 1) and 1 µL of two-fold diluted cDNA template.

Since it was the first time that mtHsp70 gene was studied in our research group, preliminary tests were necessary in order to determine the best optimal annealing temperatures for mtHsp70 and for the housekeeping. For this, their amplification efficiency was calculated using seven serial two-fold dilutions of cDNA with the threshold cycle (Ct) slope method (Pfaffl, 2001).

The different transcripts were amplified using technical triplicates per sample, under the following conditions: 95 ºC for 30 s (polymerase activation), followed by 40 cycles of denaturation at 95 ºC for 15 s, and annealing temperature of 59.7 ºC for mtHsp70, or 52 ºC for β-actin, for 1 min. A melting curve analysis was performed to verify that only specific amplification occurred and no primer-dimers were amplified. Relative expression of each transcript was normalized using the gene of β-actin as housekeeping, which was constitutively expressed and not affected by the experimental treatments, and calculated using the Pfaffl method (Pfaffl, 2001).

Table 1. Primers used for qPCR reaction

	Abbreviation	Primer sequence (5'-3')	Source
β-actin	bact-Sa-F	GGCACCACACCTTCTACAATG	Cerezuela et al., 2013
	bact-Sa-R	GTGGTGGTGAAGCTGTAGCC	
Mitochondrial 70 kDa heat shock protein	mtHsp70_Sa-F	TCCGGTGTGGATCTGACCAAAGAC	Pérez-Sánchez et al., 2015
	mtHsp70_Sa-R	TGTTTAGGCCCAGAAGCATCCATG	

Statistical analysis

All obtained data were statistically analyzed using the SPSS software. Normality and homogeneity of variances was tested by the Shapiro-Wilk and Levene tests, respectively, and data were normalized when necessary (log or arcsine transformation for percentage data).

Anesthetic induction and recovery times at different concentrations were statistically tested by a two-way ANOVA. When significant interactions were found, a one-way ANOVA was performed for each anesthetic and each anesthetic concentration. For the other data, statistical evaluation was done by one-way

ANOVA, testing the effect of the anesthetic used. The probability level of 0.05 was used for rejection of the null hypothesis. Significant differences among groups will be determined by the Tukey's multiple range test.

Results

Anesthesia induction and recovery time

Time for deep anesthesia and total recovery of gilthead seabream after exposure to different concentration of each anesthetic (2-PE; MS-222, and CO) is represented in figure 5 and 6, respectively. For all three anesthetics, the induction time followed a second order polynomial curve, gradually decreasing with the increase of anesthetic concentration. At the lowest concentration, 2-PE had the highest induction time for deep anesthesia, 6.2 minutes, while clove oil had the lowest induction time, with an average of 1.9 minutes, and MS-222 showed an average induction time of 2.5 minutes. At the two highest concentrations, all anesthetics had the same induction time of about 1 minute. For each anesthetic induction time was closely related to the anesthetic concentration (Table 2). However, recovery time was more variable in relation to anesthetic doses. Recovery time followed exposure to MS-222 was not affected by its concentration, while with 2-PE and clove oil it significantly increased from the first lowest to the second lowest concentration. After that, recovery time from 2-PE anesthesia was reduced and from clove oil was maintained irrespective the anesthesia concentration (Figure 6 and Table 2).

Based on these, the optimal concentrations of each anesthetic was found to be: 0.45 mL/L for 2-PE, 150 mg/L for MS-222 and 0.6 mL/L for CO.

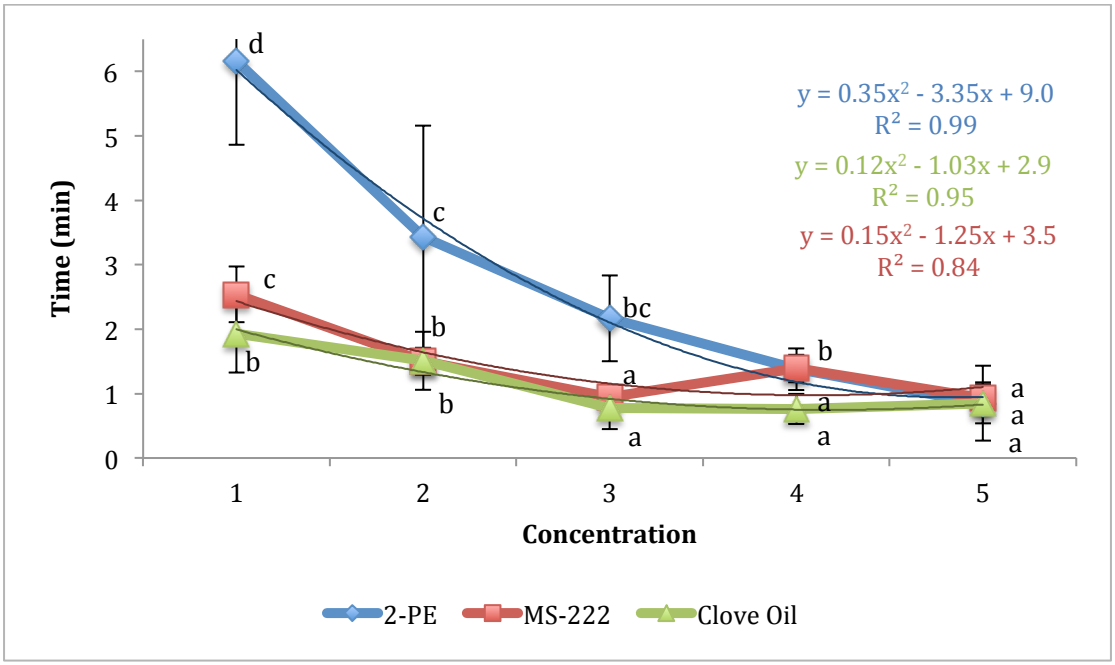

Figure 5. Induction time to deep anesthesia of gilthead seabream after exposure to different concentrations of each anesthetic. Concentrations 1 through 5: 2-PE: 0.15; 0.3; 0.45; 0.6 and 0.75 mL/L; MS-222: 50, 100, 150, 200 and 250 mg/L, and clove oil: 0.2; 0.4; 0.6; 0.8 and 1 mL/L. All values presented as mean (± S.D.); different letters denote significant differences among concentrations for each anesthetic ($p<0.05$).

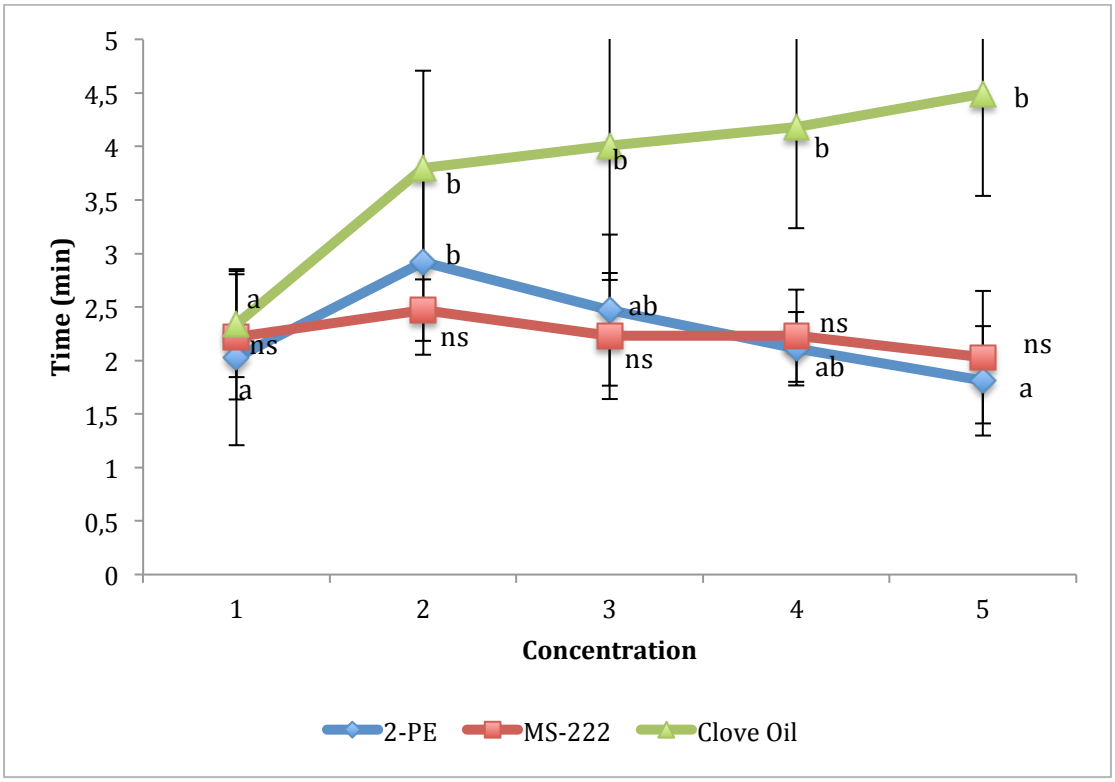

Figure 6. Time to total recovery from anesthesia of gilthead seabream after exposure to different concentrations of each anesthetic. Concentrations 1 to 5: 2-PE: 0.15; 0.3; 0.45; 0.6 and 0.75 mL/L; MS-222: 50, 100, 150, 200 and 250 mg/L, and clove oil: 0.2; 0.4; 0.6; 0.8 and 1 mL/L. All values presented as mean (± S.D.); different letters denote significant differences among concentrations for each anesthetic ($p<0.05$). ns: non-significant.

Table 2. Statistical analysis of induction and recovery times from anesthesia of gilthead seabream exposed to no anesthesia (control) or deep anesthesia with different anesthetics prior to blood sampling..

Two way Anova

	Anesthetic	Concentration	Interaction
Induction	***	***	***
Recovery	***	***	***

One way Anova

Induction time	***Concentration****				
	1	***2***	***3***	***4***	***5***
2-PE	d	c	bc	ab	a
MS-222	c	b	a	b	a
Clove Oil	b	b	a	a	a
Recovery time	***Concentration****				
	1	***2***	***3***	***4***	***5***
2-PE	a	b	ab	ab	a
MS-222	ns	ns	ns	ns	ns
Clove Oil	a	b	b	b	b

Two-way ANOVA: NS: non-significant (P ≥ 0.05); ***P < 0.001. If two-way ANOVA interaction was significant, a one-way ANOVA was performed for each anesthetic and means in the same line with different letters are significantly different (P<0.05). ns: non-significant.
*Concentrations 1 to 5: 2-PE: 0.15; 0.3; 0.45; 0.6 and 0.75 mL/L; MS-222: 50, 100, 150, 200 and 250 mg/L, and clove oil: 0.2; 0.4; 0.6; 0.8 and 1 mL/L.

Effects of deep anesthesia

Hematology

Deep anesthesia with 2-PE, MS-222 and clove oil or no anesthesia prior to the blood sampling did not affect hematocrit and hemoglobin levels (Table 3). Mean corpuscular volume and mean corpuscular hemoglobin concentration also showed no statistical differences between treatments, while mean corpuscular hemoglobin was higher in the control group that in the 2-PE group.

Total cells and red blood cell count of control and clove oil groups were lower than that observed for the 2-PE and MS-222 groups. White blood cell count had the highest values in the control and 2-PE groups, while MS-222 showed intermediate values and clove oil had the lowest value.

Table 3. Hematologic analyses of gilthead seabream exposed to no anesthesia (control) or deep anesthesia with different anesthetics prior to blood sampling.

	Control	2-PE	MS-222	Clove Oil
Hematocrit (%)	28.6 ± 4.2	30.2 ± 4.3	30.1 ± 3.8	24.7 ± 3.5
Hemoglobin (g/dl)	8.9 ± 3.9	6.6 ± 0.9	7.8 ± 2.1	5.9 ± 0.7
MCV[1]	160.4 ± 18.6	130.6 ± 24.5	127.3 ± 17.5	139.9 ± 44.1
MCH[2]	50.4 ± 23.7^{b}	28.5 ± 3.5^{a}	33.0 ± 8.5^{ab}	33.2 ± 8.0^{ab}
MCHC[3]	30.8 ± 11.0	22.1 ± 2.7	27.4 ± 13.0	24.8 ± 5.1
Total Cells (10^6/µL)	1.8 ± 0.3^{a}	2.4 ± 0.3^{b}	2.4 ± 0.1^{b}	1.8 ± 0.3^{a}
RBC[4] (10^6/µL)	1.8 ± 0.3^{a}	2.3 ± 0.3^{b}	2.4 ± 0.1^{b}	1.8 ± 0.3^{a}
WBC[5] (10^4/µL)	3.6 ± 1.6^{b}	3.8 ± 1.5^{b}	2.7 ± 1.7^{ab}	1.1 ± 0.2^{a}

Values presented as mean ± S.D. Means in the same row with different superscript letters are significantly different (Tukey's test, $P < 0.05$).
[1]Mean Corpuscular Volume (µm^3)
[2]Mean Corpuscular Hemoglobin (pg/cell)
[3]Mean Corpuscular Hemoglobin Concentration (g/100mL)
[4]Red Blood Cells (10^6/µL)
[5]White Blood Cells (10^4/µL).

Plasma analysis

Selected plasma metabolite levels of gilthead seabream exposed to no anesthesia (control) or deep anesthesia with different anesthetic, prior to blood sampling is presented in Table 4. Total protein, albumin, total cholesterol, magnesium, sodium and chlorine levels did not significantly differ among groups. However, in non-anesthetized fish (control group), glucose and potassium levels were higher than in the other groups. Also lactate and triglycerides were higher in the control than in the clove oil group while the inverse was true for inorganic phosphorus. Total calcium level was lower in the control and 2-PE groups than in the others. Regarding plasma enzymatic activity, LDH was lowest and CK highest in the clove oil group than in other groups.

Table 4. Plasma metabolites (mg/dL) of gilthead seabream exposed to no anesthesia (control) or deep anesthesia with different anesthetics prior to blood sampling.

	Control	2-PE	MS-222	Clove Oil
Glucose	63.4 ± 4.4^{b}	36.1 ± 1.4^{a}	31.1 ± 1.1^{a}	37.3 ± 1.6^{a}
Total protein (g/dL)	3.75 ± 0.14	3.81 ± 0.3	3.23 ± 0.26	3.85 ± 0.27
Albumin (g/dL)	2.82 ± 0.21	2.33 ± 0.26	2.149 ± 0.30	2.905 ± 0.33
Lactate	19.5 ± 1.9^{b}	15.6 ± 2.2^{ab}	14.1 ± 1.9^{ab}	7.6 ± 2.0^{a}
Triglycerides	41.5 ± 6.8^{b}	38.7 ± 5.6^{ab}	22.7 ± 3.6^{ab}	20.1 ± 4.6^{a}
Total cholesterol	254.4 ± 14.8	242.5 ± 18.6	237.1 ± 12.7	251.8 ± 15.5
Total calcium	10.6 ± 0.1^{a}	10.8 ± 0.3^{a}	11.7 ± 0.2^{b}	11.9 ± 0.1^{b}
Magnesium	1.8 ± 0.1	2.0 ± 0.1	1.9 ± 0.04	1.8 ± 0.1
Inorganic phosphorus	7.3 ± 0.3^{a}	7.6 ± 0.2^{ab}	8.5 ± 0.2^{ab}	8.2 ± 0.3^{b}
Sodium	180.7 ± 1.1	184.5 ± 1.2	183.7 ± 1.3	185.2 ± 1.2
Potassium	4.9 ± 0.2^{b}	4.1 ± 0.1^{a}	3.8 ± 0.2^{a}	3.9 ± 0.1^{a}
Chlorine	198.5 ± 3.5	205.8 ± 4.04	207.3 ± 2.4	208.3 ± 5.3
ALP[1](U/L)	77.5 ± 4.7	79.2 ± 15.6	87.8 ± 4.4	96.3 ± 21.2
AST[2] (U/L)	90.3 ± 22.7	71.5 ± 27.2	30.7 ± 9.4	29.2 ± 12.7
LDH[3] (U/L)	142.3 ± 23.1^{bc}	202.1 ± 41.5^{c}	47.15 ± 19.5^{ab}	30.8 ± 12.6^{a}
CK[4] (U/L)	559.4 ± 63.7^{b}	461.8 ± 150.2^{ab}	283.7 ± 126.4^{a}	709.2 ± 31.3^{b}

Values presented as mean ± S.D. Means in the same row with different superscript letters are significantly different (Tukey's test, $P < 0.05$).
[1]Alkaline Phosphatase
[2]Aspartate Aminotransferase
[3]Lactate Dehydrogenase
[4]Creatine Phosphokinase

Plasma Cortisol

Plasma cortisol levels showed an over two-fold increase in fish anesthetized with 2-PE in comparison to the control group (Figure 7). Fish anesthetized with clove oil and MS-222 showed a decrease in cortisol levels, although none of these results are statistically significant.

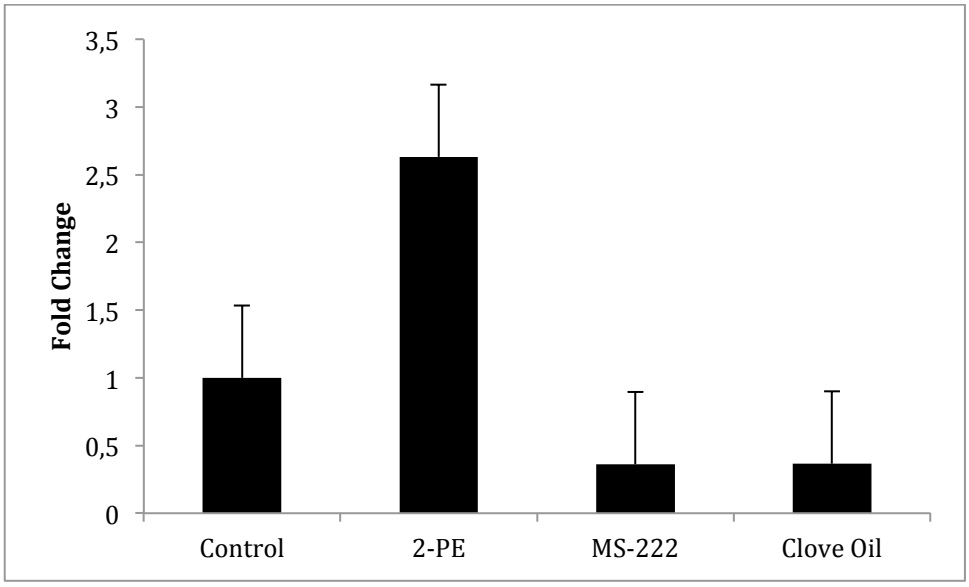

Figure 7. Fold change of plasma cortisol levels of gilthead seabream exposed to no anesthesia (control) or deep anesthesia with different anesthetic prior to blood sampling.

Hepatic Enzyme Activity

Hepatic enzyme activity results are shown in table 5. From all the enzymes that were tested, only ALT, AST, HK and G6PDH showed significant differences between treatments. For ALT and AST activity, the lowest value was found in the control group and the highest in 2-PE.

HK activity was lower in the clove oil group than that of the other groups.

Fish anesthetized with 2-PE showed the highest G6PDH activity and the clove oil group showed the lowest activity.

Table 5. Liver enzyme activity (mU/mg protein) of gilthead seabream exposed to no anesthesia (control) or deep anesthesia with different anesthetics prior to blood sampling.

	Control	2-PE	MS-222	Clove Oil
Amino acid catabolism				
ALT[1]	235.8 ± 28.8[a]	409.2 ± 142.2[b]	387.9 ± 135.1[ab]	308.6 ± 44.1[ab]
AST[2]	1048.6 ± 124.9[a]	2089.1 ± 979.3[b]	1876.6 ± 789.3[ab]	1221.7 ± 193.3[ab]
GDH[3]	132.3 ± 47.0	330.6 ± 167.9	100.5 ± 26.2	93.36 ± 23.6
Glycolysis				
GK[4]	5.86 ± 1.79	6.74 ± 4.33	5.79 ± 2.45	4.89 ± 0.78
HK[5]	5.97 ± 1.85[b]	5.94 ± 3.2[b]	3.36 ± 0.97[b]	2.65 ± 0.57[a]
PK[6]	2.89 ± 0.76	3.78 ± 1.61	3.01 ± 1.08	4.86 ± 2.01
Gluconeogenesis				
FBPase[7]	16.27 ± 12.11	29.37 ± 18.96	23.98 ± 18.42	22.8 ± 15.95
Pentose phosphate pathway				
G6PDH[8]	107.2 ± 32.2[ab]	146.5 ± 44.0[b]	126.8 ± 19.8[ab]	89.9 ± 27.9[a]

Values presented as mean ± S.D. Means in the same row with different superscript letters are significantly different (Tukey's test, $P < 0.05$).
[1]Alanine Aminotransferase
[2]Aspartate Aminotransferase
[3]Glutamate-dehydrogenase
[4]Glucokinase
[5]Hexokinase
[6]Pyruvate kinase
[7]Frutose-1,6-Biphosphatase
[8]Glucose-6-Phosphate dehydrogenase

Gene expression

Relative gene expression levels of the mitochondrial heat-shock protein 70 kDa were calculated as described by Pfaffl (2001), using β-actin as housekeeping gene that was expressed constitutively (not affected by any treatment). Fold-change units were calculated by dividing the normalized expression values (each value respect to its housekeeping level) by the normalized expression values in the controls (Figure 8).

Results showed no significant differences between the control group and each anesthetic. The mtHsp70 expression is not affected by any of the anesthetics tested.

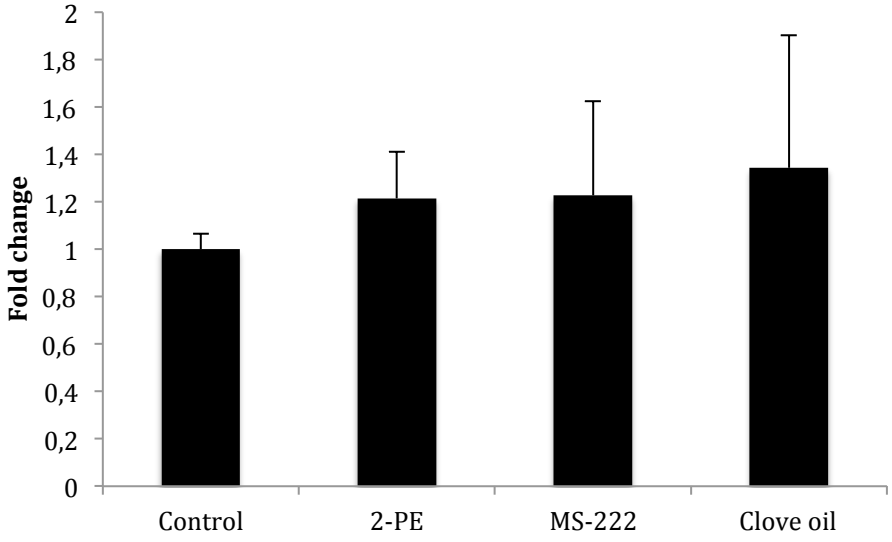

Figure 8. Expression of mitochondrial heat-shock protein 70 kDa in liver after different anesthetic treatments. The relative expression of the mtHsp70 gene was normalized to the expression of β-actin. Data are shown as mean (n=3) ± S.E.

Discussion

Induction and recovery time

Under intensive aquaculture practices, fish are subject to several practices involving anesthesia, such as handling, confinement and transportation, all of them associated to acute stress. These situations are associated to a non-specific generalized physiological response (Zahl et al., 2009). Also, under laboratorial conditions fish have been involved in several anesthesia procedures. Acute effects of sampling and anesthesia procedures may confound results, and those potential effects are not well studied.

A range of anesthetic agents is currently in use in laboratory, veterinary, and aquaculture contexts (Sneddon, 2012). The most common anesthetic drugs used in fish are MS-222 (tricaine methanesulfonate), isoeugenol, 2-phenoxyethanol, benzocaine, metomidate, and quinaldine. For each anesthetic, the induction, recovery rate, and pharmocokinetics as well as undesirable or adverse side effects needed to be accurately evaluated in order to avoid nociception or pain perception by the animal. However, this research is limited to a relatively small number of species and caution should be applied when using any of these agents on a nonvalidated

species (Sneddon, 2012). An ideal anesthetic should induce anesthesia rapidly with minimum hyperactivity or stress and the toxic dose should greatly exceed the effective anesthetic dose (Coyle et al., 2004).

For gilthead seabream juveniles, the induction and recovery time of clove oil and 2-PE were previously determined (Mylonas et al., 2005). However, for ongrowing gilthead seabream no data regarding the induction/recovery time and subsequent physiological effects of clove oil, 2-PE and MS-222 are available, even though it has been reported that fish size may affect the optimum dose of each anesthetic (Tsantilas et al., 2006; Zahl et al., 2011, 2009).

In the present study all the anesthetics tested were effective in inducing deep anesthesia in ongrowing gilthead seabream. Besides, no mortality was registered, indicating that toxic doses of the tested anesthetics were not reached. The induction and recovery times were affected by both the anesthetic and its concentration. Deep anesthesia induction time, after exposure to all the tested anesthetics, followed a second order polynomial curve being faster with higher than low anesthetic concentration. Moreover, among the anesthetic tested, 2-PE was the one that showed the greatest difference in anesthesia induction time between the lowest and highest concentration tested. These results indicated that fish treated with high doses of 2-PE were less exposed to it, due to the decreased induction times, absorbing less anesthetic, making its removal from the blood and tissues more easily achievable, decreasing induction and recovery times.

Optimal doses for anesthesia, defined as those providing the combined shortest induction and recovery times, for ongrowing gilthead seabream was determined to be 0.45 mL/L for 2-PE, 150 mg/L for MS-222 and 0.6 mL/L for clove oil. Even though several factors may affect the optimal anesthesia doses, as species, body weight, size and gill surface area to body weight ratio (Barata et al., 2016; Mylonas et al., 2005; Tsantilas et al., 2006), these results were relatively similar to those obtained for gilthead seabream juveniles (Mylonas et al., 2005), black sea bass (*Centropristis striata*), Atlantic Cod (*Gadus morhua*), Red Pacu (*Piaractus brachypomus*), salmonids, Senegalese Sole (*Solea senegalensis*), and many tropical reef species (Carter et al., 2011; King et al., 2005; Mattson and Riple, 1989; Roubach et al., 2005; Sladky et al., 2001; Weber, 2009; Woody et al., 2002).

At low concentrations, 2-PE was the less efficient to induce deep anesthesia when compared with the other anesthetics (clove oil and MS-222), requiring longer induction times at low concentrations, as previously reported by Barata et al. (2016) and Mylonas et al. (2005).

For clove oil and 2-PE administrated at concentrations up to 0.4 and 0.3 mL/L, respectively, increased recovery times, followed by a significant decrease of recovery time for 2-PE and a plateau for clove oil. Contrarily, recovery time of MS-222 was unaffected by its concentration. However, for juvenile gilthead seabream, it was observed that higher clove oil (20 to 49 mg/L) and 2-PE (250 to 450 mg/L) doses resulted in similar and shorter recovery times, respectively (Mylonas et al., 2005). Among the tested anesthesia, 2-PE was the one that showed the highest difference in induction time when administrated at low or high concentration, which may, at least in part explain the decrease of the recovery time after exposure to higher doses of 2-PE. Indeed, as it was stated by Mylonas et al. (2005), the shorter the exposure time to the anesthetic bath, the smaller the amount of anesthetic absorbed by the body, and the faster its removal from the blood and recovery of the fish once placed in clear water.

Anesthesia with clove oil, at concentration higher than 0.4mL/L, did not affect the recovery time, probably due to its lipophilic nature that is very persistent on the gills, increasing exposure time to the anesthetic (Sladky et al., 2001; Summerfelt and Smith, 1990; Javahery et al., 2012). According to Tsantilas et al. (2006), one explanation for the independence between the induction and recovery times is that the anesthetic is taken up through a concentration gradient in the gill interface until it reaches an equilibrium, regardless of the induction time. During recovery the anesthetic is lost through this gradient, as such the recovery time is dictated by the anesthetic's concentration and not the induction time.

Hematology

After deep anesthesia with 2-PE at a concentration of 0.45 mL/L, MS-222 at 150 mg/L and clove oil at 0.6 mL/L blood was sampled from the caudal vein. Another group was bleeding without prior anesthesia, used as control. White blood cell (WBC) count of the non-anesthetized control group was significantly higher than that of fish anesthetized with clove oil indicating a possible reduction of restraint stress induced by previous anesthesia before bleeding (Hrubec and Smith, 2000). On the other hand, with MS-222 only a slight reduction and with 2-PE no effect on WBC count was observed.

When compared to the control group, fish that were anesthetized had lower MCH values but an increased RBC count, with the exception of clove oil. These results may indicate that both 2-PE and MS-222 induced a respiratory depression,

due to reduced opercular movements and gill saturation with anesthetic, inducing a hypoxic state (Neiffer and Stamper, 2009). Under this state, to increase the oxygen supply to the organs, immature RBC's may have been released (Shaluei et al., 2012). Contrarily, fish anesthetized with clove oil did not show this trend, which may indicate that fish were bled before respiratory depression induced hypoxia (Hrubec and Smith, 2000).

Plasma analysis

Plasma cortisol levels are commonly used as stress indicators in fish studies (Tort et al., 2011). In the present study, plasma cortisol levels trended to be lower in the anesthetized groups with MS-222 and clove oil, while with 2-PE it was observed an increase of over two-fold comparatively to the non-anesthetized group. Similarly, Ortuño et al. (2002) observed that sedation of gilthead seabream with 2-PE (60 mg/L) induce a cortisol response similar to a density stress and for brown trout, Flodmark et al. (2002) reported that 2-PE may be stressful *per se*, increasing plasma cortisol levels. Juvenile meagre exposed to low doses of anesthetics exhibited signs of stress, when compared to non-anesthetized fish, suggesting an apparent stressor effect of the anesthetic itself (Barata et al., 2016), whereas silver catfish anesthetized with eugenol have shown a decrease in plasma cortisol levels when compared to non-anesthetized fish (Cunha et al., 2010).

Plasma biochemical profile of control group is within the reference values reported for this species, bleeding without prior anesthesia and unfed for 24 hours (Peres et al., 2013). Plasma glucose secretion is recognized as a secondary response to stress (Martinez-Porchas et al., 2009; Tort et al., 2011). Relatively to the non-anesthetized fish, glycemia was reduced in all the anesthetized groups, suggesting a reduction of blood sampling stress. Moreover, the observed trend to reduce plasma cortisol levels, combined with the significant reduction of glycemia after exposure to MS-222 and clove oil may indicate that both anesthetics may inhibit the primary and secondary stress responses. Despite the observed trend to increase plasma cortisol levels, 2-PE group did not induce an increase of glycemia. These results may be related to a more pronounced hypoxia induced by 2-PE than by the other anesthetics, that may have activated the hypothalamus-pituitary-interrenal axis rather than blocked (Bolasina, 2006). This result corroborated the observed increase in RBC count with this anesthetic but not with clove oil. Moreover, for the 2-PE group

the activity of LDH, the enzyme that catalyzes the final step in anaerobic glycolysis, was fivefold higher than that of the other anesthetics, suggesting a more pronounced anaerobic metabolism. For gilthead seabream, it was previously observed that hypoxia increased the activity of LDH (Perez-Jimenez et al., 2012) and plasma cortisol levels (Martos-Sitcha et al., 2017) during hypoxia.

Plasma lactate level has been used, for some fish species, as an indicator of anaerobic metabolism and to assess the speed of recovery after a period of anaerobic metabolism (Virani and Rees, 2000). However, for gilthead seabream maintained under hypoxia conditions, the increased activity of LDH was not reflected in the plasma lactate levels (Perez-Jimenez et al., 2012; Martos-Sitcha et al., 2017). In present study, clove oil anesthesia reduced plasma lactate levels which may suggest a decreased anaerobic metabolism rate compared to the other groups. This observation is consistent with the lower RBC count observed in this group than in the other anesthetics, as an increase of RBC usually followed an anaerobic state. Similar results were obtained for rainbow trout exposed to four different anesthesia (MS-222, clove oil, 2-PE and propiscin; (Velisek et al., 2011), while other authors observed an increased plasma lactate levels after clove oil or eugenol exposure in tambaqui (Inoue et al., 2011) and Atlantic salmon (Iversen et al., 2003).

Nonspecific plasma enzymes activities, such as ALP, AST, LDH may also be a stress indicator, associated to tissue damage, due to pathological processes, toxic chemical exposure, or traumatic fish handling (Peres et al., 2013). In the present study, LDH trend to decrease in anesthetized fish, being significant with MS-222 and clove oil. CK activity was lower in MS-222 group; no significant differences were observed in ALT and AST activity, but AST activity was three fold lower after anesthesia with MS-222 or clove oil than in non-anesthetized fish, suggesting that anesthesia did not induce tissue damage. Similarly, Velíšek and Svobodová (2004) and Velíšek et al. (2005) observed that AST activity was reduced after exposure to clove oil or 2-PE anesthesia, in rainbow trout. Also in Siberian sturgeon it was observed a decreased activity of ALP after anesthesia with MS-222 or eugenol (Gomulka et al., 2008). Contrarily, for rainbow trout an increase of AST, ALT, LDH and CK activity was observed after exposure to MS-222 (Congleton, 2006). Also, in perch, anesthesia with MS-222 and 2-PE was reported to increase ALP activity (Velíšek et al., 2009).

Lower CK activity in MS-222 group may be related to a lower energy need in the muscles, since this enzyme catalyzes the conversion of creatine, using ATP, into create phosphocreatine (PCr), that serves as an energy reservoir for rapid

regeneration of ATP, which is a sign of muscle damage (Velisek et al., 2011; Yousaf and Powell, 2012).

As aforementioned, anesthetics can cause water acidification, which in turn will cause stress to the fish. Stress has been proven to disturb the fish's water and salt balance and can increase plasma sodium and potassium levels. Similarly, the use of anesthetics, mainly MS-222 in brook trout, increases plasma potassium levels and decreases calcium (Eddy, 1981), altering the blood's electrolyte balance and, consequently, affect osmoregulation. Also, long induction time with an inappropriate dose of clove oil may increase plasma cortisol levels, and so potassium and sodium plasma levels (Javahery et al., 2012). In the present study, bleeding fish after deep anesthesia with MS-222 and clove oil increased plasma total calcium and reduced plasma potassium levels with clove oil, competitively to the 2-PE anesthetized and non-anesthetized fish. This reduction of potassium levels may be explained by the trends to decrease plasma cortisol levels in fish anesthetized clove oil . Indeed, previously Laiz-Carrión et al. (2003) and (Pierson et al., 2004) highlighted the importance of cortisol in the osmoregulation and energy metabolism, by increasing cell permeability to water and ions in the epithelia of gills. Kakizawa et al. (1995) have also demonstrated that an increase in stress and, consequently, in plasma cortisol levels also lead to an increase in plasma Ca^{2+} levels. Similar to present results, in burbot (*Lota lota* L.) it was observed that plasma calcium levels increased following anaesthesia with 2-phenoxyethanol and Propiscin, that may be attributed to acute respiratory acidosis (Svačina et al., 2016).

According to Groff and Zinkl (1999), plasma osmolarity can be indirectly determined by the ratio between sodium and chlorine, as these two electrolytes contribute more than 75% of the osmotic balance in teleosts (Cataldi et al., 1998). In this study, both sodium and chlorine levels remained unaltered, indicating that the anesthetics did not alter the plasma's osmolarity.

Enzyme activity

The liver is a key metabolic organ in body energy regulation, adaptive response to stress, somatic growth regulation, immune response, and detoxification (Ballester-Lozano et al., 2015; Moon, 2004; Tort, 2011). Generally, the hepatic enzyme activity of gilthead seabream euthanized without prior anesthesia (control) was different from those euthanized after being anesthetized with different anesthetics. Anesthesia with 2-PE almost doubled the activity of ALT, AST and GDH and trend to increase activity of G6PDH. Anesthesia with MS-222 had little effect on

the activity of hepatic enzymes, while anesthesia with clove oil reduced activity of HK and trend to reduce activity of G6PDH.

The observed increased activity of amino acids catabolic enzymes after anesthesia with 2-PE may be related to the observed increased levels of plasma cortisol. It was observed that a small increase of plasma cortisol may affect amino acid metabolism (Hopkins et al., 1995). A similar profile of hepatic enzymes activity of gilthead seabream after anesthesia with 2-PE was observed for sea raven (Vijayan et al., 1996). In that study, cortisol treatment stimulated nitrogen metabolism, increasing the activity of the amino acid catabolic enzymes, but did not affect glycogenolysis nor the hepatic glycolytic potential. In the present study, even though no significant difference were observed on the activity of FBPase, its activity in 2-PE group increased up to 80% relatively to the non-anesthetized group, suggesting that amino acid may have also been used as a substrate for gluconeogenesis.

HK and GK enzymes are glycolytic enzymes, catabolizing the first reaction of glycolysis, which consists on the phosphorylation of glucose to glucose-6-phosphate, a molecule that may be used in other metabolic pathways, such as glycolysis, glycogenesis and the pentose-phosphate pathway (Enes et al., 2009). Anesthesia with clove oil reduced the activity of HK, and trend to decrease the activity of GK, and so the glucose up-take by the cells, and, consequently, its catabolism probably due to a decreased demand of glucose for energy. Moreover, clove oil also decreased the activity of G6PDH, reducing the use of glucose-6- phosphate in the pentose phosphate pathway, suggesting a decrease in the oxidative stress of clove oil group, compared to the other groups. Indeed, G6PDH is the rate-limiting enzyme of the pentose phosphate pathway and is involved in NADPH regeneration, which plays an important role in the protection of cells from oxidative stress.

Gene expression

Heat shock proteins (HSP) are known as stress proteins. Contrary to other results, the clove oil group showed an increase in the expression of mitochondrial heat-shock proteins in the liver, whereas the 2-PE and MS-222 groups showed little to no increase, although none of these results are statistically significant. Euthanasia after deep anesthesia with clove oil group lead to an increase in plasma CK levels, compared to that of the other anesthetics, which catalyses a reaction using ATP. According to Rudneva (2013), the decrease in ATP is thought to cause accumulation

of denaturated proteins and aggregation of constitutive Hsp70 that ultimately triggers the heat shock response under non-heat temperature. However, further studies are required in order to fully understand the effect of the anesthetics on HSP.

Conclusion and further studies

The aquaculture industry shows no signs of slowing down and presents itself as a suitable and sustainable alternative to supply the world's fish demands. The industry's growth is accompanied by scientific investigation in order to increase the productivity and quality of the fish destined for human consumption. Anesthesia has been used in aquaculture, to reduce stress induce by capture, handling, and transport of fish. Also, at the research level, anesthesia is required for invasive studies such as physiological investigations. Under the actual context of rising ethical concerns regarding fish welfare, it is imperative to use an appropriate anesthetic that not only sedates and immobilizes the fish but also mitigates any pain and harmful side effects of any stressor. Moreover, the collateral biological effects of the anesthesia needed to be clarified as, at research level for example, it may confound the study results.

Optimal doses for anesthesia for ongrowing gilthead seabream, providing the combined shortest induction and recovery times, was determined to be 0.45 mL/L for 2-PE, 150 mg/L for MS-222 and 0.6 mL/L for clove oil.

Overall, results indicate that sampling procedures may affect plasma metabolites levels and liver enzyme activity, and this should be considered when designing experiments that require fish anesthesia.

The observed reduction of glycemia, plasma cortisol levels and RBC count observed in the fish anesthetized with clove oil, compared to the other anesthetics, suggests that clove oil is an effective anesthetic for ongrowing gilthead seabream. Clove oil not only sedates gilthead seabream but also inhibits the primary and secondary stress responses and its by-products. Moreover, clove oil is a natural and inexpensive product with an easy preparation and a safe handling.

Further studies are required to fully understand the anesthetic's metabolic interaction. Additional genetic and enzymatic analyses are required to deepen the

knowledge of how anesthesia may affect the oxidative status of gilthead sebaream. Moreover, other stress markers should also be analysed, such as HSP-90 and low-molecular-mass HSP, to have a more complete dataset of the physiological responses to stress and anesthetics.

Finally, all these analyses should be performed on other commercially important species, to create a database on the most suitable anesthetic and concentration and the main physiological site effects of its application, with practical application at aquaculture and research levels.

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