Some Contributions to Knowledge of Stress Response in Innovative Species with Particular Focus on the Use of the Anaesthetics

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Abstract: This study investigated some aspects of stress research, including, also, the effects of chemical anaesthesia, in two important species for diversification in aquaculture, blackspot seabream, *Pagellus bogaraveo* and greater amberjack, *Seriola dumerilii*. The first part of the research (trial 1 and 2) was designed to determine the efficacy of clove oil as an anaesthetic alternative to MS-222. In trial 1, the minimum concentration of anaesthetic producing the total loss of muscular tone within 3 min, and recovery within 10 min, was determined. The obtained results showed that MS-222 and clove oil were effective as anaesthetics for juveniles of blackspot sea-bream and greater amberjack at concentrations of 40 and 100 mg L^{-1}, respectively. The aim of trial 2 was to assess the side-effects of both anaesthetics on greater amberjack juveniles by monitoring serum cortisol and glucose levels, and haematocrit values. No significant differences between anaesthetics were found for most of the measured physiological variables. The second part of research (trial 3) was designed to establish the ability of anaesthesia to mitigate stress responses blocking activation of the HPI axis associated to handling stress. In this trial, the stress responses to handling of adult blackspot sea-bream anaesthetised with either anaesthetics were compared to those of unanaesthetised fish used as controls. Serum cortisol and glucose concentrations were used as stress indicators. Both anaesthetics were unable to block activation of the HPI axis that occurs as a consequence of handling stress in blackspot sea-bream, although anaesthesia with both anaesthetics proved to be effective in reducing the duration of stress response.

Keywords: *Pagellus bogaraveo*, *Seriola dumerilii*, stress response, anaesthesia, tricaine methanesulfonate, clove oil, physiological parameters.

1. INTRODUCTION

1.1. Fish Welfare and Stress in Aquaculture

Over the last decade, finfish aquaculture production has experienced a worldwide expansion and is projected to become an increasingly significant source of animal proteins; it is estimated that, by 2015, about 39% of world fish production will come from aquaculture [1].

The importance of animal welfare is now recognised in all animal productions including those from aquaculture. Fish welfare is an important issue for aquaculture industry for production efficiency, quantity and product quality as well as for consumer perception and marketing [2-4].

However, while there is a well established literature on welfare of terrestrial farm animals, for fish under intensive aquaculture conditions there is a paucity of scientific information on which to base future guidelines and potential legislation where necessary. There are many aquaculture practices that may compromise fish welfare (see [5] for a review).

Fish react to stress with a primary neuroendocrine response, represented by a rapid release of catecholamines (adrenaline and noradrenaline) from the chromaffin tissue (homologous to the mammalian adrenal medulla) and by the activation of hypothalamic-pituitary-interrenal (HPI) axis. Corticotropin Releasing Factor (CRF) released by hypothalamus acts on the pituitary gland to synthesise and release the Adrenocorticotropic Hormone (ACTH) which, in turn, promotes the secretion of cortisol by interrenal tissue (homologous to the mammalian adrenal cortex) [6].

As a result of high levels of these hormones in the circulatory system, a wide range of secondary responses can be observed; particularly secondary responses are defined as the subsequent actions and effects of these hormones at both blood and tissue levels, and include disturbance of the metabolic and hydromineral balance [6].

The tertiary responses, which are determined by prolonged or chronic stress, induce a significant reduction of fish welfare and influence adversely fish growth, reproduction and immune responses [7].

A variety of haematological and biochemical measurements are used as indicators of stress in fish. Among the most frequently measured variables, there are levels of circulating corticosteroid hormones, mainly cortisol; this is widely used both as a long and short term primary stress indicator [8,9].

Indicators of secondary effects of stress are very numerous but the most used is plasma or serum glucose level. Haematocrit value is, also, used as a stress index because it is simple and immediate to be determined.

Physiological responses to stress can vary depending on the type and duration of stressors [10-12] and species [8, 13, 14]. For example, elevation in plasma cortisol levels, which

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represents a typical response to stress, can differ by as much as two orders of magnitude among different species of fish following identical stressors [12, 15-17].

In this context, the AHAW (Animal Health and Welfare) Panel of European Food Safety Authority (EFSA) recommends that indicators of fish welfare are species-specific [18].

1.2. Anaesthesia and Stress Response

Anaesthesia is, by definition, a biological reversible state induced by an external agent, which results in the partial or complete loss of sensation or loss of voluntary neuromotor control, through chemical or non chemical means [19].

Anaesthesia is frequently applied in aquaculture being a valuable tool that helps to minimize fish stress and to prevent physical injuries to fish while handling them during routine practices. For example, anaesthesia is required for measuring or weighing fish, sorting and tagging, administrating vaccines, live transport, sampling for blood or gonadal biopsies and collecting of gametes, to cite some of the main applications.

A number of parameters have to be considered in the choice of an anaesthetic for fish [19]. A fish anaesthetic must, primarily, be effective: (*i.e*., it must have short induction and recovery times), be economical and harmless to both the user and environment and have limited side effects. However, when choosing an anaesthetic, it is important to take into consideration the type of the experiment and the species of fish [19, 20]. So far, a number of different anaesthetics have been used or evaluated for aquaculture applications [19, 21-25].

Tricaine methanesulphonate (better known under the trade name of MS-222) is the most frequently used fish anaesthetic. This is the only approved by Food and Drug Administration (FDA) for use with edible fish in the USA, although fish treated with MS-222 must be held for a minimum of 21 days before human consumption. MS-222 is potent and effective in low concentrations but it is expensive and has a suspected carcinogen effect although no mutagen one has been documented [26].

Among fish anaesthetics, another compound that shows interesting features, in terms of both safety and efficacy, is clove oil. Clove oil is a distillate of the herbaceous portions of the clove tree, *Eugenia caryophyllata* (fam. *Myrtaceae*); its main constituent is eugenol (4-allyl-2-methoxyphenol), known for its anaesthetic properties and for use in human dentistry. Eugenol has been shown to be effective for anaesthesia in a variety of fish species [20, 27-30].

Although it was defined by FDA as a GRAS (*Generally Recognised As Safe*) product with human intake levels established at 2.5 mg Kg^{-1} day^{-1} [31] it has not yet been approved for use in edible fish.

Another primary feature that an anaesthetic should possess is the ability to mitigate the stress response [32] reducing or blocking activation of the HPI axis associated with handling stressors. In fact, failure to suppress stress-induced activation of HPI axis results in a release of cortisol hormone which in turn causes various physiological responses and the associated consequences.

Extensive studies on Teleost species have shown that cortisol and glucose stress responses to handling are usually reduced by previous treatment with anaesthetics [33-35]. However, literature reports conflicting data on the effects and dose-response relationship of exposure to anaesthetics.

Another important aspect to consider is related to systemic side effects induced by anaesthetic itself. In fact, although anaesthesia benefits fish by minimizing the impact of more severe stressors, it is also inherently stressful [36-38].

Anaesthetics can influence fish physiology during induction of the "state of anaesthesia". This aspect has to be considered when blood or tissue samplings are made, because the values of physiological parameters could change as a result of anaesthetic effect during induction; this is especially important for physiological responses that occur rapidly (*i.e*., cortisol rise).

1.3. Research Objectives

This research was conducted on blackspot sea bream, *Pagellus bogaraveo* (Brünnich, 1768) and greater amberjack, *Seriola dumerilii* (Risso, 1810), two Teleost species considered important to diversification of farmed fish.

Moreover, while several studies on stress response to handling have been reported for some cultured marine fish (see [5] for a review), no research has been conducted on these two species in terms of comparison of various anaesthetics and their effects.

In particular the main objectives of this research were the following:

Trial 1: to compare the anaesthetic efficacy of MS-222 and clove oil in blackspot seabream and greater amberjack;

Trial 2: to compare the effects of MS-222 and clove oil on the physiology of greater amberjack juveniles after anaesthesia;

Trial 3: to determine whether MS-222 and clove oil were able to block the normal serum cortisol increase associated to handling stress in adult blackspot seabream.

Serum cortisol and glucose levels and haematocrit values were used as indicators of stress.

2. MATERIALS AND METHODOLOGY

2.1. Rearing Conditions and Experimental Animals

The study was carried out at the Experimental Aquaculture Plant of the Institute for Coastal Marine Environment (IAMC-CNR) of Messina, Italy.

Wild juveniles of blackspot sea bream, *Pagellus bogaraveo* and greater amberjack, *Seriola dumerilii* were chosen as studied species because of their importance to the diversification of farmed fish.

Prior to the study, fish were maintained in indoor tanks, provided with a flow-through supply of aerated seawater under natural photoperiodic conditions.

P. bogaraveo was fed *ad libitum* a commercial diet (Hendrix S.p.a.) while *S. dumerilii* was fed a fresh diet consisting of anchovies and sardines.

2.2. Trial 1: Comparative Efficacy of MS-222 and Clove Oil in *Pagellus bogaraveo* and *Seriola dumerilii*

The experiment was carried out in different time periods but with the same experimental procedure.

Body weight (mean ± SD) of *P. bogaraveo* and *S. dumerilii* was 10.8 ± 5.3g and 288.22 ± 96.56g respectively.

To allow acclimatisation, one month before the beginning of the experiment, fish were randomly transferred in 2 m^3 circular tanks, provided with a flow-through supply of aerated seawater.

Fish were fed to satiation daily, then they were fasted and human contact or disturbance was avoided for 24 h before the start of the experiments.

The tested anaesthetics were MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate or tricaine methanesulfonate) and clove oil, both purchased from Sigma-Aldrich (Milano-Italy).

Due to clove oil's incomplete solubility in water, it was dissolved in ethanol at a 1:10 ratio (v/v), before being mixed in the anaesthetic tank.

To standardise the concentrations of anaesthetics used throughout the experiments, all the concentrations were expressed as mg L^{-1} in relation to the concentration of active substance of each anaesthetic.

The tested concentrations were 70, 80, 90, 100 mg L^{-1} for MS-222 and 10, 20, 30, 40 mg L^{-1} for clove oil.

The minimum concentration producing in all fish the total loss of muscle tone, within 3 minutes, and recovery to normal swimming within 10 minutes or less, was considered as the effective anaesthetic concentration.

Total loss of muscle tone is equivalent to light anaesthesia (stage III - plane 1) used by Stoskopf [24] and considered as the condition suitable for external sampling and biopsies (Table **1**).

The experimental procedure was the following: each fish was transferred with a net to an anaesthetic tank consisting of 25-L glass aquaria (one for each anaesthetic). Each anaesthetic tank was filled with 20 L of sea water containing different concentrations of anaesthetics; these latter were tested from the lowest to highest one to ensure that any potential residue would influence the successive trials.

Salinity and temperature of sea water in anaesthetic tanks were 38 and 18°C and 38 and 16°C for blackspot sea-bream and greater amberjack, respectively.

The time taken by each fish to reach stage III anaesthesia ("induction time") was determined using a stopwatch.

Behavioural reaction to insertion of a hypodermic needle was observed to assess that analgesia was reached in each anaesthetised individual.

Following application of the anaesthesia, fish was immediately removed from the anaesthetic tank and placed in a tank containing only sea water; then the time needed to recover to normal conditions ("recovery time") was determined.

Fish were anaesthetised individually and a total of 60 fish were tested for each chemical.

The Shapiro-wilk test was applied to data for testing if they were normally distributed.

The correlation between induction time and fish weight for both each anaesthetic and concentration was examined using the regression analysis.

The effect of anaesthethic concentrations on both induction and recovery times was evaluated using the Analysis of Variance (ANOVA), followed by the Scheffe test when appropriate. Statistical analyses were performed with a Statistical Analysis Software (SAS).

Table 1. Stages of Anaestesia in Fish (from Stoskopf [24])

STAGES OF ANAESTHESIA IN FISH			
Stage	**Plan**	**Category**	**Behavioural Response of fish**
0		Normal	Swimming actively; Reactive to external stimuli; Equilibrium normal; Muscle tone normal.
I	1	Light Sedation	Voluntary swimming continues; Slight loss of reactivity to visual and tactile stimuli; Respiratory rate normal; Equilibrium normal; Muscle tone normal.
I	1	Light Narcosis	Excitement phase may precede increase in respiratory rate; Loss of equilibrium; Efforts to right itself; Muscle tone decreased; Still responds to positional changes weakly.
II	2	Deep Narcosis	Ceases to respond to positional changes; Decrease in respiratory rate to near normal; Total loss of equilibrium; No effort to right itself; Muscle tone decreased; Some reactivity to strong tactile and vibrational stimuli; Suitable for external sampling, fin and gill biopsies.
III	1	Light Anaesthesia	Total loss of muscle tone; Responds to deep pressure; Further decrease in respiratory rate; Suitable for minor surgery
III	2	Surgical Anaesthesia	Total loss of reactivity; Respiratory rate very low; Heart rate slow.
IV		Medullary Collapse	Total loss of gill movement followed in several minutes by cardiac arrest.

2.3. Trial 2: Comparison of MS-222 and Clove Oil Anaesthesia Effects on the Physiology of *Seriola dumerilii* Juveniles

Three groups of greater amberjack juveniles (each composed of n=15 fish with mean body weight ± SD: 288.22 ± 96.56 g) were compared in this study: control (unanaesthetized), MS-222-anaesthetised and clove oil-anaesthetised.

The concentrations of the used anaesthetic were those established in the trial 1 (MS-222: 100 mg L^{-1}; clove oil: 40 mg L^{-1}). One month before the start of the experiment, fish were acclimatised in 2-m^3 circular tanks and fed to satiation daily. Fish were fasted and left undisturbed for 24 h before the start of the experiment.

Anaesthetic baths (glass aquaria) were prepared taking into consideration the volume of water (25-L) in order to reach the final concentration of MS-222 or clove oil desired in the exposure tank. The water in the tank was mixed manually with a glass rod, to allow a homogeneous distribution of the anaesthetic before fish exposure. In order to ensure the correct exposure of specimens to chemicals, anaesthetic bath was changed every five fish.

The experimental procedure was the following: fish were placed individually in the anaesthetic concentration. Following induction of light anaesthesia (stage III – plane 1, see trial 1 and Table **1**), just before being transferred in the recovery tank, fish were immediately bled from the caudal vein. The control group was sampled in < 30 seconds and fish belonging to this group were considered as a negative control for comparison with anaesthetised fish. After bleeding, fish were placed into a tank containing fresh, aerated sea water for recovery.

Differences between groups were determined using the Analysis of Variance (ANOVA), followed by the Duncan test at a significance level of $P \leq 0.05$.

2.4. Trial 3: Comparison of MS-222 and Clove Oil Anaesthesia on Stress Response to Handling Stress in *Pagellus bogaraveo* Adults

Ninety-six adult specimens of *P. bogaraveo* (mean body weight ± SD, 302.70 ± 23.36 g) were randomly distributed in four groups of 24 fish: 1) two groups pre-treated respectively with an immobilizing dose of MS-222 (100 mg L^{-1}) or clove oil (40 mg L^{-1}) and exposed to handling stress; 2) a group subjected to the same stress but without a preliminary anaesthesia; 3) a control group was kept undisturbed.

The procedure for each experimental group was as follows: interruption of water-flow, introduction of the anaesthetic solution in tank and application of stressors after the fish were anaesthetised. The latter consisted in transferring fish from one tank to another one and exposing them to air for 2 minutes.

Blood samples were collected from the caudal vein at the time of transfer (time 0) and 15, 30 and 60 minutes after the start of exposure to stress. To avoid a sampling-induced stress response, withdrawal lasted less than 3-4 minutes for group, since it was carried out simultaneously by two operators.

Data were analysed using the Analysis of Variance (ANOVA), followed by the Duncan test at a significance of $P \leq 0.05$.

2.5. Analytical Procedures

In order to extract the serum for the determination of cortisol and glucose, blood samples not treated with heparin, were allowed to clot at 4°C, centrifuged at 1500g for 10 minutes and stored at - 80°C until analysis.

Small volumes of blood collected in heparinised (14 International Unit ml^{-1}) tubes, were used for the immediate determination of haematocrit values.

Serum cortisol concentrations were determined by use of a commercially available enzyme-linked (ELISA) immunoassay kit (*Alpha Diagnostic International*, USA).

Serum glucose levels were determined by a commercial kit based on the reaction of GOD-POD (Glucose Oxidase-Peroxidase) (*Sclavo Diagnostics*, Italy).

Haematocrit as the percentage of red blood cell to the total blood volume, was determined in heparinised capillary tubes, after centrifugation in a standard microhaematocrit centrifuge at 12.000 g for 10 minutes, and comparison of capillary tubes with a reference scale.

3. RESULTS

3.1. Trial 1: Comparative Efficacy of MS-222 and Clove Oil in *Pagellus bogaraveo* and *Seriola dumerilii*

No mortality was observed during anaesthesia or 24 hrs after it in both the anaesthetic treatments. The induction and recovery times (mean ± SD) of fish exposed to MS-222 or clove oil are shown in Table **2**.

Table 2. Induction and Recovery Times of Different Anaesthetics in *P. bogaraveo* and *S. dumerili* (Mean ± SD), n=15. For each Anaesthetic, Statistically Significant Differences (P<0.01) Recorded in the Induction and Recovery Times in Relation to the Different Tested Concentrations are Indicated By Superscripts

Anaesthetic Concentration	Induction Time (minutes)	Recovery Time (minutes)
Pagellus bogaraveo		
MS-222 (mgL^{-1})		
70	4.73 ± 1.71^{A}	1.73 ± 0.27^{A}
80	3.69 ± 0.58^{B}	1.25 ± 0.49^{A}
90	2.65 ± 0.52^{C}	1.49 ± 0.76^{A}
100	1.91 ± 0.70^{D}	1.22 ± 0.60^{A}
Clove Oil (mgL^{-1})		
10	$> 15^{A}$	6.87 ± 2.74^{A}
20	4.04 ± 0.58^{B}	2.62 ± 0.92^{B}
30	2.86 ± 0.63^{C}	3.02 ± 1.13^{B}
40	1.88 ± 0.65^{C}	3.54 ± 1.45^{B}

(Table 2) Contd…..

Anaesthetic Concentration	Induction Time (minutes)	Recovery Time (minutes)
Seriola dumerilii		
MS-222 (mgL^{-1})		
70	5.71 ± 1.65^{A}	2.62 ± 0.25^{A}
80	4.65 ± 0.78^{B}	2.20 ± 0.50^{A}
90	3.60 ± 0.70^{C}	2.46 ± 0.72^{A}
100	2.59 ± 0.55^{D}	2.39 ± 0.45^{A}
Clove Oil (mgL^{-1})		
10	$> 15^{A}$	7.77 ± 2.70^{A}
20	4.04 ± 0.58^{B}	2.52 ± 0.82^{B}
30	2.86 ± 0.63^{C}	3.00 ± 1.08^{B}
40	2.40 ± 0.45^{C}	3.50 ± 1.39^{B}

No correlations between fish weight and induction time were observed for both the species.

Both in blackspot seabream and greater amberjack, anaesthesia induction times decreased with increasing concentrations of MS-222 and clove oil.

For MS-222, induction times obtained with all the concentrations were significantly ($p \leq 0.01$) different to each other; instead, for clove oil, induction times observed at 10 and 20 mg were significantly ($p \leq 0.01$) different to each other while there were no differences between those recorded using 30 and 40 mg (Table **2**).

On the other hand, recovery times were concentration independent. However, for both the species, there were significant ($p \leq 0.01$) differences in recovery times following exposure to 10 mg of clove oil.

The concentration of MS-222 and clove oil found to be effective in blackspot seabream and greater amberjack was 100 mg L^{-1} and 40 mg L^{-1}, respectively.

3.2. Trial 2: Comparison of MS-222 and Clove Oil Anaesthesia Effects on the Physiology of *Seriola dumerilii* Juveniles

Fig. (**1**) shows the changes in serum cortisol, glucose and haematocrit values after exposure of greater amberjack juveniles to MS-222 or clove oil.

Serum cortisol levels of anaesthetised fish (with both anaesthetics) were significantly ($p \leq 0.01$) higher than control values (Fig. **1A**). However there were no significant differences in serum cortisol levels between fish exposed to MS-222 (100 mg L^{-1}) and clove oil (40 mg L^{-1}).

Clove oil group displayed a significant increase in serum glucose levels compared to controls or MS-222 anaesthetized fish (Fig. **1B**).

Finally, haematocrit levels of anaesthetised fish were significantly ($p<0.01$) higher than controls (Fig. **1C**), while

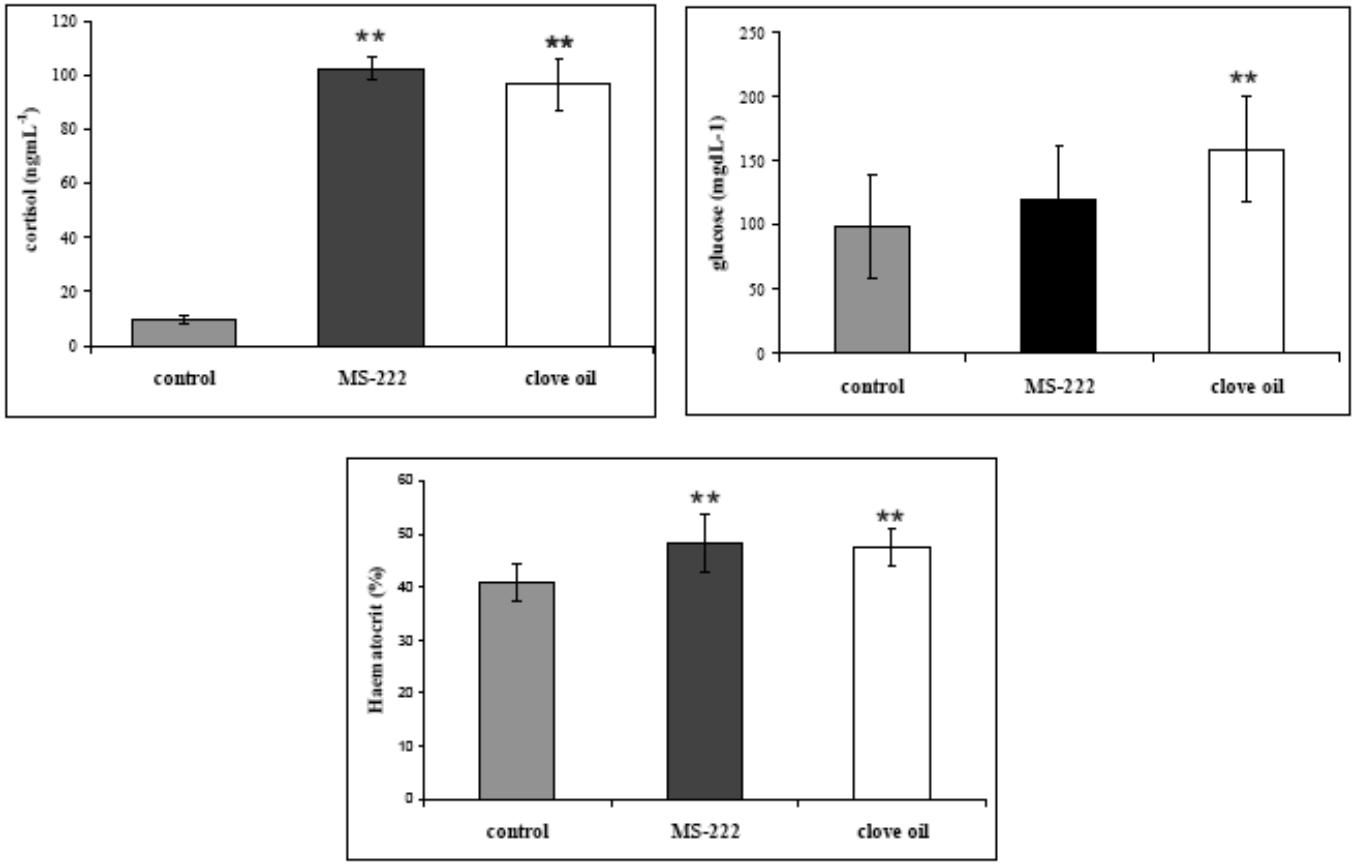

Fig. (1). Measurements of some physiological parameters (mean ± SD) in *S. dumerilii* juveniles anaesthetised in MS-222 or in clove oil. (**A**): serum cortisol; (**B**): serum glucose; (**C**): haematocrit. Asterisks indicate significant differences between groups (**P≤0.01).

no differences were found between MS-222 and clove oil-treated fish.

3.3 Trial 3: Comparison of MS-222 and Clove Oil Anaesthesia on Stress Response to Handling Stress in *P. bogaraveo* Adults

Transfer of tank and exposure to air for 2 minutes elicited in *P. bogaraveo* a marked elevation of cortisol and glucose in both the control and anaesthetised groups treated with both the tested anaesthetics.

In Figs. (**2** and **3**) the effects of anaesthetics on cortisol and glucose response to handling stress are shown.

Serum cortisol markedly increased 15 minutes after handling stress, and the peak was detected 30 minutes after handling, when cortisol reached a maximum concentration of 292.38 ± 183.31, 235.93 ± 116.02 and 213.14 ± 41.87 ng mL^{-1} in clove oil, MS-222 and control groups, respectively. There were no significant differences in cortisol levels between either of these groups.

Cortisol levels decreased significantly ($P \leq 0.01$) 60 minutes after handling, returning to the initial values measured before handling, in both anaesthetised groups. In fact, cortisol concentrations were 126.43 ± 93.41 and 104.81 ± 60.49 ng mL^{-1} in clove oil and MS-222 groups, respectively.

On the contrary, in the control group, cortisol levels continued to increase and differences between anaesthetised and control groups were significant.

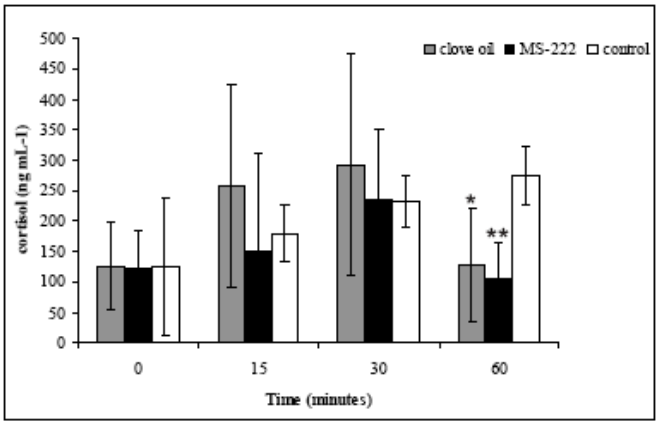

Fig. (2). Effects of anaesthetics on cortisol response in *P. bogaraveo* after handling stress. Asterisks indicate significant differences between groups (** $p \leq 0.01$; * $p \leq 0.05$).

Serum glucose markedly increased 15 minutes after stress and the peak was detected 30 minutes after stress, when glucose reached a maximum concentration of 141.02 ± 44.42, 130.01 ± 21.23 and 139.37 ± 32.18 mg dL^{-1} in clove oil, MS-222 and control groups, respectively. There were no significant differences in cortisol levels between either of these groups.

Afterward, glucose levels decreased in both the anaesthetized groups, although no recovery to initial values was observed. In fact, glucose maintained concentrations of 102.42 ± 25.73 and 113.90 ± 23.77 mg dL^{-1} respectively in clove oil and MS-222 groups, respectively. On the contrary, in the control group, glucose concentration continued to increase, reaching 155.32 ± 35.78 mg dL^{-1}.

Differences between anaesthetised and control groups were significant ($p \leq 0.05$) 60 minutes after handling.

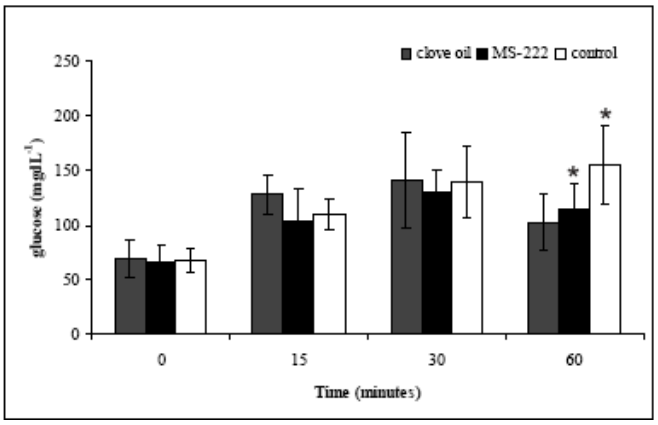

Fig. (3). Effects of anaesthetics on glucose response in *P. bogaraveo* after handling stress. Asterisks indicate significant differences between groups (*$p \leq 0.05$).

4. DISCUSSION

4.1. Trial 1: Comparative Efficacy of MS-222 and Clove Oil in *P. bogaraveo* and *S. dumerilii*

This trial examined the efficacy of clove oil, in comparison with MS-222, as an anaesthetic for wild juveniles of *P. bogaraveo* and *S. dumerilii* and assessed the minimum concentration producing the desirable anaesthetic effects.

There is no simple definition of efficacy for fish anaesthetics, although many of published papers define this as the ability to handle the fish.

Generally, an ideal anaesthetic should induce anaesthesia rapidly and allow a speed recovery, should not be toxic, should leave low tissue residues and be inexpensive [22, 23].

In our study we assumed as criteria of efficacy the ability to induce anaesthesia (stage III – plane 1) within 3 minutes and to allow recovery within 10 minutes or less.

The obtained results have shown that clove oil acts as an anaesthetic in the juveniles of both *P. bogaraveo* and *S. dumerilii.*

Blackspot sea bream and greater amberjack specimens exposed to clove oil progressed sequentially through the stages of anaesthesia outlined by Stoskopf [24].

Like MS-222, the increase in clove oil concentration, produced a reduction in the time required by fish to reach stage III-plane 1 anaesthesia.

The sequential passage of fish through the stages of anaesthesia together with the concentration and duration of the observed exposure effects, indicate that clove oil acts as "true" anaesthetic [30, 39].

The analgesic effect of clove oil comes from the inhibition of prostaglandin H synthase (PHS) by eugenol, the active ingredient of clove oil [30].

Eugenol has shown to immobilize fish at a concentration, lower than MS-222 (40 mg L^{-1} instead of 100 mg L^{-1}).

Similar results were obtained by Mylonas *et al*. [40] which showed that a concentration of 40 mg L^{-1} clove oil was able to anaesthetize both juvenile gilthead sea bream (25°C, weight 41.9 g) and sea bass (25°C, weight 32.5 g) within 3 min. Conversely, Endo *et al.* [41] showed that a concentration of 25 mg L^{-1} is required to anaesthetize rainbow trout (14°C, weight 12 g).

It seems that the highest efficacy of eugenol compared to MS-222, in this study, is due to the oil's high lipid solubility [30]. In addition to efficacy, clove oil has several benefits.

Eugenol has been widely tested for human use and consumption (as anaesthetic in dentistry, as a flavor ingredient in food) and is considered not toxic or carcinogen in mammals [29, 30] but rather, it may have anticarcinogenic properties [42].

Clove oil has not yet been approved for use in fish destined to human consumption by FDA, but AQUI-S^{TM}, an anaesthetic with chemical composition similar to clove oil (50% isoeugenol), was approved in New Zealand and Australia, as a fish anaesthetic with no withdrawal time, and work for its approval both in USA and Scandinavia was made [43].

4.2. Trial 2: Comparison of MS-222 and Clove Oil Anaesthesia Effects on the Physiology of *Seriola dumerilii* Juveniles

This trial examined the physiological responses of greater amberjack juveniles to anaesthesia with MS-222 and clove oil.

In the research on fish anaesthesia, an important aspect to be considered is whether the anaesthetic by itself generates stress.

In mammals it is known that anaesthesia, induced by certain chemical substances (*i.e.*, diethyl ether), produce stress because activation of HPI axis; conversely, other anaesthetic agents (*i.e*., barbiturates) are not stressful [44, 45].

Similarly to mammals, also in fish, anaesthesia can have a physiological impact that varies according to the anaesthetic agent [38].

Most of researches carried out to evaluate anaesthetics in relation to their stress response use cortisol and glucose as stress indicators [7, 46, 47] and a number of anaesthetic-induced increases in cortisol have been observed in several species [48-51].

Many studies were performed on MS-222, being the most used anaesthetic in aquaculture [37, 38, 52-55]; in contrast, researches on clove oil, especially on its physiological effects, are a few only [50, 51, 56].

It is known that MS-222 anaesthesia induces activation of HPI axis and its related metabolic consequences, and affects parasympathetic system resulting in an increase of both cardiac frequency and ventilatory rhythm [36, 54]. Moreover, MS-222 is an hypoxic agent; this causes a depression of medullary respiratory centre which is associated with several physiological changes including bradycardia, erythrocytes swelling and increased resistance of blood flow trough the gill lamellae [57-59].

In greater amberjack juveniles, physiological changes in serum cortisol levels and haematocrit values following exposure to clove oil, were indistinguishable from changes associated with exposure to MS-222.

Serum cortisol levels increased in greater amberjack exposed to MS-222 or clove oil.

These results are consistent with most of reports on anaesthesia in fish. Strange & Schreck [37] found that an immobilizing dose of MS-222 was capable of eliciting an immediate cortisol response; Barton & Peters [60] reported a cortisol increase in larvae after anaesthesia. Conversely, Iwama *et al*. [38] showed that anaesthesia in general produced a downward trend in plasma cortisol throughout induction and recovery.

Also haematocrit values increased in *S. dumerilii* after anaesthesia with MS-222 or clove oil.

Increase in haematocrit is a symptom of hemoconcentration that is one of the most common consequences of anaesthesia.

Literature reports haemoconcentration in fish anaesthetized with both tricaine [53-55, 61] and clove oil [50, 56].

The mechanism of action responsible for haematocrit increase is unknown, but the rapidity of the response supports the hypothesis of splenic contraction that causes an increase in red blood cell number [62].

Blood glucose concentration increased in greater amberjack anaestethized with clove oil but not with MS-222.

Sladky *et al*. [56] reported a rapid increase of blood glucose concentration in red pacu (*Piaractus brachypomus*) after anaesthesia with MS-222 or clove oil. The authors hypothesized that, similarly to what occurs in mammals, this sudden increase of glucose levels was due to catecholamine-induced gluconeogenesis.

However, a transient decrease in blood glucose concentration has also been observed by some authors [36, 63] immediately after induction of MS-222 anaesthesia. This results has led to conclude that tricaine did not alter carbohydrate metabolism in fish.

In our study is likely that this initial decrease of glucose levels occurred, while the hyperglycemia was not observed because MS-222 anaesthesia was rapidly induced (about 3 min) and therefore it probably occurred afterwards.

Although the assessment of the cardiovascular functions was not included in the aims of our study we observed that fish exposed to clove oil may have an impairment of cardiovascular system.

Collection of blood by means of caudal puncture was subjectively assessed to be more difficult in greater amberjack anaesthetised with clove oil than in those anaesthetised with MS-222.

Similar considerations were made by Sladky *et al.* [56] in red pacu (*Piaractus brachypomus*) anaesthetised with clove oil or MS-222. Authors concluded that this difficulty in blood withdrawal suggests hemodynamic instability or insufficient oxygen loading or delivery, probably associated with decreased arterial blood pressure.

One of the most common side-effects of anaesthetics is the reduced ventilation and circulation.

Moreover, clove oil has been reported to reduce gill ventilation because of depression of medullary respiratory centres, bradycardia and decreased blood flow through the gills [39].

4.3. Trial 3: Comparison of MS-222 and Clove Oil Anaesthesia on Cortisol Responsiveness to Handling Stress

This trial examined the ability of MS-222 and clove oil to avoid the normal serum cortisol increase associated to handling stress in adults of *P. bogaraveo*.

Handling is an inherently stressful event [64-66]. Removal from the water elicits a maximal physiological emergency and evokes a neuroendocrine stress response in many species of farmed fish [67]; in *S. aurata* air exposure for 3 min resulted in a 50-fold increase in plasma cortisol levels within 30 min [68].

Where handling is prolonged, such as during stripping and milking, sedation or anaesthesia is recommended [69, 70].

Therefore, when selecting an anaesthetic, it is important to evaluate its stress-reducing capacity that is the ability to block the HPI axis and make the fish unable to respond to additional stressors [30, 71].

A transitory elevation of cortisol (with a maximum concentration at 15-30 min and recovery to initial levels by 90 min) is a common response of Teleosts to an acute stress [37, 64].

The increase in cortisol levels and the hyperglycemia, typical of acute stress response, were observed in all the experimental groups exposed to handling stressor.

Cortisol values obtained in the present study indicate that anaesthetised fish are also affected by handling, but to a lesser extent than not anaesthetised fish.

However anaesthesia has proved to be effective to mitigate the duration of stress response; indeed, 60 min after handling, cortisol levels decreased in both clove oil and MS-222 groups unlike the control in which, at this time, this hormone was still high.

Similarly, blood glucose levels were different between control and anaesthetised group suggesting, also in this case, the mitigating effect of anaesthetics.

The effect of anaesthesia, however, can be considered positive if we consider, as reported in literature, the duration of stress response to handling.

Morales *at al*. in a research on physiological responses to stress of common dentex (*Dentex dentex*) [72] reported that plasma glucose and lactate levels rise, as consequence of handling, but the recovery to basal levels occurs 8 hours after stress. Similarly, Rotland *et al*. [73] reported that, in *Sparus aurata*, blood cortisol returned to normal values 4 hours after handling.

The magnitude and duration of neuroendocrine response can vary between species and depends on the intensity and type of stress [10, 13, 14].

Presumably, in this trial, the intensity of stress (transfer of tank and 2 minutes of air exposure) experienced by *P. bogaraveo* was very high and this prevented the recovery of glucose to initial levels.

Moreover, previous studies about the effectiveness of anaesthesia on handling stress in adults of *P. bogaraveo* [74], had highlighted the ability of MS-222 to prevent the activation of HPI axis; in this case, however, anaesthesia protocol was different (individual anaesthesia in 30-liters tanks *vs.* group anaesthesia in 2000-liters tanks) and the induced stress was lower (handling *vs.* handling and hypoxia).

The obtained results can be considered positive even if further studies are needed to develop efficient and reproducible anaesthetic protocols for the handling of *P. bogaraveo*.

CONCLUSIONS

This study contributes to increase knowledge on *Pagellus bogaraveo* and *Seriola dumerilii*, two species of interest for diversification of farmed fish, analysing two aspects not yet reported in literature for either the species, namely the stress response and the use of anaesthesia.

We have compared MS-222, the most popular anaesthetic used in aquaculture, with a new one, clove oil, not yet sufficiently investigated.

According to the results of induction and recovery times observed in the present investigation it can be concluded that clove oil is a good anaesthetic for both the examined species.

The minimum desirable concentration for anaesthesia which resulted in a total loss of equilibrium and muscular tone (light anaesthesia) in all the fish within 3 min, was determined to be 40 mg L^{-1} for both the species (Trial 1); to obtain the same results, 100 mg L^{-1} of MS-222 are necessary.

Moreover, the results obtained in trial 2 showed that clove oil does not affect both serum cortisol and glucose levels, differently from MS-222 in the anaesthetised fish.

According to these results clove oil seems to have interesting characteristics as an anaesthetic for both black-spot sea bream and greater amberjack. It is relatively cheap and can provide a plan of anaesthesia suitable for external sampling, fin and gill biopsies. Furthermore clove oil is easily obtained and is composed of organic substances safe for both environment and user.

One aspect that needs further researches concerns the stress-reducing capability of both MS-222 and clove oil.

Both anaesthetics proved to be unable to block activation of the HPI axis that occurs following handling stress in adults of *P. bogaraveo*, although a reduction of the duration of stress response was observed.

These results confirm that fish stress response, other than species-specific, is also affected by the duration and severity of the applied stress. In fact in previous studies MS-222

anaesthesia proved to be effective in preventing cortisol and glucose increase in *P. bogaraveo* subjected to handling stress although of a lower intensity than that experienced in the present study.

Further studies however are needed to know how *P. bogaraveo* reacts to routine practices in aquaculture and develop efficient and reproducible anaesthetic protocols.

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