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Effects of new plant based anesthetics *Origanum* sp. and *Eucalyptus* sp. oils on stress and welfare parameters in *Dicentrarchus labrax* and their comparison with clove oil

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ABSTRACT

The effects of two new anesthetics (essential oils) extracted from oregano (OO) and eucalyptus (EO) plants were studied in European sea bass (*Dicentrarchus labrax*) stress response and welfare parameters in comparison to the effects of clove oil (CO) that is one of the most used anesthetics in aquaculture. A time-course study on evolution of plasma cortisol after anesthesia was conducted. Relative expression of stress-related genes, including glucocorticoid receptor (*gr*), heat shock protein 70 and 90 (*hsp70*, *hsp90*) steroidogenic acute regulatory protein (*star*), cytochrome 11B (*cyp11b1*) and hypoxia inducible factor (*hif*) was determined in liver, gills and head kidney after anesthesia.

After anesthetic use, there was a time effect on plasma cortisol concentration, with highest values registered after 2 h for all the experimental groups. EO induced a secondary increase of plasma cortisol 24 h after the use of this anesthetic. *Hsps* expression level in gills was high at 0 h in EO fish group, whereas *hif* expression increased after 2 h of exposure in CO fish. In hepatic tissue, *gr* expression increased after 24 h in the EO group. In head kidney tissue, expression of steroidogenesis-related genes *star* and *cyp11b1* increased in EO fish group, whereas in the OO group, the expression of the same genes decreased at 2 h and 24 h of exposure. Although the three anesthetics generally showed similar patterns of variation of all analyzed parameters, results indicate that EO could be deleterious for welfare, whereas OO affects fish welfare to a lesser extent than CO and EO. Overall, these results show that OO could be a good anesthetic for fish based on greater effectiveness, lower optimum concentration and less impact on fish stress.

1. Introduction

Anesthetics in aquaculture improve fish welfare when handling procedures, such as measurements of weight and length, are necessary. Besides, anesthetic also help to prevent fish from getting hurt or feeling pain during activities such as sampling, vaccination, grading, spawning, transport or other invasive procedures such are tagging, spawning induction procedures and/or biopsy (Javahery et al., 2012). An optimum and well-applied anesthesia must induce muscle relaxation (motor reflexes) and autonomic stabilization (autonomic reflexes). Besides, it should cause analgesia (reduction of pain and suffering), must rapidly induce in fish an unconsciousness stage, and must reduce metabolic rate, oxygen consumption, and excretion (Zahl et al., 2012). It also should maintain the animal in a resting state and should allow fast recovery once the animal is removed from the anesthetic, to cause minimum struggling or stress, facilitating thus fish handling (Coyle et al., 2004).

One of the most used anesthetics in aquaculture procedures is clove oil. This is a natural and effective anesthetic obtained from the tree *Syzygium aromaticum* without negative side effects on fish (Javahery et al., 2012; Readman et al., 2013). Although clove oil is widely used for production of non-food fish and research protocols, new herbal anesthetic agents are being investigated. In this context, the anesthetic effect of Oregano and Eucalyptus oils on European sea bass

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(*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*), two important marine aquaculture species, has been very recently analyzed (Bodur et al., 2018). Those authors determined their optimal concentrations, validated their anesthetic effect without causing fish mortality and proved that the effectiveness of Oregano oil was higher than Eucalyptus and clove oils (in terms of the time to reach the anesthesia stage and post-anesthetic recovery time). However, little is known on the effects of those new plant based anesthetic agents on the stress indicators of marine fish.

Plasma cortisol, which is considered one of the best stress and welfare indicators in fish, is released into blood stream from inter-renal cells of the head kidney, triggering specific metabolic pathways in order to maintain homeostasis (Ellis et al., 2012). Cortisol is not stored at the inter-renal cells and *de novo* synthesis from cholesterol is required when a stressful condition affects the fish (Stocco et al., 2005). Steroidogenic acute regulatory protein (StAR) regulates the transport of cholesterol into the mitochondria for steroid synthesis (Stocco et al., 2005). Once in the mitochondria, steroidogenic processes induce the activation of the cytochrome P450 enzymatic cascade. The last step is regulated by the action of 11-β hydroxylase (or cytochrome P450 11β1 (CYP11B1), encoded by *cyp11b1* gene), in which 11-deoxycortisol is converted into cortisol. Both *star* and *cyp11b1* are limiting steps for cortisol synthesis (Gornati et al., 2004; Aerts et al., 2015).

Glucocorticoid receptor (GR) is a receptor protein located in the cytosol, responsible for initiating the cellular response to cortisol. GR binds heat shock proteins (HSPs) until cortisol, by entering by passive transport into the cell, induces a conformation change into another form, conferring lower affinity for HSPs (which are then released and activated). This gives GR the capability to translocate into the nucleus, dimerizes and acts as a transcription factor for stress response genes (Prunet et al., 2006). HSPs have a stress-coping function themselves and their expression dramatically increase after heat shock events. HSP synthesis is also up-regulated by a variety of stressors, and plays a fundamental role in maintaining homeostasis at cellular level by helping to fold new proteins involved in multiple processes (Gornati et al., 2004; Poltronieri et al., 2007; Roberts et al., 2010; Zhang et al., 2015). *hsp70* and *hsp90* genes are the most frequently studied since they are well-characterized and highly conserved in the genome (Roberts et al., 2010).

Cortisol-related genes are not the only genes that can be used as stress indicators. Hypoxia inducible factor (HIF) is responsible for initiating the cellular response to hypoxia and thus its expression could provide information on the effects of anesthetics on oxygen uptake and metabolism (Terova et al., 2008). It is well know that fish under a stressful situation can suffer some alterations in gills such as epithelial lifting, aneurysms, increased number of goblet cells, or fusion of some secondary lamellae, causing a decrease in gill efficiency and therefore a lack of oxygen supply (Rinaldi et al., 2005).

Most studies on fish anesthetics deal with the time-course of plasma cortisol after anesthetic use. Several studies report that anesthetics are effective in reducing the stress response but some evidences indicate anesthesia may in itself induce elevated levels of cortisol (Zahl et al., 2012). However, even when the aforementioned stress-related molecular markers are used in stress studies (Montero et al., 2015, Benítez-Dorta et al., 2017), little is known on the behavior of those indicators after the use of different anesthetics. Therefore, the aim of this study was to monitor changes in circulating plasma cortisol concentration and relative expression patterns of a set of six stress-related genes (*gr*, *hsp70, hsp90, star, cyp11b1* and *hif*) after anesthesia with Oregano and Eucalyptus oils in European sea bass, in comparison with the effects with the well-known herbal anesthetic clove oil.

2. Materials and methods

Stress experiments were carried out in the facilities of Instituto Universitario ECOAQUA, Universidad de Las Palmas de Gran Canaria (ULPGC) – Warm Water Species Selection Unit (WWSSU), following standardized procedures approved for this facility. The experimental procedures were approved by the Ethical Committee of Animal Welfare of the University of Las Palmas de Gran Canaria.

2.1. Anesthesia

Oregano (OO) (Carvacrol: 78.163%) and Eucalyptus (EO) (1.8 cineole: 80.844%) essential oils were supplied from a local company (Derdeva Herbal Oil Co, Turkey) in Antalya-Turkey, and diluted in ethanol in a 1:2 ratio before they were used. The concentrations used for each anesthetic were their optimal concentrations reported for this species: 50 $\mu L \cdot L^{-1}$ for OO and 300 $\mu L \cdot L^{-1}$ for EO (Bodur et al., 2018). Clove oil (CO) was used as reference anesthetic with the concentration determined to be effective for this and similar species (60 $\mu l \cdot L^{-1}$) (Barata et al., 2016).

The stages of anesthesia were established as follow: stage I (Sedation: motion and breathing reduced), stage II (Anesthesia: total loss of equilibrium, no reaction to touch stimuli), stage III (Recovery: gaining back equilibrium, normal swimming and breathing) and stage IV (Death: breathing and heart beat stop, overdose) (Bodur et al., 2018).

2.2. Fish and experiments

One hundred and seventeen European sea bass juveniles with a weight of 90.84 ± 1.68 g (mean ± SD) and a total length of 20.39 ± 0.13 cm were supplied by ULPGC-WWSSU and stocked in a 2 m^3 fibre-glass tank three weeks before the beginning of the experiments as acclimatization period. Temperature (20.5 ± 0.5 °C) and oxygen concentration (7.5 ± 0.1 ppm) were recorded in continuous and checked twice a day. Fish were fed with commercial extruded feed (Skretting®, Burgos, Spain) and kept under natural photoperiod (around 12:12 light/dark). Fish were fasted for 24 h before the experiment started, similarly to protocols applied in the farms when the fish are handled.

At the end of the acclimatization period, 9 fish were rapidly sampled (< 3 min of handling for the whole 9 fish population) and was considered as pre-anesthesia or control group, giving values previous to anesthetic and, consequently, were considered as basal levels of the different stress indicators measured in the present study. With the rest of the animals, a stress trial was conducted as follow: 36 fish were used for each of the anesthetic (OO, EO and CO). First, 12 fish from each anesthetic treatment were stocked in a bucket filled with 10 L sea-water (triplicate procedure for each anesthetic used). The indicated concentration of each anesthetic was applied after the fish in bucket were calmed (approximately 1 min) and 3 fish from the each bucket were sampled immediately after reaching the stage II of anesthesia (0 h sampling point). The remaining 9 fish from each three buckets per anesthetic treatment were transferred separately to 3 cylindrical tanks (500 L) for recovery and for the next time point samples (2 h, 6 h, 24 h). Three fish from each tank (triplicate for each anesthetic group) were sampled for each of the next sampling points. Fish were then not re-used and triplicate buckets and tanks were used for each anesthetic treatment, being $n = 9$ for each sampling point.

For each fish, blood was collected from the caudal vein, stocked in 1.5 mL heparinized centrifuge tubes kept on ice and immediately centrifuged for 10 min at 14000 rpm. The supernatant was stocked in 1.5 mL centrifuge tubes at −80 °C separately until cortisol analyses. After blood withdrawal, fish were immediately slaughtered by percussive stunning, and head kidney, gills and liver samples were collected. The samples of each tissue (from 3 fish for each tank and sampling point) were stored together in RNA*later* (Sigma) for 8 h at 4 °C. After that, RNA*later* was removed and tissues were kept at −80 °C until analysis.