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# Anesthetic Efficacy and Physiological Response to MS-222, Eugenol, and *Cymbopogon flexuosus* Essential Oil in Juvenile Spotted Seatrout (*Cynoscion nebulosus*)

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## ABSTRACT

For aquaculture, animal welfare constitutes a fundamental dimension of sustainability because it directly influences health, productivity, resource efficiency, and the ethical standards of production systems. Spotted seatrout (*Cynoscion nebulosus*) is a marine fish recognized as an emerging candidate for aquaculture in the Gulf of Mexico and, like other developing species, is continually subjected to experimental research to support the advancement of its culture technologies. In this context, identifying effective anesthesia conditions is essential for minimizing stress during operational procedures and improving the consistency of experimental outcomes. The aim of this study was to comparatively evaluate anesthetic effectiveness in juvenile spotted seatrout using behavioral responses and blood biochemical stress indicators. The anesthetic effectiveness of MS-222, eugenol, and *Cymbopogon flexuosus* essential oil (CFEO) was evaluated in juvenile spotted seatrout (mean ± SD: 28.2 ± 3.5 g wet weight and 14.7 ± 0.7 cm total length). MS-222 at 200, 250, and 300 mg $L^{-1}$, eugenol at 25, 30, 35, and 40 mg $L^{-1}$, and CFEO at 100 and 125 μ$L^{-1}$ induced deep anesthesia within 15 min, followed by complete recovery within 5 min. However, only MS-222 at 300 mg $L^{-1}$ and eugenol at 40 mg $L^{-1}$ achieved deep anesthesia within 3 min, 110.0 and 149.5 s, respectively. Despite this, MS-222 at 300 mg $L^{-1}$ produced the highest plasma lactate levels, whereas eugenol at 40 mg $L^{-1}$ elevated both plasma lactate and glucose relative to the control group. In contrast, eugenol at 35 mg $L^{-1}$ resulted in the lowest plasma lactate and glucose levels. These findings indicate that eugenol at 35 mg $L^{-1}$ represents a balanced anesthetic option for juvenile spotted seatrout. Moreover, rapid anesthetic induction does not necessarily imply reduced physiological stress in this species.

## 1 | Introduction

Aquaculture is an important sector for global food and nutrition security [1]. Its significance stems from sustained expansion, driven by technological advances in efficient production protocols and the development of culture packages for emerging species [2]. Similar to other animal production industries, aquaculture acknowledges animal welfare as a critical dimension of production, essential for both productivity and long-term

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sustainability [3]. In fish culture, whether experimental or commercial, organisms are regularly exposed to handling stress associated with operational procedures such as biometrics, tagging, transportation, vaccination, and blood collection, among others. In this context, protocols for sedation and anesthesia have been established to mitigate handling-induced stress, with numerous natural and synthetic anesthetics evaluated for this purpose [4–6].

Tricaine methanesulfonate (MS-222) is the most widely used synthetic anesthetic in fish aquaculture and is distinguished by its 100% purity [7, 8]. It is also the only anesthetic agent approved by the Food and Drug Administration (FDA) for use in fish in the United States, with a mandatory withdrawal period of 21 days prior to harvest [9]. Eugenol, a monoterpene with anesthetic properties, is widely distributed in plants, with the clove tree (*Syzygium aromaticum*) as its principal source [10]. Despite lacking official recognition as an anesthetic, it is extensively applied off-label in fish aquaculture, mainly through clove oil [4, 11, 12]. Lemongrass (*Cymbopogon flexuosus*) essential oil (CFEO), composed primarily of citral and geraniol—both monoterpenes with anesthetic properties—is an emerging anesthetic in fish aquaculture [13–15]. These anesthetic agents are recognized and recommended for their proven efficacy in inducing anesthesia and for their essential role in minimizing handling-induced stress in fish aquaculture.

The pharmacokinetic and pharmacodynamic properties of anesthetic agents are species-specific, and consequently, their efficacy in inducing anesthesia varies among fish species [16–19]. For this reason, it is necessary to establish sedation, anesthesia, and euthanasia protocols tailored to each species, thereby ensuring adequate animal welfare practices. Spotted seatrout (*Cynoscion nebulosus*) is a marine fish with recreational and commercial importance, distributed from New York, USA, to the Yucatán Peninsula, México [20, 21]. This species is considered emerging in the aquaculture industry of the Gulf of Mexico due to the availability of functional reproduction and larviculture techniques, the feasibility of intensive rearing under controlled systems, and its favorable biological traits (eurythermal and euryhaline) [22]. However, further research is needed on its specific nutritional requirements, the optimization of juvenile production, and the overall feasibility of commercial aquaculture.

Although MS-222 is widely used and remains the only anesthetic for food fish in several regulatory frameworks, comparative evaluations integrating behavioral and physiological stress indicators have not been conducted in juvenile spotted seatrout. Moreover, the suitability of alternative anesthetics such as eugenol and CFEO under research conditions has not been assessed in this species. The aim of this study was to comparatively evaluate MS-222, eugenol and CFEO in juvenile spotted seatrout to identify an appropriate protocol integrating behavioral responses and biochemical stress indicators.

## 2 | Materials and Methods

### 2.1 | Experimental Animals

The juvenile spotted seatrout (*C. nebulosus*) (mean ± SD: 28.2 ± 3.5 g wet weight and 14.7 ± 0.7 cm total length) were obtained from Unidad Multidisciplinaria de Docencia e Investigación (UMDI)-Sisal, Facultad de Ciencias, UNAM, following the methodology described by Arenas-Pardo et al. [23]. Prior to the experiment, the fish were reared in 3500 L fiberglass tanks equipped with a continuous water flow system and constant aeration, housed within a greenhouse facility. The seawater used for fish rearing was pretreated using a pressurized sand filter and multicartridge filters (10 and 5 microns). During the rearing period, water quality parameters were maintained at the following levels: temperature, 28.2 ± 3.5°C; dissolved oxygen, 4.6 ± 0.4 mg $L^{-1}$; pH, 7.8 ± 0.0; salinity, 35 PSU; ammonium, 0.05 ± 0.0 mg $L^{-1}$; nitrites, 0.0 ± 0.0 mg $L^{-1}$; nitrates, 2.23 ± 1.4 mg $L^{-1}$. The fish were fed *ad libitum* three times daily with an experimental diet containing 54.3% protein, 11.5% lipid, 11% carbohydrates, and 8% moisture.

### 2.2 | Anesthetics

The anesthetic efficiency of MS-222 (E10521, Sigma Aldrich, USA), eugenol (99.99%, Mirafill, Mexico), and *C. flexuosus* essential oil (CFEO) (30130608, doTerra, USA) were evaluated. The following anesthetic concentrations were tested: 150, 200, 250, and 300 mg $L^{-1}$ for MS-222; 20, 25, 30, 35, and 40 mg $L^{-1}$for eugenol; 75, 100, and 125 μL $L^{-1}$ for Lemongrass essential oil. The concentrations with anesthetic effects were established *a priori*, based on the minimum concentration producing anesthetic within a 15 min period [5].

Prior to use, eugenol was diluted in ethanol (493546, Sigma Aldrich, USA) at 1:2 (eugenol: ethanol), and CFEO was diluted in ethanol at 1:10 (CFEO: ethanol) [4, 13, 24]. The final ethanol concentrations in the anesthetic baths ranged from 0.04% to 0.08% (v/v) for eugenol treatments and from 0.075% to 0.125% (v/v) for CFEO treatments. These concentrations were below levels commonly associated with acute physiological or behavioral alterations in teleost fish during short-term exposure [25, 26]. The acidic effect of MS-222 dilution was neutralized using sodium bicarbonate at 1:2 (MS-222: sodium bicarbonate) [7]. All anesthetic solutions were prepared in seawater and aerated vigorously for 5 min before use.

### 2.3 | Experimental Design

The trial was conducted in 10 L rectangular glass aquaria (29 cm length × 18 cm width × 20 cm height). The experimental design consisted of independent dose–response evaluations for each anesthetic agent during the behavioral phase. Based on the shortest induction time to stage III anesthesia within each agent, the most effective concentration was subsequently selected for biochemical analysis.

A total of 88 fish were used in this study. Fish were individually exposed to the anesthetic baths, with each fish considered an independent experimental unit. Therefore, a total of 88 individual exposure aquaria were used (one fish per aquarium). For each anesthetic concentration, eight fish were randomly assigned and exposed individually. Of these, four fish were used for behavioral assessment and four for biochemical analyses. Fresh anesthetic solution and fresh recovery seawater were prepared for each fish to ensure consistent exposure conditions.

For biochemical evaluation, a handled control group was included. Control fish were subjected to the same handling

procedures and duration as treated fish (capture, transfer to exposure aquaria, and recovery period) but were exposed only to clean seawater for 3 min, corresponding to the maximum induction time considered acceptable for an ideal anesthetic protocol. All fish were fasted for 24 h prior to the experiment.

### 2.4 | Behavioral Analysis

The criteria for determining behavioral responses were based on Jia et al. [27] and modified (Table 1). To conduct this experiment, stage I anesthesia was characterized by the fish's lack of response to gentle pressure applied to the caudal fin using a plastic rod. Stage II was defined by the loss of equilibrium, while stage III was identified when the fish exhibited no struggling upon removal from the water with a net. No specific time criterion was established for recovery; fish were allowed to recover freely. Each anesthetic induction and recovery was recorded using a GoPro Hero 11 Black camera (GoPro, Inc., USA) to evaluate the visual behavioral responses. Fish survival was monitored for 24 h following anesthetic induction.

### 2.5 | Biochemical Analysis

Blood samples were collected from the caudal artery using a U-100 1 mL 31G × 6 mm BD Ultra-Fine syringe previously coated with heparin. A total of 80 μL of blood was collected per fish and immediately diluted in 240 μL of EDTA solution (2 mg $mL^{-1}$, E9884, Sigma Aldrich, USA) at a 1:3 ratio (blood: EDTA) to prevent coagulation. Samples were centrifuged at 5000 rpm for 10 min, and then the resulting plasma was used to measure lactate and glucose concentration. Lactate and glucose were determined using commercial assay kits L7596-50 (POINTE, USA) and GPSL-0507 (ELITechGroup Inc, USA), respectively. Biochemical assays were performed in triplicate.

### 2.6 | Statistical Analysis

Data are presented as mean ± standard error (SE). For each anesthetic, induction and recovery times were analyzed across concentrations using a one-way ANOVA ($n = 4$ per concentration). For biochemical analysis, only concentrations that effectively induced stage III anesthesia during the behavioral phase were selected within each anesthetic agent, and comparisons were performed under this effective-dose framework. This approach allowed comparison of physiological stress indicators among effective anesthetic doses within each agent ($n = 4$). Statistical analyses were performed using one-way analysis of variance (ANOVA). When significant differences were detected ($p < 0.05$), Tukey's post hoc test was applied for multiple comparisons. When only two groups were compared, a two-sided Student's *t*-test was used. Prior to ANOVA and *t*-test analyses, normality and homogeneity of variances were assessed using the Shapiro–Wilk and Bartlett tests, respectively. All statistical analyses were conducted using R statistical software (R Core Team), and graphical representations were generated using GraphPad Prism 8 (GraphPad Software, USA).

**TABLE 1** | Stages of anesthesia induction and recovery in juvenile spotted seatrout (adopted and modified from Jia et al. [27]).

| Stage of anesthesia | Behavior responses |
|---|---|
| I | Sedation: partial decrease of reactivity to extern stimuli and equilibrium normal. |
| II | Partial loss of equilibrium, signs of ataxia and lethargic swimming. |
| III | Anesthesia: complete loss of equilibrium, cessation of locomotion and no response to strong external stimuli. |
| IV | Medullary collapse: death. |
| Recovery | Total equilibrium recovery, normal swimming, and response to external stimuli. |

## 3 | Results

### 3.1 | Behavioral Analysis

Anesthesia induction and recovery times of fish exposed to different concentrations of MS-222 are presented in Figure 1. MS-222 did not induce stage I anesthesia, fish were immediately induced to stage II anesthesia upon immersion. The lowest concentration (150 mg $L^{-1}$) failed to induce stage III anesthesia after 15 min of exposure and was therefore excluded from the analysis (Figure 1A). The concentration of 300 mg $L^{-1}$ produced the shortest induction time to stage III anesthesia ($p < 0.05$), whereas 200 and 250 mg $L^{-1}$ did not differ significantly ($p > 0.05$). The fastest recovery was observed at 300 mg $L^{-1}$ ($p < 0.05$), whereas recovery times at 200 and 250 mg $L^{-1}$ did not differ significantly ($p > 0.05$) (Figure 1B).

Anesthesia induction and recovery times of fish exposed to different concentrations of eugenol are presented in Figure 2. The lowest concentration (20 mg $L^{-1}$) did not induce stage III anesthesia within 15 min of exposure and was therefore excluded from the analysis (Figure 2A). The concentrations of 30, 35 and 40 mg $L^{-1}$ resulted in the shortest induction times to stage III anesthesia, whereas 25 mg $L^{-1}$ produced the longest time ($p < 0.05$). At 40 mg $L^{-1}$, stage I anesthesia was not observed; fish were immediately induced to stage II anesthesia upon immersion. No significant differences in recovery time were observed among treatments ($p > 0.05$) (Figure 2B).

Anesthesia induction and recovery times of fish exposed to different concentrations of CFEO are presented in Figure 3. The lowest concentration (75 μL $L^{-1}$) did not induce stage III anesthesia within 15 min of exposure and was therefore excluded from the analysis (Figure 3A). No significant differences were observed between 100 and 125 μL $L^{-1}$ in either induction to stage III anesthesia or recovery time after exposure ($p > 0.05$) (Figure 3B).

The overall results for anesthesia and recovery times for the three anesthetics used in the study, including survival, are reported in Table 1.

### 3.2 | Biochemical Analysis

Plasma glucose levels in juvenile spotted seatrout exposed to different concentrations of anesthetic agents are presented in

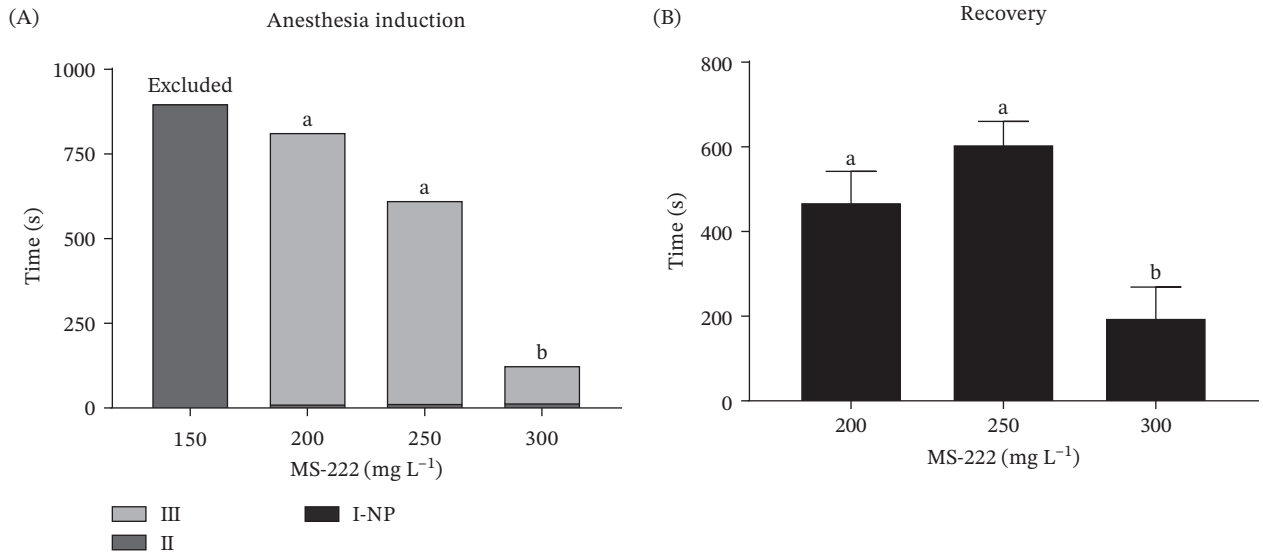



**FIGURE 1** | Anesthesia induction (A) and recovery times (B) of juvenile spotted seatrout exposed to different concentrations of MS-222. (A) Temporal progression of anesthetic stages during induction at each concentration. Mean induction times and associated variability are provided in Table S1. (B) Recovery times as mean ± SE ($n = 4$). Difference letters indicate significant differences among treatments (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Abbreviations: NP, not presented.

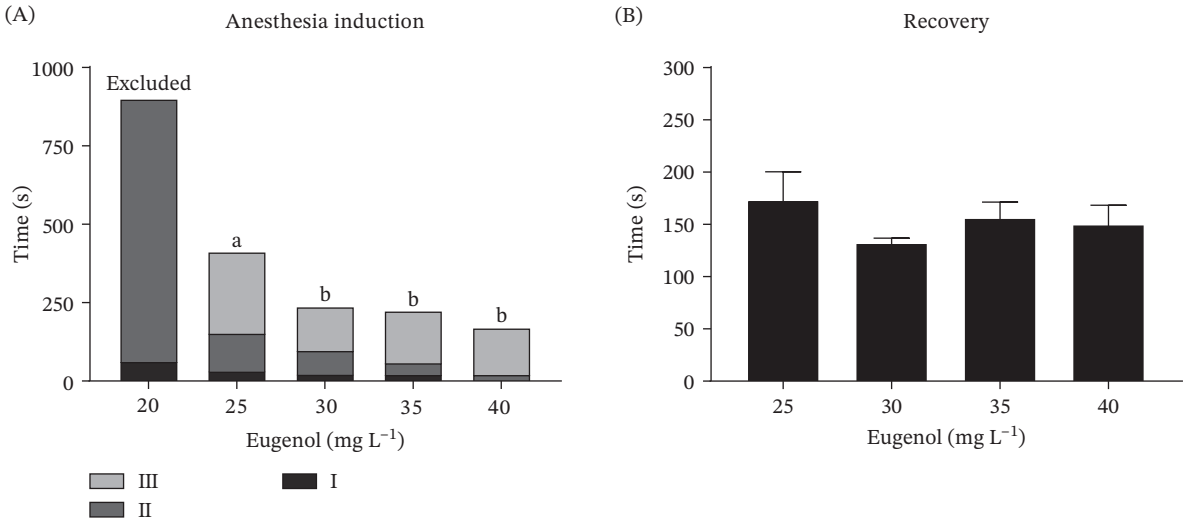



**FIGURE 2** | Anesthesia induction (A) and recovery times (B) of juvenile spotted seatrout exposed to different concentrations of eugenol. (A) Temporal progression of anesthetic stages during induction at each concentration. Mean induction times and associated variability are provided in Table S1. (B) Recovery times as mean ± SE ($n = 4$). Statistical differences were detected in panel A (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$), whereas no significant differences were observed in panel B (one-way ANOVA, $p > 0.05$). Abbreviations: NP, not presented.

Figure 4. Compared with the Control, MS-222 at 300 mg $L^{-1}$ (MS-222-300), eugenol at 35 mg $L^{-1}$ (E35), and *C. flexuosus* essential oil at 125 μL $L^{-1}$ (CFEO-125) produced the lowest glucose levels ($p < 0.05$). In contrast, glucose levels in MS-222-200, MS-222-250, E25, and E40 treatments did not differ significantly from the Control ($p > 0.05$); however, glucose levels in E30 and CFEO-100 were lower than those in Control ($p < 0.05$).

Plasma lactate levels in juvenile spotted seatrout exposed to different concentrations of anesthetic agents are presented in Figure 5. Compared to the Control, E35 produced the lowest lactate level, whereas E25 resulted in the highest ($p < 0.05$). The remaining treatments did not differ significantly from the Control ($p > 0.05$); however, lactate levels in MS-222-200, MS-222-250, E30, and CFEO-125 were lower than those in MS-222-300 and CFEO-100 ($p < 0.05$).

## 4 | Discussion

This study provides the first integrative evaluation of MS-222, eugenol, and CFEO in juvenile spotted seatrout, combining behavioral responses and blood biochemical stress indicators, with the aim of developing an appropriate anesthetic protocol for handling procedures under both experimental and farming conditions.

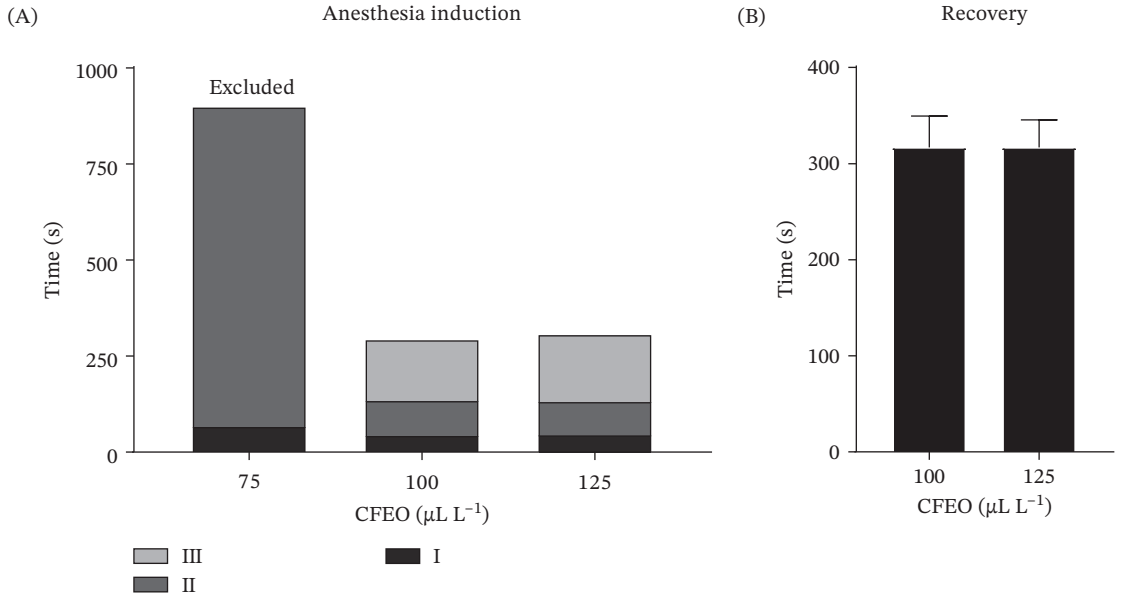



**FIGURE 3** | Anesthesia induction (A) and recovery times (B) of juvenile spotted seatrout exposed to different concentrations of *Cymbopogon flexuosus* essential oil (CFEO). (A) Temporal progression of anesthetic stages during induction at each concentration. Mean induction times and associated variability are provided in Table S1. (B) Recovery times as mean ± SE ($n = 4$). No significant differences were observed among treatments (Student's two-sided *t*-test, $p > 0.05$). Abbreviations: NP, not presented.

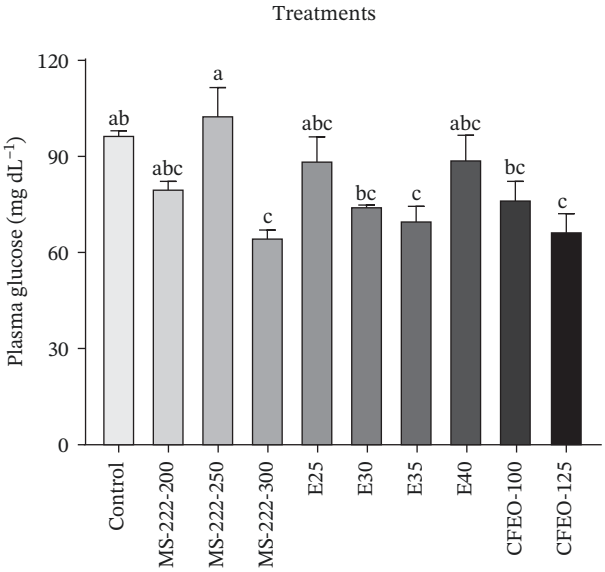



**FIGURE 4** | Changes in plasma glucose in juvenile spotted seatrout after exposure to different concentrations of different anesthetic agents. Data are presented as mean ± SE ($n = 4$). Difference letters indicate significant differences among treatments (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Abbreviations: MS-222-200, MS-222 at 200 mg $L^{-1}$; MS-222-250, MS-222 at 250 mg $L^{-1}$; MS-222-300, MS-222 at 300 mg $L^{-1}$; E25, eugenol at 25 mg $L^{-1}$; E30, eugenol at 30 mg $L^{-1}$; E35, eugenol at 35 mg $L^{-1}$; E40, eugenol at 40 mg $L^{-1}$; CFEO-100, *Cymbopogon flexuosus* essential oil at 100 μL $L^{-1}$; CFEO-125, *C. flexuosus* essential oil at 125 μL $L^{-1}$.

### 4.1 | Behavioral Analysis

In fish, the effectiveness of anesthetic agent primarily depends on species, age, physiological condition, and environmental factors, all of which influence both pharmacokinetic and pharmacodynamic properties [28–30]. The criterion for an ideal anesthetic protocol in fish is the induction of deep anesthesia (stage III) within 15 min of exposure, preferably in less than 3 min, followed by complete recovery within 5 min after cessation of the anesthetic [5, 12].

MS-222 is a local anesthetic that induces anesthesia by reversibly blocking voltage-gated sodium channels, thereby preventing cellular depolarization and blocking the propagation of nerve impulses essential for sensory transmission and muscle contraction [31]. In fish, the doses of MS-222 required to achieve effective anesthetic induction vary considerably among species, ranging from 70 to 480 mg $L^{-1}$ [8]. In this study, MS-222 at 150 mg $L^{-1}$ did not induce stage III anesthesia in juvenile spotted

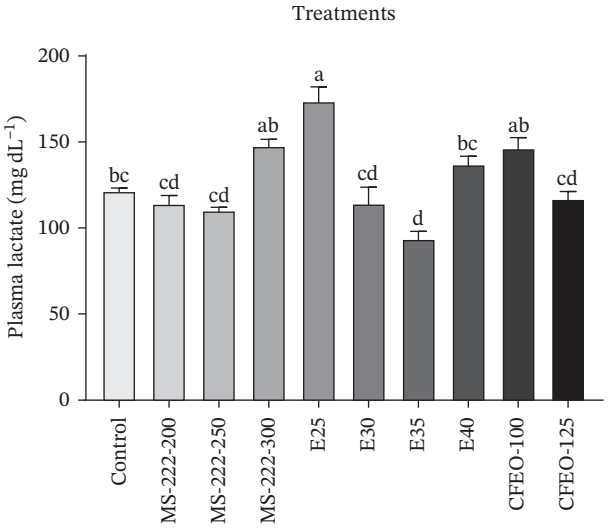



**FIGURE 5** | Changes in plasma lactate in juvenile spotted seatrout after exposure to different concentrations of different anesthetic agents. Data are presented as mean ± SE ($n = 4$). Difference letters indicate significant differences among treatments (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Abbreviations: MS-222-200, MS-222 at 200 mg L$^{-1}$; MS-222-250, MS-222 at 250 mg L$^{-1}$; MS-222-300, MS-222 at 300 mg L$^{-1}$; E25, eugenol at 25 mg L$^{-1}$; E30, eugenol at 30 mg L$^{-1}$; E35, eugenol at 35 mg L$^{-1}$; E40, eugenol at 40 mg L$^{-1}$; CFEO-100, *Cymbopogon flexuosus* essential oil at 100 μL L$^{-1}$; CFEO-125, *C. flexuosus* essential oil at 125 μL L$^{-1}$.

seatrout within 15 min of exposure. By contrast, concentrations between 70 and 150 mg L$^{-1}$ induced stage III anesthesia within 3 min and allowed full recovery within 5 min in *Hippocampus kuda*, *Labeo rohita*, and *Solea senegalensis* [32–34]. In this study, only MS-222 at 300 mg L$^{-1}$ produced an effective stage III anesthesia, with induction and recovery occurring within 3 and 5 min, respectively. However, this concentration is at the upper limit of the range reported for fish species [8, 12]. Variability in the efficacy of MS-222 among fish species may be related to differences in voltage-gated sodium channel isoforms, although the contribution of these isoforms to anesthetic sensitivity remains speculative [35, 36].

Eugenol, similar to MS-222, is a local anesthetic. In addition to acting on voltage-gated sodium channels, it reversibly inhibits potassium and calcium channels, which are critical for neural signaling, mediating membrane repolarization and neurotransmitter release, respectively [37–39]. Furthermore, in silico analyses predict that eugenol may also interact with a wide range of membrane receptors, neurotransmitters, and ionic signaling pathways in fish [40]. Except for eugenol at 20 mg L$^{-1}$, all treatments produced stage III anesthesia, with induction and recovery within 15 and 5 min, respectively. Eugenol at 40 mg L$^{-1}$ achieved induction within 3 min, while 30 and 35 mg L$^{-1}$ required 4 min, with no significant differences between them. The effective eugenol doses identified in this study are consistent with those reported for other fish species [4].

Compared with the effective concentration identified for MS-222 in the present study, eugenol achieved stage III anesthesia at lower nominal concentration under the tested conditions, a difference that may be attributed to its broad-spectrum modulatory effects on multiple receptor types and ion channels involved in neuronal signal transmission. Consistent with the present findings, MS-222 has been reported to be less effective than eugenol in other fish species, including *Acipenser baerii*, *Anisotremus scapularis*, and *H. kuda* [33, 41, 42].

CFEO is a mixture of multiple constituents, only some of which possess anesthetic activity. Among these, citral and geraniol are primary bioactive components, occurring at concentrations of ~54%–71% and 3%–7%, respectively [43]. Like eugenol, citral and geraniol are monoterpenoids with local anesthetic-like properties, as they inhibit nerve excitability [10, 44]. In general, the effective anesthesia doses of herbal essential oils with anesthetic properties are higher than those of their pure active ingredients [5], which is consistent with the findings of the present study comparing eugenol and CFEO. In this study, the effective doses for stage III anesthesia of CFEO were 100 and 125 μL L$^{-1}$, with average induction times of 4.9 and 5.1 min, respectively, and a recovery time of 5.2 min for both doses. Although these doses slightly exceed the desirable features of an anesthetic agent—recovery within 5 min—they remain within an acceptable range for practical application. Moreover, these values represent the lowest doses, as well as shortest induction and recovery times reported when compared with other fish species, such as *Rhamdia quelen*, *Oreochromis niloticus*, and tambacu (*Piaractus mesopotamicus* male X *Colossoma macropomum* female) [13–15]. The variations in effectiveness of CFEO among fish species may be associated with intrinsic plant factors, environmental conditions, cultivation practices, and the extraction process [4].

In contrast to eugenol, which showed a relatively consistent reduction in induction time across effective concentrations, MS-222 and CFEO did not exhibit a strictly proportional dose-response pattern in terms of induction and recovery. Although increasing concentration generally shortened induction time, these improvements were not consistently accompanied by faster recovery or a clear separation between doses. Such variability likely reflects non-linear pharmacodynamic dynamics and threshold-dependent effects, together with species-specific differences in anesthetic sensitivity. Non-linear concentration-effects relationships have been described in general anesthesia models [45], supporting the interpretation that anesthetic responses may not scale proportionally with dose. Therefore, increasing anesthetic concentration does not necessarily result in predictable or proportional improvements in anesthetic performance.

## 4.2 | Biochemical Analysis

Stress is the nonspecific physiological and behavioral response to disruptions of dynamic homeostasis in organisms [46]. In fish, glucose and lactate are widely recognized as secondary indicators of the stress response, reflecting downstream metabolic consequences of hypothalamic-pituitary-interrenal (HPI) axis activation [12]. Although cortisol represents the primary endocrine mediator of the stress response in teleost fish, secondary metabolic indicators provide direct insight into the functional energetic disturbance associated with acute handling and anesthetic exposure [47]. In the present study, with the exception of MS-222 at 300 mg L$^{-1}$ (MS-222-300), eugenol at 35 mg L$^{-1}$ (E35) and CFEO at 125 μL$^{-1}$ (CFEO-125), plasma glucose levels in most anesthetic treatments were comparable to those of the Control group. These findings suggest that several treatments did not

mitigate the metabolic consequence of handling stress and may have contributed additional physiological disturbance during anesthesia exposure. Although anesthetics are employed to mitigate handling stress in fish, they may also act as stressors, as reflected by alterations in biochemical stress indicators [8, 12, 48].

Under stress conditions, lactate production in vertebrates results from reduced oxygen availability or impaired cellular utilization of oxygen, both of which constrain aerobic metabolism [49]. In this study, fish exposed to E35 exhibited the lowest plasma lactate levels, whereas MS-222-300 and CFEO-125 treatments showed levels comparable to those of the Control group, similar to the other treatments. Among agents tested, eugenol at 35 mg $L^{-1}$ produced the most favorable biochemical profile in juvenile spotted seatrout. To explain the elevated lactate levels observed, two possible scenarios can be considered. First, the high lactate levels recorded in MS-222-300 and CFEO-125—treatments associated with the lowest plasma glucose—may reflect reduced oxygen availability due to anesthetic suppression of the respiratory center and impair gas exchange, as reported in vertebrates [50]. Second, the high lactate levels recorded in treatments with elevated glucose (E25, E40, and CFEO-100) reflect impaired cellular oxygen utilization, potentially linked to increased energy demand and reduced respiratory efficiency [51].

Although rapid induction has traditionally been considered a defining characteristic of an ideal anesthetic protocol in fish—particularly induction to stage III anesthesia within 3 min—the present findings suggest that induction time alone may be insufficient to determine anesthetic suitability. In this study, eugenol at 35 mg $L^{-1}$ did not consistently achieve stage III anesthesia within 3 min, yet it produced the most favorable biochemical stress profile among the evaluated treatments. This discrepancy indicates that reliance solely on behavioral endpoints may overlook relevant physiological responses. Therefore, the integration of biochemical stress indicators with behavioral criteria may provide a more comprehensive and biologically meaningful framework for defining anesthetic suitability in marine fish species.

While the number of biological replicates per concentration was modest ($n = 4$ for behavioral assessments and $n = 4$ for biochemical analyses), statistically significant differences were detected in key metabolic and behavioral endpoints, indicating adequate sensitivity to identify major physiological responses under the tested conditions. Nevertheless, future studies incorporating larger sample sizes would further strengthen the statistical robustness of these findings.

From a practical perspective, the regulatory context of anesthetic use in aquaculture must also be considered. MS-222 remains the only anesthetic formally approved for use in food fish under several regulatory frameworks [9]. Regulatory approval requires comprehensive evaluation, including residue depletion studies, pharmacokinetic characterization, toxicological assessment, environmental impact analysis, and the establishment of withdrawal periods [52]. This study provides comparative behavioral and physiological evidence under experimental conditions; however, additional regulatory-oriented studies would be required before formal approval of alternative anesthetics could be considered for commercial aquaculture applications.

## 5 | Conclusion

In summary, this study provides the first evaluation of MS-222, eugenol, and CFEO as anesthetic agents for juvenile spotted seatrout, considering both behavioral responses and biochemical stress indicators. Among the agents tested, eugenol at 35 mg $L^{-1}$ produced the most favorable biochemical profile, although it did not induce stage III anesthesia within 3 min. This finding suggests that the conventional definition of an ideal anesthetic protocol—primarily based on rapid induction criteria—may not fully capture anesthetic suitability in this species. Overall, the results indicate that eugenol at 35 mg $L^{-1}$ represents the most balanced option for juvenile spotted seatrout, underscoring the importance of integrating behavioral and physiological stress indicators when establishing anesthetic protocols. The integrative approach may contribute to improved welfare standards and more physiologically informed anesthetic practices in marine aquaculture.


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### Ethics Statement

The study was conducted according to the guidelines of Bioethics subcommittee of the Ethics and Scientific Responsibility Commission of the Facultad de Ciencias, UNAM (Folio: PD_21_06_2023_Gaxiola).

### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Supporting Information**. Table S1: Induction and recovery times (s) of juvenile spotted seatrout exposure to different concentrations of MS-222, eugenol, and *Cymbopogon flexuosus* essential oil (CFEO).