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Effects of three anesthesia and two tranquilizers' protocols on stress response during handling in tilapia *Oreochromis niloticus*

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Abstract

To determine the effect of anesthesia and tranquilization on the stress response of tilapia (*Oreochromis nilotica*) during a handling procedure. We assess three anesthetics: ketamine, MS222, and clove oil; and two tranquilizers: xylazine and medetomidine, which were administrated by submersion in adult tilapia. Our results showed a significant difference in cortisol levels between treatments after handling procedure ($P = 0.0002$), where xylazine had the highest cortisol levels compared to the other treatments. We also found differences between induction and recovery times in the different treatments. Then, animals exposed to xylazine took longer to present induction effects, than the other treatments ($P < 0.0001$). For recovery times, ketamine presents the longest recovery times, than clove oil and MS222, but not when compared to animals exposed to medetomidine and xylazine ($P = 0.0019$). We observed paleness in animals exposed to medetomidine.

Keywords: *Oreochromis* niloticus; Cortisol; Ketamine; MS222; Clove oil; Xylazine; Medetomidine.

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Study contribution

Studies directed to compare the effect of anesthetics and tranquilizers to determine their effect on the stress response during a surgical or handling procedure in fish are scarce. This is a prospective randomized experimental study that assessed the effect of three anesthetics and two tranquilizers in the stress response of tilapia (*Oreochromis nilotica*) during a handling procedure. We found that ketamine and medetomidine are the most recommended treatments in case of seeking anesthetic and tranquilizing effect and avoiding the activation of the hypothalamic-pituitary-inter-renal (HPI) axis in fish. We found that MS222 and clove oil were able to increase cortisol levels in the blood associated with stress. This is an indication that animals exposed to those treatments do not lose consciousness completely. Paleness was observed in animals exposed to medetomidine, indicating that it is a potent alpha 2 adrenergic receptor agonists, therefore, its use must be evaluated before use in fish.

Introduction

Anesthetics are widely used in commercial aquaculture and field studies in fish.[1] The ideal anesthetic should be safe for the animal, as well as the staff performing the procedure. It should produce rapid induction and recovery, be low-stress, be non-toxic, and have a reasonable cost.[2] Any anesthetic method, must guarantee loss of motility, consciousness, reflexes, and muscle relaxation.[3] Anesthesia protocols in fish should depend on the procedure type, time, and the place in which a handling method will be performed. Routes of anesthetic administration in fish can be by submersion, gill irrigation, and intramuscular administration.[4]

The drugs of choice for submersion are MS222 (tricaine methanesulfonate) and eugenol (clove oil).[4] Since their effect is variable, they are only effective for quick handling procedures, and therefore they are not recommended in surgical procedures in fish. Moreover, in the case of eugenol, it remains undetermined whether it really induces loss of consciousness in fish, or only produces immobilization,[5] therefore, clove oil is a product that is not suitable for surgical or painful procedures in fish. Inhaled anesthetic such as isoflurane, has been reported in fish, and it can be administered via direct bubbling in water.[6] There have been reported systems, in which inhaled and parenteral anesthetic can be dissolved in water and irrigated across the gills, and this allows control of anesthesia times and reduces recovery periods.[6] In the case of intramuscular administration, ketamine can be found in combination with xylazine, benzocaine, metomidate, and urethane for surgical interventions in fish.[4]

Ketamine is a dissociative anesthetic widely used in several animal species. By itself it cannot promote appropriate muscle relaxation, therefore it is necessary its combine with tranquilizers, which are usually alpha-adrenergic drugs like xylazine, medetomidine, and detomidine to achieve a deeper and long-lasting dissociative anesthesia.[7] Ketamine has been used to induce experimental anesthesia combined with fentanyl,[8] and there is also a report of its use as an anesthetic in carp combined with xylazine.[2] This combination results in prolonged anesthesia (42.7 min) with an induction time of 18 min.[2] In the case of medetomidine, there is only a report as an analgesic in golden fish.[9]

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Both xylazine and medetomidine, are from the family of alpha-adrenergic tranquilizers or sedatives.[10] The localization and function of alpha-adrenergic receptors are similar among all vertebrates. In fish, these receptors affect motor function at the medullar level and in peripheral nerves. They do not cause loss of consciousness and have a weak effect on pain reduction,[11] therefore they should be combined with anesthetics and analgesics.

To assess the effectiveness of an anesthetic, it is essential to evaluate the deepness of the surgical plane. However, its evaluation in fish is a challenge, therefore, physiological mediators of stress response can be used to determine the effectiveness of the effect of an anesthetic and nociception in fish.[12] Among the substances released during the stress response, glucocorticoids have commonly been used as biomarkers[13] and have also proved to be effective in determining the level of stress and pain during handling, and surgical interventions.[14]

In vertebrates, the stress response involves the activation of the hypothalamic-hypophyseal axis. In fish, unlike mammals, the cells responsible for producing catecholamines and glucocorticoids are found within the parenchyma of the kidney and located in clusters around the arteries and veins that supply the kidney; therefore, the axis is called hypothalamic-pituitary-inter-renal (HPI). While cortisol is the main glucocorticoid released during the stress response in teleost fish,[15] and cichliformes,[16] there is evidence that corticosterone can be found in other taxonomic class,[17] as well as during enzymatic deficiencies in fish,[18, 19] or in early developmental stages.[20]

There is evidence that handling procedures can activate the HPI axis in fish[21] and might be associated to an increase of glucocorticoids.[22, 23] Therefore, any type of procedures like restriction, immobilization, aerial exposure must be well planned to reduce times, and if a longer procedure is needed, tranquilizers or anesthetics must be used to reduce mortality associated with stress. The objective of the present study was determining the effect of three anesthetic and two tranquilizer agents commonly used in veterinary medicine, in the stress response of *Oreochromis niloticus* during a handling procedure.

Materials and methods

Ethical statement

All procedures were approved by The Veterinary School from the Universidad Autónoma de Tamaulipas, Animal Ethics Committee (CBBA_15_2021).

Animals

A total of 25 tilapias with body mass ranging between 500 g to 1 000 g were selected from the Universidad Tecnológica del Mar de Tamaulipas Bicentenario (UMART) fish farm. An experimental area was set up at the UMART, and the animals were distributed in 53 000 L fiberglass tanks with recirculation. The initial population density was 5 organisms per tank. Temperature (°C) and dissolved oxygen (mg/L) were measured daily with a Pro 1 030 PH and conductivity meter (Yellow Springs Instruments, USA). The photoperiod cycle was 12 h light: 12 h dark throughout the study.

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Experimental design

The protocol consisted of five treatments with five animals (n = 25) randomly assigned to each of the treatments. Guided by the animal ethics committee the sample size was chosen to minimize the number of experimental animals used. To reduce the error, we used sensitive measurement techniques during all the procedures, seeking 95 % of confidence level and 10 % of margin error. The treatments consisted of individually submersion in: 1) a dilution of 2.5 g of ketamine (Ketanil 200 mg/mL; Wildlife Pharmaceuticals Mexico, SA de CV) in 10 L of water; 2) a dilution of 1.5 g of MS222 (Tricaine-S; Western Chemical Inc.) in 10 L of water; 3) a dilution of 1.25 g clove oil (Clove oil 1 g/mL; Farmacia Paris) in 10 L of water; 4) a dilution of 0.75 g of xylazine (Cervicine 300 mg/ml; Wildlife Pharmaceuticals Mexico, SA de CV) in 10 L of water; 5) a dilution of 0.025 g of medetomidine (Medised 10 mg/mL; Wildlife Pharmaceuticals Mexico, SA de CV) in 10 L of water.

An initial sample of 200 µL of blood was carried out via caudal vein, using 1 mL heparinized syringes, and the animals were subsequently immersed in the designated treatment. Induction times were determined once submerged every three minutes by recording voluntary movements, and loss of the ability to swim freely. Nociception was determined by assessing the loss of skin puncture reflex by using tweezers and pricking the tail region with a needle. Once loss of nociception and voluntary swimming movements were verified, a second sampling of 200 µL of blood was drawn sublingually. After that, the fish was submerged in a recovery tank with constant aeration and was monitored every 5 min until they fully recovered their ability to swim freely, their breathing was normal (operculum movement), had flight response, and had skin puncture reflex.

Analysis of blood glucocorticoids

Once the blood samples from the two sampling periods were collected, they were transferred to microtainer tubes and centrifuged at 25 000 rpm for 5 min. Plasma was collected and kept frozen at -20 °C until the day of analysis. The analysis to determine cortisol levels was performed using a commercial ELISA test (DRG® Cortisol ELISA) in solid phase with monoclonal antibody raised in mice and conjugated to horseradish peroxidase. Reported cross-reactions are cortisol (100 %), corticosterone (45 %), progesterone (< 9 %), other steroids (< 0.01 %). The range of the standard curve is between 0-800 ng/mL and the sensitivity reported for the kit is 2.5 ng/mL as the limit of detection.

Statistical analysis

Shapiro-Wilk test was used for the determination of data normality distribution for cortisol levels in blood, as well as for the induction and recovery times for each of the treatments. The result was that, both cortisol levels and induction and recovery times to drug exposure did not meet the assumption of normality. Therefore, a logarithmic transformation of the family of Box-Cox was suggested by the statistical program to transform the data for induction and recovery times.

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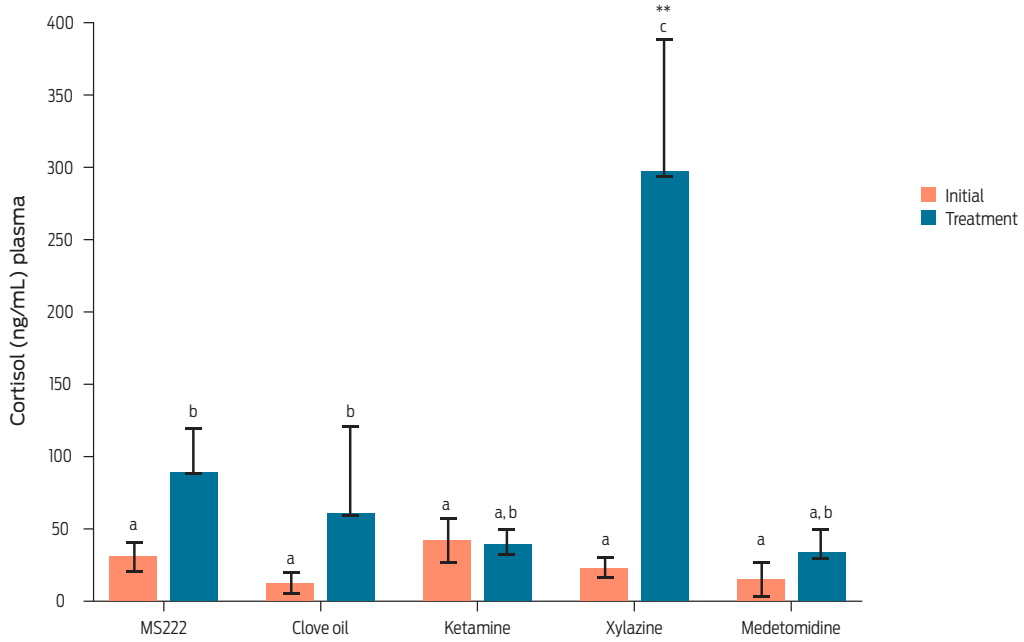

Figure 1. Plasma levels of cortisol (mean ± SD) in tilapia exposed to five drugs with anesthetic and tranquilizing effect. **Different letters indicate statistical differences between treatment, P = 0.0002.

The Box-Cox equation is as follow $(y_{ij}^{(\lambda-1)})/\lambda=\mu+\tau i+\varepsilon ij$; where y_{ij} is the variable response; μ is the statistical general mean; τ_i is the effect of the treatment and ε_{ij} is the random effect of the mean. For y_{ij} equals cortisol level, $\lambda = -0.021$, and τ_i equals evaluation times or any treatment, and for y_{ij} equals time (min) in recuperation or treatment, $\lambda = 0.086$. Once normality was confirmed after data transformation, it was decided to perform an analysis of variance, along with a Tukey test to determine differences between sampling times and treatments. Statistical analyses were performed using Prism statistical software (version 10, for Windows, GraphPad Software LLC). For descriptive statistics the means and standard deviations of the original data were used.

Results

Figure 1 shows cortisol levels between treatments and times. Statistical analysis showed that there were significant differences between cortisol levels before (initial) and after being exposed to the treatments ($F_{1, 48} = 16.11$, P =0.0002). It was found that MS222 (89.1 ± 30.8 ng/mL); clove oil (60.7 ± 60.9 ng/mL) and xylazine (298.5 ± 91.1 ng/mL) had a significant increase in cortisol levels compared to their respective initial values after handling procedure (MS222 (30.8 ± 10.3 ng/mL); clove oil (12.3 ± 7.7 ng/mL) and xylazine (22.9 ± 7.3 ng/mL)). In contrast, treatments with ketamine (38.6 ± 10.8 ng/mL) and medetomidine (33.7 ± 15.8 ng/mL) did not present significant increases in relation to their initial values (ketamine (42.4 ± 15.3 ng/mL); medetomidine (15.1 ± 11.8 ng/mL).

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It was also found that there was a significant difference in cortisol levels between treatments after exposure ($F_{4, 20} = 7.23$, $P = 0.0009$). In this case, xylazine had the highest cortisol levels compared to the other treatments. The rest of the treatments did not present significant changes in cortisol levels according to the Tukey test.

Figure 2 (2a) shows the induction times of fish exposed to different drug treatments. Statistically significant differences were observed between treatments ($F4, 20 = 23.0$; $P < 0.0001$). Animals exposed to xylazine (44 ± 10.84 min) took longer to present induction effects, compared to the other treatments. Although ketamine (18.60 ± 7.19 min) was the second treatment with the longest induction times, it only showed significant differences compared to the induction times of MS22 (4.20 ± 2.9 min), but not with the induction times of medetomidine (16.0 ± 5.6 min) and clove oil (8.00 ± 4.5 min). Neither MS222 nor the treatments with medetomidine and clove oil presented significant differences in induction times ($P \geq 0.0955$).

The results obtained from the recovery times to the treatments are summarized in Figure 2 (2b). The statistical analysis showed that there were significant differences between treatments ($F_{4, 20} = 6.3$, $P = 0.0019$). Animals exposed to ketamine (156.8 ± 80.99 min) presented the longest recovery times, however, not significant longer when compared to animals exposed to medetomidine (72.0 ± 26.60 min) and xylazine (85.0 ± 46.48 min). Animals exposed to clove oil (24.0 ± 10.37 min) and MS222 (45.0 ± 33.14 min) were the animals that showed the best recovery times.

Discussion

The use of anesthetic and tranquilizing drugs is an important practice in medical management and commercial aquaculture. Its main function is to ensure that the animals do not suffer stress and pain during these procedures. In the present study, drugs with anesthetic and tranquilizing effects previously reported in fish were used. Among the anesthetics, ketamine was shown to reduce the activity of inter-renal stress response cells, with an induction period similar to clove oil. Ketamine has been shown to increase the liberation of glucocorticoids in rats[24] however in amphibians it does not stimulate the production of glucocorticoids.[25] This last result is similar to the findings in our study; therefore, ketamine does inhibit the activation of the hypothalamic inter-renal cells axis in tilapia related to handling stress.

Concerning the recovery periods, the ketamine group had the longest period compared to the other treatments, and we observed hyperreactivity in the fish during recovery. The ketamine effect is dissociative, causing loss of consciousness and distortion of the perception of external stimuli, and hyperreactivity during recovery times is commonly observed.[26] Therefore, to avoid hallucinations during the induction or recovery times, as well as produce good relaxation during abdominal surgery, ketamine should be used with caution, and in conjunction with neuroleptics.[27]

MS222 is one of the most used drugs in aquaculture and is categorized as an anesthetic. Therefore, it would be expected that exposure to this drug would have an effect in decreasing the physiological stress response. However, our study showed that cortisol levels increased after exposure to MS222 and handling. MS22

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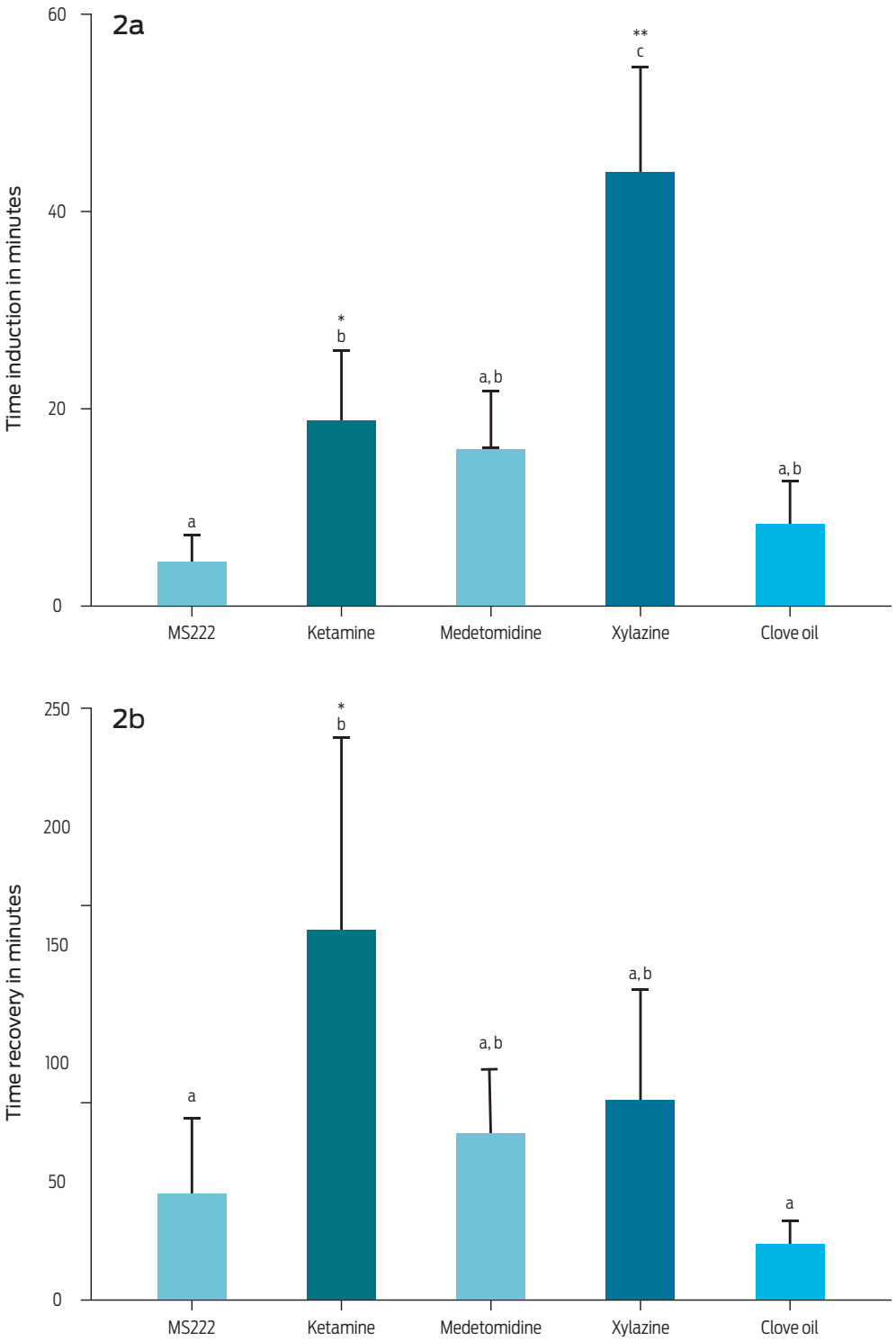

Figure 2. Times (mean ± SD) to induction (2a) and recovery (2b) in tilapia exposed to five drugs with anesthetic and tranquilizing effect.

*Different letters indicate statistical differences between treatments, $P < 0.05$; ** $P < 0.001$.

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is a benzocaine derivative with a hypnotic effect, and it has a motor sensory blockage depending on its dose.[28] In this study we used a dose of 150 mg in a litter of water, which has been previously reported as a sedative in ornamental and commercial species.[29]

Probably higher doses would have a better effect in reducing the inter-renal cell stress response in tilapia, therefore future studies must be directed to determine its effect on glucocorticoid production. Cortisol levels were similar, between the group exposed to MS222 against those exposed to clove oil. Exposure to clove oil also increased cortisol levels in tilapia.

Clove oil is rich in eugenol and has been widely used as an anesthetic in fish.[30] However, several studies have shown that eugenol is not capable of causing central nervous system depression, therefore it should not be classified as an anesthetic.[31] Our study showed that exposure to 1.25 mL of clove oil diluted in 10 L of water, did not have an effect in the reduction of cortisol by inter-renal cells, and the levels achieved were similar to those of MS222. Therefore, it did not influence the reduction of stress response during a handling procedure in tilapia. Induction and recovery times of clove oil were like those shown by MS222, thus it might be reasonable to think that clove oil would be a cheaper option for use than MS222.

However, its use should be recommended only for non-painful procedures, since there is evidence that the effect of clove oil in fish is reducing motor activity, but not sensory, or consciousness activity.[31]

Xylazine and medetomidine, α2-adrenergic tranquilizers were used for this study. Fish exposed to xylazine, had the highest levels of blood cortisol as well as the longest induction times when compared to the other treatments. In the case of recovery, fish exposed to xylazine had similar times compared to the other treatments, except for ketamine. In mammals, xylazine is combined with ketamine, and in fish has been used only experimentally.[2] Our results indicate that xylazine causes a reduction in motor activity, however, does not reduce the rise of glucocorticoids, therefore it has a poor effect in handling stress. Combining xylazine with ketamine should improve its effect in reducing inter-renal cell activity, however, it might be necessary to evaluate its effect during recovery time, since they normally present a period of excitement[32] and in fish, this could cause injury.

Finally, fish exposed to medetomidine did not present significant differences in cortisol levels before and after exposition. Induction time was similar to Ms222 and clove oil, but shorter to xylazine and ketamine. Regarding recovery times, no significant differences were found between all the other treatments, except ketamine. Something that we notice is that the animals exposed to medetomidine showed changes in skin color, observed as paleness. A study of the effect of dexmedetomidine, an analog of medetomidine, showed that it has an agonist effect on α2-adrenergic receptors in the skin of zebrafish, which caused paleness and they also found that the effect on the skin could be antagonized by atipamezole.[11]

Therefore, the effect on color changes in the animals exposed to medetomidine in our study can be attributed to the action on alpha receptors in the skin of the fish. This indicates that medetomidine is a potent α2-adrenergic receptor agonist. Therefore, its use must be evaluated before its use in fish with impairments in respiratory, and vascular capacity, as well as any environmental event that could exacerbate the hypotensive effect of medetomidine, such as low temperature and low oxygenation levels in the water. Future studies should be directed to determine the effectiveness of the use of antagonists such as atipamezole on recovery times in fish exposed to medetomidine.

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Conclusions

In conclusion, ketamine and medetomidine are the most recommended treatments in case of seeking anesthetic and tranquilizing treatments in fish that do not exert the release of glucocorticoids associated with stress. However, it should be noted that induction and recovery times are long. While treatments with MS222 and clove oil had short induction and recovery times, they were found able to increase cortisol levels in the blood associated with stress. This is an indication that the animals exposed to MS222 and clove oil do not lose consciousness completely and can continue perceiving stressors during an intervention. The use of higher doses of these anesthetics might probably improve its effect. Finally, xylazine failed to inhibit the activation of the HPI axis, producing the highest levels of cortisol as well as prolonged induction and recovery times.

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Data availability

The original data sets used in this research and, supporting information files, are deposited and available for download in the SciELO Dataverse repository doi: 10.48331/scielodata.K1ME2D.

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Conflicts of interest

The authors have no conflicts of interest to declare regarding this publication.

Author contributions

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Formal analysis: SE Hernandez, J Loredo.
Funding acquisition: SE Hernandez, P Gonzalez.
Investigation: SE Hernandez, P Gonzalez, J Loredo, AL Urbizu
Methodology: SE Hernandez, P Gonzalez.
Project administration: SE Hernandez, P Gonzalez.
Resources: SE Hernandez, P Gonzalez.
Software: J Loredo, AL Urbizu.
Supervision: SE Hernandez, P Gonzalez.
Validation: J Loredo, AL Urbizu.
Visualization: AL Urbizu.

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