CLOVE OIL AS ANESTHETIC FOR GUPPY

Larissa da CUNHA[1]; Andressa Mariza Ribeiro GERALDO[1]; Vagner Callai da SILVA[2]; Matheus dos Santos CARDOSO[2]; Alessandra Sayuri Kikuchi TAMAJUSUKU[2]; Márcio Aquio HOSHIBA[1]

ABSTRACT

The present study evaluated different concentrations of clove oil for anesthesia of guppy (*Poecilia reticulata*). Adult female, male and juveniles fish were used to assess the influence of the anesthesia. The animals were individually submitted to the following clove oil concentrations: 50, 75, 100, 125 and 150 mg L^{-1}. The induction, recovery stages and mortality 96 hours after the experiment were evaluated. All fish exposed to experimental concentrations reached the final stage of anesthesia. Adult female and male and juveniles guppies respond differently to anesthesia induction by clove oil. Thus, it is recommended the use of clove oil on concentrations of 125 mg L^{-1} for adult males and from 75 mg L^{-1} to 150 mg L^{-1} for adult females and juveniles animals.

Keywords: anesthesia; eugenol; ornamental fish

ÓLEO DE CRAVO COMO ANESTÉSICO PARA GUPPY

RESUMO

O presente estudo avaliou diferentes concentrações de óleo de cravo para anestesia de guppy (*Poecilia reticulata*). Foram utilizados fêmeas adultas, machos adultos e juvenis para avaliar a influência da anestesia. Os animais foram submetidos, individualmente, às seguintes concentrações de óleo de cravo: 50, 75, 100, 125 e 150 mg L^{-1}. Foram avaliados os estágios de indução, recuperação e a mortalidade até 96h após o experimento. Todos os peixes expostos às concentrações experimentais atingiram o estágio final de anestesia. Verificou-se que fêmeas adultas, machos adultos e juvenis de guppy respondem de formas diferentes à indução anestésica pelo óleo de cravo. Dessa forma, recomenda-se a utilização do óleo de cravo nas concentrações de 125 mg L^{-1} para machos e de 75 a 150 mg L^{-1} para fêmeas e juvenis.

Palavras chave: anestesia; eugenol; peixes ornamentais

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[1] *Universidade Federal do Pampa (UNIPAMPA), Campus Uruguaiana, Programa de Pós Graduação em Ciência Animal. BR 472 – km 592 – Caixa Postal 118 – CEP: 97508-000 – Uruguaiana – RS – Brazil. e-mail: laridacunha@gmail.com (corresponding author); andressamrgeraldo@gmail.com; tokudazoo@gmail.com*

[2] *Universidade Federal do Pampa (UNIPAMPA), Campus Uruguaiana, Curso Superior de Tecnologia em Aquicultura. e-mail: vagnercallaidasilva@gmail.com; msccardoso27@gmail.com; alessandratamajusuku@unipampa.edu.br*

INTRODUCTION

In traditional fish culture, routine management practices as biometrics, transportation, air exposure and induced reproduction expose fish to a number of stressors that may affect their performance, causing as consequences loss of appetite and weight, reduction in growth, diseases or even death of the animals (BARCELLOS *et al*., 2000). In ornamental fish culture, stress is one of main causes of animal death.

One of the practices used to minimize stressors effects during handling is the anesthesia. Accordingly, various substances with different properties have been frequently used as anesthetic for fish, in order to minimize stress and facilitate fish handling (FAÇANHA and GOMES, 2005; TEIXEIRA *et al*., 2011).

Several chemicals are currently used as an anesthetic for fish, such as: MS-222 (tricaine methane sulfonate), Benzocaine (ethyil-p-aminobenzoate), Quinaldine (2-4-methylquinoline) or Quinaldine sulfate (sulfate of 2-4-methylquinoline) and 2-phenoxyethanol (phenoxyethanol). However, studies report that these chemicals can cause harm to animals as loss of mucus, gills irritation and damage to the corneas of organisms, in addition to being expensive and difficult to obtain (GOMES *et al*., 2001; INOUE *et al*., 2003). Therefore, studies with anesthetics from natural sources began to be suggested searching for less aggressive and residual anesthetics (GONÇALVES *et al*., 2008).

Clove oil has emerged as one of the most used natural anesthetic and can be extracted from the stem, flowers and leaves of the species *Eugenia caryophyllata* and *E. aromatica*. It has as active ingredient eugenol (4-allyl-2-methoxyphenol), a depressant of the central nervous system (CNS), with concentrations that vary according to the composition of the essential clove oil (ANDERSON *et al*., 1997; KEENE *et al*., 1998). This is a low-cost anesthetic, safe, highly effective, with a broad safety margin for fish and lack of toxicity to the operator (KEENE *et al*., 1998; VIDAL *et al*., 2007).

According to WOODY *et al*. (2002), one of the advantages of eugenol is its rapid elimination from the bloodstream of the fish in less than two days after its use. It also reduced stress because *Rhamdia quelen* anesthesized with eugenol (50 mg L^{-1}) and exposed to the air for one minute presented significantly lower levels of plasma cortisol than fish not anesthetized (CUNHA *et al*., 2010). In addition, SMALL (2003) found that cortisol levels of *Ictalurus punctatus* did not change after exposure for 30 min to recommended clove oil concentration of 100 mg L^{-1}. WONG *et al*. (2014), when testing different drugs for anesthesia and euthanasia of zebrafish (*Danio rerio*), concluded that clove oil is less aversive to fish than MS-222, anesthetic usually used for euthanasia in these specie.

However, it should be noted that the concentrations needed to induce anesthesia of fish can vary within and between species. Fish with different size, age and sex can respond in a manner contrary to a particular concentration (TEIXEIRA *et al*., 2011). The efficacy and safety of clove oil have been described in the literature for many commercial fish species including, pintado (*Pseudoplatystoma corruscans*) (VIDAL *et al*., 2006), matrinxã (*Brycon amazonicus)* (VIDAL *et al*., 2007), pampo (*Trachinotus marginatus*) (OKAMOTO *et al*., 2009) and pacu (*Piaractus mesopotamicus*) (ROTILI *et al*., 2012). However, it is observed that the use of anesthetics for the management of ornamental fish is still little studied and it is hard to access.

Thus, the aim of this study was to evaluate different concentrations of clove oil for anesthesia induction of males, females and juveniles guppy (*Poecilia reticulata*).

MATERIAL AND METHODS

For the experiments, 60 adult fish were used, being 30 adult females (total length average ± standard error [SE]) = 3.63 ± 0.23 cm and weight average ± SE = 0.543 ± 0.116 g), 30 adult males (total length average ± SE = 3.58 ± 0.22 cm and weight average ± SE = 0.289 ± 0.083 g) and 60 juveniles animals (total length average ± SE = 1.58 ± 0.23 cm and weight average ± SE = 0.039 ± 0.019 g) of guppy (*P. reticulata).* After preliminary tests, differences in responses between males and females were observed. Thus, the animals were

separated into the three categories evaluated. The juveniles, in turn, have no apparent sexual dimorphism, so they were analyzed as one group.

The animals were classified into their respective categories (female, male and juveniles) and acclimated for 7 days in a system of tanks with water recirculation, where each category of fish was maintained in a 50 L tank, with the same experimental conditions. Feeding was supplied daily with commercial feed in flakes for ornamental fish (guppys), at 9:00 h and 17:00 h, *ad libitum*. Daily, siphonage was performed to remove feces, and 10% of aquarium water was renewed when necessary.

For each treatment, six females, six males and 12 juveniles, were chosen randomly and subjected one by one, at concentrations of 50, 75, 100, 125 and 150 mg L^{-1} of clove oil, in a total of 24 fish per treatment. The clove oil (Biodinâmica Química e Farmacêutica Ltda.) concentrations for this study were defined based on concentration applied in the literature for anesthesia in fish. The solutions were diluted in ethanol (99.8%) in the proportion of 1:10. Preliminary tests confirm that the higher alcohol concentrations used for clove oil dilution did not cause any visible effect on anesthetized fish.

For anesthesia induction, tanks containing 3 L water were used, with diluted eugenol concentrations established for the experiment. After the exposure time to anesthesia in which the animals reached the last stage of induction, fish were transferred to another tank containing 20 L anesthetic-free water with constant aeration for evaluating of recovery time.

The anesthesia and recovery of the fish were divided into three stages, which are distinguished by a set of signals peculiar to each stage. The stages observed for induction and recovery from anesthesia are described in Table 1.

The time to onset of behavioral patterns was monitored with a digital stopwatch. The lack of reaction to any stimulus was checked by touching the lateral of the fish with a glass rod. After full recovery, fish were returned in the recirculation system, where they were maintained with constant aeration and feeding for 96 hours for monitoring and verification of the mortality.

Table 1. Behavioral characteristics of fish in three stages of anesthesia and recovery (adapted from OKAMURA *et al*., 2010)

Stage	Induction stage	Recovery stage
1	Reduced swimming movement, reaction to external stimuli and normal balance	Slight recovery of opercular movement and swimming movements
2	Loss of muscle movement and balance, decreased opercular movement and decreased reflexes to external stimuli	Balance recovery and mild reaction to external stimuli
3	Total loss of reflexes to external stimuli and opercular movement almost absent	Normal movement and swimming balance

During the experimental period, water physical and chemical variables of experimental units were monitored by measuring the temperature, dissolved oxygen and pH using a multiparameter portable (HI 9829) at each hour. The experiment was conducted in a randomized block design. Data were checked for normality by the Shapiro-Wilk test; later it were verified for the homogeneity of variance by the Levene's test and comparison of means was performed by Tukey test (5%) (SAS 9.0). Data are presented as mean ± standard error (SE).

RESULTS

The means (± SE) of physical and chemical variables were: 8.49 ± 0.02 for pH, 6.45 ± 0.09 mg L^{-1} for dissolved oxygen and 29.95 ± 0.27 ^{o}C for temperature.

Analyzing the results independently in each anesthetic stage (time of induction and recovery) it was observed different responses between the classes (size, sex) in treatments; however, it was not possible to establish a single dose-response pattern among females, males and juveniles.

Anesthesia in females guppies

Guppy females submitted to all clove oil concentrations (50, 75, 100, 125 and 150 mg L^{-1}) were able to reach the stage 3 of anesthesia induction, i.e., failed to respond to any external stimulus (Table 2).

During the experimental period, there was no female mortality. The concentration of 50 mg L^{-1} showed significantly increased induction time to anesthesia compared at concentrations of 100 to 150 mg L^{-1}. The concentration of 75 mg L^{-1} did not differ for any of the tested concentrations. There was no significant difference (P>0.05) in recovery times for females for the different concentrations tested.

Anesthesia in males guppies

In all clove oil concentrations (50, 75, 100, 125 and 150 mg L^{-1}) males reached stage 3 of the anesthetic induction (Table 3). The concentrations of 50 mg L^{-1}, 75 mg L^{-1} and 150 mg L^{-1} resulted in the death of 4.17%, 8.33% and 4.17% of the animals, respectively. A concentration of 50 mg L^{-1}, as well as in females, showed the longest time of anesthesia. There was no significant difference (P>0.05) in recovery times for the different concentrations.

Table 2. Induction and recovery time (min) of adult females guppy exposed to different concentrations of clove oil.

Clove oil concentration	Induction time (min)			Recovery time (min)		
(mg L^{-1})	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
50	1.47 ± 0.23^{a}	2.98 ± 0.78^{a}	4.58 ± 0.47^{a}	0.65 ± 0.28^{a}	1.70 ± 0.62^{a}	5.78 ± 1.48^{a}
75	1.00 ± 0.22^{ab}	2.07 ± 0.55^{ab}	2.92 ± 0.68^{ab}	0.80 ± 0.25^{a}	2.10 ± 0.47^{a}	4.02 ± 0.58^{a}
100	0.67 ± 0.12^{b}	1.22 ± 0.17^{b}	2.77 ± 0.50^{b}	1.12 ± 0.40^{a}	2.20 ± 0.53^{a}	3.95 ± 0.80^{a}
125	0.63 ± 0.10^{b}	1.02 ± 0.10^{b}	1.72 ± 0.23^{b}	0.72 ± 0.10^{a}	1.95 ± 0.42^{a}	3.60 ± 0.48^{a}
150	0.50 ± 0.07^{b}	0.68 ± 0.07^{b}	1.32 ± 0.17^{b}	0.90 ± 0.18^{a}	2.03 ± 0.38^{a}	3.62 ± 0.50^{a}

Mean ± standard error. Means followed by the same letter in the same column do not differs each other at 5% of significance by Tukey test.

Table 3. Induction and recovery time (min) of adult males guppy exposed to different concentrations of clove oil.

Clove oil concentration	Induction time (min)			Recovery time (min)		
(mg L^{-1})	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
50	0.57 ± 0.08^{a}	3.52 ± 0.57^{a}	11.17 ± 2.70^{a}	1.32 ± 0.47^{a}	4.58 ± 1.12^{a}	9.68 ± 1.95^{a}
75	0.73 ± 0.07^{a}	1.90 ± 0.20^{b}	3.92 ± 0.37^{b}	0.98 ± 0.38^{a}	3.37 ± 0.72^{a}	6.67 ± 0.75^{a}
100	0.67 ± 0.07^{a}	1.88 ± 0.52^{b}	3.02 ± 0.22^{b}	1.57 ± 0.53^{a}	3.20 ± 0.35^{a}	7.87 ± 1.00^{a}
125	0.53 ± 0.08^{a}	1.22 ± 0.17^{b}	2.08 ± 0.20^{b}	1.37 ± 0.28^{a}	2.55 ± 0.30^{a}	5.65 ± 0.62^{a}
150	0.43 ± 0.05^{a}	0.77 ± 0.08^{b}	1.65 ± 0.18^{b}	1.43 ± 0.15^{a}	2.98 ± 0.35^{a}	6.18 ± 0.85^{a}

Mean ± standard error. Means followed by the same letter in the same column do not differs each other at 5% of significance by Tukey test.

Anesthesia in juveniles guppies

In all of clove oil concentrations (50, 75, 100, 125 and 150 mg L^{-1}), juveniles guppies reached stage 3 induction of anesthesia (Table 4). There was no mortality of animals during or after the tests. For juveniles, the concentration of 50 mg L^{-1} caused the longer anesthetic induction, differing at concentrations of 100-150 mg L^{-1}. As for males and females, there was no significant difference (P>0.05) in recovery times for juveniles for the different concentrations tested.

Table 4. Induction and recovery time (min) of juveniles guppy exposed to different concentrations of clove oil.

Clove oil concentration	Induction time (min)			Recovery time (min)		
(mg L^{-1})	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
50	0.55 ± 0.08^{a}	1.60 ± 0.07^{a}	3.05 ± 0.22^{a}	0.90 ± 0.20^{b}	3.05 ± 0.60^{a}	6.38 ± 1.00^{a}
75	0.52 ± 0.07^{a}	0.97 ± 0.07^{b}	1.87 ± 0.15^{ab}	0.77 ± 0.13^{b}	2.77 ± 0.42^{a}	5.65 ± 0.65^{a}
100	0.35 ± 0.05^{ab}	0.65 ± 0.07^{c}	1.40 ± 0.10^{bc}	1.05 ± 0.13^{b}	3.03 ± 0.45^{a}	5.63 ± 0.72^{a}
125	0.28 ± 0.03^{b}	0.48 ± 0.07^{c}	1.15 ± 0.10^{c}	1.37 ± 0.23^{ab}	3.48 ± 0.63^{a}	6.73 ± 0.95^{a}
150	0.32 ± 0.03^{ab}	0.62 ± 0.05^{c}	1.10 ± 0.08^{c}	2.12 ± 0.33^{a}	3.77 ± 0.45^{a}	7.25 ± 1.07^{a}

Mean ± standard error. Means followed by the same letter in the same column do not differs each other at 5% of significance by Tukey test.

DISCUSSION

During the experimental period, mortality was observed only for male in concentrations of 50 mg L^{-1}, 75 mg L^{-1} and 150 mg L^{-1}. One possible explanation for this mortality may be due to the long-time of exposure of the animal to anesthesia in concentrations of 50 mg L^{-1} and 75 mg L^{-1}. BITTENCOURT *et al*. (2012) have tested drug efficiency in kinguios (*Carassius auratus*) and observed a mortality of 25% of animals when exposed to concentration of 75 mg L^{-1}, besides presenting long recovery time. The concentration of 150 mg L^{-1} may have caused the mortality of animals due to a possible sensitivity of males at higher drug concentrations. In anesthetizing juveniles of pintados (*P. corruscans*) at concentrations of 25, 50, 75 and 100 mg L^{-1}, VIDAL *et al*. (2006) found 16.67% of mortality in the animals at the highest concentration tested, suggesting that at high concentration the substance can become toxic to fish. However, further studies are needed that verify the deleterious effects in the different categories of assessed guppies.

According to OKAMURA *et al*. (2010), the third stage of anesthesia is the limit between reversible anesthesia and the spinal cord collapse that leads the animal to death. It is also the stage in which the animals remain in the largest effect of the drug, with a high degree of desensitization. This behavior can be considered as recommended for handlings as biometry, where it is needed immobilization of fish, to prevent any physical injury relating to usual agitation of the animals when exposed to handling.

Furthermore, KEENE *et al*. (1998) define as recommended characteristics of an anesthetic its rapid action (about 3 min), as well as ease of use with low risk to the animals. In the present study, the concentrations which provided the anesthesia within the stated by the above mentioned authors were 125 mg L^{-1} for males and 75-150 mg L^{-1} for females and juveniles. In relation to recovery, in all clove oil concentrations females, males and juveniles recovered in less than 10 min, within the time range considered optimal by ROSS and ROSS (2008) and PARK *et al*. (2003). The concentration of 50 mg L^{-1} resulted in the highest induction time to anesthesia of the animals regardless of the size and sex, exceeding the time indicated by the above mentioned authors and is therefore not recommended for anesthesia of the species.

It is observed that the results obtained in relation to the recommended concentration for induction differ from those found for commercial fish by PEREIRA-DA-SILVA *et al*. (2009), VIDAL *et al*. (2006) and GONÇALVES *et al*. (2008). These authors had tested the clove oil in lambari larvae (*Astyanax altiparanae*), pintado juveniles (*P. corruscans*) and pacu juveniles (*P. mesopotamicus*), respectively, and recommended the concentration of 50 mg L^{-1}. This indicates a variability of responses to anesthetic between species and the importance of specific studies that define the optimal concentrations for each of them.

In the present study, we observed a reduction in the time required for anesthesia of fish as we increased the anesthetic concentration, which agrees with the results found by VIDAL *et al*. (2007); ROTILI *et al*. (2012), which tested eugenol

in *Brycon cephalus* and *P. mesopotamicus,* respectively and CHAMBEL *et al*. (2013) which tested MS-22 in *D. rerio*, *P. reticulata*, *Symphysodon discus* and *Xiphophorus helleri*. In these experiments the longest time induction was observed in the lowest concentration tested.

No significant differences were observed (P>0.05) for the anesthetic recovery, indicating that for the tested species recovery time is independent of the anesthetic concentration. Differently, BITTENCOURT *et al*. (2012) tested the clove oil in goldfish juvenile (*C. auratus*) and found that recovery times were dependent on the concentration, so the lower was the anesthetic concentration, the higher were the induction time to anesthesia and the lower were the animal recovery. GOMES *et al*. (2001) also observed that the fish recovery time is influenced by the exposure time to the drug and the recovery time increases significantly with increasing anesthetic concentration.

Studies with anesthetics for ornamental fish are still scarce, demonstrating a growing interest from researchers in recent years. In studies with zebrafish (*D. rerio*), guppy, discus (*S. discus*) and espada (*X. helleri*) with MS-222 as anesthetic agent, it was found that in concentrations of 75, 100, 125, 150, 200 and 250 mg L^{-1} there was difference in optimal concentrations from one species to another. For example, for zebrafish it was indicated concentrations of 75, 100 and 125 mg L^{-1}; for guppy 125, 150 and 200 mg L^{-1}; for discus 75 and 100 mg L^{-1}; and for espada 125 and 150 mg L^{-1} (CHAMBEL *et al*., 2013). While for *Pterophyllum scalare* the ideal clove oil concentration found was 40 mg L^{-1} after 40 min of induction (MILLÁN-OCAMPO *et al*., 2012).

Among the various options of available anesthetics, clove oil was efficient in anesthesia of guppies because it did not cause mortality of females, males and juveniles at recommended concentrations, and the animals quickly returned to its natural condition after exposure.

CONCLUSIONS

Clove oil was efficient in latency of guppies and the recommended concentrations were: 125 mg L^{-1} for males and 75 to 150 mg L^{-1} for females and juveniles animals.

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