Aquaculture 433 (2014) 491–495

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Short communication

The efficacy and effect of repeated exposure to 2-phenoxyethanol, clove oil and tricaine methanesulphonate as anesthetic agents on juvenile Angelfish (*Pterophyllum scalare*)

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ARTICLE INFO

Article history:
Received 16 December 2013
Received in revised form 17 June 2014
Accepted 15 July 2014
Available online 22 July 2014

Keywords:
Angelfish
Anesthetic
Fish handling
2-Phenoxyethanol
MS-222
Clove oil

ABSTRACT

The use of anesthetics in Angelfish (*Pterophyllum scalare*) has not been fully explored. The aim of this study is to determine the lowest effective dose (LED) of three anesthetics (2-phenoxyethanol, clove oil and tricaine methanesulphonate (MS-222)) and their effect after multiple exposures at 24 and 48 h. Each agent was tested on ten juvenile angelfish at four different doses. Considering the effect criteria of complete anesthetic induction time within 3 min and recovery time within 5 min, LEDs were established at 800 mg L^{-1} for 2-phenoxyethanol, 100 mg L^{-1} for clove oil and 140 mg L^{-1} for MS-222. Different anesthesia and recovery times should be considered when repeated exposures during consecutive days are performed on juvenile angelfish.

1. Introduction

The freshwater angelfish (*Pterophyllum scalare*) is one of the most popular ornamental fish species. However, this species is very sensitive to stress during handling and transport (Pramod et al., 2010). It is known that stress can affect their growth, behavior, coloration and risk of illness, especially at a juvenile stage. Low survival rates in juvenile angelfish are responsible for significant financial loss in the ornamental fish industry (Norouzitallab et al., 2009).

Anesthesia in fish is necessary in order to achieve enough sedation to allow the manipulation of individuals, measurement, weighing, vaccination, transportation, and blood or biopsy sampling (Iversen et al., 2003; Mylonas et al., 2005).

Tricaine methanesulphonate (referred to hereafter as MS-222), 2-phenoxyethanol and clove oil are the most commonly used anesthetics in aquaculture. Anesthesia is typically induced by immersing the fish in an anesthetic solution of a specific concentration (Javahery et al., 2012; Topic Popovic et al., 2012; Weber et al., 2009). To choose the appropriate anesthetic for fish, several important factors must be considered: rapid effect, quick recovery, low toxicity (for the fish, staff and the environment) (Soto and Burhanuddin, 1995), low tissue residues, low cost (Marking and Meyer, 1985), ease of use, availability and type of procedure (Cho and Heath, 2000).

2-Phenoxyethanol (ethylene glycol monophenylether) has been suggested for short-term immobilization in fish (Ortuño et al., 2002; Tsantilas et al., 2006). This drug has a short anesthesia induction time, rapid recovery and low price (Weyl et al., 1996). However, adverse effects such as decreased heart rate and low blood pressure (Fredricks et al., 1993), or respiratory depression (Ortuño et al., 2002), have been described in fish.

Clove oil is obtained from the distillation of the stems, leaves and flowers of *Eugenia aromatica* and *Eugenia caryophylata* trees (its active substance is eugenol) (Soto and Burhanuddin, 1995). Its advantages include low price, little environmental impact, relatively few adverse reactions, such as photosensitivity for both fish and amphibians, and safety for staff (Cho and Heath, 2000).

MS-222 is a water-soluble anesthetic, commonly used for fish and other cold-blooded animals. However, MS-222 has been proven to affect cardiac function in the zebrafish, causing bradycardia (Huang et al., 2010).

The concentration at which these three anesthetics are effective have been reported in some fish species: 2-phenoxyethanol (Josa et al., 1992; Ortuño et al., 2002; Ross and Ross, 2008; Tsantilas et al., 2006; Weyl et al., 1996), clove oil (Iversen et al., 2003; King et al., 2005; Mylonas et al., 2005) and MS-222 (Cho and Heath, 2000; King

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http://dx.doi.org/10.1016/j.aquaculture.2014.07.013
0044-8486/

et al., 2005; Weber et al., 2009). However, comparative studies regarding these anesthetics in *P. scalare* are lacking.

Furthermore, little information is available in the literature about the use of anesthetics on juvenile and immature fish, except for some species like salmonid (Cho and Heath, 2000), sole (Weber et al., 2009) and zebrafish embryos (Huang et al., 2010).

The aims of this study were to determine the lowest effective dose (LED) of these three anesthetic agents (2-phenoxyethanol, clove oil and MS-222) on juvenile angelfish, and the effect of multiple anesthetic exposures on induction and recovery times.

2. Materials and methods

2.1. Fish and experimental facilities

This study was approved by the Ethical Committee of the University of Zaragoza, Spain. One hundred and thirty juvenile angelfish were purchased from a commercial supplier (total length 4.45 ± 0.39 cm; body weight 2.46 ± 0.60 g). The fish were allowed to become acclimatized for 7 days prior to the experiment, in two 120 L glass aquariums with appropriate aeration. They were fed with a commercial pellet diet formulation (Sera® discus granules, Germany) for 1 week, and feeding was stopped 24 h before the experiment began. A control group of 10 juveniles was kept in a separate aquarium throughout the study in order to check the morbidity and mortality levels of this species.

Water conditions were monitored throughout the experiment by measuring the pH (using a pH meter; YSI 100), dissolved oxygen (oxygen meter; Crisol YSI 55) and temperature (SCT meter; YSI 30) in the tanks using hand-held equipment, in order to maintain constant and suitable parameters ($T^a = 25 \pm 1$, pH = 7.7 ± 0.2, dissolved oxygen = 6.6 mg L^{-1}).

2.2. Anesthetic agents

Three anesthetic agents were used: 2-phenoxyethanol (Ethylene glycol monophenyl ether, Sigma-Aldrich, Madrid), MS-222 (Tricaine methanesulphonate; Sigma-Aldrich, Madrid) and clove oil (Sigma-Aldrich, Madrid). The 2-phenoxyethanol and MS-222 were added directly to water. Clove oil is insoluble in water. Therefore, and in order to facilitate mixing, a stock solution (100 mg mL^{-1}) was prepared by dissolving clove oil in absolute ethanol (1:9 v/v) as described by Woody et al. (2002). No toxic or anesthetic effects have been previously observed with the use of ethanol at these doses in juvenile angelfish.

2.3. Determination of the lowest effective dose (LED)

In a pilot experiment, two observers monitored the behavior changes (opercular movements, equilibrium and absence of response of tactile movements) in response to the anesthetics in juvenile Angelfish. Anesthetic stages were consistent with those described by Summerfelt and Smith (1990) and Keene et al. (1998). No significant difference was found between the times registered by both observers, we conclude that they monitored the data properly.

Four different doses of the anesthetic agents were prepared a few minutes prior to anesthetic induction (2-phenoxyethanol: 400, 600, 800, 1000 mg L^{-1}, clove oil: 20, 60, 80, 100 mg L^{-1} and MS-222: 120, 140, 160, 180 mg L^{-1}). The angelfish were divided into groups so that 10 individuals were included in each group of anesthetic agent and concentration as presented in the literature (Cunha and Rosa, 2006; Josa et al., 1992; Weber et al., 2009). Each angelfish was transferred into a 4 L aquarium, where the anesthetic agent had been already added.

The experiment was run blind; the observers had no knowledge of which treatment they were evaluating. The time was recorded with electronic chronometers for each animal to reach stage A3 (loss of equilibrium) and A5 (deep anesthesia) (Table 1). Once stage A5 was reached, fish were removed from the water, individually weighed, measured for length and photographed. The specimens were placed into a recovery aquarium with aeration and no anesthetic, where the recovery time (R5) was also recorded (see Table 1). Subsequently, each batch of 10 angelfish was transferred to a separate 120 L stock aquarium, and fed with a maintenance diet after each anesthetic exposure. The experiment (same anesthetic and dose) was repeated with the same group of individuals 24 and 48 h later.

Table 1
Stages of sedation (A3), deep anesthesia (A5) and recovery (R5) times from anesthesia employed in the present study.Modified from Keene et al. (1998) and Summerfelt and Smith (1990).

Stage	Description	Notable behavior
Anesthesia		
A3	Light sedation	Total loss of equilibrium, pectoral fins moving regular opercular ventilation
A5	Deep anesthesia	No movement, loss of responsiveness to tactile stimuli, slow and irregular opercular ventilation
Recovery		
R5	Total recovery	Responsiveness to visual stimuli, normal swimming

It was considered that an anesthetic was efficient if deep anesthesia was reached under 3 min after exposure, and recovery under 5 min after the exposure was stopped.

2.4. Effect of multiple anesthetic exposure on induction to anesthesia and recovery time

In this second experiment, the anesthetic induction and recovery was repeated at 24 and 48 h, following the same conditions and methodology as for the first experiment. Two observers also recorded the time to reach stage A3, A5 and R5 for each animal. The fish were weighed and measured again at 24 h and 48 h, looking for possible variation in these parameters.

2.5. Statistical analysis

All values in the text and tables are expressed as arithmetic means ± standard deviation of the mean (SD), except for the figures where means ± standard error (SEM) are presented. Data were statistically analyzed using SPSS.18 software. The relevant variables for each experimental group were compared by one-way analyses of variance (ANOVA), followed by the Bonferroni's post hoc test to detect significant differences. In order to reject the null hypothesis, a $p < 0.05$ was required.

3. Results

3.1. Lowest effective dose

Considering the accepted efficacy criteria of complete anesthetic induction time or deep anesthesia (A5) within 3 min and recovery time (R5) within 5 min, the lowest effective doses for each anesthetic agent were established at 800 mg L^{-1} for 2-phenoxyethanol (A5 = 2.36 ± 0.87 min; R5 = 4.67 ± 1.63 min); 100 mg L^{-1} for clove oil (A5 = 2.31 ± 0.67 min; R5 = 3.31 ± 0.59 min) and 140 mg L^{-1} for M-222 (A5 = 3.17 ± 0.62 min; R5 = 3.38 ± 0.99) (Table 2). Except for MS-222, which decreased the pH to 6.6 ± 0.1 as described in other studies, no changes in the quality of the water were detected.

3.2. Effect of multiple anesthetic exposure on induction to anesthesia and recovery time

Despite the fact that all concentrations were tested at 0, 24 and 48 h (data not shown), only data for the LED of each anesthetic are described.