Aquaculture 454 (2016) 171–175

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Short communication

Clove extract: A potential source for stress free transport of fish

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ARTICLE INFO

Article history:
Received 7 September 2015
Received in revised form 18 December 2015
Accepted 18 December 2015
Available online 19 December 2015

Keywords:
Anesthetic agent
Clownfish
Clove extract
Induction time
Recovery time

ABSTRACT

Ornamental aquaculture is one of the fastest growing industries that require stringent economical protocols for the effective and stress-free transportation of aquarium fishes. In the present study, the efficacy of clove extract as a low-cost and effective sedative agent for use in shipping of clownfish, *Amphiprion sebae* (Bleeker) is reported. The time required to induce complete anesthesia using clove extract (100 mg l^{-1}) and the subsequent recovery in *A. sebae* were 1245 ± 6 s and 119 ± 4 s and 4200 ± 10 s and 480 ± 5 s, respectively. The induction and recovery times varied significantly ($P < 0.05$) as a function of extract concentrations. An inverse exponential relationship was observed between the dosage and anesthesia induction time, whereas there is a direct relationship between dosage and recovery time. The LC_{50} value of the extract was 50 mg l^{-1}; the optimum dosage with no mortality was found to be 17.5 mg l^{-1}. Correlation coefficient and Chi-square test analysis showed a significant association ($P < 0.001$) between the concentration and incubation period in terms of mortality rate. Furthermore, histopathology observation of gills revealed that the extracts do not affect the gill tissue as compared to control. Having explored the positive roles of clove extract, this study recommends it as can be used as an ideal anesthetic agent for aquaculture practices especially in ornamental fish transport.

1. Introduction

Anesthetic agents have practical relevance in diverse husbandry manipulations such as selection of fish, measurement, sampling, tagging, artificial reproduction, and surgery (Roubach et al., 2005; Weber et al., 2009; Gatica et al., 2010). In aquaculture, fish is exposed to stress during handling and transportation; hence anesthetics are used to reduce stress-induced damages and attenuate the associated adverse physiological responses (Weber et al., 2009).

Some chemicals have proven to be effective anesthetic agents for fish but each has its own advantages and disadvantages (Velíšek et al., 2006). Various studies have documented the efficacy of different anesthetic agents and the dosages in a variety of cultured fish species such as rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Anderson et al., 1997; Keene et al., 1998; Taylor and Roberts, 1999), white sturgeon, *Acipenser transmontanus* (Richardson) (Taylor and Roberts, 1999), Asian sea bass, *Lates calcarifer* (Bloch) (Afifi et al., 2001), and Atlantic salmon, *Salmo salar* L. (Chanseau et al., 2002). Studies have also been made on the physiological effects of different anesthetics, their efficacy and swimming performance in different fishes (Soivio et al., 1977; Iwama et al., 1989; Cho and Heath, 2000; Munday and Wilson, 1997; Keene et al., 1998; Peake, 1998; Anderson et al., 1997).

The clove oil is a pale yellow liquid derived from the leaves, buds, and stems of *Syzygium aromaticum* used for many years as a food additive that recognized as a GRAS (Generally Recognized As Safe) substance by the US FDA for human being. It has recently been suggested as an alternative fish anesthetic agent (http://www.ccac.ca/Documents/Standards/Guidelines/Add_PDFs/Fish_Anesthetics.pdf). However, there was no detailed study conducted on the clove extract on anesthetic activity in aquatic animals. Therefore, the clownfish, *Amphiprion sebae* (Bleeker) is the most popular species of global ornamental fish trade; it was used as an experimental specimen in this study. This is the first report of its kind in marine ornamental fish to determine the optimum concentration of clove extract to anesthetize and transport the fish under packed conditions.

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http://dx.doi.org/10.1016/j.aquaculture.2015.12.020
0044-8486/

Table 1
Stages of anesthetic induction and recovery studied by using clove extract and clove oil in *A. sebae*.

Stages	Behavioral responses
IS1	Lethargic swimming
IS2	Crawling at bottom
IS3	Slow down of caudal fin
IS4	Inverted swimming
IS5	Slowdown of reflex action
IS6	Unconscious state
RS1	Swimming at water surface
RS2	Settling at bottom
RS3	Normal swimming
RS4	Active swimming against water current

IS: induction stage; RS: recovery stage.

2. Materials and methods

2.1. Preparation of clove extract and oil

Dried clove (*S. aromaticum*) was crushed into a fine powder using Hammer-Miller (I.L.E Ltd., India). One percent (1000 mg l^{-1}) of the extract was prepared using distilled water boiled at 80 °C for 20 min and filtered through a 150 μm filter paper. Desired concentrations were prepared from this extract and used for the experiments. The excess of clove extract was stored in a refrigerator at 4 °C further use.

Commercially available clove oil (Sigma Aldrich Co. USA) was mixed few min prior to the experiment with ethanol 1:9 for stock solution of 100 mg l^{-1}. Aliquots from the stock solution were used for the preferred experimental concentration (Chanseau et al., 2002).

2.2. Experimental design

One hundred and ten healthy, marketable size clownfishes (average length 2.8 ± 0.3 cm; weight 6.7 ± 0.01 g) were collected from the marine ornamental fish hatchery at the Centre of Advanced Study in Marine Biology, Annamalai University, Tamil Nadu, India. The fishes were not fed for 12 h prior to the experiment. Then they were packed in polythene bags (15 × 30 cm) each holding 1 l of seawater. The maintained water quality parameters were: temperature 28 ± 0.5 °C, salinity 30 ± 1 psu, pH 7.6 ± 0.1, dissolved oxygen 5.3 ± 0.5 mg ml^{-1}, and ammonia <0.01 ppm. Seven concentrations of clove extract and oil (10.0, 17.5, 25.0, 40.0, 50.0, 75.0, and 100.0 mg l^{-1}) were added in each polythene bag, keeping positive (fishes in bags with no additives) and negative (fishes from culture tanks) controls. Each bag in triplicate was stocked with five fishes.

2.3. Induction and recovery stages of anesthesia

Anesthetized fishes were monitored for behavioral changes during induction and recovery periods developed from criteria outlined by Stoskopf (1993). Fish behavior changes and its time interval were noted in each concentration (http://www.ccac.ca/Documents/Standards/Guidelines/Add_PDFs/Fish_Anesthetics.pdf).

2.4. LC_{50} optimization of clove extract

After 60 min of behavioral observations, the packed fishes were kept under the dark in cartons simulating the transportation environment and monitored at different time intervals (6, 12, 24, and 48 h). Based on the survival rate of fishes, the clove extracts and oil concentrations were optimized for an effective transportation.

2.5. Post-treatment survival

The optimized concentration of clove extract from the above experimental trials was tested for the post-treatment survival of fish to find out the toxicity effect of the given dosage. After recovery, the anesthetized fishes were transferred to post-treatment tanks (200 l FRP tanks) for 30 days to assess behavioral changes and/or mortality. The treated fishes were fed with commercial diets (Gene Eleven-Marine Carni flakes, India) and *Artemia* mass thrice daily. About 30% of water was exchanged daily so as to siphon out the waste materials and replace the removed water level.

2.6. Histology study

After 48 h of post-exposure to the extract inducing anesthesia at a concentration of 17.5 mg l^{-1} both the positive and negative controls were sacrificed immediately. Gill tissues were fixed in Bouin's solution (Roberts, 2001) for histological examination, dehydrated with graded series of ethanol solutions, cleared in xylene, embedded in paraffin blocks, and cut with a WESWOX-OPTIK MT1090A microtome. Then the sections were stained with Hematoxylin–Eosin stain. Histopathological changes were examined microscopically (Leica Microsystems, Germany).

2.7. Statistical analysis

One-way analysis of variance (ANOVA), regression, Chi-square, and correlation coefficient were applied to find out statistical significance of data using SPSS version 16 (Norusis, 2009).

3. Results

3.1. Stages of anesthesia

Induction and recovery stages of anesthesia with clove extract significantly differed in relation to the concentrations. Overall, six induction stages (IS) and four recovery stages (RS) were identified in the clownfish (Table 1). The IS6 for clove extract with different

Table 2
Induction and recovery times (s) for *A. sebae* anesthetized with seven concentrations of clove extracts. Data are represented as mean ± SD.

Stages	Concentration (mg l^{-1})							F-value	*P*-value
	10	17.5	25	40	50	75	100		
IS1	951 ± 20^{a1}	1292 ± 21^{a2}	805 ± 8^{a3}	515 ± 8^{a4}	384 ± 7^{a5}	207 ± 6^{a6}	106 ± 10^{a7}	3470.21	<0.001**
IS2	1406 ± 14^{a8}	1532 ± 6^{a9}	1228 ± 9^{a10}	756 ± 11^{a11}	643 ± 9^{a12}	516 ± 8^{a13}	352 ± 8^{a14}	7254.89	<0.001**
IS3	1631 ± 11^{a15}	1824 ± 5^{a16}	1464 ± 13^{a17}	921 ± 4^{a18}	717 ± 9^{a19}	687 ± 14^{a20}	631 ± 9^{a21}	7657.89	<0.001**
IS4	2299 ± 6^{a22}	2428 ± 6^{a23}	2181 ± 5^{a24}	1659 ± 7^{a25}	1060 ± 7^{a26}	955 ± 11^{a27}	813 ± 4^{a28}	32,584.94	<0.001**
IS5	2982 ± 6^{a29}	2718 ± 9^{a30}	2500 ± 5^{a31}	1970 ± 4^{a32}	1703 ± 5^{a33}	1211 ± 4^{a34}	977 ± 7^{a35}	52,832.00	<0.001**
IS6	4037 ± 5^{a36}	3447 ± 4^{a37}	3021 ± 6^{a38}	2234 ± 5^{a39}	1951 ± 3^{a40}	1634 ± 4^{a41}	1245 ± 6^{a42}	132,326.62	<0.001**
RS1	6 ± 2^{b1}	10 ± 2^{b2}	15 ± 1^{b3}	16 ± 2^{b3}	20 ± 1^{b4}	24 ± 2^{b5}	27 ± 2^{b6}	61.36	<0.001**
RS2	12 ± 2^{b7}	25 ± 3^{b8}	29 ± 3^{b8}	41 ± 3^{b9}	49 ± 2^{b10}	57 ± 4^{b11}	63 ± 5^{b12}	112.81	<0.001**
RS3	25 ± 3^{b13}	32 ± 3^{b14}	36 ± 2^{b14}	48 ± 3^{b15}	57 ± 2^{b16}	67 ± 5^{b17}	82 ± 3^{b18}	130.47	<0.001**
RS4	65 ± 3^{b19}	72 ± 3^{b20}	81 ± 3^{b21}	87 ± 5^{b22}	95 ± 4^{b23}	106 ± 4^{b24}	119 ± 4^{b25}	89.45	<0.001**

IS: induction stage; RS: recovery stage. ** indicates the significant level at 1%, $^{a1–a42}$ and $^{b1–b25}$ represent the significant value of induction time at 5% level.