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Effects of 2-phenoxyethanol anaesthesia on juvenile yellow tail anemone fish *Amphiprion clarkii* (Bennett, 1830), Pomacentridae

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Abstract

The present research was performed to confirm the efficient concentration of the anaesthetic 2-phenoxyethanol (2-PE) on juvenile ($1.3 \pm 0.03\text{g}$) yellow tail anemone fish (*Amphiprion clarkii*) and analysis the LC50 and LT50 of 2-PE at $28 \pm 0.5^\circ\text{C}$, salinity 34 psu, pH 8.0-8.3 and dissolved oxygen 4-6 mg/l. The initiation point decreased and the revival moment increased with escalating concentrations. The results were conflict in revival moment and there were no significant differences among the revival moment from all concentrations. The most suitable concentration of 2-PE was 0.3 ml/l for about or over 10 min exposure time. The LC50 and LT50 for the 3-60 min exposure periods were estimated for juvenile *A. clarkii*. The toxic effect of 2-PE on survival rates of *A. clarkii* juveniles increased depending on the exposure period. In addition, 2-phenoxyethanol LT50 (median survival time) values, slope function (S) and lower and upper 95% confidence limits were estimated. The present work is applied first time in Indian aquaculture.

Keywords: 2-phenoxyethanol, anaesthetic, *Amphiprion clarkii*, LC50, LT50.

1. Introduction

MS222 (Tricaine methanesulfonate), benzocaine and 2-phenoxyethanol (2-PE) are the most widely used anaesthetics in aquaculture (Gilderhus and Marking, 1987; Mylonas *et al.*, 2005; Weber *et al.*, 2009) [2, 11, 18], with anaesthesia usually being induced by immersing the fish in a solution of a given concentration. After MS222, 2-PE is the most commonly used anaesthetic in aquaculture because of their low toxicity level and user friendly.

One of the important members of the Pomacentridae family inhabiting coral reefs, the yellow tail anemone fish (*Amphiprion clarkii*) is a symbiotic fish with sea anemones inhabiting the reef and lagoon of the tropical water, being an interesting species with high marine ornamental aquaculture potential. Over the past few years, production of this species has increased considerably, in particular in South Asian countries (Swagat *et al.*, 2011), and in India since 1999 (Gopakumar *et al.*, 2001) [3]. Existing commercial hatcheries are capable of growing massive quantities of this species and the response to captive rearing has produced excellent results. Furthermore, *A. clarkii* can live on the same feed that is used for others aquarium fishes. No significant pathological outbreaks have been observed, even when it has been farmed at high stocking densities.

The effective anaesthetic concentrations of 2-PE have been reported for a number of fish species, ranging from 0.2 to 0.6 ml/l (Mattson and Ripple, 1989; Josa *et al.*, 1992; Hseu *et al.*, 1996, 1997, 1998; Weyl *et al.*, 1996; Kaminski *et al.*, 2001; Ortuno *et al.*, 2002; Tsantilas *et al.*, 2006; Basaran *et al.*, 2007; Ross and Ross, 2008, Seerzli *et al.*, 2012) [10, 7, 4, 5, 6, 19, 8, 12, 17, 1, 13, 14], but there is no literature on *A. clarkii* and records involving toxicity or safety exposure times and concentrations of 2-PE in fisheries procedures such as transporting, measuring, weighing and sorting *A. clarkii* juveniles are missing and highly needed. The aims of the study were to establish the concentration ranges of 2-PE that provide efficient and safe anaesthesia in juvenile *A. clarkii*, and to evaluate both initiation point and revival moment.

2. Materials and methods

2.1. Fish

The experiments were performed in February, 2013, using juvenile *A. clarkii* produced at the CMLRE, ornamental fish hatchery (Lakshadweep Island, India). Three-month old juveniles

(n = 1000), (total length 4.84 ± 0.04 cm; weight 1.3 ± 0.03 g) of the same brood fish were used in the study. Fish were stocked in 5 re-circulatory aquarium glass tank (500-lit volume, flow rate 10% of volume per hour; water oxygenated, established in indoor research facilities receiving filtered seawater. Natural photoperiod was maintained (12/12, dark/light). During the 3-day acclimation period the fish were fed on a commercial diet (Lucky Star, 1.0-1.5 mm pellet) and all fish were starved for 24 h prior to the experiments.

2.2. Water quality

Water quality was monitored by measuring pH (pH meter; YSI 100), dissolved oxygen (oxygen meter; YSI 55), salinity and temperature (SCT meter; YSI 30) in the tanks using hand-held equipment. Nutrients (nitrite-nitrogen, $\text{NO}_2\text{-N}$, nitrate-nitrogen, $\text{NO}_3\text{-N}$, ammonium-nitrogen, $\text{NH}_4\text{-N}$, phosphate-phosphorus, $\text{PO}_4\text{-P}$) and suspended solids were determined once prior to all treatments. Nutrients were determined using a HACH DR 2000 spectrophotometer.

2.3. Experimental design

Fish (n=500) were exposed to 2-PE anaesthetic concentrations to determine anaesthesia dosage and recovery time. Each test series used 10 fish in triplicate (n = 30 per concentration/exposure duration combination). These were exposed to seven different concentrations of 2-PE, including 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, and 0.5 ml/l as well as a control group for each triplicate test group. Oxygen levels were adjusted to saturation in the induction and recovery buckets (up to 10 mg/l) using a liquid oxygen source while the water was aerated during the tests. The experimental groups were placed in aerated 15-L buckets; before each treatment the fish were left undisturbed for 60 min, with 10 fish in each of 21 buckets containing 10-L of anaesthetic solution, plus three buckets with control fish. 2-PE is a colourless oily liquid with a distinctive rose scent and a density of 1.11 kg/dm^3 . Its water solubility at 25°C is 26.7 g/L but it is readily soluble in ethanol. In the present study, 2-PE with a purity of over 99% (Merck-Schuchardt) was used.

The anaesthetic solution was made up of water from the acclimation tank and thoroughly mixed with the respective volumes of 2-PE. A fresh anaesthetic solution was prepared for each triplicate. Fish of each group were taken from the acclimation tanks and transferred by netting into the anaesthetic buckets for the selected times. Fish were then taken out of buckets to the recovery buckets with the same temperature and salinity for 60 min. After placing the fish in the anaesthetic or recovery buckets, mortality was recorded. Furthermore, following the last 60-min period, the juveniles were transferred to identical quarantine tanks without anaesthetics and observed to record mortality for 24h.

To determine the efficacy of each concentration of 2-PE, induction and recovery times were estimated as by Sumner and Smith (1990) [15], Keene *et al.* (1998) [9] and Tsantilas *et al.* (2006) [17]. Time elapsed until 50% of the fish had lost their balance was called 'first initiation point'; disappearance of all fin movement (no reflex stimuli) was 'full initiation point'; 50% of fish having regained their balance was 'first revival moment'; and all fish with normal swimming and reflex to stimuli were entitled 'full revival moment'. When fish did not lose their full equilibrium or reflex to stimuli, the exposure

time was increased by 60 min for estimating the induction time.

2.4. Estimation of LC50 and LT50

According to induction and recovery time experiments, fish were exposed to five anaesthetic doses for different exposure times. Exposure time was selected according to exposure dose. A low dosage requires longer exposure time such as 5 or 10 min; high doses need a shorter exposure time interval such as 0.5 or 1 min. Thus, ten exposure times were defined for each concentration to determine LT50 (the time lethal to 50% of test animals after 3.0 to 60 min using eighteen different time periods) and LC50. In this experiment 100 fish were used in each treatment (300 fish for triplicates plus 100 fish for control). Fish were exposed to 2-PE concentrations, 10 fish were caught by net and transferred into the recovery tank. The same procedure was performed for the control group and fish mortalities recorded.

2.5. Statistical analysis

The LC50, LT50 and 95% confidence limits were calculated by probit analysis using US EPA software (Version 1.5, 1999). Mann-Whitney's U tests following Kruskal-Wallis's test were used to determine the statistical significance of the differences among the induction times and among the recovery times for each concentration. Statistical analyses were performed using the SPSS (Version 20.0, SPSS Chicago, IL) statistical software package and significance was accepted at $P < 0.05$.

3. Results

All fish were initially healthy as indicated by their normal activity and exterior appearance observed in samples of 10 fish from each tank. No mortality was recorded during the acclimation period. Values of temperature, salinity, pH and dissolved oxygen were measured separately in all trials as follows $20 \pm 0.5^\circ\text{C}$, 38 g/L , $8.2\text{-}8.4$ and $>7 \text{ mg/L}$, respectively. Total suspended solids (TSS), nitrite-nitrogen ($\text{NO}_2\text{-N}$), nitrate nitrogen ($\text{NO}_3\text{-N}$), ammonium-nitrogen ($\text{NH}_4\text{-N}$) and total ortho-phosphate ($\text{PO}_4\text{-P}$) were detected at 13, 0.004, 0.028, 0.021 and 0.006 mg/L, respectively. Seven different concentrations of 2-PE were studied. Induction time decreased and recovery time increased with increasing concentrations but changes in recovery times were significantly different. While full induction time was too long at 0.2 and 0.25 ml/L 2-PE concentrations, at a concentration of 0.5 ml/L full anaesthesia was found to be 1' 48".

Although longer exposure time to the anaesthetics was observed in the treatments, recovery times change significantly. Revival moment time never exceeded 3 min in all concentrations (Table 1). No mortalities occurred within 96 h following recovery from anaesthesia. No mortality or other adverse effects occurred in the control, 0.2 and 0.25 ml/L 2-PE groups, during the 60 min exposure time. The 0.3 ml/L 2-PE treatment of 60 min exposure time caused death in 53.3% of the population. At a concentration of 0.3 ml/L the first death was observed after 35 min. After 6 min exposure 1% death in the concentration of 0.35 ml/L, while all fish died at 0.5 ml/L 2-PE concentration (Table 2).

Table 1: Efficacy of 2-PE concentrations on anaesthetizing juvenile *A. clarkii* first and full revival moment in triplicate groups (n = 5, per group) (exposure time up to 60 min)

Concentration (ml/ L)	Initiation point (min'and s'')		Revival moment (min'and s'')	
	First initiation	Full initiation	First revival	Full revival
0.20	5.12±0.03 ^a	20.30±1.27 ^a	0.30±0.05 ^a	1.25±0.05 ^a
0.25	2.30±0.31 ^b	11.26±4.60 ^b	0.35±0.10 ^a	1.30±0.30 ^b
0.30	1.31±0.07 ^c	5.50±1.10 ^c	0.55±0.15 ^b	1.12±0.14 ^b
0.35	1.20±0.08 ^{cd}	4.25±0.60 ^c	1.05±0.17 ^{bc}	1.20±0.08 ^a
0.40	1.11±0.26 ^{cd}	3.40±0.70 ^c	1.20±0.22 ^{bc}	2.10±0.70 ^a
0.45	0.40±0.18 ^{de}	2.04±1.20 ^c	1.25±0.20 ^{cd}	2.20±0.16 ^a
0.50	0.25±0.06 ^e	1.40±0.64 ^c	1.35±1.18 ^d	2.08±0.76 ^b

Data in same column with same superscript letter not different from each other (P > 0.05).

Data among columns are significantly different for initiation point and revival moment (P < 0.05). Data given as mean (±SD).

Table 2: Percentage of cumulative mortality of experimental juvenile *A. clarkii* depending on 2-PE concentrations and exposure time.

Exposure time (min)	Concentrations (ml/ L) and percent mortality (%)				
	0.3	0.35	0.4	0.45	0.5
3.0	-	-	-	-	9.5
3.5	-	-	-	-	11.6
4.0	-	-	-	1.5	14.9
4.5	-	-	3.5	12.5	17.5
5.0	-	-	16.5	20.0	20.0
5.5	-	-	26.5	32.5	50.0
6.0	-	1.0	50.0	55.0	66.7
7.0	-	35.0	60.0	65.0	75.8
8.0	-	45.0	75.5	85.0	90.0
10.0	-	60.0	90.0	93.0	100.0
15.0	-	75.0	100.0	100.0	-
20.0	-	84.0	-	-	-
25.0	-	92.0	-	-	-
30.0	10.56	100.0	-	-	-
40.0	30.73	-	-	-	-
50.0	38.93	-	-	-	-
60.0	51.65	-	-	-	-

The LC50 and LT50 for exposure periods ranging from 3 to 60 min were estimated for juvenile *A. clarkii* fish (Tables 3 and 4). Effective and non-toxic concentrations of 2-PE within the 60 min. Exposure time in all groups ranged between 0.20 and 0.50 ml/L. At 0.5 ml/L, 20% sudden mortality and 80% fishes were very weak within the 6 min exposure period and after 30 min all fishes not able to recover and died. At 0.45 and 0.4 ml/L, 100% deaths occurred during 15, 15 and 30 min exposure time respectively. At concentration of 0.3 ml/L, 100% mortalities were not found in 60 min exposure time.

The 0.3, 0.35, 0.4, 0.45 and 0.5 ml/L acute LT50 (95% confidence limits) values of 2-PE for juvenile *A. clarkii* were 60.25, 7.80, 6.50, 5.50 and 3.5 min, respectively (Table 3). The 4, 5, 6, 7 and 8 min LC50 (95% confidence limits) values of 2-PE for the juvenile *A. clarkii* were found to be 0.55, 0.50, 0.45, 0.35 and 0.30 ml/L, respectively (Table 4). The toxic effect of 2-PE on survival rates of *A. clarkii* juveniles increased depending on the exposure period (Tables 3 and 4).

Table 3: LT50 (median survival time, min) values, slope function (S) and lower and upper 95% confidence limits for tested concentrations of 2-PE

Concentrations (ml/L)	LT ₅₀	slope function	Lower confidence limits	Upper confidence limits
0.30	60.25	4.5	50.09	.0
0.35	7.80	10.0	a	a
0.40	6.50	15.0	a	a
0.45	5.50	14.2	5.80	6.3
0.50	3.50	4.10	3.30	4.4

Table 4: LC50 (median survival concentration, ml · L⁻¹) values, slope function (S) and lower and upper 95% confidence limits for tested exposure periods of 2-PE

Exposure time (min)	LC ₅₀	slope function	Lower confidence limits	Upper confidence limits
4	0.55	0.25	0.52	0.53
5	0.50	0.20	0.50	0.50
6	0.45	0.20	0.45	0.45
7	0.35	0.15	0.40	0.35
8	0.30	0.24	0.35	0.30

4. Discussion

A fish species new to aquaculture, the *A. clarkii* was used to determine the effective anaesthetic concentrations of 2-PE, known in a number of fish species to range from about 0.2 to 0.6 ml/L (Table 5). Water quality needs to be carefully controlled during an anaesthesia procedure; the main problems involved being those faced by all aquatic animals: control of temperature, dissolved oxygen concentration, ammonia levels and other solids in the baths (Ross and Ross, 2008) [13]. An ideal anaesthetic for fish should induce anaesthesia in <3-5 min, with total loss of equilibrium and muscle tone for ease of handling in particular when fish need to be measured. The anaesthetic should allow an uneventful and rapid (i.e. Effects of 2-phenoxyethanol anaesthesia on juvenile *A. clarkii* 89 <10 min) recovery, leave low tissue residues and be safe to users as well as being inexpensive and easy to use (Gilderhus and Marking, 1987; Summer felt and Smith, 1990; Hseu *et al.*, 1998; Tsantilas *et al.*, 2006; Ross and Ross, 2008) [2, 15, 6, 17, 13]. In the present study, concentrations of 0.3 ml/ L 2-PE seem to fulfil these requirements for juvenile *A. clarkii*. Similar results were observed in some marine fishes such as sea bass, black sea bass and gilthead sea bream (Table 5). Concentrations of 0.35 ml/L can be seen as useful for anaesthesia, however first mortalities appear at 6 min, with a mortality of 6.5%.

Although the efficacy and recovery of anaesthetic agents vary with fish size, Zahl *et al.* (2009) [20] found no differences in induction or recovery times associated with body weight for 2-PE in their study of Atlantic cod (*Gadus morhua*). The present study has shown the induction time to decrease and the recovery time to increase with higher concentrations, which is in agreement with other reports on the efficacy of 2-PE in various species (Mylonas *et al.*, 2005; Tsantilas *et al.*, 2006; Basaran *et al.*, 2007) [11, 17, 1]. Conflicting results in the present study were found only in recovery time but the general tendency was the same as in the other studies. However, there few significant differences were found among the revival moment times of 0.45 and .050 ml/L concentrations; this phenomenon provided us with advantages in aquaculture applications. High concentrations may be useful for short time applications to reduce the working time.

Table 5: Comparison of lowest effective 2-PE concentrations reported for some marine aquaculture fishes

Fish	Weight (g)	Dose (ml/L)	Temp (°C)	Reference
Sea bass (<i>Dicentrarchus labrax</i>)	2.7 ± 0.03	0.3–0.45	19 ± 0.5	Basaran <i>et al.</i> (2007)
Sea bass (<i>Dicentrarchus labrax</i>)	32.8 ± 1.2	0.35	25	Mylonas <i>et al.</i> (2005)
Sea bass (<i>Dicentrarchus labrax</i>)	0.86 ± 0.1	0.32	18 ± 1	Marsic-Lucic <i>et al.</i> (2005)
Gilthead sea bream (<i>Sparus aurata</i>)	44.1 ± 2.2	0.30	25	Mylonas <i>et al.</i> (2005)
Black sea bass (<i>Centropristis striata</i>)	76.0–81.7	0.3	18–21	King <i>et al.</i> (2005)
Senegalese sole (<i>Solea senegalensis</i>)	99 ± 2.5	0.6	14 ± 1	Weber <i>et al.</i> (2009)
White sea bream (<i>Diplodus sargus</i>)	30–60	0.167	20 ± 1	Tsantilas <i>et al.</i> (2006)
Sharp snout sea bream, (<i>Diplodus puntazzo</i>)	15–30	0.167	20 ± 1	Tsantilas <i>et al.</i> (2006)
Meagre (<i>Argyrosomus regius</i>)	1.3 ± 0.03	0.30	20 ± 1	Serezli <i>et al.</i> (2012)
Yellowtail anemone fish (<i>Amphiprion clarkii</i>)	1.5 ± 0.05	0.30	28 ± 1	Present study

In conclusion, results indicate that 2-PE can be used as an effective and efficient agent for anaesthesia in the aquaculture of juvenile *A. clarkii*. The most suitable concentration of 2-PE was 0.3 ml/ L up to 15 min. On the other hand, long-term or short time exposure limits can be predicted according to LT50 and LC50 values for transportation, vaccination, measuring and sorting.

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