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Size-relative Effectiveness of Clove Oil as an Anaesthetic for Rainbow Trout (*Oncorhynchus mykiss* Walbaum, 1792) and Goldfish (*Carassius auratus* Linnaeus, 1758)

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Abstract

The purpose of this work was to investigate the size-relative effectiveness of clove oil as an anaesthetic for rainbow trout and goldfish. In total, 128 rainbow trout (*Oncorhynchus mykiss*) (two groups of 20-23 and 30-33 cm mean fork length) and 160 goldfish (*Carassius auratus*) (four size groups of 1.5-2.5, 5-7, 11-15 and 20-25 cm) were anaesthetized at different clove oil concentrations of 50, 100, 150 mg·l^{-1} for trouts and 75, 100, 150 mg·l^{-1} for goldfish. Rainbow trout exhibited total loss of balance and no response to external stimuli with shorter induction time as dosage increased (120.5 s, 64.4 s and 44.3 s, respectively). Goldfish exhibited total loss of balance and no response to external stimuli after induction time that varied with dosage used and body size of fish. The small fish (1.5-7 cm) exhibited shorter induction time which ranged from 84.28 s at 75 mg·l^{-1} clove oil to 41.14 s at 150 mg·l^{-1} clove oil. The larger fish had a longer induction time inversely related to the dosage. Recovery time was longer than induction time in both species. Both species recovered within 6 min after anaesthesia at 150 mg·l^{-1} clove oil. Clove oil did not produce marked changes ($P < 0.05$) in the physiological indicators of goldfish compared to the control. However, marked changes ($P < 0.05$) were exhibited in the haematocrit of treated rainbow trout that also exhibited hyperkalaemia and hyperglycaemia ($P > 0.05$). For both fish species, clove oil was effective, producing minimum stress and zero mortalities, and can be recommended as an effective anaesthetic.

Syzygium aromaticum, fish anaesthesia, animal welfare

Rapid expansion of the aquaculture industry that occurred in previous decades prompted scientific debates on the potential suffering of fish being handled during common aquaculture procedures or during slaughtering. Research aimed at lessening the suffering of cultured fish is vital to meet the concern for farmed fish welfare (Ashley 2007). Handling stress and various manipulations in aquaculture can have a negative impact on fish health and their growth (Hoskonen and Pirhonen 2006). Anaesthetics are therefore applied to reduce these negative effects to the minimum. The dosage required to induce general anaesthesia varies according to the anaesthetic used and other factors such as water temperature, hardness, salinity, oxygen concentration, length of exposure, body weight, the ratio of gill area/body surface area and the species of fish. In general, small fish are more sensitive to anaesthesia than larger fish (Ross and Ross 1999).

An ideal anaesthetic for fish should induce anaesthesia in less than 3 to 5 min, with total loss of balance and muscle tone, allowing an uneventful and rapid (i.e. less than 10 min) recovery with low tissue residues after recovery, thus being safe to users and consumers. The anaesthetic should be inexpensive and easy to use (Gilderhus and Marking 1987;

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Summerfelt and Smith 1990; Hseu et al. 1998; Ross and Ross 1999; Tsantilas et al. 2006; Klimankova et al. 2008).

Some traditionally used anaesthetics in aquaculture include tricaine methanesulphonate (MS-222), benzocaine, etomidate, metomidate, 2-phenoxyethanol and quinaldine. In farmed fish, MS-222 appeared to be the most widely used anaesthetic. However, recently there has been considerable interest in another fish anaesthetic, clove oil extracted from the clove tree, *Eugenia caryophyllus* (syn. *Syzygium aromaticum*) which has traditionally been used as human anaesthetic (Soto and Burhanuddin 1995). Chaieb et al. (2007) reported that the main active ingredients of clove oil are: eugenol (88.58%), eugenyl acetate (5.62%), β-caryophyllene (1.39%), 2-heptanone (0.93%), ethyl hexanoate (0.66%), humulenol (0.27%), α-humulene (0.19%), calacorene (0.11%) and calamenene (0.10%). Clove oil is generally considered a reasonable alternative of fish anaesthetic with low cost and of no risk to human health (Velisek et al. 2005a,b). In fact, clove oil is used for local application to reduce pain and promote healing and exhibits antimicrobial, antioxidant, antifungal and antiviral properties (Chaieb et al. 2007). According to the U.S. FDA, the constituting ingredients of clove oil are considered safe (substance that can be used in food industry), but none of them has been approved for fish anaesthesia yet (FDA/CVM, 2009).

Clove oil has recently been studied as a potential anaesthetic for some ornamental fish (Kaiser et al. 2006; Macova et al. 2008) and several farmed cold and warm water fish species (Wagner et al. 2003; Holloway 2004; Velisek et al. 2005a,b; Hajek et al. 2006, Gomulka et al 2008). However, results of several studies suggest that the physiology of fish can be affected by changes in the haematology and biochemistry of exposed fish. Therefore it is important to assess the extent of physiological changes of fish exposed to an anaesthetic (Inoue et al. 2005; Park et al. 2008). Results of the numerous studies on clove oil revealed that effective concentrations for anaesthesia vary with fish body size and water temperature (Oikawa et al. 1994; Hoskonen and Pirhonen 2006; Mylonas et al. 2005; Zahl et al. 2009) with the smaller fish being more responsive than the larger one (Holloway et al. 2004; Velisek et al. 2005a,b). Velisek et al. (2005b) studied the effect of clove oil on juvenile and brood stock of rainbow trout (*Oncorhynchus mykiss*) but there are no data for larger sizes of this species, in particularly for the final growing stages (200-300 g) where size grading prior to harvesting may be required. Goldfish (*Carassius auratus*) is a warm water ornamental fish species, which is frequently subjected to anaesthesia for spawning and vaccination and could benefit from the use of clove oil.

The aim of this study was to observe the response of two fish species of different sizes to clove oil used as an anaesthetic and to observe its effect on selected haematological and biochemical variables.

Materials and Methods

Experimental animals

Male rainbow trout (n = 128) with an average fork length 31.06 cm (± 2.47, SD) and goldfish (n = 160) with mean body weight of 6.8 g (± 1.11, SD) were used for independent experiments. The trout were maintained in a circular 2 m^3 tank connected to a re-circulating system at mean temperature of 12 ± 0.6 °C. Goldfish were kept in a circular 200 l tank connected to a second re-circulating system at a mean temperature of 18 ± 0.7 °C. The trout were fed daily with trout pellets at 2% of body weight; the goldfish were provided carp pellets at a 3% daily ratio. Oxygen concentrations were maintained above 7 and 8.5 ppm in the tanks for goldfish and trout, respectively. In both groups, concentrations of pH, nitrate and ammonia were within the physiological/optimal range for these species (pH: 7.5-7.7; nitrate ($P < 0.01$ $mg \cdot l^{-1}$), ammonia ($P < 0.03$ $mg \cdot l^{-1}$); photoperiod 12 L:12 D. All fish were starved for 24 h before the experiment.

Before the experiment, the fish were observed daily to evaluate their health condition as indicated by their activity and external appearance. Four days before the experiment, ten fish were randomly selected from each tank and sampled for bacteriological and parasitological examinations. Kidney and spleen samples were inoculated onto Tryptone Soy Agar (TSA) and Thiosulphate Citrate Bile Salt Agar (TCBS) for bacteriology assessment (Roberts and Shepherd 1997). Squash imprints of gill, skin, gall bladder, liver, spleen, kidney, muscle, brain and gut tissues from freshly killed fish were examined for the presence of parasites.

Prior to anaesthesia, the goldfish were divided into four fork length classes (1.5-2.5, 5-7, 11-15 and 20-25 cm) and placed in separate glass aquaria (volume 70 l). The trout were divided into two size groups (20-23 and 30-33 cm) and maintained in different holding tanks. Seven to ten fish of each species and size group were exposed to three different concentrations of clove oil (n = 7-10 fish per size group/dosage tested).

Anaesthesia preparation and procedures

Clove oil (Sigma Aldrich Co., St. Louis, USA) was initially dissolved in 95% ethanol (clove oil:ethanol at a ratio 1:9). In pilot experiments, it was confirmed that the volume of ethanol used in each experiment did not have a visible anaesthetic effect on fish. The various concentrations (50, 100 and 150 $mg \cdot l^{-1}$ for trout and 75, 100 and 150 $mg \cdot l^{-1}$ for goldfish) were prepared and mixed in 20 l observation tanks filled with water from the respective re-circulating systems. Fish were randomly caught by hand-net in the holding tanks and transferred immediately into the anaesthetic in the observation tank. The fish were observed for opercular movements, balance and thereafter loss of response to stimulus. The fish were removed when they exhibited loss of equilibrium, no spinal reflexes and imperceptible opercular movements.

Induction and recovery times were obtained using a digital chronometer for fish. Induction of anaesthesia was assumed as complete when fish lost equilibrium and reflex reactivity (stage 5). Once a fish reached stage 5 of anaesthesia, it was removed from the experimental tanks, dried and measured. Then the fish was transferred to fresh water in identical tank without rinsing to remove traces of the anaesthetic. Recovery was considered complete in all groups when fish were able to regain upright position and swim normally.

Recovery and induction times were recorded for each fish. Following recovery, the fish were transferred to maintenance tanks and observed for 48 h for potential mortality. Control groups were fish that remained in the rearing tanks and left undisturbed until they were collected for blood sampling.

Blood sample collection

Blood was sampled from trout (30-33 cm group) and goldfish (20-25 cm group) with heparinized syringes from the caudal vein at stage 5 of anaesthesia. Haematocrit was calculated after centrifugation of microhaematocrit tubes (4,000 *g* for 5 min, Sigma 1-15 microcentrifuge). For plasma biochemical analysis, blood was collected (0.5-1.5 ml) in Eppendorff vials and centrifuged (9,000 *g* for 13 min, Sigma 1-15 microcentrifuge). The plasma was collected and stored at -80 °C for further analysis. Glucose and calcium plasma concentrations in trout and goldfish plasma were measured by Olympus 600 Medicon Analyzer, using Hexokinase enzymatic UV and Arsanazo III photometric colour tests, respectively.

Statistical analysis

Box plots were plotted for induction and recovery times for the various sizes of fish using SPSS 15.0 software. The above data and haematocrit, glucose and calcium were subjected to a one-way ANOVA, and Duncan multiple range test used to separate the means (Zar 1984). In all cases, confidence levels were set at 95%.

Results

All fish used in the present study were healthy as was indicated by their activity and exterior appearance and the bacteriological and parasitological examinations carried out in samples of ten fish from each holding tank. No mortality was observed during the acclimatization period. Furthermore, no deaths or other adverse effects occurred within 48 h following recovery from anaesthesia.

Anaesthetic effect of clove oil on rainbow trout

Figs 1a and 1b present the results of induction (1a) and recovery (1b) time of rainbow trout exposed to different doses of the anaesthetic, and the same results are presented in Figs 2a and 2b with fish grouped according to their size. Anaesthesia progressed rapidly but steadily in all treated groups increasing with the concentration of clove oil. Induction time was faster (120 s) and similar for all groups with concentration 50 $mg \cdot l^{-1}$. Induction time decreased with the higher concentration used in large (30-33 cm) size group, whereas the reverse effect was observed for the smaller (20-23 cm) size group (Fig. 3a, Table 2). Compared to the large size class, the small size class exhibited longer induction time at all dosages tested ($P < 0.001$).

Recovery time was more variable at 100 $mg \cdot l^{-1}$ ($P < 0.001$) compared to the other concentrations (Fig. 3b). This time increased with higher concentration used, ranging between 2 - 6.6 s (Table 2), but did not differ with size, $P > 0.05$ (Fig. 4b, Table 2). The higher dose (100 $mg \cdot l^{-1}$) induced deep anaesthesia within 45 s, i.e. much faster compared to the other doses ($P < 0.001$). All fish recovered within 6 min, although in the lower doses recovery progressed much more rapidly ($P < 0.001$).

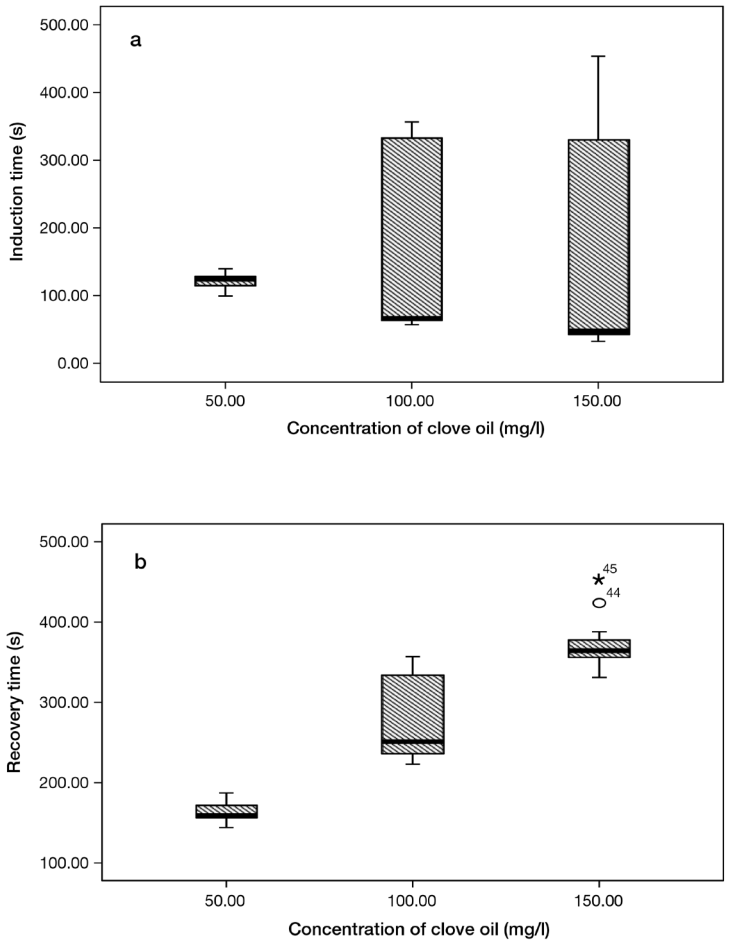

Fig. 1. Box plots of (a) induction and (b) recovery times for rainbow trout anaesthetized with various concentrations of clove oil. The upper and lower hinges of each plot represent the maximum and times, middle line represents the median, the shaded inner box represents the 25th and 75th percentiles and the whiskers represent lower and upper limits.

Anaesthetic effect of clove on goldfish

Figs 3a and 3b present the results of induction (3a) and recovery (3b) of goldfish exposed to different doses of the anaesthetic, and the same results are presented in Figs 4a and 4b for fish grouped according to size. Induction time of anaesthesia varied ($P < 0.01$) with clove concentration, decreasing with the increase of clove concentration (Fig. 1a, Table 1) and was more variable at 75 and 150 mg·l^{-1} compared to 100 mg·l^{-1} (Fig. 1b, Table 1). An increase in recovery time with clove concentration was recorded for the largest size class, a decrease for the smallest, and a variable response for the medium (Table 1). Induction time was rapid in small-size goldfish, irrespective of the clove oil dose applied. Anaesthesia was more size-dependent at the lower dose, compared to the higher dose ($P < 0.001$). Smaller fish required less time to lose the upright position than larger ones at the dose of

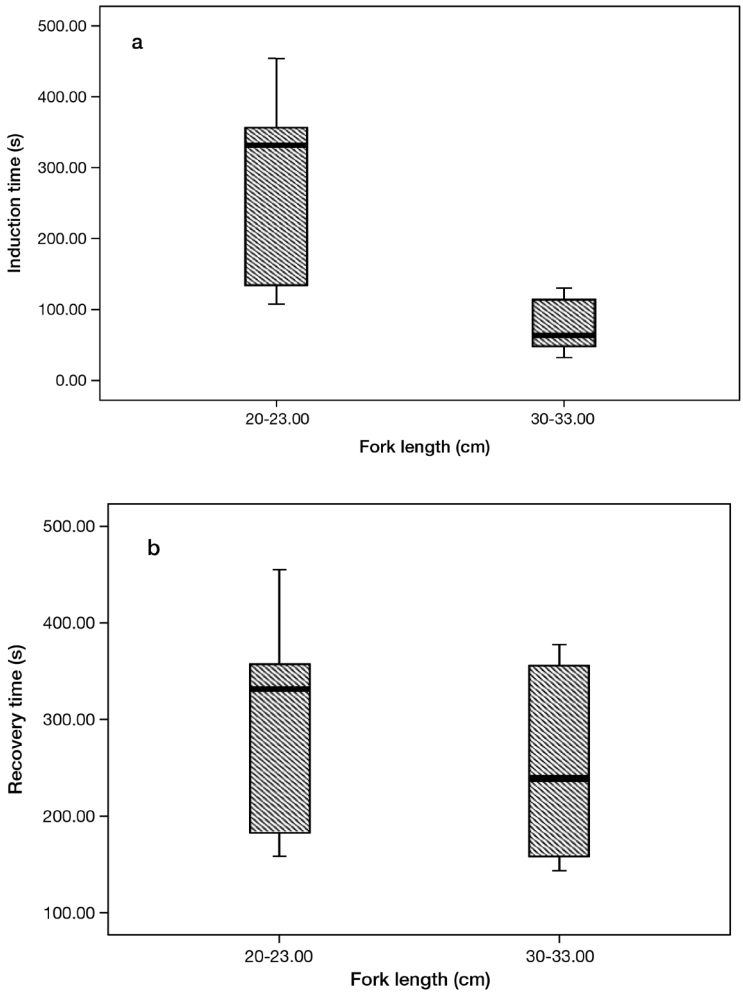

Fig. 2. Box plots of (a) induction and (b) recovery times for different sizes of rainbow trout anaesthetized with clove oil. The upper and lower hinges of each plot represent the maximum and times, middle line represents the median, the shaded inner box represents the 25th and 75th percentiles and the whiskers represent lower and upper limits.

75 mg·l^{-1}($P < 0.001$). For the higher dose, induction time was rather uniform in all size groups ($P > 0.05$).

Recovery time was more size-dependent at the lower dose (75 mg·l^{-1}), with larger fish requiring less time to recover ($P < 0.001$) (Table 1). However, smaller fish recovered earlier compared to the other size groups ($P < 0.001$).

Physiological responses in trout and goldfish

Clove oil did not produce marked changes ($P < 0.05$) in the physiological indicators of experimental goldfish compared to control (Table 3). However, it produced significant changes between haematocrit of the control for rainbow trout and the treated group; and

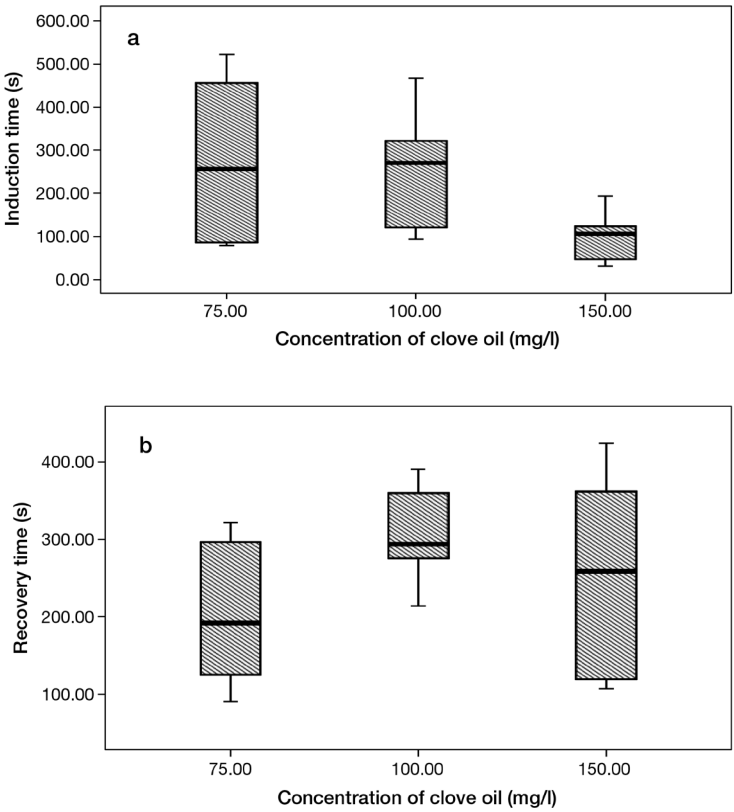

Fig. 3. Box plots of (a) induction and (b) recovery times for goldfish anaesthetized at various concentrations of clove oil. The upper and lower hinges of each plot represent the maximum and times, middle line represents the median, the shaded inner box represents the 25th and 75th percentiles and the whiskers represent lower and upper limits.

bewteen the treated groups ($P < 0.001$). It also caused hyperkalaemia and hyperglycaemia ($P > 0.05$) in the trout compared to control, but not in the goldfish (Table 3).

Discussion

In this study, clove oil was found to be effective anaesthetic for both rainbow trout and goldfish. Both fish species exhibited normal behavior and remained calm during the induction time with no struggling or rapid swimming, which was a positive sign of their welfare. Additionally, no mortalities were observed for a 24 h post-recovery period.

Some species-specific differences were evident in the induction time. Rainbow trout exhibited a more rapid induction time compared to goldfish at all the doses tested. This may be the result of a higher uptake of eugenol via the gills (Keene et al. 1998) in rainbow trout which is a fish species with higher respiratory activity compared to the more sluggish ornamental goldfish. In goldfish, induction time was related to the size of fish, with more time needed for larger fish to be anaesthetized. This may be because of higher rate of uptake of the anaesthetic through the gills in the smaller fish compared to the larger ones. This is in accordance with the results reported by Woody et al. (2002) for sockeye

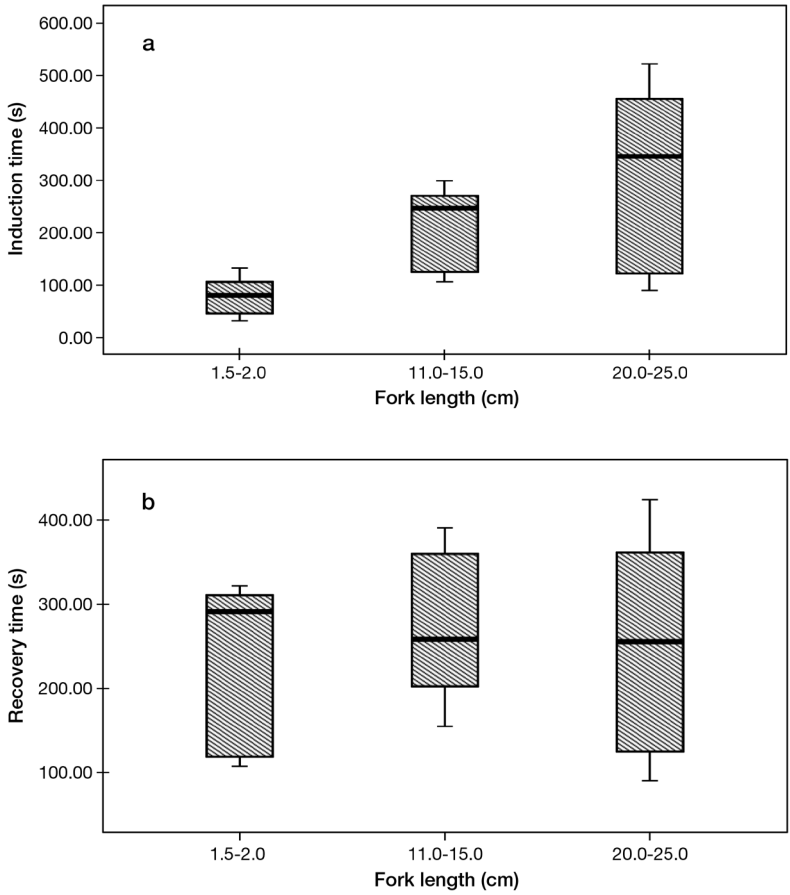

Fig. 4. Box plots of (a) induction and (b) recovery times for different sizes of goldfish anaesthetized with clove oil. The upper and lower hinges of each plot represent the maximum and times, middle line represents the median, the shaded inner box represents the 25th and 75th percentiles and the whiskers represent lower and upper limits.

salmon (*Oncorhynchus nerka*). The higher dose produced a rapid and uniform response in all size classes, suggesting that a dose of 150 mg·l^{-1} might be well over the effective concentration.

For rainbow trout, recovery time increased with increased doses of clove. In goldfish, recovery time varied with the concentration of clove oil for various size groups. An increase in recovery time relative to clove concentration was recorded in roach (*Rutilus rutilus*) (Sudagara et al. 2009), cobia (*Rachycentron canadum*) (Gullan and Villanuera 2009) and rainbow trout (Velisek et al. 2005b), and to 2-phenoxyethanol in rainbow trout (Velisek et al. 2004). Woody et al. (2002) and Inoue et al. (2003) found no direct relationship between clove concentration and recovery times in adult sockeye salmon (*Oncorhynchus nerka)* and matrinxã (*Brycon cephalus*). Results of this study seem to confirm species-specific differences in recovery times of anaesthetized fish. In rainbow trout, recovery time was slightly longer 1.3 - 8.1 × compared to induction time. This could be explained by the fact that eugenol exerts an inhibitory effect on respiration, reducing the ability of fish to remove the excess of eugenol from the system via the

Table 1. Induction and recovery time of goldfish anaesthetized with clove oil

Fish length (cm)	Dosage of clove oil (mg·l^{-1})				Dosage of clove oil (mg·l^{-1})			
	75	100	150	Mean (± SD) for size	75	100	150	Mean (± SD) for size
	Induction time (s)				Recovery time (s)			
1.5-2.0	84.28 (9.39)	111.0 (13.67)	41.15 (5.63)	78.81 (31.01)^{c}	306.86 (12.90)	294.14 (16.43)	115.00 (4.90)	238.67 (90.52)^{c}
11.0-15	256.43 (34.22)	270.43 (16.66)	119.43 (7.16)	78.81 (31.01)^{b}	189.00 (25.76)	364.00 (24.11)	257.71 (14.24)	270.23 (76.66)^{b}
20.0-25	475.00 (34.22)	366.14 (56.93)	117.14 (35.14)	215.43 (71.00)^{a}	110.86 (23.17)	256.00 (28.33)	381.71 (38.94)	249.52 (117.12)^{a}
Mean (± SD) for each dosage	271.90 (165.19)^{c}	249.19 (112.87)^{b}	92.5714 (42.25)^{a}		202.23 (85.00)^{a}	304.71 (50.95)^{b}	251.47 (114.00)^{c}	

(n = 10 for each data point, figures in parenthesis, SD; Mean in the respective row or column under induction or recovery with similar superscripts are not significantly different, $p > 0.05$)

Table 2. Induction and recovery time of rainbow trout anaesthetized with clove oil

Fish length (cm)	Dosage of clove oil (mg·l^{-1})				Dosage of clove oil (mg·l^{-1})			
	75	100	150	Mean (± SD) for size	75	100	150	Mean (± SD) for size
	Induction time (s)				Recovery time (s)			
20.00-23.00	120.50 ± 10.55	340.17 ± 15.77	399.50 ± 53.24	283.53 ± 123.23^{a}	176.00 ± 10.34	340.17 ± 15.77	399.50 ± 53.24	301.27^{a} ± 98.67
30-33.00	120.50 ± 0.56^{c}	64.40 ± 3.20^{b}	44.30 ± 6.24^{a}	76.4000 ± 33.55^{b}	156.80 ± 5.88^{c}	240.40 ± 13.25^{b}	363.50 ± 9.32^{a}	253.57 ± 86.88^{a}
Mean (± SD) for each dosage	121.27 ± 0.83^{c}	167.81 ± 138.21^{b}	145.79 ± 168.55^{a}		163.20 ± 11.86^{c}	277.81 ± 51.76^{b}	373.79 ± 31.61^{a}	

(n = 10 for each data point, figures in parenthesis, SD; Mean in the respective row or column under induction or recovery with similar superscripts are not significantly different, $p > 0.05$)

Table 3. Haematocrit and biochemical variables of rainbow trout and goldfish anaesthetized with clove oil

Concentration of clove (mg/l)	Haematocrit (%)	Calcium (mmol/l)	Glucose (mmol/l)
Rainbow trout			
0.00	45.58 ± 3.23^{c}	8.23 ± 1.28^{b}	4.94 ± 0.72^{b}
50.00	50.90 ± 4.48^{b}	10.38 ± 0.79^{a}	6.05 ± 0.20^{a}
100.00	58.97 ± 2.67^{a}	9.99 ± 0.62^{a}	5.27 ± 1.35^{ab}
150.00	54.58 ± 2.97^{b}	10.51 ± 0.48	5.00 ± 0.42^{ab}
Goldfish			
0.00	36.08 ± 1.48^{a}	8.36 ± 1.23^{a}	5.08 ± 0.76^{a}
75.00	37.06 ± 3.22^{a}	8.48 ± 0.59^{b}	5.97 ± 0.29^{a}
100.00	36.03 ± 2.44^{a}	9.3457 ± 1.43^{ab}	5.27 ± 0.67^{a}
150.00	35.79 ± 2.32^{a}	8.9367 ± 503^{ab}	5.47 ± 0.828^{a}

Means in the respective column for each species with similar superscripts are not significantly different, $p > 0.05$

gills (Keene et al. 1998; Guénette et al. 2007). Our results indicate that clove concentration of 50 mg·l^{-1} induces the minimum induction and recovery times in rainbow trout; thus, this dose may be suitable for sedation and for standard on-farm procedures that require anaesthesia. However,

the recommended concentrations for both rainbow trout and goldfish may be influenced by environmental factors quite different from those under which this study was conducted.

In some fish species exposed to clove oil, alteration of some blood biochemical and haematocrit values were detected (Cho and Heath 2000; Velisek et al. 2005ab). However, increased haematocrit concentration in the experimenal group of rainbow trout compared to control does not suggest toxicity in haematopoietic centres (spleen and head kidney) of fish (Van der Weiden et al. 1994). The effects of toxicants on various tissues may vary with dosage, exposure, body size and species. Tort et al. (2002) reported clove oil altering haematocrit concentrations in rainbow trout. Velisek et al. (2005b) observed the same in rainbow trout and carp, both of them exhibited also significantly increased plasma glucose concentrations. Nevertheless, these findings were observed after longer exposure periods (10 min) compared to 0.69-7.92 min in the present study. These results reveal the significance of exposure time and dosage on some physiological indicators of anaesthetized fish. The short induction time after which blood was collected could explain the lack of biochemical alteration and post-exposure mortality in this study. These results indicate the need to study possible physiological changes occurring in different fish species exposed to different doses of clove oil.

Glucose and calcium plasma concentrations along with cortisol concentrations have been used as stress indicators in fish (Schreck 2000; Sink et al. 2006). In previous studies, clove oil did not induce changes ($P > 0.05$) in plasma glucose and calcium concentrations in trout and goldfish used for experiments (Andersen et al. 1991; Cho and Heath 2000; Wagner et al. 2003). However, mild hyperglycaemia and hypercalcaemia were recorded in some treated groups of the two species. A rise in glucose concentration is a second order reaction under stress (Barton and Iwama 1991) and is mediated by the rise in cortisol concentration induced by stress. Clove was found to block the activity of cortisol, although not completely, in matrinxã (*Brycon cephalus*) (Inoue 2005). Although the mechanism is not well known, Iverson (2003) suggested that it blocks transmission of impulses to the hypothalamus-pituitary interrenal axis (HPI). Cortisol is considered one of the mediators of the increase in plasma glucose under stress (Barton et al. 2002) and the hyperglycaemia recorded in rainbow trout may be explained by the increase in cortisol concentration.

In conclusion, clove oil was found to be safe and can be effectively and easily applied at 150 $mg \cdot l^{-1}$ to anaesthetize various size groups of rainbow trout and goldfish with minimal disruption in the physiological indicators studied and with zero mortality. Clove oil was easy to handle, without producing unpleasant or irritating odor. Hence, serious considerations should be given to the use of clove oil as a replacement for synthetic forms of anaesthetics.

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