

Comparison of acute toxicity of 2-phenoxyethanol and clove oil to juvenile and embryonic stages of *Danio rerio*

Stanislava MÁCOVÁ, Petra DOLEŽELOVÁ, Vladimíra PIŠTĚKOVÁ,
Zdeňka SVOBODOVÁ, Iveta BEDÁŇOVÁ, Eva VOVLÁŘOVÁ

Department of Veterinary Public Health and Toxicology, Faculty of Veterinary Hygiene and Ecology,
University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic

Correspondence to: Stanislava Mácová, DVM.
University of Veterinary and Pharmaceutical Sciences Brno
Palackého 1/3, 612 42 Brno, Czech Republic
TEL.: +420-541 562 783, FAX: +420-541 562 790
E-MAIL: smacova@vfu.cz

Submitted: 2008-06-30 Accepted: 2008-09-02

Key words: *Danio rerio*; 2-phenoxyethanol; clove oil; fish; age; LC50

Neuroendocrinol Lett 2008; 29(5):680–684 PMID: 18987607 NEL290508A04 © 2008 Neuroendocrinology Letters • www.nel.edu

Abstract

OBJECTIVES: Anaesthetics are used in aquaculture to prevent stress and mechanical damage to fish during handling or the treatment of fish in breeding, blood sampling and other veterinary interventions. Clove oil and 2-phenoxyethanol are used in the Czech Republic in a water bath for the short-term immobilization of the fish.

DESIGN: Acute toxicity tests were performed on aquarium fish *Danio rerio*, which is considered to be one of the model organisms most commonly used in toxicity testing. The semi-static method according to OECD No. 203 (Fish acute toxicity test) was used for testing juvenile fish. Embryo toxicity tests were performed in zebrafish embryos (*D. rerio*) in compliance with the OECD No. 212 methodology (Fish, short-term toxicity test on embryo and sac-fry stages). The results obtained (the number of dead individuals at particular test concentrations) were subjected to a probit analysis using the EKO-TOX 5.2 programme in order to determine LC50 clove oil and 2-phenoxyethanol values. The statistical significance of the difference between LC50 values in juvenile and embryonic stages of *D. rerio* was tested using the Mann-Whitney non-parametric test implemented in the Unistat 5.1 programme.

RESULTS: The LC50 clove oil mean value was 18.8 ± 5.52 mg.L⁻¹ in juvenile *D. rerio*, and 15.64 ± 3.30 mg.L⁻¹ in embryonic stages of *D. rerio*. The LC50 2-phenoxyethanol mean value was 338.22 ± 15.22 mg.L⁻¹ in juvenile *D. rerio*, whereas in embryonic stages of *D. rerio* it was 486.35 ± 25.53 mg.L⁻¹.

CONCLUSIONS: The study proved statistically significantly higher ($p < 0.01$) sensitivity in juvenile fish to 2-phenoxyethanol compared to the embryonic stages. Acute toxicity values of clove oil for juvenile and embryonic stages were comparable.

INTRODUCTION

Anaesthetics are commonly used in aquaculture to reduce handling stress and mechanical damage that fish may suffer during manipulation, breeding or veterinary interventions. Anaesthetics are administered in cases of the artificial propagation of fish, the injection of medicinal drugs and hormonal preparations, blood sampling, measuring, the weighing and tagging of fish, photographing and in the long-distance transport of especially aquarium fish (Ross & Ross, 1999; Svoboda & Kolarova, 1999; Svobodova et al. 2007). The common aquarium fish *Danio rerio* was used in our acute toxicity tests. Zebrafish (*D. rerio*) is a popular aquarium fish, and is a species that is most commonly used in toxicity testing (Grush, 2004; Mikula 2006).

The use of anaesthesia helps to decrease stress-induced complications, e.g. reduced food intake and the decrease in immune functions of the fish (Ross & Ross, 1999). Effects of stress induced by fish manipulation and anaesthesia may even affect certain haematological and biochemical blood parameters. The effects of anaesthetics on these parameters have been studied by a number of authors (Velisek et al. 2005a; 2005b; 2006; 2007; Velisek & Svobodova, 2004a; 2004b).

Fish sedation is most frequently carried out in an anaesthesia bath (Svobodova et al. 2007). Guilderhus & Marking (1987) define three criteria that anaesthetics which are used in fish rearing must meet. They must be efficient, safe and inexpensive. The most commonly used fish anaesthetics include clove oil and 2-phenoxyethanol (Svoboda & Kolarova, 1999). The resulting effect of the anaesthesia bath depends on a number of factors including anaesthetic concentration, water temperature, and the size and species of the fish (Hamackova et al. 2004; Myszkowski et al. 2003; Soto & Burhanuddin, 1995; Weyl et al. 1996).

2-phenoxyethanol (ethylene glycol monophenyl ether) is used for short-term immobilization of fish at a generally recommended concentration of 0.30–0.40 mL.L⁻¹ (Barton & Helfrich, 1981; Hamackova et al., 2001; Svoboda & Kolarova, 1999; Svobodova et al. 2007). The advantages of phenoxyethanol include the quick induction of anaesthesia, the rapid recovery of the fish once they are transferred to clean water, the ease of preparation and low cost (Weyl et al. 1996). At the recommended concentration, anaesthesia is induced within 5 to 10 minutes. The anaesthesia subsides and physiological reflexes are restored within 10 minutes after the fish are transferred to clean water (Hamackova et al. 2001; Svoboda & Kolarova, 1999; Svobodova et al. 2007). Anaesthetics are taken up by the fish through a concentration gradient at the gill surface, and enter the arterial circulation until a balance with the outside environment is reached. This concentration gradient is also responsible for the drain of the anaesthetic when the fish are transferred to clean water (Ross & Ross, 1999). The recovery time is therefore affected

by the concentration of the anaesthetic, but unaffected by the length of anaesthesia. This indicates that 2-phenoxyethanol can be used over a longer period of time without any adverse effects on the degree of recovery (Weyl et al. 1996). When 2-phenoxyethanol is being administered, it is necessary to observe the rules for its safe use because 2-phenoxyethanol in poorly ventilated rooms may cause fatigue, drowsiness, and irritation of the skin and eyes of the persons handling it (Svoboda & Kolarova, 1999; Svobodova et al. 2007).

Clove oil is a dark-brown liquid of natural origin, obtained by the distillation of *Eugenia aromatica* and *Eugenia caryophyllata* plants (Soto & Burhanuddin, 1995; Svobodova et al. 2007). It contains a number of terpene compounds that are responsible for its characteristic smell and taste (Ross & Ross, 1999). Clove oil's active substance is eugenol (4-allyl-methoxyphenol), which makes up about 80–90% of clove oil (Keene et al. 1998). The natural origin of this anaesthetic is has proven to be a disadvantage, because it is not possible to accurately define the composition of individual clove oil batches (Svobodova et al. 2007). In the Czech Republic, clove oil is used for the short-term immobilization of fish at a recommended concentration of 30–40 mg.L⁻¹ (Hamackova et al. 2001; Svobodova et al. 2007). At this concentration, anaesthesia is induced within 5–10 min, and the period of recovery is somewhat longer than in the case of other anaesthetics (Svobodova et al. 2007). The use of clove oil as a potent anaesthetic agent for *D. rerio* was reported by Grush et al. (2004).

The advantages of clove oil are its low price, the potentially little or no side effects for fish and for the safety of the staff administering it (Keene et al. 1998; Soto & Burhanuddin, 1995).

The aim of the present study was to compare the acute toxicity of clove oil and of 2-phenoxyethanol, and to compare the toxicity of the two anaesthetics to embryonic and juvenile stages of zebrafish (*Danio rerio*).

MATERIAL AND METHODS

Acute toxicity tests were performed on juvenile stages of the aquarium fish *Danio rerio* in accordance with OECD 203 guidelines (Fish Acute Toxicity Test). The experimental fish were 2–3 months old, weighed 0.3 ± 0.1 g, and their total length was 30 ± 5 mm. In the experiment, each anaesthetic was tested in five different concentrations consisting of an approximate geometric progression. Two series of 5 tests each were conducted, one series with clove oil and the other with 2-phenoxyethanol. The tests were made using a semi-static method with solution replacement after 48 hours. During the tests, records of the temperature, pH, the concentration of oxygen dissolved in test tanks and fish mortality rate were noted. Ten fish picked randomly from the spare

stock were placed in each tank. Then 96-hour-long acute toxicity tests were conducted.

The temperature of the experimental bath was $24 \pm 1^\circ\text{C}$, the dissolved oxygen concentrations did not fall below 60% (80–94%), and the pH was between 7.89 and 8.62. No fish died in the control tanks during the experiments.

Embryonic toxicity tests were performed on embryos of *D. rerio*. The tests were made according to OECD guideline 212 (Fish, short-term toxicity test on embryo and sac-fry stages). Two series of 5 tests each were con-

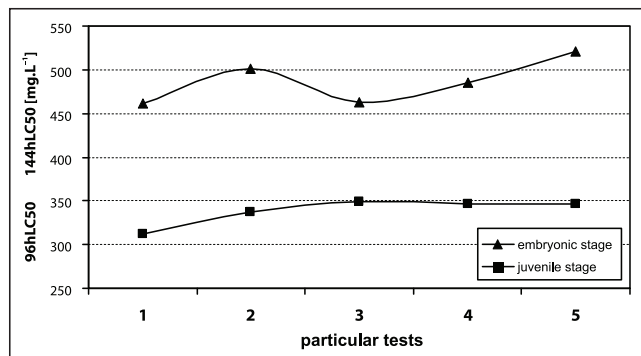


Figure 1. 96hLC50 and 144hLC50 2-phenoxyethanol values in juvenile and embryonic development stages of *D. rerio*

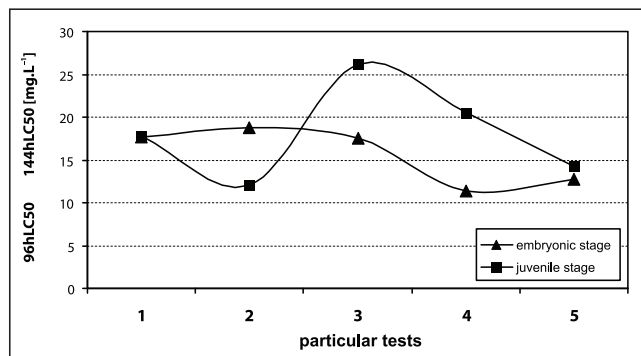


Figure 2. 96hLC50 and 144hLC50 clove oil values in juvenile and embryonic development stages of *D. rerio*

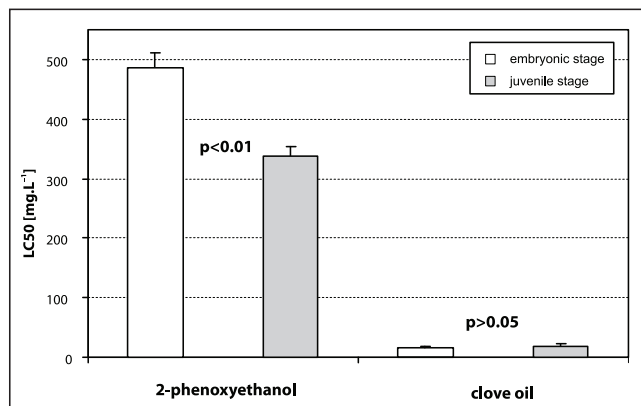


Figure 3. Comparison of the mean values of LC50 2-phenoxyethanol and clove oil for the embryonic and juvenile stages of *D. rerio*

ducted, one series with clove oil and the other with 2-phenoxyethanol. A series of 5 ascending concentrations of the tested substance was used in the tests. Twenty fertilized eggs in a Petri dish were tested at each concentration and in one control. The eggs were placed in Petri dishes within 8 hours at the latest after fertilization. The tests were terminated after hatching and the absorption of the yolk sack in all individuals in the control dish (144–168 h after placement onto the dish). The bath was replaced at 24 h intervals. During the tests, the numbers of dead embryos in individual concentrations were recorded. The mortality rate of the control embryos did not exceed 20%. Test bath temperatures were between 24.5 and 25.5 °C.

The basic physical and chemical parameters of diluting water used in toxicity tests on embryonic and juvenile stages were: $\text{ANC}_{4.5}$ 3.6–3.7 mmol.L^{-1} ; COD_{Mn} 1.4–1.9 mg.L^{-1} ; total ammonia below the limit of determination; NO_3^- 24.5–31.4 mg.L^{-1} ; NO_2^- below the limit of determination; Cl^- 18.9–19.1 mg.L^{-1} ; $\Sigma \text{Ca} \pm \text{Mg}$ 14 mg.L^{-1} .

The probit analysis (EKO-TOX 5.2 software) was applied to the results obtained (the number of fish dying at individual test concentrations) and the LC50 values for clove oil and 2-phenoxyethanol were calculated. The statistical significance of the difference between LC50 values for the juvenile and the embryonic stages of *D. rerio* was calculated using the non-parametric Mann-Whitney test and the Unistat 5.1 software.

RESULTS AND DISCUSSION

Because anaesthetics are routinely used in aquacultures, it is necessary to test the toxicity of the anaesthetics to determine the suitable treatment concentrations of anaesthetic baths.

In our tests, we monitored the toxic effects of two anaesthetics most frequently used for the sedation of the embryonic and the juvenile life stages of the aquarium fish *D. rerio*. The first anaesthetic tested was 2-phenoxyethanol. The LC50 of 2-phenoxyethanol for the embryonic life stage of zebrafish and expressed as the 144h LC50 was calculated to be in the 461.52–521.55 mg.L^{-1} range (mean 144hLC50 = $486.35 \pm 25.53 \text{ mg.L}^{-1}$). The LC50 for the juvenile life stage expressed as a 96h LC50 was calculated to be in the 312.10–349.02 mg.L^{-1} range (mean 96hLC50 = $338.22 \pm 15.22 \text{ mg.L}^{-1}$) (Figure 1).

In the study on sheatfish (*Silurus glanis* L.), Velisek *et al.* (2007) reported a comparable LC50 value of 2-phenoxyethanol that was also found in our study on the juvenile stage of *D. rerio*. The acute toxicity as expressed by the 96hLC50 value was 0.29 ml.L^{-1} (321 mg.L^{-1}). A higher sensitivity to 2-phenoxyethanol compared with *D. rerio* was found in the case of a 6-month old rainbow trout (*Oncorhynchus mykiss*). The 96hLC50 for the rainbow trout was calculated at 0.25 ml.L^{-1} (277 mg.L^{-1}) (Velisek & Svobodova, 2004b). Velisek & Svobodova (2004a) studied carp fry (*Cyprinus carpio* L)

sensitivity to the acute toxicity of 2-phenoxyethanol. The reported 96hLC50 value 0.17 mL.L^{-1} (188 mg.L^{-1}) is lower than that found in toxicity tests of the two life stages of *D. rerio*, which indicates that sensitivity to anaesthetics is species-dependent. Interspecies differences in anaesthetic tolerance are attributed to differences in metabolisms of individual fish species. Differences in 96hLC50 values calculated may also be due to differences in external environment conditions under which the acute toxicity tests were made. One of the most important parameters of the external environment that affect 2-phenoxyethanol toxicity was water temperature: the higher the temperature, the higher the efficacy of the anaesthetic (Hamackova *et al.* 2001; Svobodova *et al.* 2007; Weyl *et al.* 1996). Weyl *et al.* (1996) also reported an increased tolerance of fish to 2-phenoxyethanol when anaesthetics were used repeatedly.

The acute toxicity of clove oil on the embryonic life stage of zebrafish expressed as a 168hLC50 in our tests was calculated at $11.4\text{--}18.7 \text{ mg.L}^{-1}$ (mean 168hLC50 = $15.64 \pm 3.30 \text{ mg.L}^{-1}$), and at $12.1\text{--}26.2 \text{ mg.L}^{-1}$ (mean 96hLC50 = $18.18 \pm 5.52 \text{ mg.L}^{-1}$) for the juvenile life stage of zebrafish (Figure 2). A higher value of 96h LC50 for a one-month old *D. rerio* were reported by Grush *et al.* (2004) (96h LC50 for eugenol = 21 mg.L^{-1}). The acute toxicity of clove oil comparable to our results were reported by Velisek *et al.* (2005b) for the common carp (*Cyprinus carpio*) (96hLC50 = 18.10 mg.L^{-1}), by Velisek *et al.* (2006) for the European catfish (*Silurus glanis*) (96hLC50 = 18.40 mg.L^{-1}) and by Velisek *et al.* (2005a) for the rainbow trout (*Oncorhynchus mykiss*) (96hLC50 = 14.1 mg.L^{-1}). A lower LC50 for the rainbow trout was found by Keene *et al.* (1998). They estimated the acute toxicity of eugenol (as the active ingredient of clove oil) expressed as 8–96h LC50 at 9 mg.L^{-1} . We may, however, consider that value is also comparable with the 96hLC50 values ascertained in toxicity tests on *D. rerio*, and particularly the tests on its embryonic life stage. The same relationship between water temperature, anaesthetic concentration and the course of anaesthesia (the higher the water temperature, the faster the onset of anaesthesia) as described for 2-phenoxyethanol were also reported for clove oil (Hamackova *et al.* 2004, Walsh & Pease, 2002). The anaesthetic response to clove oil may also be influenced by biological factors such as stress, individual fitness and skin thickness (Walsh & Pease, 2002).

Another important factor influencing the sensitivity of fish to anaesthetics is their age and size (Guilderhus & Marking, 1987; Oikawa *et al.* 1994). Younger fish are generally assumed to be more sensitive to chemical substance toxicity. Barton & Helfrich (1981) reported a higher sensitivity of young fish to 2-phenoxyethanol, and they recommended that lower anaesthetic concentrations be used when young fish are being anaesthetized. Velisek & Svobodova (2004a), who studied the acute toxicity of 2-phenoxyethanol in the common carp (*Cyprinus carpio*), reached the same conclusions.

They found that the recommended concentration of 0.30 mL.L^{-1} is not completely safe for carp fry and two-year old carp, but in view of the differences between the sensitivity of different age groups, they consider that particular concentration to be safe for broodstock carp. A comparison between the results of toxicity tests of 2-phenoxyethanol in the embryonic and juvenile life stages of *D. rerio*, revealed a statistically significantly higher sensitivity ($p < 0.01$) in the juvenile life stage compared to the embryonic stage (Figure 3). One reason for this difference could be the embryonic stages' uptake and metabolism of the anaesthetic compared with the juvenile and adult life stages of fish. Myszkowski *et al.* (2003), who compared 2-phenoxyethanol efficacy on the tench (*Tinca tinca*) from different age groups, reported a decrease in safe concentrations in relation to the increased age of the fish. Weyl *et al.* (1996) found no dependence between the induction time of phenoxyethanol and the size of the fish *Carassius auratus*.

After these series of acute toxicity tests with clove oil were conducted, the sensitivity of the two life stages of *D. rerio* to that compound was compared. The evaluation of the results confirmed that the sensitivity of the two life stages to the toxicity of the compound test was comparable (Figure 3). The comparable sensitivity of the life stages of the longfinned eel (*Anguilla reinhardtii*) to clove oil was also reported by (Walsh & Pease, 2002). A comparison between the results of our clove oil toxicity tests on juvenile two to three-month-old fish *D. rerio* and results obtained on one-month-old *D. rerio* (Grush *et al.* 2004) showed a higher sensitivity in the two to three-month-old fish.

ACKNOWLEDGEMENTS

The work was realized with the support of IGA 34/2007/FVHE and MSM Project No. 6215712402 Veterinary Aspects of Foodstuff Safety and Quality.

REFERENCES

- Barton, BA, Helfrich, H (1981). Time dose responses of juvenile rainbow trout to 2-phenoxyethanol. *Prog Fish Cult.* **43**: 223–223.
- Grush J, Noakes DLG, Moccia RD (2004). The efficacy of clove oil as an anesthetic for the zebrafish, *Danio rerio* (Hamilton). *Zebrafish.* **1**: 46–53.
- Guilderhus PA, Marking LL (1987). Comparative efficacy of 16 anaesthetic chemicals on rainbow trout. *North. Am. J. Fish. Manage.* **7**: 288–292.
- Hamackova J, Lepicova A, Kozak P, Stupka Z, Kouril J, Lepic P (2004). The efficacy of various anaesthetics in tench (*Tinca tinca* L.) related to water temperature. *Vet Med-Czech.* **49**: 467–472.
- Hamackova J, Sedova MA, Pjanova SV, Lepicova A (2001). The effect of 2-phenoxyethanol, clove oil and Propiscin anaesthetics on perch (*Perca fluviatilis*) in relation to water temperature. *Czech J Anim Sci.* **46**: 469–473.
- Keene JL, Noakes DLG, Moccia RD, Soto CG (1998). The efficacy of clove oil as an anaesthetic for rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquac Res.* **29**: 89–101.

- 7 Mikula P, Dobsikova R, Svobodova Z, Jarkovsky J (2006). Evaluation of xenoestrogenic potential of propylparaben in zebrafish (*Danio rerio*). *Neuroendocrinol Lett.* **27**: 104–107.
- 8 Myszkowski L, Kaminski R, Wolnicki J (2003). Response of juvenile tench *Tinca tinca* (L.) to the anaesthetic 2-phenoxyethanol. *J Appl Ichthyol.* **19**: 142–145.
- 9 Oikawa S, Takeda T, Itazawa Y (1994). Scale effects of MS-222 on a marine teleost, porgy *Pagrus major*. *Aquaculture.* **121**: 369–379.
- 10 Ross LG, Ross B (1999). Anaesthetic and sedative techniques for aquatic animals. 2nd ed. Oxford, Blackwell Science Ltd.
- 11 Soto CG, Burhanuddin (1995). Clove oil as a fish anaesthetic for measuring length and weight of rabbitfish (*Siganus lineatus*). *Aquaculture.* **136**: 149–152.
- 12 Svoboda M., Kolarova J (1999). Review of anaesthetics used in fish culture. In: Vajcova V, Svobodova Z, editors. Health protection of aquarium fish. VÚRH JU Vodňany. p. 49–72 (In Czech)
- 13 Svobodova Z, Kolarova J, Navratil S, Vesely T, Chloupek P, Tesarcik J *et al* (2007). Diseases of freshwater and aquarium fish. Informatorium Praha (In Czech)
- 14 Velisek J, Svobodova Z (2004a). Anaesthesia of common carp (*Cyprinus carpio* L.) with 2-phenoxyethanol: Acute toxicity and effects on biochemical blood profile. *Acta Vet Brno.* **73**: 247–252.
- 15 Velisek J, Svobodova Z (2004b). Anaesthesia of rainbow trout (*Oncorhynchus mykiss*) with 2-phenoxyethanol: Acute toxicity and biochemical blood profile. *Acta Vet Brno.* **73**: 379–384.
- 16 Velisek J, Svobodova Z, Piackova V (2005a). Effects of clove oil anaesthesia on rainbow trout (*Oncorhynchus mykiss*). *Acta Vet Brno.* **74**: 139–146.
- 17 Velisek J, Svobodova Z, Piackova V, Groch L, Nepejchalova L (2005b). Effects of clove oil anaesthesia on common carp (*Cyprinus carpio* L.) *Vet Med-Czech.* **50**: 269–275.
- 18 Velisek J, Wlasow T, Gomulka P, Svobodova Z, Novotny L (2007). Effects of 2-phenoxyethanol anaesthesia on sheatfish (*Silurus glanis* L.). *Vet Med-Czech.* **52**: 103–110.
- 19 Velisek J, Wlasow T, Gomulka P, Svobodova Z, Novotny L, Ziomek E (2006). Effects of clove oil anaesthesia on European catfish (*Silurus glanis* L.) *Acta Vet Brno.* **75**: 99–106.
- 20 Walsh CT, Pease BC (2002). The use of clove oil as an anaesthetic for the longfinned eel, *Anguilla reinhardtii* (Steindachner). *Aquac Res.* **33**: 627–635.
- 21 Weyl O, Kaiser H, Hecht, T (1996). On efficacy and mode of 2-phenoxyethanol as an anesthetic for gold fish, *Carassius auratus* (L.) at different temperatures and concentrations. *Aquac Res.* **27**: 757–764.