Evaluation of the developmental toxicity of 2-phenoxyethanol and clove oil anaesthetics using the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX)

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ABSTRACT: The developmental toxicity of two anaesthetics, 2-phenoxyethanol and clove oil, used in aquaculture was evaluated using the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) and the results were compared to outcomes in fish. *Xenopus laevis* embryos were exposed to 50, 100, 300, 500, 700 and 1000 mg/l of 2-phenoxyethanol or 1, 5, 10, 20, 30 and 40 mg/l of clove oil. Values of 96 h LC50, 96 h EC50 (malformation) and teratogenic index (ratio of 96 h LC50 and 96 h EC50) were determined and the types and severities of the induced malformations and minimal concentration inhibiting the growth of embryos were estimated. Teratogenic index values for 2-phenoxyethanol and clove oil were estimated at 1.69 and 0.61 respectively. The most frequently observed malformations produced by 2-phenoxyethanol were axial flexure and oedema and for clove oil, axial flexure, gut malformation, microphthalmia and oedema. 2-phenoxyethanol was found to induce growth inhibition of frog embryos at concentrations above 300 mg/l and clove oil at concentrations above 20 mg/l. In summary, both 2-phenoxyethanol and clove oil affected the growth of *Xenopus* embryos, while only 2-phenoxyethanol represented a teratogenic risk.

Keywords: eugenol; amphibian; anaesthesia; teratogenic index; malformation; African clawed frog

Anaesthetics such as 2-phenoxyethanol or clove oil are used in aquaculture to prevent stress or mechanical damage in fish during manipulation, veterinary intervention, artificial spawning or measuring (Ross and Ross 1999). The right use of anaesthesia prevents stress-induced complications in fish such as decreased immune functions or food intake (Ross and Ross 1999).

With the growing popularity of amphibians in research and as pets a need for high-quality anaesthesia that could be used to manage amphibians for diagnostic and surgical procedures without side-effects, arose. A large number of anaesthetics for parenteral administration have been evaluated in amphibians; tricaine methanesulfonate, isoflurane or ketamine rank among the most frequently used (Whitaker and Wright 2001). In consideration of the small size of some amphibians and difficulty with injectable administration, the possibility of anaesthetic immersion baths for water amphibians represents an ideal route of anaesthetic administration. Clove oil, tricaine methanesulfonate (MS 222) and propofol rank among the most efficacious compounds for immersion bath anaesthesia in amphibian medicine (Mitchell 2009).

Clove oil is a naturally occurring compound obtained from clove plants *Eugenia aromatica* or *Eugenia caryophylatta*. The active substance, eugenol, comprises from 80% to 90% of clove oil. Clove oil's advantages include low price, relatively little adverse reactions for both fish and amphibians and safety for staff (Svobodova et al. 2007).

2-phenoxyethanol (ethylene glycol monophenyl ether) has been suggested as a good anaesthetic for short term immobilisation of fish (Ortuno et al. 2002; Tsantilas et al. 2006). The advantages of 2-phenoxyethanol include short anaesthesia induction phase, rapid recovery and low price (Weyl

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et al. 1996). Compared to clove oil, 2-phenoxyethanol is less safe for staff; in closed rooms it can cause skin and eye irritation in people (Svobodova et al. 2007).

The African clawed frog *Xenopus laevis* is a popular worldwide amphibian model and is one of the amphibian species most commonly used in toxicity testing. Embryos of *X. laevis* are sensitive indicators for evaluation of the developmental toxicity of substances in the water environment.

The purpose of this study was (1) to evaluate the teratogenic risk of 2-phenoxyethanol and clove oil to *Xenopus laevis* embryos and (2) to estimate the risk for amphibian embryos in the field when subjected to an anaesthetic immersion bath for fish containing 2-phenoxyethanol or clove oil.

MATERIAL AND METHODS

In this study, clove oil supplied by the Kulich Company (Hradec Kralove/Ricany, Czech Republic) and 2-phenoxyethanol supplied by MERCK (Hohenbrunn, Germany) were used. The substances were dissolved in standard FETAX solution (625.0 NaCl, 96.0 $NaHCO_3$, 30.0 KCl, 15.0 $CaCl_2$, 60.0 $CaSO_4.H_2O$ and 70.0 $MgSO_4$ all in mg/l dissolved in distilled water) (ASTM 1998).

The South African clawed frog (*Xenopus laevis*) embryos were obtained from an adult pair injected with human chorionic gonadotropin (Pregnyl 1500, N.V. Organon, Oss, The Netherlands) into the dorsal lymph sac (females 600 IU, males 300 IU).

Embryonal tests were conducted using the Standard guide for the frog embryo teratogenesis assay-*Xenopus* (FETAX) (ASTM 1998). Normally developed embryos in mid-blastula (stage 8) to early gastrula (stage 11) were selected for testing (Nieuwkoop and Faber 1994). Groups of 25 embryos were randomly placed in plastic Petri dishes (60 mm) with 10.0 ml of the tested solution and incubated in Zanussi ZT 155 BR at 24 ± 1 °C. A photoperiod of 12 : 12 LD was maintained throughout the test. The control groups of embryos exposed to FETAX solution were tested four times and 2-phenoxyethanol-treated groups (50, 100, 300, 500, 700, 1000 mg/l) and clove oil-treated groups (1, 5, 10, 20, 30, 40 mg/l) were tested twice. Subsequently a series of three tests were performed using embryos produced by different parental pairs. All 2-phenoxyethanol and clove oil concentrations (stock solutions) were prepared immediately before changing the bath. The test medium was changed daily and dead embryos were removed. At the end of the experiment (after 96 h), surviving embryos were euthanized with carbon dioxide-saturated water and fixed in 3.0% formaldehyde. Head-tail length and an assessment of the morphological abnormalities of fixed embryos were determined under a dissecting microscope according to the Atlas of Abnormalities (Bantle et al. 1991).

The results of the FETAX assay were analysed using the statistical package Unistat® v. 5.1 (Unistat Ltd., Great Britain). Concentrations causing 50% lethality (96 h LC50) and concentrations eliciting malformations in 50% of surviving embryos (96 h EC50) were estimated using a probit model with 95% confidence. The homogeneity of variances prior to ANOVA was assessed using Levene's test. The teratogenic index (TI) was calculated as a ratio of 96 h LC50 and 96 h EC50 for tested samples. Differences among head-tail lengths were evaluated using one-way ANOVA and Fischer LSD test. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Controls for the FETAX assay met the criteria for the test acceptance – mortality and malformation rates were lower than 10% (ASTM 1998).

In 2-phenoxyethanol, statistically significant increases in mortality were observed at concentrations of 500, 700 and 1000 mg/l and increases in malformation at concentrations of 300, 500 and 700 mg/l (Table 1). 96 h LC 50 and 96 h EC50 were estimated at 588 mg/l and 348 mg/l, respectively. Severe oedema and axial abnormality were the most frequent malformations observed. The calculated teratogenic index (TI; ratio of 96 h LC50 and 96 h EC50) for 2-phenoxyethanol was 1.69. Statistically significant decreases in growth were found in embryos exposed to 2-phenoxyethanol at concentrations of 300, 500 and 700 mg/l compared to the control (Figure 1). The minimal concentration that inhibited growth (MCIG) was estimated to be 300 mg/l.

In clove oil, statistically significant increases in mortality were observed at concentrations of 20, 30 and 40 mg/l, and increases in malformation were seen at concentrations of 10, 20 and 30 mg/l (Table 2). 96 h LC50 and 96 h EC50 values were esti-

Table 1. Effects of 2-phenoxyethanol on the survival and malformation of *Xenopus laevis* embryos after 96 h FETAX (mean of three tests)

Concentration (mg/l)	Mortality (%)			Malformation (%)		
	mean	SD	*P*	mean	SD	*P*
50	5.25	2.55	> 0.05	1.25	2.12	> 0.05
100	4.63	5.66	> 0.05	2.50	3.04	> 0.05
300	6.14	2.83	> 0.05	40.00*	1.91	< 0.001
500	26.22*	2.83	< 0.001	83.00*	5.87	< 0.001
700	80.36*	5.66	< 0.001	86.50*	4.95	< 0.001
1000	100.00*	0.00	< 0.001			
Control	5.00	3.83		8.00	2.33	

*indicate statistically significant difference from the control (ANOVA plus LSD post-test)

mated to be 21.60 mg/l and 35.71 mg/l, respectively. Axial flexure, gut malformation, microphthalmia and oedema were the most frequent malformations observed. The TI for clove oil was 0.61. Statistically significant decreases in growth were found in embryos exposed to clove oil at concentrations of 20 and 30 mg/l compared to the control (Figure 2). The MCIG was estimated to be 20 mg/l.

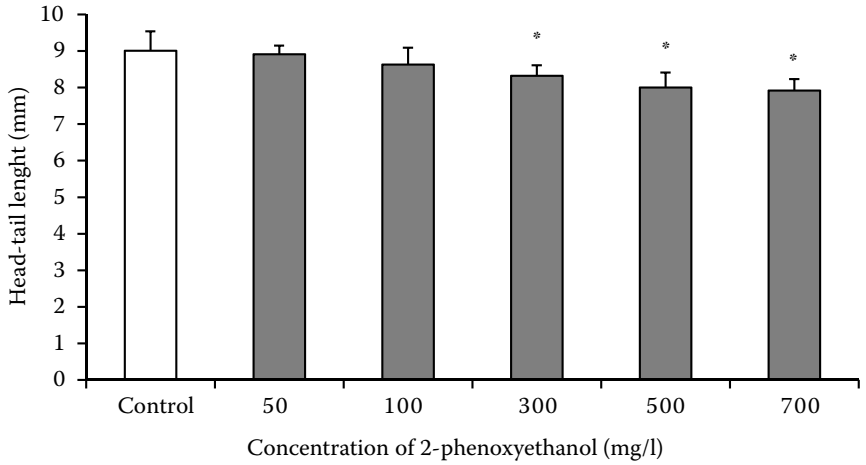

Figure 1. Effects of 2-phenoxyethanol on the growth of *Xenopus laevis* embryos in 96 h FETAX (mean of three tests)

*indicate a statistically significant difference from the control (ANOVA plus LSD post-test)

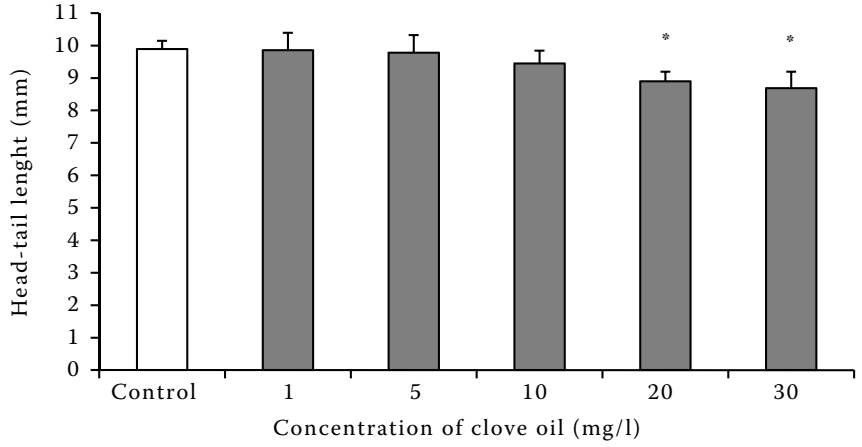

Figure 2. Effects of clove oil on the growth of *Xenopus laevis* embryos in 96 h FETAX (mean of three tests)

*indicate a statistically significant difference from the control (ANOVA plus LSD post-test)

Table 2. Effects of clove oil on the survival and malformation rate of *Xenopus laevis* embryos in 96 h FETAX (mean of three tests)

Concentration (mg/l)	Mortality (%)			Malformation (%)		
	mean	SD	*P*	mean	SD	*P*
1	2.36	1.54	> 0.05	0.50	1.21	> 0.05
5	12.50	2.83	> 0.05	11.25	6.24	> 0.05
10	26.75	6.36	> 0.05	20.41*	2.12	< 0.05
20	34.69*	3.54	> 0.05	24.62*	4.49	< 0.01
30	50.00*	3.87	< 0.001	64.57*	3.65	< 0.001
40	100.00*	0.00	< 0.001			
Control	6.50	5.72		2.25	1.38	

*indicate statistically significant difference from the control (ANOVA plus LSD post-test)

DISCUSSION

There exists only a small number of reports dealing with the toxic effects of xenobiotics on amphibians in different developmental stages (Pauli et al. 2000; Richards and Cole 2006; Gungordu et al. 2010) and according to our knowledge, there are no data on the embryotoxicity and teratogenity of 2-phenoxyethanol or clove oil.

According to the ASTM (1998), three separate criteria have been considered for the identification of teratogens using the FETAX assay: a TI higher than 1.5, the occurrence of severe malformations at concentrations near the 96 h LC50 and growth inhibition (the growth should be significantly affected at concentrations below 30% of the 96 h LC50). A substance presents a teratogenic risk when any one of the three criteria is met.

FETAX assay with 2-phenoxyethanol gained TI value 1.69, and the occurrence of malformations in 96 h LC50 reached over 80%, so our study met two criteria for estimating teratogenic hazard. The MCIG of 2-phenoxyethanol was estimated at 300 mg/l, the 30% value of 96 h LC50 was 177 mg/l, so this criterion for teratogenic risk was not met.

Acute toxicity of 2-phenoxyethanol for juvenile fish is higher than for embryonal stages of fish. Values 96 h LC50 for juvenile fish range from 188 mg/l (0.17 ml/l) (*Cyprinus carpio*) to 338 mg/l (*Danio rerio*) (Velisek and Svobodova 2004a). Embryotoxicity for 2-phenoxyethanol in zebrafish embryo according OECD No. 212 method yield 168 h LC50 values 486 mg/l (Macova et al. 2008). Compared to these results embryos of *X. laevis* are less sensitive to effects of 2-phenoxyethanol than *Danio rerio* embryos.

According to our knowledge, there is only one experimental paper reporting the effect of 2-phenoxyethanol on the adult amphibians, because 2-phenoxyethanol is not used in amphibian clinical practice. Pitkin and Pettyjohn (1992) described sinus bradycardia in the red-spotted newt (*Notophthalmus viridescens)* after the bath with 2-phenoxyethanol at concentration of 5000 mg/l. The generally recommended concentration of 2-phenoxyethanol for fish anaesthetic bath varies depending on genus from 167 mg/l to 442 mg/l (Barton and Helfrich 1981; Velisek and Svobodova 2004a,b) and anaesthetic effect differs according fish age, weight, gender and physical condition of water (Weyl et al. 1996). In our experiment 96 h EC50 was estimated at 348 mg/l, TI at 1.69 and MCIG at 300 mg/l, so we concluded, that direct affecting of amphibian embryos by 2-phenoxyethanol immersion bath solution at commonly used concentration would cause increase in malformation rate and it would also affect the larval growth.

The FETAX assay with clove oil gave a TI value of 0.61 and the occurrence of severe malformations at concentrations near the 96 h LC50 reached about 25% (this value was statistically significant). The MCIG of clove oil was estimated to be 20.0 mg/l while the 30% value of the 96 h LC50 was 6.5 mg/l. Our results did not meet the criteria for the identification of a teratogenic hazard and it is thus proven that clove oil does not represent a teratogenic risk for *Xenopus laevis* embryos.

The acute toxicity of clove oil for fish according to the OECD No. 203 method in juvenile common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) gave 96 h LC50 values of 18.1 mg/l, 14.1 mg/l and

18.8 mg/l (Velisek et al. 2005). The embryotoxicity of clove oil for zebrafish embryos according to the OECD No. 212 method yielded a 168 h LC50 value of 15.6 mg/l (Macova et al. 2008). The 96 h LC50 value for clove oil obtained using FETAX (21.6 mg/l) indicated that *X. laevis* embryos are less sensitive than fish embryos and juvenile fish.

Although the recommended concentrations of clove oil for the short-term immobilization of fish range from 40 up to 100 mg/l (Keene et al. 1998; Waterstrat 1999), convenient clove oil concentrations for immersion anaesthesia for amphibians differ according to the genus and size of the animal and range from 300 to 450 mg/l (Lafortune et al. 2001; Guenette et al. 2007; Mitchell et al. 2009). Interspecies differences in anaesthetic effects, induction time and analgesia in amphibians are attributed to differences in metabolisms of individual species, the route of administration and in the case of immersion or topical administration, to the rate of absorption of the drug across the skin (Mitchell et al. 2009). Clove oil is generally considered a safe anaesthetic agent in amphibians (Guenette et al. 2007), but Goulet et al. (2011) reported side effects in adult *X. laevis*, where a single 10 minute anaesthetic bath containing clove oil at a concentration of 350 mg/l caused renal distal tubular apoptosis, hepatic necrosis, formation of lung hyaline membranes and adipose tissue haemorrhages. Cutaneous necrosis after topical administration of clove oil at concentrations of 60 and 100 mg/l were reported by Ross et al. (2006). Mitchel et al. (2009) described other side effects of baths with clove oil at a concentration of 450 mg/l that included gastric prolapse and respiratory depression in leopard frogs (*Rana pipiens*) and bradycardia in leopard frogs and tiger salamander (*Ambystoma tigrinum*).

It is suggested that when used in fish, clove oil be administered in anaesthesia baths at concentrations between 25–100 mg/l, according to the genus and size of the fish (Hikasa et al. 1986; Walsh and Pease 2002; Hamackova et al. 2004). When we compare our experimental results (21.60 mg/l for 96 h LC50, 35.71 mg/l for 96 h EC50, 20.0 mg/l for MCIG) and the recommended concentrations for fish anaesthesia, it is clear that although the TI of clove oil is low (0.61), the effect on amphibian embryos of a clove oil immersion bath solution could result in higher mortality, malformation and growth inhibition.

In conclusion, our study showed a lower sensitivity of *X. laevis* embryos to both tested anaesthetic agents compared to fish embryos. Furthermore, our study proved that, in contrast to clove oil, 2-phenoxyethanol represents a teratogenic risk for amphibian embryos. An extended period of exposure could increase the toxicity of anaesthetics. Our results indicate that the risk to amphibian embryos is high when they are exposed to anaesthetic concentrations of 2-phenoxyethanol and clove oil for 96 hours.

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