Toxicity of PAHs and jelly protection of eggs in the Common frog *Rana temporaria*

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Abstract. Polycyclic Aromatic Hydrocarbons (PAHs) are damaging for aquatic organisms such as amphibians. In this study, toxicity of a mixture of three PAHs (naphthalene (2 rings), phenanthrene (3 rings) and pyrene (4 rings)) was tested on Common frog (*Rana temporaria*) embryos. The protective role of the jelly coat surrounding the eggs was studied by exposing embryos with and without jelly coat to PAHs dissolved in an aqueous solution without organic solvent. Results showed that the mixture of these three PAHs significantly increase embryonic mortality rate after a few hours of exposure. Embryos with jelly coat tend to suffer a lower mortality rate than embryos without jelly. The jelly surrounding eggs is filled by water of the breeding site, which can contain pollutants. Because jelly characteristics vary among species, sensitivity to environmental pollutants and levels of embryonic protection could be different among amphibian species.

Polycyclic aromatic hydrocarbons (PAHs) are transported through the atmosphere over long distances and are now widely distributed in the ecosphere (Aceves et al., 1993, Fernandez and Grimalt, 2003). PAHs are naturally produced by volcanic eruptions, forest fires and decomposing organic matter in sediment (Blumer, 1976). Human activities, especially urbanisation, as well as incomplete combustion of living or fossil organic matter, exhaust fumes from vehicles, gas- and oil-fired heating and steel mills, for example (Nykolaou et al., 1984; Van Schooten et al., 1997) are major sources of PAH emissions into the environment. Despite their hydrophobic nature, PAHs can be highly damaging to aquatic organisms (Bowling et al., 1983; Eisler, 1987; Landrum et al., 1987). Because of their unique physiology and habitat requirements, amphibians are often regarded as potentially more vulnerable to changes in their environment than many other vertebrates (Sparling et al., 2000, 2001).

Amphibians suffer from the spread of toxic substances in the environment (Carey and Bryant, 1995; see Blaustein et al., 2003 for a review). The toxicity of PAHs, and especially the effect of photo-induced toxicity on amphibian embryos and larvae, has been reported by several authors (Fernandez and L'Haridon, 1992; Hatch and Burton, 1998). Enzymatic activity within the organism can, with the influence of electrophilic metabolites (e.g. diol epoxide), transform PAHs to react with the nucleophilic sites of macromolecules such as DNA. This binding on nucleotides disturbs the three-dimensional DNA structure and can lead to single or double strand breaks in DNA (Moller and Wallin, 1998; Braithwaite et al., 1999).

To our knowledge, the toxic effects of PAH exposure on eggs, tadpoles or adults of *Rana temporaria* have not been reported in the literature. This species is widespread in Europe (from lowland plains to 2500 m above sea level in the Alps) and is often considered a biological indicator of pollution (Oldham et al., 1997; Johansson et al., 2001; de Wijer et al., 2003).

Tests for the potential effects of PAHs on aquatic organisms should consider the pollutant's solubility. Because of their very low solubility in water, PAH solutions are often prepared in an organic solvent before being diluted in water. However, organic solvents can themselves

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be toxic to eggs and tadpoles (Marquis et al., 2006). The aim of this work is to evaluate the toxicity of PAHs dissolved in water without an organic solvent and the protective effect of the jelly coat surrounding the embryos of *R. temporaria*.

A mixture of three PAHs was tested: naphthalene (2 rings), phenanthrene (3 rings) and pyrene (4 rings). One hundred mg of each crystallized PAH was added to 2 litres of dechlorinated tap water (dissolved oxygen: 95% at 21.2°C; pH = 6.91; conductivity = 302 μS/cm) and stirred for 24 hours at 20°C. The solution was then filtered to remove non-dissolved remnants. The exact concentration of the three compounds was determined by High Performance Liquid Chromatography (HPLC) analysis. Analyses were carried out with a Waters 996 HPLC equipped with a photodiode array detector operated at 240 nm. The column was a Varian Chronospher 5 (25 cm, 4.6 cm, 5 μm) with an acetonitrile/water (80%/20%) mixture as a mobile phase at 1 mL min^{-1}. The solution composition was 30 mg L^{-1} of naphthalene, 1.15 mg L^{-1} of phenanthrene and 128.8 μg L^{-1} of pyrene. These concentrations are close to the solubility of these three PAHs, i.e. 30 mg L^{-1}, 1.29 mg L^{-1} and 0.135 mg L^{-1} respectively, at 20°C.

Eggs were collected from 10 different clutches in the same population. Their embryonic development indicated that no more than 24 h had passed since fertilization. Two embryonic conditions were established: normal eggs (with jelly) and eggs without jelly (jelly was manually removed). All experiments were performed with 4 replicates of PAHs solution and 4 controls of dechlorinated tap water without added PAHs. Each replicate and control test was performed on a set of 20 eggs (2 from each of the 10 clutches). Eggs were exposed to 100 ml of solution in a 150 ml glass bottle that had been washed, rinsed with distilled water and dried at 200°C. Experiments were performed in a dark room at a constant temperature of 15°C. The number of dead embryos was recorded after 96 h of exposure. The effect of removed jelly, the effect of PAHs treatment and their interaction were tested with GLM procedure with binomial error distributions. Significance was assessed with likelihood ratio chi-square.

Eggs exposed to dissolved PAHs suffered a higher mortality rate than eggs in tap water ($\chi^2 = 57.84$, $P < 0.001$, df = 1) (fig. 1). The mortality rate of eggs exposed to PAHs was 0.488 ± 0.056 (mean $\pm$ SE) for normal eggs and 0.638 ± 0.054 for eggs without jelly. The mortality rate of eggs in tap water was 0.075 ± 0.042 for normal eggs and 0.10 ± 0.047 for eggs without jelly. When exposed to PAHs, mortality rate of eggs with jelly was not significantly different from eggs without jelly ($\chi^2 = 3.71$, $P = 0.054$, df = 1). The interaction of treatment and jelly was not significant ($\chi^2 = 0.120$, $P = 0.729$, df = 2).

Earlier studies have found that pollutants such as nitrogenous fertiliser (ammonium nitrate) and PCBs can affect embryonic survival, larval development and adult survival of the Common frog *R. temporaria* (Oldham et al., 1997; Gutleb et al., 2000; de Wijer et al., 2003). Further, experimental exposure of amphibians to pyrethroids has found differences in sensitivity among species and life stages (Cole and Casida, 1983; Berrill et al., 1998). Our study found that in *R. temporaria* exposure to three PAHs (naphthalene, phenanthrene and pyrene) dissolved in water without solvents is clearly toxic to eggs at the early stages of embryonic development. Dead embryos were close to stage 3 (Gosner, 1960), indicating that they died in the first hours of exposure. In future studies it would be interesting to test each PAH individually to estimate the synergistic effect between PAHs.

The thick jelly coat surrounding amphibian eggs is composed of glycoproteins, mucoproteins, carbohydrates and mucopolysaccharids (Salthe, 1963) and may be a protective agent against some chemicals (Berrill et al., 1997; Jung and Walker, 1997) and UV-b radiation (Grant and Licht, 1995; Marquis, 2006). To our knowledge, nothing is known about the role of egg jelly in protecting against PAHs. Our experiment addresses this knowledge deficit and found a strong trend of greater sensitivity to PAHs, expressed as higher mortality, in eggs stripped of their jelly. We can assume that at least a part of the PAHs may be retained in the different layers of the jelly coat. Under natural conditions, eggs would be exposed to pollutants when the jelly is filled with water from the breeding site when the eggs are laid. The jelly coat differs among amphibian species (e.g. in number of layers and composition) (Duellman and Trueb, 1994), which may mean that embryonic protection by the jelly coat, and therefore sensitivity to pollutants, varies among amphibian species.

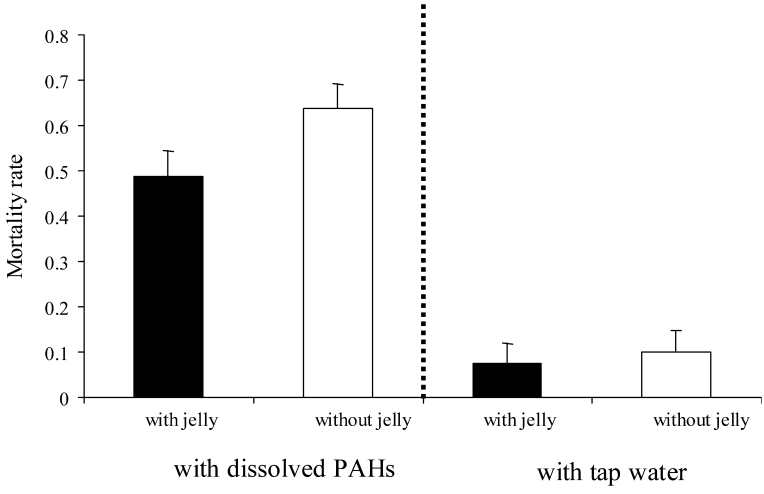

Figure 1. Mortality of *Rana temporaria* embryos exposed to a mixture of three PAHs dissolved in water. Mortality rate was the proportion of dead eggs after 96 h of exposure. Dark bars: normal eggs with jelly (mortality rate + 1 SE, $n = 80$). Open bars: eggs without jelly (mortality rate + 1 SE, $n = 80$).

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