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Susceptibility of Embryonic and Larval African Catfish (*Clarias gariepinus*) to Toxicants

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Fish Early Life Stage (ELS) tests have been proposed as faster and more cost-efficient bioassays for testing the potential toxicity of chemicals. However, the various embryonic and larval stages of fish differ in their susceptibility due to physiological and biochemical differences (McKim 1995).

The chorion or egg case of fish is permeable to water and small molecules during fertilization and hardens later on in the development. Consequently the permeability of the chorion decreases and the eggs become more resistant to external conditions (Blaxter 1969). The influence of water-hardening or the permeability of the chorion on the uptake of toxicants of chemicals by fish has been reported by a number of authors. The higher susceptibility of eggs before water-hardening has been noted in different fish species (Blaxter 1977; Middaugh et al. 1988). However, several authors reported that the change in permeability of the chorion after water-hardening had no effect on the uptake of toxicants (Van Leeuwen et al. 1985; Kevan and Dixon 1991). Early larval stages, especially newly hatched larvae, are considered the critical life stages for exposure to toxicants. The reason for this is that the embryos lose their' protective membrane and therefore are fully exposed to potential toxicants at hatching (Engel and Sunda 1979). In addition, after hatching, the larvae are not yet fully developed and the organ systems continue to differentiate. The development of organ systems of the larvae may alter their toxicological response after hatching (Weis and Weis 1989). It has been reported that fish larvae and juveniles are more sensitive to some toxicants than the embryos (Middaugh and Dean 1977; Kristensen 1991; Barry et al. 1995). However, other studies indicate that embryos are more sensitive than larvae (Ingersoll et al. 1990).

The results of this ELS study with the African catfish (*Clarias gariepinus*) are therefore expected to be dependent on the susceptibility of the developmental stages at which the toxicant exposure started. In the present study, the effects of heavy metals and organic compounds on the susceptibility of *C. gariepinus* exposed to toxicants (1) immediately after fertilization; (2) 3 hr after fertilization, when the chorion is assumed to have hardened; and (3) upon hatching, were examined.

MATERIALS AND METHODS

Eggs and sperm of *Clarias gariepinus* (Burchell, 1822) were obtained from adult fish bred under standardized conditions at the Laboratory of Aquatic Ecology (Katholieke Universiteit Leuven, Belgium). The eggs were stripped from the mature females 9 hr after induced ovulation at 26°C, and were fertilized dry with 1:10 extender diluted

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sperm; 1 min was allowed for fertilization. *C. gariepinus* were exposed to the toxicants at different developmental stages, in three experiments, as follows:

	Time at exposure	*Corresponding developmental stage*
1	0 - 1 hr after fertilization	2 - 4 cell stage (according to Volckaert et al. 1994)
2	3 hr after fertilization	Late blastula stage (according to Volckaert et al. 1994)
3	≥ 10 hr after hatching	Larval stage (according to Volckaert et al. 1994)

Fertilized eggs/larvae were randomly transferred into the 24-well test plates (1 organism/well containing 2 mL of the test solution). Each test comprises one control and five toxicant concentrations, with four replicates per concentration. The exposure conditions were based on the "standardized developmental" conditions for *C. gariepinus* described by Huisman and Richter (1987). Main test conditions were: temperature 27±1°C; photoperiod 0 hr Light - 24 hr Dark. Test plates were removed from the incubator daily for approximately one hr while embryos and larvae were examined for survival and development using a dissecting microscope (10-40x magnification). No food was provided during the test period.

The dilution water in all tests was aerated tap water (hardness: 200 mg/L as $CaCO_3$, dissolved oxygen concentration (DO): 7.3±0.1l mg/L; pH: 7.7±0.2; mean±s.d.). DO, pH and temperature of each concentration were measured daily. The test solution was renewed every 24 hr. Five chemicals were used as toxicants in this study: $K_2Cr_2O_7$(pro analysi, UCB,); $CdCl_2.2,5H_2O$ (pro analysi, UCB,); $CuSO_4.5H_2O$ (UCB); NaPCP (>98%, Merck) and malathion, (98% analytical standard, Riedel-de Haën).

The effects of toxicants on both the embryos and the endogenously feeding larvae were monitored daily throughout the 5-day exposure period. The number of dead eggs/embryos was recorded 24 hr after fertilization. This criterion was based on the total number of exposed eggs including the death of unfertilized eggs which can not be distinguished from toxicant-induced mortality of the embryos. Hatching was defined as rupture of the egg membrane. Fully as well as partially hatched larvae were counted. The total hatching percentage based on the number of surviving embryos, was calculated for each replicate, from 24 until 72 hr after fertilization. Survival and malformation of the larvae were observed and recorded daily. Larvae were considered dead when no heart beat was observed. The cumulative percentage of the dead larvae was obtained at the end of the tests. The effects of toxicants on the growth of *C. gariepinus* was evaluated by comparing the standard length (from the top of the premaxilla to the root of the caudal fin) in the treatments with the control at the end of the 5 days test period.

Prior to statistical analysis, data on hatching, survival and malformation were transformed using arsine square roots and tested for the homogeneity of the variance using Bartlett's test. The effects of toxicants on survival, hatching, morphological development and growth of *C. gariepinus* were evaluated using one-way analysis of variance following by Duncan's multiple comparison test (Sokal and Rohlf 1995). The significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

At the concentrations tested, only Cr and NaPCP caused detectable effect on embryo survival (Table 1). Mortality of eggs/embryos exposed at the 2- to 4-cell

stage was significantly higher than that of the control at ≥ 36 mg/L Cr. In contrast, no toxicant-related effect on survival was observed in embryos exposed at the late blastula stage. For NaPCP, in both tests initiated with the 2- to 4-cell and the late blastula stage eggs, the development of the embryos was delayed at > 1 mg/L and all embryos exposed to these concentrations died before hatching. The embryo mortality of *C. gariepinus* exposed to $K_2Cr_2O_7$ and NaPCP at the 2- to 4-cell stage may be attributed not only to Cr and PCP but also to K^+ and Na^+. Before water-hardening, the egg membrane has been reported to be selectively permeable to K^+ and Na^+, resulting in the combined effects of K^+ and Na^+ with thiocyanate on *Oncorhynchus mykiss* eggs (Kevan and Dixon 1991). A high influx of K^+ and Na^+, combined with Cr and PCP, respectively, may also be suspected to increase mortality of newly fertilized *eggs* of *C. gariepinus* exposed to high concentrations of $K_2Cr_2O_7$ and NaPCP.

Table 1. Egg/embryo mortality and hatching of *C. gariepinus* exposed to Cr and NaPCP at the 2- to 4-cell stage and the late blastula stage (Mean ± sd., n=4).

Cr (mg/L)	Egg/embryo mortality (%)		NaPCP (mg/L)	Egg/embryo mortality (%)	
	2- to 4-cell	late blastula		2- to 4-cell	late blastula
control	16.0 ± 0	18.8 ± 12.5	control	12.3 ± 0.4	2.1 ± 2.4
11	25.9 ± 16.4	6.1 ± 4.1	0.03	12.8 ± 0.4	7.3 ± 6.3
20	30.9 ± 15.3	10.5 ± 10.5	0.1	4.2 ± 5.9	4.1 ± 3.4
36	32.7 ± 0.9*	18.8 ± 17.2	0.3	18.4 ± 3.4	6.3 ± 7.2
64	38.9 ± 9.8*	8.1 ± 6.3	1	100.0 ± 0*	100.0 ± 0*
114	56.3 ± 2.9*	12.6 ± 4.8	3	100.0 ± 0*	100.0 ± 0*

*: significantly different from the control ($p \leq 0.05$).

Survival of larvae exposed to Cr at the 2- to 4-cell stage was adversely affected at ≥ 20 mg/L, while survival of the larvae exposed after hatching was affected at ≥ 64 mg/L, and that of larvae exposed at the late blastula stage was only affected at the highest concentration tested. The number of abnormality in the test with the 2- to 4-cell eggs was significantly higher than that of the control at ≥ 36 mg/L Cr. In contrast, no differences in morphological development were found between controls and treated larvae exposed at the late blastula and after hatching. The growth of larvae exposed at the 2- to 4-cell stage was significantly affected at the lowest concentrations tested, while growth of larvae exposed at the late blastula stage was significantly reduced at ≥ 36 mg/L Cr, and growth of larvae exposed after hatching was not affected (Fig. 1 A).

For Cd, exposure at the 2- to 4-cell stage revealed a significant decrease in larval survival at ≥ 0.5 mg/L, while exposure at the late blastula stage and after hatching only resulted in larval mortality at 5 mg/L. Deformation occurrence in the test starting with the 2- to 4-cell stage eggs, was significantly higher than that of the control at 2 0.5 mg/L Cd. In both tests using the late blastula and after-hatching stage, significant deformities occurred at ≥ 1.5 mg/L Cd. The growth of *C. gariepinus* was not affected by Cd for any of the three different developmental stages tested (Fig. 1 B).

Survival of larvae exposed to Cu in three tests was not affected up to the highest concentration tested. The percentage of abnormal larvae exposed at the 2- to 4-cell

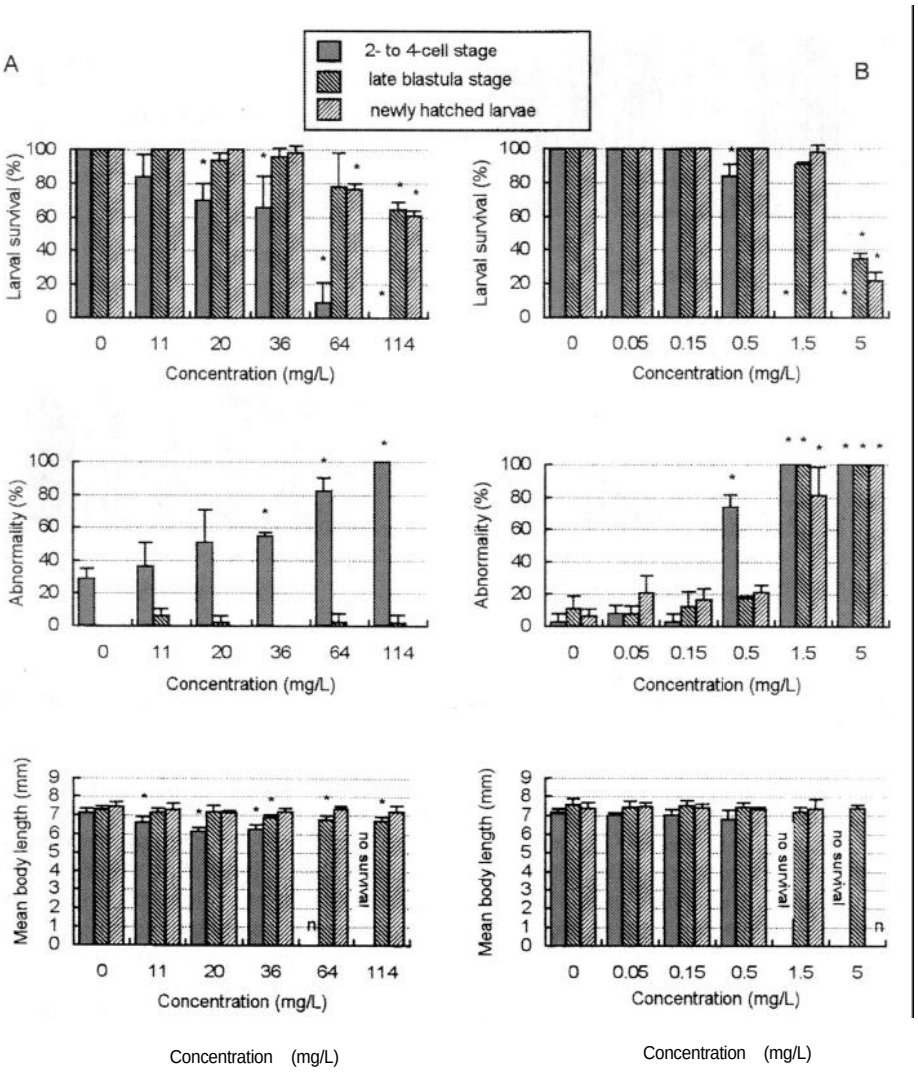

Figure 1. Larval survival, abnormality and growth of *C. gariepinus* exposed to Cr (A) and Cd (B) at different developmental stages. Data (n=4) are given as mean±sd. (*): $p<0.05$ compared to control; (n): not measured due to low survival (120%).

stage was significantly higher than the control at ≥ 0.6 mg/L Cu, while for larvae exposed at the late blastula stage and after hatching it was significantly different from the controls at ≥ 1.25 mg/L Cu. A significant difference in growth of larvae exposed at the 2- to 4-cell stage was observed at ≥ 0.6 mg/L Cu. No effect of Cu on the growth of larvae exposed at the late blastula and after hatching was observed (Fig. 2).

The results obtained show that the effect of Cr, Cd and Cu on larvae reduced when the organisms were exposed 3 hr after fertilization. This phenomenon is probably due to water-hardening of the chorion which acts as an effective barrier for heavy metals. In many fish species, most of the Cd absorbed by the eggs was located in the chorion (Rombough and Garside 1982; Meteyer et al. 1988). Similarly, Cu has

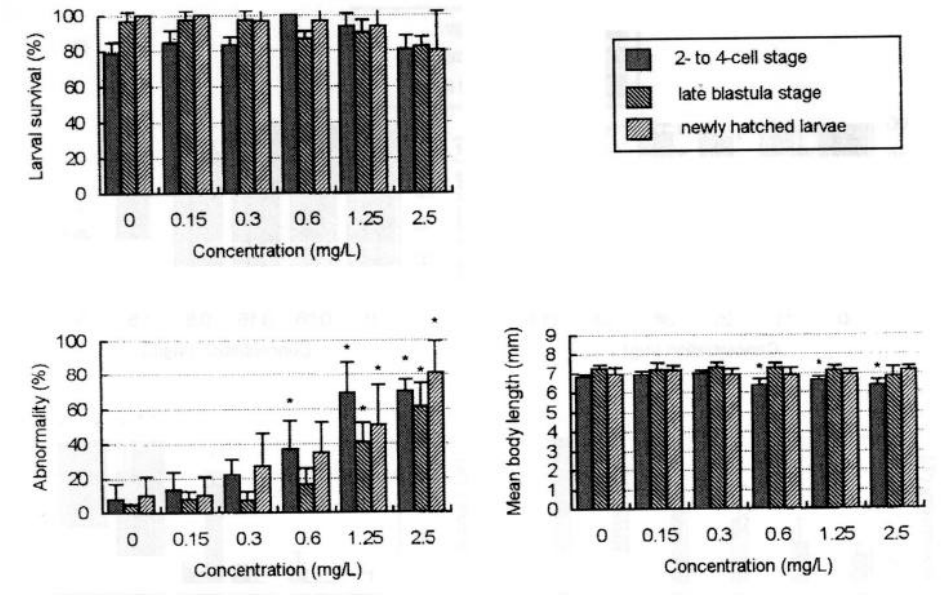

Figure 2. Larval survival, abnormality, and growth of *C. gariepinus* exposed to Cu at different developmental stages. Data (n=4) are given as mean±sd. (*): $p \leq 0.05$ compared to control.

been noted to accumulate mainly in the chorion of *Cyprinus carpio* (Stouthart et al. 1996). The relatively high resistance of *C. gariepinus* to Cr, exposed 3 hr after fertilization, may be related to both the protective capacity of the chorion and the ability of the perivitelline fluid to concentrate this metal. Stouthart et al. (1995) reported that Cr was neither found in the chorion nor in the embryo of *C. carpio.* The authors suggested that the association of Cr with perivitelline compounds (e.g., proteins, gelatins, collagens) was the reason of the reduction for the toxicity.

There was no effect of NaPCP on larval survival in the test initiated using the late blastula eggs and newly hatched larvae. Whereas the percentage of larvae surviving, decreased significantly at 0.3 mg/L NaPCP in the tests starting with the 2- to 4-cells stage eggs. Larvae exposed to 0.3 mg/L NaPCP at the 2- to 4-cell and late blastula stage exhibited severe malformations. Morphological development of larvae exposed after hatching, on the other hand, was not affected by any of the NaPCP concentrations tested. The growth of larvae exposed at the 2- to 4-cells stage was significantly reduced in all NaPCP concentrations tested. Whereas, growth of fish exposed at the late blastula stage and after hatching was inhibited at 0.3 mg/L and at ≥ 0.1 mg/L NaPCP, respectively (Fig. 3A). Statistical analysis revealed a significant decrease in survival at 5 mg/L for the larvae in all three experiments with malathion. The number of abnormal larvae in the tests with the 2- to 4-cell stage eggs and newly hatched larvae was significantly higher than that of the control at ≥ 1.25 and ≥ 2.5 mg/L malathion, respectively. Whereas no toxicant-deformity relationship was found in the test started with the late blastula stage eggs. Growth of larvae exposed to malathion at the 2- to 4-cell stage was strongly

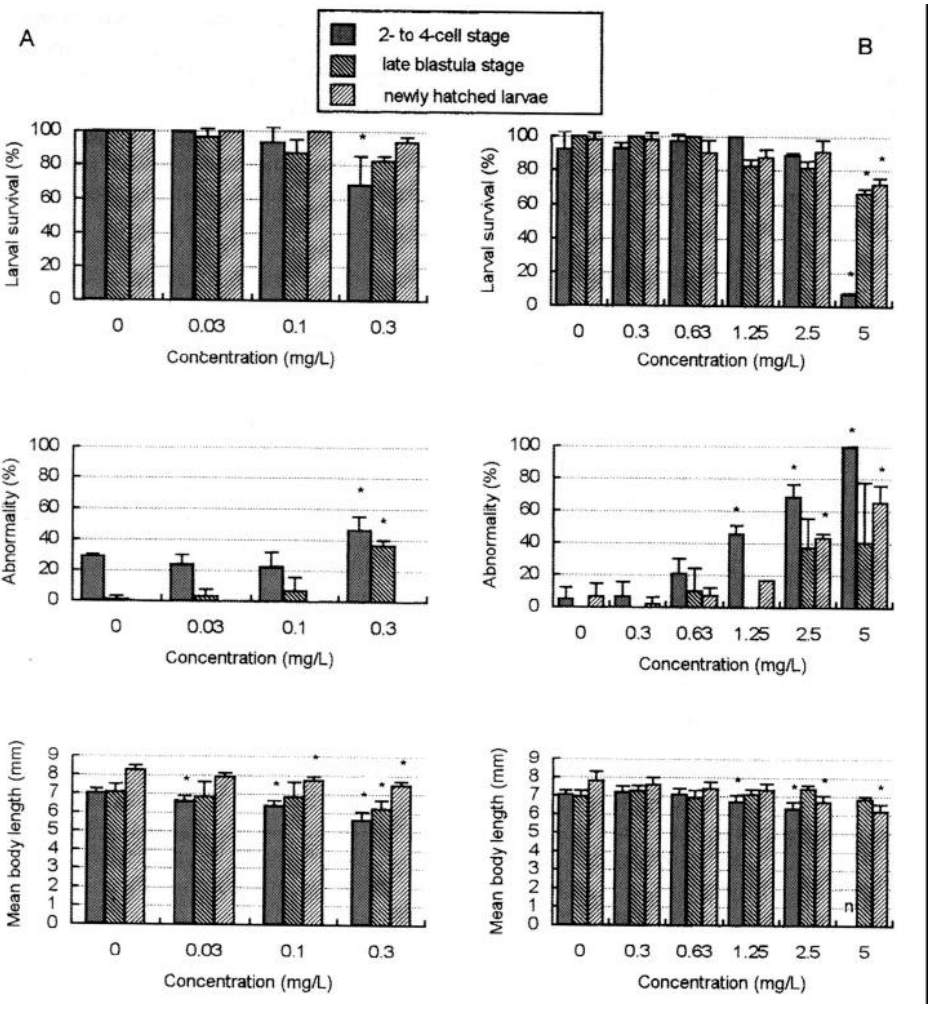

Figure 3. Larval survival, abnormality and growth of *C. gariepinus* exposed to NaPCP (A) and malathion (B) at different developmental stages. Data (n=4) are given as mean±sd. (*): $p \leq 0.05$ compared to control; (n): not measured due to low survival (≤20%).

inhibited, followed by larvae exposed after hatching. Growth was, however, not affected by any malathion concentrations in late blastula stage tests (Fig. 38).

The toxicity of PCP and malathion on *C. gariepinus* exposed at different embryonic stages, is likely to be linked to the physico-chemical properties of these chemicals. The amount of organic compounds that penetrate fish eggs is controlled by their lipid solubility (Helmstetter and Alden III 1995). These authors have demonstrated that PCP can easily penetrate the chorion (after water-hardening) due to its physico-chemical characteristics and have consequently calculated a permeability factor (PF) of 70%. Thus, the slight difference in toxicity of PCP on *C. gariepinus* exposed at the

2- to 4-cell and the late blastula stage, could be due to the penetration power of this compound irrespective of the membrane hardening. Taking in account the correlation between the PF and the *n*-octanol-water partition coefficient (K_{ow}): PF = 11.1 $\log K_{ow}$ + 3.97 (Helmstetter and Alden III 1995), and a K_{ow} of malathion of 2.89, an approximative permeability factor of 30% for malathion was estimated. This may suggest that a small proportion of malathion can penetrate the chorion after water-hardening, resulting in less toxicity of this compound on *C. gariepinus* exposed 3 hr after fertilization.

Overall, the results obtained from the present study show that the susceptibility of *C. gariepinus* is dependent on the developmental stage at the time of exposure. For all toxicants tested, exposure at the 2- to 4-cell stage resulted in a greater effect in comparison with exposure at later stages. Especially for Cr, deformations were only observed when the tests started with the 2- to 4-cell stage eggs. The period from fertilization until the 4 cell stage of *C. gariepinus* includes the development of the chorion prior to the water-hardening process of the eggs. Exposure initiated at this moment enables toxicants to pass easily through the membrane and induces the effects e.g. mortality, malformations or other developmental effects, expressed at later stages. The uptake of toxicants before and after water-hardening has been discussed by numerous researchers. Water-hardening has also been reported not to induce great differences in toxicity to *O. mykiss* for manganese ethylenebisdithiocarbamate, PCP and Cd (Van Leeuwen et al. 1985). Kevan and Dixon (1991) found that rainbow trout eggs were more sensitive to thiocyanate after water-hardening. In contrast, higher susceptibility of eggs before water-hardening has also been demonstrated. Blaxter (1977) noted that teratogenicity only showed up when *Clupea harengus* eggs were exposed to Cu before water-hardening. *Salvelinus fontinalis* exposed before water-hardening also appeared to be the most sensitive to acid stress (Hurley et al. 1989). Middaugh et al. (1988) observed that in *Menidia beryllina* exposed to naphthalene, the 2- to 4-cell stage exhibited a higher teratogenicity than the blastula stage. This was explained by the high chorionic permeability of the eggs and by the effect of the longer exposure time. In *C. gariepinus,* since the difference of exposure time between the 2- to 4-cell stage and the late blastula stage is only 3 hr, the higher sensitivity of the former stage probably points to the high permeability of the chorion rather than the effect of a longer exposure time. The results of the present study also show that newly hatched larvae of *C. gariepinus* were less sensitive than the 2- to 4-cell stage embryos, although they were equally or more sensitive than the late blastula *stage* embryos. Some investigators have presented similar findings when comparing the sensitivity of newly hatched larvae with embryos exposed after water-hardening. *Pimephales promelas* exposed as embryos to four different chemicals did not show greater sensitivity than larvae exposed after hatching (Norberg-King 1989). Embryos of *Melanotaenia fluviatilis* were found to be less sensitive to esfenvalerate compared to newly hatched larvae (Barry et al. 1995).

In summary, *C. gariepinus* exposed at the 2- to 4-cell stage were more sensitive than those exposed at the late blastula stage and after hatching. Although the determination of the actual amounts of toxicants that penetrate the chorion before and after water-hardening still remain to be investigated, the difference in sensitivity of *C. gariepinus* exposed at the 2- to 4-cell stage and at the late blastula stage suggests that after the hardening of the chorion, the fish embryo was better protected from the toxicants. Therefore, in order to gain maximal sensitivity, the exposure to toxicants should start as soon as possible after fertilization.

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