Effects of Metazachlor on Vitellogenin Induction in Zebrafish (*Danio rerio*)

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Abstract

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The influence of metazachlor on vitellogenesis in juvenile (20 days old) zebrafish (*Danio rerio*) was investigated after ambient water exposure to concentrations of 0.1, 1.0 and 5.0 mg l^{-1} of the chloroacetanilide herbicide Butisan 400 SC containing approximately 35.6% (w/w) metazachlor. After 20 days of exposure, vitellogenin concentrations in whole-body homogenates of the fish were measured by direct sandwich ELISA. The results were compared to vitellogenin concentrations in fish from both negative (no exposure) and positive (exposed to natural oestrogen 17β-oestradiol) control groups. Exposure to Butisan 400 SC at a concentration of 5.0 mg l^{-1} induced vitellogenin synthesis significantly compared to the control fish ($p < 0.05$). The oestrogenic effect of 17β-oestradiol was confirmed.

Butisan 400 SC; Chloroacetanilide herbicides; Endocrine disruptors; 17β-oestradiol; Fish

Many chemicals in the environment have been shown, or are suspected, to have endocrine disrupting potential that may adversely affect the reproductive capabilities of fish (Shioda and Wakabayashi 2000). Endocrine disrupting chemicals (EDCs) are, broadly defined, natural and man-made agents present in the environment that interfere with normal endocrine function (Arcand-Hoy and Benson 1998). The best characterised group of EDCs are environmental oestrogens or xenoestrogens. These compounds can mimic the effects of natural endogenous oestrogens (Colborn et al. 1993). Effects of xenoestrogens on fish include stimulating synthesis of vitellogenin, the yolk precursor protein, in males and juveniles (Jobling et al. 1996; Harries et al. 1997; Flammarion et al. 2000); affecting the development of the gonads (Lee et al. 2003); inducing hermaphroditism, as evidenced by the presence of both testicular and ovarian tissues, (Gimeno et al. 1998; Jobling et al. 1998; Nolan et al. 2001); and reducing plasma testosterone concentrations (Folmar et al. 1996).

An important biomarker used to detect fish exposure to oestrogenic endocrine disruptors is the occurrence of phospholipoglycoprotein vitellogenin in blood plasma, liver, or whole-body homogenates of juvenile and male fish (Sumpter and Jobling 1995). Vitellogenin synthesis in fish liver is oestrogen-dependent and, hence, is normally limited to reproductive females. Vitellogenin released from the liver is transported by the blood and taken up by developing oocytes, where it is cleaved into major constituents of yolk, lipovitellin and phosvitin (Mommsen and Walsh 1988). Male and juvenile fish possess the vitellogenin gene, which remains inactive under normal conditions, but can be activated by exposure to exogenous oestrogens or xenoestrogens. Vitellogenin concentrations in male and juvenile fish can therefore provide a biomarker of exposure to environmental oestrogens (Kime 1999).

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Herbicides are intended to be uniquely toxic to plants. Although plant cells differ from animal cells, it is difficult to produce herbicides that are not toxic to animals (Saunders and Harper 1994). One of the most widely and extensively used chloracetanilide herbicides is metazachlor. It is used for pre-emergence and early post-emergence control of annual grasses and broad-leaved weeds in a broad spectrum of crops including maize, soybeans, peanuts, sugarcane, cotton, tobacco, oilseed rape and transplanted brassicas (Roberts 1998). Based on the results of tests of acute toxicity, metazachlor was classified as toxic to trout and moderately toxic to carp (Gangolli 1999). Since Keith (1997) included alachlor and metolachlor, the other members of the chloroacetanilide herbicide family, in the list of EDCs, the endocrine disrupting potential of metazachlor could be expected.

In the present study, the xenoestrogenic potential of metazachlor was evaluated by measuring vitellogenin concentrations in whole-body homogenates of control and experimental juvenile zebrafish (*Danio rerio*).

Materials and Methods

Maintenance of fish and experimental protocol

The experiment was carried out as a semistatic test, using juvenile zebrafish. After an adaptation period, juvenile zebrafish, 20 days post hatching (dph), were placed in 10 glass tanks (15 l) each containing 10 l of water, and about 30 fish per tank. Fish were divided into 5 groups: three metazachlor treated, one 17β-oestradiol treated (positive control) and one negative control. Experimental fish were exposed to the preparation Butisan 400 SC containing 35.6% (w/w) metazachlor in concentrations of 0.1, 1.0, and 5.0 $mg \cdot l^{-1}$ of tank water. For the positive control group, a stock solution of 17β-oestradiol was prepared by diluting 0.1 mg of 17β-oestradiol (97%, Sigma-Aldrich) in 100 ml of 96% ethanol. One ml of this solution was added to the water in the tank to give a concentration of 100 $ng \cdot l^{-1}$. The final concentration of ethanol in the tank was not greater than 0.1 $ml \cdot l^{-1}$. The fish from the negative control group were not exposed to metazachlor or 17β-oestradiol. The baths in all tanks were changed 3 times a week. The test was replicated for each treatment, i.e. approximately 60 specimens per treatment group.

Basic physical and chemical parameters of water were measured as follows: pH 7.4-7.8, $ANC_{4.5}$ (alkalinity) 3.7-3.8 $mmol \cdot l^{-1}$, COD_{Mn} 1.6-1.9 $mg \cdot l^{-1}$, BOD_5 1.02-1.36 $mg \cdot l^{-1}$, NH_4^+ 0 $mg \cdot l^{-1}$, NO_3^- 24.5-31.4 $mg \cdot l^{-1}$, NO_2^- 0 $mg \cdot l^{-1}$, Cl^- 17.7-18.5 $mg \cdot l^{-1}$. The water was continuously aerated, at an oxygen saturation above 60%, and maintained at 25±1.5 °C.

A photoperiod of 12:12LD was maintained throughout the test. Fish were fed 3 times a day at approximately 2% of their body weight per day. During the first week fish were fed with freshly hatched brine shrimps (*Artemia salina*, Sanders Brine Shrimp Company Inc.). From the second week until the end of the test, fish were fed decapsulated brine shrimp eggs (Discus CZ).

Homogenisation of zebrafish and vitellogenin analysis

The concentration of vitellogenin was measured in whole-body homogenates of fish using the zebrafish vitellogenin EIA kit (Biosense Laboratories AS, Norway). At 40 dph, 12 randomly selected fish from each of the 5 groups (i.e. 6 fish from each tank) were used for vitellogenin measurement. The fish were killed in carbon dioxide-saturated water, weighed, and stored at -80 °C for later analysis of vitellogenin. The fish were homogenized in homogenate buffer (50 mM Tris-HCl pH 8.0, 0.02% aprotinin, 0.1 mM phenylmethanesulfonyl fluoride) at a ratio of 1:2 (w/v). After centrifugation (14 585 *g*, 60 min, 4 °C), the supernatants were removed and analysed for vitellogenin by direct sandwich ELISA according to instructions provided by the manufacturer. For detailed description of parameters of the detection method you can also see Nilsen et al. (2004). The fish remaining in the tanks were used for the subsequently performed test.

Statistical analysis

The data were statistically processed using standard descriptive statistics, Kruskal–Wallis test, and a subsequent multiple comparison of the mean ranks.

Results

The vitellogenin concentrations in whole-body homogenates of juvenile zebrafish exposed to metazachlor-based herbicide Butisan 400 SC and 17β-oestradiol were measured at 40 dph. In the whole-body homogenates of fish from the negative control group, the median vitellogenin concentration was 616 $ng \cdot ml^{-1}$. Medians of vitellogenin concentrations in whole-body homogenates of fish exposed to Butisan 400 SC at concentrations of 0.1, 1.0 and 5.0 $mg \cdot l^{-1}$ were 916; 556 and 1254 $ng \cdot ml^{-1}$, respectively. The median value of

vitellogenin concentration in whole-body homogenates of fish exposed to 17β-oestradiol at a concentration of 100 ng·l^{-1} was 10 562 ng·ml^{-1}. (Fig. 1 and Fig. 2).

Among the groups tested, significant differences were found (Kruskal–Wallis test: Q = 33.68; N = 60; $p < 0.001$). Multiple comparison of the mean ranks confirmed a significant ($p < 0.05$) increase in vitellogenin concentration in fish exposed to 17β-oestradiol compared to fish from the control group and groups exposed to 0.1 mg·l^{-1} or 1.0 mg·l^{-1} of the preparation tested (see Fig. 1).

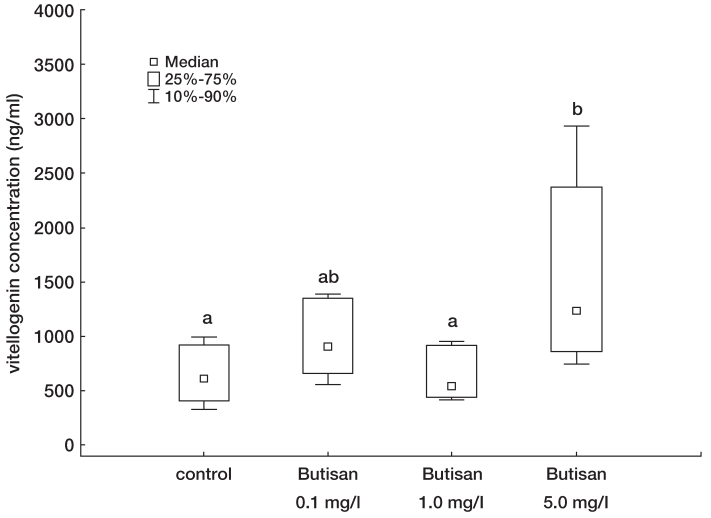

Fig. 1. Vitellogenin concentrations in whole-body homogenates of juvenile zebrafish (*Danio rerio*) after exposure. Groups with different alphabetical letters differ significantly at $p < 0.05$.

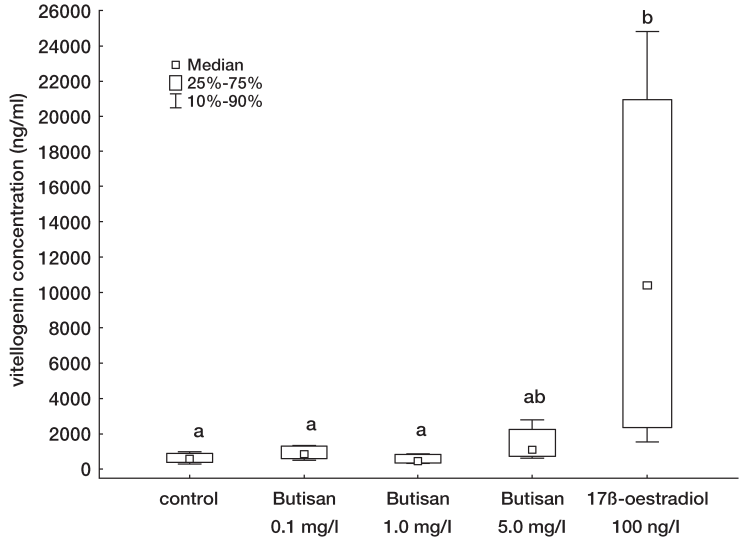

Fig. 2. Vitellogenin concentrations in whole-body homogenates of juvenile zebrafish (*Danio rerio*) after exposure. Groups with different alphabetical letters differ significantly at $p < 0.05$. The 17β-oestradiol exposed group was excluded from the statistical analysis.

Due to markedly higher vitellogenin concentrations in fish from the positive control group, this group was excluded from subsequent statistical analyses. Analysis of the remaining 4 groups showed significant differences (Kruskall–Wallis test: Q = 12.57, N =48; $p = 0.006$). In a multiple comparison of the mean ranks, significantly ($p < 0.05$) increasing levels of vitellogenin concentration in whole-body homogenates of fish exposed to 5.0 mg·l^{-1} of Butisan 400 SC compared to fish from both the control group and the group exposed to 1.0 mg·l^{-1} of Butisan 400 SC were observed (Fig. 2).

Discussion

Environmental pollution caused by pesticides has been increasing, due to their extensive use in agriculture. Dispersal of pesticides from cultivated fields to surrounding surface waters occurs through runoff or drainage of rain or irrigation water (Larson et al. 1995). Varying levels of herbicides in the aquatic environment are common. Chloroacetanilide herbicides frequently detected in water samples include alachlor, acetochlor, metolachlor, however metazachlor was detected in the Rhine River and other large rivers in Germany at concentrations exceeding 1 µg l^{-1} (Haberer 1992).

Chloroacetanilide herbicides are among suspected environmental endocrine disruptors. Previous studies have established the ability of chloroacetanilide herbicides to bind to human recombinant oestrogen receptors (hERs) *in vitro* (Scippo et al. 2004) and to possess a weak oestrogenic effect (Klotz et al. 1996). Another i*n vitro* study has demonstrated hER_{α}-antagonistic and hAR- (human androgen receptor) antagonistic actions of alachlor (Kojima et al. 2004). Metolachlor induced activity of a key steroidogenetic enzyme, aromatase (i.e. CYP19A1), changing endogenous testosterone to 17β-oestradiol in the human choriocarcinoma JEG-3 cell-line (Laville et al. 2006). On the other hand, the spectrum of *in vivo* studies dealing with the issue of endocrine disruption caused by this group of herbicides is limited. Chang et al. (2005) reported no induction of vitellogenin mRNA expression in liver of male carp (*Cyprinus carpio*) exposed to alachlor at concentrations of 10, 25, 50, and 100 µg·l^{-1} compared to control fish.

In the present study, Butisan 400 SC, a chloroacetanilide herbicide containing metazachlor as the active substance, was used to assess the oestrogenic potential of chloroacetanilide herbicides in zebrafish. Our results suggested that the exposure of juvenile zebrafish to metazachlor could induce oestrogenic responses. This is the first evidence of oestrogenic effects of chloroacetanilide herbicides demonstrated in fish *in vivo*. However, the oestrogenic response in our study was observed only in fish from the group treated with 5.0 mg·l^{-1} of preparation, while in lower concentrations (i.e. 0.1 and 1.0 mg·l^{-1} of Butisan 400 SC) the effect was not evident. Responsiveness of zebrafish to oestrogens or xenoestrogens was confirmed, since the exposure of fish to the natural oestrogen 17β-oestradiol in the concentration of 100 ng·l^{-1} elicited vitellogenin production.

The world production and use of chloroacetanilide herbicides in agriculture are still high, and data dealing with the potential for these substances to act as EDCs are limited. Further research, with studies on other vertebrate species, is therefore necessary.

Vliv metazachloru na indukci tvorby vitellogeninu u dania pruhovaného (*Danio rerio*)

Vliv metazachloru na proces vitellogeneze u juvenilních danií pruhovaných *(Danio rerio)* ve věku 20 dnů byl zkoumán po expozici ryb chloroacetanilidovým herbicidem Butisan 400 SC obsahujícím přibližně 36.5% hmotnosti metazachloru. Dania byla exponována přípravkem přidaným do vody v koncentracích 0.1; 1 a 5 mg/l. Po 20 dnech expozice byly pomocí přímé sendvičové ELISY změřeny koncentrace vitellogeninu v homogenátech těl exponovaných ryb a výsledky byly srovnány s koncentracemi vitellogeninu v rybách

z kontrolní skupiny a ze skupiny pozitivní kontroly exponované přirozeným estrogenem 17β-estradiolem. Expozice ryb přípravkem Butisan 400 SC o koncentraci 5 mg/l vedla k signifikantní indukci procesu vitellogeneze u exponovaných ryb ($p < 0.05$). Potvrzen byl také estrogenní účinek17β-estradiolu.

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