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A synchronized amphibian metamorphosis assay as an improved tool to detect thyroid hormone disturbance by endocrine disruptors and apolar sediment extracts

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Abstract

Amphibian metamorphosis assays are used to evaluate potential effects of endocrine disrupting compounds on the thyroid hormone axis. In this study, *Xenopus laevis* tadpoles are kept in a solution of 0.2% thiourea (TU) to arrest and synchronise them in their development.

The advantage of this synchronized amphibian metamorphosis assays is that synchronised tadpoles are available at any time to start metamorphosis experiments, and experimental groups are much more homogenous at the start of experimental exposure compared with groups selected from an untreated pool of animals. The water volume per animal was kept constant throughout the experimental period to overcome the influence of declining numbers of animals per aquarium due to metamorphosis and mortality on the density dependent development of the remaining tadpoles. Clophen A50 (a technical PCB mixture), the single congener 3,3′,4,4′-tetrachlorobiphenyl (PCB 77) and apolar sediment extracts that were previously tested positive in the T-Screen, an *in vitro* proliferation assay for thyroid hormone disruption, were tested in the Synchronized Amphibian Metamorphosis Assay. Endpoints studied were mortality, malformations, body weight, and percentage of metamorphosed froglets at the end of the 60-day experimental period, percentage of tadpoles in different developmental stages, and developmental stage-dependent awarded penalty points. Dietary exposure to Clophen A50 (0.2–50 mg/kg food) resulted in a significant increased percentage of tadpoles that did not pass metamorphosis at concentrations higher than 2 mg/kg food. Time until metamorphosis in those animals that were able to metamorphose after the 60-days experimental period was significantly decreased. Dietary exposure to PCB 77, a congener that can be readily metabolised, did not result in significant effects in any exposure group (2–500 μg/kg food). Apolar sediment extracts from two of the three sites that are contaminated with a wide variety of chemicals significantly decreased the percentage of metamorphosed animals and significantly increased the number of tadpoles that remained in early and late metamorphic stages. These effects already occurred when the extracts where diluted more than 1000 times (on an organic carbon base) compared to environmental concentrations. The rank of potency was comparable to results obtained with the T-screen. This suggests the presence of thyroid hormone disrupting compounds in the aquatic environment and possible effects of such compounds on animal development in the wild.

Keywords: In vivo functional assay; Dietary exposure; PCBs; Thiourea; Xenopus; Tadpole

1. Introduction

The presence of chemicals in the environment with the potential to disrupt endocrine systems, endocrine disrupting compounds (EDCs), has become a major focus of research

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during the last years, as both wildlife and humans may be affected (Tyler et al., 1998; Vos et al., 2000). Whereas a lot of studies deal with (anti)estrogenic effects and related test systems (Sumpter, 1998; Legler et al., 2000), far less effort has been put in the identification of thyroid hormone disrupting compounds (Brouwer et al., 1998). It has been suggested that thyroid hormone disrupting compounds may contribute to the observed global decline of amphibian species (Blaustein, 1994; Pechmann and Wilbur, 1994).

Thyroid hormones, 3,3′5-triiodo-L-thyronine (T_3) and 3,3′,5,5′-tetraiodo-L-thyroxine (T_4), have a wide range of biological effects in vertebrates both in fetal and prenatal development (Porterfield and Hendrich, 1993; Tata, 1999; Power et al., 2001). Thyroid hormones play an important role in many vertebrate classes such as in the development of sexual organs and the central nervous system in mammals (Bernal and Nunez, 1995), or in the metamorphosis of amphibians (Gudernatsch, 1912; Kanamori and Brown, 1996). Therefore, the necessity to pay more attention to compounds that possibly interfere with this hormone system has been clearly formulated (EDSTAC, 1998; DeVito et al., 1999; Colborn, 2002). A number of *in vitro* tests already exist such as the T-Screen (Gutleb et al., 2005), thyroid peroxidase assay (DeVito et al., 1999; Baker, 2001), deiodinase assays (DeVito et al., 1999; Baker, 2001), and T_4-TTR-competition binding studies (Lans et al., 1993; Janosek et al., 2006). However, these assays only focus on single aspects of thyroid hormone dependent metamorphosis and like all *in vitro* assays do not take complex feedback mechanisms and toxicokinetics into account. Therefore, there is a need for functional *in vivo* screening assays for thyroid active compounds (Brouwer et al., 1998; Fort et al., 1999, 2000; Herkovits et al., 2002).

A process where thyroid hormone plays a very specific and crucial role is completion of amphibian metamorphosis (Bray and Sicard, 1982; Galton, 1992). The first stage of tadpole development is characterized by development of larval structures and body growth (early metamorphosis, up to NF stage 55 (Nieuwkoop and Faber, 2005; further referred to as NF), followed by further growth and development of extremities (metamorphosis, up to NF stage 60). This metamorphic development up to NF stage 60 is dependent on a surge of thyroid hormone. The following stages up to completion of metamorphosis are characterised by remodelling of the body shape such as completion of extremities, tail resorption and decreasing thyroid hormone levels (late metamorphosis, up to NF stage 66) (Etkin, 1932; Nieuwkoop and Faber, 2005).

Test systems based on thyroid hormone dependent processes using tadpoles have been suggested as useful tools to study the thyroid hormone disrupting effects of single compounds, mixtures or environmental extracts (EDSTAC, 1998; Opitz et al., 2005). Examples are 30-day limb development, 14-day tail resorption studies or a 28-day Xenopus Metamorphosis Assay (Fort et al., 1999, 2000; Christensen et al., 2005; Opitz et al., 2005).

However, until now, *in vivo* assays with tadpoles require a relative large number of animals (Gutleb et al., 2000; Goleman et al., 2002). Furthermore, the rate of tadpole development differs greatly, even between individuals from the same clutch. Also the most careful selection procedure cannot completely overcome the problem that slight differences in individual development at the start of such experiments will introduce a large variation in the final outcome. To overcome this intrinsic problem of introducing variation during the process of animal selection and to make the planning of experiments more convenient by having a constant pool of animals in the right developmental stage, we designed the Synchronized Amphibian Metamorphosis Assay. For the Synchronized Amphibian Metamorphosis Assay early metamorphic *Xenopus laevis* tadpoles are reversibly arrested in their development in NF stage 54. This is done by inhibiting thyreoperoxidase by exposure to 0.2% thiourea (TU) in NF stage 50–51 (Gorbman and Evans, 1943; Gordon et al., 1943, 1945; Capen, 1994). Animals can be kept in this stage until they are required for an experiment. Exposure to the compounds starts 7 days before allowing them to develop further by removing the TU. Functional endpoints of the Synchronized Amphibian Metamorphosis Assay are mortality, rate of malformations, body weight, duration and percentage of completed metamorphosis at the end of the 60-day experimental period, percentage of tadpoles in different developmental stages at the end of the experimental period, and developmental stage dependent awarded penalty points. As tadpole growth is density dependent (Werner, 1986; Scott, 1994) it is required that the water volume is corrected for the number of remaining tadpoles per experimental group when tadpoles are removed because they completed metamorphosis or died.

The thus newly developed protocol for the Synchronized Metamorphosis Assay was applied to test the effects of the known thyroid hormone disrupting compounds Clophen A50 (a technical PCB mixture), 3,3′,4,4′-tetrachlorobiphenyl (PCB 77), and apolar sediment extracts, that altered thyroid hormone dependent cell growth *in vitro* in the T-Screen (Gutleb et al., 2005). Dietary exposure was chosen, as this is more realistic for the field situation for many lipophilic compounds with low concentrations in the water phase and higher concentrations in feed (Patyna et al., 1999).

2. Animals, materials and methods

2.1. Chemicals

The technical PCB mixture Clophen A50 was a kind gift of Jan Boon (NIOZ, Den Burg, The Netherlands). PCB 77 (99% purity) was obtained from Promochem (Wesel, Germany). All other chemicals used throughout the experiments were of analytical grade and were obtained from Merck (Darmstadt, Germany).