In vivo testing of PBDE flame retardants using the *Xenopus* tail regression model

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Introduction

Polybrominated diphenyl ethers (PBDEs) have been quantified in most environmental compartments, having been identified in sediment, invertebrates, fish, marine mammals and human breast milk (1). Most studies investigating temporal trends indicate increasing levels of BDE-47, 99 and 100 since the 1970s, and in North America, these trends indicate rapid increases since the 1990s with no tendency to level off or decline (1). Short term toxicity testing has demonstrated that these compounds are not acutely toxic. However, interest in sub-acute thyroidogenic toxicity has been increasing since some congeners such as BDE-47, 99 and 100 are known to possess thyroid hormone-like, bromo-substitution patterns and hyroxylated metabolites of these compounds can compete with T4 for binding to the thyroid hormone transport protein transthyretin (2).

The thyroidogenic potency of a commercial PBDE mixture (DE-71) and PBDE congeners BDE-47, BDE-99 (major constituents of DE-71) was assessed using a modified version of the African clawed frog tail regression model described by Fort et al. (3). Tail resorption is one of the final changes in tadpole metamorphosis and is under the control of thyroid hormones (THs). Disruption to the circulating concentration of THs can influence the rate at which the tail regresses and hence can be used to indirectly identify xenobiotics that have the potential to alter the production, concentration or signaling of THs. The toxicity of the BFR compounds was tested under two separate routes of exposure. A food amended feeding study was conducted with the commercial mixture DE-71. A second method utilizing a single intraperitoneal (i.p.) injection was used to study the thyroidogenic potency of the congeners BDE-47 and BDE-99 along with re-testing of the commercial mixture DE-71.

Methods and Materials

African clawed frogs (*Xenopus laevis*) were artificially induced to spawn by administrating subcutaneous injections of gonadotropin. In the feeding trials tadpoles were fed DE-71 amended food beginning approximately one week after hatch until termination of the experiment eight weeks later. Stock concentrations of DE-71 were prepared by dissolving this compound into acetone (dissolved in glass grade) and then adding to a commercial frog food (frog brilltle, NASCO™, Fort Atkinson, WI, USA). This procedure was followed to create the DE-71 treatments of 1, 1000 and 5,000 µg/g DE-71, plus a

carrier solvent (acetone) control, a clean control (non-amended food) and dieldrin (1000 µg/g) which has been shown in previous studies in our laboratory to inhibit tail resorption. Each treatment received an identical amount of food two to three times per day. Treatments were separated into four replicate groups containing 20 tadpoles in 5 L of water to unsure that tadpole growth was not stunted because of high densities. Tadpole growth (head, trunk & tail length) was monitored with the aid of a digital camcorder and images were measured using the Sigma Scan Imaging Software 5.0.0 (SPSS Inc). Measurements were performed two to three times per week during the first month of growth and nearly every day thereafter until the end of the experiment.

During the i.p. injection trials tadpoles were not exposed to the test material until they had reached Stage 58 in the development of *Xenopus* (4), which corresponded to an age of approximately six weeks post hatch. The congener specific compounds BDE-47 and BDE-99 had not been tested using the food amended route, primarily because of the large milligram quantities required. All test compounds were dissolved into the carrier solvent triolein. Congeners BDE-47 and BDE-99 were each tested at 1 and 100 µg per tadpole. The upper limit of 100 µg per tadpole represented the maximum mass that could be dissolved into a 5 µL injection volume, a volume which was considered appropriate for the size of the approximately 1 gram tadpole. The commercial mixture DE-71 was tested at 0.6, 6 and 60 µg per tadpole. Each treatment consisted of four replicates with 10 tadpoles per replicate. The study design also included a negative control group of sham injections (triolien) and a group injected with dieldrin at a concentration know to disrupt tail resorption (1 µg / tadpole). Tadpole images were recorded immediately following injections and continued for approximately two weeks when control groups had fully resorbed their tails.

Tadpole growth was uneven within each tank despite efforts to control density and feeding rates. This factor is a traditional problem in these types of studies and one which introduces greater variability into the measurements. In order to address this problem we recorded the rate of tail resorption as a cumulative resorption ratio (CRR) which was derived from a morphometric ratio of tail length to total length (TTL). The CCR measurement reflects the relative rate of tail resorption and therefore eliminates problems associated with natural variation in frog sizes. Differences among treatments were analyzed using analysis of covariance (ANCOVA). Selected treatments from the feeding trials were histologically surveyed for anatomical alterations to thyroid tissue and for chemical analysis of BFR body burden.

Results and Discussion

Under normal (non-exposed) conditions, tail resorption generally begins shortly after Stage 58 in the development of *Xenopus* and is completed approximately two weeks later when frogs metamorphose (Stage 66). Treatment differences in tail resorption during this two week period (Stage 58 to 66) indicated that the lowest concentration of DE-71 (1 µg/g food) did not statistically alter resportion in comparison to the tadpoles fed clean non-amended food or those fed food spiked with the acetone carrier solvent. In contrast,

tail resportion was significantly (ANCOVA; $F_{5,29}$=9.56; p<0.0001) inhibited in tadpoles administered DE-71 at a concentration of either 1000 or 5000 µg/g food suggesting that the threshold level is between 1 and 1000 µg/g food. Interestingly, the response observed with dieldrin (1000 µg/g food) suggests that it had less effect on tail resportion than the commercial mixture DE-71 when tested at 1000 µg/g food. Analysis of congener profile differences between the parent DE-71 mixture and tadpoles fed the commercial mixture suggest that *Xenopus* may eliminate congener BDE-47 faster from tissues than congener BDE-99 or BDE-100.

A single i.p. injection of either congener BDE-47 or BDE-99 did not statistically alter tail resportion at the highest concentration tested (100 µg/tadpole) when administered at Stage 58. However, the highest concentration (60 µg/tadpole) of the commercial mixture DE-71 when administered with a single i.p. injection (Stage 58) significantly inhibit tail resportion. This suggests that compounds other than congener BDE-47 or BDE-99 are contributing to the effects observed with this commercial mixture.

A histological review of the thyroid gland in *Xenopus* from the feeding trials did not reveal any anatomical differences between the clean control group and those fed DE-71 at a concentration of 5000 µg/g. However, this concentration, along with dieldrin (1000 µg/g food), was associated with spinal curvatures and erratic swimming behaviour in the exposed tadpoles. Figure 1 illustrates that tadpoles fed DE-71 at a concentration of 5000 µg/g DE-71 experience not only inhibition of tail resorption (lower CCR'), but were overall smaller in size (body length) indicating that growth was also inhibited in comparison to those fed a normal un-amended food ration. In addition, tadpoles fed a high concentration of DE-71 were very pale in colour and appeared to be lacking normal pigmentation. Interestingly, cessation of exposure nearly always resulted in a resumption and completion of metamorphosis (Figure 3) however, it appears that this compensatory growth may be at the sacrifice of normal body size.

Conclusions

Results from this study suggest that the commercial BFR mixture (DE-71) has the potential to disrupt growth, likely through the disruption of the thyroid system. Concentrations investigated in this work are well above environmental concentrations but may indicate the potential for BFR compounds to disrupt thyroid function and growth in longer lived animals where chronic exposure begins at early stages of development.

References

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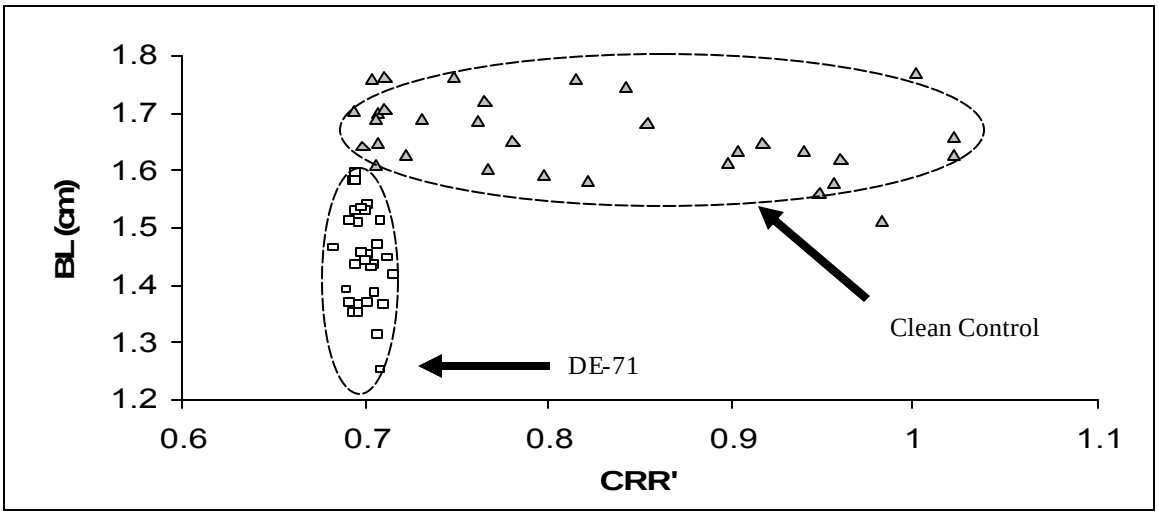

Figure 1. Relationship between cumulative resorption ratios (CRR') and body length (BL cm) of Clean (triangles) and DE71-5mg/g (squares) feeding treatments. Each point represents an individual trial (i.e. replicate) on a specific day.

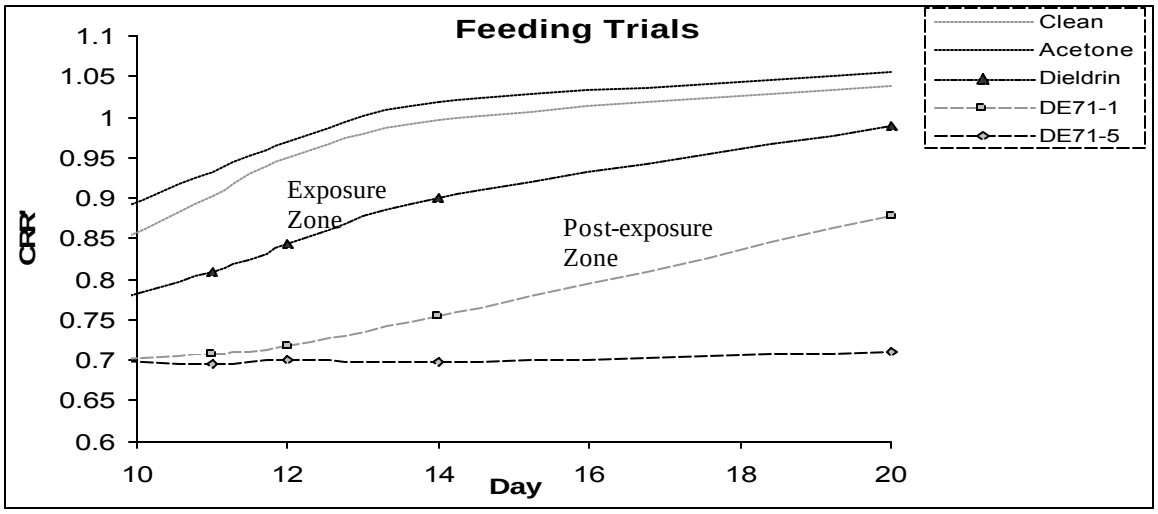

Figure 2. Cumulative resorption ratios in *Xenopus* during and after exposure.