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Effects of methoprene, nonylphenol, and estrone on the vitellogenesis of the mysid *Neomysis integer*

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Abstract

The induction of the female-specific protein, vitellogenin, in male fish is a well-established endpoint to assess exposure to estrogen-like chemicals. The use of vitellogenesis as a biomarker for xenobiotic exposure in egg-laying invertebrates, however, is still relatively unexplored. Recently, we developed a quantitative enzyme-linked immunosorbent assay (ELISA) for vitellin in *Neomysis integer* (Crustacea: Mysidacea) to study mysid vitellogenesis and its potential disruption by xenobiotics. In this study, gravid mysids were exposed to methoprene, nonylphenol, and estrone for 96 h. All methoprene-exposed (0.01, 1, and 100 μg/L) animals had lower vitellin levels compared to the control animals, though this effect was not statistically significant. Exposure to nonylphenol resulted in significantly induced vitellin levels in the lowest exposure concentration (0.01 μg/L), whereas no effects were observed at higher concentrations. Estrone significantly decreased vitellin levels at the highest test concentration (1 μg/L). These results indicate that mysid vitellogenesis can be disrupted following chemical exposure. Difficulties in the interpretation of the observed chemical-specific and concentration-specific responses in this study highlight the need for a better understanding of hormone regulation of crustacean vitellogenesis.

Keywords: Crustacea; Endocrine disruption; ELISA; Mode-of-action; Invertebrate

1. Introduction

Vitellogenesis involves the production of the yolk protein vitellin that acts as a nutrient source for the developing embryo. Consequently, any event that affects the synthesis of vitellin and the yolk precursor vitellogenin will also modify reproductive success. A number of anthropogenic chemicals are known to have the potential to disrupt vitellogenesis in vertebrates. A well-known example of endocrine disruption is the induction of vitellogenin in male fish exposed to xeno-estrogens (Fenske et al., 2001; Tyler et al., 1999; Versonnen and Janssen, 2004). Little is known about the potential effects of endocrine-disrupting chemicals on vitellogenesis in invertebrates, and few studies have evaluated endocrine toxicity to vitellogenesis in crustaceans (Billinghurst et al., 2000; Lee and Noone, 1994; Oberdörster et al., 2000; Sanders et al., 2005; Tsukimura, 2001; Volz and Chandler, 2004). Recently, we purified and characterized vitellin from the mysid *Neomysis integer* (Ghekiere et al., 2004) and subsequently developed a quantitative enzyme-linked immunosorbent assay (ELISA) (Ghekiere et al., 2005). The present study validates the use of the *N. integer* vitellin ELISA to detect potential effects of three reported endocrine-disrupting chemicals on mysid vitellogenesis.

ENDIS-RISKS is a multidisciplinary project that studies the occurrence, distribution, and potential effects of endocrine disruptors in the Scheldt estuary (Belgium/The Netherlands), one of the most polluted estuaries in the world (ENDIS-RISKS project, http://www.vliz.be/projects/endis). Our first studies found high exposure to endocrine-disrupting substances and potential effects on the resident mysid population in this estuary

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(Noppe et al., 2005; Verslycke et al., 2004b, 2005). A number of priority substances have been identified based on these field studies, and their potential effects on hormone-regulated processes in the mysid *N. integer* are currently being evaluated through laboratory studies. To that end, we have been researching a number of hormone-regulated processes in mysids that could be used as endpoints to evaluate endocrine disruption, e.g., energy and steroid metabolism (Verslycke et al., 2004b,c), molting (Ghekiere et al., in press), embryogenesis (Ghekiere et al., accepted), and vitellogenesis (present study). Mysid shrimp have been used extensively in regulatory toxicity testing, and it is the only invertebrate species included in US-EPA's endocrine disruptor screening and testing program (Verslycke et al., 2004a).

One of the test compounds that has been used in validation studies of these endpoints is the insecticide methoprene. Methoprene is an insect growth regulator that is generally used to control mosquitos. This insecticide has been shown to disrupt normal development in non-target organisms, such as crustaceans (Celestial and McKenney, 1994; McKenney and Celestial, 1996; McKenney and Matthews, 1990; Mu and Leblanc, 2004; Olmstead and LeBlanc, 2001; Templeton and Laufer, 1983; Walker et al., 2005). The two other chemicals used in the present study are nonylphenol (a breakdown product of alkylphenol ethoxylates, APEs) and estrone, which have been reported to be present in the Scheldt estuary and are known endocrine disruptors (Noppe et al., 2005; Verslycke et al., 2005). APEs are synthetic surface-active agents (surfactants), commonly used in industrial detergents and plastic manufacturing (Blackburn et al., 1999). Around 80% of all manufactured APEs are nonylphenol ethoxylates (Naylor, 1998), which degrade to nonylphenol in sewage treatment plants (Ahel et al., 1994). Reported nonylphenol concentrations in UK rivers are <0.2–12 μg/L, although concentrations as high as 180 μg/L have been detected in water receiving effluent directly from sewage treatment works (Allen et al., 2002; Blackburn et al., 1999; Blackburn and Waldock, 1995). These levels of nonylphenol correspond with concentrations measured in the Scheldt estuary (Verslycke et al., 2005; Vethaak et al., 2002). From the large group of substances that are suspected or known to be environmental endocrine disruptors, the natural and synthetic estrogens are suggested to have high estrogenic potency (Noppe et al., 2005). Synthetic estrogens are used in birth-control pills and for the management of menopausal syndromes, and cancer (De Alda and Barcelo, 2001). Of the natural female sex hormones, estrone is detected most frequently in the Scheldt estuary at concentrations of up to 8 ng/L (Noppe et al., 2005; Vethaak et al., 2002).

Natural estrogens and xeno-estrogens like APEs cause a number of well-documented estrogenic effects in fish, such as disruption of vitellogenesis (Fenske et al., 2001; Korsgard and Pedersen, 1998; Sumpter and Jobling, 1995). The effect of (xeno-) estrogens on the reproduction of crustaceans, however, remains controversial and is still poorly understood (Billinghurst et al., 2000; Sanders et al., 2005; Tsukimura, 2001). This study is the first to report effects of environmental endocrine disruptors on the vitellogenesis of the mysid *N. integer*.

2. Materials and methods

2.1. Chemicals

Methoprene (CAS # 40596-69-8) and estrone were obtained from Sigma–Aldrich (Bornem, Belgium). Nonylphenol was obtained from Acros Organics (Geel, Belgium). Stock solutions of the test compounds were prepared in absolute ethanol. The ethanol concentration in the solvent control and in the different test concentrations was 0.01%.

2.2. Test organisms

The mysid crustacean, *N. integer*, was collected in March 2005 by handnet in the Braakman, a lake (10 psu) near the Scheldt estuary in Hoek (The Netherlands). After a 24 h acclimation period to the maintenance temperature, the organisms were transferred to 200-l glass aquaria. Culture medium was artificial seawater (Instant Ocean, Aquarium Systems, France), diluted with aerated deionized tap water to a final salinity of 5 psu. A 14 h light:10 h dark photoperiod was used during culturing and water temperature was maintained at 15 °C. Cultures were fed daily with 24- to 48-h-old *Artemia* nauplii *ad libidum*. Hatching of the *Artemia* cysts was performed in 1-L conical-cylinder vessels under vigorous aeration and continuous illumination at 25 °C.

2.3. Acute toxicity test with estrone

Ninety-six hours LC_{50}s for methoprene and nonylphenol to juvenile *N. integer* were previously published by Verslycke et al. (2004c). Juvenile mysids of similar size (visual selection of organisms with a size of 2–4 mm) were taken from the laboratory culture and randomly distributed to 400 ml glass beakers containing 200 ml of the desired test concentration in water with a salinity of 5 psu and temperature of 15 °C. For each test concentration, two replicate beakers containing five mysids each were used. Mysids were exposed for 96 h to 1–100–1000–10,000 μg estrone/L. Exposure solutions were renewed every 24 h and juveniles were fed daily with 24-h-old *Artemia* nauplii *ad libidum*.

2.4. Test design for vitellin assessment

Gravid females of approximately the same size (27.5 ± 4.9 mg wet weight), carrying stage I embryos in their marsupium, were selected and exposed to the test compounds. Stage I carrying females were used as this stage can be determined easily and it is a short embryonic stage (±4 days), minimizing the intra-stage variability between individual animals. A detailed description of the different developmental stages of *N. integer* embryogenesis is given in Fockedey et al. (in press). Females were exposed to the sublethal concentrations 0.01–1–100 μg methoprene/L; 0.01–1–100 μg nonylphenol/L; 10–100–1000 ng estrone/L. Females were randomly put in 400 ml beakers containing 200 ml of the desired test concentration in water with a salinity of 5 psu and a temperature of 15 °C. For each test concentration, two replicate beakers with six females were used and the mysids were exposed for 96 h. Exposure solutions were renewed every 24 h and test organisms were fed daily with 24-h-old *Artemia* nauplii *ad libidum*. After 96 h, the females were shock-frozen in liquid nitrogen and kept at −80 °C until analysis of the vitellin levels using the ELISA. All vitellin analyses were performed within two weeks after exposure to reduce the risk of vitellin degradation.

All individual animals were homogenized in 200 μl Tris–HCl, pH 7.2, and diluted 10,000 times in this buffer for vitellin quantification. Concentrations are expressed in 1 ml of this homogenate.

2.5. Competitive enzyme-linked immunosorbent assay for Neomysis integer vitellin

The *N. integer* vitellin ELISA assay was recently developed (Ghekiere et al., 2005). Purified vitellin was thawed on ice and diluted in coating buffer (0.05 M sodium carbonate buffer, pH 9.6). The wells of 96-well microtiter plates (Nunc F96 Maxisorp Immuno Plate) were coated with 100 µl of vitellin solution (100 ng vitellin/ml coating buffer), sealed and incubated overnight at 4 °C. For determination of non-specific binding (NSB) effects, three wells per plate were treated with coating buffer only.

For the standards, purified vitellin was diluted in PBS-T blocking buffer (0.01 M phosphate-buffered physiological saline solution with 0.05% Tween 20 and 1% fatty acid-free BSA) to a concentration of 2000 ng vitellin/ml. From this stock solution, serial dilutions were prepared in PBS-T blocking buffer. In parallel, samples with an unknown vitellin content were diluted in PBS-T blocking buffer. The vitellin standards and unknown samples (60 µl/well) were incubated in non-coated 96-well microtiter plates with vitellin antibody (60 µl/well, 1:10,000 in PBS-T blocking buffer). For the NSB, 60 µl/well of blocking buffer was mixed with 60 µl of the antibody solution only. The incubates were mixed on a rotary shaker, and the plates were sealed and incubated overnight at 4 °C.

The coated plates were washed three times with 100 µl PBS-T washing buffer (0.01 M phosphate-buffered physiological saline solution with 0.05% Tween 20, pH 7.4). To reduce background, the plates were blocked with 150 µl of PBS-T blocking buffer/well for 30 min at 37 °C. After this blocking step, the plates were washed another three times with PBS-T, before 100 µl of the sample/antibody or standard/antibody incubates were pipetted into the wells. The plates were sealed and incubated for 120 min at 37 °C. The first antibody incubates were then removed and the plates were washed three times with PBS-T. Second antibody (125 µl) against rabbit IgG (goat anti-rabbit IgG, whole molecule, peroxidase conjugate; Sigma) was added to each well at a dilution of 1:2000 in PBS-T blocking buffer and the plates were sealed and incubated at 37 °C for 60 min. The plates were washed three times with PBS-T and then 125 µl of the enzyme substrate solution was added to each well. This solution was prepared by dissolving 0.5 mg of *o*-phenylenediamine dihydrochloride (OPD) (Sigma–Aldrich) in 0.05 M phosphate–citrate buffer, pH 5.0 (0.051 M dibasic sodium phosphate and 0.024 M citric acid). After addition of 0.5 µl/ml H_2O_2 (30%; Merck), the substrate solution was immediately pipetted into the plates (125 µl/well). The enzyme reaction was allowed to proceed for 10 min in the dark, at which point the color reaction was stopped by the addition of 30 µl of 3 N H_2SO_4. The absorbance of the reaction product was read at 490 nm using a microtiter plate reader (Multiskan Ascent, Thermo Labsystems, Helsinki, Finland). The absorbance values obtained with the ELISA are inversely proportional to the amount of vitellin present in the sample. Vitellin content in samples was quantified from a standard curve with concentrations log-transformed.

2.6. Statistics

All data were checked for normality and homogeneity of variance using Kolmogorov–Smirnov and Levene's test, respectively, with an $\alpha = 0.05$. The effect of replication was tested for significance using a two-way analysis of variance. The effect of the treatment was tested for significance using a one-way analysis of variance (Dunnett's test; Statistica, Statsoft, Tulsa, OK, USA). All box-plots were created with Statistica and show the mean (small square), standard error (box), and the standard deviation (whisker).

3. Results

3.1. Acute toxicity of methoprene, nonylphenol, and estrone

To establish relevant test concentrations for subsequent sublethal vitellogenesis testing, the acute toxicity of estrone was determined. No significant mortality was observed at any of the tested exposure concentrations of estrone, i.e., the 96 h-LC_{50} of estrone to *N. integer* is >10 mg/L. Mortality in the controls was ⩽20%. The 96 h-LC_{50} of methoprene

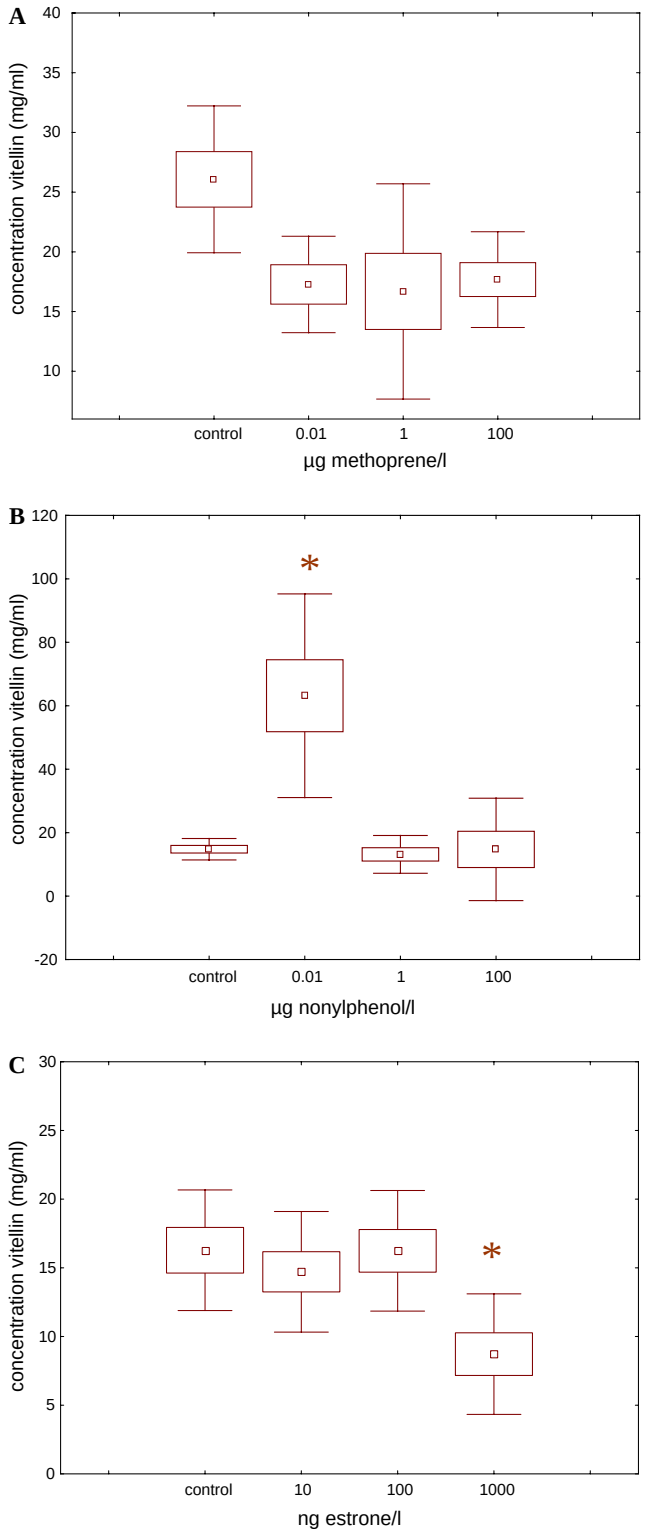

Fig. 1. Levels of vitellin (mg/ml) in females exposed to different concentrations methoprene (A), nonylphenol (B), and estrone (C). The box-plots show the mean (small square), standard error (box), and the standard deviation (whisker) of 12 replicate measurements per treatment. ∗ significantly different from control (Dunnett's; $p < 0.05$).

and nonylphenol to juvenile *N. integer* were previously determined to be 320 and 590 μg/L, respectively (Verslycke et al., 2004c).

3.2. Sublethal effects of methoprene, nonylphenol, and estrone on vitellogenesis

The effect of 96 h exposure to sublethal concentrations of methoprene, nonylphenol, and estrone on the vitellin levels in gravid *N. integer* are shown in Fig. 1. The effect of replication was tested for significance using a two-way analysis of variance and no significant differences were found between the two replicate beakers for the three different exposure treatments ($p > 0.2$). As such, we used one way analysis to determine significant differences from the control (12 mysids per concentration). Although methoprene-exposed females exhibited lower vitellin levels than control animals, these reductions were not statistically significant. Only animals exposed to the lowest nonylphenol exposure concentration, 10 ng nonylphenol/L, had significantly induced vitellin concentrations ($p < 0.001$). Finally, only the highest estrone exposure concentration (1000 ng/L) resulted in significantly lower vitellin concentrations ($p < 0.05$).

4. Discussion

The best documented examples of endocrine disruption in the aquatic environment are the estrogenic effects of discharges of treated sewage effluents on fish (Harries et al., 1996, 1997; Vos et al., 2000). Alkylphenols, natural hormones, and synthetic hormones, amongst others, have been suggested as the most likely responsible for the 'feminization' detected in male fish (Desbrow et al., 1998; Vos et al., 2000). Existing studies on the effects of endocrine disruptors on crustacean vitellogenesis, however, are fragmented and contradictory. Billinghurst et al. (2000) reported that cypris major protein (CMP), which is related to barnacle vitellin, is elevated in larvae of the barnacle *Balanus amphitrite* exposed to both nonylphenol and 17β-estradiol at a concentration of 1.0 μg/L. They concluded that CMP and perhaps other vitellin-like proteins are potential biomarkers of low level estrogen exposure in crustaceans. In another study by Tsukimura (2001), ridgeback shrimp *Sicyonia ingentis* were injected with 1.0 μg of 17β-estradiol, but no significant changes in hemolymph vitellogenin levels were observed. A recent study by Sanders et al. (2005) found that 17β-estradiol and nonylphenol had contrasting effects on the expression of a vitellin-like protein in the glass prawn *Palaemon elegans*. Relatively high concentrations of 17β-estradiol (0.2 μg/L) significantly reduced expression of the protein, while nonylphenol produced a concentration-independent increase. The lowest concentration of nonylphenol tested, 0.2 μg/L, exerted the most consistent stimulatory effect.

In an effort to further explore the potential effects of chemicals on crustacean vitellogenesis, we recently developed a quantitative vitellin enzyme-linked immunosorbent assay (ELISA) for the mysid shrimp *N. integer* (Ghekiere et al., 2005). Here, we present the first validation study of the *N. integer* vitellin ELISA following exposure to toxicants with suspected endocrine activity. All methoprene-exposed (0.01, 1, and 100 μg/L) mysids had lower vitellin levels compared to the control animals, but this effect was not statistically significant. A significant increase in mysid vitellin was observed at the lowest nonylphenol exposure concentration (0.01 μg/L), whereas mysid vitellin levels decreased in the highest estrone exposure (1 μg/L), compared to vitellin levels in the controls. These effect levels on mysid vitellogenesis are above environmental concentrations measured in the Scheldt estuary for estrone (8 ng/L; Noppe et al., 2005), and below reported levels for nonylphenol (<0.2–12 μg/L; Verslycke et al., 2005; Vethaak et al., 2002). The focus of ongoing field studies in the Scheldt estuary is to correlate endocrine disruptor exposure levels and mysid vitellin levels (ENDIS-RISKS project, http://www.vliz.be/projects/endis), and will be helpful in evaluating potential effects in the field. It should be noted that vitellin levels in the control were different between the three different experiments. Specifically, control animals in the methoprene exposure had significantly ($p < 0.01$) lower vitellin levels than control animals in nonylphenol and estrone experiments. These differences are most likely related to both biological (e.g., small differences in the time of the vitellogenine cycle at which the animals are sampled for analyses) or methodological factors (e.g., sampling strategy).

We have recently developed in vivo assays to study growth and embryonic development in *N. integer* and have evaluated the potential disruption of these processes by methoprene at the same concentrations used in the present study (Ghekiere et al., in press; Ghekiere et al., accepted). In these studies, methoprene caused a concentration-dependent decrease in hatching success (significant at 1 and 100 μg/L), whereas growth of *N. integer* was significantly reduced at 100 μg/L. As such, embryogenesis and growth of *N. integer* seem to be more sensitive to the effects of methoprene than vitellogenesis. The observed differences in the effect concentration of methoprene on these physiological processes in mysids could be due to differences in the developmental stage of the test organisms, differences in the exposure duration, and differences in the mode-of-action or hormonal regulation of the respective physiological process. Females with stage I embryos, eggs, and <24-h-old juveniles were used for the vitellogenesis, embryogenesis and growth assay, respectively. Animals in the vitellogenesis study were exposed for 96 h, whereas animals in embryogenesis study were exposed from oviposition until hatching (~2 weeks). Finally, animals in the growth assay were exposed during five successive molts (~3 weeks). With respect to the toxic mode-of-action, methoprene is known to mimic juvenile hormone and can directly disrupt early stages of embryonic development in developing insects (Dhadialla et al., 1998; Hoffmann and Lorenz, 1998), whereas nonylphenol and estrone are estrogenic. Effects on crustacean growth through molting are most likely an

indirect effect that is caused through cross-communication between the ecdysteroid and juvenoid hormone regulatory pathways as recently suggested by Mu and Leblanc (2004) in daphnids. While a juvenile hormone receptor has not been identified to date in arthropods, the anti-ecdysteroidal activity of juvenile hormone, or chemicals with juvenile hormone activity, has been demonstrated (Celestial and McKenney, 1994; McKenney and Matthews, 1990; Olmstead and LeBlanc, 2001; Templeton and Laufer, 1983). Finally, we previously examined the effect of methoprene on the energy and testosterone metabolism of *N. integer* (Verslycke et al., 2004c). Mysids exposed to 100 μg methoprene/L had significantly altered energy and steroid metabolism. Based on the above studies with methoprene and mysids, we suggest that chronic exposure to juvenoids in mysids could result in effects on reproduction via different pathways, i.e., by interaction with energy allocation and the metabolic machinery of mysids (Verslycke et al., 2004c), by disruption of embryonic development (Ghekiere et al., accepted), and by disruption of molting and growth (Ghekiere et al., in press) leading to reduced fecundity as size and fecundity are linked in mysids. A recent transgenerational exposure study by McKenney (2005) found that second generation adult mysids, which were exposed to the juvenile hormone analog phenoxycarb only as embryos, produced fewer young and had altered sex ratios. Future chronic exposure studies should focus on determining which life stages and/or physiological processes are critical in leading to reproductive and ultimately population effects in mysids.

The present study further adds to the weight-of-evidence that (xeno)estrogens appear to be less effective in causing disruption of normal vitellogenesis in crustaceans than they are in oviparous vertebrates. Most likely, this is a result of the different hormonal control strategies for vitellogenesis in crustaceans compared with oviparous vertebrates. Future studies should be aimed at the identification and quantification of the hormones, the hormone receptors and downstream hormone-responsive genes and gene products involved in the control of vitellogenesis and other hormone-regulated processes in crustaceans. These studies will lead to a better understanding of the mode-of-action of chemicals on crustacean hormone-regulated processes.

While it can be concluded that vitellogenesis is an interesting physiological process to study endocrine toxicity in crustaceans, future studies should focus on understanding hormonal regulation of vitellogenesis and other hormone-regulated processes (vitellogenesis, molting, embryogenesis, energy metabolism, and steroid metabolism) in mysids and other invertebrates. In addition, priority should be given to exposures with chemicals that are more likely to interact with hormones that are unique to invertebrates, such as ecdysteroids and juvenile hormones. To date, the uniqueness of hormonal regulation in invertebrates as compared to vertebrates, is not reflected in proposed regulatory screening and testing programs that only focus on vertebrate estrogen, androgen, and thyroid hormones.

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