Effects of Ethinylestradiol and the Fungicide Prochloraz on Metamorphosis and Thyroid Gland Morphology in *Rana temporaria*

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Abstract: Several environmental xenobiotics have been found to affect the metamorphosis of amphibians. In this study we exposed tadpoles of the Common frog *Rana temporaria* from hatching to metamorphosis to two known endocrine disruptors, the estrogenic pharmaceutical 17α-ethinylestradiol and the antiandrogenic/antiestrogenic fungicide prochloraz to determine their effect on 1) days to metamorphosis and size at metamorphosis, 2) body concentrations of triiodothyronine (T3) and corticosterone, and 3) thyroid morphology. We found effects of both compounds on each of these response variables. A low dose of prochloraz (115 μg/l) and all doses of ethinylestradiol also caused a delay in metamorphosis. T3 levels were elevated in metamorphs exposed to high concentration of prochloraz (252 μg/l) but the group showed a delay in metamorphosis. A low dose of prochloraz (115 μg/l) and all doses of ethinylestradiol also caused a delay in metamorphosis but no changes in T3 levels. The delayed metamorphs weighed more than controls. Thyroid histology revealed significant differences in the high prochloraz exposure group only. Ethinylestradiol and prochloraz, however not in environmentally relevant doses, may therefore impact the thyroid axis, and may cause other sublethal effects especially in combination with other stressors likely encountered.

Keywords: Endocrine disruptors, Ethinylestradiol, Metamorphosis, Prochloraz, *Rana temporaria*, Thyroid gland.

INTRODUCTION

Amphibian populations are declining in several regions of the world [1-3]. Anthropogenic chemicals have been suggested as one possible cause [4-6]. Endocrine-disrupting chemicals (EDCs) or xenobiotics encompass a large group of environmental pollutants that includes pesticides, herbicides, pharmaceuticals and chemicals from the industry. Exposure to these chemicals during development and differentiation of major organ systems may result in irreversible physiological, morphological and behavioural changes by altering the hormones that control the course of development. While most attention has been given to potential interactions of environmental chemicals with the sex steroid hormone system, there also exists evidence that some classes of chemicals interacts with the thyroid hormone system and stress hormone axis and there is growing knowledge how the molecular mechanisms mediates the subsequent effects.

Probably the most critical process in amphibian life history is the metamorphosis from aquatic tadpole to terrestrial frog. Many amphibian species, especially those inhabiting unpredictable environments, exhibit phenotypic flexibility in growth rate prior to metamorphosis and time to metamorphic climax. This adaptive strategy allows individuals to optimize the probability of successful emergence from the larval environment [7-9]. Phenotypic plasticity in developmental time results from trade-offs between physiological mechanisms regulating growth and development [9]. Size at metamorphosis has significant subsequent fitness consequences.

Environmental and internal stimuli are translated into hormonal signals, thyroid hormone and corticotrophins, by the central nervous system, which modulates the rate and course of metamorphosis [9, 10]. Endocrine disrupting chemicals may act as metamorphic cues by stimulating a hormonal response similar to that caused by natural environmental stressors or by altering hormone transport, hormone-receptor interactions or metabolism.

Thyroid hormones (TH) are essential in stimulating all aspects of amphibian metamorphosis [10-14]. The metamorphosis is accompanied by an increase in the synthesis and secretion of TH, an increase in iodide uptake by the thyroid gland and hypertrophic and hyperplastic changes in the thyroid and pituitary glands [15-18]. Chronic TH deficiency retards or prevents metamorphosis often leading to oversized larvae [15]. The action of thyroid hormone is modulated by various other hormones including adrenal steroids [14]. Larson et al. [19] suggests a model for how corticosterone and thyroxine interact to regulate metamorphosis. Corticosterone may retard or accelerate metamorphosis depending on species, developmental stage and TH concentration [20-23].

Amphibian metamorphosis and active TH synthesis and secretion are accompanied by changes in parameters of thyroid histology and growth. Thyroid gland histology has also been judged to be one of the most sensitive parameters for detection of compounds that adversely affect thyroid function in mammals [24,25], even more sensitive than measure of TH levels [26].

It is obvious that amphibian larvae undergoing dramatic metamorphic changes in a short period of time may be very sensitive to any impact on the thyroid system. Several studies have focused on the effects of endocrine-disrupting
chemicals on the thyroid system by interfering with one or several of the steps in the TH pathway and/or affect time of metamorphosis [27,28], including ethinylestradiol used in this experiment [29,30].

17α-ethinylestradiol (EE2) is a synthetic estrogen and the main ingredient in the contraceptive pill. A large percentage of the ingested ethinylestradiol is excreted and has at least been partially held responsible for the estrogenic activity of many effluents (e.g. <0,053-62 ng/l in Denmark) and feminization in fish and frogs [31-35]. Prochloraz (Pro) is a contact fungicide of the imidazole group acting by inhibiting ergosterol synthesis in the target organism through inhibition of the cytochrome P450-dependent 14α-demethylase activity [36]. This fungicide is bio concentrated in aquatic organisms and has been shown to be both antiestrogenic and antiandrogenic in rats and fish by antagonizing these hormones receptors or down-regulate the receptors, as well as being a potent aromatase inhibitor [36-38] with reduced fecundity as a consequence [39]. Prochloraz is also a known thyroid disruptor in rats [36, 40]. Both compounds are enco-unted in the aquatic environment in Europe.

In this study we exposed tadpoles of the Grass frog Rana temporaria from hatching to metamorphosis to two known endocrine disruptors, the estrogenic pharmaceutical ethinylestradiol and the antiandrogenic/antiestrogenic fungicide prochloraz. Both environmentally relevant doses and high doses were used. The sex steroid-disrupting effects of these compounds are discussed elsewhere [41]; here we focus primarily on potential interference with the thyroid hormone axis and metamorphosis. We ask if exposure to these compounds results in changes in thyroxine concentrations in tadpoles and if these changes are associated with changes in growth rate and metamorphic progress. If Rana tadpoles respond to the compounds as a stressor we would predict elevated thyroxine concentrations, decreased body condition and accelerated metamorphosis. If the compounds interfere directly with metamorphosis, work directly and negatively on thyroxin or interfere with other hormones involved in metamorphosis we should expect a delay in metamorphosis. Finally, we also looked at the histology of the thyroid gland to relate the biochemical results to morphological effects.

**MATERIALS AND METHODOLOGY**

**Frogs**

Egg masses from Grass frog (Common frog), Rana temporaria, were sampled in the beginning of April 2005 from a temporary freshwater pond located in Odense, Denmark. The clutches were stored at 15°C until hatching from a temporary freshwater pond located in Odense, temporaria, Egg masses from Grass frog (Common frog), Frogs results to morphological effects. delay in metamorphosis. Finally, we also looked at the directly and negatively on thyroxin or interfere with other the compounds interfere directly with metamorphosis, work decreased body condition and accelerated metamorphosis. If stressor we would predict elevated thyroxine concentrations, ergosterol synthesis in the target organism through inhibition contact fungicide of the imidazole group acting by inhibiting feminization in fish and frogs [31-35]. Prochloraz (Pro) is a many effluents (e.g. <0,053-62 ng/l in Denmark) and of the ingested ethinylestradiol is excreted and has at least decreased body condition and accelerated metamorphosis. If these compounds results in changes in thyroxine concentrations in tadpoles and if these changes are associated with changes in growth rate and metamorphic progress. If Rana tadpoles respond to the compounds as a stressor we would predict elevated thyroxine concentrations, increased body condition and accelerated metamorphosis. If the compounds interfere directly with metamorphosis, work directly and negatively on thyroxin or interfere with other hormones involved in metamorphosis we should expect a delay in metamorphosis. Finally, we also looked at the histology of the thyroid gland to relate the biochemical results to morphological effects.

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**Thyroid Gland Histology**

Five metamorphs from each treatment group (Gosner stage 44-45) were euthanized by immersion in MS-222. They were then fixed in Bouin’s fixative for 48 hours and then transferred to 70% ethanol and stored. The sample animals (minus the limbs) were then dehydrated in a graded series of alcohols, cleared in TissueClear (Sakura) and embedded in paraffin. The whole animal was sectioned
transverse (5 μm) and stained using Mayer's haematoxylin and eosin procedure. The sections where examined under a light microscope and at least 7 sections through both lobes of the thyroid gland were photographed from each animal. Area occupied by the thyroid was measured using an image analysis software (Image Pro Plus, Media Cybernetics) and total volume was calculated by multiplying the area with number of section through the gland and the thickness of the sections. The volume is expressed per gram animal to correct for differences in size. In addition the follicular cell height was measured from random chosen cells in the section. A single mean value for epithelial cell height was calculated for each animal. From two sections in approximately the middle of the thyroid the percent area occupied by colloid was calculated. An evaluation of colloid depletion, follicular cell morphology or hypertrophy according to description by Hooth [49] and Patiño et al. [50] was done. Hyperplasia was ranked positive according to the presence of follicular cell masses or irregular clusters not forming follicles. Colloid depletion was evaluated according to presence of reduced colloid volume or collapsed follicles. Finally the maximum number of follicles per thyroid gland lobe was counted.

Data Analysis

One-way analysis of variance was used to determine overall levels of significance and Fishers LSD method for a pairwise multiple comparison with the control group.

For follicle number and days to metamorphosis, normality test failed and Kruskal-Wallis one way analysis of variance on ranks was used, and Dunn's pairwise multiple comparison procedure was used to separate the means.

The analysis was carried out using SigmaStat (Systat Software Inc.). Significance level was P = 0,05.

RESULTS

Days to Metamorphosis

Tadpoles exposed to ethinylestradiol and the highest doses of prochloraz took significantly longer to reach metamorphosis compared with the control group (P < 0,001, df = 6, n = 141-189, on ranks) (Fig. 1). Tadpoles exposed to 6 and 61 ng EE2/l took on average 3,9 and 4,1 days longer until metamorphosis, while tadpoles exposed to 115 ng EE2/l was delayed 11,4 days compared to controls. For the prochloraz exposed groups the delay was 0,8 and 6,4 days for 11 μg Pro/l and 115 μg Pro/l respectively. The delay for the 252 μg/l group was 8,3 days compared to the control animals.

The groups receiving the highest doses of EE2 and prochloraz weighed significantly more at metamorphosis (P < 0,001, df = 6, n = 141-182, on ranks) (Fig. 2).

The difference in size at metamorphosis is due not only to a longer larval period as tadpoles exposed to the two highest concentrations of prochloraz and the highest concentration of ethinylestradiol were also heavier at day 91 (P = 0,046, F = 2,202, df = 6, n = 20-22) (Fig. 3). However, this is not true for the group exposed to 115 ng EE2/l that was heavy at day 81 but metamorphosed at a relative low weight.

Mortality was not related to treatment and the survival rates lay between 71,3 % to 84,3 %.

Thyroid Hormones

Fig. (4) shows total and free T3 levels in the tadpoles at metamorphosis. Tadpoles exposed to 252 μg prochloraz /l had higher total T3 than the rest of the groups (P < 0,001, F = 5,828, df = 6, n = 11) reaching an average of 5,13 ng/g frog. However, there was no difference in free T3 levels (P = 0,527), and the difference in the total amount compared to the other groups was small.

Corticosterone

Measurement of corticosterone levels in extracted whole-body homogenate did not reveal any significant differences between the exposure groups (P = 0,326).
Fig. (2). Effect of different treatments on larval weight of *Rana temporaria* at metamorphosis (Gosner stage 44-45) (n = 141-182). Data are means (grams) (± SEM). * indicates a significant difference from controls. The tadpoles exposed to the highest doses of ethinylestradiol (EE₂) and prochloraz (Pro) were heavier at metamorphosis than controls.

Fig. (3). Effect of ethinylestradiol (EE₂) and prochloraz (Pro) treatments on larval growth of *Rana temporaria* during the exposure period (n = 8/exposure group). Data are means (g) (± SEM). The points to the right (where lines are not connected to) are the average time for metamorphosis and weight at metamorphosis. The groups exposed to 115 ng EE₂/l, 115 μg Pro/l and 252 μg Pro/l are significantly heavier than the control group at day 81.

Fig. (4). Effect of different treatments on larval total and free Triiodothyronine (T₃) of *Rana temporaria* at metamorphosis (Gosner stage 44-45) (n = 11). Data are means (ng/g) (± SEM) for total T₃ and means (pg/g) (± SEM) for free T₃. * indicates a significant difference from controls. The tadpoles exposed to the highest dose of prochloraz (Pro) had higher total T₃ levels. There was no effect from treatment with ethinylestradiol (EE₂).
Thyroid Histology

Morphological examination of the thyroid gland from control animals (Fig. 5A) showed that each thyroid lobe consisted of on average 22.7 follicles (middle section) of varying size and shape (i.e. very irregular in shape). Normal inactive thyroid structure is characterized by regular shaped follicles, a single layer of cuboidal epithelial cells with large centrally placed nucleus with single nucleolus and a lumen filled with smooth colloid [51-53]. In this study, the epithelial cells were columnar in shape in some follicles but cuboidal in others. The nucleus in the columnar cells was large and situated basally. Also diffuse epithelial tissue with no lumens was present. Moreover, hyperplasia was present in some follicles. Signs of hyperplasia include the proliferation of follicle cell clusters in extracellular spaces or follicular cells clusters projecting outwards from the follicles or inwards into the lumen. Vacuolisation of the colloid was evident and some follicles contained no colloid. Follicular collapse was also noted. All these morphologies are characteristic of an active secreting gland.

Histological endpoints of the thyroid gland in tadpoles exposed to the endocrine disrupting chemicals ethinylestradiol and prochloraz were evaluated (Fig. 5B). Morphologically the glands from the exposed frogs showed the same as for controls. They also contained small follicles with rather thick epithelia, follicular cell hyperplasia and colloid depletion. In the 251.5 µg/l prochloraz group (B) follicular cell hyperplasia appeared to be more extensive than the other groups.

DISCUSSION

Days to Metamorphosis

High concentrations of both ethinylestradiol and prochloraz in this study decreased the developmental rates of Rana temporaria tadpoles so these tadpoles took significantly longer to reach metamorphosis compared with the control group. Alteration of timing of metamorphosis may be due to either cumulative physiological damage or a disruption of the thyroid hormone or adrenal axis. Developmental plasticity could be less adaptive in the current aquatic environment if novel stimuli such as endocrine disruptors activate stress response or cause alteration in the stage specific levels of active thyroid hormone that influence growth, development and time of metamorphosis, uncoupling the link between environmental variability and developmental plasticity [10, 14, 19]. In the field a maladaptive delay in metamorphosis could be lethal to tadpoles in temporary ponds which dry up, like the pond where the eggs for this study were collected, due to subsequent increasing predation pressure, food resource limitation and osmotic stress.

Several studies show effects of xenobiotics on metamorphosis [19, 54-58], and size at metamorphosis [59, 60]. Exposure of Rana clamitans tadpoles to 0,1 mg E2/l for 273 days caused initiation of metamorphosis at a younger age [55], while EE2 exposure of Rana pipiens during mid-metamorphosis caused developmental delay [61]. The effects of xenobiotics on metamorphosis, however, seem to vary with exposure concentration, species/populations and age [61-64]. As mentioned in the introduction and discussed below thyroid hormone function can be disrupted at many different places in the pathway and the mechanistic explanation for the delay is still unknown.

In the present study there was a trend of the delayed metamorphs to be larger at metamorphosis in the 115 and 159 ng/l EE2 group and the 252 µg/l prochloraz group. The same groups also weighed more at day 81 reflecting a greater growth rate. All in all the xenobiotics used in this study surprisingly seemed to enhance growth. Hogan et al. [64]...
also found increased body weight in tadpoles exposed to high concentrations of ethinylestradiol. The rate of development generally is inversely related to larval growth rate and therefore to size at metamorphosis. Wilbur and Collins [7] hypothesized that changes in recent growth rate, together with overall larval size, determines age and size at metamorphosis; rapidly growing larvae (due to favourable environment) should begin metamorphosis later and at larger size than larvae growing slowly. However, experimental support has been varied [19, 65-67] and several other models have been proposed [65, 66]. As mentioned, if favourable conditions consist metamorphosis may be delayed, resulting in emergence of larger size metamorphs. However, it is more plausible that the delay may be due to disruption of the hormones involved in metamorphosis than the tadpoles experiencing their contaminated environment as favourable. Premetamorphic development is TH-independent due to a lack of functional thyroid gland but probably regulated by prolactin and growth hormone [13]. It is therefore likely that ethinylestradiol may stimulate production of one or both of these [68].

Hormones

Several studies have focused on the effects of endocrine-disrupting chemicals on the thyroid system. Most work has been done on mammals or fish and in the laboratory and includes effects of not only environmental xenobiotics but also clinical drugs. Relative few studies have been conducted on amphibians and they are often limited to effects on metamorphosis and not the mechanisms of action on the thyroid gland. From the present experiments it was expected that the differences in time at metamorphosis, in this study would be reflected in the levels of T3. However, only tadpoles exposed to a high concentration of prochloraz had slightly elevated total T3 levels.

Plasma concentrations of TH are regulated by a balanced action of synthesis, secretion, peripheral deiodination and metabolic inactivation. Normal TH homeostasis and action can be disrupted at several sites in the pathway [27]. Vinggaard et al. [36] found that prochloraz reduced T4 and thyroid stimulating hormone levels in rats. Prochloraz has also been shown to interfere with cell proliferation of TH-dependent rat pituitary GH3 cell line [40], suggesting inhibition of TH. The mechanism for the prochloraz-induced inhibition of TH levels found is unknown. It is speculated that the effect is due to prochloraz' effect on cytochrome P450 enzymes or through an effect on the CNS regulation of the hormones [36]. However, prochloraz in this study increased total T3 levels. Similarly, Larson et al. [19] found an increase in T3 in Ambystoma tigrinum exposed to the pesticide atrazine despite delayed metamorphosis. Wade et al. [69] found that various organochlorines reduced T4 levels at high concentration but increased T3 levels. How these compounds stimulate the thyroid hormones is still unknown. Possible pathways known to elevate T3 levels is an increase in corticosterone levels [70, 71] (see below), a decrease the conversion of T3 to T2 by 5-deiodinases increasing T3 concentrations [72, 69] or an increase of prolactin secretion that could antagonize the thyroid hormone [10, 14]. The elevated T3 levels seem paradoxal in the light of the delayed metamorphosis unless its effect is blocked, so the above explanations seem unlikely. However, various chemicals have been shown to inhibit T3 uptake into cells by transmembrane transporters [29, 73]. Moreover, a disruption of TH binding to transthyretin could, for example, strongly affect total and free concentrations of TH in the plasma and thereby the cellular uptake [10, 74]. Prochloraz may have a stimulatory effect on either transthyretin concentration or binding affinity for T3, elevating total levels, but making the hormone unavailable. Another possibility may be direct interference of prochloraz with the TH receptor. Certain chemicals block binding to the TH receptor or the interaction of the TR complex with its target [29, 75].

There is cross-talk between the steroid hormone axis and the thyroid axis, and testosterone and estradiol may affect development and growth by antagonizing the effects of TH [23, 55, 76-79]. Experimental results have been varied and may be stage-dependent [23], but most have reported an inhibition of the sex steroids on development. Administration of estradiol reduced plasma thyroid hormone
levels are known to be higher in females than in males [95] and may explain the large variation in concentrations in this study. No differences in mortality in the groups were observed. If the compounds had caused a stress response we might also have seen an earlier onset of metamorphosis instead of delayed.

**Thyroid Histology**

The thyroid tissue comprises mainly of follicle cells which consist of a single layer of epithelial cells occupying a lumen filled with proteinaceous colloid secreted by the epithelial cells. The TH is assembled in the follicular lumen from essential raw materials trapped by the follicular cells and stored as the colloid [52, 96]. In the nonstimulated thyroid tissue the follicles are large and their epithelial cells are squamous or cuboidal in appearance, the ratio of nucleus to cytoplasm is high and the colloid in the lumen homogenous [53, 96]. In the present study histological endpoints of the thyroid gland in tadpoles exposed to the endocrine disrupting chemicals ethinylestradiol and prochloraz were evaluated and the thyroids of all tadpoles including controls showed typical characteristics for an active secreting gland. A statistically significant difference was found between epithelial cell height of treated animals and controls. In anurans, increases in epithelial cell height (hypertrophy), a more columnar appearance of the epithelial cells, decreases in colloid volume and appearance of vesicles, as well as decrease in follicular cell number and size of follicles have been reported in response to increasing TH levels [24, 97, 98]. The follicle epithelium of control animals also showed columnar active cells. More pronounced hyperplasia in the high prochloraz group may represent abnormal activity. Follicular cell height is used as a morphological indicator of TSH response; the taller the cells, the higher the pituitary TSH activity [18]. When the height of the follicular cells is increased the follicular colloid in the lumen tends to be reduced by being absorbed by the follicular cells [18]. The changes in size and volume of follicular cells appear to be a function of an increased amount of endoplasmic reticulum, size of Golgi complex, number of mitochondria and cytosomes [18, 99, 100]. This change in ultrastructure reflects a more functionally active gland with also altered enzyme activity for thyroglobin and TSH synthesis [99].

Decreased TH levels and subsequent increased TSH levels also may cause hyperplasia in the pituitary [101, 102]. When the thyroid in unable to keep up with the demand, the follicular cells hypertrophy and cells divide, leading to hyperplasia (multiple layers of epithelium protruding into the lumen) [52]. Histological analysis of TH inhibited tadpoles have shown larger, disorganized, irregular or empty follicles and reduced colloid [17, 52, 98, 103, 104]. Moreover, the nuclei become basally located and ovoid in the follicular cells when activated and vacuoles become present in the follicular lumen and reduced colloid [52]. Histological analysis of TH inhibited tadpoles have shown larger, disorganized, irregular or empty follicles and reduced colloid [17, 52, 98, 103, 104]. Moreover, the nuclei become basally located and ovoid in the follicular cells when activated and vacuoles become present in the follicular lumen (hypertrophy), a more columnar appearance of the epithelial cells, decreases in colloid volume and appearance of vesicles, as well as decrease in follicular cell number and size of follicles have been reported in response to increasing TH levels [24, 97, 98]. The follicle epithelium of control animals also showed columnar active cells. More pronounced hyperplasia in the high prochloraz group may represent abnormal activity. Follicular cell height is used as a morphological indicator of TSH response; the taller the cells, the higher the pituitary TSH activity [18]. When the height of the follicular cells is increased the follicular colloid in the lumen tends to be reduced by being absorbed by the follicular cells [18]. The changes in size and volume of follicular cells appear to be a function of an increased amount of endoplasmic reticulum, size of Golgi complex, number of mitochondria and cytosomes [18, 99, 100]. This change in ultrastructure reflects a more functionally active gland with also altered enzyme activity for thyroglobin and TSH synthesis [99].

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Environmental Protection Agency produced thyroid follicular cell tumours in rodents [108]. Hyperplastic thyroid has also been described as having many microfollicles [51]. Microfollicles were not evident in this study. Neither was there any evidence of neoplasia. Many empty follicles were also observed in this study, but they where just as common in control animals as in treated animals and may be an artefact of the fixation procedure.

Thyroid gland morphology can be used to detect abnormal TH response, but the toxicological significance is unclear. In other words, it is not known where the threshold between compensatory effects and adverse effects is.

CONCLUSION

Anuran metamorphosis represents a valuable biological model to investigate the effects of endocrine disruptors on the TH system because the regulatory role of TH in amphibian metamorphosis has been extensively studied on all levels [28, 109-111]. Inhibition of metamorphosis has profound implications on the fitness of frogs and thyroid dysfunction is capable of producing a myriad of potentially deleterious effects because it is paramount for resorption of tail, limb development, organ development, morphogen activity, and maturation of the skin. Thus the possibility of a larva with thyroid dysfunction surviving in the wild may be very slim.

Amphibians are generally considered to be more sensitive to aquatic contaminants than other aquatic vertebrates. Here we showed that age and size at metamorphosis can be influenced by xenobiotic compounds. This is reflected in altered hormone levels and thyroid morphology. However, it is not clear if the changes in thyroid morphology are merely compensatory or may result in chronic alterations in TH levels. Likewise, it is not known at what threshold alterations in TH levels become adverse. More studies are necessary to answer these questions.

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