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 (T_3) 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. T_3 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 T_3 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.

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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

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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 (EE_2) 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-untered 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 where after they were randomly separated out into experimental groups. The tadpoles were raised in 10 l aerated tanks with approximately 150 tadpoles in each. The number of animals per tank was continuously reduced throughout the experimental period to about half the original number at the end of the experimental period, due to sampling and mortality.There were 7 experimental groups (Control, 6 ng EE_2/l, 61 ng EE_2/l, 115 ng EE_2/l, 11 µg Pro/l, 115 µg Pro/l and 252 µg Pro/l, actual values). Each group consisted of two replicate tanks. The actual concentrations were measured once by Liquid Chromatography Mass Spectrometry by a method described in Örn *et al*. [32]. The tadpoles were kept at 15°C on a 12:12 h light cycle and fed a diet of fish flakes and frozen spinach 3 times a week. The water in the tanks was renewed 3 times a week and subsequently the treatment solution was added. The ethinylestradiol and prochloraz concentrations were dissolved in 0,5 ml isopropanol/ 10 l. and the control group received only 0,5 ml isopropanol.

Days to Metamorphosis

All the metamorphs were sampled at the end of metamorphic climax at stage 44-45 [42]. The metamorphs were euthanized by immersion in 0,1 % MS 222, weighed, snap frozen in liquid nitrogen and stored at -80°C. The days to reaching this stage (days to metamorphosis) was noted.

Furthermore, to obtain information on tadpole growth, 8 tadpoles from each group was sampled at the beginning of the experiment and 5 times at regular intervals during the larval period until metamorphosis. The sampled tadpoles were randomly chosen to be representative of the group.

Thyroid Hormone and Corticosterone Measurement

Because of the small size of the *Rana temporaria* tadpoles and the difficulties associated with collecting sufficient amounts of blood, the whole-body content of the hormones was measured. This method has been used in several other studies [16, 43-46]. Niinuma *et al*. [16] demonstrated that removal of the thyroid gland did not appreciably affect whole-body T_3 or T_4 in tadpoles, suggesting that the whole-body measurements of TH reflect extra-thyroidal concentrations.

First, the whole body of tadpoles was weighed and homogenized without any liquid with a handheld mechanical homogenizer/dispersion instrument (Polytron PT1200, Kinematica Ag). The extraction method used was modified from a method described in Feist *et al*. [47]. Total triiodothyronine (T_3) and Corticosterone (Cort) were measured by enzyme immunoassay (ELISA) kits (DRG Diagnostics, Germany). Thyronine is not species specific and commercial assay kits can be used. The quality criteria for the application of kits produced for human plasma were verified by testing the values in a serial dilution. The working range of the tests was 0-10 ng/l for T_3 and 0-240 nmol/l for Cort. The T_3 kits cross-reacts with several other compounds with a likelihood of less than 0,0002.

Free T_3 was also measured from the extracted samples. It is generally considered that the activity of circulating thyroid hormones correlates better with the level of free hormone than total level [48]. However, this will depend on changes in binding of the hormone. Changes in free hormone levels have been found to follow the pattern of total hormone in plasma [48]. Also here an ELISA kit (DRG Diagnostics, Germany) was used, ranging from 0-19 pg/ml.

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 microscopeand 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) (df = degrees of freedom, n = number of animals) (Fig. **1**). Tadpoles exposed to 6 and 61 ng EE_2/l took on average 3,9 and 4,1 days longer until metamorphosis, while tadpoles exposed to 115 ng EE_2/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 EE_2 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 EE_2/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 T_3 levels in the tadpoles at metamorphosis. Tadpoles exposed to 252 μg prochloraz /l had higher total T_3 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 T_3 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).

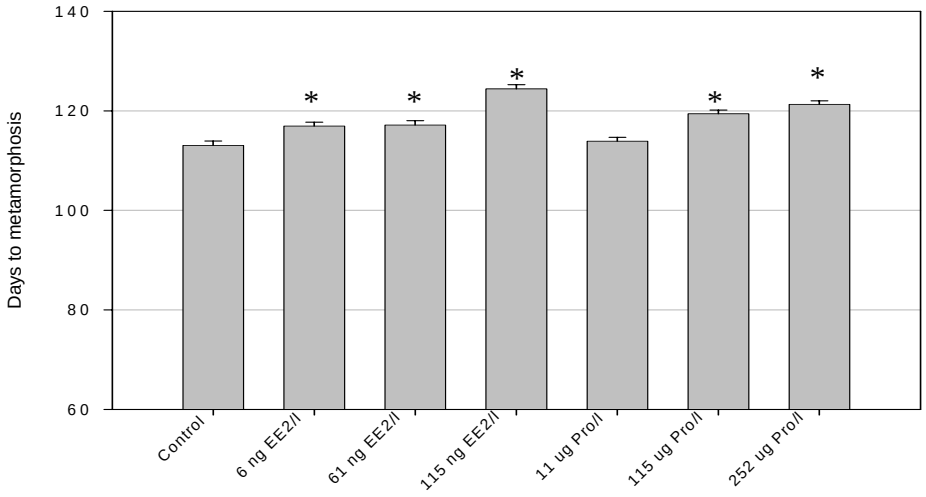

Fig. (1). Effect of different treatments on larval period of *Rana temporaria*(n = 141-189). Data are mean (± SEM) number of days from hatching to metamorphosis (Gosner stage 44-45). * indicates a significant difference from controls. Tadpoles exposed to ethinylestradiol (EE_2) and the highest doses of prochloraz (Pro) took significantly longer to reach metamorphosis compared with the control group.

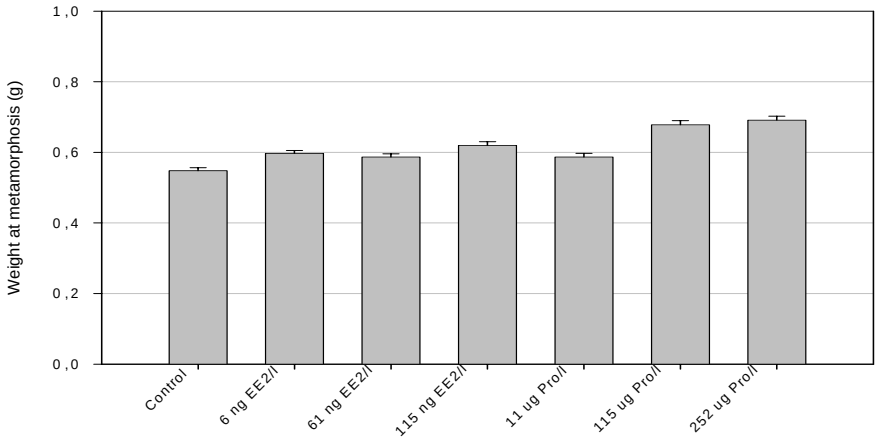

Fig. (2). Effect of different treatments on larval weight of *Rana temporaria* at metamorphosis (Gosner stage 44-45)(n = 141-182). Data are means (grams) (± SEM). * indicates a significant difference from controls. The tadpoles exposed to the highest doses of ethinylestradiol (EE_2) and prochloraz (Pro) were heavier at metamorphosis than controls.

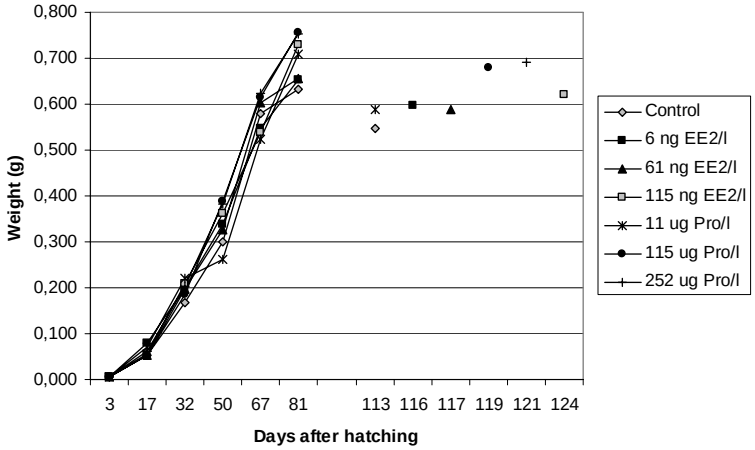

Fig. (3). Effect of ethinylestradiol (EE_2) and prochloraz (Pro) treatments on larval growth of *Rana temporaria* during the exposure period (n = 8/exposure group). Data are means (g) (± SEM). The points to the right (where lines are not connected to) are the average time for metamorphosis and weight at metamorphosis. The groups exposed to 115 ng EE_2/l, 115 µg Pro/l and 252 µg Pro/l are significantly heavier than the control group at day 81.

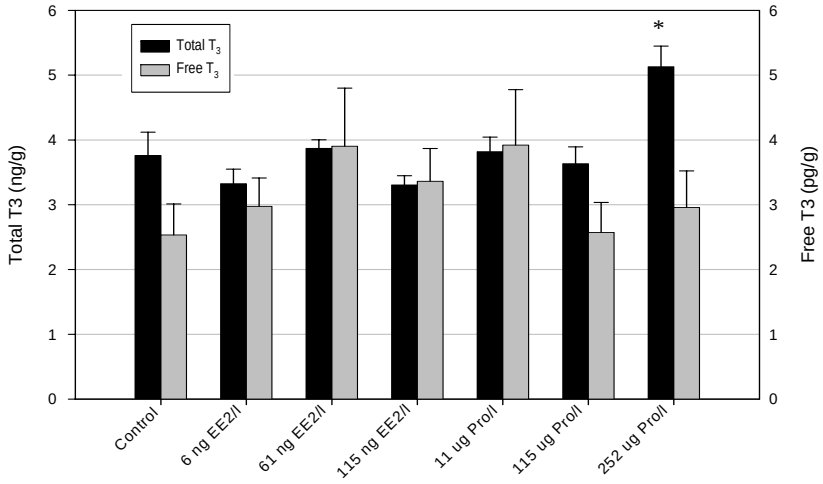

Fig. (4). Effect of different treatments on larval total and free Triiodothyronine (T_3) of *Rana temporaria* at metamorphosis (Gosner stage 44-45)(n = 11). Data are means (ng/g) (± SEM) for total T_3 and means (pg/g) (± SEM) for free T_3. * indicates a significant difference from controls. The tadpoles exposed to the highest dose of prochloraz (Pro) had higher total T_3 levels. There was no effect from treatment with ethinylestradiol (EE_2).

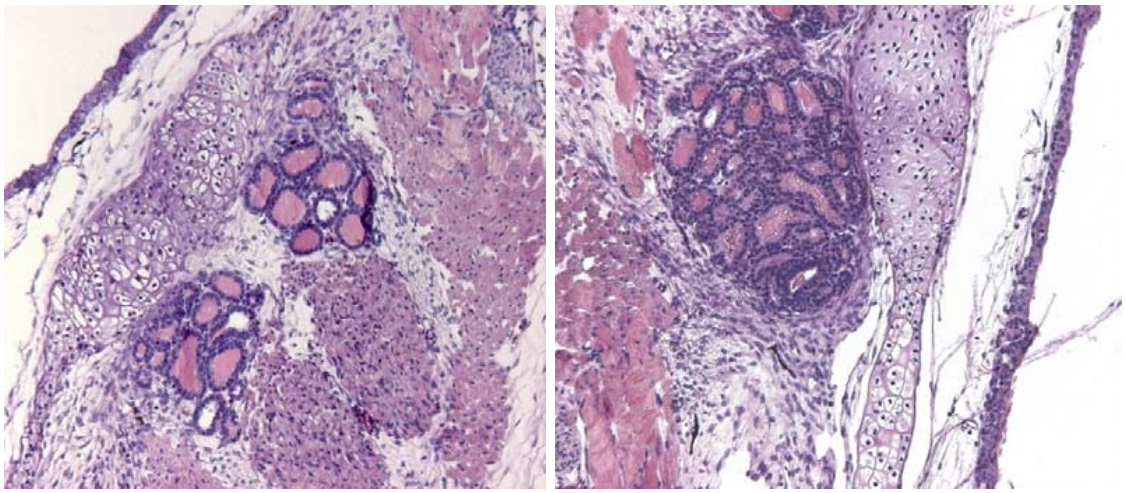

Fig. (5). Histological pictures of the thyroid gland of *Rana temporaria*. Control animal (**A**) and an animal from the 252 µg/l prochloraz group (**B**). Both have follicles with rather thick epithelia, follicular cell hyperplasia and colloid depletion. In the 251,5 µg/l prochloraz group (**B**) follicular cell hyperplasia appeared to be more extensive than the other groups.

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 follicles containing little or no colloid material. However, the changes described from an nonstimulated thyroid gland seemed more pronounced for the 252 µg Pro/l group.

Thyroid volume (per gram frog) did not differ between control and treatments ($P = 0{,}566$) (data not shown). Neither did percent colloid ($P = 0{,}113$) averaging 32,1 % or follicle number ($P = 0{,}006$) (data not shown). A statistically significant difference in follicular cell height, however, was evident (Fig. **6**). The follicular epithelial cells in the highest prochloraz exposed group (252 µg Pro/l) was slightly taller and more columnar in shape than in the other groups, while the cell height for the other exposure groups was decreased compared to controls ($P < 0{,}001$, $F = 12{,}348$, $df = 6$, $n = 65$-93).

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 E_2/l for 273 days caused initiation of metamorphosis at a younger age [55], while EE_2 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 EE_2 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]

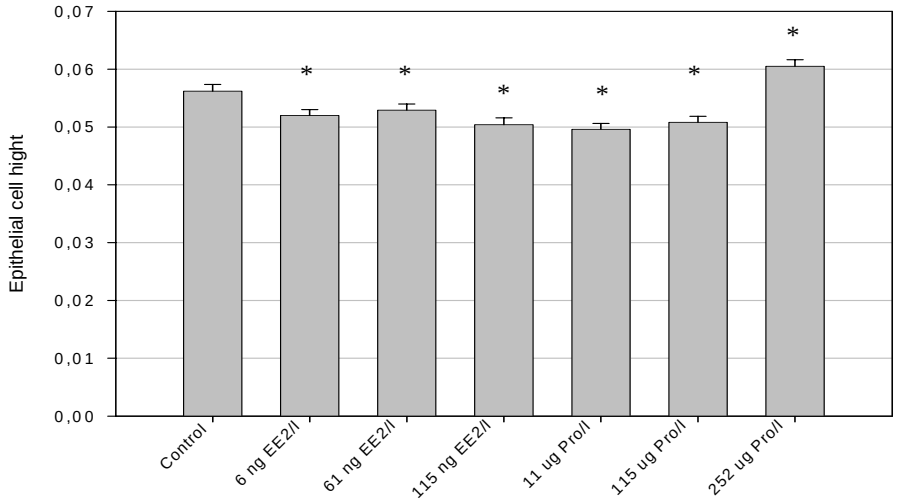

Fig. (6). Effect of treatments with ethinylestradiol (EE_2) and prochloraz (Pro) on thyroid follicle cell height in *Rana temporaria* at metamorphosis (44-45)(n = 65-93). Data are means (± SEM). * indicates a significant difference from controls. The tadpoles exposed to the highest doses of prochloraz had taller follicular cell height while the other exposure groups had lower cells compared to controls.

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 T_3. However, only tadpoles exposed to a high concentration of prochloraz had slightly elevated total T_3 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 T_4 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 T_3 levels. Similarly, Larson *et al*. [19] found an increase in T_4 in *Ambystoma tigrinum* exposed to the pesticide atrazine despite delayed metamorphosis. Wade *et al*. [69] found that various organochlorines reduced T_4 levels at high concentration but increased T_3 levels. How these compounds stimulate the thyroid hormones is still unknown. Possible pathways known to elevate T_3 levels is an increase in corticosterone levels [70, 71] (see below), a decrease the conversion of T_3 to T_2 by 5-deiodinases increasing T_3 concentrations [72, 69] or an increase of prolactin secretion that could antagonize the thyroid hormone [10, 14]. The elevated T_3 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 T_3 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 T_3, 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

concentrations in frogs [80], inhibited tail resorption [76] and at high concentrations induced developmental abnormalities and delayed organogenesis in tadpoles [78]. In a study by Park and Kidd [30] where ethinylestradiol was added to an experimental lake at a concentration of approx. 5ng/l it was found that growth and development was inhibited in the Green frog, *Rana clamitans*. However, in a couple of studies, estradiol has been found to accelerate metamorphosis [55, 81]. Many different mechanisms has been proposed how the sex steroids potentially regulate TH activity [23, 29, 76, 80, 82-84]. In this study EE_2 had apparently no inhibitory effect on TH but did increase time to metamorphosis consistent with the studies above. This point to a blockage of the function of TH by EE_2 rather than effects on synthesis or metabolism. Hogan *et al.* [61] suggests that EE_2 suppresses or blocks the ability of TH to induce the expression of target genes involved in mediating tissue-specific TH sensitivity during metamorphosis.

Moreover, disruption of normal thyroid function during larval development can have permanent effects on the reproductive system in frogs [23, 79, 85, 86]. In several studies disruption in the thyroid axis impaired testis or ovary differentiation and resulted in a skewed sex ratio [45, 85-87] while other studies have show no role of TH in gonadal differentiation [88]. In a parallel study to this prochloraz treated tadpoles showed higher percentage of male phenotypes at metamorphosis [41]. If this is a direct effect of the compound on the steroid hormone axis or gonads or the effect is mediated through TH is not known, however, but effects on the estrogen receptor and aromatase expression has been confirmed [79].

Compared to total T_3 levels of whole body homogenate reported from other studies the levels measured in this study by ELISA is comparable to or higher to those measured by RIA [16, 43, 44]. Total T_3 levels measured in whole body homogenates in different anuran species have been found to range between 0,009-10 ng/g depending on method, species and developmental stage [16, 89, 43-46]. Free T_3 levels has only been measured in plasma of *Rana catesbeiana* by equilibrium dialysis in the range of 5,6-6,9 pg/ml at climax compared to 570-780 pg/ml for total [48]. So approximately 1 % of the total hormone is in the free form [48, 90].

Amphibian larvae have the ability to accelerate metamorphosis in response to deteriorating conditions in their environment such as the presence of chemical contaminants as stressors [9,71]. Corticotropin-releasing hormone (CRH) from the hypothalamus controls the activity of both the thyroid and interrenal glands in amphibians [19]. However, experimental studies have yielded varied response where corticosterone has been shown to retard or accelerate metamorphosis [20-23]. Laboratory studies of aquatic animals exposed to many toxic substances often elicit hypersecretion in the interrenals as expected in response to noxious stimuli [91-93]. It might therefore be expected that the differences in time at metamorphosis would be reflected in the levels of corticosterone measured in the present work. However, measurement of corticosterone levels in whole-body homogenate did not reveal any significant differences between the exposure groups. However, the levels measured in this study correspond well with the levels measured by Glennemeier and Denver [94]. Moreover, corticosterone 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 singe layer of epithelial cells occupying a lumen filled with proteinaceus colloid secreted by the epithelial cells. The TH is assemblaged 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. However, 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 TH 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 apical end [97]. Some xenobiotics have caused similar effects in anurans [18, 26, 98, 105, 106]. Many of these reactions function to re-establish TH homeostasis and these effects are often reversible upon removal of the stimulus, at least in the early phases. However, if the stimulus continues it can lead to neoplasia, growth of adenomas or tumours (goiters) [107,108]. In a review from 1998, 24 pesticides out of 240 screened for carcinogenicity by the U.S.

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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