Endocrine disruption of the fungicide propiconazole in the frog *Xenopus tropicalis*

Effects on the aromatase activity and egg development

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Abstract

Amphibian populations around the world are decreasing, and endocrine disrupting chemicals have been suggested as a part of the problem. The fungicide propiconazole is used in agriculture in many countries. It works as a fungicide by inhibiting CYP51, affecting the fungi cell membrane, but it has also been proven to inhibit aromatase, CYP19. Aromatase converts androgens to estrogen and is important for sexual differentiation in the early development and in reproduction in vertebrates. The aim of this study was to investigate the effect of propiconazole on the aromatase activity in brains from the West African clawed frog (*Xenopus tropicalis*), after larval and adult exposure and on aromatase activity in ovaries after adult exposure. Furthermore, effects on egg development was determined after adult exposure.

Tadpoles were exposed to propiconazole (0, 25, and 250 µg/L) from three days after hatching until metamorphosis. In a former study, female adult *X. tropicalis* were exposed to 0 and 250 µg/L propiconazole during 4 weeks. The result of the aromatase activity measurements showed that 250 µg/L propiconazole increased brain aromatase activity after larval exposure. Brain and ovarian aromatase activity was also increased compared to the controls after adult exposure to 250µg/L propiconazole. The increased aromatase activity could reflect a compensatory response to decreased estrogen levels. Propiconazole had a significant impact on egg development, with an increased proportion of atretic oocytes and a decreased proportion of vitellogenic oocytes compared to the controls. The effect on egg development seems to be the result of a low estrogen level. The results suggest that exposure to propiconazole 250 µg/L could have a negative impact on *X. tropicalis* fertility. Further studies to determine impacts of environmental exposure concentrations are needed to evaluate the risk of azoles to amphibian reproduction.
**Introduction**

Amphibians are identified as the vertebrate group that is the most threatened. The IUCN (International Union for Conservation of Nature) evaluation, from 2008, of all today known amphibians, 6260 species, found over one third of them to be globally threatened (Vie et al. 2008). Also, 42.5% of the species are declining (Vie et al. 2008). It was at the First World Congress of Herpetology in 1989 that the problem of worldwide declining amphibian populations were first recognized (Stuart et al. 2004). There are many theories, for example Blaustein and Wake (1990) suggested that factors such as use as food or pets, exotic predators, destruction of habitats and pollution are parts of the problem. Other proposals are climate changes (Herman and Scott 1992), increased levels of ultraviolet radiation (Blaustein et al. 1995) and disease (Berger et al. 1998). Endocrine disrupting pesticides have also been suggested to be part of declines in amphibian populations (Hayes et al. 2003).

**Azoles**

Azole fungicides are a group of chemicals that are found in rivers and lakes worldwide (Battaglin et al., 2011, Khale et al., 2008, Castillo et al., 2000, Chau et al. 2015). The azoles act as fungicides by inhibiting the enzyme 14α-demethylase, encoded by CYP51, a gene in the cytochrome P450-family (Henry and Sisler 1984, Zarn et al. 2003), which is necessary for synthesis of ergosterol from lanosterol (Joseph-Horne and Hollomon 1997). Ergosterol is an essential constituent of the cell membrane in fungi (Zarn et al. 2003). The inhibition results in either cell death or a powerful growth inhibition (Roberts and Hutson 1999). However, the azoles do not specifically act on the fungi 14α-demethylase, but also bind to other CYP enzymes involved in steroid synthesis in different organisms, including vertebrates (Mason et al. 1987). In mammals lanosterol is involved in the development of male and female germ cells, as 14α-demethylase converts lanosterol to meiosis-activation sterols (Zarn et al. 2003).

Azoles’ endocrine disruptive properties and adverse effects on reproduction have been shown in zebrafish (Danio rerio) (Kinnberg et al. 2006), Japanese medaka (Oryzias latipes) (Sun et al. 2007) and the West African clawed frog (Xenopus tropicalis) (Olmstead et al. 2009). Exposing zebrafish to 0, 16, 64 and 202 µg/L prochloraz for 60 days, starting 24 h post fertilization, gave a sex ratio of 77% males and intersex gonads in 12 % of the individuals in the 202 µg/L exposure group (no intersex gonads were seen in the control group) (Kinnberg et al. 2006). In O. latipes exposed to 0, 1, 5, 25, 125 and 625 µg/L letrozole, fertility and hatching were significantly reduced at 25 µg/L (Sun et al. 2007). No hatching occurred at 125, and at 625 µg/L there were no spawning. The sex ratio was significantly biased towards males, 65 %, in the 25 µg/L letrozole exposure group (Sun et al. 2007). In a larval exposure study on X. tropicalis, testing fadrozole (0, 1, 4, 16 and 64 µg/L), all individuals in the 16 and 64 µg/L exposure groups were males (Olmstead et al. 2009). There are concerns that azoles also cause adverse effects in wild populations of roach (Rutilus rutilus) and perch (Perca fluviatilis) (Noaksson et al. 2001).

**Propiconazole**

Propiconazole (CAS-number 60207-90-01) is a chemical compound belonging to the azols (PPDB, 2013). The structures of azoles vary but they share the presence of a triazole (3 nitrogen and 2 carbons) or an imidazole ring (Roberts and Hutson 1999). They are used as
fungicides in agriculture and as pharmaceuticals to treat fungal infections (Henry and Sisler 1984). Some azoles are also used to treat human cancer (Zarn et al. 2003). Propiconazole is commonly used in agriculture to inhibit fungal growth in fruits, vegetables and cereals (Henry and Sisler 1984).

In agriculture it is common to use large amounts of propiconazole to cover widespread areas, and it can be applied several times per year. For example in Costa Rica, airplanes are used to spray propiconazole over large areas of banana plantations 10-20 times per year (Castillo et al. 2000). As other pesticides and fungicides, propiconazole can spread with run-off from treated areas and contaminate the environment (Battaglin 2011). The two most important factors controlling the proportion of the runoff are the time from the pesticide is applied to rainfall, and also intensity of pesticide use (Wauchope et al. 2004).

Propiconazole has been detected in US streams with a maximum concentration of 1.15 µg/L and mean concentration 0.29 µg/L, in an agricultural area and was found in 17% out of 29 investigated streams (Battaglin et al. 2011). In Swiss lakes, it has been measured to a maximum concentration 2.0 ng/L, mean concentration 0.053 ng/L, and was detected in 9 of 11 investigated lakes (Kahle et al. 2008). In a German agricultural area around Braunschweig, propiconazole has been detected in several streams with a maximum concentration 0.8 µg/L and a mean of 0.6 µg/L (Liess & Von der Ohe 2005). Neumann et al. (2003) found 5.5 µg/L in a water sample from an agricultural drainage channel, and 0.9 µg/L in a rainwater sewer located in the catchment area of the river Nette, Germany (Neumann et al. 2003). In an area of the Mekong Delta in Vietnam, propiconazole is one of the most commonly used fungicides and was found in almost 40% of all collected surface water samples, with a maximum concentration of 4.76 µg/L in surface water in the vicinity to rice cultivation (Chau 2015). Highest measured concentrations have been found near Costa Rican banana plantations. Mortensen et al. (1998) reported concentrations up to 24.20 µg/L in planttion drainage water collected after rainfall. Castillo et al. (2000) studied Suerte River in Costa Rica, a river that drains into the national conservation area Tortuguero. The study was conducted 1993-1997 and propiconazole was found in >40% of 21 samples from the conservation area, with concentrations up to 1.0 µg/L.

As most amphibians breed and deposit their eggs in water, in for example ditches, streams or lakes (Dyson and Gerhardt 2008) the risk of exposure to various contaminations are high, as well as for adults and the offspring. The larval stage has been identified as sensitive to endocrine disruption (reviewed in Hayes 1998). Also the stage when egg and sperm mature and are released should be considered as vulnerable to exposure to endocrine disrupting chemicals (Duellman and Trueb 1994, Säfholm et al. 2014).

**Aromatase**

Azoles are known to act as inhibitors on aromatase, also known as P450$_{arom}$ or CYP19 (Zarn et al. 2003, Milnes et al. 2006). Aromatase is an enzyme that catalyzes the last step in converting androgens to estrogens (Simpson et al. 1994). It is found among all vertebrates, such as birds, reptiles, amphibians, mammals and fish (Callard et al. 1978), and is expressed in various tissues like liver, gonads and brain (Milnes et al. 2006). Aromatization in the brain plays an important role in sex differentiation of the brain in the early development (Hutchison 1993). Aromatization regulates gonadal activities via the hormone gonadotropin and also
regulates male sexual behavior (reviewed in Lephart 1996, Balthazart and Ball 1998, and Forlano et al. 2006).

Previous in vitro studies using gonadal and/or brain tissue from fish (Oncorhynchus mykiss) and human placenta microsomes, have demonstrated propiconazole’s inhibitory effect on aromatase activity (Vinggaard et al. 2000, Hinfray et al. 2006). The study on O. mykiss using gonadal and brain microsomes, found equal inhibition of aromatase activity in microsomes from both organs (Hinfray et al. 2006). There are few in vivo studies conducted on propiconazole, but results from a study using adult fathead minnows (Pimephales promelas) found upregulation of the mRNA expression of CYP19 at 500 and 1000 µg/L. Besides the effects on CYP19 mRNA, an increase in atretic (degenerating) oocytes (1000 µg/L) and a decrease in egg production were seen (500 and 1000 µg/L), however no effect on fertility and hatching success were found in the deposited eggs (Skolness et al. 2013). To my knowledge the effects of propiconazole, on aromatase activity and ovary composition in amphibians, after developmental or adult exposure have not been characterized.

**The test species**

The test species used in the experiment is the West African clawed frog Xenopus tropicalis. More than twenty species of Xenopus is known today, naturally occurring in Africa, sub-Saharan (Hellsten et al. 2010). X. tropicalis used in laboratory originate from Nigeria and the Ivory Coast (Jafkins et al. 2011). X. tropicalis is a relative to Xenopus laevis, which have a long history as a model animal. For example, X. laevis normal development was described by Niewkoop and Faber in 1956 (Niewkoop and Faber, 1956) and the Frog Embryo Teratogenesis Assay: Xenopus (FETAX) was described as a standard protocol in 1998 (ASTM, 1998). Laevis is the most commonly studied Xenopus (Sparling et al. 2010), but due to its long generation time of 1-2 years it is not optimal for studies of developmental reproductive toxicity in life cycle studies (Berg et al. 2009).

X. tropicalis is an amphibian with several eligible properties: It is a small aquatic frog living in groups, easy to keep in aquaria (Song et al. 2003). The generation time is short compared to other amphibians; it takes approximately 4 months for males and 6 months for females to become sexually mature (Hirsh et al. 2002). They are able to produce embryos for more than a decade (Jafkins et al. 2012). A female generally lays more than 1000 eggs per mating occasion, and could be reused after a resting period of 2-3 months. These properties make X. tropicalis useful in life cycle studies (Hirsh et al. 2002). The species is also suitable for genetic studies due to its diploid genome. The genome consists of 1.5×10⁹ base pairs, the smallest known genome of tetrapods (Jafkins et al. 2012) that have been sequenced (Hellsten et al. 2010) and mapped (Wells et al. 2011).

X. tropicalis have shown to be a good model animal to study effects of endocrine disrupters on the reproductive system (Berg et al. 2009, Säfholm et al. 2014). The neuroendocrine system of anurans (frogs and toads), the hypothalamus-pituitary-gonadal and the hypothalamus-pituitary-thyroid axes, are similar to mammals. Also the androgens, testosterone and dihydrotestosterone are similar, in opposite to fish that have 11-ketotestosterone as main functioning androgen (Kloas and Lutz 2006).
The ovary of X. tropicalis

The ovary of adult female Xenopus is considered to be the frogs’ largest organ. The two ovaries, one right and one left, are divided into approximately 24 lobes containing hundreds of oocytes in different stages of maturation. Amphibian oogenesis, the process where oogonia (germal stem cells) transform to oocytes, is continuous in the adult ovary (Rasar and Hammes 2006). The oogenesis starts in the larva as the gonads begin to sex differentiate in NF-stage (Niewkoop and Faber 1994) 54-59 (Takase and Iguchi 2007).

The so-called vitellogenesis is an important part of the oocyte maturation. It is a process where vitellogenin produced in the liver is transformed to yolk and stored in yolk platelets in the oocyte (Rasar and Hammes 2006). The vitellogenin production is induced by estradiol-17β, which is produced by oocytes and follicle cells surrounding the oocytes (Gohin et al. 2011). The oocytes are divided in different stages of development. In the pre-vitellogenic stage I-II the oocytes are small (50-250 µ in diameter) and transparent (Dumont 1972). Studies have shown though, that vitellogenin is incorporated into the oocytes already at these stages (O’Brian et al. 2010). In the vitellogenic stage III-V the oocytes are growing larger as they are accumulating vitellogenin, also pigmentation starts. When the oocyte enters the post-vitellogenic stage VI, it has reached its maximum size, the yolk accumulation has stopped and the oocyte is ready for ovulation. Unovulated, degenerating oocytes, so called atretic oocytes are seen in the ovary as spheres of densely packed pigment (Dumont 1972).

Aims

The aim of this study was to determine the effects of propiconazole on aromatase activity in frogs, using the Xenopus tropicalis test system. The frogs were exposed to propiconazole at two life stages, larval period and adulthood. The goals were to 1) evaluate effects of the propiconazole exposure on the aromatase activity in ovaries and brain after adult exposure. 2) evaluate effects on aromatase activity in brain after larval exposure. 3) compare the sensitivity to propiconazole exposure between the different life stages. 4) evaluate effects of propiconazole on egg development in adult females.

Material and methods

Larval exposure

Propiconazole (CAS-number 60207-90-01) was purchased from Sigma-Aldrich, Saint-Louis, USA. Stock solutions of 12.5 g/L and 1.25 g/L were prepared, with acetone as a solvent. Exposure aquaria (38×26×22 cm) filled with 15 L of water, were saturated with the test compound 7 days before the experiment started. Concentrations of propiconazole were 25 µg/L and 250 µg/L and the concentration of acetone was 0.002% in all aquaria, including the control aquaria. In this pilot study, the chosen test concentrations were high compared to most measured environmental concentrations, though 25 µg/L are of environmental relevance, as it is close to the highest measured concentration in nature. 250 µg/L was chosen to facilitate comparisons to an earlier preformed exposure of adult X. tropicalis females, using 250 µg/L. 25 µg/L was chosen as a tenth of the high test concentration.
Five male and five female *X. tropicalis* (Xenopus 1, Dexeter, USA) were injected in the dorsal lymph-sack with a low dose (0.1 mL, 20 IU) of human chorionic gonadotropin hCG (Sigma-Aldrich, Saint-Louis, USA), which induces mating behavior in both sexes. The first dose was given one day prior to mating and a second, high dose of hCG (0.1 mL, 100 IU) was given to the frogs on the day of the mating. After the second injection, pairs of male and female *X. tropicalis* were placed in coated tanks, as mating naturally occurs during night time. They were observed every second hour. When the mating was over, after approximately 7 hours, the frogs were removed from the tanks.

All tadpoles used in the experiment originate from one mating. Three days after fertilization, at NF-stage 47 tadpoles were transferred to 9 exposure aquaria (15 L) with concentrations of 25µg/L and 250 µg/l propiconazole and controls, with 3 replicas of each concentration. The number of tadpoles was 65 per tank. Exposure was undertaken using semi static conditions, half of the water (7.5 L) was renewed five days per week. After water change, 150 µL propiconazole stock solution was added to the aquaria, and 150 µL acetone to the controls. They were exposed until completed metamorphosis, NF-stage 66, approximately 7 weeks.

The tadpoles were maintained in a 12:12 h light:dark cycle. The temperature was monitored daily. pH, conductivity, oxygen saturation, levels of nitrite and ammonia/ammonium were monitored weekly. The measurements of nitrite and ammonia/ammonium were done using standard kits from Sera (Gibbon, Sweden). The frogs were fed three times per day, weekends once, with Sera Micron (Sera, Heinsberg, Germany), Fish flakes (Sera, Heinsberg, Germany) and Tropical Fishfood Excel (Aquatic Nature, Roeseler, Belgium).

**Dissection of metamorphosed frogs**

Dissection took place at three occasions as the time to reach metamorphosis differed between individuals. Totally 45 metamorphosed frogs were anesthetized in 0.3 % tricaine (Sigma-Aldrich, Saint-Louis, USA) (dissolved in sodium bicarbonate buffer, pH 7) and killed by decapitation. Weight, length (snout to vent) was measured. They were opened from the ventral side, gonads were exposed and a preliminary sex determination was done, using a dissection microscope. Spheroid shaped gonads, ventral to the anterior half of the kidney, were classified as testis. The gonads were classified as ovaries if elongated and covering the whole length or parts of the kidneys. (Duellman and Trueb, 1994).

The gonad-kidney complex was put in formaldehyde 4 % with phosphate buffer, for later histological analysis and determination of gender. The brain was excised, snap frozen in liquid nitrogen and stored in a -80°C freezer.

**Exposure and dissection of adult female *X. tropicalis***

In a previous study, sexually mature female *X. tropicalis* (Xenopus 1, Dexeter, MI, USA) had been exposed via the ambient water in individual tanks to 0 µg/L or 250 µg/L propiconazole during four weeks (Brande-Lavridsen et al. in preparation). Ovarian tissue and brain was sampled, snap frozen in liquid nitrogen and stored in a -80 °C freezer.

**Aromatase activity**

The aromatase activity in brain and gonads was measured using a modified triated water-release method developed by LePhart and Simpson (1991) (Gyllenhammar et al. 2009). The
The principle is that isotope-labeled androstendione release 1β^-3[H] when aromatization occurs. The 1β^-3[H] is released into the waterphase and can be used to quantify the aromatase activity.

The tissue was homogenized with 300 µL buffer (10 nM KPO₄, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) (KCl: VWR, Radnor, USA, EDTA and dithiothreitol: Sigma-Aldrich, Saint-Louis, USA) and for the ovarian tissue, the homogenate was further diluted 1:10 with buffer. 5.2 µL of 0.96 µM 1β^-3[H] androstendione (Perkin-Elmer, Waltham, USA) dissolved in ethanol, was transferred to 2-ml tubes and was left to evaporate, duplicates were used. When no liquid was left, 5 µL propylene glycol (Sigma-Aldrich, Saint-Louis, USA), 45 µL buffer, 100 µL of homogenized tissue and 40 µL of 1 mM NADPH (100 mg NADPH dissolved in 25.26 mL 10 mM NaOH) (Sigma-Aldrich, Saint-Louis, USA and VWR, Radnor, USA) was added. The left over homogenate was put in a freezer to be used later for protein measurements. After shaking with Vortex, the tubes were incubated in 37° C for 30 minutes, with the lids opened. The reaction was stopped by placing the samples on ice and 100 µL of trichloroacetic acid, TCA 30 % (TCA 100% diluted in deionized water) (VWR, Radnor, USA) was added. The tubes were then centrifuged at 1500 g for 10 minutes. The supernatant was removed to a new tube. Unmetabolized androstendione was extracted by adding 1250 µL chloroform (VWR, Radnor, USA), shaken for 60 seconds and centrifuged during 25 minutes in 1500 g. The water phase was transferred to a new tube, 1250 µL water was added and the sample was centrifuged for 5 minutes (1500 g). 1 mL of the isotope-labeled supernatant was added to a tube containing 1 mL suspension of 5% charcoal (VWR, Radnor, USA) and 0.5% dextran T-70 (Sigma-Aldrich, Saint-Louis, USA). The sample was shaken for 40 seconds and centrifuged for 30 minutes at 10000 g. 1 mL of the 3H₂O was put in a scintillation container together with 4 mL of scintillation fluid (Perkin-Elmer, Waltham, USA) and the aromatase activity was then measured in a scintillation counter (Tri-carb 2100TR, CiAB Chemical Instruments AB, Lidingö, Sweden). The aromatase activity is expressed as fmol/h/mg protein.

**Protein content**

The protein content of the homogenate was measured using a BCA-kit (Nordic Biolabs AB, Stockholom, Sweden) and a spectrophotometer. The principle of the bicinchoninic acid, BCA, assay is based on the formation of a Cu²⁺-protein complex under alkaline conditions, followed by a reduction of Cu²⁺ to Cu⁺. The reduction occurs in the presence of protein in alkaline environments, resulting in BCA molecules forming a purple-blue complex with Cu⁺. The reduction is proportional to the amount of the protein present.

The common protein bovine serum albumin, BSA, was used to create a standard curve. Dilutions of BSA (Fisher Scientific, Waltham, USA), so-called standards, were prepared according to the BCA assay kit: 0, 500, 1000 and 2000 µg/L BSA diluted in BCA buffer (50 mL deionized water, 0.34 g KPO₄ and 0.019g EDTA) (EDTA: Sigma-Aldrich, Saint-Louis, USA). The protein homogenates from the aromatase measurement were diluted 5 times with BCA buffer. 10 µL BSA standards or homogenate were loaded, in triplets, in a 96 well plate. 200 µL of so-called working reagent (Pierce, Waltham, USA) from the kit were added to each well. The well plate were covered with laboratory film and incubated in room temperature for 30 minutes. The plate was read spectrophotometrically in a Wallac workstation (Victor3 1420 Multilabel counter, Perkin-Elmer, Waltham, USA). An equation was obtained from the standard curve and was used for calculation of the protein content.
Histology of adult female X. tropicalis ovary

Ovarian tissue from totally 14 adult females, 7 exposed and 7 controls, was dehydrated in increasing concentrations of ethanol (70%, 95% and 99%) and then embedded in hydroxyethyl methacrylate (Leica Historesin). A thin 2 µm-layer was cut out at three levels of one ovary per individual and stained with hematoxylin-eosin. The three sections per individual was put on coded slides. One section per individual was evaluated by counting the number of oocytes in different stages, a method described by Hausen and Riebesell (Hausen and Riebesell 1991). The oocytes were divided into pre-vitellogenic (stage I-II), vitellogenic (stage III-V), post-vitellogenic (stage VI) and atretic (degenerating). The result is presented as distribution of oocytes in various stages.

Statistics

Statistics was performed using GraphPad Prism 5.04 (GraphPad Software, San Diego, USA). For the metamorphosed frogs, length, weight and time to metamorphosis, lacked normal distribution and was analyzed using Kruskal-Wallis, with Dunn’s Multiple Comparison test as post-hoc. Fisher’s Exact test was used to compare sex ratio and mortality. Unpaired t-test was used to test for sex differences in brain aromatase activity in the control. Treatment related effects on brain aromatase activity were compared using one-way Anova and Tukey’s Multiple Comparison test.

For the adult females, unpaired t-test was used to compare the treatment groups with respect to aromatase activity in brain. The Mann-Whitney test was used to compare the aromatase activity in ovaries, as this groups failed the normal distribution test. The frequency of oocytes in different stages was compared between the exposure groups using unpaired t-test, except for the comparison of the atretic stage. The atretic stage failed the normal distribution test, and therefore the Mann-Whitney test was used.

Results

Health status and testing conditions in the larval exposure

The mortality was 54 % for controls, 43 % for 25µg/l exposure group and 46 % for the 250 µg/L exposure. The mortality was significantly higher in the control group compared to the 25 µg/L exposure group. There were no visual signs of acute toxicity, causing for example weight loss or behavioral changes.

The temperature were 26.4 ± 0.5 °C (mean ± SD), pH 8.0 ± 0.3 (mean ± SD), conductivity was between 472-504 µS/cm and oxygen saturation 78 ± 15% (mean ±SD in %). The nitrite levels were 0.0- >25.0 mg/L (at 3 dates no exact value were given for 12 aquaria above range, >25 mg/L, and 1 date no exact value were given for 8 aquaria >5 mg/L) and ammonia levels were 0.0- >10.0 mg/L (at one date no exact values were given for three aquaria above range, >10 mg/L). The measured levels of nitrite and ammonia/ammonium are showed in appendix A.
Weight, length and time to reach metamorphosis

The time to reach metamorphosis, NF-stage 66, was (mean ± SD) 46 ±9 days for the controls, 43 ±6 days for the 25 µg/L group and 44 ±6 days for the 250 µg/L group. There were no significant differences between the groups regarding the time to reach metamorphosis. There were no differences between groups in sex ratio, as determined by gross gonadal morphology (figure 1) or in body weight and length (table 1).

![Sex ratio %](image)

**Figure 1.** Sex ratios based on gross gonadal morphology in metamorphosed (N.F. 66) *X. tropicalis* after larval exposure of propiconazole. Numbers of individuals are shown above the bars.

**Table 1.** Weight and length of metamorphosed *X. tropicalis* exposed to propiconazole between N.F. stages 47-66. Data are presented as mean ± SD.

<table>
<thead>
<tr>
<th>Propiconazole (µg/L)</th>
<th>Weight (g) mean ± SD</th>
<th>Length (cm) mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (n=15)</td>
<td>0.45 ± 0.06</td>
<td>1.56 ± 0.06</td>
</tr>
<tr>
<td>25 (n=15)</td>
<td>0.43 ± 0.09</td>
<td>1.52 ± 0.06</td>
</tr>
<tr>
<td>250 (n=15)</td>
<td>0.45 ± 0.12</td>
<td>1.56 ± 0.12</td>
</tr>
</tbody>
</table>

Aromatase activity in metamorphosed *X. tropicalis*

There were no significant sex differences in aromatase activity in the control group (figure 2). Aromatase activity in brains from metamorphosed *X. tropicalis* was significantly increased in the 250 µg/L group compared to the control (figure 3).
Figure 2. Aromatase activity in brains from metamorphosed (NF 66) *X. tropicalis*, females and males from the control group. Sex was determined by gross gonadal morpholgy. Each point represents one individual and the number of individuals is presented in the figure. Mean value ± SD is shown. Unpaired t-test.

Figure 3. Aromatase activity in brains from metamorphosed (NF 66) *X. tropicalis* after larval exposure to propiconazole. Each point represents one individual, mean value of the exposure group and ± SD is shown. ** = significantly different from the control group (p<0.01), one-way Anova and Tukey’s Multiple Comparison test.

**Aromatase activity in adult female *X. tropicalis***

Aromatase activity in ovaries and brains from adult females was significantly increased in propiconazole exposed animals compared to control (figure 4 and 5).
Figure 4. Aromatase activity in ovaries from X. tropicalis females after adult exposure to propiconazole for 4 weeks. Each point represents one individual and the number of animals is shown in the figure. Mean value of the group and ± SD is shown. ** = significantly different from the controls (p<0.01), the Mann-Whitney test.

Figure 5. Aromatase activity in brains from X. tropicalis females after 4 weeks of adult exposure to propiconazole. Each point represents one individual and the number of animals is presented in the figure. Mean value of the group and ± SD is shown. ** = the propiconazole group is significantly different from the controls (p<0.01), unpaired t-test.

Histology of ovary from adult female X. tropicalis

Evaluation of the ovarian histology showed that treated females had a significantly reduced proportion of vitellogenic oocytes and an increased proportion of atretic oocytes compared to the controls (table 2).
Table 2. Distribution of oocyte stages in ovary of female *X. tropicalis* exposed to propiconazole (0 or 250 µg/L), presented as mean (± SD). * = significantly different from control (P˂0.05), Mann-Whitney test. ** = significantly different from control (P˂0.01), t-test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-vitellogenic oocytes</th>
<th>Vitellogenic oocytes</th>
<th>Post-vitellogenic oocytes</th>
<th>Atretic oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=7)</td>
<td>58 (10)</td>
<td>36 (8)</td>
<td>5 (3)</td>
<td>0.82 (0.37)</td>
</tr>
<tr>
<td>Propiconazole (n=7)</td>
<td>66 (6)</td>
<td>25 (3)**</td>
<td>6 (2)</td>
<td>3.23 (2.13)*</td>
</tr>
</tbody>
</table>

Discussion

This pilot study is to my knowledge the first to investigate effects of propiconazole exposure in amphibians. As propiconazole is commonly found in water samples worldwide (Khale *et al.* 2008, Battaglin 2011, Chau 2015), it is of interest to study effects in reproduction in aquatic and semi aquatic species. Environmental concentrations of propiconazole are considered as low, often <6 µg/L (Neumann *et al.* 2003, Leiss and Von der Ohe 2005, Khale *et al.* 2008, Battaglin 2011, Chau 2015), but 24.20 µg/L have been measured in a water sample from a plantation drainage canal (Mortensen *et al.* 1998). Even though propiconazole is not found in high environmental concentrations, it is one of several azole fungicides that are frequently found in water, contributing to the mix of chemicals that aquatic organisms are commonly exposed to (Khale *et al.* 2008, Battaglin 2011, Chau 2015).

The results from this study showed that both developmental and female adult exposure to 250 µg/L propiconazole increased the brain aromatase activity compared to the control. Adult exposure also increased the ovarian aromatase activity compared to control. In adult females, egg development was affected, showing increased frequency of atretic oocytes and decreased frequency of vitellogenic oocytes compared to control.

In the present study, the mortality of the tadpoles could be perceived as high, with 54% overall mortality in the control group, 43 % in the 25µg/L and 46 % for 250 µg/L exposure. On the other hand, the *X. tropicalis* life strategy, with thousands of deposited eggs during each spawning, and no parental care, indicate that mortality at this level might not be unusual during early life stages. The mortality was significantly higher in the control group compared to the 25µg/L group. The reason for this is unclear, and could not be explained other than a coincidence. It seems as the tadpoles were most vulnerable in the beginning of their life, as 75% (controls and 250 µg/L exposure) and 94 % (25µg/L exposure) of all deaths occurred during the two first weeks of exposure. Two periods of increased mortality was seen; during the first day of exposure, 23-29 % of the tadpoles died (25 % of controls, 29 % of 25 µg/L exposure and 23 % of 250 µg/L exposure). These deaths could possibly be stress-related, as the tadpoles were transferred to exposure aquaria the day before. The other event of increased mortality took place during day 9-13 of the exposure, with a mortality of 44 % (controls and 250 µg/L exposure), and 57 % (25 µg/L exposure). This mortality might have been caused by disease, or the tadpoles entering a sensitive stage of the development. No correlations between mortality and increased nitrite or ammonia/ammonium levels could be found.
Previous studies have found changes in sex ratio in both fish and amphibian after
developmentalazole treatment (Kinnberg et al. 2006, Sun et al. 2007, Olmstead et al. 2009,
Chardard and Dournon, 1999). The preliminary results in the present study did not find any
skewed sex ratio, as determined by gross gonadal morphology. However, more detailed
evaluations of the gonads using histological methods are needed in order to determine effects
such as intersex or other malformations.

The result regarding the increased brain aromatase activity in metamorphosed X. tropicalis
differs from a previous study on the imidazole clotrimazole (0, 1.72, 17.2 and 172 µg/L), in
which an inhibition of brain aromatase in X. tropicalis tadpoles exposed to 172 µg/L
clotrimazole has been shown at stage 56, whereas no effect on brain aromatase activity was
seen at metamorphosis (Gyllenhammar et al. 2009). These results suggest that depending on
when brain aromatase is analyzed, different effects can be seen. The different results at
metamorphosis might be due to differences in chemical properties of clotrimazole and
propiconazole.

Brain aromatase activity was compared between control females and males, as determined by
gross gonadal morphology. No sex difference in brain aromatase activity was found in the
control group. As there were no sex difference in brain aromatase activity in the control
group, and the histological sexing of the metamorphosed X. tropicalis was not yet performed,
which might differ from the macroscopic determination; I chose to test all individuals in each
exposure as one group. Further evaluations will show if there are gender differences in brain
aromatase activity in metamorphosed X. tropicalis after exposure to propiconazole.

The present results show that aromatase activity in brain and ovaries in adult female X.
tropicalis was increased after exposure to 250 µg/L propiconazole. In a study by Ankley et al.
2005, testing prochloraz using adult fathead minnows (Pimephales promelas), brain
aromatase activity in females was unaffected at concentrations up to 300 µg/L. However,
decreased levels of plasma estradiol (at 300 µg/L) and plasma vitellogenin (at 100 and 300
µg/L) were seen (Ankley et al. 2005). In a 21-day study testing propiconazole (0, 5, 50, 100
and 1000 µg/L) in female P. promelas, mRNA expression of several genes involved in
endocrine function of the gonads, for example CYP19a1a, were tested (Skolness et al. 2013).
CYP19a1a, the gene encoding gonadal aromatase in fish (Chiang et al. 2001) was upregulated
in ovarian tissue at 500 and 1000 µg/L propiconazole. Other upregulated genes in female P.
promelas gonadal tissue were CYP11, CYP17 and fshr (follicle stimulating hormone receptor)
(Skolness et al. 2013). Plasma estradiol and vitellogenin was also investigated in female P.
promelas, the estradiol levels in females were decreased at 500 and 1000 µg/L and the
vitellogenin were decreased at 50, 500 and 1000 µg/L (Skolness et al. 2013). The
upregulation of CYP19a1a in adult female P. promelas ovarian tissue is thought to be a
response and/or an adaption to compensate for the aromatase inhibition of estradiol
production caused by propiconazole (Skolness et al. 2013). The result in the present study
suggests a compensatory effect of propiconazole also on the enzyme activity, as a result of
low estrogen levels.

In the present study the results of the oocyte staging showed a decrease in the proportion of
vitellogenic oocytes and an increase of atretic oocytes in the adult frogs. This indicates there
has been an inhibition of aromatase, decreasing the estradiol production, as seen in other
studies of azoles in female P. promelas (Ankley et al. 2005 and Skolness et al. 2013). A
reduced estradiol level can reduce the liver synthesis of vitellogenin. Estradiol in combination with gonadotropin prevents the conversion of pregnenolone to progesterone in follicle cells, thereby preventing atresia (Gohin et al. 2011). Depletion of estradiol might therefore cause an increased proportion of atretic oocytes. The result from the present study is partly confirmed by studies of egg development in female fish, *P. promelas* after adult exposure to azoles by Ankley *et al.* (2005) and Skolness *et al.* (2013). They found increased oocyte atresia in adult female *P. promelas* exposed to prochloraz 300 µg/L (Ankley *et al.* 2005) or propiconazole at 500-1000 µg/L (Skolness *et al.* 2013). They did not find any effects on the proportion of vitellogenic oocytes (Ankley *et al.* 2005 and Skolness *et al.* 2013), which might indicate that *X. tropicalis* is more sensitive to propiconazole exposure than the fish *P. promelas*. The result suggests that exposure to propiconazole 250 µg/L could impair egg development and thereby decrease fertility in *X. tropicalis*.

**Conclusions**

The increased aromatase activity shows that propiconazole exposure affects the hormone system in *X. tropicalis*. The increase in aromatase activity was probably a response to compensate for low estrogen levels. Both larval period and adulthood is sensitive periods for effects of propiconazole exposure on the hormone system. Adult exposure to propiconazole can impair the egg development, suggesting a negative impact on fertility. Further investigation to determine impacts at environmental exposure concentrations is warranted to elucidate the risk of azoles to amphibian reproduction.

**References**


Hinfray, N., Porcher, J-M., Brion, F. 2006. Inhibition of rainbow trout (Oncorynchus mykiss) P450 aromatase activities in brain and ovarian microsomes by various environmental substances. Comparative Biochemistry and Physiology, part C 144:252-262.


Appendix A. Levels of nitrite, NO₂, and ammonia/ammonium, NH₃/NH₄ measured weekly. — indicating that the aquarium is not in use.

<table>
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<th>Aquaria/date</th>
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<th>150302</th>
<th>150309</th>
<th>150316</th>
<th>150323</th>
<th>150330</th>
<th>150406</th>
<th>150413</th>
<th>150420</th>
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<td>NO₂ (mg/L)</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1 (control)</td>
<td>0.0</td>
<td>1.0</td>
<td>0.0</td>
<td>0.5</td>
<td>1.0</td>
<td>&gt;5.0</td>
<td>&gt;25</td>
<td>&gt;25</td>
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<td>2.5</td>
</tr>
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<td>—</td>
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<tr>
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</tr>
<tr>
<td>4 (25 µg/L)</td>
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<td>1.5</td>
<td>0.0</td>
<td>&gt;5.0</td>
<td>&gt;25</td>
<td>&gt;25</td>
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<tr>
<td>5 (25 µg/L)</td>
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<td>&gt;25</td>
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<tr>
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<td>2.0</td>
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<td>5.0</td>
<td>&gt;5.0</td>
<td>&gt;25</td>
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<tr>
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<td>&gt;25</td>
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<tr>
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<td>1.5</td>
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<tr>
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<tr>
<td>8 (250 µg/L)</td>
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<tr>
<td>9 (250 µg/L)</td>
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