Endocrine Disruption in Amphibians

Developmental Effects of Ethynylestradiol and Clotrimazole on the Reproductive System

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Abstract


Amphibian populations are declining world-wide and one of the suggested reasons is environmental pollutants. Studies of long-term effects on the reproductive system in frogs following larval exposure to environmental pollutants are scarce. It is therefore important to develop methods to study developmental reproductive toxicity in amphibians. In this thesis the usefulness of *Xenopus tropicalis* (the West African clawed frog) as a model species for a test system was investigated. Effects on the reproductive system after larval exposure to the pharmaceuticals ethynylestradiol (EE) and clotrimazole were evaluated. The susceptibility to EE exposure was compared between the model species and a wild species, the European common frog (*Rana temporaria*). Larval exposure to EE caused female-biased sex ratios in both examined frog species, indicating male-to-female sex-reversal. In adult *Xenopus tropicalis*, male frogs that were not sex-reversed had reduced fertility and decreased amount of mature spermatozoa in the seminiferous tubules. The proportion of frogs with ovaries but lacking oviducts increased with increasing EE2-concentrations. A female frog without oviducts is sterile. The development of ovaries in sex-reversed male frogs was implied to be similar to control females. The combination of a reduced number of males, due to sex-reversal, and impaired fertility could have severe effects on frog populations. Larval exposure to clotrimazole modulated aromatase activity in gonads and brain in *Xenopus tropicalis*. Brain aromatase activity was decreased at the time for gonadal differentiation and gonadal aromatase activity was increased at metamorphosis. The findings in this thesis indicate that reproduction in wild frogs might be impaired by estrogenic compounds in the environment. The results combined with the short generation time supports the use of *Xenopus tropicalis* as a model species when evaluating long term effects of endocrine disruptors on the reproductive system in amphibians.

Keywords: Test system, Xenopus tropicalis, Rana temporaria, sex ratio, sex differentiation, fertility, aromatase, ovary, testis, oviduct

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List of papers

The present thesis is based on the following papers, which will be referred to by their Roman numerals:


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* Irina Gyllenhammar, formerly Pettersson.
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>CYP</td>
<td>Cytochrom P450</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DES</td>
<td>Diethylstilbestrol</td>
</tr>
<tr>
<td>EDC</td>
<td>Endocrine disrupting chemical</td>
</tr>
<tr>
<td>EE2</td>
<td>Ethynylestradiol</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>GSI</td>
<td>Gonadosomatic index</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>LOEC</td>
<td>Lowest observed effect concentration</td>
</tr>
<tr>
<td>NF</td>
<td>Niewkoop and Faber</td>
</tr>
<tr>
<td>OviSI</td>
<td>Oviducalsomatic index</td>
</tr>
<tr>
<td>PBDE</td>
<td>Polybrominated diphenyleter</td>
</tr>
<tr>
<td>PC</td>
<td>Pars convoluta</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCDF</td>
<td>Polychlorinated dibenzofuran</td>
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</tbody>
</table>
Introduction

During the last decades amphibian populations have dramatically declined all around the world (Griffiths and Beebee 1992; Houlahan et al. 2000; Alford et al. 2001). Today one third of the amphibian species are reported to be threatened (Stuart et al. 2004). There are several possible reasons for this decline and one of them is environmental pollutants (Alford and Richards 1999; Corn 2000; Gibbons et al. 2000; Gardner 2001; Kiesecker et al. 2001; Beebee and Griffiths 2005). Amphibians have a very important role in the functioning of ecosystems as both prey and predators.

Observations of high incidences of hermaphroditic male frogs with testicular oocytes and skewed sex ratio towards males in wild populations of frogs in the U.S. have been suggested to result from exposure to environmental pollutants (Reeder et al. 1998; Hayes et al. 2003). This raised the question of whether disrupted sex differentiation is a large-scale problem among amphibians and if this phenomenon is caused by contaminants. Studies of long-term effects of larval exposure to environmental pollutants on the reproductive system in frogs are scarce. It is therefore important to develop methods to study developmental reproductive toxicity in amphibians.

Endocrine disruption

Many environmental pollutants can interfere with development and function of the endocrine system and have in some cases been linked to reproductive disorders in both wildlife and humans (Helle et al. 1976; Guillette et al. 1994; Toppari et al. 1996; Jobling et al. 1998; Toft et al. 2003; Olesen et al. 2007). These compounds are called endocrine disrupting chemicals (EDCs), defined as exogenous substances that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function. Hormones play an important role during reproduction and development, and during these processes the organism is most sensitive to EDCs. The main recipient of EDCs is surface waters, making fish and frogs the main potential targets among vertebrates. Exposure to EDCs can result in organisational or activational effects. Organisational effects causes permanent changes and can only be induced at critical periods during the development. Activational effects are transient and can occur throughout life. Exposure to EDC in adult animals might for example induce activational effects that disappear if the
exposure ends. However, if the exposure occurs during critical periods of early development it can cause irreversible structural and functional effects, e.g. on sex organ development and reproductive behaviour. There is a need to develop test systems in order to evaluate consequences of EDC exposure in wild animal species, and especially long-term effects after exposure during the critical early life-stages.

Endocrine disruption in amphibians

It is known that larval exposure to estrogenic compounds disrupt gonadal differentiation which results in female-biased sex ratios in amphibians (Chang and Witschi 1956; Richards and Nace 1978; Kloas et al. 1999; Mackenzie et al. 2003). In a whole lake experiment with wild Rana septrionalis it was shown that 28.6% of the first year tadpoles had testicular oocytes after exposure to 17 pM EE2 (corresponding to 5 ng/L). In the reference lakes no intersex gonads were found (Park and Kidd 2005). It is also known that larval exposure to androgens can cause male-biased sex ratios in amphibians (Saidapur et al. 2001; Bögi et al. 2002). Metamorphosis, the transformation of a tadpole into a frog, is stimulated by thyroid hormones. In laboratory studies on tadpoles, exposure to mixtures of polychlorinated biphenyls (PCB), and to a single polybrominated diphenylether flame retardant (PBDE-99), caused effects that can be linked to thyroid disruption (Gutleb et al. 2000; Lehigh Shirey et al. 2006; Carlsson et al. 2007). There are also examples of EDCs that affect both the thyroid system and gonadal differentiation. Environmentally relevant concentrations of ammonium perchlorate (0.5 μM, 59 μg/L), a compound used as an oxidizer in rocket fuels by the military and aerospace industry, has been shown to inhibit thyroid function and cause a female biased sex ratio in Xenopus laevis (Goleman et al. 2002). Exposure to the pesticide methoxychlor (0.3 μM, 100 μg/L) resulted in a female biased sex ratio, in Xenopus tropicalis (Fort et al. 2004b) while lower concentrations (0.03 μM) caused delayed development and enlarged thyroid glands.

In wild frogs, findings of testicular oocytes and skewed sex ratios towards males have been proposed to be linked to environmental pollution (Reeder et al. 1998; Hayes et al. 2003; Reeder et al. 2005; McDaniel et al. 2008). In cricket frogs (Acris crepitans) from Illinois, USA, male-biased sex ratios were correlated to areas contaminated with the industrial chemicals PCB and polychlorinated dibenzofuran (PCDF) (Reeder et al. 1998). A later study with Cricket frogs from museum collections in Illinois, showed that the frequency of testicular oocytes correlated with major trends in exposure to PCBs and the pesticide p,p-dichlorodiphenyltrichloroethane (DDT) throughout the century (Reeder et al. 2005). McDaniel et al. (2008) evaluated Northern leopard frogs (Rana papiens) in southwestern Ontario, USA, and
found that the occurrence of testicular oocytes was significantly higher in agricultural areas (42%) than in reference sites (7%). From field studies in agricultural areas of the Midwestern USA, it was reported that up to 92% of wild male *Rana pipiens* had testicular oocytes (Hayes et al. 2003). Hayes *et al.* associated the feminised frogs in the wild with exposure to the pesticide atrazine and have in laboratory experiments shown that low concentrations of atrazine (0.1 μg/L) affect testicular development, in *Xenopus laevis* and *Rana pipiens* (Hayes *et al.* 2002; Hayes *et al.* 2003). Giesy and collaborators were, however, unable to confirm the results from Hayes’s group (Coady *et al.* 2005; Murphy *et al.* 2006).

**Test species**

Two amphibian species have been used in this thesis: the West African clawed frog, *Xenopus (Silurana) tropicalis* and the European common frog, *Rana temporaria*. *Xenopus tropicalis* has several advantages as a model species in amphibian tests on endocrine disruption of the reproductive system. It is a small aquatic frog that is easy to keep and breed (all year round) in aquaria. It also has a short generation time, as metamorphosis is reached in 40-50 days and sexual maturity as soon as 4-6 months after metamorphosis (Hirsch *et al.* 2002; Song *et al.* 2003; Grammer *et al.* 2005). The South African clawed frog, *Xenopus laevis*, which is related to *Xenopus tropicalis*, has been widely used in amphibian studies. However, *Xenopus laevis* is larger and has a longer generation time (1-2 years) and is therefore not suitable for life cycle studies (Hirsch *et al.* 2002; Song *et al.* 2003). The normal larval developmental process, with 66 developmental stages from fertilization until completed metamorphosis, has been defined for *Xenopus laevis* (Nieuwkoop and Faber 1956). These developmental stages are found to be applicable also on *Xenopus tropicalis* (Fort *et al.* 2004a; Fort *et al.* 2004b; Mitsui *et al.* 2006).

In order to assess the risk for endocrine disruption in European frog populations there is a need to investigate the sensitivity of frogs native to Europe. The European common frog, *Rana temporaria* is a terrestrial species, widespread in Europe, which could be useful as a test species. Therefore, also this species was examined in this thesis.

**Sex determination and differentiation in amphibians**

Sex determination in amphibians is thought to be controlled genetically, although environmental factors can affect the sex determination and differentiation (Schmid and Steinlein 2001). There are uncertainties about the mechanisms by which gonadal sex differentiation is controlled (Hayes 1998;
The sex differentiation is likely initiated by sex determination genes. Steroid hormone exposure can affect gonadal differentiation resulting in genotypic males developing ovaries and genotypic females developing testes (reviewed in Hayes 1998). Therefore, all individuals may have the ability to develop into either sex. In most amphibians the gonadal differentiation starts during the larval development (Lofts 1974). In this thesis, sex ratios and disrupted sex organ morphology were used as endpoints for endocrine disruption.

Gonadal and oviducal differentiation in *Xenopus*

In *Xenopus laevis* the females are considered to be heterogametic (ZW) and the males homogametic (ZZ) (Chang and Witschi 1955), which could be expected also in *Xenopus tropicalis*. The sex chromosomes are not morphologically distinguishable. In *Xenopus tropicalis*, the gonadal sex differentiation occurs between larval developmental stage (Nieuwkoop and Faber 1956) NF 54 and 59 (Takase and Iguchi 2007). In *Xenopus laevis* the primitive gonads are formed in the first week after fertilization, during early larval stages, NF 48. The differentiation of testes and ovaries starts at NF 51 and a ovarian cavity can be seen at NF 56 (Witschi 1971). In *Xenopus laevis*, the feminising effects of estradiol benzoate depend on the exposure period (Villalpando and Merchant-Larios 1990). Early larval exposure, NF stage 44-50, resulted in total sex reversal as all frogs developed ovaries. When exposure started at NF stage 51-54, 50 % of the frogs had ovotestes and 50 % ovaries. Late larval exposure, NF stage 55-56, did not affect the sex ratio (Villalpando and Merchant-Larios 1990). It is known that the oviducts start to differentiate, from the Müllerian ducts, during late larval stages (NF 57-66) in *Xenopus laevis* (Witschi 1971).

Gonadal differentiation in *Rana temporaria*

In *Rana temporaria*, the males are considered to be heterogametic (XY) and the females homogametic (XX) (Witschi 1929b). Three different races of *Rana temporaria* have been found based on sex differentiation patterns: differentiated, semidifferentiated and undifferentiated races (Witschi 1929b). In the differentiated race, the undeveloped gonads differentiate into ovaries and testes during larval development. In the semidifferentiated and undifferentiated races, the gonads in genetic males first differentiate into ovaries and then transform into testes. The transformation passes through an intersex stage when the gonads have both female and male characteristics (Witschi 1929b). This transition of the gonad, from female into male, occurs during metamorphosis in the semidifferentiated race and after metamorphosis at the
end of the first year in the undifferentiated race. Therefore, populations of these two races have high percentages of individuals with ovaries at metamorphosis. Moreover, intersex individuals are naturally found in populations of these races (Witschi 1929b). Witschi (1929c) has presented the geographical distribution of different races of *Rana temporaria* in Europe. It is not known, however, which races of *Rana temporaria* that are present in Sweden.

**Aromatase**

The aromatase enzyme (CYP 19) converts androgens into estrogens and is therefore a key enzyme in the regulation of estrogen levels. Aromatase is suggested to be involved in sexual differentiation of brain and ovary in *Xenopus laevis* (Kelley 1996; Urbatzka *et al.* 2007), but its specific function in these processes is not yet fully understood. It has been shown that inhibition of aromatase during gonadal differentiation results in testicular development in amphibians, reptiles, and fish (Piferrer *et al.* 1994; Chardard and Dournon 1999; Pieau *et al.* 1999). Aromatase mRNA is expressed in various tissues of adult *Xenopus laevis*, including testis, ovary, and brain (Miyashita *et al.* 2000). During larval development, aromatase mRNA has been detected in gonads and brain in *Xenopus laevis* (Urbatzka *et al.* 2007). Aromatase is a target for several endocrine disrupting chemicals (Monod 1993; Monteiro *et al.* 2000; Sanderson *et al.* 2000; Sanderson *et al.* 2002; Kazeto *et al.* 2004), and has been shown to be modulated by estrogen exposure in fish and amphibians (Kishida *et al.* 2001; Ohtani *et al.* 2003; Kazeto *et al.* 2004; Akatsuka *et al.* 2005; Coady *et al.* 2005; Lyssimachou *et al.* 2006). Aromatase activity is used as an endpoint for endocrine disruption in the present thesis (paper IV and V) in order to evaluate effects of EDC exposure in brain and gonads.

**Estrogen receptors**

Estrogen receptors (ERs) are ligand-activated transcription factors belonging to the nuclear receptor superfamily. Estrogens bind to ERs and form a complex that can attach to estrogen responsive elements (ERE) on DNA and modulate gene transcription. Increased ER mRNA levels are commonly used as a biomarker for estrogenic exposure, so also in amphibians (Bögi *et al.* 2002; Lutz 2002). ERs are present in, for example, gonads, accessory sex organs, and the brain (Kuiper *et al.* 1997). At first only one ER was known but in 1996 Kuiper *et al.* (1996) found another type of ER. The first ER was named ERα and the second ERβ. Wu *et al.* (2003) have been able to isolate both ERα and ERβ in *Xenopus tropicalis*. It has been shown that both ERα
and ERβ mRNA is expressed in brain, liver, and gonad/kidney in *Xenopus tropicalis* during gonadal differentiation (Takase and Iguchi 2007). In this thesis (paper I) mRNA levels of ERα are used as an endpoint for estrogen exposure.

**Ethynylestradiol (EE₂)**

EE₂ is a widely prescribed pharmaceutical used for example in contraceptive pills. Users of products containing EE₂ excrete both the parent compound and conjugated metabolites in their urine (Abdel-Aziz and Williams 1970; Reed *et al.* 1972; Ranney 1977). In figure 1 the chemical structures of EE₂ and the endogenous estradiol are shown. Though chemical structures are similar, EE₂ has proved to be about 30 times more potent than estradiol in studies with fish, using vitellogenin induction and OSI (ovarian somatic index) as endpoints (Thorpe *et al.* 2003; Van den Belt *et al.* 2004). EE₂ is also more resistant to biodegradation than estradiol (Jürgens *et al.* 2002).

In Sweden, Larsson *et al.* (1999) detected concentrations of 15 pM EE₂ (4.5 ng/L) in sewage effluent water, on the west coast. In rivers downstream sewage treatment plants in the UK, Germany, and the Netherlands maximum concentrations of, respectively, 51 pM EE₂ (15 ng/L), 17 pM (5.1 ng/L), and 15 pM (4.3 ng/L) have been detected (Aherne and Briggs 1989; Belfroid *et al.* 1999; Ternes *et al.* 1999; Kuch and Ballschmiter 2001). Concentrations of EE₂ ranging from 7 to 420 pM (2-125 ng/L) have been found in the canals and the lagoon outside Venice, Italy (Pojana *et al.* 2004). In the U.S., Kolpin *et al.* (2002a; 2002b) detected EE₂ concentrations up to 920 pM (273 ng/L) with a median concentration of 320 pM (94 ng/L). Ternes *et al.* (1999) measured EE₂ concentrations in sewage effluent water from ten Canadian sewage treatment plants and found a median concentration of 30 pM (9 ng/L) and a maximum concentration of 140 pM (42 ng/L). EE₂ is used in this thesis both as an environmental pollutant and a model compound to explore estrogen-induced developmental toxicity.
Clotrimazole belongs to the group of imidazole fungicides (Fig. 2). These pharmaceuticals are used for treatment of fungal infections, acting by inhibiting cytochrome P450 (CYP) 51 in the ergosterol synthesis (van den Bossche et al. 1978). The reduced ergosterol synthesis will result in damage of the cell membrane and inhibition of fungal growth. It is known that clotrimazole and other imidazole fungicides are also efficient aromatase inhibitors (Mason et al. 1985; Monod 1993). In vitro studies have shown that clotrimazole inhibits aromatase activity in fish ovaries (Monod 1993; Shilling et al. 1999; Hinfray et al. 2006). In the amphibian Rana dybowskii, oocyte maturation and ovulation were inhibited after exposure to clotrimazole in vitro (Choi et al. 2007). Clotrimazole is discharged from municipal waste water treatment plants and may be present in surface waters. In the UK, clotrimazole has been detected in water from five rivers at concentrations from <3 to 99 pM (<1 to 34 ng/L) (Thomas and Hilton 2004; Roberts and Thomas 2006; Peschka et al. 2007). In Germany, clotrimazole has been found in two rivers at concentrations ranging from 9 to 12 pM (3 to 4 ng/L) (Peschka et al. 2007). Very little information is available regarding effects of clotrimazole in aquatic animals.
Exposure period

Most amphibian species are aquatic during larval development and becomes terrestrial after metamorphosis. During larval development amphibians can be exposed to water-borne environmental pollutants. It is also during this period that sex organ differentiation starts in most frog species (Lofts 1974). They can therefore be exposed to EDCs found in waters during this critical period of reproductive development. That is why the period from hatching until metamorphosis has been chosen as exposure time in this thesis. The frogs were evaluated immediately after exposure but also at later developmental stages in order to investigate persistent effects.
Objectives

The overall objective of this thesis was to develop and apply methods that can be used to study effects of environmental pollutants on the reproductive system in amphibians. A main purpose was to evaluate whether *Xenopus tropicalis* is a useful model species when evaluating long-term effects of endocrine disruptors on the reproductive system. In order to investigate whether the model species is representative for other frog species, the susceptibility to estrogen in *Xenopus tropicalis* was compared with a wild European species, *Rana temporaria*.

Specific aims

- To investigate effects of EE2- and clotrimazole exposure during larval development on gonadal differentiation in the model species *Xenopus tropicalis*.

- To examine effects of larval exposure to EE2 on reproductive organ development, fertility, sexual behaviour, aromatase activity in brain and testis, and ERα mRNA levels in brain and ovary in sexually mature *Xenopus tropicalis*.

- To evaluate effects of larval exposure to EE2 on testicular structure and function in *Xenopus tropicalis* males that were not sex-reversed.

- To investigate effects of larval exposure to environmentally relevant concentrations of EE2 on gonadal differentiation in *Xenopus tropicalis* and *Rana temporaria*, and to compare the susceptibility to EE2 for the two species.

- To examine effects of clotrimazole and EE2-exposure during larval development on aromatase activity in brain and gonads in *Xenopus tropicalis*. 
Methods and experiments

Test system

In the test system used in this thesis, frogs were exposed during their larval development. *Xenopus tropicalis* was evaluated at four different life-stages; during gonadal differentiation, at metamorphosis, one month after metamorphosis, and at sexual maturity. *Rana temporaria* was evaluated at metamorphosis and one month after metamorphosis. Figure 3 shows an overview of the test system. All experiments were approved by the Local Ethics Committee for Research on Animals.

*Figure 3. An overview of the test system used in the present thesis.*
Exposure systems

Two different types of exposure systems were used in this thesis: a semi-static exposure system in paper I, IV, and V and a flow-through system in paper II. The water in all experiments was prepared from nine parts deionised water and one part copper-free Uppsala tap water. Survival rates were recorded from start of exposure until metamorphosis (paper II and V) or one-month after metamorphosis (paper I), and from metamorphosis or one-month after metamorphosis until adulthood (paper I and III). The EE2 concentrations were 7 and 90 pM in the *Rana temporaria* experiment (paper II), 6, 60, and 600 pM in paper II, III, and IV for *Xenopus tropicalis*, and (nominal) 1, 10, 100 nM for *Xenopus tropicalis* in paper I. The clotrimazole concentrations used were 6, 41, and 375 nM in paper V and (nominal) 8.7, 87, and 870 nM in paper IV.

Semi-static system

In the semi-static exposure system water was changed every second day (paper I), three times a week (paper IV), or every day (paper V). Water and new substance diluted in ethanol was added in the test tanks. Ethanol concentrations in the tanks, including controls, were 0.001 % in paper I, 0.0008 % in paper IV, and 0.0005 % in paper V. The total water volume in each tank was 4 L in paper I and IV, and 15 L in paper V.

Flow-through system

In the flow-through exposure system the water flow was 60 ml/min and the EE2-solution flow was 50 ml/min. The EE2-solutions were changed every 5th day, with EE2 from acetone stock solutions. Acetone concentrations in the tanks, including controls, were 0.0001 % and the total water volume in each tank was 10 L.

Animals

*Xenopus tropicalis*

*Xenopus tropicalis* frogs were obtained from Carolina Biological Supply Company (Burlington, NC, USA) in paper I and from Xenopus 1 (Dexter, MI, USA) in paper II-V. The frogs were held at 26 ±1°C and a 12:12-h light:dark cycle. Spawning was induced by injecting human chorionic gonadotropin (hCG) into the dorsal lymph sac. The exposure started from developmental NF stage 47-49 (4-5 days after hatching) due to high mortality.
rate of tadpoles during the first stages. The exposure ended at complete metamorphosis (NF stage 66).

After metamorphosis the frogs were kept in aquaria containing only water. In paper I, frogs were kept in aquaria for one month or until adulthood, 9 months after metamorphosis. In paper III and IV, frogs were kept until 8 months after metamorphosis. On termination of the experiments frogs were weighed and the snout-vent length and width were measured. Both ovaries, the left testis and the right oviduct were weighed. Gonadosomatic index (GSI) was calculated as gonad weight/body weight x 100. Oviducalsomatic index (OviSI) was calculated as oviduct weight/body weight x 100.

**Rana temporaria**

Newly laid eggs were collected from a pond out side of Uppsala (Sweden) and kept in aerated water at 18°C until hatching. Immediately after hatching, at Gosner larval developmental stage 22-23 (Gosner 1960), tadpoles were transferred to the exposure tanks. The tadpoles were exposed until the end of metamorphosis when just a short tail stub was remaining (Gosner stage 44-45). They were thereafter transferred to terraria. The frogs were held at a water temperature of 20.8 ±0.1°C and a light/dark cycle corresponding to latitude 54°N and longitude 10°E (16:8 h light:dark).

At completed metamorphosis about one-third of the *Rana temporaria* frogs were killed from each group and the remaining frogs were kept in terraria for one month.

**Time to metamorphosis**

The time to metamorphosis was measured from the day of fertilization until complete metamorphosis, NF stage 66, in *Xenopus tropicalis*. In *Rana temporaria*, the time to metamorphosis was measured from hatching until Gosner stage 44-45, when the frogs were transferred to terraria.

**Fixation and histology**

Juvenile frogs were fixed in formaldehyde (4 % in phosphate buffer, pH 7) for 72 h. In adults the right testis, oviduct and a part of the ovary were fixed in glutaraldehyde (2 % in phosphate buffer, pH 7) for 24 h, and thereafter transferred to phosphate buffer. Gonads and oviducts were dehydrated through a graded series of ethanol and embedded in hydroxyethyl methacrylate (Leica Historesin, Heidelberg, Germany). Oviducts were divided into one, two, or three different parts depending on the size. Sections (2μm) from
oviducts and gonads were stained with hematoxylin and eosin (paper I-III and V) or toluidine blue (paper V).

Histological evaluation of sex organs in *Xenopus tropicalis*

In juvenile *Xenopus tropicalis*, gonads with an ovarian cavity and a cortex containing oocytes were classified as ovaries and gonads with a medulla containing spermatogonia but lacking a cavity were classified as testes.

At metamorphosis (paper V), gonadal area, ovarian cavity area, and number of germ cells/gonad were determined. Juvenile females had mostly leptotene oocytes in their ovaries and males had spermatogonia.

One month post-metamorphosis (paper I), juvenile females had pachytene and diplotene oocytes in their ovaries. One month old males had spermatogonia and most of the individuals also had spermatocytes in the testes. Gonads containing both testicular tissue and oocytes were classified as intersex.

In adult *Xenopus tropicalis*, oocyte maturation stages were classified (stages 1-6) (Dumont 1972) in paper I. Testes containing oocytes, found in adult males, were classified as ovotestis in paper I and testicular oocytes in paper III. Individuals with one ovary and one testis were classified as intersex. Testes from adult males were evaluated in paper III. The testicular area and perimeter were measured, the total number of seminiferous tubules was determined, and the shape of the testis was classified as round or irregular. Seminiferous tubular area, luminal area, and epithelial area were measured and the shape of the seminiferous tubules was noted. The number of germ cell nests per seminiferous tubule was recorded and all nests were classified using criteria established by Kalt (1976). The number of spermatozoa in the lumen of the seminiferous tubules was estimated and converted to score numbers, zero to three. The luminal area of all visible efferent ducts between the testis and kidney in one section was measured and an average luminal area was determined for each individual. The number of efferent ducts containing sperm cells was noted. The right oviducts from frogs in paper I were histologically evaluated and the height of the glandular cells and the surface epithelial cells was measured. Three glandular and three surface epithelial cells at three different areas were measured for each oviduct. All measurements were made in the pars convoluta section 1-3 (PC1-PC3) of the oviduct (Yoshizkaki 1985).
Histological evaluation of gonads in *Rana temporaria*

In *Rana temporaria* as in *Xenopus tropicalis*, ovaries have a cavity and oocytes in the cortex, while testes have no cavity and the germ cells, spermatogonia, are located in the medulla. Because of the transition from ovary to testis in this species, oocytes are normally found in the testis at the time of metamorphosis. Therefore, gonads containing oocytes were classified as testes as long as the gonad had a distinct testicular structure. Some gonads contained testis-like tissue and lacked a cavity but contained a high frequency of oocytes (> 50 % of the germ cells as estimated by the histological evaluation). These gonads could not be unambiguously sexed and were therefore classified as intersex. Individuals with one ovary and one testis were also classified as intersex. The ovaries in *Rana temporaria* were classified into different maturation stages using the criteria established by Ogielska and Kotusz (2004).

Fertility studies

In paper III fertility studies were performed of adult *Xenopus tropicalis* after larval exposure to EE2.

Breeding success

For studies of breeding success exposed males and females were mated with unexposed frogs that had not been mated before. Mating was induced by injecting of hCG in both females and males. One female and one male frog were placed in a mating aquarium (4L) and were observed every 45th min during at least six hours. Amplexus success, the time from the last hCG injection until amplexus, and the duration of amplexus were recorded. After the mating the eggs were counted. One day after the mating the developing embryos were counted and the fertilization rate was calculated (number of developing embryos/number of laid eggs x 100).

Sperm count and motility

Half of the left testis was minced in simplified amphibian ringers solution (see paper III) at 0.1 mL/mg tissue. The sample was centrifuged and 50 μL of the supernatant was diluted 1:4 with deionised water before it was placed in a hemacytometer. Sperm cells in eight squares (half a chamber) were recorded and the moving sperm cells were counted. The percentage of moving sperm cells (moving sperm cells/total number of sperm cells x100) was calculated. In order to determine sperm concentration, sperm cells in three chambers were counted and a mean value was calculated.
Aromatase activity

Aromatase activity in brain and gonads was analysed in paper IV and V using a modified method of the triated water-release assay for aromatase activity (Lephart and Simpson 1991). In short, the release of $^3$H from the $^3$H-labelled substrate androstenedione during aromatisation was quantified after incubation with homogenate samples supplemented with NADPH (see paper V). Protein concentrations were measured using BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). The aromatase activity was expressed as the number of fmol androstendione transformed per hour and per mg protein.

Quantitative (real time) - PCR

ERα mRNA expression was analysed in brain and ovaries in paper I using a Mx3000P Real-Time PCR System (Strata-gene, La Jolla, CA, USA) and SYBR Green mix (Bio-Rad Laboratories, Hercules CA, USA). The ERα PCR product had been cloned and sequenced, and from known amounts of plasmid containing the ERα amplicon a standard curve was made in order to quantify gene expression (Arukwe 2005; Mortensen et al. 2006). The EE2-exposed phenotypic females were separated depending on whether or not they had oviducts, for comparison of ERα mRNA levels in brain and ovaries.

Chemical analysis

In order to determine the actual concentrations of EE2 and clotrimazole in the exposure tanks, chemical analysis of water samples was performed. The analytical method applied was liquid chromatography and mass spectrometry (LC/MS/MS). Chemical analysis of the EE2 concentrations was made by Analycen Nordic AB, Lidköping, Sweden. The detection limit was 0.0017 nM EE2. The analysis of clotrimazole concentrations was performed by Drs Jerker Fick and Richard Lindberg, Department of Chemistry, Umeå University. The detection limit was 0.03 nM clotrimazole.

Statistics

Sex ratios were compared using the G-test (Chi-Square test for likelihood) in paper I (SPSS 13.0 software, SPSS Inc., Chicago, USA) and the Fisher’s exact test in paper II and III using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). Survival rates were compared using Chi-square test (paper I and II) or Fisher exact test (paper III) (GraphPad Prism 5.0).
Frequencies of oviducal anomalies (paper I and III), amplexus success, and testes shape (paper III) were compared using Fisher’s exact test (GraphPad Prism 5.0). ERα levels (paper I) and all data from the male frogs in paper III were compared using unpaired t-test if the data passed the Kolmogorov-Smirnov normality test, otherwise Mann-Whitney test was used (GraphPad Prism 5.0). Time to metamorphosis, body weight, body size, GSI, OviSI, aromatase activity, all data from fertility studies of female frogs in paper III, and data from gonadal histology in paper V, were compared using ANOVA with Bonferroni’s post-test, if the data passed the Kolmogorov-Smirnov normality test, otherwise Kruskal-Wallis test with Dunn’s Multiple Comparison Test was used (GraphPad Prism 5.0). The correlation between fertilization rate and sperm concentration (paper III) was tested using Pearson correlation test (GraphPad Prism 5.0). If replicates were used, the data from the replicates were statistically compared for all endpoints and if not different the data were pooled.

In paper I multivariate data analyses were made using the software SIMCA-P 10.0.4 (Umetrics AB, Umeå, Sweden).
Results and discussion

Effects on sex organ differentiation

Sex ratios

In this thesis it was shown that larval exposure to environmentally relevant concentrations of EE₂ causes female-biased sex ratios in *Xenopus tropicalis* and *Rana temporaria* (Fig. 4, paper II and III). The skewed phenotypic sex ratios are assumed to result from ovarian development in genotypic males. Sex-reversal could not be confirmed through genotypic sexing of the frogs due to lack of available methods. In *Xenopus tropicalis* it was shown that the sex-reversal persisted into adulthood (Fig. 4, paper I and III). Male to female sex-reversal was implied at a concentration as low as 6.2 pM EE₂ (1.8 ng/L) in one of the replicates in *Xenopus tropicalis* (Fig. 4, paper III). The cause for the different sex ratio in the two replicates of the 6-pM group is not known but may be related to the slight difference in EE₂ concentration (5.2 and 6.2 pM). Six pM EE₂ is the lowest concentration of an estrogenic substance reported to disrupt gonadal differentiation in amphibians and it might be close to LOEC (lowest observed effect concentration) for induction of sex ratio skew. Larval exposure to higher concentrations of EE₂ (3 and 33 nM) has been shown to cause a female-biased sex ratio in *Rana pipiens* but not in *Rana sylvatica* (Mackenzie et al. 2003). It has been shown that males of *Rana pipiens* and *Xenopus laevis* undergo persistent gonadal sex-reversal following larval exposure to high concentrations of estradiol (Chang and Witschi 1955; Richards and Nace 1978). Whether the sex-reversal in *Rana temporaria* persists into adulthood remains to be clarified.

Among fish there is species variation in the susceptibility to sex-reversal induced by EE₂. A female-biased sex ratio was caused by EE₂ exposure to concentrations of 1 pM (0.32 ng/L) in *Pimephales promelas* (Parrott and Blunt 2005), 3 pM (1 ng/L) in *Danio rerio* (Örn et al. 2003), 170 pM (50 ng/L) in *Gasterosteus aculeatus* L. (Hahlbeck et al. 2004), and 340 pM (100 ng/L) in *Oryzias latipes* (Scholz and Gutzeit 2000). In this thesis it was shown that the susceptibility to estrogen-induced sex-reversal of *Xenopus tropicalis* and *Rana temporaria* is comparable to that observed in several fish species.
Figure 4. Sex ratios after larval exposure to EE$_2$ in a) Xenopus tropicalis at metamorphosis, b) Rana temporaria at one month after metamorphosis, and c) Xenopus tropicalis at sexual maturity. Sample size (n) above the bars. $A =$ significantly deviated from control ($p<0.001$), $B =$ significantly deviated from 5.2- or 6-pM group ($p<0.001$), and $b =$ significantly deviated from 5.2-, 6-, or 7-pM group ($p<0.05$), Fisher’s exact test.
No effects were seen on sex ratio at completed metamorphosis in *Xenopus tropicalis* after exposure to the aromatase inhibitor clotrimazole (paper V). Exposure to the aromatase inhibitor fadrozole at a concentration of 1.3 μM (300 μg/L) during gonadal differentiation resulted in female-to-male sex-reversal in the newt *Pleurodeles waltl* (Chardard and Dournon 1999). In studies of tadpoles, exposure to 40 nM (10 μg/l) fadrozole during larval development resulted in an increased frequency of intersex individuals in *Rana pipiens*. In *Rana sylvatica*, no significant effects were seen after exposure to 40 and 450 nM fadrozole (10 and 100 μg/l), although in the highest concentration group there were more females (65 %) than males (35 %) (Mackenzie *et al.* 2003).

### Ovarian differentiation

The ovarian GSI of adult phenotypic female *Xenopus tropicalis* exposed from 6 pM to 100 nM EE₂ did not differ from those of control females (paper I and III). The histological evaluation of ovaries in adult *Xenopus tropicalis* did not show any differences in proportion of immature and mature oocytes between EE₂–exposed and control females (paper I). The differentiation stages of the ovaries were classified in *Rana temporaria* (paper II). The results obtained did not show any differences in ovarian maturity between control and EE₂-exposed groups. This indicates that development of ovaries in sex-reversed male frogs is similar to control females. Larval exposure to EE₂ did, in contrast to our results, increase ovarian differentiation rate in *Rana pipiens*, at 3 and 33 nM, and in *Rana sylvatica*, at 33 nM (Mackenzie *et al.* 2003). In a recent study, however, no abnormalities or altered developmental rate were found in ovaries of *Rana pipiens* exposed to 5 nM EE₂ during larval development (Hogan *et al.* 2008). In *Rana temporaria*, the sex ratio in the control groups varied depending on the developmental stage of the frogs (paper II). There were higher proportions of phenotypic females at metamorphosis than at one month later, indicating that some female gonads had differentiated into testes at the latter time-point. This suggests that the sampled populations likely belong to the semidifferentiated race of *Rana temporaria* (Witschi 1929c).

No effects were seen on ovarian differentiation at completed metamorphosis in *Xenopus tropicalis* after exposure to the aromatase inhibitor clotrimazole (paper V).

### Testicular differentiation

The score values representing the amount of spermatozoa in seminiferous tubular lumen were significantly lower in the EE₂-exposed males compared to the control group (Table 1). An example of two seminiferous tubules is
seen in figure 5. This result could imply that the EE2-exposed males have a reduced production of mature spermatozoa. No differences were, however, seen between exposed and control males in the amount of maturing germ cells in the epithelial nests. An alternate explanation would be that more spermatozoa had been ejaculated from the EE2-exposed frogs during mating compared to the controls. The evaluation of testicular histology also showed that 62.5% of the EE2-exposed males had irregularly shaped testes, whereas all control testes had a regular, round form (Table 1). In frogs exposed to 6 pM EE2, the testicular area, perimeter, and number of seminiferous tubules per testicular cross section were reduced compared to control frogs, indicating a reduced size of the right testis (paper III). The GSI, based on the weight of the left testis, was however not reduced in these males. A reduced amount of sperm cells in testis or in ejaculate and/or volume of ejaculate has been observed in fish (van Aerle et al. 2002), birds (Blomqvist et al. 2006), rodents (Wilson et al. 1986; Goyal et al. 2003; Newbold 2004), and humans (reviewed in Toppari et al. 1996) after exposure to estrogenic compounds during early development. In wild mosquitofish, fewer sperm cells in testis have been observed in fish from Lake Apopka, known to be contaminated with numerous chemicals, compared to fish from reference waters (Toft et al. 2003). Feminized male roach in UK rivers polluted with estrogenic compounds were found to have reduced milt production with fewer sperm cells in the ejaculate as well as decreased sperm quality compared to normal males (Jobling et al. 2002). In humans, the number of cases of testicular dysgenesis syndrome (Skakkebaek et al. 2001), with symptoms including poor semen quality have increased during the last 60 years (Toppari et al. 1996; Swan et al. 1997). The testicular dysgenesis syndrome is suggested to be caused, at least partly, by exposure to estrogenic chemicals during fetal or childhood development (Toppari et al. 1996; Olesen et al. 2007). The results in this thesis are consistent with previous studies of vertebrates.

EE2-exposure from hatching or shortly after hatching until metamorphosis induced sex-reversal but not intersex or testicular oocytes in Xenopus tropicalis and Rana temporaria males. In contrast to our findings, larval exposure to estrogenic compounds resulted in an increased frequency of intersex frogs or male frogs with testicular oocytes in Rana species (Mackenzie et al. 2003; Park and Kidd 2005) and in Xenopus laevis (Chang and Witschi 1956; Hu et al. 2008). In this thesis testicular oocytes were present in control frogs, which is in agreement with other studies in Xenopus tropicalis and Xenopus laevis (Witschi 1971; Coady et al. 2005; Knechtges et al. 2007). The varying results from these studies may be due to species differences and different exposure conditions.

No effects on testicular differentiation were observed at completed metamorphosis in Xenopus tropicalis following exposure to the aromatase inhibitor clotrimazole (paper V).
Table 1. Histomorphometrical evaluation of testes and seminiferous tubules in adult male *Xenopus tropicalis* exposed to EE2 during larval development. SG=spermatogonia, SC=spermatocytes, ST=spermatids, and SZ=spermatozoa.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Testis area</th>
<th>Seminiferous tubules / testis</th>
<th>Testis shape</th>
<th>Seminiferous tubule area</th>
<th>Cell nests / seminiferous tubule</th>
<th>Germ cell stages of nests in seminiferous tubules</th>
<th>Spermatozoa in lumen^b^</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n mm^2 (±SD)</td>
<td>mean (±SD)</td>
<td>Round</td>
<td>Irregular</td>
<td>n mm^2 (±SD) mean (±SD)</td>
<td>% (±SD)</td>
<td>% ±SD</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>3.4 (0.9)</td>
<td>53 (3)</td>
<td>100</td>
<td>8 0.063 (0.019)</td>
<td>13.5 (2.9)</td>
<td>26 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EE2-exposed^a</td>
<td>8</td>
<td>2.4 (1.3)</td>
<td>47 (20)</td>
<td>37.5</td>
<td>10 0.052 (0.016)</td>
<td>12.1 (2.3)</td>
<td>29 (11)</td>
</tr>
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<td></td>
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</tbody>
</table>

^a EE2-exposed phenotypic males i.e. 7 or 9 frogs from the 6-pM group and the single one from the 60-pM group.

^b the number of spermatozoa in the seminiferous tubular lumen was estimated using score numbers 0-3.

* Significantly different from control (p<0.05), Fisher’s exact test.

** Significantly different from control (p<0.01), Mann-Whitney test.
Figure 5. Seminiferous tubules in adult Xenopus tropicalis a) control male containing a large number of spermatozoa in the lumen and b) male exposed to 6 pM EE2 during larval development with few spermatozoa in the lumen. L = lumen; SZ = spermatozoa in the lumen. Bar = 50 μm.
Oviducal differentiation

In this thesis it was shown that the frequency of frogs with ovaries but lacking one or both oviducts increased with increasing EE$_2$ concentration (Fig. 6). Frogs exposed to 600 pM and 100 nM EE$_2$ had a significantly higher proportion of individuals which had normal ovaries but lacked one or both oviducts compared to the controls (paper I and III). Female frogs without oviducts will be sterile. Two phenotypic females lacking one or both oviducts were tested for reproductive success and they did not lay eggs after hCG stimulation (paper III). Eggs were found in their body cavity upon dissection, confirming that they had ovulated. It is not known if frogs are affected by having eggs in their body cavity. Previous studies in birds have shown that embryonic exposure to EE$_2$ or $o,p'$-DDT resulted in oviducal malformations, and egg yolk in the abdomen which was associated with adverse health effects (Berg et al. 2001; Holm et al. 2006).

![Figure 6. Frequencies of individuals with ovaries but lacking one or both oviducts in adult Xenopus tropicalis following larval exposure to EE$_2$. The number of individuals with ovaries above the bars (n). a = significantly deviated from respectively control ($p$<0.01) and b = significantly deviated from 0.006-nM group ($p$<0.05), Fisher’s exact test.](image-url)
Larval exposure to EE2 both inhibited and stimulated oviducal differentiation in adult *Xenopus tropicalis*. In the highest concentration of EE2 (paper I) the OviSI was significantly higher (p<0.001) than in the control group. An earlier report has shown that estradiol exposure of juvenile *Rana cyanophlyctis* results in an increased oviduct weight (Pancharatna *et al.* 2001). In the present work, the height of the glandular cells and the surface epithelial cells (in PC1-PC3) in the oviducal wall did not differ between groups, indicating that there were no differences in thickness of the oviducts. As the OviSI was found to be increased while the oviduct thickness remained unaffected, the oviducts might have been longer in frogs in the highest concentration of EE2 compared to the control oviducts. Unfortunately, it was difficult to measure the length of the oviducts because of their coiled structure. Alternatively, another part of the oviduct might have been affected by EE2-exposure resulting in increased thickness. The measurements were, however, made in PC1-PC3 which constitute the major part of the oviduct (Yoshizakaki 1985).

In male fish, gonadal ducts develop ovarian-like attachments to the testis after exposure to estrogenic compounds (Gimeno *et al.* 1996; van Aerle *et al.* 2002). In birds, differentiation of Müllerian ducts (embryonic oviducts) is affected in both males and females by embryonic exposure to EE2 and the synthetic estrogen diethylstilbestrol (DES) (Berg *et al.* 1999; Berg *et al.* 2001). In female mice, prenatal exposure to DES results in malformations of the oviducts (Newbold *et al.* 1983), and possibly also in women (DeCherney *et al.* 1981). The results in this thesis show that the differentiating oviduct is a sensitive target for estrogen exposure in amphibians.

### Reduced fertility

In paper III, fertility was evaluated in adult *Xenopus tropicalis* after larval exposure to low concentrations of EE2. The EE2-exposed males that were not sex-reversed had a significantly reduced fertilization rate compared with control males (Fig. 7). No effects of larval EE2 exposure were seen in the fertility studies of females. Studies in fish has shown that exposure to EE2 during early stages impaired reproductive success at adulthood (Hill and Janz 2003; Maunder *et al.* 2007; Schäfers *et al.* 2007). Reduced fertilization rates have also been observed in wild roach in UK rivers receiving estrogenic sewage effluents (Jobling *et al.* 2002). Reports have also shown reduced reproductive success in male rats neonatally exposed to DES (Atanassova *et al.* 2000; Goyal *et al.* 2003). Possible explanations for the reduced fertility rate in the EE2-exposed male frogs are reduced sperm production or impaired sperm quality.

No effects of EE2 exposure on sperm concentration in testis homogenates were detected (paper III) although the histological evaluation of testis...
showed a reduced amount of spermatozoa in the seminiferous tubular lumen. A likely reason for the lack of effect on sperm concentration is that, in addition to luminal spermatozoa, spermatids and spermatozoa from the seminiferous tubular epithelium were counted as well. Since there were no differences between exposed and control males in the amount of maturing germ cells in the epithelial nests, this may hide possible differences in concentrations of spermatozoa. It cannot be excluded that the reduced fertility in the EE2-exposed male frogs was also due to impaired fertilizing ability of spermatozoa.

\[ \text{Figure 7. Fertilization rate in adult male Xenopus tropicalis after larval exposure to EE2. The EE2-exposed group includes 10 frogs from the 6-pM group and the single one from the 60-pM group. Sample size (n) above the bars. Data is given as mean \pm SD.}^* \text{ = significantly different from control (p<0.05), Mann-Whitney test.} \]

Aromatase activity after clotrimazole exposure

Larval exposure to clotrimazole modulated aromatase activity in gonads and brain in *Xenopus tropicalis* (paper IV and V). These are the first studies reporting *in vivo* effects of clotrimazole in amphibians. During gonadal differentiation no significant differences were found in aromatase activity in the gonad/kidney complex between groups, although aromatase activity increased with increasing exposure time in the 41- and 375-nM groups (paper V). At metamorphosis aromatase activity was significantly increased in the gonad/kidney complex of both sexes exposed to 41 and 375 nM clotrimazole.
compared with the controls (Fig. 8). Aromatase activity was measured in gonads attached to the kidneys. As only extremely low levels of aromatase mRNA have been found in adult *Xenopus laevis* kidneys (Miyashita *et al.* 2000), the aromatase activity of the gonad/kidney complex is expected to originate mainly from the gonads. The aromatase assay was based on tissue homogenate prepared from tadpoles exposed to clotrimazole *in vivo*. Aromatase activity measured *in vitro* does not necessarily mirror the aromatase activity in the gonads during clotrimazole exposure *in vivo*, where the enzyme inhibition should be expected to be more significant because of clotrimazole extraction during homogenate preparation. The increased aromatase activity *in vitro* could consequently reflect a compensatory up-regulation of aromatase gene expression and protein translation as a result of enzyme inhibition *in vivo*. At NF stage 56, during gonadal differentiation, brain aromatase activity was significantly lower in the 375-nM group than in the control group (Fig. 9), but at metamorphosis no differences were seen between exposure groups (paper V). The reason for the discrepant effects of clotrimazole on the aromatase activity in brain and gonad is presently not understood, but may be due to differences in regulation of the aromatase gene expression in the two organs. Similar results have been shown in adult fish (*Pimephales promelas*) following exposure to the aromatase inhibitor fadrozole (Villeneuve *et al.* 2006). In that study, aromatase activity in brain decreased whereas ovary aromatase activity showed an inverted U-shaped concentration-response. Complementary studies of aromatase mRNA expression and protein levels would have been valuable for the interpretation of the present results.

At metamorphosis, aromatase activity in brain was higher in females than in males in paper IV, however, no sex differences were found in paper V. At sexual maturity no differences was seen between sexes (paper IV). No gender differences were found in brain aromatase mRNA levels in *Xenopus laevis* at NF stages 56 to 66 (Urbatzka *et al.* 2007). In 2-3 months old *Xenopus laevis* females had higher brain aromatase activity than males (Coady *et al.* 2005). More research in *Xenopus* is needed in order to establish whether there are gender differences in brain aromatase activity and, if so, at what life stages.

We did not detect any effect of clotrimazole on gonadal differentiation as evaluated by sex ratio and gonadal histology at metamorphosis. This lack of effect on morphological endpoints is in agreement with the interpretation above that aromatase inhibition by clotrimazole was at least partly compensated for by an up-regulation of aromatase gene expression during the developmental stages examined. Further studies are needed to investigate the long-term effects of developmental clotrimazole exposure on the reproductive system in amphibians.
Figure 8. Aromatase activity in gonad/kidney complexes from a) female and b) male Xenopus tropicalis frogs at metamorphosis (Niewkoop-Faber stage 66) after exposure to clotrimazole from NF stage 47-48. n = 6. Each sample (n) consisted of two pooled gonad/kidney complexes. Data are given as mean ±SD. A = significantly different from control (p<0.001), a = significantly different from control (p<0.05) and b = significantly different from 6-nM group (p<0.05), Kruskal-Wallis test and Dunn’s multiple comparison test.
Figure 9. Aromatase activity in brains from Xenopus tropicalis tadpoles at Niewkoop-Faber stage 56 after exposure to clotrimazole from NF stage 47-48. n = 4. Each sample (n) consisted of 10 pooled brains. Data are given as mean ±SD. * = significantly different from the control group (p<0.05), Kruskal-Wallis test and Dunn’s multiple comparison test.

Aromatase activity after EE2 exposure

During gonadal development (paper V) no significant effects were found in aromatase activity of brain between EE2-exposed tadpoles and controls (Fig. 10). Aromatase activity in gonad/kidney complex of EE2-exposed tadpoles appeared to be lower than that of controls but the difference was not significant (Fig. 10). The EE2-exposure resulted in 100 % female frogs. These findings are in disagreement with studies in amphibians and fish, reporting increased aromatase expression and/or activity during gonadal differentiation following exposure to estrogen concentrations that resulted in male-to-female sex-reversal (Kobayashi et al. 2003; Kuntz et al. 2003; Ohtani et al. 2003; Kuhl et al. 2005). All EE2-exposed frogs developed ovaries and these results indicate that estrogen-induced ovarian differentiation is not paralleled by increased gonadal aromatase activity in Xenopus tropicalis. In frogs exposed to EE2 during larval development no changes were seen in aromatase
activity in adult testis and brain (paper IV). In fish, exposure to estrogens increased brain aromatase activity as measured at the end of the exposure period (Melo and Ramsdell 2001; Lyssimachou et al. 2006). It is not known if aromatase activity was affected in the frogs at metamorphosis, directly after EE₂ exposure.

**Figure 10.** Aromatase activity in gonad/kidney complexes and brain from Xenopus tropicalis tadpoles at Niewkoop-Faber stage 56 after exposure to 100 nM EE₂ from NF stage 47-48. n = 3. Each sample (n) consisted of 10 pooled brains. Data is given as mean ±SD.

Organisational effects on ERα mRNA levels

In paper I it was shown that some of the frogs exposed to the highest concentration of EE₂ had decreased ERα mRNA levels in the brain compared to the control females (Fig. 11). Interestingly, this effect was only observed in frogs which had developed ovaries but lacked one or both oviducts. No effect was found in females with normal oviducts. This finding shows that EE₂-exposure during larval development can result in an organisational effect on the adult brain. A similar organisational effect has been seen in male rats, where ERα mRNA levels were permanently decreased in the pituitary after neonatal exposure to estrogen (Tena-Sempere et al. 2001).
There were no differences in ERα mRNA levels in the ovaries between controls and the EE2-exposed groups (paper I). In frogs, the mechanisms of steroid hormone action on the brain and gonads are not well known. It has been reported, however, that estrogenic compounds cause increased ERα mRNA levels in whole-body homogenates of tadpoles and juvenile *Xenopus laevis* (Bögi *et al.* 2002; Levy *et al.* 2004). Whether these studies are showing activational or organisational effects remains unknown as ERα mRNA levels were measured immediately after the exposure period.

![Graph showing ERα mRNA levels in the brain of adult Xenopus tropicalis after larval exposure to EE2. Individuals with or without oviducts were separated. Data is given as mean ±SD. * = significantly different (p<0.05), t-test.](image)

**Figure 11.** Expression of ERα mRNA in the brain of adult *Xenopus tropicalis* after larval exposure to EE2. Individuals with or without oviducts were separated. Data is given as mean ±SD. * = significantly different (p<0.05), t-test.

**Time to metamorphosis**

EE2 and clotrimazole exposure during larval development did not affect time to metamorphosis. In paper II, 60 and 600 pM EE2 caused a slightly prolonged time to metamorphosis in *Xenopus tropicalis*. However, higher concentrations of EE2 did not affect the time to metamorphosis in paper I and V. Therefore, the prolonged time to metamorphosis was likely not caused by EE2.
Test system

Test species

Because of its shorter generation time, *Xenopus tropicalis* has advantages compared to *Xenopus laevis* as a model species for reproductive toxicity studies. It is sensitive to estrogenic exposure (paper II and III) and the effects persist into adulthood (paper I and III). The sensitivity to EE₂ was similar for the two test species, *Xenopus tropicalis* and *Rana temporaria* (paper II), which favours *Xenopus tropicalis* as a model species. The two test species seem to be more sensitive to estrogen exposure than *Rana pipiens* and *Rana sylvatica* (Mackenzie *et al.* 2003; Hogan *et al.* 2008). The estrogen-induced developmental reproductive effects observed in *Xenopus tropicalis* are comparable with those found in fish, birds and mammals (Wilson *et al.* 1986; Gimeno *et al.* 1996; Toppari *et al.* 1996; Berg *et al.* 2001; van Aerle *et al.* 2002; Goyal *et al.* 2003; Hill and Janz 2003; Newbold 2004; Blomqvist *et al.* 2006), implying that *Xenopus tropicalis* could be used as a complementary vertebrate model in research on developmental reproductive toxicity. The results in this thesis together with the short generation time compared with other frog species (Witschi 1929a, b; Ryan 1953; Hirsch *et al.* 2002), support the use of *Xenopus tropicalis* as a model species when evaluating long term effects of endocrine disruptors on the reproductive system in amphibians. The effect on gonadal differentiation (skewed sex ratio) in *Rana temporaria* was not detected until one month after metamorphosis (paper II). This suggests that one month after metamorphosis is a more suitable time point than at metamorphosis for detection of effects on the sex ratios in semidifferentiated *Rana temporaria*.

Exposure

The skewed sex-ratios observed in the present studies suggest that the exposure periods from NF stage 48 until metamorphosis in *Xenopus tropicalis* and from hatching until metamorphosis in *Rana temporaria* were appropriate for studies on gonadal sex-reversal and developmental reproductive toxicity in these species. The flow-through exposure system showed advantages compared to the semi-static system. The water changes that are needed in the semi-static system are time consuming. The flow-through system also resulted in higher water quality.

Endpoints

In the present work endpoints were evaluated at gonadal differentiation, after metamorphosis, and at sexual maturity. In order to assess effects on the reproductive system following larval exposure, it is necessary to evaluate ef-
fects both on developing tadpoles and in adult frogs (Kloas and Lutz 2006). The endpoints used varied from ERα mRNA levels and aromatase activity, to sex organ development with sex ratio and macroscopic and histological evaluation of gonads and oviducts, up to reproductive function with evaluation of breeding success and sperm concentration.

Sex organ development is an important endpoint when evaluating ecological risks in wild animals. Effects on the reproductive organs can reduce the reproductive capacity or result in infertile animals, as was seen in paper I and III. The present results show that in our test system a female-biased sex ratio, rather than increased frequency of testicular oocytes, is a sensitive endpoint reflecting an estrogenic effect. Aromatase activity has a central role in the sex hormone system and has in the present thesis been shown to be modulated by clotrimazole exposure. It might therefore be a useful endpoint indicating endocrine disruption in amphibians. The decreased levels of ERα mRNA imply an organisational effect in the brain in paper I. Time to metamorphosis is an important endpoint because a prolonged developmental period can decrease survival (Hayes et al. 2006) and can also indicate that the thyroid system is affected.

The most sensitive endpoints for estrogenic exposure used in this thesis were sex-ratio, fertilisation rate, and amount of spermatozoa in the seminiferous tubular lumen. Female-biased sex ratio and reduced fertilisation success have been reported to be the most sensitive endpoints following exposure to low concentrations of EE2 in fathead minnow (Pimephales promelas), when compared to changes in GSI and decreased male secondary sex characteristic index (Parrott and Blunt 2005).
Summary and overall conclusion

In this thesis it has been shown that frogs are sensitive to EE2-exposure. Environmentally relevant concentrations of EE2 affected gonadal development, resulting in a female biased sex ratio, both in Xenopus tropicalis and Rana temporaria. Male-to-female sex-reversal was implied at a concentration as low as 6 pM EE2. These estrogen concentrations are lower than those earlier reported to give rise to skewed sex ratios in amphibians. The skewed sex-ratios observed are assumed to result from male-to-female sex reversal. Rana temporaria frogs were only evaluated as juveniles but in Xenopus tropicalis the sex-reversal was shown to be persistent into adulthood.

In adult Xenopus tropicalis, testicular and oviducal development were disrupted after larval exposure to EE2. For the first time it was demonstrated that larval exposure to environmentally relevant concentrations of an environmental pollutant can cause reproductive toxicity in adult amphibians. The EE2-exposed males that were not sex-reversed showed impaired fertility as fertilization rate and amount of mature spermatozoa in the seminiferous tubular lumen were reduced. The proportion of frogs with ovaries but lacking oviducts increased with increasing EE2-concentrations. A female frog without oviducts will be sterile. The differentiating oviduct seems to be a sensitive target for estrogen exposure in frogs. The ovarian differentiation and development were not affected in EE2-exposed groups. This indicates that development of ovaries in sex-reversed male frogs is similar to control females. Larval exposure to EE2 caused a reduced ERα expression in the brain of adult frogs with ovaries but without oviducts. This indicates that larval exposure to EE2 caused persistent organisational effects on the reproductive and central nervous systems. The combination of a reduced number of males, due to sex-reversal, and an impaired fertility could have severe effects on frog populations. Our results therefore indicate that wild amphibian populations might be at risk from exposure to estrogenic environmental pollutants.

In Xenopus tropicalis tadpoles, exposed to a high concentration of EE2, all individuals developed ovaries but no effects were seen on aromatase activity in gonads and brain. These results indicate that estrogen-induced ovarian differentiation is not paralleled by increased gonadal aromatase activity in Xenopus tropicalis. No effects were seen on aromatase activity in brain and testis in adult frogs after larval exposure to EE2.
Larval exposure to clotrimazole modulated aromatase activity in gonads and brain during development in *Xenopus tropicalis*, as recorded using tissue homogenates *in vitro*. The altered aromatase activity did not affect sex ratio or gonadal differentiation at the time of completed metamorphosis. Further studies are needed in order to investigate the long-term effects of developmental clotrimazole exposure on the reproductive system in amphibians.

The exposure period used in this thesis was appropriate for studies on gonadal sex-reversal and developmental reproductive toxicity in *Xenopus tropicalis* and *Rana temporaria*. The susceptibility to EE$_2$ was similar for the two test species. Clotrimazole exposure affected the endocrine system in *Xenopus tropicalis*. The results in this thesis combined with the short generation time supports the use of *Xenopus tropicalis* as a model species when evaluating long term effects of endocrine disruptors on the reproductive system in amphibians.


att vilda grodor kan vara påverkade av östrogena miljögifter. Kombinationen av att östrogenexponeringen leder till färre antal hanar som samtidigt uppvisar försämrad fertilitet skulle kunna leda till allvarliga konsekvenser för vilda grodopopulationer.


De viktigaste slutsatserna i denna avhandling är att 1) grodor är känsligare för östrogen än vad man tidigare har trott, 2) exponering för miljörelevanta koncentrationer av etinylöstradiol under yngelperioden orsakar könsbyte och en minskad fertilitet hos vuxna grodor, samt att 3) klotrimazol-exponering kan leda till förändrad aktivitet hos enzymet aromatas i gonader och hjärna. Den väst-afrikanska klogrodan är således en passande modellart för att studera effekter på reproduktionssystemet hos grodor efter exponering för miljögifter.
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References


Berg, C., Holm, L., Brandt, I., and Brunström, B. (2001). Anatomical and histological changes in the oviducts of Japanese quail, Coturnix ja-


Sanderson, J. T., Boerma, J., Lansbergen, G. W., and van den Berg, M. (2002). Induction and inhibition of aromatase (CYP19) activity by


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