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The *Xenopus tropicalis* model for developmental endocrine and reproductive toxicity

Histological and molecular endpoints for disrupted spermatogenesis

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Abstract
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Endocrine disrupting chemicals (EDCs) are linked to adverse effects in both humans and wildlife. There are however, large knowledge gaps regarding cause-effect and dose-response relationships between the interference with endocrine pathways and adverse effects in the organism, especially at puberty and in subsequent generations. Using the frog model *Xenopus tropicalis*, endocrine and adverse effects were determined for environmentally relevant concentrations of imazalil, propiconazole and linuron. Flutamide was used as an anti-androgenic model substance. Sexual development was analysed at three juvenile ages and the pubertal onset in males was determined to five weeks post metamorphosis. Pale and dark spermatogonial stem cells (SSCs) were histologically characterized, these sperm stages are not previously described in Xenopus, to the best of my knowledge. Dark SSC, secondary spermatogonia and their ratio were shown to be sensitive endpoints for both flutamide and imazalil juvenile exposure. Imazalil decreased mRNA levels of ID4, increased DDX4 and decreased their ratio. These genes were associated with the germ cell stages affected and are suggested as new endpoints for disrupted spermatogenesis. The number of early spermatogonia is linked to fertility, hence the histological effects of imazalil can be seen as adverse outcomes. In the testes of the imazalil males, the levels of CYP19 and 3β-HSD mRNA were decreased and these changes were associated with the altered numbers of SSCs and secondary spermatogonia. Juvenile propiconazole exposure resulted in increased Aldh1a2 mRNA levels in the testes, but no histological effects on spermatogenesis were observed. These results demonstrate different modes of action of propiconazole and imazalil in the juvenile gonads. Chronic tadpole propiconazole exposure increased brain CYP19 activity at metamorphosis and decreased the time to metamorphosis. Two months after, the testis and Müllerian ducts were smaller and less mature compared with the controls, indicating that propiconazole can induce endocrine and adverse effects, but differently depending on timing of exposure (life stage) and exposure duration. Male offspring of fathers developmentally exposed to linuron demonstrated altered growth and were less fertile, whereas the main findings in the grand-offspring were altered growth and metabolism demonstrating transgenerational effects after developmental exposure to a pesticide. The findings in this thesis contribute with methods and knowledge on how EDCs induces adverse effects via endocrine pathways in juvenile animals and in subsequent generations. The results from this thesis thereby further increase the understanding how EDC exposure can affect humans and wildlife.

Keywords: Endocrine disrupting chemicals, Reproductive toxicology, Developmental toxicology, Spermatogenesis, Transgenerational, Xenopus tropicalis

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“Everything must be taken into account. If the fact will not fit the theory – let that theory go.”

- *Hercule Poirot, The Mysterious Affair at Styles*

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Svanholm, S**[*]., Säfholm, M[*]., Brande-Lavridsen, N., Larsson, E., Berg, C. (2021) Developmental reproductive toxicity and endocrine activity of propiconazole in the *Xenopus tropicalis* model. *Science of the Total Environment,* 753, 141940.

II. **Svanholm, S**., Roza, M., Marini, D., Brouard, V., Karlsson, O., Berg., C. (2022) Pubertal sexual development and endpoints for anti-androgenic changes in spermatogonial differentiation in the model *Xenopus tropicalis*. *Manuscript.*

III. **Svanholm, S**., Brouard, V., Roza, M., Marini, D., Karlsson, O., Berg, C. (2022) Impaired spermatogenesis and associated endocrine effects of azole fungicides in peripubertal *Xenopus tropicalis*. *Manuscript.*

IV. Karlsson, O., **Svanholm, S**., Eriksson, A., Chidiac, J., Eriksson, J., Jernerén, F., Berg, C. (2021) Pesticide-induced multigenerational effects on amphibian reproduction and metabolism. *Science of the Total Environment,* 775, 145771.

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Contents

Abbreviations

16:0	Palmitic acid
16:1	Palmitoleic acid
18:0	Stearic acid
18:1	Oleic acid
3β-HSD	3β-hydroxysteroid dehydrogenase
Aldh1a2	Aldehyde dehydrogenase 1 family, member A2
AMH	Anti-Müllerian hormone
AMHR2	Anti-Müllerian hormone receptor 2
AR	Androgen receptor
BMI	Body mass index
CYP	Cytochrome P450
CYP17	Cytochrome P450 family 17
CYP19	Cytochrome P450 family 19 (aromatase)
CYP26	Cytochrome P450 family 26
CYP51	Cytochrome P450 family 51
DDX4	DEAD-box helicase 4
EDC	Endocrine disrupting chemical
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Estrogen receptor
ESR1	Estrogen receptor 1
ESR2	Estrogen receptor 2
F0	Parental generation
F1	First generation offspring
F2	Second generation offspring
F3	Third generation offspring
FSH	Follicle-stimulating hormone
FSI	Fat body somatic index
GSI	Gonadosomatic index
HPG	Hypothalamic–pituitary–gonadal
ID4	Inhibitor of DNA binding 4
LSI	Liver somatic index
MD	Müllerian duct
MoA	Mode of action
MIE	Molecular initiating event
NF	Nieuwkoop and Faber

PCA	Principle component analysis
PM	Post metamorphosis
Rsbn1	Round spermatid basic protein 1
SSC	Spermatogonial stem cell
SCD-1	Stearoyl-CoA desaturase-1
Sox9	SRY-box transcription factor 9
SVL	Snout-vent length
T3	Triiodothyronine

Introduction

Endocrine disrupting chemicals

Numerous chemicals have the ability to interfere with endogenous endocrine systems in both humans and wildlife. Such endocrine disrupting chemicals (EDCs) have been shown to cause disrupted metabolism and reproductive development in experimental studies and epidemiological links to metabolic diseases and fertility problems in humans exist (Casals-Casas and Desvergne, 2011; Green et al., 2021; Heindel et al., 2017; Holley et al., 2020). There are different ways in which a chemical can interfere with the endocrine systems. These endocrine modes of actions (MoAs), include receptor binding and inhibition of key enzymes. It has proven to be difficult to classify chemicals as EDCs according to the World health organization's definition: "An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (IPCS, 2002; WHO/UNEP, 2013), as this requires a casual association between the endocrine and adverse effect.

Pesticides (biocides and plant protection products) are chemicals that are used to control all kinds of pests, mainly in agriculture, but also in other fields. In 2020, the usage of pesticides alone was estimated to be 3.5 million tonnes (Zhang, 2018). Both humans and wildlife are exposed to pesticides and biocides directly during application (inhalation or dermal absorption during overspray etc.) as well as indirectly via contaminated soil, water and residues in food. This can for example result in exposure of wild amphibians to concentrations of pesticides that in laboratory studies have been shown to cause adverse effects (EFSA, 2018). All pesticides on the European market shall be assessed for endocrine disruptive properties and their potential linkages to adverse effects according to the criteria determined by the European Commission. A guidance document was published to provide guidance regarding implementation of the scientific criteria for the hazard-based identification of EDCs in the context of the pesticide regulations (ECHA et al., 2018). Several widely used pesticides have endocrine disruptive activity *in vitro* but data regarding potential adverse impacts are scarce and even less is known about peripubertal, multigenerational and transgenerational effects. The knowledge gaps on potential endocrine disruptive effects of pesticides *in vivo* needs to be addressed to protect humans and wildlife from potential harm.

Azoles

Azoles are a group of fungicides used to prevent and treat fungal growth. They are cyclic compounds classified according to the number and position of nitrogen in the ring. The anti-fungal properties of azoles come from the inhibition of Cytochrome P450 (CYP) 51, an enzyme important in fungal cell wall formation. Like many azoles, the imidazole imazalil, and the triazole propiconazole have several MoAs including inhibition of CYP1, CYP17 and CYP19 (aromatase) and androgen receptor (AR) antagonization (Aït-Aïssa et al., 2010; Beijer et al., 2018; Kjærstad et al., 2010; Kjeldsen et al., 2013; Kojima et al., 2004; Orton et al., 2011; Roelofs et al., 2013; Trösken et al., 2006, 2004; Vinggaard et al., 2000). In addition, imazalil weakly antagonizes the estrogenic receptor (Kojima et al., 2004). Some azoles have also been shown to target retinoic acid signaling by increasing transcription of genes involved in the synthesis (Dimopoulou et al., 2017). To our knowledge, no interactions with the retinoic acid pathway have been reported for neither propiconazole nor imazalil.

Propiconazole has been detected in run-off water at concentrations of a few µg to tens of µg/L, whereas imazalil concentrations range up to a hundred µg/L (Castillo et al., 2000; Ministry of the Environment Government of Japan, 2004). Both these azoles have been detected as residues in fruit and vegetables at levels of 0.47-1.55 mg/kg (Andersson et al., 2010; Jansson and Fogelberg, 2018). Despite the concerns of endocrine activity *in vitro* and presence in food, water and the environment, there is still limited information regarding reproductive toxicity of propiconazole and imazalil in amphibians and higher vertebrates, including humans.

Linuron

Linuron is a herbicide acting by inhibiting photosystem II. It is broadly used in both orchards and fields and has been found in water systems in concentrations up to 2.8 mg/L (Caux et al., 1998). Linuron has an anti-androgenic MoA and is classified as an EDC. Developmental linuron exposure has resulted in testicular abnormalities in rats and a female-biased sex ratio, demasculinization and reduced fertility in adult male frogs (Lambright et al., 2000; Orton et al., 2018). Linuron has also been shown to suggested to exert anti-estrogen and thyroid disrupting properties in amphibian larvae (Spirhanzlova et al., 2017).

The *Xenopus tropicalis* model

X. tropicalis is a good toxicological model for studies on reproduction and endocrine disruption for several reasons. Laboratory studies have shown *X.*

tropicalis to be sensitive to environmentally relevant concentrations of EDCs, including pesticides and pharmaceuticals (Gyllenhammar et al., 2009b; Säfholm et al., 2014, 2012). Both the development of the reproductive organs as well as the hypothalamic–pituitary–gonadal (HPG) and the hypothalamic–pituitary–thyroid axes are similar to those in higher vertebrates. The diploid genome is sequenced and the generation time is shorter compared to its commonly used relative, the tetraploid *X. laevis*, which facilitates life cycle studies (Berg, 2019; Pettersson and Berg, 2007). *X. tropicalis* also has 66 clearly defined developmental stages by Nieuwkoop and Faber (NF stages), which describes development from fertilization (NF stage 1) until completed metamorphosis (NF stage 66). This makes it possible to expose and analyse during specific developmental key events e.g. sex determination (Nieuwkoop and Faber, 1956).

X. tropicalis is not only an important toxicological model for studies on reproduction and EDCs in general. Biodiversity is decreasing globally and amphibians is the vertebrate group most affected. As many as 40% of amphibian species are threatened with extinction (IUCN, 2020). EDCs have been suggested as one of the factors contributing to the global amphibian decline (Mann et al., 2009). It is therefore of great importance to further understand the effects of EDCs leading to adverse outcomes in amphibians specifically. *X. tropicalis* has been shown to be comparable to the European common frog (*Rana temporaria*) in sensitivity to estrogenic EDC exposure, indicating its usefulness as a model for wild amphibians (Pettersson and Berg, 2007).

Previous studies in the *X. tropicalis* model have been able to link endocrine effects during development to adverse effects at adulthood after exposure to various EDCs (Gyllenhammar et al., 2009b; Kvarnryd et al., 2011; Orton et al., 2018; Pettersson et al., 2006). However, life cycle studies are very time and resource consuming, and faster *in vivo* toxicity test methods need to be developed as a compliment to meet the demand for increased EDC testing.

Reproductive development

Hormones regulate the development of the reproductive system and exposures to EDCs during this sensitive period can therefore have severe consequences (WHO/UNEP, 2013). Links between early EDC exposure, disrupted testis development including reduced sperm quality, and decreased reproductive success later in life have been established in humans and wildlife populations (Delbes et al., 2022; Marlatt et al., 2022; WHO/UNEP, 2013). Increasing the knowledge on sexual development and consequences of EDC exposure on the developing reproductive system is therefore important.

The development of the reproductive system starts in early life with the sex determination. The sex in *X. tropicalis* is determined genetically and is manifested phenotypically at NF stage 51-53 (Figure 1) (Takase and Iguchi, 2007).

However, exogenous hormones e.g. from the environment, have the ability to interfere with this process and override the genetic sex resulting in phenotypic sex reversal. Thus sex ratio in *X. tropicalis* has been shown to be a sensitive endpoint for both estrogenic and anti-androgenic chemicals (Orton et al., 2018; Pettersson et al., 2006, Pettersson & Berg 2007).

Sex differentiation refers to the whole period of sex characteristics development, including gonads, brain and secondary sex characteristics. The sex differentiation continues up to sexual maturation, which is 3 months for male and 4.5 months post metamorphosis (PM) for female *X. tropicalis* (Hirsch et al., 2002). At sexual maturation mature germ cells (spermatozoa in males and mature oocytes in females), oviducts (females), vas deference (males) and secondary sex characteristics are present. Secondary sex characteristics include pear shaped body in females and nuptial pads (breeding "glands" situated on the fore limbs) in males (Sever and Staub, 2011). The development of the nuptial pads and the width of the fore limbs are highly androgen dependent and have previously been shown to be affected by developmental linuron exposure in an anti-androgenic fashion (Orton et al., 2018). The information regarding peripubertal sexual development, i.e. first occurrence of secondary sex characteristics and mature sperm, is however limited. Toxicological studies often focus on the very early establishment of germ cells, so information on other critical periods e.g. peripuberty, is therefore scarce (WHO/UNEP, 2013). To further develop *X. tropicalis* as a model and adequately address these knowledge gaps, studies on peripubertal development and effects of EDC exposure are needed.

There are several key enzymes and receptors involved in endocrine pathways for reproductive development. CYP19 is present in both brain and gonads where it converts androgens into estrogens (which binds to estrogen receptor 1, ESR1). CYP17 and 3β-hydroxysteroid dehydrogenase (3β-HSD) are also involved in sex steroid synthesis (Piprek et al., 2018). Anti-Müllerian hormone (AMH), together with its receptor AMH receptor 2 (AMHR2) regulates estrogen and androgen production, whereas SRY-box transcription factor 9 (Sox9) regulates AMH (Jamil et al., 2008; Munsterberg and Lovell-Badge, 1991; Racine et al., 1998; Roco et al., 2021). CYP26 and aldehyde dehydrogenase 1 family, member A2 (Aldh1a2) are involved in synthesis and degradation of retinoic acid (Endo et al., 2019). Chemical interference with the expression or activity of these enzymes could therefore be used as indication of endocrine disturbance.

Spermatogenesis

Spermatogonial stem cells (SSCs, sometimes referred to as primary spermatogonia), secondary spermatogonia, primary spermatocytes, spermatids and spermatozoa have previously been found in *Xenopus* (Gyllenhammar et al., 2009a; Kalt, 1976; Orton et al., 2018; Säfholm et al., 2016; Takamune et al.,

2001) although morphological details on some stages are lacking for *X. tropicalis*. Haczkiewicz et al. (2017) and Ogielska and Bartmańska (1999) have characterized additional stages in *R. esculenta, Pelophylax lessonae* and *P. ridibundus*: gonocytes and pale and dark SSCs. No such information is available for *Xenopus* to the best of the author's knowledge. As the sperm development has been shown to be targeted by EDCs (Orton et al., 2018), histological characterization of the early sperm stages is therefore important to enable a better understanding of EDC targets of the spermatogenesis.

Sex hormones are involved in the regulation of spermatogenesis, with testosterone being the main androgen in amphibians and higher vertebrates (Singh and Handelsman, 1996). It is produced by Leydig cells in the testis and acts on Sertoli cells via the AR and other transcription factors (Walker, 2009). Production is stimulated by gonadotropins via negative feedback on the HPG axis. A lack of the AR or the hormone itself results in inhibited germ cell maturation and no mature sperm in adult mice and amphibians (Ogielska and Bartmańska, 2009; Yeh et al., 2002). There are several chemicals shown to have an anti-androgenic MoA *in vitro* (Aït-Aïssa et al., 2010; Fang et al., 2003; Kojima et al., 2004; Orton et al., 2011), which thereby pose a risk for spermatogenesis in vertebrates *in vivo*.

In addition to the sex steroids, thyroid hormones are suggested to play a role in amphibian testis development, and could therefore also affect spermatogenesis (Duarte-Guterman and Trudeau, 2011; Hayes, 1998; Holsberger et al., 2005; Mendis-Handagama and Ariyaratne, 2005). There is cross-talk between the HPG and hypothalamic-pituitary-thyroid axes (Flood et al., 2013; Flood and Langlois, 2014) further supporting the role of thyroid hormones in gonadal maturation.

Some genes are only expressed in specific germ cell stages and their mRNA levels could therefore be explored as endpoints for disturbed spermatogenesis. Important genes are: Inhibitor of DNA binding 4 (*ID4*) which is expressed in SSCs; DEAD-box helicase 4 (*DDX4*, or Xenopus Vasa Homolog, *XVLG1*), expressed in both SSCs and spermatocytes and; Round spermatid basic protein 1 (*Rsbn1*), expressed in spermatids and spermatozoa (Komiya et al., 1994; Oatley et al., 2011; Takahashi et al., 2004).

Müllerian ducts

The oviducts in vertebrates (except teleost fish) originate from the Müllerian ducts. The regression of the Müllerian ducts in males is stimulated by AMH, but the timing of the regression in amphibians is not clear (Jansson et al., 2016; Säfholm et al., 2016). Azole pesticides have been shown to affect Müllerian duct development in amphibians (Haselman et al., 2018).

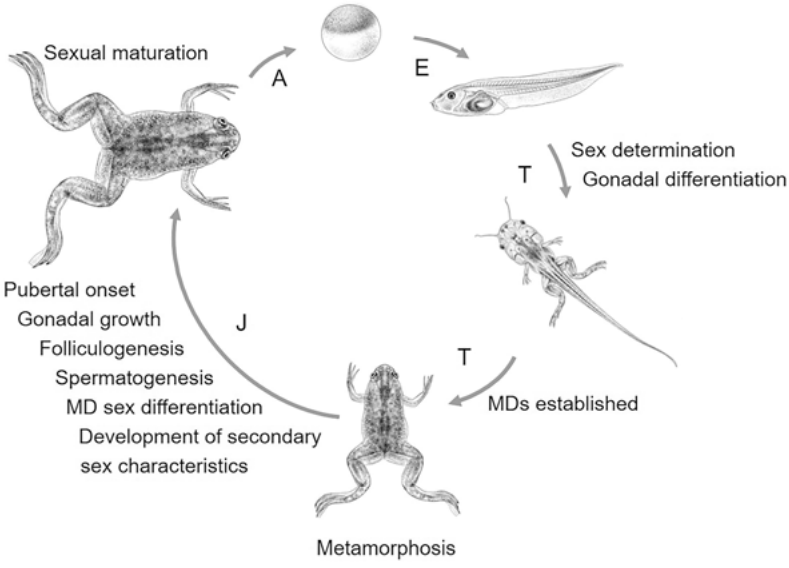

Figure 1. Reproductive cycle of *Xenopus tropicalis* with important developmental events indicated. E: embryo stage.T: tadpole stages. J: Juvenile stages. A: Adult stage. MD: Müllerian duct. Adapted from Xenbase, (2022) and Zahn et al. 2022. *Xenopus* illustrations © Natalya Zahn (2022).

Transgenerational effects

There is an increasing interest in the epigenome, in the context of chemical exposure. EDCs have been suggested to induce epigenetic alterations in humans, rodents and fish amongst others (Baker et al., 2014; Nilsson et al., 2018; Xin et al., 2015). The most common epigenetic mechanisms are DNA methylation, histone modification and non-coding RNAs. Epigenetic modifications survive cell division within the organism, but questions are raised as to whether they can also be transferred to the offspring. Transgenerational epigenetic inheritance refers to epigenetic changes in germ cells in response to a stimulus, leading to effects in a generation which has not been in contact with that stimulus. In mammals, the developing embryo (F1) is dependent on the mother (F0) and may therefore be exposed *in utero*. The next generation (F2) consists of the developing germ cells in F1, which may be indirectly exposed. Therefore, to ensure that the epigenetic changes studied are not a result of direct exposure, transgenerational effects are studied in the F3 generation in mammals. In frogs, on the other hand, the external embryo development, ensuring no direct or indirect exposure, makes it possible to study transgenerational effects in the F2 generation.

Developmental exposure to EDCs has been shown in both mammals and fish to cause reproductive failure as an effect of impaired spermatogenesis in subsequent generation offspring (Anway et al., 2005; Bhandari et al., 2015;

Hao et al., 2016; Meyer et al., 2018; Nilsson et al., 2018; Song and Yang, 2018). Several studies have also demonstrated metabolic changes in following generations after parental developmental EDC exposure (Rajesh and Balasubramanian, 2014; Susiarjo et al., 2015; Zhang et al., 2014; Zhao et al., 2021). There are however, several questions remaining to be elucidated within the field of transgenerational toxicology. It is still uncertain if transgenerational inheritance after chemical exposure occurs in frogs and the respective influence of paternal and maternal exposure.

Aims

The overall aim of this thesis was to develop methods to increase the understanding of how environmental chemicals interfere with endocrine pathways during development leading to adverse effects later in life, and in subsequent generations, using *Xenopus tropicalis* as a model organism. The specific objectives were to:

- Identify molecular and histological endpoints for developmental reproductive toxicity in juveniles (paper I, II, and III).
- Determine endocrine and adverse effects of chronic larval exposure to propiconazole using a partial life cycle assay (paper I).
- Determine apical and detailed histological effects of juvenile exposure to the anti-androgenic model substance flutamide on sexual maturity (paper II).
- Increase the understanding of peripubertal sexual development, including pubertal onset, gonadal maturity and gametogenesis (paper II and III).
- Determine endocrine and adverse effects of propiconazole and imazalil on development, reproduction and metabolism after short-term juvenile exposure (paper III).
- Characterise transgenerational adverse effects and underlying pathways of paternal developmental linuron exposure on male reproduction and metabolism (paper IV).

Methods

Experimental design

The endpoints for endocrine and adverse effects used within this thesis are presented in Table 1.

Table 1. Endpoints for endocrine and adverse effects used in thesis.

Endpoint	System	Paper
Endocrine effects		
Time to metamorphosis	Thyroid	I
Hind limb length	Thyroid	I, II and IV
mRNA levels of key genes	Sex steroid	III
Presence/morphology of secondary sex characteristics	Sex steroid	II, III and IV
Fore limb morphology	Sex steroid	III and IV
Brain CYP19 activity	Sex steroid	I
Estradiol and AMH whole body levels	Sex steroid	I
Plasma glucose levels	Metabolic	IV
Liver and fat body fatty acid composition	Metabolic	IV
Adverse effects		
Sex ratio	Reproductive	I
Gonadal area	Reproductive	I, II and III
Number of germ cells per stage (related to area)	Reproductive	I, II, III and IV
Germ cell associated mRNA levels	Reproductive	III
Müllerian duct maturity	Reproductive	I, II and III
Müllerian duct length	Reproductive	I
Fertility and mating behaviour	Reproductive	IV
Gonadosomatic index	Reproductive	IV
Body weight/BMI	Metabolic	IV
Liver and fat body somatic index	Metabolic	IV

CYP19: Cytochrome P450 19. AMH: Anti-Müllerian hormone. BMI: body mass index

Peripubertal reproductive development was assessed in control animals in paper II and III. The number of germ cells per stage, gonadal area and their relationship as well as gonadal (most mature germ cell present) and Müllerian duct maturity (scoring system) were used as a measures of reproductive organ development. Presence of mature germ cells and secondary sex characteristics was used to evaluate pubertal onset in paper II. If either mature germ cells (spermatozoa/mature oocytes) or nuptial pads/pear-shaped body were present, the animal was determined as having entered puberty. To characterise the reproductive development over different juvenile ages, the number of germ cells, and gonadal and Müllerian duct maturity was compared.

Chronic, partial life-cycle exposure

In paper I, *X. tropicalis* were developmentally exposed to 0, 33 or 384 µg propiconazole/L from NF stage 47/48 until completed metamorphosis (Table 2). A subset of 15 animals from each treatment was sampled at metamorphosis, and the rest transferred to clean water until eight weeks PM. The exposures were semi-static with half of the water changed three times a week. Triplicate tanks with acetone (0.002%) as a solvent was used for all treatments, including control.

Short-term peripubertal exposures

Three different short-term juvenile *X. tropicalis* exposures were conducted in papers II and III (Table 2). In paper II, 17.5±4 days PM old animals were exposed to 0 (control), 250 (low), 500 (mid) or 1000 (high) µg flutamide/L for 17±2 days. In paper III 12.5±2.5 days PM old animals were exposed to 0 (control), 17 (low) or 178 (high) µg propiconazole/L for 16±1 days and 45±5 days PM old animals were exposed to 0 (control), 1 (low), 12 (mid) or 154 (high) µg imazalil/L for 15.5±0.5 days. Acetone (0.0008 or 0.002%) was used as a solvent in all tanks, including controls in all exposures. The exposures were semi-static, with half of the water changed three times per week. Triplicate (imazalil, propiconazole and flutamide control) or duplicate (flutamide low, mid and high) tanks were used.

Larval exposure for transgenerational study

Paper IV is based on frogs originating from the Orton et al. (2018) study. In short, *X. tropicalis* was developmentally exposed to a mean linuron concen-

tration of 26 µg/L from NF stage 40 until metamorphosis (NF 66) using acetone (0.0008%) as solvent (Table 2). The exposure was semi-static, with water change three times a week. After metamorphosis, the frogs were moved to clean water and reared until sexually mature, when the males were mated with naïve females generating the F1 generation. The F1 frogs were raised until adulthood at which time the males were mated with naïve females in the same way as F1, generating the F2 generation. Throughout the whole transgenerational study, all treatments were kept in a minimum of three replicate tanks.

Table 2. Overview of the exposure scenarios and time points of analysis used in this thesis.

Test substance	Concentration	Exposure period	Duration	Age at analysis
Propiconazole (Paper I)	0 µg/L 33 µg/L 384 µg/L	Tadpole	NF stage 47/48-66	Metamorphosis and eight weeks PM
Flutamide[a] (Paper II)	0 µg/L 250 µg/L 500 µg/L 1000 µg/L	Juvenile	17±2 days	Five weeks PM
Propiconazole (Paper III)	0 µg/L 17 µg/L 178 g/L	Juvenile	16±1 days	Four weeks PM
Imazalil (Paper III)	0 µg/L 1 µg/l 12 µg/L 154 µg/L	Juvenile	15.5±0.5 days	Eight weeks PM
Linuron[b] (Paper IV)	0 µg/L 26 µg/L	F0 tadpole	NF stage 40-66	Adult

[a]Nominal concentrations. [b]Exposure conducted in Orton et al., (2018).
PM: post-metamorphosis

Mating behaviour and fertility analysis

F1 and F2 generation males were mated with unexposed females (paper IV) according to Berg (2019). One male and one female were placed in an aquarium and checked every 45th minutes. Time to enter amplexus, total time in amplexus, time to spawning and the total time spent in amplexus were noted. Photos of the tanks containing eggs were taken right after the mating was discontinued and 26 hours later. Fertility and fecundity were calculated comparing the two photos using ImageJ (NIH, Bethesda, MD, USA).

Analysis of apical endpoints

The time to metamorphosis was measured as the number of days between fertilization and NF stage 66 (paper I). During dissection, the body weight, snout-vent length (SVL) (paper I, II, III and IV), hind limb length (paper II, III and IV) and forelimb width and length were measured (paper III and IV). In addition, fat body weight (paper III and IV), liver weight (paper I and III) and gonadal weight (paper IV) were recorded. Fat somatic index (FSI), liver somatic index (LSI) and gonadosomatic index (GSI) were calculated as (weight of organ/weight of body)*100 and body mass index (BMI) calculated as body weight/SVL^2. The presence of secondary sex characteristics was noted (paper II, III and IV) and a photo of the nuptial pads was taken (paper IV) in which the nuptial pad area was measured using ImageJ (NIH, Bethesda, MD, USA).

Histological analysis

Histological processing

Either the whole, or the right part of the gonadal-kidney-complexes (metamorphs/juveniles) or left testis (adults) were put in formalin (4%, buffered), and later dehydrated in increasing concentrations of ethanol and imbedded in hydroxyethyl methacrylate. In paper I, the complexes were divided transversally (to fit in the embedding mould) and embedded in two parts, whereas in paper II and III, they were embedded in one part.

The juvenile gonadal-kidney-complexes were sectioned transversally either throughout the whole complex (paper I) or at three levels (paper II and III) starting from the anterior part with as distance of 300 µm in-between. Sections from the adult testis (paper IV) were taken from the anterior part. All sections were 2 µm thin and stained with either toluidine blue or haematoxylin and eosin depending on the purpose of the evaluation.

Histological evaluation

All histological slides were analysed by one person using coded slides. The slides were either evaluated using light microscopy (paper I and IV) or scanned with a histological scanner (NanoZoomer 2.0-H, Hamamatsu) and evaluated using NDP.view (Hamamatsu Photonics K.K, version 2.7.52, 2019) (paper II and III).

Characterization of sperm stages

In control animals from paper II sperm stages were characterized using detailed histology and a combination of criteria from Haczkiewicz et al. (2017), Kalt (1976), Ogielska and Bartmańska (2009) and Takamune et al. (2001). The stages being characterized were: Gonocytes (large, single cells occupying the major part/space of the sex cords), pale SSCs (large, single cells with light pink cytoplasm and homogenous chromatin), dark SSCs (smaller than SSCs and with a darker cytoplasm and more heterochromatin. Present individually or in clusters of two), secondary spermatogonia (clearly organized into spermatocysts. Irregular shaped nucleus with visible nucleoli and chromatin patches), primary spermatocytes (chromosome appearance depends on the phase of meiosis. In prophase, the cells have a large nucleus with loosely packed chromatin), secondary spermatocytes (round, smaller than primary spermatocytes, with a small, round, condensed nucleus), spermatids (completely round or slightly elongated. Dissolved cytoplasm with cells connected via a spider net-like structure) and spermatozoa (elongated cells, completely lacking cytoplasm).

Sex determination

At metamorphosis, gonads with an ovarian cavity and oocytes were scored as oocytes, and as testis if there was a lack of cavity at metamorphosis (paper I). In juvenile animals, individuals were scored as females if containing a cavity and oocytes and as testis if lacking a cavity and containing seminiferous cords/tubuli and sperm (paper I, II, III and IV).

Testicular evaluation

In paper I and IV, the number of spermatogonia and number of individual (paper I) or nests of (paper IV) spermatocytes, spermatids and lumen with spermatozoa were counted. In paper IV, the number of spermatocytes in the largest nest was also counted. A more detailed testis morphology was analysed in paper II and III, where gonocytes, pale SSCs, dark SSCs, secondary spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and lumen containing spermatozoa were quantified. A ratio between secondary spermatogonia:dark SSCs was also calculated in paper III. In the juveniles, the maturation of the testis was scored by noting the most mature germ cell: 1. Spermatogonium (pale, dark and secondary), 2. Spermatocyte (primary and secondary), 3. spermatid or 4. spermatozoon (paper I and II) or pre-meiotic cells/meiotic cells (paper III).

Ovarian evaluation

In paper I, II and III, the oocyte maturation was scored using criteria by Hausen and Riebesell (1991). The number of non-follicular (premeiotic oogonia, primary oogonia, secondary oogonia, preleptotene oocytes, leptotene oocytes, pachytene oocytes and early diplotene oocytes) or follicular (pre-vitellogenic oocytes stage I, II, III, IV, V and VI) were counted and the most mature germ cell present was noted. A ratio between follicular/non-follicular oocytes was calculated for paper II.

Gonadal area

The cross-section area of both (paper I and II) or the left (paper III and IV) gonads were measured using ImageJ (National Institute of Health, Bethesda, MD, USA). In paper I, II and III the area was related to number of germ cells.

Müllerian duct maturity

Müllerian duct maturation (paper I, II and III) was evaluated using the scoring by Jansson et al. (2016): "1 - a small rounded bulge of irregularly packed mesenchymal cells at the lateral side of the kidney, 2– a small bud protruding from the kidney, 3 – a distinct structure attached to the lateral side of the kidney, 4 – a distinct tubular structure without a cavity, and 5 – a distinct tubular structure with a cavity lined by elongated epithelial cells." The length of the Müllerian ducts was calculated as the number of slides where the ducts were visible multiplied with the length in between the sections (paper I).

Biochemical analyses

Whole body hormone concentrations

In paper I, the whole body (without intestines, head, gonads, and liver) was crushed in a mortar using liquid nitrogen. Phosphate-buffered saline was added and the samples mixed with a homogenizer, sonicated, and centrifuged. The supernatant was saved for further analysis. AMH (both sexes) and estradiol (females) in whole body homogenate was measured using commercial ELISA kits (MyBioSource, San Diego, Califonia, USA), following the instructions of the manufacturer (paper I). In short, all standards, samples and blanks were added in duplicates to the plate. Horseradish peroxidase-conjugate reagent, chromogen solutions A and B and stop solution was added in turns after periods of incubation and washing. The plates were read spectrophotometrically at 450 nm.

CYP19 activity

Brain CYP19 activity was quantified in paper I by a modified tritiated water-release method (Gyllenhammar et al., 2009a; Lephart and Simpson, 1991), and is based on quantification of 1β-3[H] released from labelled androstenedione added to the samples. Protein content was measured using a BCA-kit (Nordic Biolabs AB, Stockholm, Sweden) and read spectrophotometrically.

Fatty acid analysis

Fatty acids in liver and fat bodies were analysed in paper IV using gas chromatography-mass spectrometry (Scion TQ, Bruker Daltonics Inc., Billerica, MA, USA). A series of fatty acids were used to create a standard curve and a modified Folch procedure was used to extract lipids for the tissue and an internal standard mix was added to all the samples (Jernerén et al., 2015).

Blood plasma analysis

Plasma was sampled during dissection (Paper IV) and plasma glucose levels were analyzed using enzymatic colorimetric methods at the Clinical Pathology Laboratory, Swedish University of Agricultural Sciences (SLU).

Molecular analysis

mRNA analysis

Gonadal mRNA was extracted, cDNA synthesised, and quantitative real time PCR (qPCR) preformed using a commercial kit following the manufacturer's instructions (Bio-Rad Laboratories Inc. Hercules, CA, USA). The quantity and quality were checked spectrophotometrically and a melting curve analysis was made. The measured genes were: *Sox9, CYP17a1, 3β-HSD, ID4, Rsbn1, DDX4, CYP19a1, Aldh1a2, CYP26b1, ESR1, AMH* and *AMHR2* with eukaryotic translation elongation factor 1 alpha 1 (EF1) and Ribosomal protein L8 (RPL8) as reference genes to which the other genes were standardized to. Additional primer information is presented in paper III. A ratio of *ID4:DDX4* was calculated as a molecular endpoint for disturbed spermatogenesis.

Chemical analysis

The propiconazole and imazalil concentrations in the exposure aquariums in paper I and III, were analysed using automated on-line solid phase extraction-liquid chromatography-tandem mass spectrometry (Jansson and Kreuger,

2010) by the Centre for chemical pesticides, Department of Aquatic Sciences and Assessment, SLU.

Statistics

The data from the different replicate tanks were visually examined to estimate the variability, and as there were no clear difference, the data were pooled according to treatment. Fisher's exact test or Chi square test was used to analyze all frequencies/proportions in all papers. Mann-Whitney U-test were used for all continuous data in paper IV as well as for comparisons between sexes in paper I and II and between ages in paper III. Linear regression was used to assess the relationship between number of germ cells and gonadal area in paper II. Continuous data for the treatments in paper I were compared using one-way ANOVA with Tukey's Multiple Comparison Test if the data passed the Shapiro-Wilk test, if not, the Kruskal-Wallis test with Dunn's Multiple Comparison Test was used. Treatment effects in paper II and III were analysed using generalized linear models, specifying different distribution and link functions (Gaussian, Gamma and Quasi-binomial distributions and identity, log and logit link functions) depending on the distributions of the residuals. The exception was the *ID4*:*DDX4* ratio which was compared using Kruskal-Wallis test with Dunn's Multiple Comparison Test. The Holm-Bonferroni method was used to adjust the p-value to compensate for multiple comparisons. A principle component analysis (PCA) was conducted to assess any associations between the affected genes (involved in gonadal development and expressed in specific sperm staged) and the histological changes in imazalil exposed animals in paper III.

Fisher's exact test, Chi square, Mann-Whitney, ANOVA and Kruskal-Wallis tests were conducted using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) whereas model diagnostics, generalized linear models and the PCA was conducted using RStudios 1.3.1093 and 4.0.2 (R Core Team, Vienna, Austria and RStudio Team, Boston, MA, US). Data was considered statistically significant if $p<0.05$ in all papers.

Results and discussion

Gonadal maturation and spermatogenesis

Spermatogenesis

Both pale and dark SSCs were characterized in the testis at five weeks PM along with secondary spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa (Figure 2). The SSCs and the mature spermatozoa were the stages easiest to characterize as their morphology is very distinct. Later stages of secondary spermatogonia and early primary spermatocytes, were similar but the packing of the chromatin and nucleoli differed. As dark SSCs is suggestive of an active spermatogenesis, gonocytes were not identified at five weeks PM. This characterization harmonizes the spermatogenesis with other amphibians and higher vertebrates (Haczkiewicz et al., 2017). SSCs in amphibians would equal A_{dark} and A_{pale} spermatogonia in monkeys and humans or A_{single} in rodents. B spermatogonia in monkeys and humans and A_{paired}, $A_{aligned}$ and B spermatogonia in rodents, would equal to secondary spermatogonia in amphibians. However, the occurrence of different A spermatogonia in mammals is low and identification is difficult (Chen and Liu, 2015; Oatley and Brinster, 2006), hence, *X. tropicalis* is suggested as a useful model for studies on early spermatogenesis.

Testis maturity

The sperm stage composition of the testis was similar at five (**paper II**) and eight (**paper I and III**) weeks PM, where approximately half of the animals had mature spermatozoa as the most mature germ cell stage and the others secondary spermatogonia, secondary spermatocytes and spermatids as the most mature germ cells. The testis at eight weeks PM were significantly more mature compared to four weeks PM (**paper III**), where secondary spermatogonia was the most mature germ cell in the majority of the animals, and only one individual had germ cells in the first meiotic division. Furthermore, the number of primary spermatocytes and the testis area was increased at eight weeks compared to four weeks PM, further supporting that the male reproductive maturity was more advanced at the later age. No significant difference between the two age groups with regard to gonadal mRNA levels of the analysed genes was however found. These results help to determine important

growth periods during reproductive development, and guide further studies on molecular processes, thus advancing *X. tropicalis* as a model in reproductive toxicology.

The numbers of both total and dark SSCs were positively correlated to testis area at five weeks PM, but not the other sperm stages evaluated (too few observations of secondary spermatocytes, spermatids and spermatozoa were made for meaningful regression analysis). The results in the present thesis therefore indicate that differentiation of pale to dark SSCs i.e. activation of spermatogenesis and the proliferation of dark SSCs constitute the main contribution to the testis growth during peripuberty.

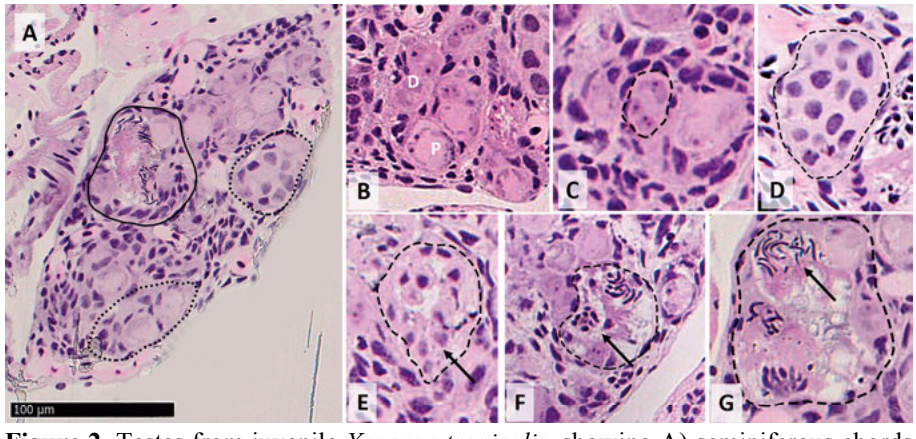

Figure 2. Testes from juvenile *Xenopus tropicalis*, showing A) seminiferous chords (dotted line) and seminiferous tubule containing a lumen (solid line), B) spermatogonial stem cells (SSCs); pale SSCs (P) and dark SSCs (D), C) nests with proliferating secondary spermatogonia, D) nest with primary spermatocytes, E) secondary spermatocytes starting to differentiate in to spermatids (arrow), F) spermatids (arrow), G) fully mature spermatozoa (arrow). Sections are stained with haematoxylin-eosin. Dashed lines encircle germ cell nests.

Ovary maturity

Females at four, five and eight weeks PM had ovaries containing non-follicular and follicular oocytes in stages I, II and III. Follicular oocytes were the most mature oocytes in all animals at five and eight weeks PM as well as in the majority of the females at four weeks PM. Only a few animals had ovaries containing non-follicular oocytes at four weeks PM and the ovary maturity did not differ between four and eight weeks PM (**paper III**). The area of the ovaries were larger at eight compared to four weeks PM (**paper III**) and there was a positive correlation between total number and follicular oocytes to ovar-

ian area. Although there was no significant difference in mRNA concentrations of the studied genes between the ovaries four and eight weeks PM (**paper III**), this period seem to be important for the ovarian growth.

Müllerian duct maturity

At five and eight weeks PM, the females had longer and more mature ducts compared to the males (**paper I and II**) which is in line with previous research finding sexual dimorphism of the Müllerian ducts at four weeks PM (Säfholm et al., 2016). This suggests that the weeks after metamorphosis is an important growth period for the Müllerian ducts either by developing in females or regressing in males.

Pubertal onset

Mature sperm was present in the testes of both five and eight weeks PM old animals, suggesting the start of puberty in males. The presence of spermatozoa but absence of nuptial pads in males suggest that circulating testosterone is still low at these ages. Additionally, no mature stage VI oocytes nor secondary sex characteristics were observed in any of the females implying low levels of circulating estradiol levels in the juveniles. These finding further precise the pubertal onset in males to five weeks PM in *X. tropicalis*. Moreover, the results show that gonadal histology was the earliest and most reliable method for establishing pubertal onset, as compared with secondary sex characteristics used in **paper II**.

Sex hormone disruption

Fertility and mating behaviour

The average male fertility in **paper IV** did not differ between the control and the linuron frogs in the F1 generation (44.9% and 30.8% respectively), but there was a significantly larger frequency of animals with low fertility (< 15%) in the linuron group (10/32) than in the control group (0/12). There was no difference between the F1 linuron and the control males regarding time to amplexus or time spent in amplexus, but the F1 linuron frogs started to spawn earlier than the controls. No differences between F2 linuron and F2 control males on breeding was observed. Decreased fertility was also seen in the males of the parental generation as an effect of direct exposure to linuron (Orton et al., 2018) and in F2 and F3 generation offspring to female rats exposed to vinclozolin (Nilsson et al., 2018). The results in the present thesis add to the increasing body of evidence showing that several EDCs can induce decreased fertility after paternal developmental exposure.

Secondary sex characteristics and gonadal weight

The size of the nuptial pad and the forelimb width (**paper IV**) were decreased in the F1, but not in the F2 linuron males (Table 3). These results combined with the changes seen in testis composition (increased number of spermatogonia and smaller spermatocyte nests), suggest interference with the androgen system in the F1 generation, similar to what was seen in the parental generation (Orton et al., 2018). No effects were detected on fore limb morphology after short-term juvenile imazalil exposure (**paper III**). As no nuptial pads were observed, and both characteristics are androgen dependent, the results indicate that fore limb morphology is not a sensitive endpoint for anti-androgenic exposure at eight weeks PM. The results from **paper IV** are suggestive of anti-androgenic effects resulting from indirect linuron exposure.

Sexing

No significant difference in sex ratio after the propiconazole exposure was observed compared with the control group (**paper I**). The azole fadrozole induced a female biased sex ratio in newts after developmental exposure to 300 µg/L (Chardard and Dournon, 1999). The lack of consistent effects of azoles on amphibian sex ratio may result from their many different MoAs, which complicate comparisons between the compounds. Since propiconazole has been shown to interfere with both CYP19, ER and ARs (Aït-Aïssa et al., 2010; Kjærstad et al., 2010; Kjeldsen et al., 2013; Kojima et al., 2004), it is possible that any endocrine disrupting effects of propiconazole on sex hormones are counteracted during the short window of sex determination.

Testis histology

Tadpole exposure to linuron and propiconazole affected testis composition later in life (**paper I**) as well as in the F1 and F2 generation males (**paper IV**). Chronic propiconazole exposure to 33 µg/L (**paper I**) caused less mature testis (when comparing the most mature sperm stage, Figure 3A) as well as an increased proportion of testis smaller than the mean of controls ($\leq$194mm^2) (Figure 3B). There was an increase in size of spermatocyte nests as well as more spermatogonia in the F1 linuron frogs compared to the F1 controls, whereas there was a decrease in the number of germ cell nests in the F2 linuron frogs compared to the controls (Table 3). There were also several testes (8/22) in the F1 males and one control with disorganized tubules. The F1 linuron group also had thickened interstitial connective tissue in the testis. Because of the lack of clear nests, these individuals were excluded from the detailed analysis of the spermatogenesis. Similar observations have been made in frog and fish after exposure to anti-androgenic chemicals (Lee and Veeramachaneni, 2005;

Rimayi et al., 2018) as well as in F2 and F3 generation rats after developmental vinclozolin exposure (Nilsson et al., 2018). Propiconazole acted on the testis in an anti-androgenic fashion after early developmental exposure (**paper I**). The offspring and grand-offspring of linuron exposed males did also show alterations in testis composition (**paper IV**). This suggests that commonly used pesticides have the ability to impair testis development after direct exposure as well as indirectly, in subsequent generations.

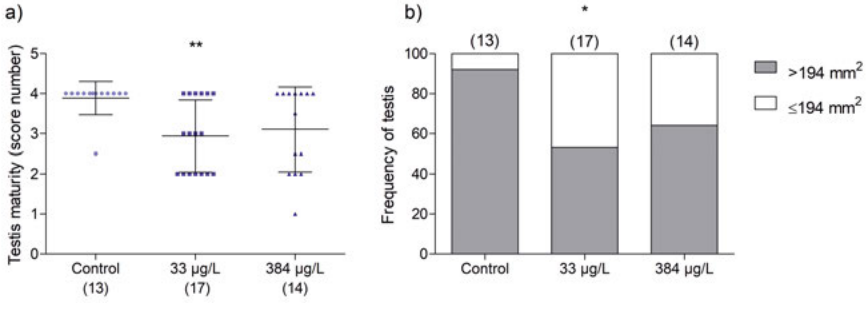

Figure 3A) Testis maturation score and B) frequency of males with smaller than average cross-section area in juvenile *X. tropicalis* after tadpole exposure to 0 (control), 33 or 384 µg propiconazole/L **(paper I)**. *significantly different from control ($p<0.05$). **significantly different from control ($p<0.01$).

The results in the present thesis show that spermatogonia in general (**paper IV**), and dark SCCs and secondary spermatogonia in particular (**paper II and III**) seem to be sensitive targets for EDCs. Flutamide and imazalil altered the number of dark SSCs (decreased and increased respectively) per testis area after juvenile exposure to 500 µg flutamide/L and 1, 12 and 154 µg imazalil/L (Figure 4), suggesting interference of these chemicals on the spermatogenesis. The total number of SSCs was also increased in all imazalil treatments compared to the control. In addition, the number of secondary spermatogonia was increased by flutamide and decreased by imazalil (Figure 4). Similarly, the azole clotrimazole (196 µg/L) increased the number of spermatogonia A and decreased spermatogonia B, and estrogen (300 ng/L) increased spermatogonia A after short term exposure in zebrafish (*Danio reiro*) (Baudiffier et al., 2013; de Waal et al., 2009). There was no other difference in germ cell stages nor was there any effects of flutamide or imazalil on cross-section area (**paper II and III**) implying that dark SSCs and secondary spermatogonia are the most sensitive endpoints for short-term EDC exposure in this thesis. No histological effects were observed after short-term juvenile propiconazole exposure (**paper III**) (Figure 4). Follicle-stimulating hormone (FSH) and androgens stimulates and inhibit SSC differentiation respectively (Meehan et al., 2000; Sofikitis et al., 2008). Since flutamide is an AR antagonist, thus inhibiting the

negative feedback on the HPG axis, a stimulatory effect on SSC differentiation, as seen by the increased differentiation of dark SSCs to secondary spermatogonia in **paper II**, is suggestive of increased FSH secretion. The opposite effects of juvenile imazalil compared to flutamide exposure on spermatogenesis may have resulted from the different MoAs of the compounds. In addition to the anti-androgenic MoA of both chemicals, imazalil is an ER receptor antagonist and CYP19 inhibitor. These multiple MoAs resulted in decreased FSH levels in the imazalil exposed males as indicated by the decreased mRNA levels of sex steroid enzymes. Clotrimazole has also been shown to alter FSH expression and related genes after short-term exposure in zebrafish (Baudiffier et al., 2012). It is therefore plausible that the disrupted spermatogenesis following juvenile exposure to flutamide or imazalil (**paper II and III**), as well as less mature testis, as a result of chronic larval propiconazole exposure (**paper I**), is a result of endocrine disturbance during pubertal gonadal development.

The main effects of the chemicals studied in this thesis were on spermatogenesis. These effects could all be attributed to imbalance in sex hormones during different critical periods of testicular development, which is reflected in the increase in brain CYP19 activity at metamorphosis (**paper I**) and change in mRNA levels of sex steroid enzymes during puberty (**paper III**). Developmental exposure to anti-androgenic and estrogenic EDCs have previously been shown to impair spermatogenesis leading to decreased fertility at adulthood in *X. tropicalis* (Gyllenhammar et al., 2009b; Orton et al., 2018). Links between spermatogonial arrest and fertility have been established in humans (Bar-Shira Maymon et al., 2003; Steger et al., 1998). Hence, the altered testis morphology and effects on spermatogenesis seen in **paper I, II and III** can be interpreted as an adverse outcome, resulting from endocrine disruption during development, supporting the classification of propiconazole and imazalil as EDCs.

Within this thesis, pale and dark SSCs were identified in *Xenopus* (**paper II**), and dark SSCs were shown to be targeted by an anti-androgen and an azole fungicide (**paper II and III**). In the standardized OECD guideline LAGDA (Larval Amphibian Growth and Development Assay) (OECD, 2015), there is a lack of fully quantitative and objective histological endpoints (as the assessment is based on scoring). Including quantification of dark SSC would therefore increase the sensitivity and objectivity of LAGDA, thereby contributing to the 3Rs (replace, reduce, refine) since more detailed and conclusive information could be obtained from one animal.

Effects on the early spermatogenesis were observed in all four papers. The paternal developmental exposure to linuron in **paper IV** increased the number of spermatogonia in F1 males. Chronic larval propiconazole exposure (**paper I**) resulted in smaller and less mature testis, juvenile propiconazole exposure (**paper III**) did not. While the juveniles already had established SSCs, the

larvae are exposed during gonadal differentiation when the stem cells are established. The exposure concentrations (although similar) and length as well as timing of exposure differed between the two studies. **Paper I** thereby show that propiconazole has the ability to induce adverse effects via endocrine pathways during early development. These studies therefore highlight the importance of choice of life stage and length of the exposure period to ensure that the most sensitive developmental stages are evaluated. For the two azole exposures in juveniles in **paper III**, both the concentrations and length of exposure are comparable. Hence, the difference in testicular effects of these exposures could therefore possibly have resulted from the difference in timing of analysis since the propiconazole males were four weeks younger compared to the imazalil males. However, the effects of imazalil exposure were seen on early sperm (i.e. dark SSCs and secondary spermatogonia), which were present in the juvenile propiconazole exposed animals as well. Therefore, it is more probable that the different testicular responses are a result of the difference in MoAs for these azoles. In **paper II**, flutamide was used as a model substance for AR antagonization. The effects of flutamide on spermatogenesis (i.e. decreased number of dark SSCs and increased number of secondary spermatogonia) is therefore the result of anti-androgen action during pubertal testicular development. Although imazalil also has an anti-androgenic MoA, its different effects compared with flutamide implies that imazalil also has other MoAs *in vivo*. The studies from this thesis show that several chemicals, with different and multiple endocrine MoAs, are able to interfere with spermatogenesis in juvenile *X. tropicalis* and subsequent generations.

Ovarian histology

There were no effects on ovarian histology in any of the studies (**paper I, II and III**). Adult propiconazole exposure (to 500 and 1000 µg/L) resulted in decreased fecundity in female fathead minnow (*Pimephales promelas*) (Skolness et al., 2013). As androgens are involved in the final maturation, the early oocytes do not seem to be the main target for anti-androgenic chemicals. No vitellogenic oocytes were found in the juveniles, hence it cannot be excluded that effects on the ovary can be seen later in life.

Müllerian duct histology

The males in the 33 µg propiconazole/L exposure group had less mature Müllerian ducts after tadpole exposure compared to the controls suggesting propiconazole has the ability to interfere with early developing Müllerian ducts in males (**paper I**). No significant treatment effects were seen in the females regarding maturity or in either sex regarding length (**paper I**). Short-term juvenile exposure did not result in altered Müllerian duct maturity after either flutamide or azole exposure (**paper II and III**). Prochloraz, another azole, has

also been shown to target Müllerian duct development in *X. laevis* by accelerating maturation in both sexes after chronic developmental exposure starting from the tadpole stage (Haselman et al., 2018). The previous and present results therefore suggest that Müllerian ducts are sensitive targets for azoles during tadpole development.

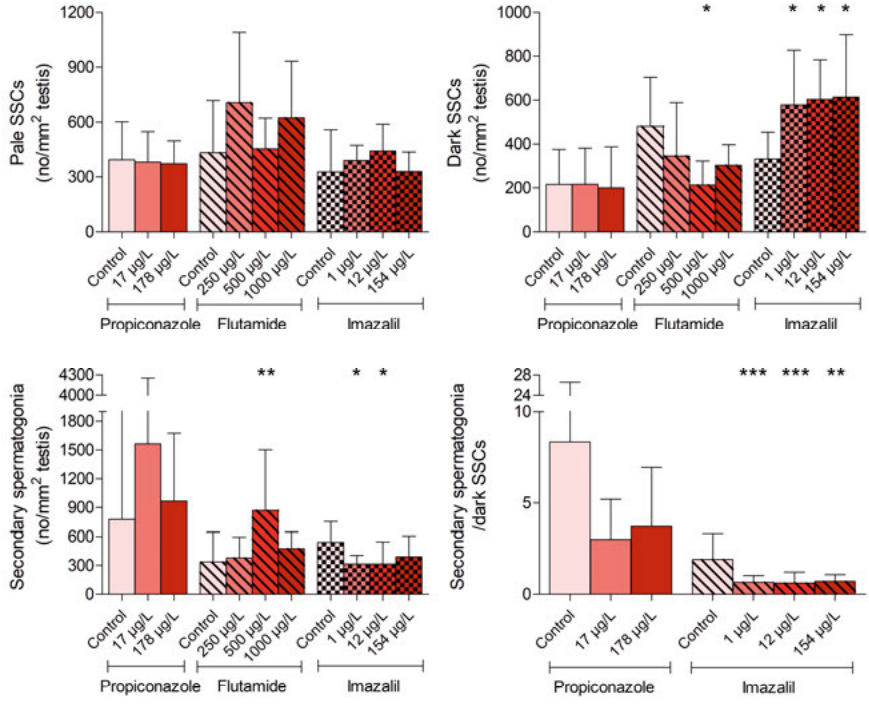

Figure 4. Number of sperm cells related to testis area in male juvenile *Xenopus tropicalis* after short term peripubertal exposure to either flutamide, propiconazole or imazalil. SSCs: Spermatogonial stem cells. n=5-15. *Statistically significant from respective control ($p<0.05$). **Statistically significant from respective control ($p<0.01$). ***Statistically significant from respective control, ($p<0.001$).

Whole body hormone concentrations

Both AMH (males and females) and estradiol (females) was detectable in whole body homogenate (**paper I**), but no statistical differences between propiconazole and control with regard to the levels of these hormones were seen in the juveniles. The estrogenic compound ethinyl estradiol significantly reduced plasma estradiol concentrations, while the anti-estrogenic compound tamoxifen increased estradiol concentrations in adult female *X. laevis* directly after chronic exposure (Urbatzka et al., 2007). Hence, it cannot be excluded that the increase in brain CYP19 activity at metamorphosis also affected AMH and estradiol at that timepoint. Using whole body homogenate to detect small

changes in hormone levels would further refine the model. However, the relationships between hormonal levels in whole body (or carcass after organs of interest have been excised) and other tissues remains to be elucidated.

CYP19 activity

Tadpole exposure to 384 µg propiconazole/L significantly increased the brain CYP19 activity at metamorphosis in pooled sexes (Figure 5A) (**paper I**). No such effects were seen in the juveniles, two months PM neither in males nor in females. Increased brain CYP19 mRNA has been seen in both sexes of zebrafish after developmental propiconazole exposure. This resulted in a decrease in plasma testosterone and an increase in estradiol level (Teng et al., 2020) a finding also seen in rats after developmental propiconazole exposure (Costa et al., 2015; Vieira et al., 2017). As propiconazole binds to the AR receptor, it is possible that negative feedback in the HPG axis caused an up-regulation of CYP19 as a compensation. It is therefore likely that increased CYP19 activity during gonadal development seen in **paper I** may have led to the adverse testis effects seen two months after the propiconazole exposure was discontinued.

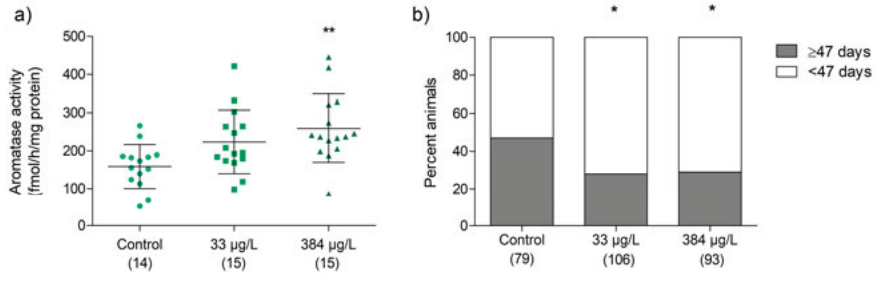

Figure 5A) Brain CYP19 (aromatase) activity at metamorphosis and B) time to metamorphosis in *X. tropicalis* after tadpole exposure to 0 (control), 33 or 384 µg propiconazole/L. The graphs represent pooled sexes. *significantly different from control ($p<0.05$). **significantly different from control ($p<0.01$). The number of individuals (n) is shown within the parentheses.

Gene expression and relationship to gonadal histology

Short-term propiconazole exposure increased the testicular *Aldh1a2* mRNA levels in juvenile males (**paper III**), without affecting any other genes, suggesting disruption of the retinoic acid pathway (Figure 6). No effects on gonadal histology were detected in these males. However, the number of meiotic sperm cells was still low at this age, and therefore it cannot be excluded that inhibited meiosis as a result of retinoic acid imbalance could be detected later in life. FSH is regulating *Aldh1a2* via the HPG axis (Nourashrafeddin and Rashidi, 2018), and propiconazole has previously been shown to increase the

number of FSH receptors in zebrafish (Teng et al., 2020). *In vitro* and *in vivo*, other azoles have been shown to increase mRNA levels of CYP26, the enzyme involved in metabolism of retinoic acid (Robinson et al., 2012; . Tiboni et al., 2009), but no other studies have reported disrupted retinoic acid synthesis after azole exposure, to the best of the author's knowledge. The difference between propiconazole and imazalil in *Aldh1a2* mRNA levels seen within **paper III** suggests that the two azoles have different endocrine MoAs in the juvenile testis with propiconazole acting on the retinoic acid pathway while imazalil acts via the sex steroid pathway.

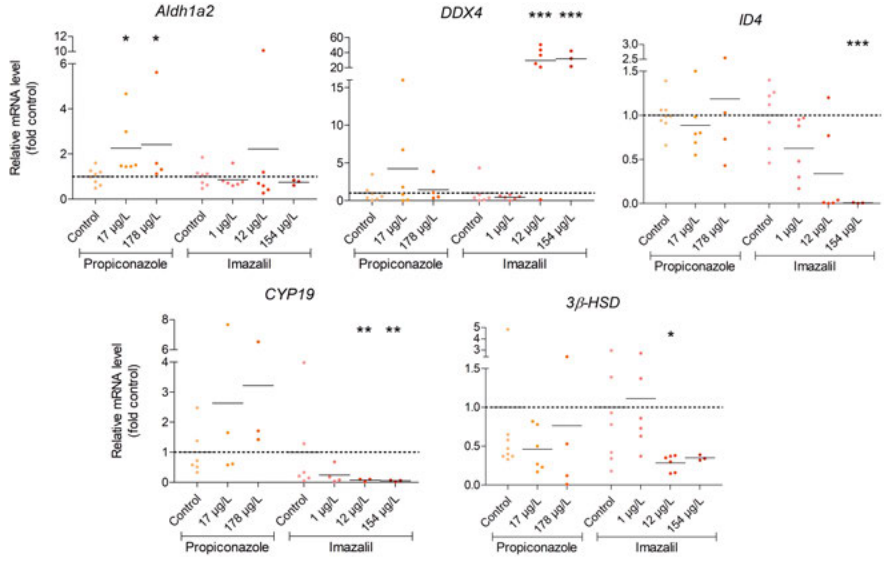

Figure 6. Testis mRNA levels in juvenile *Xenopus tropicalis* after short-term azole exposure. *Aldh1a2*: *DDX4*: DEAD-box helicase 4, *ID4*: Inhibitor of DNA binding 4, *CYP19*: Cytochrome P450 19, *3β-HSD*: 3β-hydroxysteroid dehydrogenase. All values are relative control mean (dashed lines) *Significantly different to respective control, ($p<0.05$). **Significantly different to respective control ($p<0.01$). *** Significantly different to respective control, ($p<0.001$).

In the females, ovarian *ESR1* levels were decreased after juvenile exposure to 17 µg propiconazole/L compared to the control (**paper III**). In contrast, juvenile exposure to imazalil (154 µg/L) increased *ESR1* mRNA levels and decreased *Rsbn1* levels in ovaries (**paper III**). No other significant changes in ovarian mRNA levels were detected for either treatment in the females. *Rsbn1* has been suggested to be involved in meiosis in female mice (Wang et al., 2021), but its function in female amphibians remains to be elucidated. Proper estrogen signalling is crucial for the incorporation of vitellogenin and maturation of the oocytes and the disrupted estrogen signalling in the juveniles is therefore a cause for concern.

In **paper III**, testicular mRNA concentrations of *ID4* were decreased, *DDX4* increased, and the ratio between the two genes was lower after exposure to 12 and 154 µg imazalil/L compared to the controls (Figure 6). In these exposure groups, *CYP19* and *3β-HSD* (12 µg/L only) mRNA levels were also decreased compared to the controls (Figure 6). Both these genes are involved in the sex steroid pathway, further implying that the observed adverse effects of imazalil on spermatogenesis was a result of endocrine disturbance. The PCA plot based on the affected genes and histological alterations after imazalil exposure (**paper III**) explained 66.6% of the data variability (Figure 7). The number of dark SSCs/testis area and *DDX4* were grouped together on the negative side of PCA1, whereas *CYP19*, *3β-HSD* and *ID4* were grouped together with number of secondary spermatogonia/testis area on the negative side of PCA1. Only *ID4* and secondary spermatogonia were negatively associated with PCA2, as the other data were positively associated with PCA2. *DDX4* and *ID4* are expressed in mammalian spermatogonia A and spermatogonia A_{single}, respectively and *DDX4* is also expressed in amphibian spermatocytes (Komiya et al., 1994; Oatley et al., 2011). The observed changes in mRNA levels of these genes in the frog testes are in line with the histological alterations in germ cell composition of the testes, indicating that the altered mRNA levels of these genes are a result of changed sperm cell composition, rather than an increased cellular expression. The mRNA levels of *ID4* (marker for SSCs) and *DDX4* (marker for more mature cells), and their relation to each other, therefore give insight to the testicular germ cell composition. These results suggest that a ratio of *ID4:DDX4* should be further explored as a molecular marker for disrupted spermatogenesis.

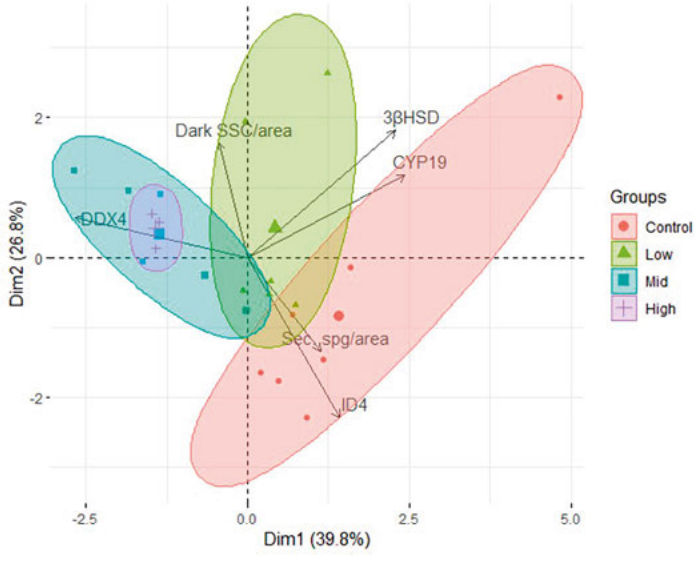

Figure 7. Relationship between significantly changed mRNA levels and sperm stages in *Xenopus tropicalis* males after short-term juvenile exposure to 0 (Control), 1 (Low), 12 (Mid) or 154 (High) µg imazalil/L. *DDX4*: DEAD-box helicase 4, *ID4*: Inhibitor of DNA binding 4, *3βHSD*: 3β-hydroxysteroid dehydrogenase, *CYP19*: Cytochrome P450 19. Dark SSC: dark spermatogonial stem cells, Sec. spg: secondary spermatogonia.

Thyroid and metabolic disruption

Early developmental propiconazole exposure (33 and 384 µg/L) significantly increased the number of animals to reach metamorphosis faster than 47 days (control mean), suggesting a stimulatory effect on the thyroid system (Figure 5B) (**paper I**). Propiconazole was also shown to reduce serum levels of thyroid hormones and thyroid stimulating hormone in adult rats in a non-monotonic dose dependent way (Wolf et al., 2006). In contrast, using the amphibian metamorphosis assay, propiconazole (0.0056, 0.056 and 0.57 mg/L) was not shown to alter developmental rate (Lee, 2012). Although no effects were seen on the endpoint for thyroid disruption (hind limb length) after short-term juvenile propiconazole (17 and 178 µg/L) and imazalil (1, 12 and 154 µg/L) exposure, other azoles have also been shown to interfere with the thyroid system in amphibians after tadpole exposure (Brande-Lavridsen et al., 2008; Haselman et al., 2018). The results from this thesis show that azoles, including propiconazole, can interfere with the thyroid system after early developmental exposure.

The shorter hind limbs seen in the F1 linuron frogs also suggest an inhibitory effect on the thyroid system, whereas the longer hind limbs in the F2

linuron frogs suggest a stimulatory effect, possibly as a compensatory response resulting from shorter hind limbs in the previous generation (Table 3).

The azole exposures did not significantly affect either body weight or length of the frogs, at metamorphosis or as juveniles (**paper I and III**). The LSI in males was increased after exposure to both 33 and 384 µg propiconazole/L groups compared to control after chronic larval exposure (**paper I**), but not after short-term juvenile exposure to 17 or 178 µg propiconazole/L or 1, 12 or 154 µg imazalil/L (**paper III**). In contrast to **paper I**, adult exposure to 500 µg propiconazole/L decreased the LSI in rainbow trout (*Oncorhynchus mykiss*) after 30 days (Li et al., 2010). Previous studies have demonstrated propiconazole's ability to induce vitellogenin (an estrogenic biomarker) in fish (Kinnberg et al., 2007; Teng et al., 2020). Since an increased LSI has been associated with vitellogenin induction, it is plausible that the increase in LSI seen in **paper I** also can be associated to an estrogenic action of propiconazole.

The juvenile males exposed to the highest concentration of flutamide were longer compared to the control males (**paper II**). This is not in agreement with flutamide studies in juvenile fish where no effects of flutamide under comparable exposure conditions on body length has been reported for body length (Bhatia and Kumar, 2016; Nakamura et al., 2014). The information on anti-androgenic effects on body length in amphibians is scarce and needs to be further investigated.

In **paper IV**, the body weight and BMI in both generations of linuron offspring were affected compared to the respective control (decreased in F1, increased in F2). Several mammalian studies have reported altered methylation of *ppar-γ* (peroxisome proliferator- activated receptor gamma) and other obesity related genes as a result of parental and grandparental environmental chemical exposure (Maradonna and Carnevali, 2018). It is possible that the methylation of such genes also were affected in **paper IV**, however further studies are needed to elucidate the underlying mechanisms of the body changes in F1 and F2. The frogs in the F1 linuron frogs also had a decreased fat body weight, shorter snout-vent length (SVL) and shorter hind limbs compared to the F1 controls (Table 3). In contrast, the F2 generation linuron frogs had longer hind limbs compared to control, but no treatment effects were observed on SVL or fat body weight (Table 3). There was a significant decrease in the plasma glucose levels in the F2 linuron frogs compared to the controls. These results together with the increase in BMI suggests an altered metabolic activity in the F2 generation after linuron exposure. In addition, there was a significant increase in desaturation index (fatty acids 16:1/16:0 (palmitoleic acid/palmitic acid)) in the fat bodies in the linuron frogs in the F2 generation compared with F2 control. No other significant treatment related effects were seen in desaturation index of fatty acids 16:1/16:0 or 18:1/18:0 (oleic acid/stearic acid) in either tissue or generation. The 16:1/16:0 desaturation index is used as an estimate of the enzyme stearoyl-CoA desaturase-1 (SCD-1).

SCD-1 converts saturated fatty acids into monounsaturated fatty acids and an increased activity and palmitoleic acid has been associated with obesity in both mammals and humans (Gong et al., 2011; Ntambi et al., 2002; Warensjö et al., 2006). Our results suggest similar associations in frogs. An increase in SCD-1 activity in fat was also observed in male rats after developmental bisphenol A exposure (Dunder et al., 2018). This adds to the similarities of *X. tropicalis* to higher vertebrates and thereby the advantages of *X. tropicalis* as a model to study endocrine disruption. In addition, the results of the present thesis indicate that SCD-1 may be useful as a marker for metabolic disruption.

The treatment effects on development (decreased time to metamorphosis, decreased/increased hind limb length) and metabolism (increased BMI, increased plasma glucose and increased SCD-1 activity) seen in **paper I and IV** suggest that the chronic larval or paternal/grandpaternal exposures to propiconazole (33 and 384 µg/L) and linuron (26 µg/L), interfered with the thyroid system. Thyroid hormones are involved in proper development, and metabolism. More specifically, they regulate lipid homeostasis in fat tissues and insulin sensitivity (Mullur et al., 2014), both of which were affected in **paper IV**. *In vitro*, it has been shown that the thyroid receptor represses genes regulated by triiodothyronine (T3) if no T3 is present (Buchholz et al., 2003). There are very low concentrations of T3 in pre-metamorphic frog, hence, antagonization of the thyroid receptor most likely result in stimulated thyroid signalling. This as well as compensatory responses in subsequent generations might explain how the pesticides studied in this thesis interacted with development and metabolism in *X. tropicalis*.

Non-monotonic dose response relationships

The non-monotonic dose response relationships for the endocrine and adverse effects observed in **paper I, II and III** add to the body of evidence showing that for EDCs, the response may be limited to specific and sometimes narrow dose ranges. In addition, the response to EDC exposure is life-stage specific. Non-monotonic dose responses are not uncommon for EDCs since hormones are tightly regulated via compensatory feedback, up/down regulation of receptors and dose range-specific binding as well as crosstalk between hormonal axes (Vandenberg et al., 2012).

Table 3. Measures from male *Xenopus tropicalis* after parental (F1) or grandparental (F2) developmental linuron exposure.

Variable	$Control_{F1}$		$Linuron_{F1}$		$Control_{F2}$		$Linuron_{F2}$	
	Mean (SEM)	**n**	**Mean (SEM)**	**n**	**Mean (SEM)**	**n**	**Mean (SEM)**	**n**
Growth/metabolism								
SVL^2 (mm)	45.69 (0.64)	7	43.47 (0.34)**	30	41.94 (1.52)	7	42.57 (0.58)	16
BMI (kg/m^2)	5.43 (0.13)	7	4.85 (0.05)***	30	3.83 (0.19)	7	4.24 (0.05)*	16
Fat bodies (g)	1.00 (0.05)	7	0.71 (0.04)***	30	0.43 (0.05)	7	0.44 (0.03)	16
Hindlimb length (mm)	59.13 (0.83)	7	56.39 (0.57)*	30	51.87 (2.66)	7	58.65	16
Reproductive system								
GSI	0.261 (0.022)	7	0.354 (0.013)**	30	0.282 (0.03)	7	0.236 (0.01)	16
Forelimb width (mm)	3.94 (0.11)	7	3.51 (0.06)**	30	2.99 (0.18)	7	3.04 (0.12)	16
Nuptial pad size (mm^2)	18.41 (0.87)	7	14.18 (0.69)**	30	6.96 (0.66)	7	7.25 (0.46)	16
Time to spawning (min)	217.5 (9.3)	12	167.3 (13.9)*	32	218.57 (22.88)	7	185.63 (9.96)	16
Fertility rate <15% (no. animals)	0	12	10*	32	3	7	4	16
Germ cell analysis								
Germ cell nests per seminiferous tubule	8.6 (1.2)	6	8.6 (1.8)	22	7.9 (0.2)	7	6.4 (0.5)*	16
Spermatogonia per seminiferous tubule	15.4 (1.6)	6	23.6 (1.9)*	22	12.1 (0.8)	7	12.1 (1.3)	16
Size of spermatocyte nests	2.7 (0.07)	6	2.9 (0.03)**	22	2.7 (0.04)	7	2.7 (0.02)	16

GSI: Gonadosomatic index. SVL: Snout-vent length. BMI: body mass index (body weight/SVL^2). $^{*}p<0.05$ compared to control. $^{**}p<0.01$ compared to control. $^{***}p<0.001$ compared to control.

Adverse outcome pathways

To help the establishment of associations between endocrine MoAs and adverse effects, the concept of adverse outcome pathways (AOPs) has been developed. Observed effects are connected via biologically plausible links from a molecular initiating event (MIE), through a series of key events on different organisational levels (cellular to tissue to organ) to an adverse effect on the individual or population level (Vinken, 2013). Based on the observations from the studies within this thesis (**paper I and III**) and the *in vitro* MIEs, AOPs were constructed for both azoles including interactions with CYP19, the AR, ER and negative feedback on FSH via the HPG axis causing sex hormone imbalance, altered spermatogenesis and testis morphology, plausibly linked to reduced reproductive success later in life (Figure 8 and 9).

Since the molecular and apical effects differed between the F1 and F2 generation (**paper IV**), direct effects of the exposure on the DNA are not likely. Instead, the proposed AOP for transgenerational toxicity of linuron suggests epigenetic alterations in the F0 and/or F1 generation (Figure 10).

The results of early developmental propiconazole exposure also suggested interference with the thyroid and metabolic systems **(paper I)**. It is possible that the thyroid disruption involves binding and inhibiting thyroid receptor (Ghisari et al., 2015) leading to stimulation of thyroid hormones (as a consequence of T3, and the thyroid receptor supressing T3 dependent genes (Buchholz et al., 2003)), decreased time to metamorphosis and ultimately altered development.

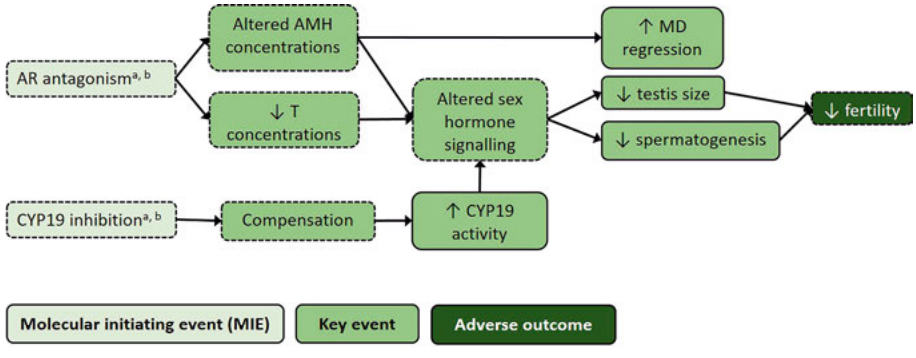

Figure 8. Proposed adverse outcome pathway in *Xenopus tropicalis* after tadpole exposure to propiconazole. Whole lines are observed effects in the present thesis and dashed lines are suggested outcomes/key events based on literature. AR: androgen receptor. CYP19: Cytochrome P450 19. AMH: Anti-Müllerian hormone. MD: Müllerian duct. [a]Kjeldsen et al., 2013. [b]Kojima et al., 2004.

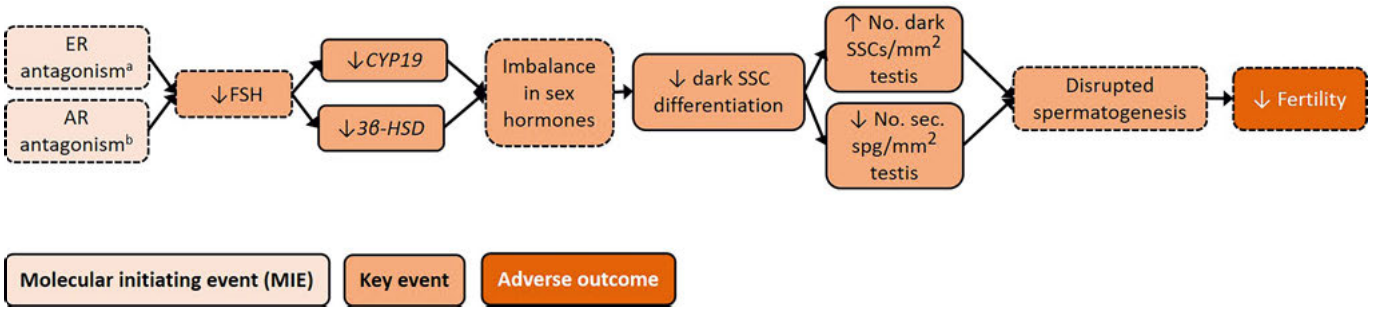

Figure 9. Proposed adverse outcome pathway for short-term juvenile exposure to imazalil in *Xenopus tropicalis*. Whole lines are observed effects in the present thesis and dashed lines are suggested outcomes/key events based on literature. ER: estrogen receptor. AR: androgen receptor. FSH: Follicle-stimulating hormone. *CYP19*: Cytochrome P450 19. *3β-HSD*: 3β-hydroxysteroid dehydrogenase. Dark SSC: dark spermatogonial stem cell. Sec. spg: secondary spermatogonia. [a]Kojima et al., 2004. [b]Orton et al., 2011.

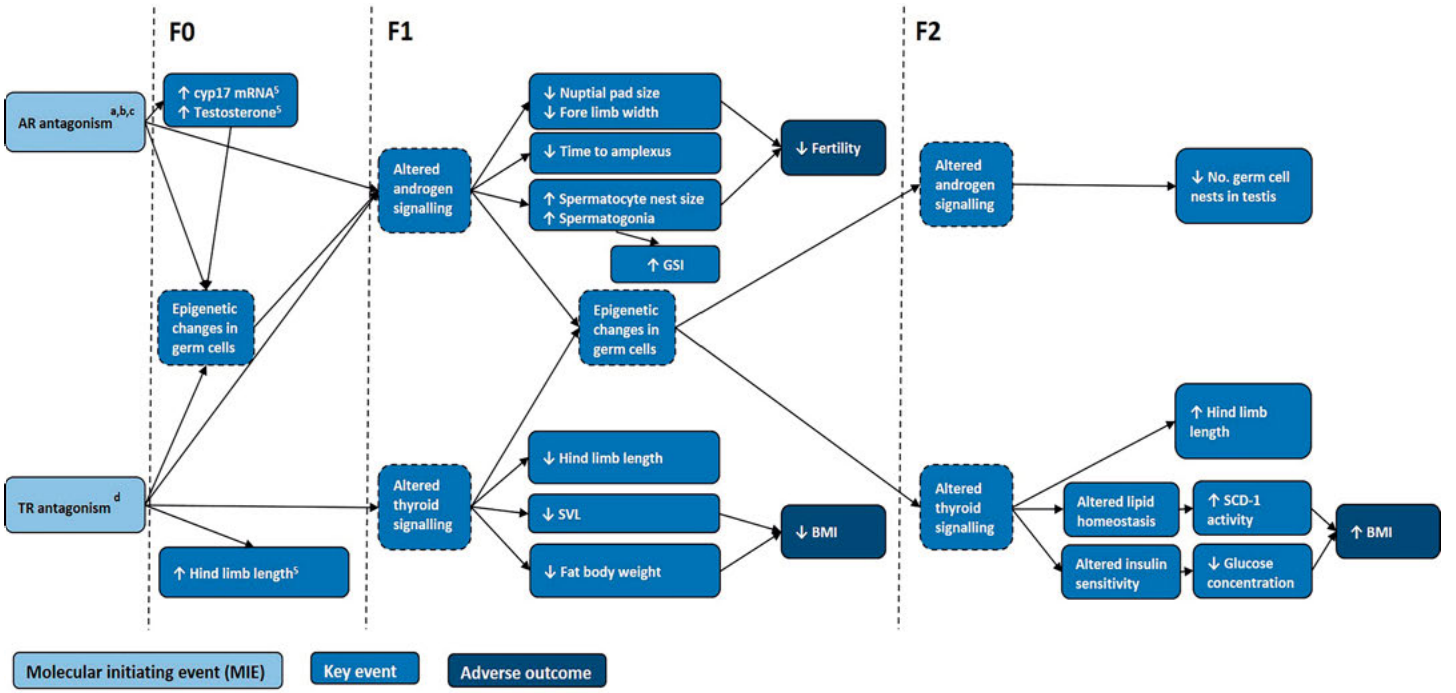

Figure 10. Proposed adverse outcome pathway in F1 and F2 generation *Xenopus tropicalis* males after F0 male developmental exposure to linuron. CYP17: Cytochrome P450 17. GSI: gonado-somatic index. SVL: snout-vent length BMI: body mass index. SCD-1: stearoyl-CoA desaturase-1. [a]Kojima et al., 2004. [b]Orton et al., 2009. [c]Wilson et al., 2009. [d]Spirhanzlova et al., 2017. [e]Orton et al., 2018.

Xenopus tropicalis as a model in developmental reproductive and transgenerational toxicity

The well characterized NF developmental stages in *X. tropicalis* is an advantage compared to mammalian models. This allows for targeted chemical exposure and analysis during specific developmental stages (e.g. sex determination and gonadal differentiation). In **paper I and IV**, delayed effects of exposure during the critical periods of sex determination and differentiation were characterised months after the exposure was discontinued as well as in two subsequent generations. Knowing the processes that occur during the exposure window facilitates the elucidation of underlying pathways of early exposure and delayed effects. In addition, compared to mammals, amphibians have external development of embryos, which facilitates specific sampling and analysis of early life stages. The main limitation of the model is the generation time. Although it is shorter in *X. tropicalis* compared to other amphibians and humans, it is still longer compared to zebrafish, mice and rats. Zebrafish also have external embryo development, and has gained increasing interest as a substitute for mammalian models in reproductive toxicology. However, compared with fish, amphibians are genetically and physiologically more similar to mammals (Hellsten et al., 2010). For example, testosterone is the main androgen in both mammals and amphibians, unlike in fish, which use 11-Ketotestosterone. Furthermore, in **paper I**, as well as in previous studies, effects were seen on the Müllerian ducts, structures crucial to female reproduction which are found in all vertebrates, except teleost fish (Gyllenhammar et al., 2009b; Kvarnryd et al., 2011). Developmental reproductive studies in teleost fish (e.g. zebrafish) risk missing effects of EDCs on sensitive and important organs and are therefore are not as well translated to higher vertebrates as those in *X. tropicalis*. This thesis further provides evidence to the usefulness of *X. tropicalis* as a model in both ecotoxicology and human toxicology.

In **paper IV** grandpaternal exposure to the anti-androgen linuron led to metabolic effects in the F2 males indicating transgenerational inheritance. Similarly, grandmaternal exposure to 50 ng benzo[a]pyrene/L resulted in disrupted metabolism and fertility in F2 female *X. laevis* (Usal et al., 2021), suggesting that altered metabolism is an important effect for grandparental transgenerational exposure to EDCs. In rats, exposure to the anti-androgenic pesticide vinclozolin, reduced sperm concentration and motility after developmental exposure (100 mg/kg/day) in the F1-F3 generation, and exposure to the antiandrogen flutamide (5 and 20 mg/kg/day) decreased sperm motility in F1 and sperm concentration in F1 and F2 males (Anway et al., 2008). These results are in line with the testicular alterations, disturbed spermatogenesis and reduced fertility in the F1 generation offspring to the linuron exposed frogs in **paper IV**. Zebrafish has also been used in several transgenerational studies of EDC effects (Cavalieri and Spinelli, 2017). However, during embryonic development, important epigenetic mechanisms such as DNA methylation are

similar in amphibians and mammals both in terms of genes regulated, patterns and enzymes (Bogdanović et al., 2011), and differ from zebrafish which do not have imprinted genes (Goll and Halpern, 2011). The results from **paper IV** not only contribute with knowledge on transgenerational effects of EDCs in amphibians, but also further highlight the risk of EDC exposure to subsequent generations in general. In conclusion, the results in the present thesis strengthen the usefulness of *X. tropicalis* as a model in developmental reproductive toxicology and transgenerational studies.

Summary and concluding remarks

The results in this thesis demonstrate disrupted spermatogenesis and endocrine perturbance in juvenile male *X. tropicalis* following exposure to common EDCs or the model substance flutamide (AR antagonist) during different critical developmental periods. The results further show male reproductive toxicity and metabolic disturbance after indirect exposure (i.e. as germ cells in the exposed fathers) and through transgenerational inheritance. The observed adverse reproductive and metabolic effects were detected at environmentally relevant exposure levels, and are likely linked to the endocrine perturbance during sensitive periods of development as illustrated in the proposed AOPs.

In **paper II**, pale and dark SSCs were morphologically characterized, male pubertal onset in *X. tropicalis* was specified to five weeks PM, and the period of four to eight months PM was identified as an important period for Müllerian duct development. **Paper III** demonstrated that the time between four and eight weeks PM is important for testis maturation and ovarian growth. The results from **paper II and III** characterize new important and sensitive sperm stages in *Xenopus* and identify the timing of central processes, thereby increasing the understanding of peripubertal sexual development in *X. tropicalis*.

Propiconazole exposure during the tadpole period induced endocrine effects at metamorphosis (increased brain CYP19 activity) which was likely linked to the delayed adverse effects (decreased testis size and maturity) observed two months after the exposure was discontinued (**paper I**). On the other hand, short-term juvenile propiconazole exposure led to endocrine effects on retinoic acid signalling but not on CYP19 expression (**paper III**). These studies highlight the importance of the timing and length of exposure during development when stablishing cause-effect relationships for EDCs.

Short-term juvenile exposure to imazalil caused an increased number of dark SSCs and decreased number of secondary spermatogonia in the testis. These changes were associated with decreased testis mRNA levels of *CYP19* and *3β-HSD,* key genes in the sex steroid pathway (**paper III**). In contrast, juvenile exposure to the anti-androgen flutamide, instead resulted in decreased dark SSCs and increased secondary spermatogonia (**paper II**). No effects on testis morphology were found after juvenile exposure to propiconazole (**paper III**). The different effects of the compounds imply other MoAs than AR antagonism for imazalil as compared with flutamide and different MoAs for the

two azoles after juvenile exposure. In **paper IV,** paternal developmental linuron exposure resulted in reproductive and metabolic effects in the F1 generation and mainly metabolic effects in the F2 generation in *X. tropicalis*, likely via epigenetic mechanisms. The results from all studies **(papers I – IV)** demonstrate that chemicals with different endocrine MoAs can disrupt the earliest stages in spermatogenesis.

In all four studies, the early spermatogenesis was a sensitive endpoint for EDC exposure. Specifically, the number of dark SSCs, secondary spermatogonia and their ratio (**paper II and III)**; testicular levels of the sperm specific genes *ID4* and *DDX4* **(paper III)**; and Müllerian ducts and testis maturity (**paper I)** were identified as sensitive endpoints for developmental reproductive toxicity. Spermatogonial arrest has previously been linked to reduced fertility, indicating that the results from **paper II and III** are to be considered as adverse effects. Based on the findings in this thesis it is suggested to include quantification of dark SSCs into the standard OECD test guideline LAGDA to increase its sensitivity and objectivity.

The increasing use of chemicals calls for sensitive methods and models for toxicity testing to adequately protect humans and wildlife. **Paper I** suggests a partial life-cycle assay (exposure during gonadal differentiation and analysis of endocrine disruption and delayed toxicity in juveniles) used to evaluate male reproductive toxicity. **Paper II and III** propose new endpoints (both histological and molecular) in juveniles which contribute to the development of assays for EDC research and reproductive toxicity testing. **Paper IV** is the first study to demonstrate transgenerational inheritance after male pesticide exposure in amphibians. Multigenerational studies are currently not a part of risk assessments. Therefore, studies like this may give an insight into the underlying pathways, key events and their linkage to adverse effects. Knowledge on dose-response relationships facilitates further studies on the underlying molecular mechanisms and may contribute to developing guidelines to assess multigenerational toxicity.

All four chemicals studied within this thesis exerted endocrine activity via different endocrine systems (the retinoic acid, sex hormone, thyroid and metabolic systems) during development. By using a number of different methods, all of these endocrine changes (except in the retinoic acid pathway) were shown to be associated to adverse effects on sexual development, reproduction, metabolism and growth. Using the *X. tropicalis* model, the present thesis thereby contributes with new knowledge on how environmental chemicals can interfere with endocrine pathways via key events, resulting in adverse effects in juvenile animals and in subsequent generations. Furthermore, the findings in the thesis contribute to the development of assays for EDC investigation and of chemical risk assessment. This is urgently needed given the increasing incidences of reproductive disorders in humans (e.g. impaired sperm quality) in which chemical causes are implied (but data on cause-effect relationships are inadequate), and the escalating global extinction rates for vertebrates in

general. It is particularly needed for amphibians as they constitute the group of organisms suffering the highest extinction rates. In conclusion, the present thesis provides methods and knowledge that contribute to increasing our understanding of how chemicals in the environment may affect humans and wildlife.

Amphibian *in vivo* models are foreseen to be essential for the validation of new and developing non-animal methods for risk assessment. Further detailed histological characterization of gonads and Müllerian ducts (e.g. the timing of regression in males) at additional juvenile ages are therefore of interest. Studies (both histological and molecular) on somatic testicular cells involved in development and spermatogenesis i.e. Leydig and Sertoli cells are also needed to advance the understanding of effects of EDC exposure. In addition, elucidating the underlying molecular mechanisms resulting in the multi- and transgenerational effects observed in the present thesis, are also of importance to further develop *X. tropicalis* as a model in developmental reproductive toxicology.

Populärvetenskaplig sammanfattning

Både människor och vilda djur utsätts för en mängd olika kemikalier dagligen. Många av de här kemikalierna har egenskaper som gör att de kan störa hormonsystem, de är så kallade hormonstörande kemikalier. Det finns kopplingar mellan att ha utsatts för de här kemikalierna under utvecklingen och negativa effekter på bland annat fortplantningsförmåga och ämnesomsättning senare i livet. Hormonstörande kemikalier har också föreslagits vara en av orsakerna till minskningen av groddjur som sker över hela världen. Många bekämpningsmedel har visat sig kunna binda till olika receptorer för hormoner i kroppen och verka hormonstörande när de har testats på celler, men kunskapen om vad som sker inne i kroppen är mycket mindre. Ännu mindre vet man om konsekvenserna i vuxna, och kommande generationer om man utsatts för de här kemikalierna tidigt i livet.

I den här avhandlingen har grodan den västafrikanska klogrodan *Xenopus tropicalis* använts som modell för att finna orsakssamband mellan hormonstörande och negativa effekter på fortplantning, ämnesomsättning och utveckling. Det är en bra modell för att undersöka effekten av hormonstörande kemikalier eftersom utvecklingen av reproduktionssystemet och hormonsystemen är lika den hos andra ryggradsdjur, vilket gör det till en lämplig modell för både grodor och däggdjur. För att få mer kunskap om känsliga perioder under utvecklingen beskrevs också utvecklingen av fortplantningsorgan och spermiestadier tiden runt puberteten i *X. tropicalis*.

De ämnen som undersöktes i den här avhandlingen var propikonazol, imazalil och linuron, de är kemikalier som bland annat används som bekämpningsmedel. De har visat sig binda till flera olika hormonreceptorer och enzymer (proteiner som hjälper till i olika biologiska processer) som finns i kroppen. Eftersom de hanliga könshormonerna androgener är mycket viktiga för utvecklingen av spermier, gjordes också ett försök med flutamid, ett läkemedel som har som syfte att blockera receptorerna i cellerna dit androgener ska binda. Alla kemikalier förutom flutamid, som bara användes som modellsubstans, undersöktes i koncentrationer som finns i miljön och som människor och djur därför skulle kunna utsättas för via till exempel förorenat vatten.

I individer som var runt puberteten beskrevs två spermiestadier, ljusa och mörka spermatogonia-stamceller, som inte har beskrivits i *X. tropicalis* tidigare. De visade sig också vara känsliga för flutamid (där de minskade i antal) och imazalil (där de ökade i antal) jämfört med grodor som inte utsatts för

något av ämnena. Andra studier har visat att hanar med ett förändrat antal spermier i tidiga stadier också har sämre fortplantningsförmåga, vilket skulle kunna hända dessa djur då de blir vuxna. Genom att identifiera känsliga stadium under utvecklingen av fortplantningsorganen i unga grodor bidrar studierna i den är avhandlingen till att öka på kunskapen om *X. tropicalis* som en modell inom toxikologi med fokus på störning av fortplantningssystemen.

För grodor som utsatts för propikonazol under hela yngelutvecklingen gick det snabbare att gå igenom omvandlingen från yngel till groda. De hade också en ökad aktivitet av aromatas, ett enzym som omvandlar androgener till de kvinnliga könshormonen östrogener, i hjärnan jämfört med de grodor som inte hade utsatts. Två månader senare (när de inte varit utsatta längre) hade de ökad levervikt, och testiklar som var mindre och inte lika utvecklade jämfört med de grodor som inte varit utsatta för propikonazol. De Müllerska gångarna (organ som ska utvecklas till äggledare i honor) var också mindre utvecklade i propikonazol-hanarna.

Efter att ha utsatts för propikonazol en kortare tid under puberteten sågs effekter på nivåer av en gen som är involverad i utvecklingen av spermier, men inga effekter sågs på testikelutvecklingen. I djuren som utsatts för imazalil däremot var det flera gennivåer som påverkades. En del av generna är involverade i tillverkningen av könshormoner, medan andra uttrycks specifikt i de spermiestamcellerna som minskade i antal. Eftersom de är specifikt uttryckta i vissa celler, skulle kunna göra dem till en bra markör för att se olika hormonstörande kemikaliernas påverkan på den tidiga spermieutvecklingen.

Hanliga grodor vars pappor utsatts för linuron som yngel (F1) vägde mer, hade kortare bakben (vilket är ett mått på störning av sköldkörtelhormon) och fler omogna spermier jämfört med grodor vars pappor inte utsatts för linuron. De var också många fler F1 grodor som hade låg befruktningsgrad av ägg. De hanar vars farfäder blivit utsatta under yngelstadiet (F2) vägde mer, hade ändrad ämnesomsättning och längre bakben. Eftersom effekterna inte var samma i båda generationerna skulle det kunna tyda på att linuron påverkade epigenetiken (mekanismer som sitter på DNA och påverkar vilka gener som ska uttryckas) i förfäderna som sedan fördes vidare till nästa generationer. De här resultaten är de första som visar på effekter i flera generationer efter att papporna/farfäderna utsatts för ett bekämpningsmedel under utvecklingen. I den riskbedömning som görs för hormonstörande kemikalier är det inte standard att se vad som sker i flera generationer. Resultaten från flergenerationsstudien skulle därför kunna vara användbara för att utveckla riktlinjer som skulle kunna användas för riskbedömning.

Sammanfattningsvis så visade studierna i den här avhandlingen på att vanligt använda bekämpningsmedel kunde ha hormonstörande effekter på olika hormonvägar under utvecklingen då de utsätts för ämnena och negativa effekter på fortplantningsorgan och ämnesomsättning både i djur i puberteten (direkt efter att de utsatts, och två månader senare), och även i senare generationer.

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