Assessing the impact of xenoestrogen exposure during larval development on the reproductive potential of the amphibian *Silurana tropicalis* in a partial life-cycle model.

A thesis submitted for the degree of Master of Philosophy

By

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Declaration

The work submitted for this thesis was carried out between 2006 and 2010 at Brunel University (Uxbridge, Middlesex, *UK*) and at the Zoological society of London (London, *UK*). This work has been carried out independently and has not been submitted for any other degree.

Abstract

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Evidence suggests that chemicals acting as endocrine disruptors can modulate amphibian sex differentiation and induce sex reversal and gonadal abnormalities. However, consequences of gonadal abnormalities such as testicular oocytes on amphibian reproductive potential remain unclear. Assessing this linkage requires suitable experimental models, such as partial life-cycle exposure, in order to correlate accessible endpoints with apical endpoints indicative of reproductive fitness. Larvae of the emerging amphibian model *Silurana tropicalis* were exposed to EE_2 in the ng/L range from NF stage 51-52 until completion of metamorphosis, and endpoints related to the reproductive function were assessed on completion of metamorphosis and at sexual maturity. Exposure to EE_2 skewed the sex ratio toward phenotypic females in the treated groups, but also affected growth and development of the tadpoles. At sexual maturity, absence of one or both oviducts was observed in some female frogs that had been exposed to EE_2. Regressed testicular oocytes were observed in most males, including males from the control group. The occurrence of regressed testicular oocytes seems to be dependent on genetic background, rather than xenoestrogens exposure. Mature testicular oocytes and presence of oviduct-like ducts in frogs displaying testis were only seen in treatment groups exposed to EE_2. The incidence and the severity of gonadal abnormalities observed in *Silurana tropicalis* males and females exposed to EE_2 were dependent on the nominal dose but also dependent on parentage. No link could be made between gonadal abnormalities

and the reproductive potential of the frogs. These findings suggest that further development of partial life cycle models using *Silurana tropicalis* may be warranted in order to support identification and characterization of endocrine disrupters in chemical safety testing, for risk assessment and registration.

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GLOSSARY

AR	Androgen receptor
BPA	Bisphenol A
DES	Diethylstilbestrol
DDT	Dichlorodiphenyltrichloroethane
DHT	Dihydrotestosterone
EDC	Endocrine disrupting chemical
EE_2	Ethynylestradiol
ER	Estrogen receptor
FSH	Follicle-stimulating hormone
GSD	Genetic sex determination
hCG	Human chorionic gonadotropin
LH	Luteinizing hormone
LIN	Linearity
SBP	Sex steroid binding protein
NF	Niewkop and Faber
PCB	Polychlorinated biphenyl
PGCs	Primordial germ cells
RIA	Radioimmunoassay
STR	Straightness
T3	Triiodothyronine
TH	Thyroid hormones
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity

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1 . Introduction

Amphibian populations have undergone worldwide declines over the last century and this decline has not yet been fully explained. Around one third of amphibian species are threatened with extinctions, and 43% are in decline (Stuart et al., 2004; Hayes et al., 2010). This decline has been attributed to several factors, including habitat destruction, climate change, increased UV exposure, introduction of non-indigenous species, the spread of virulent pathogens and adverse effects of chemical contaminants such as pesticides (Carey & Bryant, 1995). The variety of stressors contributing to amphibian population declines may also interact with each other (Kiesecker, 2010). A striking example of interactions between stressors has been observed in *Rana sylvatica,* where the occurrence of limb deformities has been linked to the synergistic effects of trematode infection and exposure to chemical contaminants (Kiesecker, 2002). Although habitat loss and destruction seem to be the biggest threat to amphibians, it appears that pollution is the second biggest threat to amphibian populations, as reported by the IUCN (International Union for Conservation of Nature and Natural Resources) Red List of Endangered Species of 2008 (IUCN, 2008). In the latest analysis, pollution threats reported by scientists were described as exposure to domestic and urban waste water, industrial and military effluents, agricultural and forestry effluents, garbage and solid waste, air-borne pollutants and excess energy (i.e. light, thermal, and/or noise pollution).

Habitat loss is often linked with agricultural expansion (Mann et al., 2009) and this raises the issue of amphibians being exposed to chemicals used in agriculture. Agricultural chemicals have been reported to impair growth, development, delay metamorphosis, impair behavioral responses, induce deformities, induce immunosuppression, and alter sexual differentiation (reviewed in Mann et al., 2009). Some chemicals impairing growth, development, and delaying metamorphosis are

inhibitors of thyroid activities. Indeed, thyroid hormones regulate amphibian metamorphosis (Galton, 1992; Kollros, 1961; Etkin, 1935), and disruption of the thyroid function can prevent metamorphosis completely (Rapola et al., 1965). Contaminants known to disrupt thyroid activity include the herbicides acetochlor and amitrole (Helbing et al., 2006; Alexander, 1959), the DDT metabolite DDE, but also nonylphenols (break down products of alkylphenol, a surfactant used in some pesticide formulations) (Yang, Xu & Wen, 2005). Some chemicals such as organophosphorus and carbamate insecticides disrupt neurological systems through inhibition of acethylcholineestherase (AChE) (Venturino & Pechen de D'Angelo, 2005), whereas other contaminants induce deformities by interfering with retinoid signaling pathways (Gardiner & Blumber, 2003). Mixtures of pesticides including endosulfan, lindane, and dieldrin, can alter the amphibian immune system (Christin et al., 2004).

Some environmental contaminants are able to disrupt hormonal signaling in receptor organisms, and this may result in an alteration of sex ratios and/or induction of gonadal abnormalities (Bogi et al., 2003; Cevasco et al., 2008; Harris et al., 2000). Contaminants having the ability to interact with the amphibian endocrine system such as these, pose a potential threat to amphibian reproductive fitness.

1.1 *The endocrine system*

General introduction

The vertebrate endocrine system is made of three main components; endocrine glands, hormones, and specific hormone receptors that interact with each others in

order to achieve homeostasis. It regulates important functions such as reproduction, growth, and digestion. The endocrine system is connected to the nervous system, especially in the hypothalamus where neurohormones are secreted and induce the production of tropic hormones by the pituitary gland. Tropic hormones travel through the blood stream to endocrine glands such as the thyroid gland, the adrenal gland or the gonads (Figure 1). Endocrine glands are ductless and are specialized in the production of hormones travelling in the bloodstream bound to plasma proteins or free as soluble molecule. Once they reach the target organ, hormones bind to specific receptors. Water soluble hormones cannot go through cell membranes and need to bind to cell surface receptors whereas hydrophobic hormones can diffuse across cell membranes and bind to cytoplasmic or nuclear receptors. Activated receptors induce specific responses in the cell like the synthesis of specific proteins for example (Figure 2).

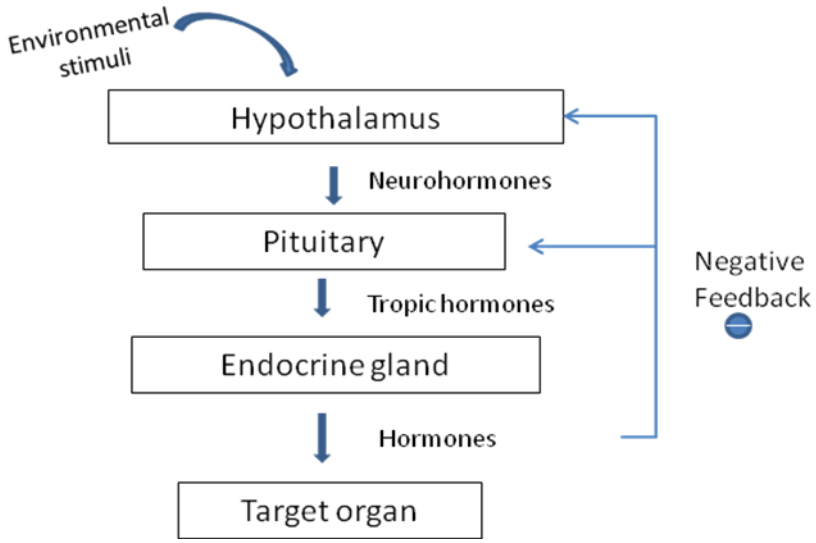

Figure 1.Hypothalamus-pituitary system.

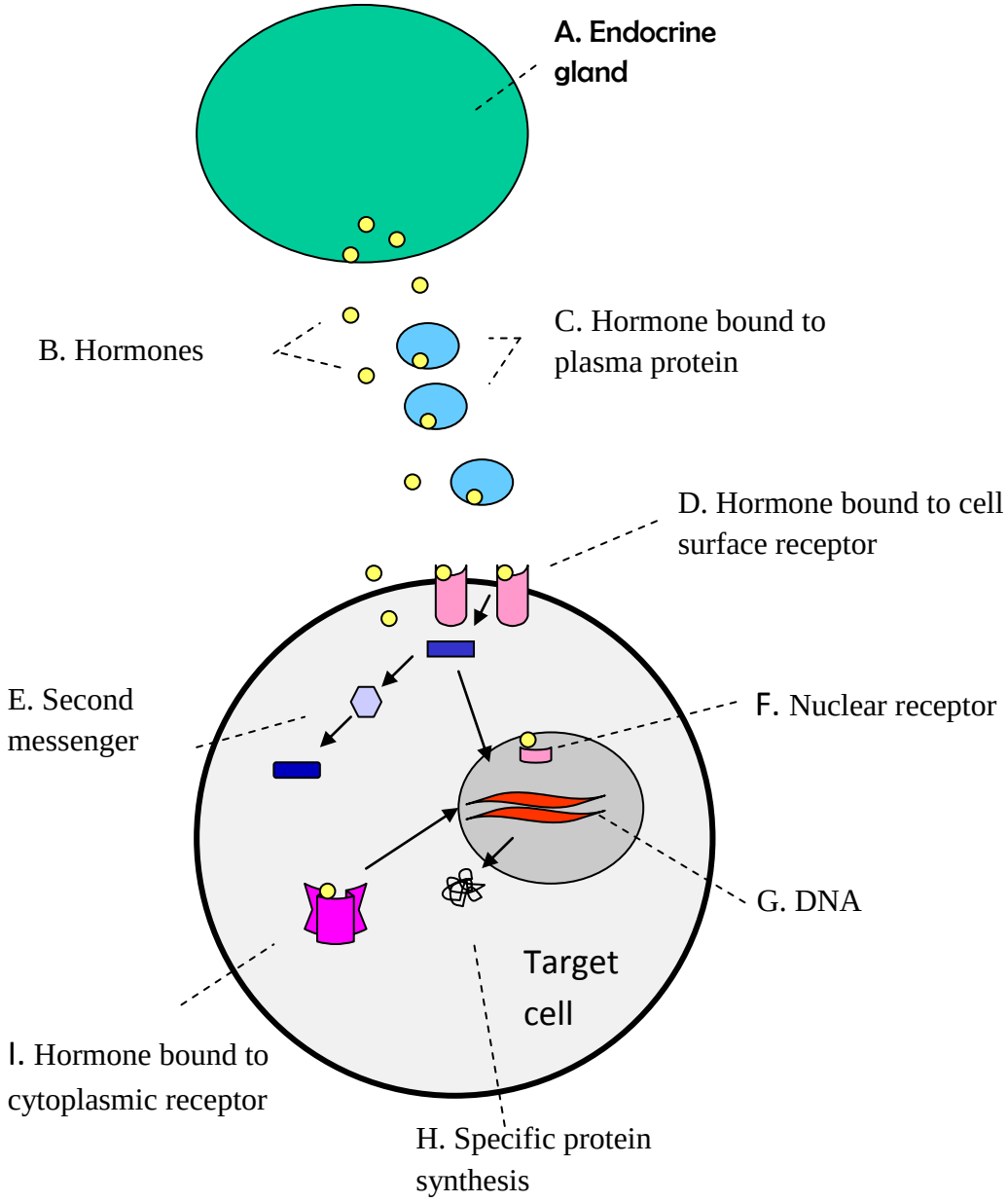

Figure 2. Diagrammatic representation of the vertebrate hormonal system. An endocrine gland (A) release hormones in the blood stream that travel under their free form (B) or bound to plasma proteins (C). Hormones then bind to specific receptors (D, I, F). Activated cell surface receptor (D) can induce a cascade of reactions leading to the production of cytoplasmic enzymes (E) or a change in the DNA expression in the nucleus (G,H). Lipophilic hormones can travel through cell membranes and bind to cytoplasmic receptors (I) or nuclear receptors (F). Those activated receptors induce DNA transcription leading to the production of specific proteins (G,H).

Reproductive endocrinology

In vertebrate reproduction, the pituitary releases gonadotropins such as Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH), which then stimulate the production of sex steroid hormones by the gonads such as estrogens, androgens and progestogens. Sex steroid hormones affect development, growth and function of the reproductive organs, development of secondary sex characteristics and behavioral patterns. They derive from cholesterol, are mostly lipophilic and can diffuse across the plasma membrane and into the cells. Steroid hormones travel in the blood stream and most of them are bound to Sex Steroid Binding Protein (SBP), which protects the bound steroids from metabolic degradation, regulates their bioavailability and their tissue distribution and facilitates their cellular uptake (Norris, 2006). The main steroids involved in amphibian reproduction are 17α-estradiol, testosterone, dihydrotestosterone and progesterone (Van Tienhoven, 1983).

Different enzymes are involved in steroid biosynthesis such as cytochrome P450 enzymes and cytosolic proteins like Δ^5,3β-hydroxysteroid dehydrogenase (3β-HSD) (Figure 3). P450 enzymes and 3β-HSD convert cholesterol into pregnenolone, which is transformed into progesterone by 3β-HSD. Progesterone is subsequently converted into androstenedione by 17α-hydroxylase/17-20 desmolase, and androstenedione is changed into testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD) or into estrone by aromatase ($P450_{aro}$). Testosterone is converted into DHT by 5α-reductase or into estradiol by aromatase. Estrone is transformed in estradiol by 17β-HSD.

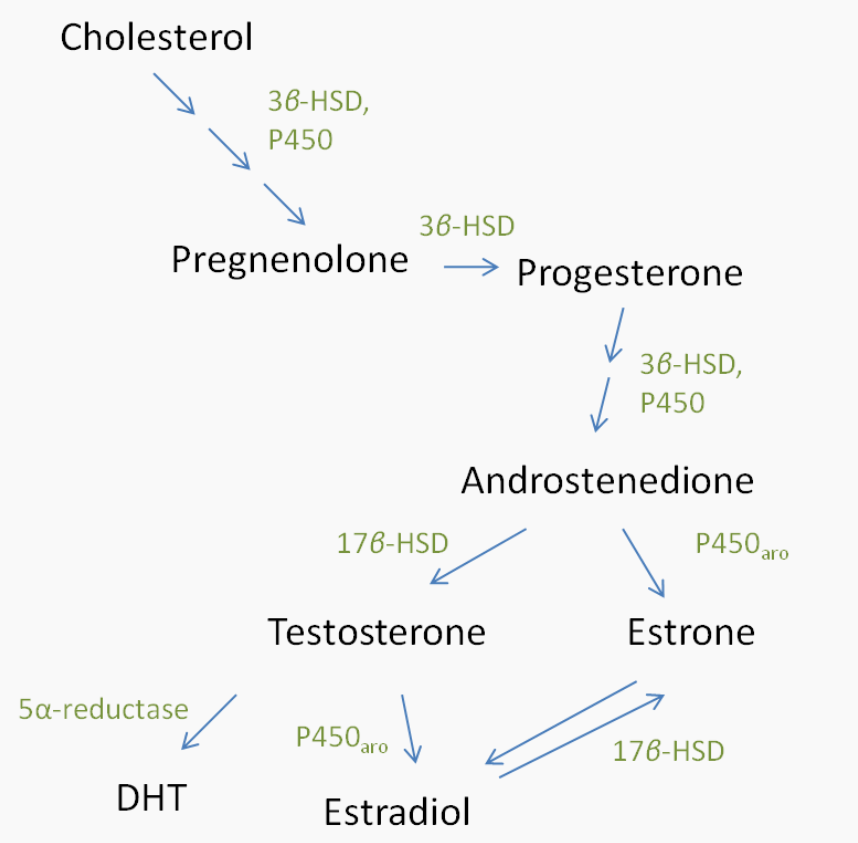

Figure 3.Simplified representation of steroid biosynthesis. Series of arrows indicate that the transformation is not direct. See text for explanations.

In the testes, steroids are produced by interstitial Leydig cells which are located in the interstices between the semineferous tubules. Leydig cells are activated by LH and secrete androgens. Sertoli cells are attached to the basement membrane of the seminiferous tubules, are activated by FSH and nurture the developing sperm in the seminiferous tubules. They produce aromatase, as well as inhibin and activin, two protein complexes regulating FSH secretion at the level of pituitary. In the ovary, steroids are produced by the ovarian follicles (Moore, 1987).

Amphibian reproduction

Amphibian mating behavior is controlled by environmental factors such as day length, temperature and rainfall which can stimulates the hypothalamus-pituitary complex to release LH/FSH, which then stimulate the gonads to produce steroid hormones (Moore, 1987).The steroids produced then stimulate mating behavior such as migration to the breeding sites (eg: ponds, streams), calling and amplexing for males and finally mating (Van Tienhoven, 1983). Male frogs and toads have nuptial pads on the ventral side and digits of their forearms that help them clasp onto the females during amplexus. Nuptial pads are characterized by keratinized epidermal hooks and androgen-dependent breeding glands (van Wyk et al, 2003a). Breeding glands secrete a substance with gluing qualities made of glycoprotein (van Wyk et al., 2003a). When males and females are in amplexus, males release spermatozoa while the female spawn. When spermatozoa enter the water, the osmotic shock activates sperm motility (Browne & Zippel, 2007). Amphibian sperm have a helical head that allows them to swim in a corkscrew-like manner. Analysis of *Xenopus laevis* sperm motility in egg jelly revealed that sperm display a stop-and-start behavior when swimming in egg jelly, but successful sperm that go through the different jelly layers exhibit fewer cases of stopping and starting (Reinhart et al., 1998) . Sperm competition trials showed that in the polyandrous species *Crinia georgiana*, males having a higher proportion of motile sperm and motile sperm with a lower average path velocity (VAP) are advantaged when competing for fertilizations (Dziminski et al., 2009).

1.2 Amphibian sex determination and differentiation

Sex determination

Sex determination is most likely under genetic control in amphibians although no sex determining gene has been identified yet. Exposure to extreme temperatures during larval development can alter the sex ratio that is usually 1:1 (Hsu et al., 1971; Witschi, 1930). Consequently we can say that in amphibians sex is determined by genetic sex determination (GSD) but extreme temperatures (not naturally experienced by the species) can modulate the sex determination system. Most amphibians do not exhibit morphologically distinguishable chromosomes. In fact sex chromosomes seem to have evolved several times among amphibians (Nakamura, 2009). The ancestral state seems to be female heterogamety (ZZ/ZW) but the two types of heterogamety can be found within the same family and in the case of *Rana rugosa*, even within the same species (Hayes, 1998; Ogata et al., 2003). Endogenous steroids are not believed to be normally involved in sex determination, but are clearly essential in amphibian sex differentiation since exposure to exogenous steroids can reverse the initial presumably genetic sex determination signal at the level of gonadal differentiation (Wallace et al., 1999). Genes involved in sex differentiation in other vertebrates such as *Sox 9, Dmrt1, WT1, CYP19*, and *CYP17* have been isolated in *Xenopus laevis*, *Rana rugosa* and *Silurana tropicalis*, but none of these are likely to be the sex determining gene in those species because they were not mapped to the sex chromosomes (Uno et al., 2008)

Sex differentiation

Amphibians display three different patterns of gonadal differentiation; the differentiated type, the undifferentiated type, and the semi-differentiated type (Gramapurohit et al., 2000). In the differentiated type, the bipotential gonad differentiates directly into a testis or an ovary (eg: *Xenopus laevis*, *Rana nigromaculata, Bufo orientalis*), whereas in the undifferentiated type, all gonads first differentiate into ovaries and then after metamorphosis, genetic male gonads differentiate into testis (eg: *Rana temporaria, Rana esculenta, Bufo bufo*) (Saidapur et al., 2001). In the semi-differentiated type, the testes differentiate after a phase of intersexuality (eg: *Rana curtipes, Rana arboreus*) (Saidapur et al., 2001). Also, there are differences in the ovarian developmental rate between species. Ogielska and Kotusz (2004) observed that species from the genus *Bufo* have a retarded gonadal development compared with most species (eg: *Xenopus laevis* and *Rana temporaria*), whereas the green frogs of the genus *Rana*, subgenus *Pelophylax* (eg: *Rana lessonae, Rana nigromaculata* and *Rana ridibunda*), display an accelerated ovarian differentiation rate relative to somatic development (Ogielska & Kotusz, 2004).

The undifferentiated gonad comprises a cortex and a medulla and is located on the ventral surface of the kidney (Villalpando & Merchant-Larios, 1990). Gonad primordial germ cells (PGCs) originate from the endoderm (Blacker, 1958) and migrate to the bipotential gonad until NF stage 55 (Nieuwkoop & Faber, 1994) in *Xenopus laevis* (Villalpando & Merchant-Larios, 1990).

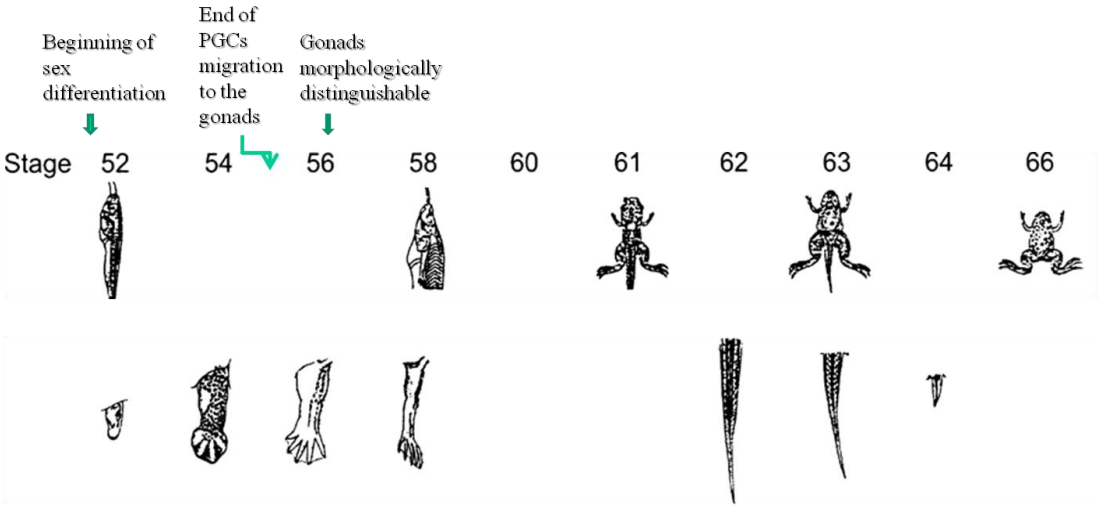

Figure 4. Staging system of *Xenopus laevis* laevae adapted from Niewkop and Faber, 1994. Schematic representation helps assessing the stage of the tadpole (growth of hind limbs is used until stage 58/59 and resorbtion of the tail is used until stage 66.). In *Xenopus laevis*, sex differentiation start at NF stage 51/52. PGCs stop migrating to the gonads at NF stage 55. Gonads are morhologically distinguishable from NF stage 56.

The sex of *Xenopus laevis* larvae is morphologically distinguishable from NF stage 56 (Villalpando & Merchant-Larios, 1990) (see Figure 4). In genetic males, the medulla develops into testes whereas in genetic females, the cortex develops into ovaries and the medulla degenerates forming an ovarian cavity (Hayes, 1998). In *Silurana tropicalis*, testes begin to differentiate at NF stage 49-50 and ovaries at NF stage 52 (El Jamil et al., 2008). An early study involving transplants of mesoderm in *Rana sylvatica* suggested that PGCs do not stimulate gonadal differentiation, but that it is the gonad that induces the germ cells to develop into sperm or oocyte (Humphrey, 1933). Also, germ cells originate exclusively from PGCs since transfer of *Xenopus mulleri* PGCs into *Xenopus laevis* at the neurula stage after removal of

the host PGCs, and subsequent mating of those experimental *Xenopus laevis* females with *Xenopus mulleri* males resulted in a progeny presenting all characteristics of *Xenopus mulleri* (Blackler & Gecking, 1972).

A more recent study on *Bufo bufo* gonadal morphogenesis suggests that amphibian sex differentiation is similar to other vertebrates sex differentiation in the way that germ cells tend toward female differentiation, and that in males, medullary somatic cells induce the germ cells to differentiate into spermatogonia (Falconi et al., 2004). These authors suggest that PGCs in the cortex primarily initiate ovarian differentiation, but migration of the PGCs to the medulla and interaction between PGCs and the medullary somatic cells leads to testicular differentiation (Falconi et al., 2004). In addition, in *Silurana tropicalis*, somatic cells are in close contact with germ cells at the beginning of testicular differentiation, suggesting that pre-sertoli cells are involved in the male germ cells differentiation (El Jamil et al., 2008). In *Xenopus laevis*, implantation of testicular grafts in undifferentiated gonads resulted in masculinisation of the genetic females (Mikamo & Witschi, 1963). This masculinisation cannot be attributed to the production of testosterone by the testis because exposure to testosterone during sex differentiation does not masculinise *Xenopus laevis* (Gallien, 1962). This result is explained by the fact that testosterone is aromatizable and thus can be converted into estradiol. These findings lead to the hypothesis that ovarian development is the default pathway and that in males, the testes secrete a testicular factor that inhibits ovarian development by blocking the aromatization of androgens to estrogens (Kelley, 1996). The theory that aromatase is essential in ovarian differentiation was reinforced by a multitude of examples in the literature showing that in amphibians, exposure to aromatase inhibitors during sex

differentiation lead to masculinisation of the gonads (Olmstead et al., 2009b; Petrini & Zaccanti, 1998; Duarte-Guterman et al., 2009; Miyata & Kubo, 2000; Yu et al., 1993; Ohtani et al., 2003). There are also numerous examples in the literature of the permanent feminizing effect of exogenous estrogens when exposed during sex differentiation in *Xenopus laevis*, suggesting that estradiol plays an important role in ovarian differentiation (Villalpando & Merchant-Larios, 1990; Miyata et al., 1999; Chang, 1955).

Steroidogenic gene expression during sex differentiation

Research on steroidogenic gene expression during ontogeny corroborated the key role of aromatase in female differentiation and also the function of the enzyme 5α-reductase in male differentiation. In *Xenopus laevis*, aromatase mRNA is expressed from NF stage 39, and is expressed 10 fold higher in female gonads than in male gonads from NF stage 56, whereas 5α-reductase type 2 mRNA is expressed two fold higher in male gonads compared to female gonads from stage 56 (Urbatzka et al., 2007). Previously, Miyashita *et al*. (2000) found that aromatase and estrogen receptor mRNA were expressed in *Xenopus laevis* gonads during sex differentiation (NF stage 50-51), suggesting that estrogen involvement in sex differentiation is mediated by the estrogen receptor (ER) (Miyashita et al., 2000). Furthermore, 5α-reductase type 1 mRNA is expressed from NF stage 12, but there are no sex-specific differences in its expression in gonads (Urbatzka et al., 2007). Exposure of *Silurana tropicalis* larvae to finasteride, an inhibitor of 5α-reductase type 1 and 2, induced feminisation of the gonads (Duarte-Guterman et al., 2009), suggesting that 5α-reductase is involved in testicular differentiation. In addition, estradiol and androgens

(testosterone and DHT) are present at high concentrations in *Xenopus laevis* eggs, embryos, and hatched larvae but the concentrations decrease dramatically around NF stage 50 (Bogi et al., 2002). This finding implies a maternal transfer of steroids and could be related to the expression of 5α-reductase type 1 mRNA at such an early stage. Also, in *Xenopus laevis*, estrogen and androgen receptor mRNA transcripts are expressed just after hatching, which suggest that maternal sexual steroids may induce expressions of their corresponding receptors, leading to a stage that is sensitive for sexual differentiation (Bogi et al., 2002). On the other hand, in *Silurana tropicalis*, estrogen and androgen receptors transcripts were found before gastrulation, indicating that they are maternally transferred (Duarte-Guterman et al., 2010). In addition, androgen receptor (AR) and ER-α mRNA increase significantly at NF stage 34 (time of hatching), whereas ER-β mRNA levels increase only at NF stage 46 (beginning of feeding), suggesting ER-α and ER-β play different roles during embryogenesis (Duarte-Guterman et al., 2010).

In female heterogametic ZZ/ZW *Rana rugosa*, AR expression begins in the gonads before sex determination and is higher in the gonads of male tadpoles, whereas ER-β expression significantly increases in the tadpole gonad after sex determination (Yokoyama et al., 2009). The role of steroid receptors in amphibian sex differentiation has also been studied in *Rana rugosa* by exposing the larvae to different types of endocrine disrupters, including estrogenic, androgenic, and antiandrogenic chemicals, plus an aromatase inhibitor, and subsequently analyzing the expression of estrogen and androgen receptors, and of aromatase genes (Ohtani, Miura & Ichikawa, 2003). The results led the authors to conclude that elevated androgen receptor expression suppresses aromatase expression directly or indirectly,

resulting in testicular differentiation due to an androgen accumulation (Ohtani et al., 2003). More recent research on *Rana rugosa* steroidogenic gene expression before sex differentiation indicated that P450 17α-hydroxylase/C17-20 lyase, an enzyme involved in the transformation of progestogens into androgens, is up regulated in male gonads before, during and after sex differentiation (Maruo et al., 2008). The study also found that aromatase is up regulated in female gonads (Maruo et al., 2008).

Thyroid hormones and sex differentiation

Thyroid hormones (TH) regulate amphibian metamorphosis, but evidence also suggests that they are involved in gonadal differentiation. Indeed, *Xenopus laevis* exposure to ammonium perchlorate, a TH synthesis inhibitor, during larval development, induced a skewed sex ratio toward females (Goleman et al., 2002). In *Rana pipiens*, exposure to triiodothyronine (T3) during metamorphix climax increases expression of ER-α mRNA but decreases aromatase mRNA levels in the brain (Hogan et al., 2007), whereas exposure to EE_2 during mid-metamorphosis, significantly delays metamorphosis (Hogan et al., 2008). Those studies indicate the existence of a cross-talk between TH and steroid hormones. Recent work on *Silurana tropicalis* shows that TH can regulate androgen related genes. Indeed, exposure to T3 increased AR and 5α-reductase type 1 mRNA levels in *Silurana tropicalis* at NF stage 46, which is prior to the start of gonadal differentiation (Duarte-Guterman et al., 2010). In a different experiment, these authors found that exposure to T3 increased AR, both 5α-reductase mRNA levels, and decreased ER-β mRNA levels in the gonad-mesonephros complex, during gonadal differentiation (Duarte-Guterman

& Trudeau, 2011). These findings suggest that TH could play a key role in testicular differentiation.

Overall, it seems that amphibian ovarian differentiation has some similarities with other vertebrate ovarian differentiation (ex: birds, fish, reptiles) since aromatase seems to play a key role in ovarian differentiation. There are more uncertainties concerning testicular differentiation, but at this stage, we know that P450 17α-hydroxylase/C17-20 lyase, 5α-reductase type 2 and AR are implicated (Ohtani et al., 2003; Urbatzka et al., 2007; Maruo et al., 2008). Most of the recent research on amphibian sex differentiation has been done on *Xenopus laevis*, *Rana rugosa*, or *Silurana tropicalis*. It is difficult to extrapolate for all amphibian species from these findings because there may be some differences among species. Indeed, we know that exposure to exogenous steroids has different consequences on gonadal differentiation depending on the species involved (Hayes, 1998). This finding suggests that there might be some dissimilarity between amphibian species with regard to sex differentiation. In anurans, exposure to estrogens usually feminizes larvae and exposure to androgens either masculinise (eg: Ranid, Hylid) or has no effect on larvae gonadal differentiation (eg: testosterone on *Xenopus* and *Bufo*) (Wallace et al., 1999; Gallien, 1962). Sex reversal caused by hormones can be temporary or permanent, depending on the species (Chang, 1955). All these laboratory experiments on frog sex differentiation led to the conclusion that exposure to exogenous steroids during larval development can alter gonadal differentiation and imply that wild frog populations could be at risk if their breeding ponds happen to be contaminated by chemicals with endocrine disrupting activity, such as xenoestrogens. What is less clear is the extent to which larval sexual differentiation

can be disrupted by such agents, before there are impacts on reproductive potential after sexual maturation.

1.3 Endocrine disrupting chemicals (EDCs)

Definitions and different modes of actions

EDCs are natural or synthetic chemicals that can interfere with the endocrine system. The International Programme on Chemical Safety (IPCS) define an endocrine disruptor as "an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (Damstra et al., 2002). EDCs can be found in man made products such as pesticides, drugs, and chemicals used by diverse industries. Excretion of hormones from animals (e.g. livestock) and the natural phytoestrogens present in plants can also act as EDCs (Cooper & Kavlock, 1997).

EDCs can disturb the endocrine system by different pathways, for example through binding steroid hormone receptors as an agonist (binding to a receptor and triggering a response in the cell) (e.g. nonylphenol (NP) which binds to the ER (Folmar et al., 2002)). But also as an antagonist by inhibiting the normal physiological function of a receptor (e.g. the metabolite of DDT, p,p'-DDE, which is an AR antagonist; Kelce et al., 1995). Some EDCs can interact directly with cellular enzymes that are involved in steroid synthesis or action, and one of the consequences of this is a hormonal overproduction or underproduction (e.g. glyphosate which disrupt the steroidogenic acute regulatory (StAR) protein expression, a protein involved in the transport of cholesterol (Walsh et al., 2000)). EDCs can also interact with SBD and affect the

ratio of free hormone present in the bloodstream (e.g. some PCBs can interact with sex hormone binding globulin (SHBG) (Persky et al., 2001)). Research has focused mainly on EDCs affecting steroid receptors such as ERs and ARs, and those interacting with thyroid function, but also those binding to retinoid receptors (RAR, RXR) or the Aryl hydrocarbon (AhR) receptor.

Some compounds seem to exhibit multiple modes of actions. For example, Bisphenol A (BPA) and NP (two phenols widely used by diverse industries) can both bind to the ER, but also present some anti-androgenic activity *in vitro* (Lee et al., 2003). Moreover, BPA and other halogenated derivates also appear to interfere with thyroid receptor signaling (Iwamuro et al., 2006; Heimeier et al., 2009; Goto et al., 2006). Some trace elements can also behave as EDCs. Indeed, cadmium (Cd), copper (Cu) and lead (Pb) can induce estrogenic activity *in vitro* and *in vivo* (Isidori et al., 2010).

Effect of EDCs on vertebrates

One of the most documented cases concerning the effects of EDCs on the reproductive system is found in humans. Diethylstilbestrol (DES), a synthetic estrogen, was prescribed by doctors during the 20th century to pregnant women in order to prevent miscarriages. Male and female humans exposed to DES in utero presented a higher rate of abnormalities on the reproductive function. Infertility, gonadal structural abnormalities, cancers, miscarriages, and pregnancy problems such as pre-term birth where found in higher incidence in DES sons and daughters (Kaufman et al., 2000; Palmer et al., 2006). In light of these findings, use of DES

was banned in 1971 in the U.S.A, in 1975 in the UK, and in 1977 in France (Rubin, 2007).

In UK rivers, where treated effluents from waste sewage treatment plants are released, scientists observed an increase of feminized male fish (Sumpter, 1995). Treated effluent contains a mixture of EDCs and among them is 17α-ethynylestradiol (EE_2), found at concentrations between 0.7 to 5 ng/L in Europe (Nash et al., 2004). Estrogens, such as EE_2, have been held responsible for the occurrence of intersex in fish for many years, but recent studies suggest that antiandrogens also contribute to widespread sexual disruption in wild fish (Jobling et al., 2009). Presence of intersex gonads has been linked to lower fertility in wild roach (Jobling et al., 2002). Furthermore, long term exposure to environmentally relevant concentrations of EE_2 can threaten fish populations. Indeed, 3 years of chronic exposure to environmentally relevant concentrations of EE_2 (5-6 ng/L) in a Canadian lake resulted in male fathead minnows (*Pimephales promelas)* feminization, altered female oogenesis and ultimately induced a collapse of the population (Kidd et al., 2007). Moreover, life-cycle exposure of fathead minnow to environmental concentrations of EE_2 under laboratory conditions produced feminized males and reduced egg fertilization success (Parrott & Blunt, 2005). Also, exposure of zebrafish (*Danio rerio*) over multiple generations to environmentally relevant concentrations of EE_2 induced a 56% reduction in fecundity in the F_1 generation, and complete population failure with no fertilization (Nash et al., 2004).

An example of a more widely used and pervasive group of chemicals with endocrine disrupting activity are the polychlorinated Biphenyls (PCBs). PCBs are organic compounds that were widely used in a variety of applications including in electrical

equipment, as coolant, in paint and lubricating oil, but which were withdrawn and then been banned in the 1970s over concern about their environmental persistence and health impact (Ross, 2004).

PCBs induce diverse health effects in wildlife, including teratogenesis (Barron, 1995), immune impairment (Ross et al., 1996), and reproductive failure in marine mammals (Reijnders, 1986; Vos et al., 2000). Like many pollutants, they are lipophilic and accumulate in adipose tissues, and can also undergo transplacental movement into the fetus (Park et al., 2008). PCBs are a large and complex group of environmental contaminants that may exert deleterious effects on receptor species through a variety of mechanisms. Some PCBs are known to interact with the Aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor (Kamata et al., 2009), which mediates dioxin-like effects, but which also exhibits some ability to modulate the estrogen receptor signaling (Swedenborg & Pongratz, 2010). Other PCBs (the less highly chlorinated congeners) may also exert estrogenic effects through interaction with ERs, while others have been shown to displace plasma hormones from carrier proteins, and to up-regulate hepatic metabolizing enzymes and increase clearance of endogenous hormones (Persky et al., 2001; Korach et al., 1988; Heinrich-Hirsch et al., 1997; Kester et al., 2000).

Correlations have been made between high concentrations of PCBs and death by infectious disease found in dead sea otters (*Enhydra lutrisnereis*) on the Californian coast (Kannan et al., 2007), and in harbour porpoises (*Phocoena phocoena*) in United Kingdom waters (Jepson et al., 2005). In addition, negative correlations were found between retinol, the thyroid hormone triiodothyronine (T3), and plasma levels of PCBs in California sea lions (*Zalophusca lifornianus*) (Debier et al., 2005). PCBs

have been linked to the otter (*Lutra lutra*) population crash seen in the UK and in Europe in the 1950s (Mason & Macdonald, 2004).

1.4 Can EDCs affect amphibian reproduction?

The response of amphibians to EDC exposure may be highly species dependent. Indeed, exposure of a whole lake to EE_2 induced no intersex individuals in *Rana clamitans* tadpoles, but induced up to 12.5% intersex individuals in *Rana septentrionalis* tadpoles (Park & Kidd, 2005). Also, the incidence of gonadal abnormalities in *Rana pipiens* was correlated to exposure to pesticides and nutrients mixtures, whereas it was not in *Rana clamitans* (McDaniel et al., 2008). These differences could be explained by variations in somatic and gonadal developmental rates in the different species. It has been established that species with slower somatic developmental rate and fast gonadal developmental rate are more susceptible to estrogenic contamination (Storrs & Semlitsch, 2008). A species such as *Rana sphenocephala*, which is beyond sexual differentiation at metamorphosis, exhibits significant treatment effects (i.e. a slower ovarian developmental rate), when exposed to estrogenic chemicals during larval period, whereas species that are at or near sexual differentiation at metamorphosis like *Bufo americanus* and *Hyla versicolor* do not demonstrate significant treatment effects (Storrs & Semlitsch, 2008). In addition, ovarian differentiation is dependent of the age of the tadpole, not of its somatic stage (Chang & Hsu, 1987), which means that tadpoles metamorphosing slower due to external factors such as lower temperature would hypothetically be more susceptible to xenoestrogens exposure.

Moreover, the impact of exogenous steroids on gonadal differentiation depends very much on the window of exposure. Under laboratory conditions, when *Xenopus laevis* larvae are exposed to estradiol from early stages (NF44-50), genetic males fully sex reverse, when exposed from the middle of larval development (NF51-54, at the beginning of gonadal differentiation), genetic male gonads develop into ovotestes, and finally when exposed from later stages (NF56, after PGCs stop migrating to the gonads), the gonads of genetic males differentiate into functional testes (Villalpando & Merchant-Larios, 1990).

Mechanism of testicular oocytes development

Histological analysis of the gonads of *Xenopus laevis* exposed to estradiol during sex differentiation revealed that exogenous estradiol prevents PGCs migration to the medulla of developing gonads in a concentration-dependent manner (Hu et al., 2008). Hence, exposure to low concentrations of exogenous estrogens to genetic males during gonadal differentiation would result in the partial blockage of PGCs migration to the medulla, resulting in the incidence of intersex, whereas exposure to higher estrogens concentrations would completely block PGCs migration to the medulla, resulting in complete sex reversal to the female phenotype (Hu et al., 2008).

Intersex gonads in frogs, a natural phenomenon during ontogeny?

Evidence that exogenous exposure to EDCs can affect amphibian gonadal differentiation have been reviewed in section 1.2. Indeed, exposure to exogenous steroids (Villalpando & Merchant-Larios, 1990; Hogan et al., 2008; Coady et al.,

2005) and anti-estrogenic or anti-androgenic compounds (Olmstead et al., 2009b; Duarte-Guterman et al., 2009; Hayes et al., 2006) can alter sex ratio and/or induce intersexuality. It seems that amphibians respond similarly to fish when exposed to xenoestrogens, since a study exposing *Rana pipiens* larvae to wastewater effluents reported an increased incidence of male testicular oocyte in the exposed treatment groups (Sowers et al., 2009). Also, correlations have been found between the occurrence of gonadal abnormalities and agricultural land use in *Rana pipiens* (McDaniel et al., 2008) and *Bufo marinus* (McCoy et al., 2008).

In the case of the *Rana pipiens* study noted above, the correlations observed indicate that the higher the concentration of atrazine and nitrate and the number of pesticides hits, the higher the proportion of testicular oocytes (McDaniel et al., 2008). In *Rana clamitans*, a great number of intersex frogs were found in suburban, urban and agricultural landscapes whereas none were found in undeveloped landscapes (Skelly et al., 2010). It must be noted that the incidence of intersex *Rana clamitans* was much higher in suburban and urban landscapes respectively, than in agricultural landscapes (Skelly et al., 2010).

Even if evidence suggests that exposure to EDCs induces gonadal abnormalities in amphibians, it is also possible that testicular oocytes could be a natural phenomenon in amphibians. Indeed, the occurrence of testicular oocytes has been observed in wild frogs before the introduction of anthropogenic EDCs in the environment. In wild frogs and toads collected before the 1930s, which is before the industrial era, intersexual individuals were reported in *Bufo japonicus, Rana esculenta, Rana limnocharis, Rana fusca, Rana pipiens, Rana catesbeiana, Rana cantabrigensis, Rana tigrina, Rana viridis,* and many cases were reported in *Rana temporaria, Bufo*

vulgaris and *Bufo bufo* (Witschi, 1929; Crew, 1921; Cheng, 1929). In *Bufo bufo*, the incidence of intersex toads is 1% (Cheng, 1929). Also, histological analysis of *Acris crepitans* gonads collected before 1930s, revealed an incidence of 1.2 % intersex frogs (Reeder et al., 2005). However, the percentage of intersex *Acris crepitans* increased between 1930-1945, which correspond to the period of industrial growth and initial uses of PCBs, and was at its highest (11.1%) between 1946-1959, the period of the greatest manufacture and use of *p,p*-dichlorodiphenyltrichloroethane (DDT) and PCBs (Reeder et al., 2005). The incidence of intersex frogs then decreased between 1980-2001, when the use of organochlorines declined (Reeder et al., 2005). The incidence of intersex in *Acris crepitans* is clearly correlated with the use of organochorines, and in breeding sites that had been contaminated by organochlorines, the sex ratio was skewed toward males (Reeder et al., 1998). Overall, it appears that the occurrence of testicular oocytes in frogs is increased by EDCs exposure, but on the other hand, there are still some contradictory results concerning the incidence of testicular oocytes in controls of exposure studies, especially regarding the specie *Xenopus laevis*. In several studies, authors reported testicular oocytes in *Xenopus laevis* controls (Coady et al., 2005; Jooste et al., 2005), when others have not (Hayes et al., 2002; Carr et al., 2003). In wild *Xenopus laevis*, a recent study suggested that the occurrence of testicular oocytes is population specific (Du Preez et al., 2009). Another study even suggests that a phase of intersex during gonadal differentiation may be common regardless of ovarian differentiation rate in amphibians (Storrs-Mendez & Semlitsch, 2010). Nevertheless, under normal conditions, the intersex phase would be temporary whereas amphibians exposed to EDCs would permanently show sign of intersexuality (Storrs-Mendez & Semlitsch, 2010).

The case of atrazine

There has been much controversy about the potential effect of atrazine (CAS # 1912-24-9; one of the most widely used herbicides), on amphibian gonadal differentiation. In 1970, Hazelwood reported that the reproductive success of a *Rana temporaria* population was affected following a contamination of breeding ponds by atrazine (Hazelwood, 1970). Laboratory experiments exposing *Rana pipiens* and *Xenopus laevis* larvae to atrazine during gonadal differentiation found that atrazine at environmentally relevant concentrations can induce the development of testicular oocytes in males (Hayes et al., 2002 a,b), however the results of the study on *Xenopus laevis* did not appear to be reproducible (Coady et al., 2005; Carr et al., 2003; Kloas et al., 2009). The occurrence of testicular oocytes in male wild *Xenopus laevis* in South-Africa was not correlated with atrazine concentrations in breeding ponds (Du Preez et al., 2009) and neither was it in Illinois (U.S.A) in *Acris crepitans* (Reeder et al., 1998), nor in Michigan (U.S.A), in *Rana catesbeiana*, *Rana pipiens*, and *Rana clamitans* (Murphy et al., 2006). Nevertheless, exposure to low levels of atrazine during gonadal differentiation seems to alter sex ratio in *Rana pipiens* (Langlois et al., 2010; Orton et al., 2006) and *Xenopus laevis* (Hayes et al., 2010). The latest study involving all-ZZ male *Xenopus laevis* tadpoles had been previously done using similar concentrations of atrazine and no feminization effect had been observed in the atrazine treated groups (Oka et al., 2008), yet it can be argued that *Oka et al.* sampled the tadpoles at NF stage 66 while *Hayes et al.* let the frogs become sexually mature before sampling them, which produced a more accurate assessment of the sex ratio and allowed the authors to screen for endpoints related to the reproductive function. Despite the amount of research done, the effect of atrazine

on amphibian sex differentiation is still unclear, especially on the specie *Xenopus laevis*. However, studies on *Rana pipiens* provide some evidence that atrazine can skew the sex ratio in Ranids. Very few studies have looked at the impact of this herbicide on the reproductive function of the adult frog after exposure during larval development, hence, at this stage the impact of atrazine use on amphibian populations is unknown.

Evidence of the effects of EDCs exposure on the reproductive function

Although there are evidences that EDCs exposure can alter gonadal differentiation, surprisingly, very few studies have looked at the reproductive consequences of EDCs exposures on the adult amphibian. Atrazine-exposed *Xenopus laevis* males have lower testosterone plasma concentrations, smaller dermal breeding glands, feminized larynges, reduced spermatogenesis, and a lower fertility compared to control males (Hayes et al., 2010). Male *Silurana tropicalis* exposed to EE_2 during larval development have a reduced amount of spermatozoa in the testis and reduced fertilization rates compared with control males (Gyllenhammar et al., 2009). Hence recent studies suggest that exposure to EDCs during gonadal differentiation have negative effects on amphibian reproductive fitness. Nevertheless, although there is substantiation that EDCs can increase the occurrence of testicular oocytes, the impact of the presence of oocytes in the gonads on male fertility is not known, and neither are the consequences of the incidence of testicular oocytes at population level.

A laboratory experiment using a model amphibian where ontogeny has already been studied, and a model EDC of a known mechanism of action, would be a realistic

approach to gather more evidences on the impact of EDCs on the amphibian reproductive potential.

1.5 Aim of study

The aim of the research presented here was to investigate the consequences of exposure of an endocrine disrupting chemical during sex differentiation for reproductive potential in adult frogs. EE_2 was chosen as a model for EDC since it is a stable molecule, and there is already some evidence it can disrupt amphibian gonadal differentiation (Pettersson & Berg, 2007). *Silurana tropicalis* was used as an amphibian model as it presents the advantage of having a relatively short life cycle. It can reach reproductive maturation at 22 weeks post metamorphosis for males and 30 weeks post metamorphosis for females (Olmstead et al., 2009a). The sex ratio is approximately 50.1% males and 49.9% females (Grammer et al., 2005). The optimal temperature range for the larvae development varies from 22°C to 25°C. The developmental of *Silurana tropicalis* tadpoles can be staged using the Nieuwkoop and Faber staging system for *Xenopus laevis* as the developmental rate is very similar (Khokha et al., 2002). Also *Silurana tropicalis* respond similarly to *Xenopus laevis* in toxicology tests (Fort et al., 2004), and to thyroid disrupters (Mitsui et al., 2006), and to *Rana temporaria* when exposed to estrogenic chemicals (Pettersson & Berg, 2007; Berg et al., 2009). *Silurana tropicalis* is thought to have ZW sex chromosomes although no morphologically distinguishable chromosomes have been found (Uno et al., 2008). Overall, there is already some evidence that *Silurana tropicalis* is a suitable model for endocrine disruptor bioassays.

In the study described in this thesis, *Silurana tropicalis* tadpoles were exposed to concentrations of EE_2 in the ng/L range (10-3000ng/L) during larval development (NF52-NF66), incorporating the window of gonadal differentiation. Since tadpoles reared at lower temperature are known to metamorphose more slowly and have a longer aquatic phase, a decision was made to explore whether temperature also had an impact on the incidence of gonadal abnormalities at metamorphosis. Half of the replicates of all treatment groups (but for the 3000ng/L group) were set at 25˚C and the other half at 28˚C. At completion of metamorphosis, half of the froglets were sampled while the other half were allowed to grow to sexual maturity in the absence of any further exposure to EE_2. The sexually mature *Silurana tropicalis* were then sampled and endpoints linked to the reproductive function were measured (Figure 5).

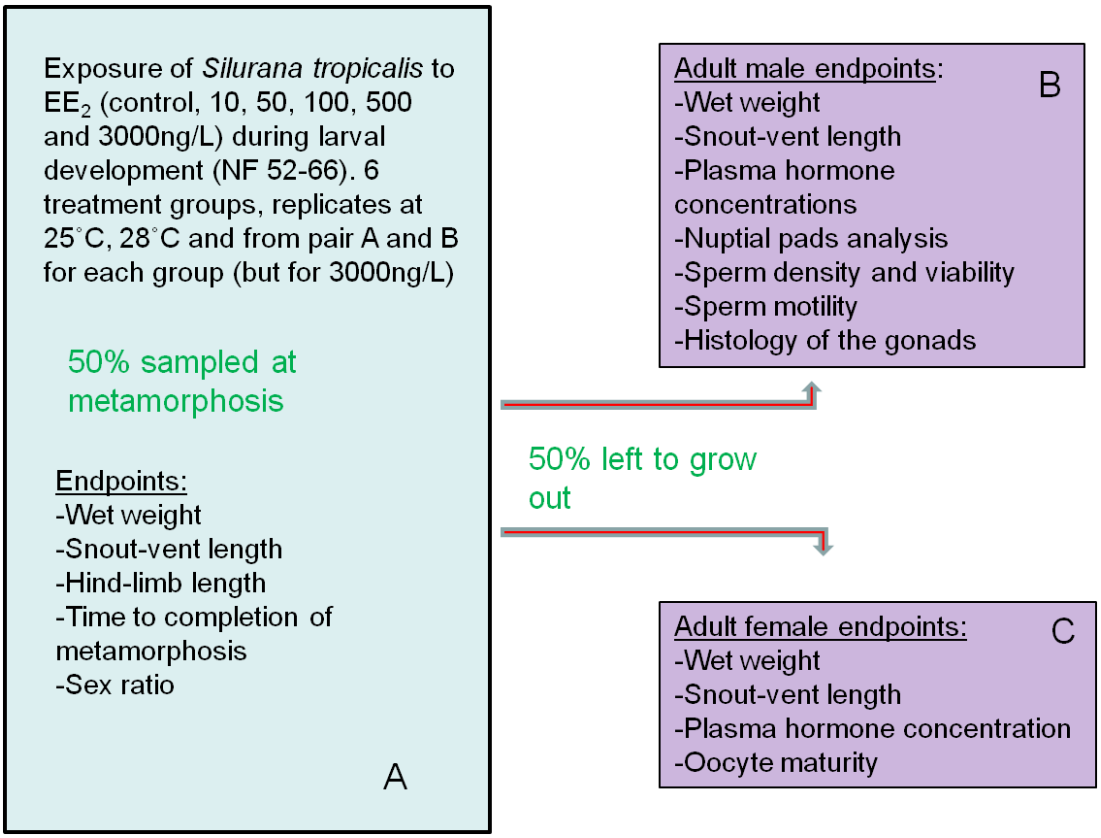

Figure 5. Schematic showing the design of the experiment, which was divided into 2 phases. The first phase was the exposure of *Silurana tropicalis* larvae to EE2 during sexual differentiation and until completion of metamorphosis. Half of the froglets were then sampled and different endpoints were measured (A), and the other half were left to grow out until sexual maturity. Adult females and males were then sampled, and different endpoints related to the reproductive function were assessed (B, C).

2 . Materials and method

2.1 Silurana tropicalis larval exposure to Ethynylestradiol

2.1.1 Experimental animals

The adult *Silurana tropicalis* used to produce larvae were kindly donated by the research group of Harvey Isaacs and Betsy Pownall from the University of York (York, United Kingdom). The frogs were fed with blood worms, Reptomin (Tetra), and Xenopus pellets (Blades Biological, UK) and were kept in a flow through system of dechlorinated tap water at 25˚C. All animal procedures were conducted in accordance with the UK Government Animals (Scientific Procedures) Act, 1986'.

2.1.2 Induction of spawning

Two adult pairs (pair A and pair B) were injected with exogenous gonadotropin hormones to induce mating behavior. Frogs were injected intravascularly, in the dorsal lymph-sac. Males received 20 IU of PMSG (Pregnant mare serum gonadotropin, Sigma: FSH-like activity) to increase sperm maturation, and then five days later, males and females were primed with 10 IU of HCG (Human chorionic gonadotropin, Sigma: LH-like activity). The next day, males were injected with 100 IU of HCG and females with 120 IU of HCG. Female/male pairs were placed in shaded glass tanks with 3 litres of dechlorinated tap water, with 5 ml/L of gentamicin at 10 mg/ml to prevent bacterial growth and left overnight. Adults were removed from the mating chambers the next morning. Dead eggs were removed everyday and the water changed every 2 to 3 days.

2.1.3 Pre-exposure study and feeding regime

When the larvae reached NF stage 42 (beginning of feeding), they were transferred into six 30 litres glass tanks filled with dechlorinated tap water. *Silurana tropicalis* larvae from the two pairs were kept in separate tanks in this pre-experimental period. The exposure study began when the tadpoles reached stage 51-52. Larvae were fed twice daily with Sera Micron (Sera, Heinsberg, *Germany*), following the OECD guidelines for the AMA (Amphibian Metamorphosis Assay) (

Table 1). A Sera Micron stock of 10g/L was made in dechlorinated tap water, kept in the fridge, and renewed every other day. After day 21 and until the end of the exposure study, larvae were fed 90 mg of Sera Micron per animal per day, and after day 25 and until the end of the exposure study, blood worms were added to the tanks every other day in order to lower risks of cannibalism.

Study day	Daily food ratio (mg Sera Micron per animal)
0-4	30
5-7	40
7-10	50
11-14	70
15-21	80

Table 1. Feeding schedule using Sera Micron for the AMA. Taken from "Proposal for phase-3 of the Validation of the Amphibian Metamorphosis Assay", OECD, 2007.

2.1.4 Design of the exposure study

Larvae were exposed to EE_2 at five nominal concentrations: 10, 50, 100, 500 and 3000 ng/L, plus dilution water control (DWC). There were four replicate tanks for the concentrations 10, 50, 100 and 500 ng/L, six replicates for the DWC and 2 replicates for the treatment group 3000ng/L. Every tank had a starting stocking rate of 30 tadpoles. Half of the replicates of each treatment group were stocked with larvae from pair A and the other half with larvae from pair B (i.e. two replicate vessels per pair per treatment for the groups exposed to EE_2, three replicate vessels per pair per controls). For each treatment group, half of the replicate tanks were maintained at 25˚C and half at 28˚C (Figure 6**Error! Reference source not found.**). Thermostatically controlled vivarium heat mats were used to maintain temperature of the test solutions.

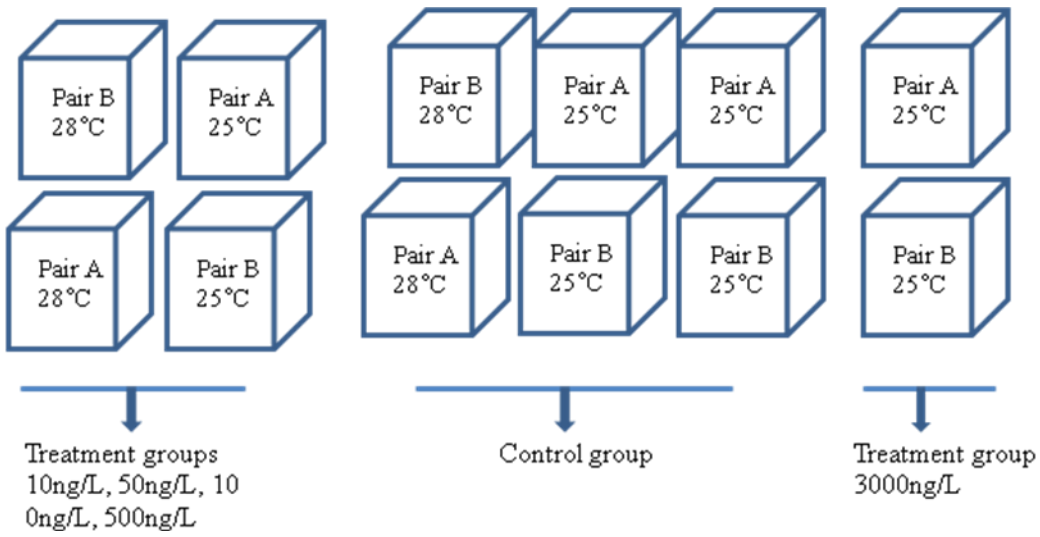

Figure 6. Schema representing the different replicate tanks for each treatment group during the exposure of *Silurana tropicalis* to EE_2 during larval development. Half of the replicates hold larvae from pair A and half from pair B. Test solutions were

maintained at 25 ˚C and 28˚C within each treatment group except for treatment group 3000ng/L.

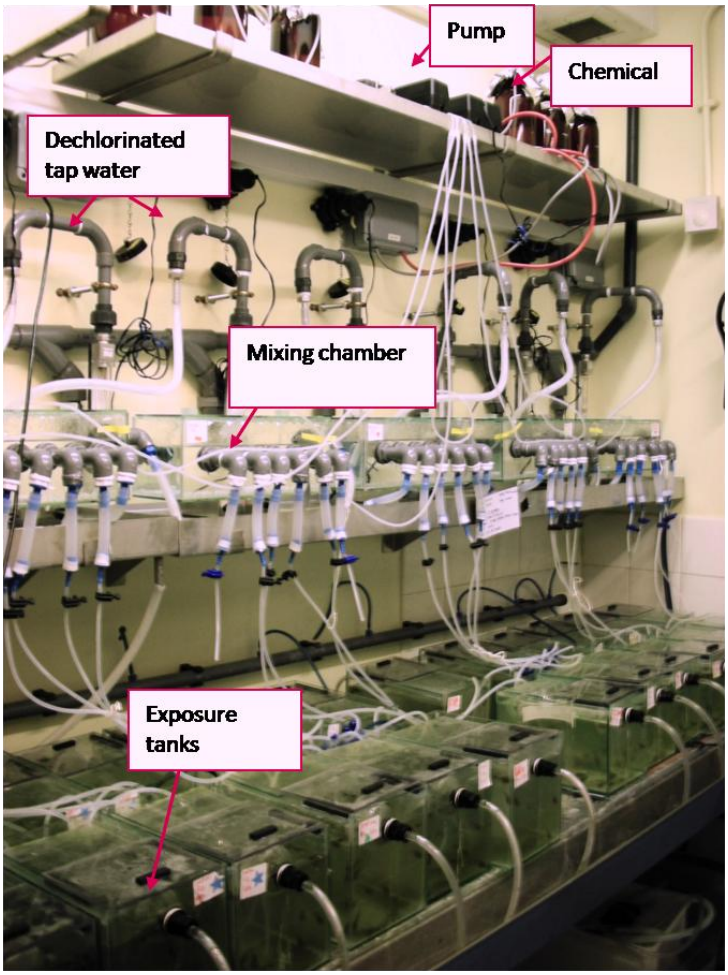

Figure 7. Photograph of the exposure equipment.

2.1.5 . Preparation of test solutions

A super-stock was prepared by dissolving EE_2 in absolute ethanol at 1mg/ml and stored at 4°C. A 3ml aliquot of this super stock was added to 1litre glass conical

flask containing 100g of 4 mm diameter glass beads. EE_2 was spread on the glass beads by manual agitation using circular motions. The solvent was evaporated under a stream of nitrogen for 40 min. EE_2 was then resuspended gradually in 1litre of distilled water by agitation of the EE_2-coated beads to generate a 3mg/L stock. Stocks of lower concentrations were prepared by diluting the 3 mg/L stock. These stocks were transferred into 2.5 L brown glass bottles with magnetic stir bars and placed on stir plates. DWC stock solution was prepared following the same protocol using an aliquot of 3ml of absolute ethanol in the first step of the procedure. A peristaltic pump transferred the stock solutions into 2.2 litre capacity rectangular glass mixing chambers (one per treatment group) where the stock solutions were blended with filtered dechlorinated tap water at 25˚C using magnetic stir bars, resulting in a x 1000 dilution of the stock preparation. This diluted test solution was subsequently gravity-fed to 4 litre test vessels at a nominal flow rate of 30 ml/min (Figure 7). All tubing used in this exposure study was made of medical grade silicone (VWR, UK) and all connectors were made of ABS (Watson and Marlow, UK). Stock solutions were changed every 3 days.

2.1.6 Daily recording

Mortalities were checked and recorded everyday for each tank and excrement was removed using a fish net. All tanks were cleaned by scraping and siphoning every three days. Dissolved oxygen and temperature were measured in each tank every week. Flow rates of stock solutions, de-chlorinated tap water and test solutions were checked and adjusted every week.

2.1.7 Preparation of water samples.

Samples of test solution from half of the replicates for each treatment group were taken once weekly for analytical verification of test concentrations. 100 ml of test solution was sampled using a glass flask and passed through an "Oasis" solid phase extraction cartridge (Waters, UK) to pre-concentrate the samples for measurement by specific radioimmunoassay for EE_2. Cartridges were first conditioned with 5 ml of methanol then rinsed with 5ml of distilled water before the water sampled went through the cartridge under vacuum. The cartridges were dried in air at ambient temperature and subsequently stored at -20°C until further elution and analysis. The cartridges were defrosted for 30 min at ambient temperature before elution with 5ml methanol. The eluant was then dried down under a stream of nitrogen and eluted residues were resuspended in 1ml ethanol (Figure 8). These samples were then further diluted in ethanol according to nominal EE_2 concentrations to achieve a final concentration in the region of 0.5ng/ml for measurement by RIA (See below), whereas controls were further diluted with 9 ml ethanol.

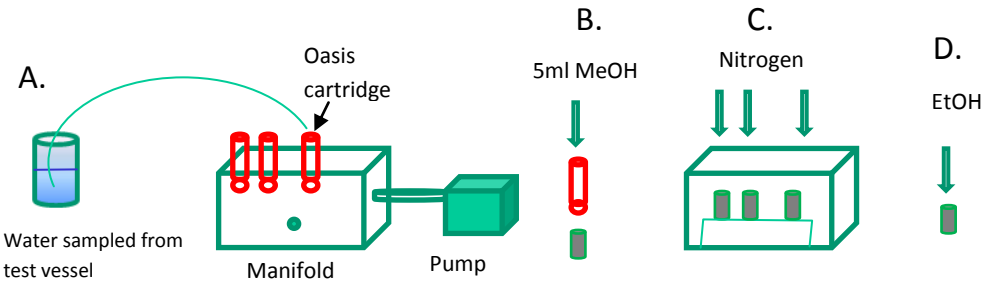

Figure 8. Illustration showing water sample preparations. A; the solution goes through a conditioned cartridge placed on a Manifold linked to a pump. B; residues are eluted in 5ml methanol (MeOH). C; MeOH is dried with nitrogen.

2.1.8 Radioimmunoassay

Aliquots of 100 µl of conditioned tank water extract (section 2.1.7.) were dried in a centrifugal evaporator Gyro Vap (Howe, UK), and were resuspended in 200 µl BSA Buffer (50 mM Na_2HPO_4, 26 mM KH_2PO_4, 150 mM NaCl, 2.5 mM Thimerosal, and 0.015 mM BSA). 100 µl of EE_2 antibody (Schering, AG Berlin, *Germany*, diluted at 1:40,000 in BSA Buffer) and 100 µl of radiolabelled EE_2 were added before the tubes were incubated for 24 hours at 4˚C. Radiolabelled EE_2 radioactivity was approximately 5000 DPM/100 µl, and it was diluted in PBSM Buffer (61 mM Na_2HPO_4, 45 mM KH_2PO_4, 150 mM NaCl, and 2.5 mMThimerosal). 100 µl of 0.5%gelatine type A from porcine skin (in PBSM Buffer) and 200µl dextran-coated charcoal (0.5% activated charcoal and 0.05% dextran in BSA Buffer) were then added to the tubes and incubated on ice for 10 min. Tubes were spun at 2500 x g for 15 min and the bound fraction was transferred into scintillation vials containing 4 ml scintillation fluid (Gold Star, Meridian). Radioactivity in the tube was counted for 5 min with a β-counter. Actual EE_2 concentrations were calculated by comparing results to a standard curve made from known concentrations of EE_2 (Sigma, *UK*) ranging from 0.01ng/ml up to 10ng/ml.

2.1.9 Sampling regime

One week after the start of the experiment five larvae were sub-sampled from each tank in order to reduce stocking density to compensate for increased biological loading with larval growth (resulting in n=25). Subsampling at this stage was not random, as this reduction in stocking density was used as an opportunity to remove

the larvae most affected by curvature of spine, as the small number of severely affected individuals may not have survived larval phase due to compromised swimming/equilibrium and feeding activity. The sub-sampled larvae were anaesthetized in MS-222 (Ethyl 3-aminobenzoate methanesulfonate salt, Sigma-Aldrich, UK) at 2g/L and buffered with $NaHCO_3$ to ph7. Euthanized larvae were subsequently weighed and fixed in Bouin's solution for 24H before being transferred into 70% IMS. When the remaining surviving *Silurana tropicalis* larvae completed metamorphosis (NF stage 66), the froglets were either sampled or transferred into larger tanks for grow-out (with no exposure to EE_2). Froglets belonging to the two replicates from each pair of each treatment group were regrouped in single tanks, which reduced the number of replicates by half for each treatment group. For instance, in the 10ng/L, 50ng/L, 100ng/L and 500ng/L treatment groups, the tank "pair A at 25˚C" were pooled with the tank "pair A at 28˚C". In the control group, 4 tanks were kept and frogs were sorted by sizes (Control pair A large froglets/Control pair A small froglets/Control pair B large froglets/Control pair B small froglets) in order to reduce risks of cannibalism. In the 3000ng/L, froglets were transferred into larger tanks. The juvenile frogs were kept on a flow-through system at 25˚C, fed everyday with blood worms and every second day with *Xenopus* pellets. The bottom of the tanks was siphoned and the water partially changed every 3 days. After six months, the feeding regime was decreased to three times a week.

Froglets sampled at metamorphosis were weighed, and sexed according to gross gonadal morphology. Gonads, still attached to the kidneys, were removed, fixed in Bouin's fixative for 4 h, rinsed in tap water and finally transferred into 70% IMS.

The froglet carcasses were stored in 10% formalin. Snout vent and hind limb lengths of the frogs were measured using a digital calliper.

2.1.10 Histology of the gonads of Silurana tropicalis at NF stage 66

Fixed gonads were dehydrated overnight through a series of increasing IMS (Industrial Methylated Spirit) concentrations followed by three histoclear bath, and finally immersed in hot paraffin (Appendix 1). Gonads were then embedded singly into paraffin wax blocks and serial-sectioned with a rotating microtome at 5µm. Sections were briefly transferred into a hot water bath and then placed onto histology glass slides. Slides were stained with haemotoxylin and eosin (Appendix 1) and mounted using histomount. All sections were examined under a microscope and froglets were sexed as females after observation of an ovarian cavity (Figure 9).

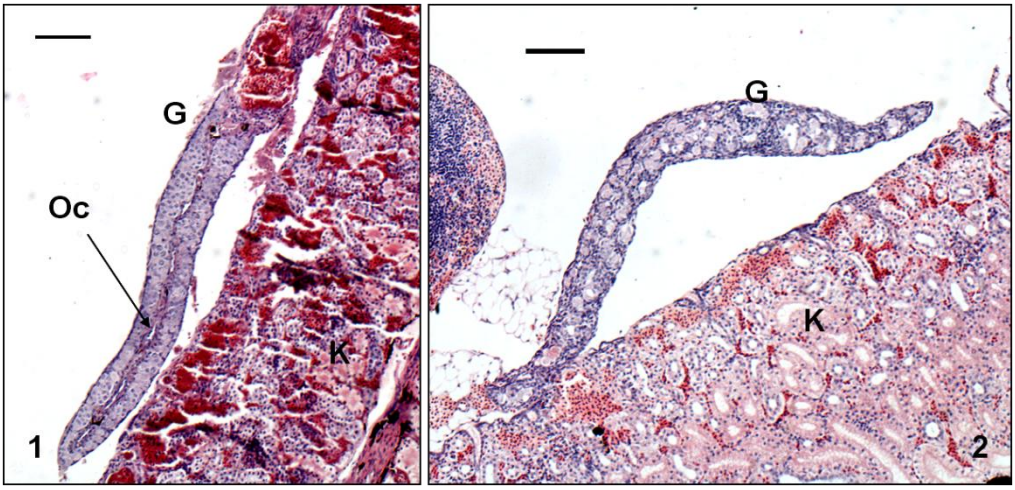

Figure 9. Histological section of *Silurana tropicalis* gonads at NF stage 66 stained with haemotoxylin and eosin. 1; Gonad of a female with an ovarian cavity. 2: Immature gonad presenting a male structure. G: Gonad. Oc: Ovarian cavity. K: Kidney. Bar represents 100µm.

2.1.11 Statistics

The statistical software SPSS v. 15 for Windows (Chicago, *U.S.A*) was used for all data analysis. Sex ratios were compared using the Chi-squared test, and the expected proportions were taken from the controls. Time to completion of metamorphosis was compared using the log rank test, and the non parametric test Kruskall-Wallis, followed by the post-hoc Mann-Whitney test. Data on the mortality were compared using the log rank test. Wet weights, snout vent lengths, and hind-limb lengths were compared using ANOVA and Dunnet's t-test as post-test if the data passed the Kolmogorov-Smirnov normality test.

2.2 ***Reproductive potential of sexually mature Silurana tropicalis***

Adult males and females were sampled 24 months and 26 months after completion of metamorphosis respectively. Adult males were sampled on seven different days. 17 males were sacrificed on a first sampling, and the remaining males were sacrificed on six different sampling days with only 6 males terminated per day. Sperm motility analyses were done at the Zoological Society of London (London, *UK*) on the males sampled on the six last sampling days. Males sampled belonged to the control group and EE_2 treated groups on each sampling day. Female frogs were sampled on 7 different days and 15 to 20 frogs from different treatment groups were sampled each time.

2.2.1 Termination and dissection of adult frogs

Adult male frogs were euthanized by an anaesthesis MS-222 (2g/L buffered at ph7 with $NaHCO_3$) until complete cessation of movements followed by destruction of the brain-sterm by pithing. Excess moisture was removed with tissue prior to weighing the frogs and measuring snout-vent length using digital callipers. Blood was collected from the heart in Sodium-heparinised haematocrit capillary tubes (Hawkley's and Son's, *UK*). Blood samples were transferred into microfuge tubes, stored on ice until the end of the sampling (less than 4 hours) and spun at 4000 rpm for 5 min at 4ºC. The plasma supernatant was collected and stored at -80ºC until further analysis. Livers and fat bodies were dissected out and weighed. Testes of the males were dissected, and weighed. The left testis was used to analyze the quality of the sperm (sperm density, sperm viability and sperm motility) whereas the right testis was fixed in Bouin's for further histological analysis. The forearms of the males were cut off and photographed under a stereo dissecting microscope to enable counting of the density of keratinized epidermal hooks per mm^2 of skin. Ovaries and oviducts were dissected from females and weighed.

2.2.2 Plasma hormones analysis

Frozen plasma samples were defrosted at ambient temperature and the volume of plasma was assessed for each sample with a pipette. Samples were then diluted in ethyl acetate (1/10 dilution) in polypropylene tubes (Luckham). Tubes were shaken manually and spun at 2500 g for 2 min to separate the two solvent phases. The volume of supernatant needed for the RIA was pipetted and transferred into tubes

placed in a Gyro Vap for 30 min or until complete evaporation of the solvent. Tubes containing plasma and ethyl acetate were stored at -20˚C, and were spun again the next time ethyl acetate was pipette for a different assay. Steroids concentrations were measured by radioimmunoassay (section 2.1.8). The volume of ethyl acetate in each sample was sufficient for multiple radioimmunoassays. We measured testosterone and estradiol concentrations in females and males frogs as well as progesterone in females. Dried extracted plasma samples were resuspended in 100 µl SAB (Steroid Assay Buffer). One litre of SAB was prepared with 0.05M PBS (Oxoid), 1g of gelatin (type A from porcine skin, Sigma) and 1g of Sodium azide. The resuspended samples were then incubated with 100 µl of specific antibody and 100 µl of specific radiolabelled hormone for 24 hours. Testosterone antibody was used at a 1:50 000 dilution, oestradiol at 1:40 000, and progesterone at 1:4000 (AbDSerotec, Morphosys AG, Germany). Radiolabelled hormones were diluted to achieve 5,000-10,000 DPM (Disintegrations Per Minute) per 100 µl (2,4,6,7-^{3}H oestradiol, 1,2,6,7 -^{3}H testosterone, Amersham International; 1,2,6,7-^{3}H progesterone, Perkin Elmer, Massachusetts, USA). The mixture was then incubated for 10 min with dextran-coated charcoal (0.5% activated carbon and 0.05% dextran in SAB). Tubes were then spun at 2500 g for 15 min and the bound fraction was transferred into scintillation vials containing 4 ml scintillation fluid. Radioactivity in the tube was counted for 5 min with a β-counter. Intrassays coefficients of variation were respectively 4.7% for estradiol, 14.3% for testosterone, and 26.5% for progesterone.

2.2.3 Measuring density of keratinized epidermal hooks

The forearms were positioned under a microscope (x10 magnification) linked to a camera. A nylon thread of a known diameter was placed on top of the forearms and used as a scale for the image analysis (

Figure 10). A picture of the wrist and the second finger (index) of each arm were taken and the number of epidermal hooks per mm^2 was subsequently calculated using the software Imagetool version 2.00 (The University of Texas Health Center in San Antonio, *U.S.A*).

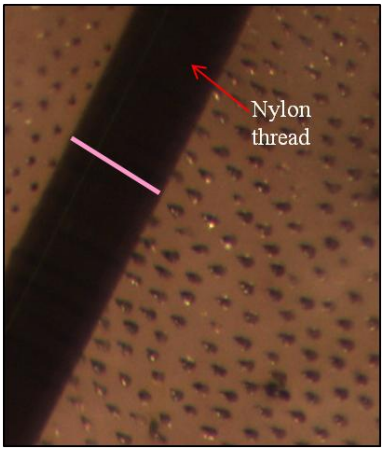

Figure 10. Picture of the ventral side of a sexually mature male *Silurana tropicalis* forearm (nuptial pad) at x10 magnification. A nylon thread (0.13mm diameter) was

used as a scale to calculate a small area on the nuptial pad using the software Imagetool and the number of epidermal hooks per square millimeter was subsequently assessed. Pink bar represents 0.13mm.

2.2.4 Testis macerate preparation

The left testis was dissected out under a dissecting microscope, and trimmed to remove vestiges of fat body and blood vessels. The testis was transferred into a De Boer's solution (110 M NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2$, buffered to ph = 7.2 with $NaHCO_3$). 15µl of media was added per mg of tissue. The testes were macerated manually with disposable pestles and the resulting macerate was then filtered through a 70 µm cell strainer. Samples were kept on ice slurry until further analysis.

2.2.5 Sperm density and viability

<u>Sperm density</u>

A 10 µl aliquot of testis macerate was allowed to flood a chamber of a Neubauer haemocytometer and the sperm cells were counted in 5 squares of the central grid. The numbers of sperm per ml in the testis macerates were found by multiplying the number of sperm counted by 5 x 10^4. Sperm density was expressed as counts $x10^5$ cells per gram of testis.

<u>Sperm viability</u>

Testis macerate (2 µL) was incubated with an equal volume of the live/dead stains, SYBR-14 and propidium iodide (10 µL of a 20 µM stock solution and 1µL of a 2.4 mM stock solution diluted in 1mL PBS, respectively (Invitrogen, UK)), for 10 min at room temperature. Stained spermatozoa were transferred to a slide, a coverslip was placed on top and 200 spermatozoa were classified as live (green) or dead (red) using fluorescence microscope (Olympus IX70; Olympus UK.) (Figure 11)

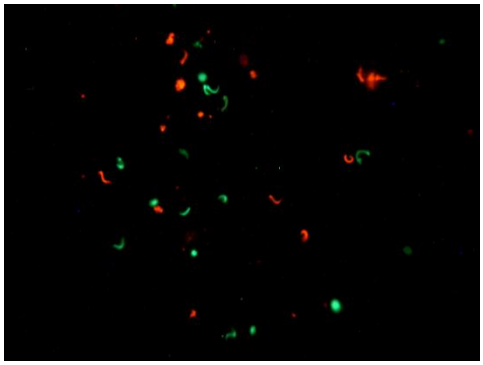

Figure 11. Picture of stained *Silurana tropicalis* spermatozoa. Spermatozoa with intact membranes show green fluorescence whereas spermatozoa with disrupted membranes show red nuclear fluorescence

2.2.6 Sperm motility analysis

Sperm activation

Testis macerate (5 µl) was diluted with 15 µl distilled water to induce motility. A 1 µl aliquot of activated sperm was immediately placed onto a single well of a 12 well multi-test slide (ICN Biomedicals Inc.), and covered by a coverslip. Sperm motility was recorded on recordable compact discs for 3 min at x10 magnification. Four

tracks were made per male and only the tracks showing no signs of cell drift were analyzed.

Sperm motility analysis

The percentage of motile sperm was assessed manually by counting the number of progressively motile sperm, the number of motile sperm, and the sperm not moving within the first 10 sec of each track. Tracks were analyzed afterwards using the Hobson Sperm Tracker (Hobson Vision Systems, Sheffield, *UK*) with settings adjusted to fit amphibian's sperm swimming pattern. The minimum and maximum tracking time were respectively 3 and 9 seconds. The Hobson tracker analyzed 5 parameters; VCL (Curvilinear velocity expressed in µm/seconds), which represents the sum of incremental distances moved along a path for each time frame divided by the time taken for the spermatozoa to cover that trajectory; VSL (Straight line velocity in µm/seconds), the straight line distance between the start and end of the sampled path divided by the total track time; VAP (Average path velocity expressed in µm/seconds), which is the velocity over a calculated smooth path; LIN (Linearity expressed as a percentage), is the ratio of the straight-line distance between the start and end points of the track divided by the actual track length and STR (Straightness expressed in percentage), which described the straightness of the average path (i.e., VSL divided by VAP).

2.2.7 Histological analysis of the gonads

The right testis was placed in Bouin's fixative for 12 to 15 hours, rinsed in tap water and transferred in 70% IMS. Testes were embedded in hot paraffin using the method previously described in paragraph 2.1.10. A section every 150 µm was placed onto glass slides and stained with haematoxylin and eosin. 4 sections per males were analyzed overall. All sections were screened for testicular oocytes.

2.2.8 Oocyte maturity assessment

Ovaries dissected from the left side of females were placed in Petri dishes with some Hanks balanced solution (HBSS) (Ca^{2+} -free, phenol red-free Hanks balanced salts diluted in 1.3 L distilled water, supplemented with Polyvinylpyrrolidone (PVP, 1 g/L), and buffered at ph=7.6 with NaOH), and the oocytes sacs were counted. The sac located in the middle was removed, weighed, rinsed in HBSS and transferred in a second Petri dish with some fresh media. The dish was left overnight at 4˚C. The next day the sacs were placed in 15 ml centrifuge tubes with 10 ml HBSS supplemented with 0.2 % collagenase (Type II, Worthington Biochemical Corporation) and shaken for 45 min at 23 ˚C. The freed oocytes where then rinsed 4 times in HBSS, and placed in multi-well plates under a dissecting microscope where the number of vitellogenic and pre-vitellogenic oocytes was assessed.

2.2.9 Statistical analysis

Statistical analysis were done using the software SPSS v.15, STATISTICA v. 7 for Windows (Statsoft, Tulsa, Oklahoma, *U.S.A*), and PATN (original DOS version, Blatant Fabrications Pty Ltd, *Australia*). Sex ratios were compared using the Chi-squared test. Morphometric data, sperm density, density of epidermal hooks, and oocytes maturity were analysed with ANOVA and a Dunnet's t-test as post-test if the data passed the Kolmogorov-Smirnov normality test. General Linear Model (GLM) analyses were used to search for the impact of the differences on days and time of sampling on the hormones and sperm motility analysis. Sperm motility data were analyzed using ANOVA but also using Nested design ANOVA where the factor "sperm" was nested within the factor "individual". Subpopulations of sperm where defined using a cluster analysis on PATN. All data generated by the Hobson sperm tracker (VCL, VSL, VAP, LIN and STR) were used in PATN to define sperm subpopulations through a complex cluster analysis. The impact of the replicates for different endpoints was assessed using GLM. Correlations between variables were established using Pearson's test. Non parametric data were analyzed with Kruskall-Wallis test, followed by the Mann-Whitney post-hoc test.

3 . Results

3.1 *Silurana tropicalis larval exposure to EE_2*

3.1.1 Water parameters

The water temperature and the DO of each tank vessel were measured seven times throughout the exposure study. Differences in water temperature observed among some treatment groups are due to the location of those tanks in the exposure room (Table 2).

Treatment group	Temp 25˚C	Temp 28˚C	DO (mg/L)
Control	25.57 (0.13)	27.8 (0.12)	6.91 (0.33)
10 ng/L	25.24 (0.18)	26.78 (0.14)	6.21 (0.41)
50 ng/L	25.58 (0.13)	27.62 (0.2)	6.34 (0.46)
100 ng/L	25.31 (0.15)	27.08 (0.1)	6.49 (0.49)
500 ng/l	25.32 (0.13)	27.2 (0.13)	6.71 (0.46)
3000 ng/L	24.55 (0.19)		6.57 (0.56)

Table 2. Mean water temperature (˚C) in replicate tanks set at 25˚C and 28˚C for each treatment group and mean dissolved oxygen (ppm) in tanks for each treatment group during the exposure of *Silurana tropicalis* larvae to EE_2 (nominal doses). Number in brackets represents ±1 SEM.

3.1.2 EE_2 concentrations in tanks

Dilution factors in the mixing chambers remained close to 1000 all the way through the exposure study which indicate that the EE_2 concentrations in the tank vessels should have remained fairly constant (Figure 12). Although water samples were taken every week, for some unforeseen reasons, only the water samples taken on day

seven of the exposure study could be analyzed at the end of the study, in order to check EE_2 concentrations in the tank vessels. According to the results, it seems the control group had been contaminated by some EE_2 (Table 3

Table 3. EE_2 concentrations in tank vessels (in ng/L) and stock bottles (in µg/L) on the seventh day of *Silurana tropicalis* larval exposure to EE_2.

), although it is not possible to say if the contamination happened in the exposure tank or somewhere during the sample preparation for the RIA. Further findings resulting from the frogs sampling suggest the contamination occurred during the water sample preparation but this remains a hypothesis since it was not possible to replicate the analysis (see chapter 4 for further explanations). Replicate water samples were not taken during the exposure study thus making it impossible to verify the accuracy of those results.

	[EE_2] in tanks (ng/L)	[EE_2] in stock bottles µg/L
Control	37.9	Not tested
10 ng/L	3.36	3.8
50 ng/L	7.4	12.8
100 ng/L	40.9	29.8
500 ng/L	293	547
3000 ng/L	5202	5813

Table 3. EE_2 concentrations in tank vessels (in ng/L) and stock bottles (in µg/L) on the seventh day of *Silurana tropicalis* larval exposure to EE_2.

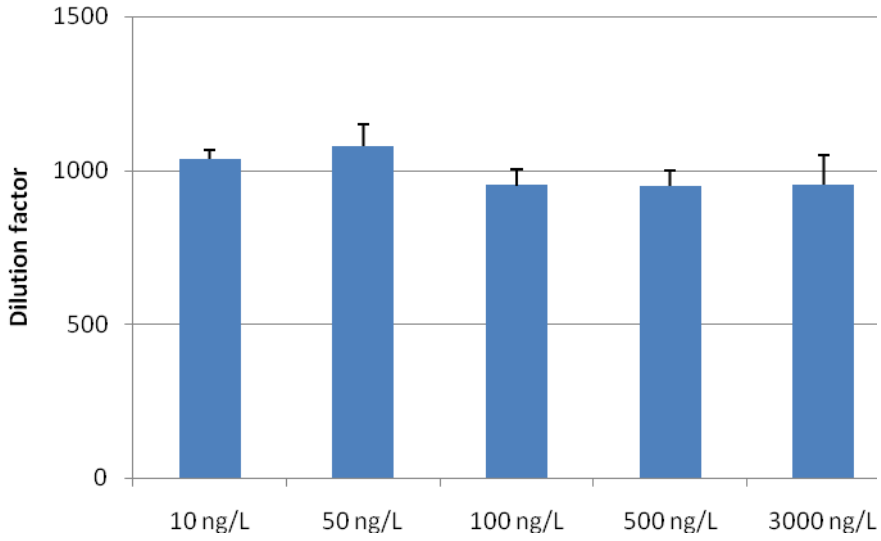

Figure 12. Mean of dilution factors weekly calculated between EE_2 stock and dechlorinated tap water in the mixing chamber for each treatment group during *Silurana tropicalis* larval exposure to EE_2. Vertical bars represent ±1 SEM.

3.1.3 Tadpole mortality

Although some significant differences were found among treatment groups (at 100 and 500 ng/L), this was due to the abrupt death of a large number of tadpoles in one of their replicate tanks (Figure 13). Larvae from pair A had a higher mortality rate than larvae from pair B (33% and 19% respectively) (Figure 14).

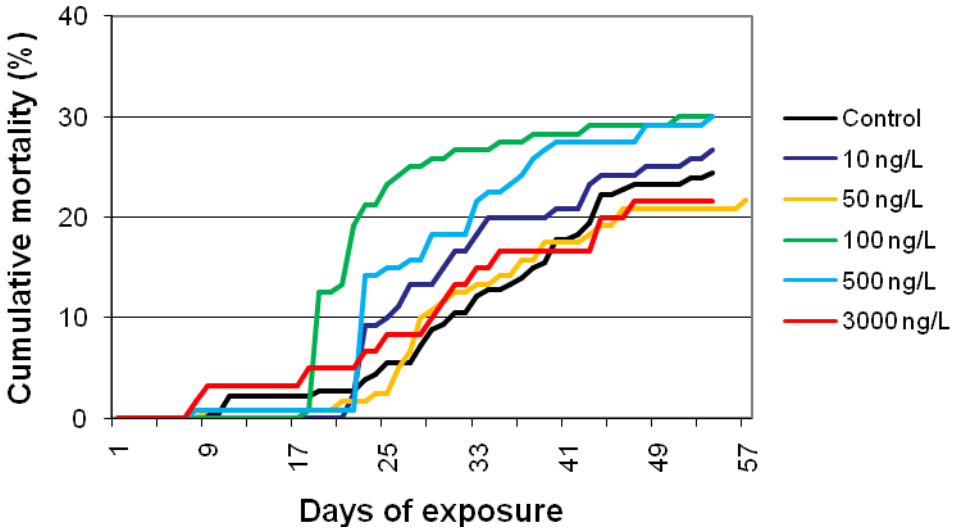

Figure 13. Cumulative % mortality of *Silurana tropicalis* exposed to EE_2 (presented as nominal doses in ng/L). Data represents the mean of all individuals from Pair A and Pair B at both 25°C and 28°C.

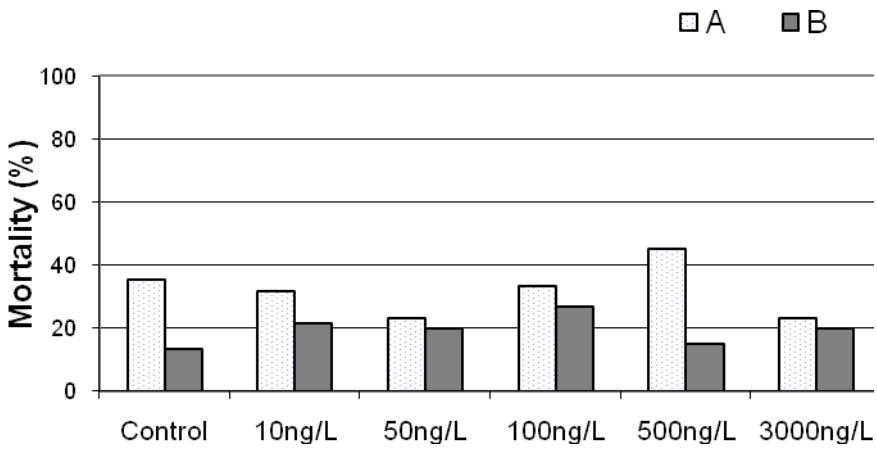

Figure 14. Percentage mortality of *Silurana tropicalis* larvae from Pair A and B following exposure to EE_2 (nominal doses in ng/L). Offspring have been pooled irrespective of rearing temperature (at 25˚C and 28˚C).

3.1.4 Time to completion of metamorphosis

Significant differences in time to complete metamorphosis were found among the different treatment groups (Log rank test; $p<0.05$, pairs and temperatures pooled). Tadpoles belonging to the control group metamorphosed faster than tadpoles exposed to EE_2 (Figure 15 and Figure 18). In addition, a higher water temperature in tanks seemed to accelerate metamorphosis (In average tadpoles from the control groups held at 28˚C metamorphosed 6 days earlier than the ones at 25˚C). At 28˚C the differences among treatment groups were bigger than at 25˚C (Figure 16, Figure 17).

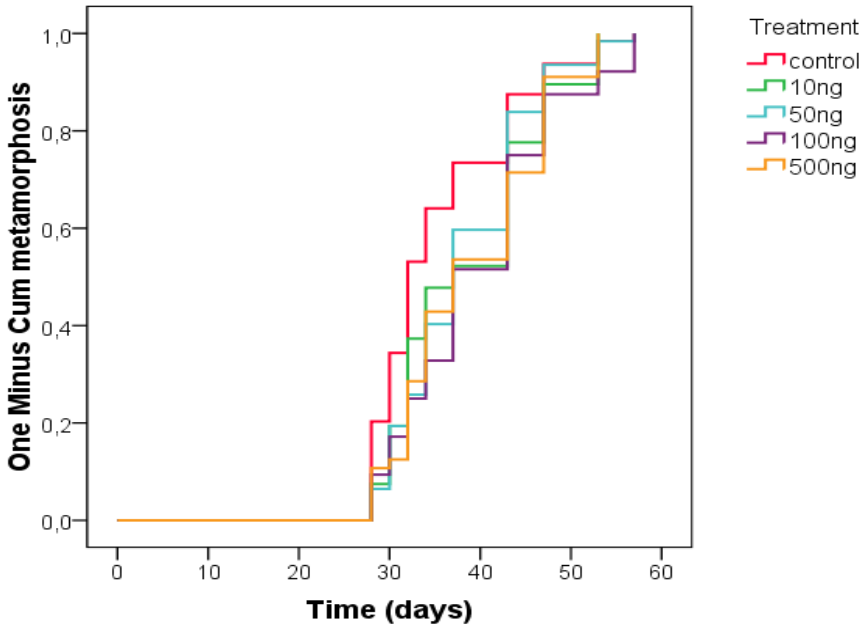

Figure 15. Kaplan-Meier curves, showing cumulative proportion of *Silurana tropicalis* larvae in each treatment group (nominal doses of EE_2 in ng/L) completing metamorphosis through the course of the study. Data from each treatment represent pooled individuals from pair A, B reared at 25˚C and 28˚C. Significant differences were found among the different treatment groups, Log rank test; $p<0.05$.

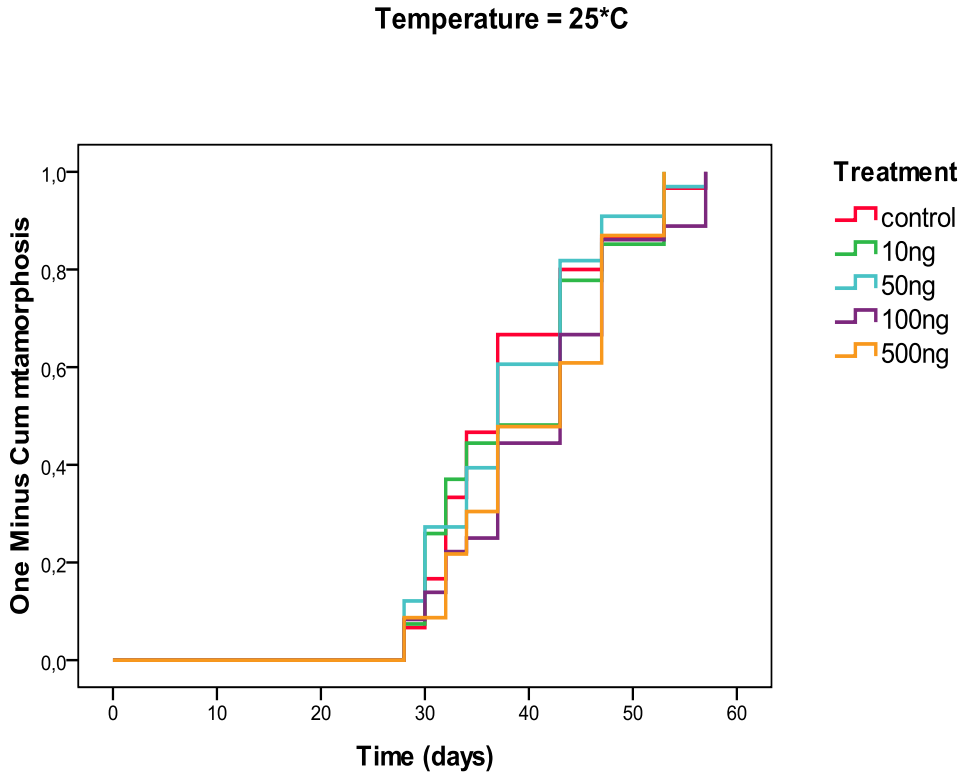

Figure 16. Kaplan-Meier curves, showing cumulative proportion of *Silurana tropicalis* larvae reared at 25C in each treatment group (nominal doses of EE_2 in ng/L) completing metamorphosis through the course of the study. Data from each treatment represent pooled individuals from pair A and B.

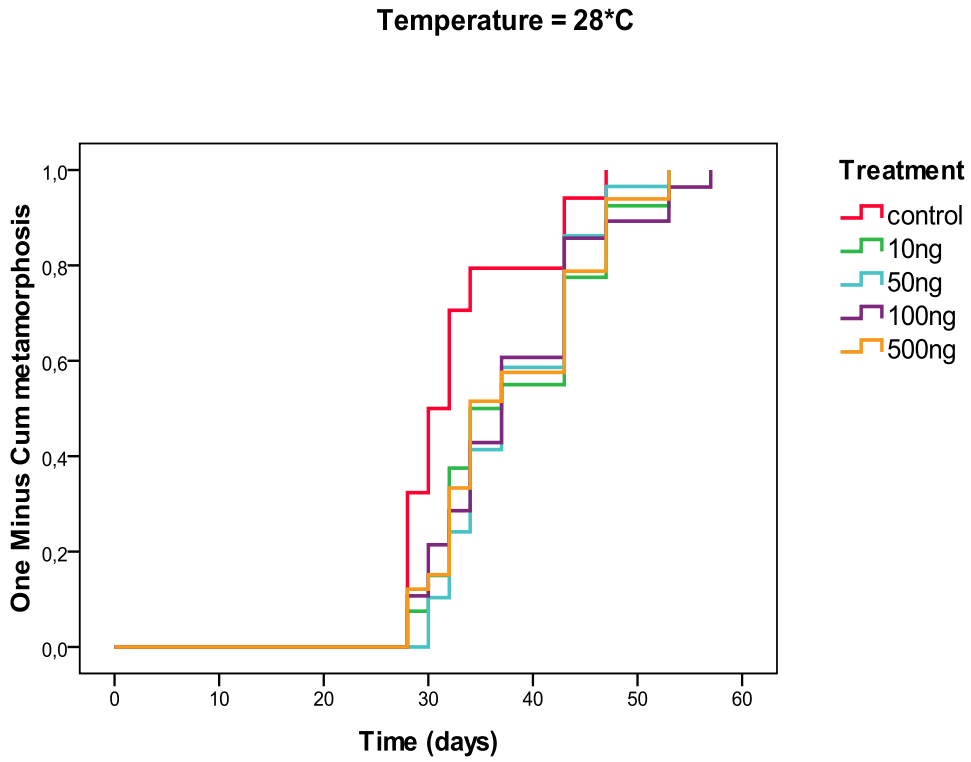

Figure 17. Kaplan-Meier curves, showing cumulative proportion of *Silurana tropicalis* larvae reared at 25C in each treatment group (nominal doses of EE_2 in ng/L) completing metamorphosis through the course of the study. Data from each treatment represent pooled individuals from pair A and B. Significant differences were found among the different treatment groups, Log rank test; $p<0.05$.

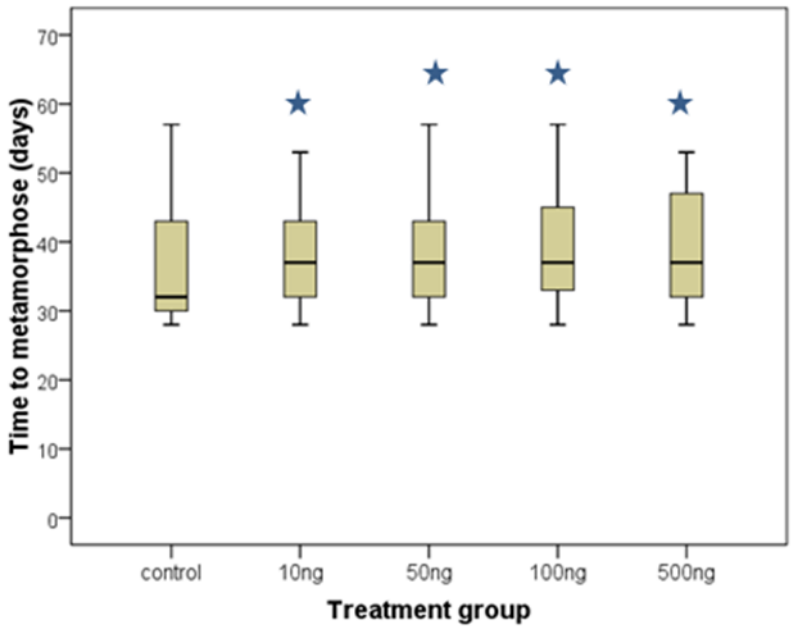

Figure 18. Time for completion of *Silurana tropicalis* metamorphosis (days) in each treatment group (pairs and temperatures pooled, nominal doses of EE_2 in ng/L). The box represent 75th and 25th percentiles, the horizontal black line shows the median. Whiskers represent highest and lowest values. Stars indicate a significant difference from the control group (Mann-Whitney; $p<0.05$).

3.1.5 Gross morphometry

Larvae that had been exposed to EE_2 were smaller than larvae from the control group at NF stage 66. EE_2 affected the froglet's weight and their hind limb length in a concentration dependent manner (Jonckheere-Trepsta, $p<0.05$) (Figure 19,**Error! Reference source not found.** Figure 20 and Figure 21). Tadpoles exposed to 100 and 500 ng/L of EE_2 had a lower condition factor (K) than tadpoles from the control

group (Figure 22 and Figure 23). Froglets that metamorphosed earlier were smaller than the one that metamorphosed later on (Figure 24). The water temperature seems to be linked with the way EE_2 affects larval growth. Indeed, EE_2 had an impact on tadpole growth in a concentration dependent manner (Joonckheere-Trepsta, $p<0.05$) at 25°C whereas it had none at 28°C (Figure 25).

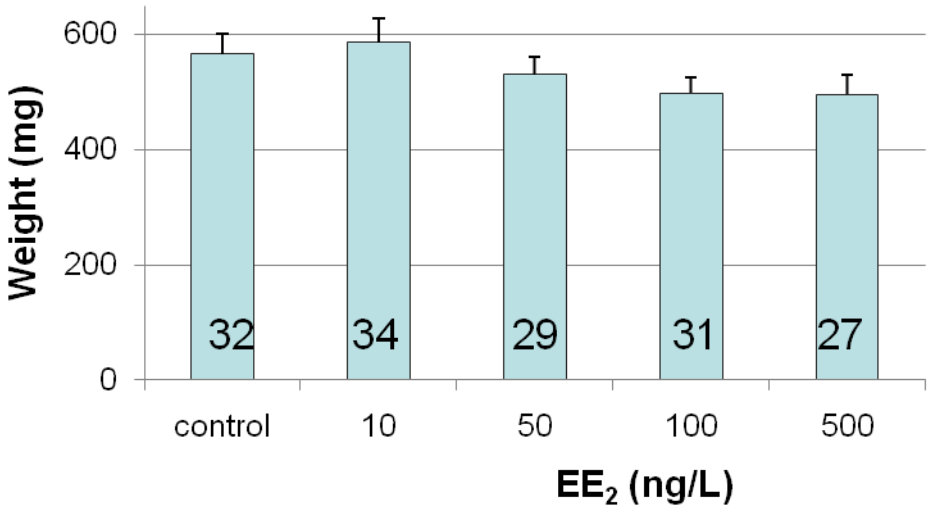

Figure 19. Mean wet weight in mg of *Silurana tropicalis* at NF stage 66 for each treatment group (nominal doses of EE_2 in ng/L). Data are the mean response of pooled individuals from pairs A and B pooled reared at both 25˚C and 28˚C. Vertical bars represent +1SEM. Samples size shown on bars for each treatment group. Joonckheere-Trepsta, $p<0.05$.

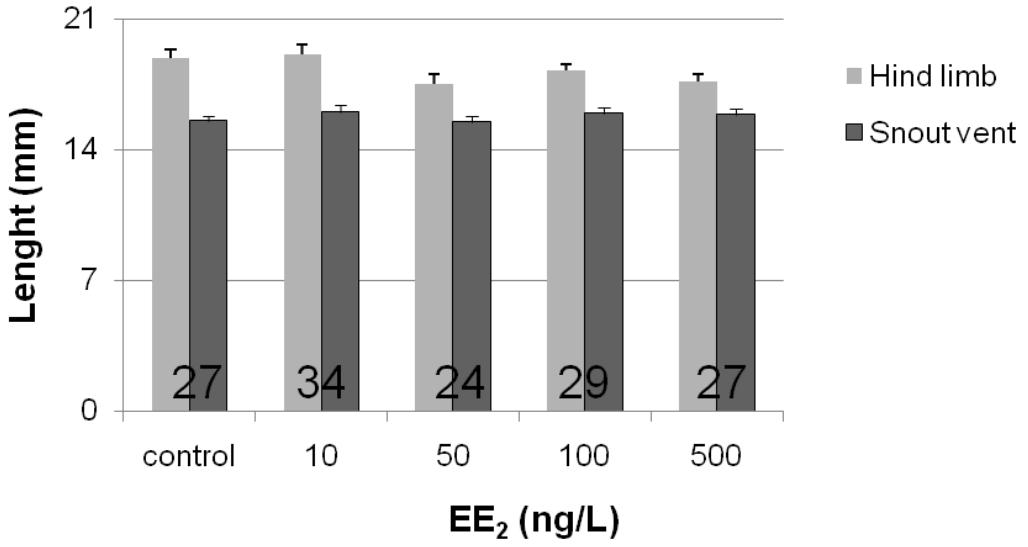

Figure 20. Mean hind-limb and snout-vent length in mm of *Silurana tropicalis* at NF stage 66 for each treatment group (nominal doses of EE_2 in ng/L). Data represent pooled individuals from Pair A and B reared at 25˚C and 28˚C. Vertical bars represents +1SEM. Sample size shown on bars for each treatment group. Joonckheere-Trepsta, $p<0.05$ for hind-limb length.

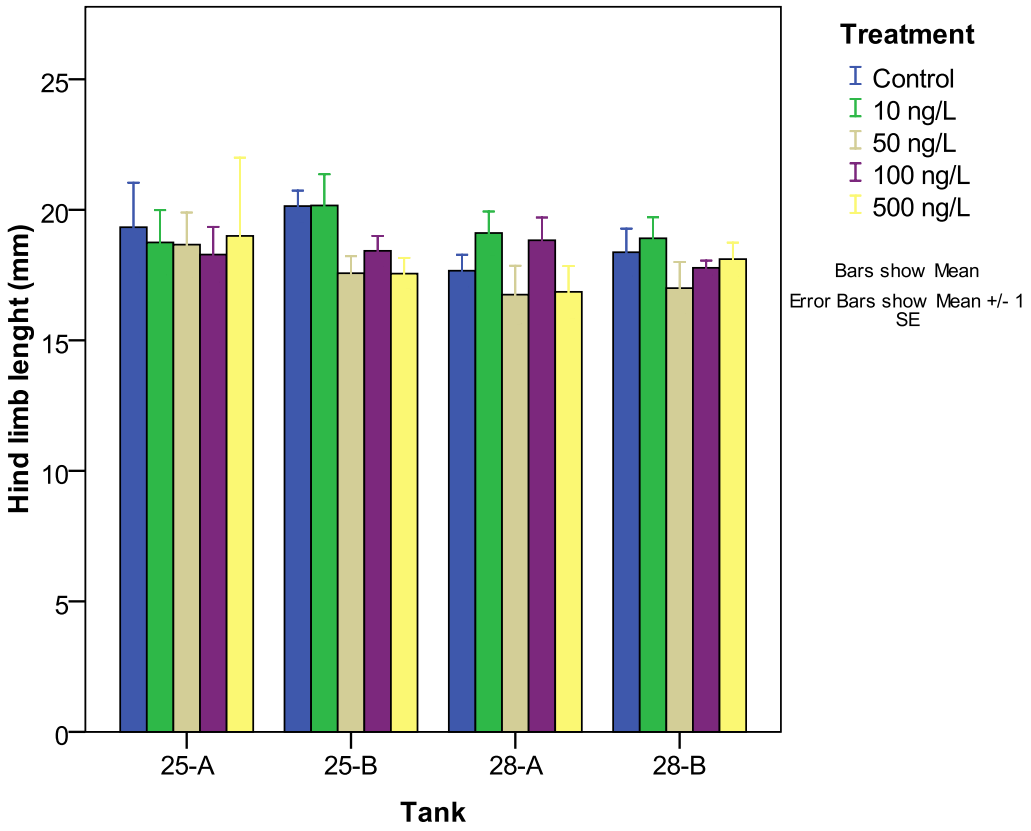

Figure 21. Mean hind-limb length in mm of *Silurana tropicalis* at NF stage 66 for each treatment group (nominal doses of EE_2 in ng/L). Vertical bars represents +1SEM. $2 \leq n \geq 11$ for each bar.

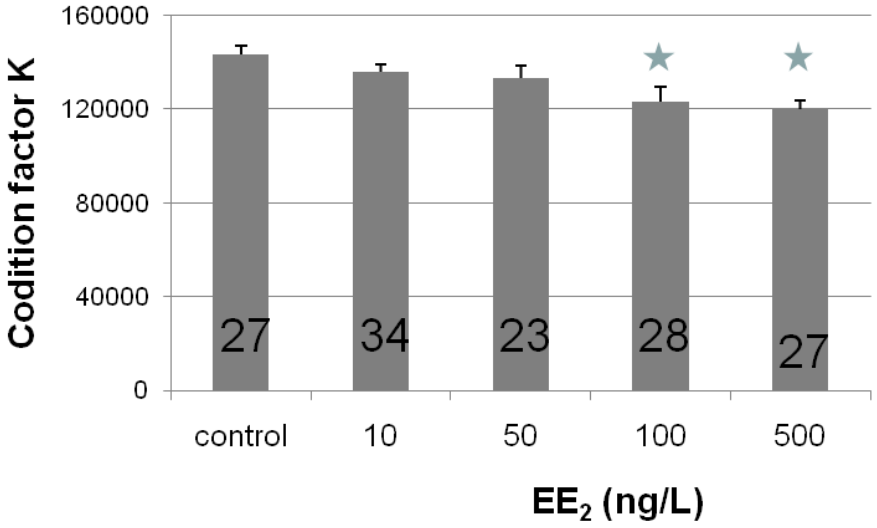

Figure 22. Mean Condition factor (+1 SEM) of *Silurana tropicalis* at NF stage 66 for each treatment group (nominal doses of EE_2 in ng/L). Data represent pooled individuals from Pair A and B reared at 25˚C and 28˚C. Vertical bars represents +1SEM. Sample size shown on bars for each treatment group. Stars above bars indicate a significant difference from the control group (Dunnett t test, $p<0.05$).

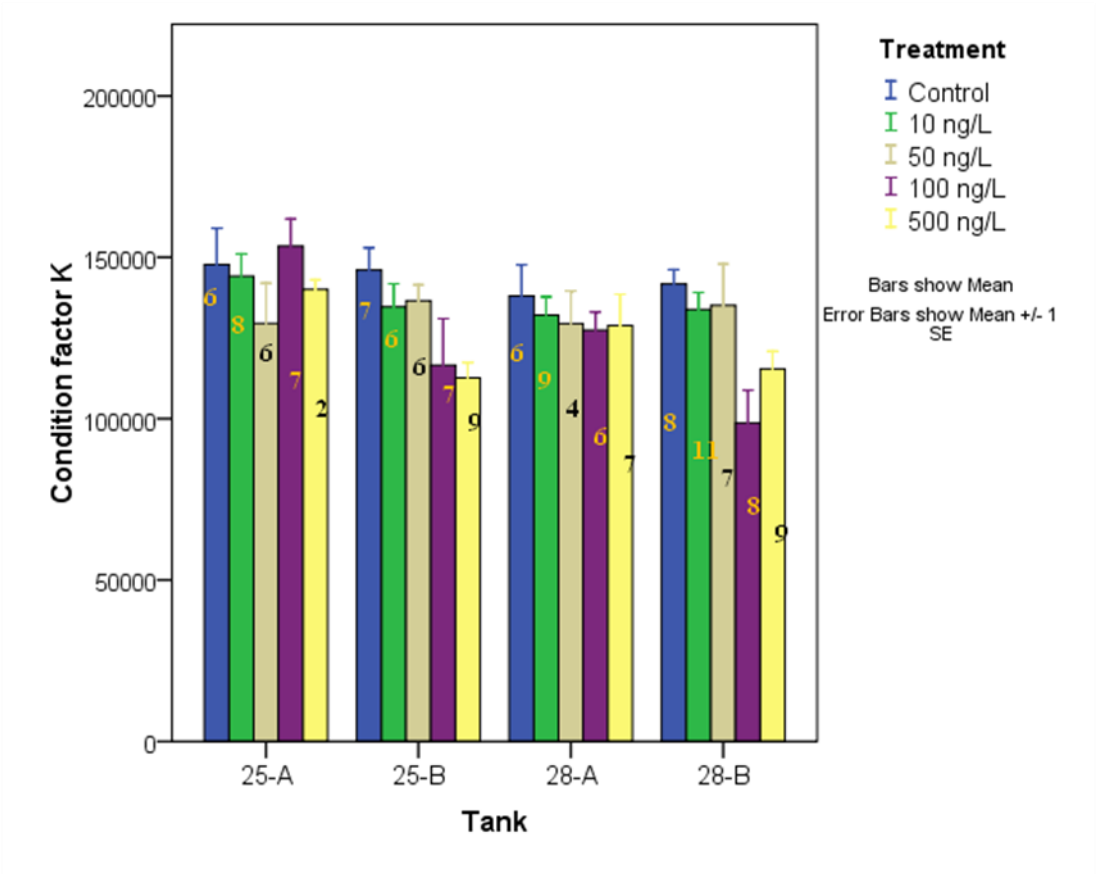

Figure 23. Mean Condition factor (+1 SEM) of *Silurana tropicalis* at NF stage 66 for each tank of each treatment group (nominal doses of EE_2 in ng/L). Sample size shown on bars for each tank. K= weight/ ((snout-vent length)^3) x10^5.

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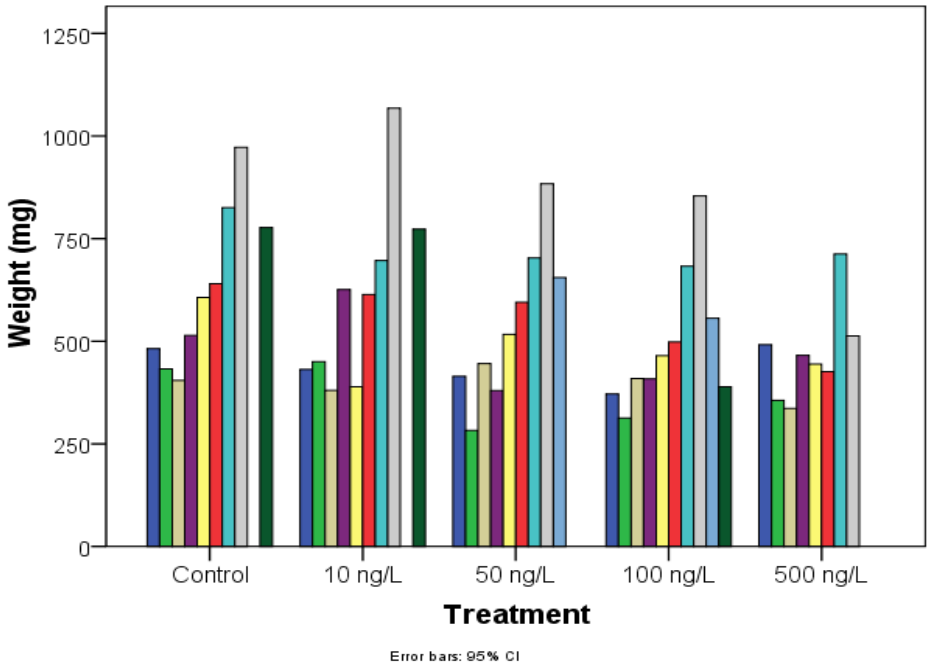

Figure 24. Mean wet weight in mg of *Silurana tropicalis* at NF stage 66 for each treatment group (Pair A, B, temperature 25˚C, 28˚C, nominal doses of EE_2 in ng/L) at different sampling dates (ascending). For each treatment group, the bar on the left end side represents the mean wet weight of the frogs that were the first to complete metamorphosis and the bar on the right end side represents the mean wet weight of the frogs that metamorphosed the latest. Error bars are not displayed. $2 \geq n \leq 6$ for all bars.

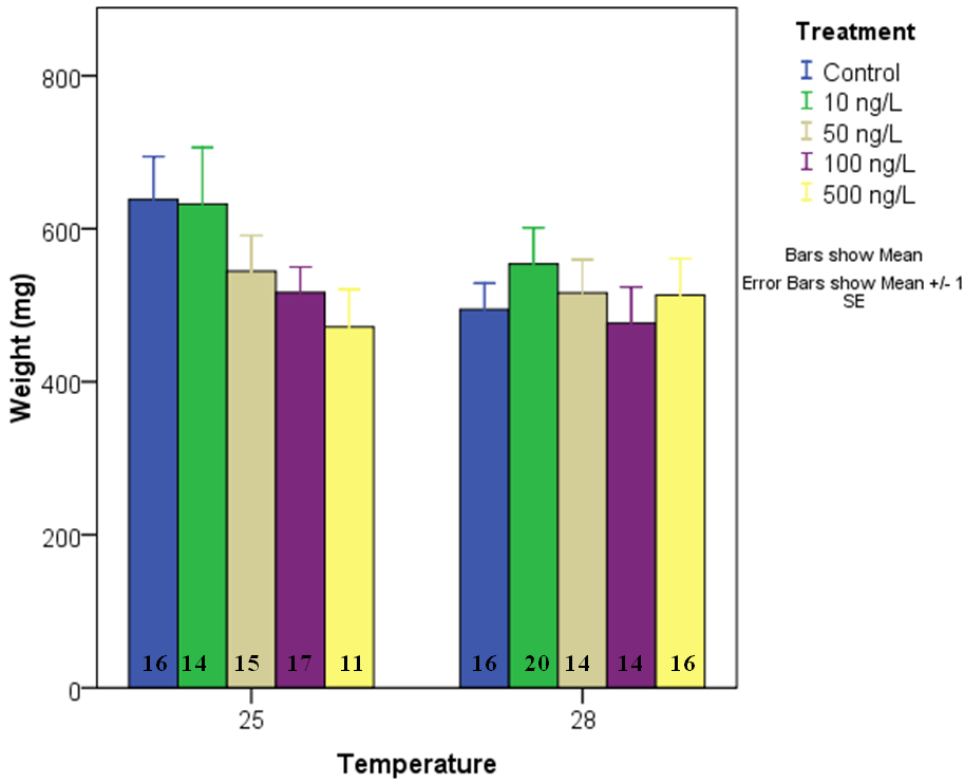

Figure 25. Mean wet weight (mg) of *Silurana tropicalis* at NF stage 66 for each treatment group (Animals from Pair A and B are pooled; nominal doses of EE_2 in ng/L) reared at 25˚C and 28˚C. Vertical bars represent +1SEM. Sample size shown on bars.

3.1.6 Sex ratio

Some of the gonads at stage 66 were not differentiated thus some of the frogs could not be sexed. Gross morphological and histological analysis of the gonads revealed that sex ratio of *Silurana tropicalis* larvae exposed to EE_2 was different from the control group (Chi square, $p<0.05$, the sex ratio of each EE_2 treatment group were compared individually to the sex ratio of the control group) (Figure 26 and Figure

27), although at this stage of the study it was not possible to accurately determine the sex ratio for each treatment group. Gross morphological analysis of the gonad provided different results than their histological analysis, suggesting that gonadal gross morphological analysis is an approximate method to assess sex ratios.

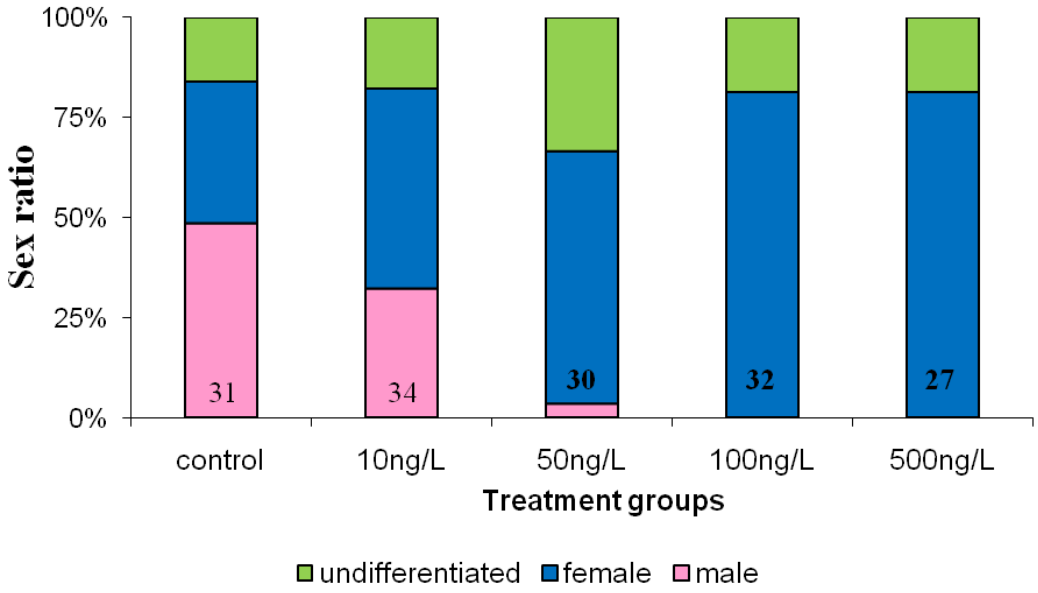

Figure 26. Sex ratio of *Silurana tropicalis* assessed at the gross morphological level at NF stage 66 after exposure to EE_2 in each treatment group (nominal doses of EE_2 in ng/L). Data represent pooled individuals from Pair A and B reared at both 25˚C and 28˚C). Sample size shown on bars for each treatment group.

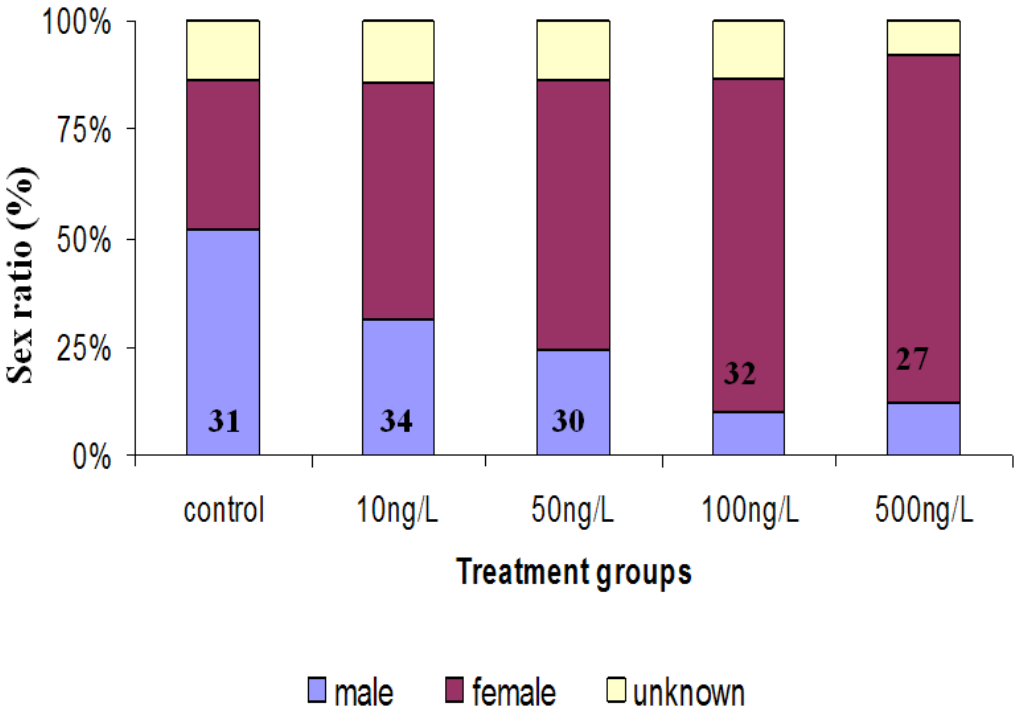

Figure 27. Sex ratio of *Silurana tropicalis* assessed after histological analysis of the gonads at NF stage 66 after exposure to EE_2 in each treatment group (nominal doses of EE_2 in ng/L). Data represent pooled individuals from Pair A and B reared at both 25˚C and 28˚C. Sample size shown on bars for each treatment group.

3.2 ***Reproductive potential of sexually mature Silurana tropicalis***

3.2.1 Sex ratio at sexual maturity

Sex ratio in the control group was not different from the 50:50 expected ratio (Chi squared, $p>0.05$). Sex ratios in the treatment groups exposed to EE_2 during larval development were statistically different from the expected 50:50 sex ratio (Chi squared, $p<0.05$, the sex ratio of each EE_2 treatment group were individually compared to 50:50) (Figure 28**Error! Reference source not found.**).

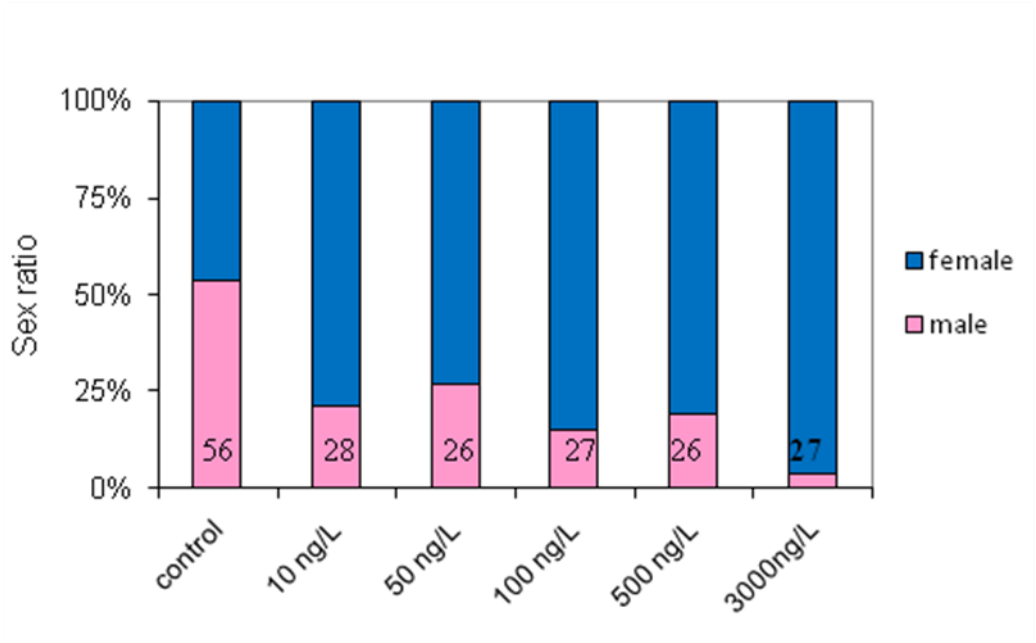

Figure 28. Sex ratio of *Silurana tropicalis* at sexual maturity in each treatment group (nominal doses of EE_2 in ng/L). Data represent pooled individuals from pair A and B. Frogs had been exposed to EE_2 during larval development. Sample size shown on bars for each treatment group. Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis.

3.2.2 Females

3.2.2.1 Gross morphometry

Gross morphometry analysis of the females revealed some differences at the lowest doses (Table 4 and Table 5). Compared to the females from the control group, the snout-vent length was significantly lower in the females from the 50ng/L group, the hepatosomatic index was significantly higher in the females from the 10 ng/L, the oviduct weight was lower in the 10 ng/L group and ovary weight was significantly

lower in the 50 ng/L group. Some females had missing oviducts, therefore only oviduct weight from females displaying both oviducts where used in the statistical analysis comparing the females oviduct weight between the different treatment groups. All females from the replicate 100 ng/L pair A accidentally drowned due to a blockage in the outflow of their tank, hence the treatment group 100ng/L hold approximately half of the number of females of the other treatment groups.

	Wet weight (mg) (± 1SEM)	Snout-vent (mm) (±1 SEM)	K (±1 SEM)
Control	22.14 (2.7)	54.1 (0.7)	140.8 (3.8)
10 ng/L	20.33 (0.6)	51.8 (0.8)	148.2 (5.5)
50 ng/L	20.16 (0.7)	51.4 (0.8)*	148.8 (4.4)
100 ng/L	21.13 (1.2)	53.7 (0.9)	135.6 (4.5)
500 ng/L	20.2 (0.8)	51.9 (0.6)	144.5 (5.5)
3000 ng/L	20.91 (0.5)	52.2 (0.6)	147.5 (3.3)

Table 4. Wet weight in mg, snout-vent length in mm and condition factor of Sexually mature female *Silurana tropicalis* that had been exposed to EE_2 during larval development for each treatment group (nominal doses of EE_2 in ng/L. Data represent pooled individuals from pair A and B. * indicate a significant difference from the control group (Dunnett t test, $p<0.05$). Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. K= weight/ ((snout-vent length) ^{3}) x10^5.

	Liver (± 1SEM)	Fat (± 1SEM)	Oviduct (± 1SEM)	Ovaries (± 1SEM)
Control	2.35 (0.05)	4.87 (0.3)	2.29 (0.1)	18.78 (0.7)
10 ng/L	2.7 (0.1)*	5.71 (0.3)	1.81 (0.1)*	16.47 (0.7)
50 ng/L	2.39 (0.08)	6.07 (0.7)	2.08 (0.2)	14.87 (1.3)*
100 ng/L	2.66 (0.1)	5.3 (0.3)	2.09 (0.2)	17.16 (1.2)
500 ng/L	2.52 (0.1)	4.09 (0.3)	2.2 (0.1)	17.24 (0.6)
3000 ng/L	2.59 (0.1)	4.89 (0.3)	2.03 (0.1)	16.45 (0.8)

Table 5. Gross morphometry of sexually mature female *Silurana tropicalis* that had been exposed to EE_2 during larval development for each treatment group (nominal doses of EE_2 in ng/L). Data represent pooled individuals from pair A and B. Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Values represents the organs weight relative to body weight. * indicates a significant difference from the control group (Dunnett t test, $p<0.05$).

3.2.2.2 Plasma hormones analysis

Although progesterone, testosterone and estradiol concentrations were strongly correlated in each animal (Pearson's, $p<0.01$), only progesterone concentrations presented some significant differences between treatment groups (Figure 29). Testosterone and estradiol concentrations in plasma were very similar (Figure 30 and Figure 31).These data are difficult to interpret because hormones concentrations were strongly correlated to the day of sampling (Pearson's $p<0.01$). For example, testosterone levels seem to follow the same trend on sampling day 1 and 6 but the levels are 4 times higher on day 6 (Figure 32).

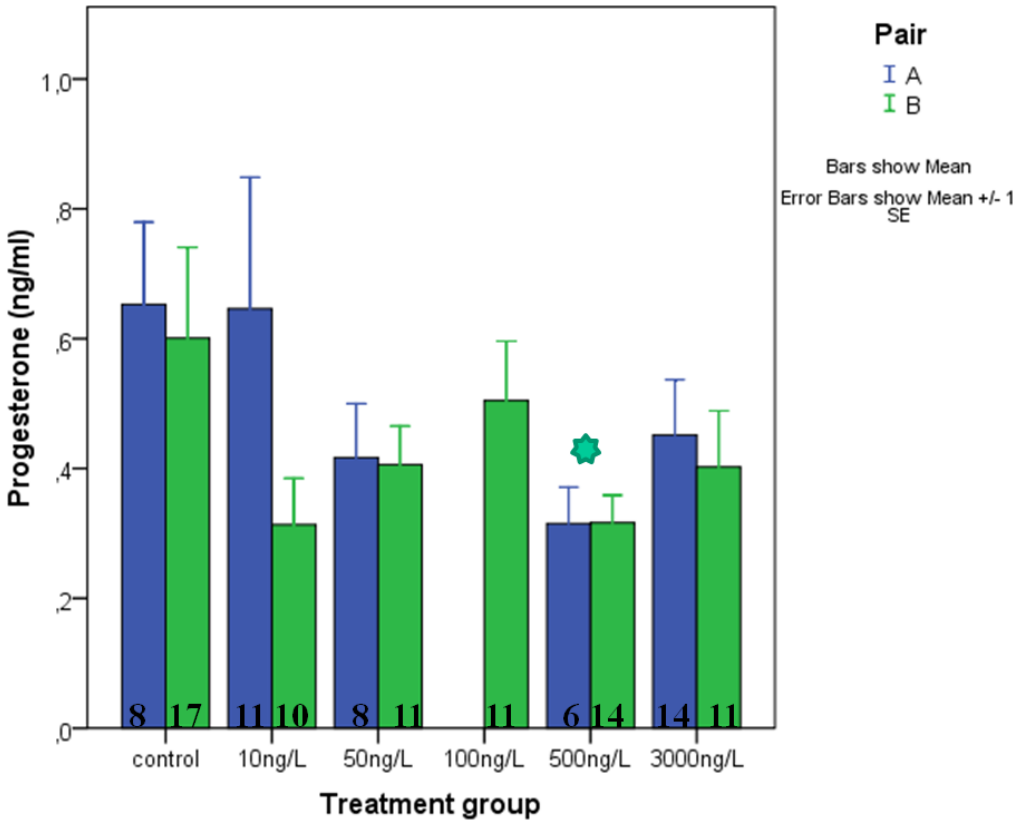

Figure 29. Progesterone concentration in ng/ml in plasma of *Silurana tropicalis* sexually mature female that had been exposed to EE_2 during larval development for each pair of each treatment group (nominal doses of EE_2 in ng/L). Vertical bars represents +1SEM. Sample size shown on bars. Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Star indicates a significant difference from the control group (Dunnett t test, $p<0.05$, data of pair A and B pooled for statistical analysis).

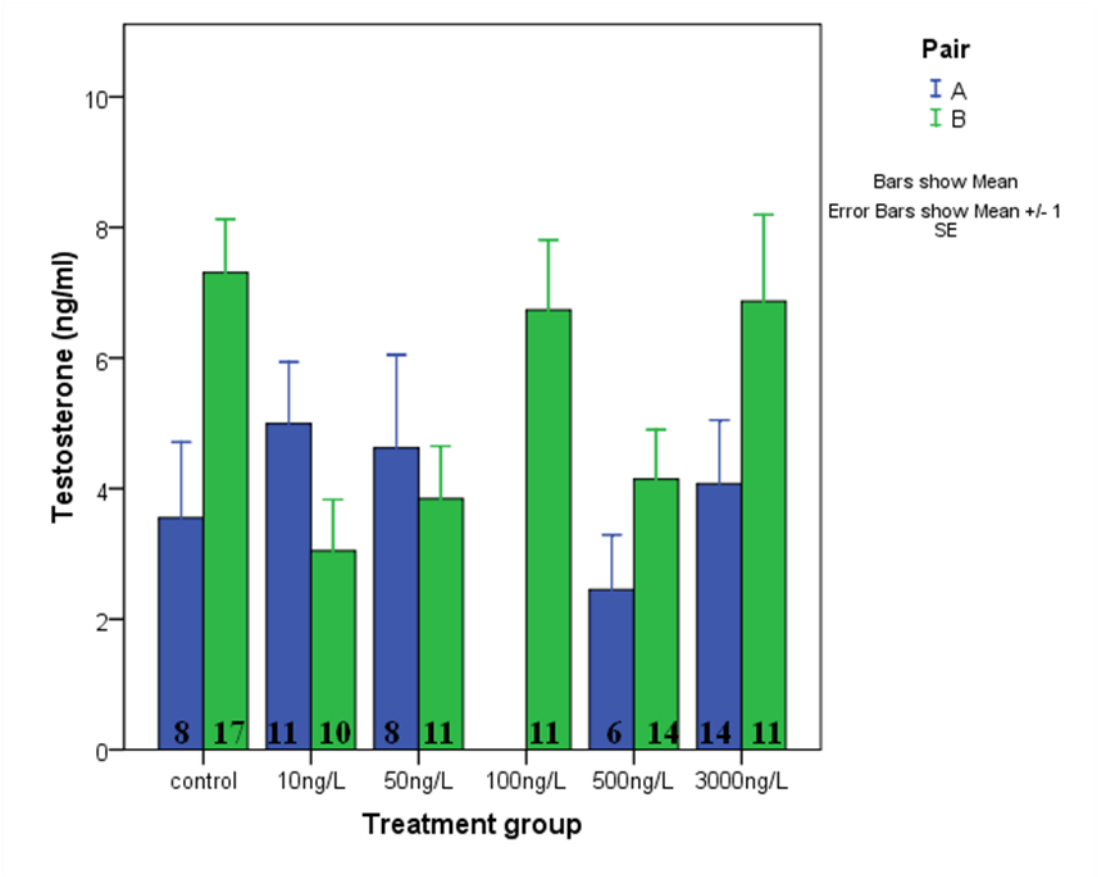

Figure 30. Testosterone plasma concentration in ng/ml in sexually mature female *Silurana tropicalis* for each pair of each treatment group. Frogs had been exposed to EE_2 during larval development (nominal doses of EE_2 in ng/L). Animals were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Vertical bars represent +1SEM. Sample size shown on bars.

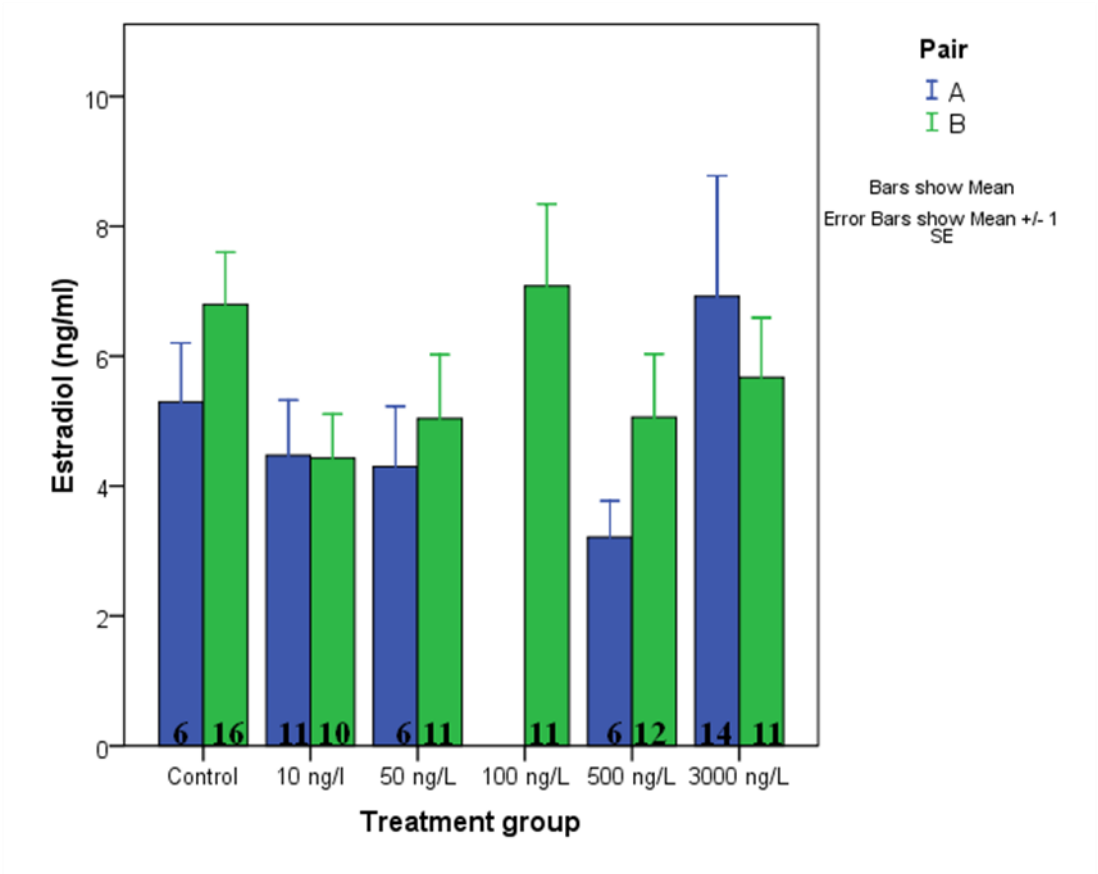

Figure 31. Estradiol concentration in ng/ml in plasma of sexually mature *Silurana tropicalis* female for each pair of each treatment group. Frogs had been exposed to EE_2 during larval development (nominal doses of EE_2 in ng/L). Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Vertical bars represent +1SEM. Sample size shown on bars.

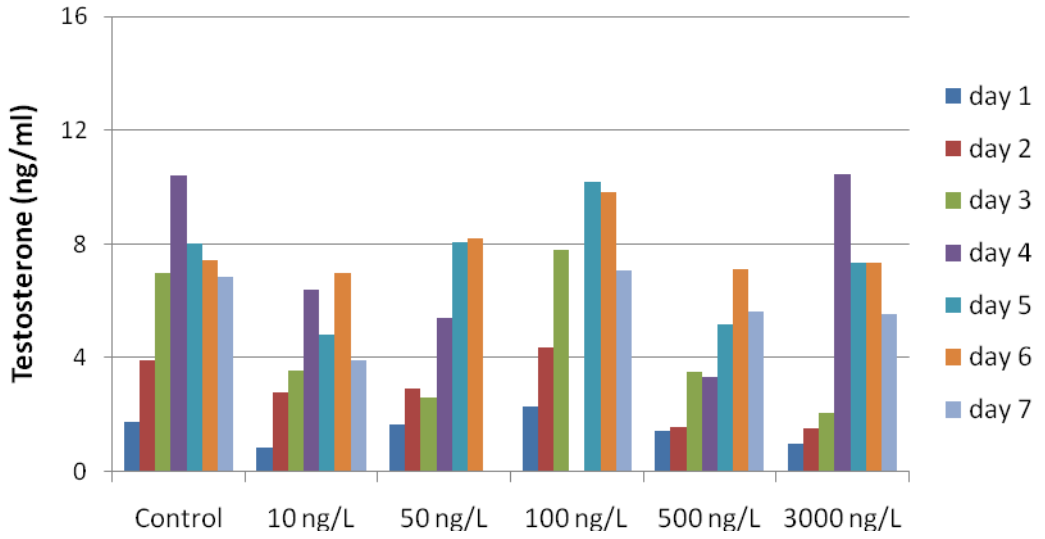

Figure 32. Each series represents testosterone concentration in ng/ml in plasma of sexually mature *Silurana tropicalis* female that had been exposed to EE_2 during larval development, for each treatment group (nominal doses of EE_2 in ng/L, animals from pair A and B are pooled) on different sampling days. Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. $n \leq 3$ for each data point.

3.2.2.3 Oocyte maturity

Proportion of pre-vitellogenic and vitellogenic oocytes in sampling of ovaries was not significantly different among treatment groups (Chi square, $p>0.05$) (Figure 33).

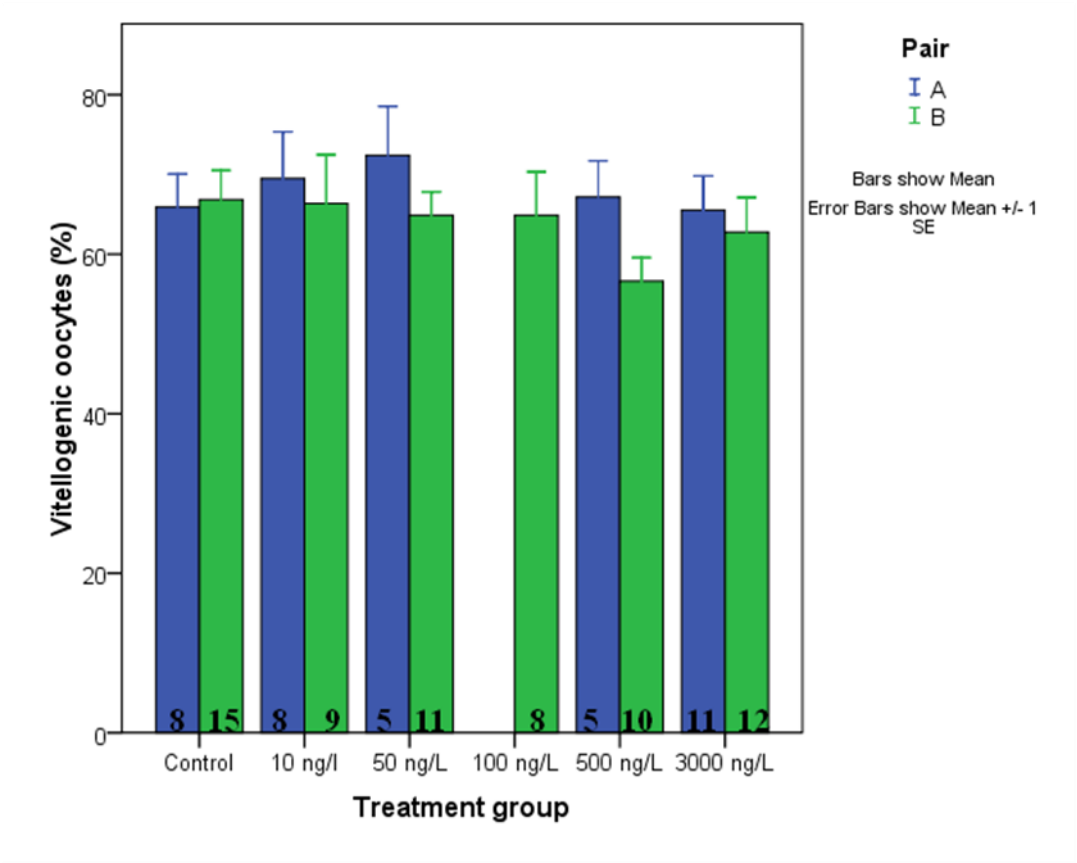

Figure 33. Percentage of vitellogenic oocytes in an oocyte sac of sexually mature *Silurana tropicalis* female ovaries for each pair of each treatment group (nominal doses of EE2 in ng/L). Frogs had been exposed to EE_2 during larval development. Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Vertical bars represent +1SEM. Sample size shown on bars.

3.2.2.4 Effect of EE_2 on oviductal growth

Some females that had been exposed to EE_2 during larval phase had not developed oviducts, or had only developed one. All the females in the control group presented both oviducts (Figure 34).

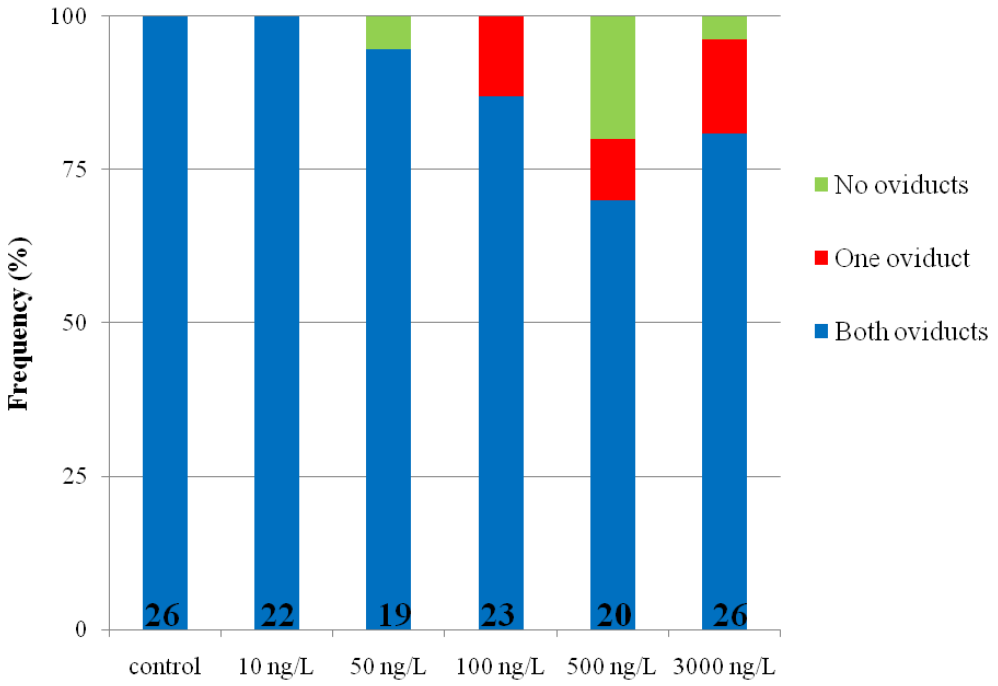

Figure 34. Frequencies of female sexually mature *Silurana tropicalis* that had been exposed to EE_2 during larval development, with both oviducts, one oviduct and no oviducts for each treatment group (nominal doses of EE_2 in ng/L). Data represent pooled individuals from Pair A and B. Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Sample size shown on bars for each treatment group.

There were also frogs with undeveloped ovaries in some of the treatment groups exposed to EE_2; 10.5 % of females frogs exposed to 50 ng/L nominal dose and 3.8 % of females exposed to 3000 ng/L nominal dose of EE_2 had undeveloped ovaries, and all belonged to the replicates pair A (data not shown). Among females that had been exposed to EE_2 during gonadal differentiation, the frequency of oviduct effects was

greater in females belonging to pair A compared with females from pair B (Chi squared, $p<0.05$) (Figure 35) although it occurred in females from both pairs.

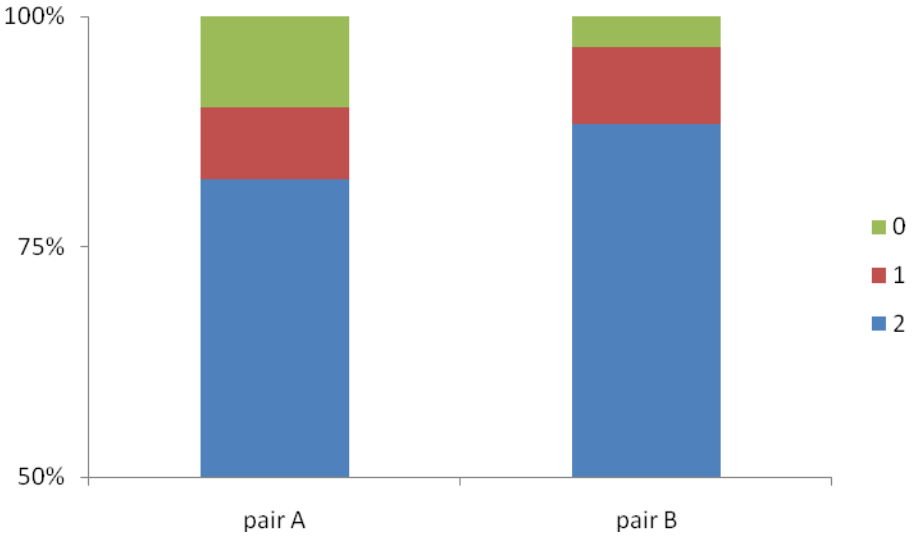

Figure 35. Oviduct frequency in sexually mature female *Silurana tropicalis* from pair A and B. Incidence of oviducts on female *Silurana tropicalis* exposed to EE_2 during larval development. 0 = no oviduct; 1 = one oviduct; 2 = both oviducts**.** Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis.

3.2.2.5 Correlations

Ovary weights were correlated with testosterone ($p = 0.000$, $R^2 = 0.128$), and estradiol plasma concentrations ($p = 0.003$, $R^2 = 0.077$). Oviduct weights were correlated with testosterone plasma concentrations ($p = 0.010$, $R^2 = 0.063$). Oocyte maturity was linked with testosterone ($p = 0.000$, $R^2 = 0.227$) (Figure 37), estradiol

(p = 0.001, R^2 = 0.11), and progesterone plasma concentrations (p = 0.015, R^2 = 0.059). Day of sampling in all frogs irrespective of treatment was correlated with oocyte maturity (p = 0.000, R^2 = 0.317) (Figure 36), testosterone (p = 0.000, R^2 = 0.337) (Figure 38), progesterone (p = 0.000, R^2 = 0.19), and estradiol plasma concentrations (p = 0.001, R^2 = 0.093) (Figure 36) Time of sampling was not correlated with hormones plasma concentrations.

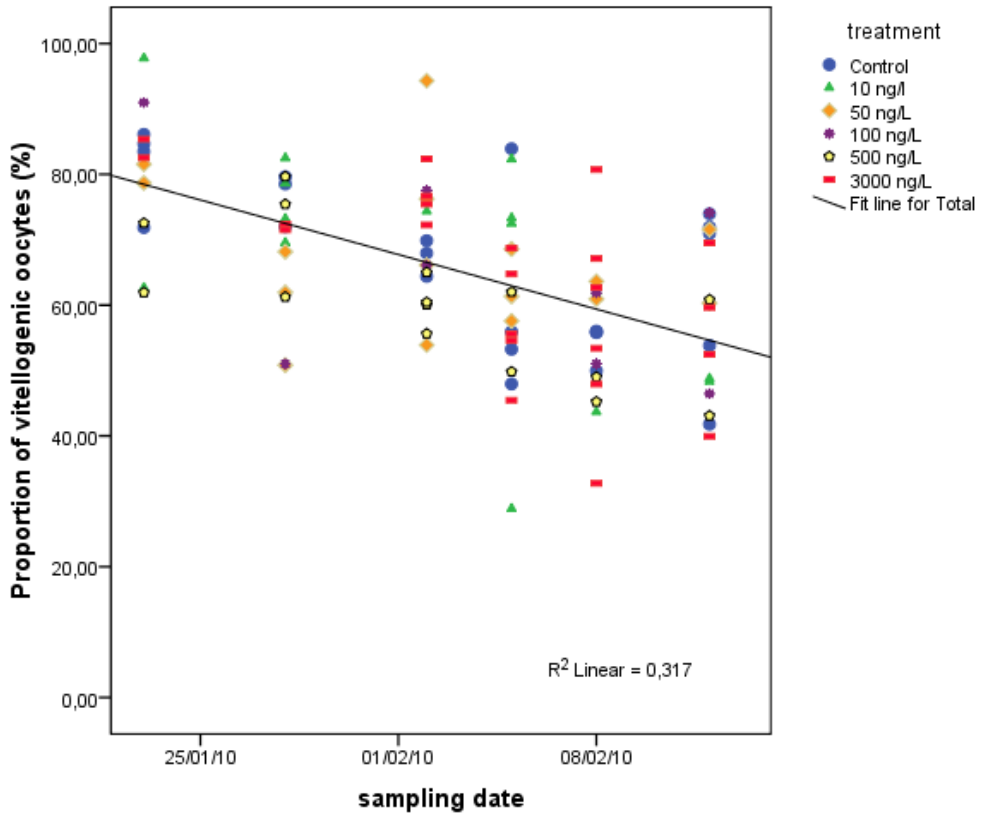

Figure 36. Proportion of vitellogenic oocytes in *Silurana tropicalis* female ovaries on sampling days. Treatment groups (nominal doses of EE_2 in ng/L) are color coded. Each data point represents one female (include females from pair A and B reared at both temperatures during larval development). Line shows linear regression.

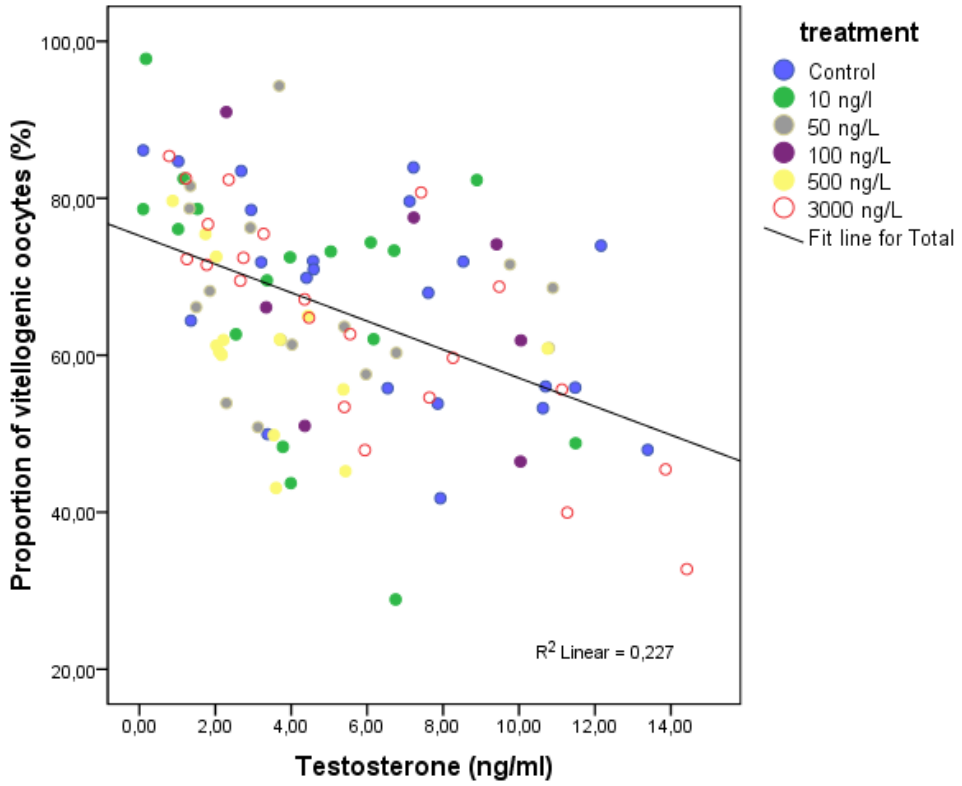

Figure 37. Correlations between the proportion of vitellogenic oocytes in sexually mature *Silurana tropicalis* ovaries and testosterone plasma concentration in ng/ml. Treatment groups (nominal doses of EE_2 in ng/L) are color coded. Each circle represent one female (include females from pair A and B). Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Line shows linear regression.

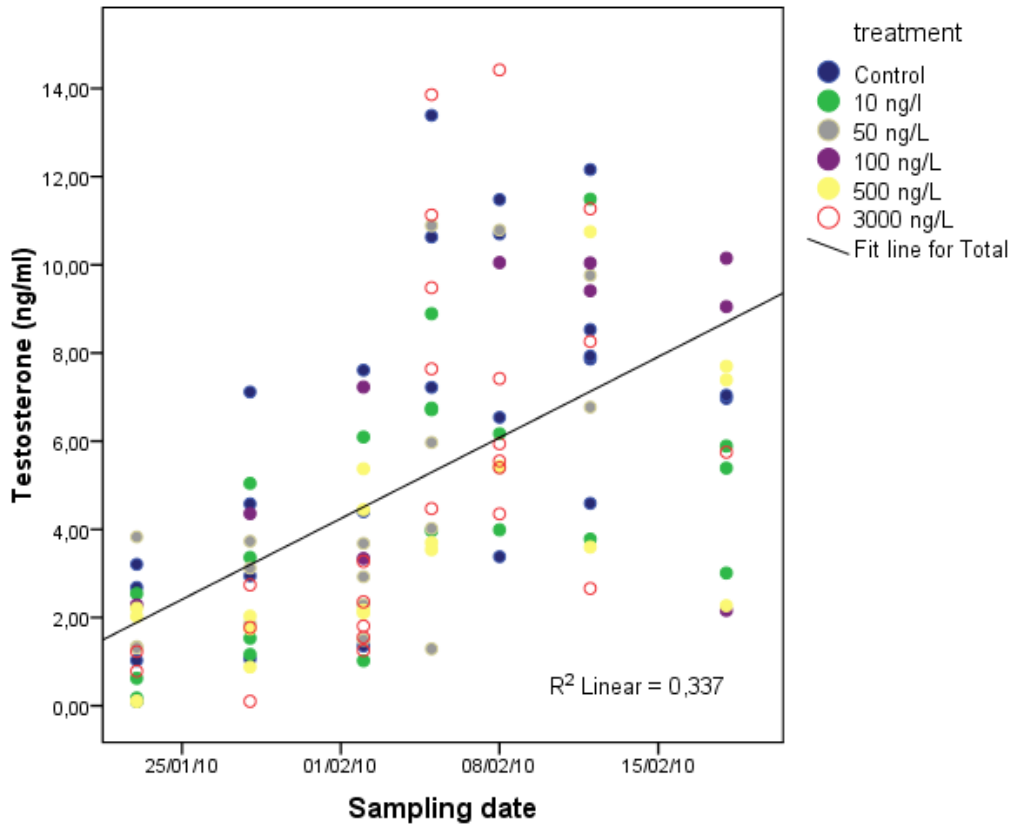

Figure 38. Testosterone plasma concentrations of sexually mature *Silurana tropicalis* females that had been exposed to EE_2 during larval development. Treatment groups (nominal doses of EE_2 in ng/L) are color coded. Each data point represents one female (include females from pair A and B reared at 25 C and 28 C during larval development). Line shows linear regression.

3.2.3 Males

3.2.3.1 Gross morphometry

Neither the wet weight, the liver weight, the testis weight, the body fat weight, nor the snout-vent length revealed any differences among males in the different treatment groups (

Table 6). Exposure to EE_2 during larval development did not affect frog growth at sexual maturity (Anova, $p>0.05$).

	control	10ng/L	50ng/L	100ng/L	500ng/L
Wet weight (mg ± sem)	11.36 (0.21)	10.93 (0.9)	10.79 (0.48)	10.64 (0.25)	10.93 (0.67)
Snout-vent (mm ± sem)	43.08 (0.56)	43.67 (1.62)	44.33 (0.53)	42.11 (1.67)	43.32 (1.14)
K (± sem)	144.6 (4.9)	134.6 (15.6)	124.3 (6.6)	146.1 (16.7)	134.4 (7.3)
Liver (± sem)	2.25 (0.06)	2.23 (0.9)	2.3 (0.09)	2.19 (0.25)	2.32 (0.09)
Fat (± sem)	11.4 (0.4)	10.67 (0.8)	9.9 (0.4)	10.6 (0.3)	8.6 (2.1)
Gonad (± sem)	0.197 (0.007)	0.185 (0.013)	0.178 (0.015)	0.179 (0.025)	0.206 (0.023)

Table 6. Gross morphometry of *Silurana tropicalis* sexually mature males that had been exposed to EE_2 during larval development (nominal doses of EE_2 in ng/L). Data represent pooled individuals from Pair A and B pooled reared at both 25˚C and 28˚C during larval development). Values for liver, fat, and gonad weight represent the organs weight relative to body weight. K= weight/ ((snout-vent length)^3) x10^5.

3.2.3.2 Plasma hormone analysis

There was no significant difference in male plasma testosterone concentrations from the different treatment groups (Figure 39).

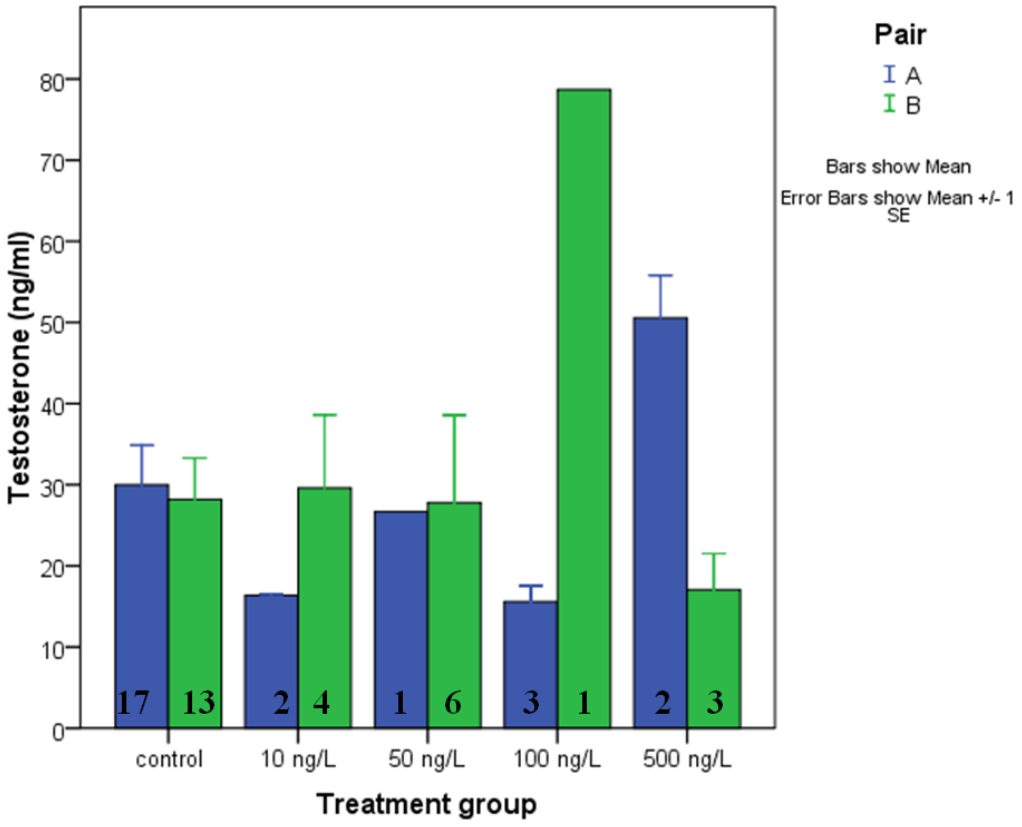

Figure 39. Mean plasma testosterone concentrations in ng/ml in sexually mature *Silurana tropicalis* males that had been exposed to EE_2 during larval development for each pair of each treatment group (nominal doses of EE_2 in ng/L). Sample size shown on bars. Data represent individuals reared at both 25˚C and 28˚C during larval development. Vertical bars represent +1SEM.

3.2.3.3 Density of keratinized epidermal hooks

No differences among treatment groups were found when comparing density of keratinized hooks on nuptial pads on forelimb or finger (Figure 40 and Figure 41).

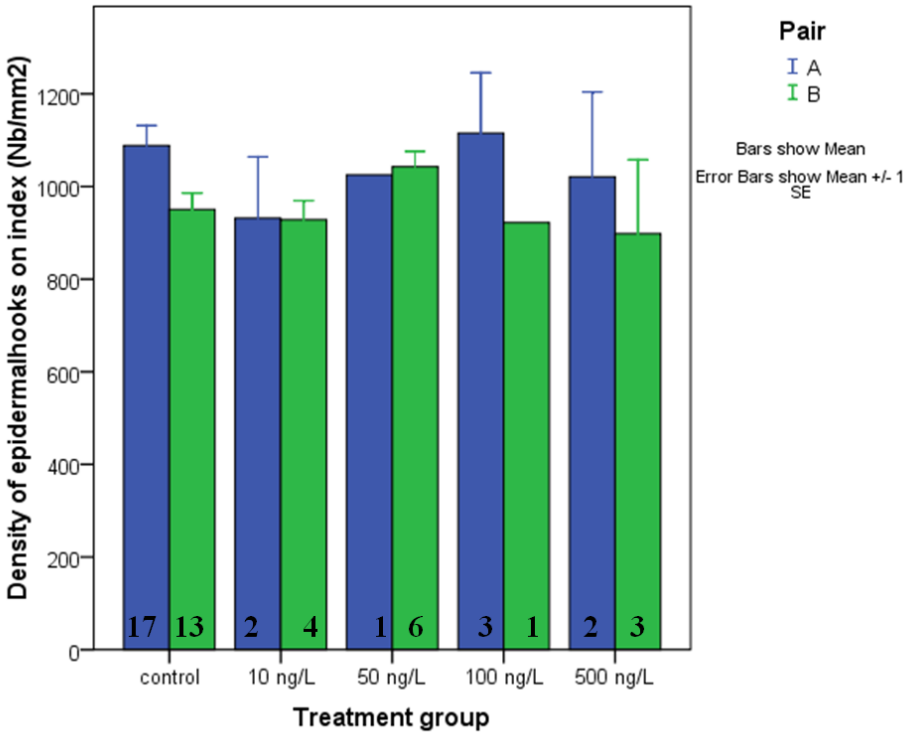

Figure 40. Keratinized hooks density in number of hooks per square millimeter of skin on sexually mature *Silurana tropicalis* male ventral side of the index for each pair of each treatment groups (nominal doses of EE_2 in ng/L). Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Animals had been exposed to EE_2 during larval development. Sample size shown on bars. Vertical bars represent +1 SEM.

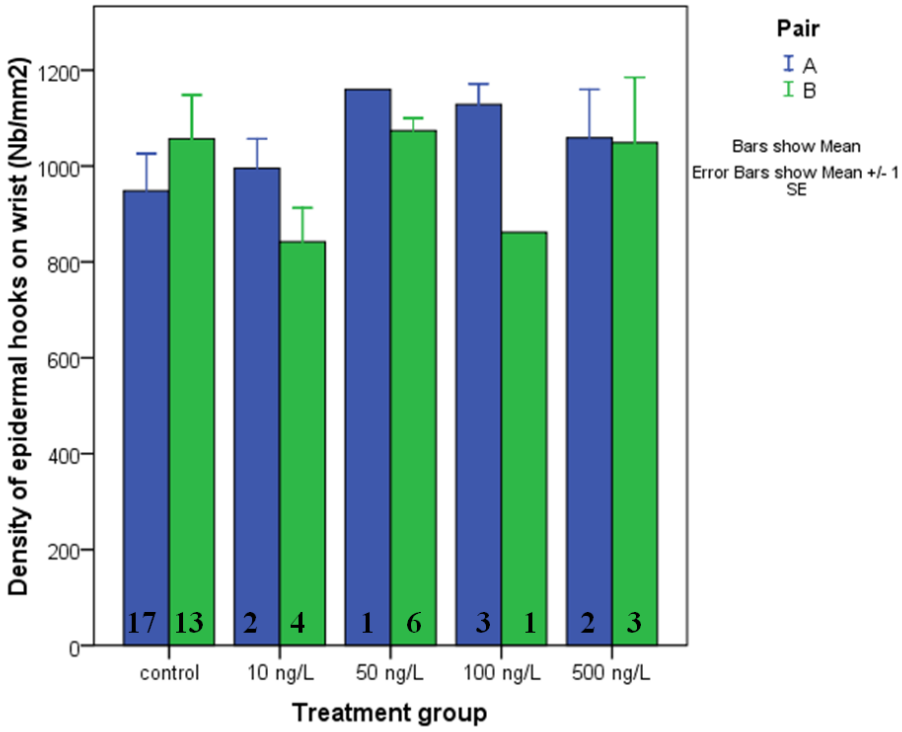

Figure 41. Keratinized hooks density in number of hooks per square millimeter of skin on sexually mature *Silurana tropicalis* male ventral side of the forelimb for each pair of each treatment groups (nominal doses of EE_2 in ng/L). Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Animals had been exposed to EE_2 during larval development. Sample size shown on bars. Vertical bars represent + 1 SEM.

3.2.3.4 Sperm density and viability

There were no statistical significant differences among the different treatment groups for sperm viability and sperm density (Figure 42 and Figure 43). The sperm viability

was not correlated to the macerated testis storage time before analysis (Pearson's, $p>0.05$).

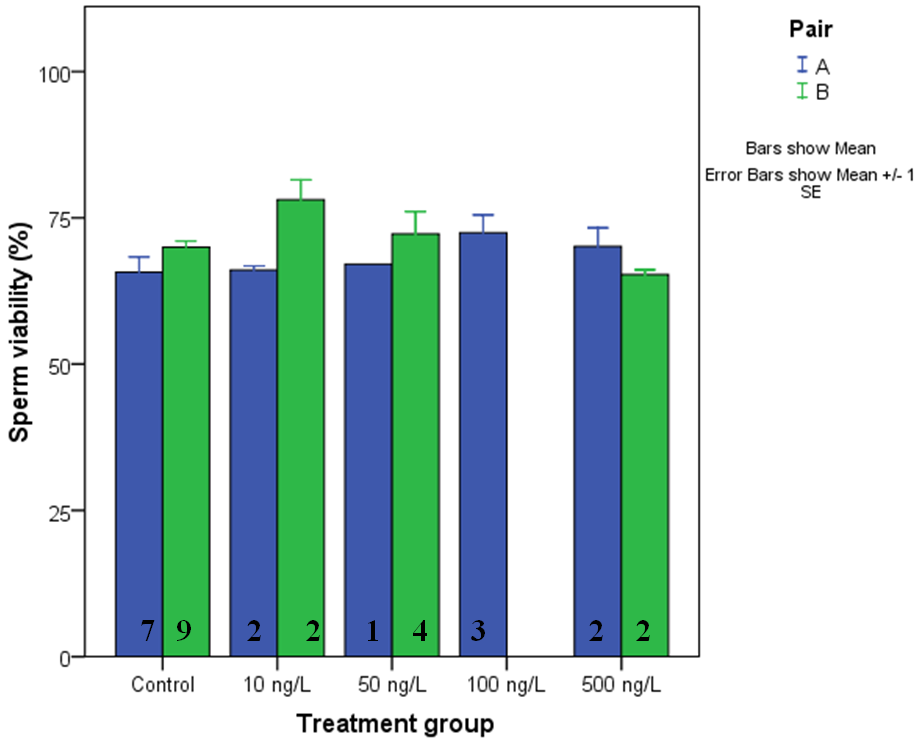

Figure 42. Percentage of viable sperm of sexually mature *Silurana tropicalis* for each pair of each treatment group (nominal doses of EE_2 in ng/L). Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Animals had been exposed to EE_2 during larval development. Sample size shown on bars. Vertical bars represent +1 SEM.

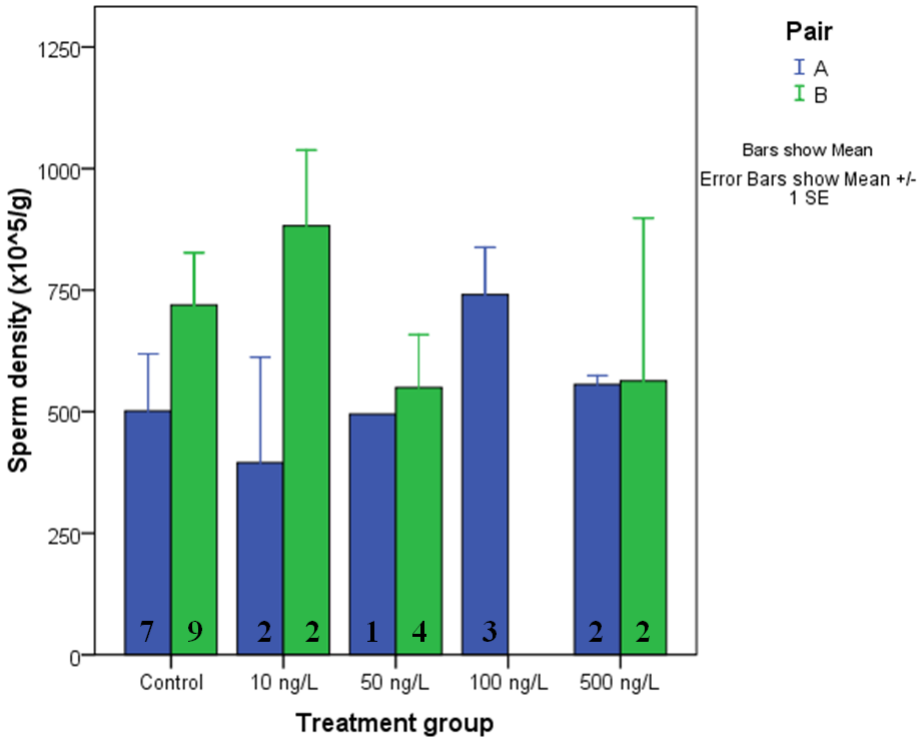

Figure 43. Sperm density expressed in $x10^5$ cells per gram of testis of sexually mature *Silurana tropicalis* males that had been exposed to EE_2 during larval development for each pair of each treatment group (nominal doses of EE_2 in ng/L). Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Vertical bars represent +1 SEM. Sample size shown on bars.

3.2.3.5 Sperm motility

No differences were found among the treatment groups in the percentage of motile spermatozoa (Anovas, $p>0.05$) (Figure 44).

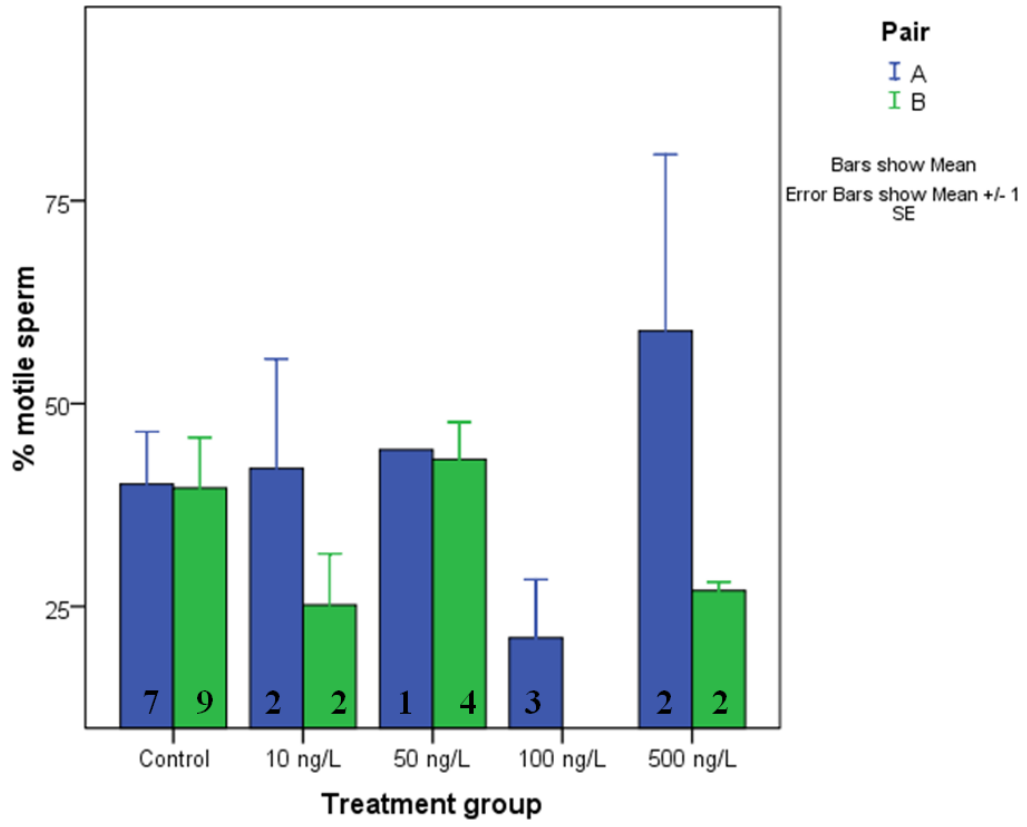

Figure 44. Percentage of motile sperm of sexually mature *Silurana tropicalis* (that had been exposed to EE_2 during larval development) for each pair of each treatment group (nominal doses of EE_2 in ng/L). Sample size shown on bars. Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Vertical bars represent +1 SEM.

STR, VSL and LIN results presented similar trends (Figure 45-47). Statistical analysis of the data offered different results depending on the method used. Table 7. Dunnett t test results using 2 methods; 1: all sperm data pooled for each treatment group (nominal doses of EE_2, pair A and B pooled). 2: for each variable, the mean for each frog was first calculated and then used to assess the mean for each treatment group. Data are compared with the control group. +: $p<0.05$, - : $p>0.05$.

shows these variations and we can see that the lowest dose seems to present the most difference with the control group. If all sperm data are pooled regardless of the factor "individual" for each treatment group, then the group 500 ng/L is also statistically different from the control group.

	10 ng/L		50 ng/L		100 ng/L		500 ng/L	
	1	2	1	2	1	2	1	2
VCL	+	-	-	-	-	-	-	-
VSL	+	-	-	-	-	-	+	-
VAP	+	-	-	-	-	-	-	-
STR	+	+	-	-	-	-	+	-
LIN	+	+	-	-	+	-	+	-

Table 7. Dunnett t test results using 2 methods; 1: all sperm data pooled for each treatment group (nominal doses of EE_2, pair A and B pooled). 2: for each variable, the mean for each frog was first calculated and then used to assess the mean for each treatment group. Data are compared with the control group. +: $p<0.05$, - : $p>0.05$.

There was a lot of variation between and within individuals for all measurements (Figure 45-47) and there were no differences between sperm parameters from frogs spawned by pair A versus pair B (Independent t test, $p>0.05$). Sperm motility of the control frogs varied a lot (**Error! Reference source not found.**). Spermatozoa from frogs exposed to 10 ng/L and 500 ng/L appear to swim in a slightly different manner to those from the other treatment groups. A cluster analysis revealed the presence of two sperm subpopulations, Group 1 and 2. The characteristics of those 2 groups are shown in Table 9.Table 8. Sexually mature *Silurana tropicalis* sperm subpopulations characteristics. Subpopulations were established by cluster analysis (all sperm data

from all males were pooled (all treatment groups, pair A and B)). Means curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN) and straightness (STR) for sperm group 1 and 2 (±SEM) .

Sperm belonging to the Group 1 have a lower VSL and STR, and a higher VAP than the sperm from Group 2. This means Group 2 tends to swim fast and straight whereas Group 1 sperms swim in circles and travel small distances. Analysis of subpopulation frequencies for each treatment group revealed that the frogs belonging to the treatment group 10 ng/L had a higher incidence of Group 1 spermatozoa compared to the other treatment groups (Figure 49).

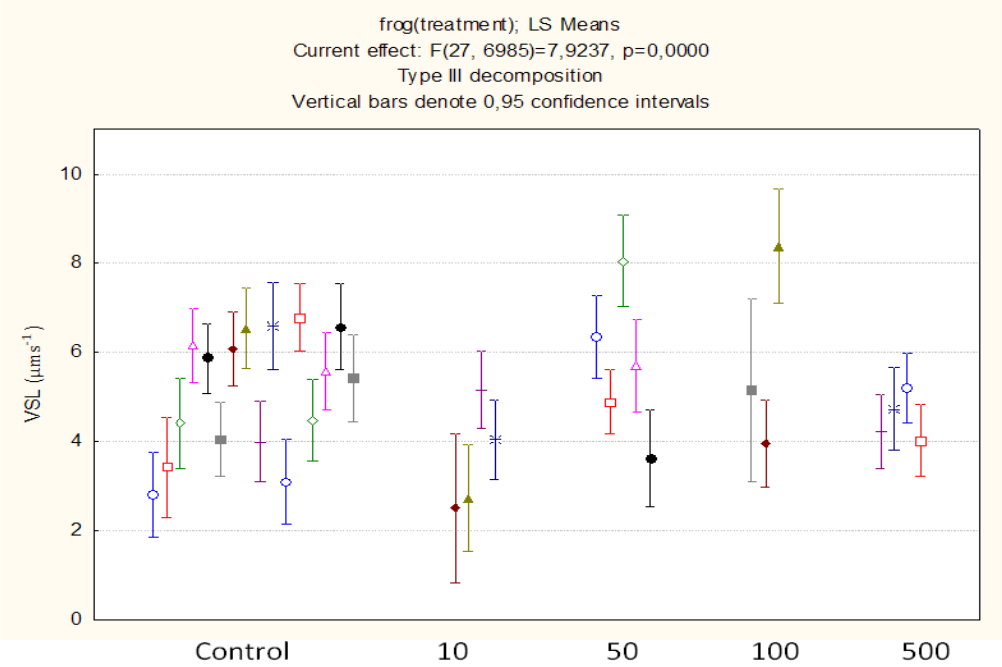

Figure 45. Mean Straight line velocity (VSL in µm/sec) of sperm of sexually mature *Silurana tropicalis* males in each treatment group (nominal doses of EE_2 in ng/L). Frogs (from Pair A and B reared at 25°C and 28°C) had been exposed to EE_2 during larval development. Number of spermatozoa analyzed per male varies between 200 and 400. Vertical bars represent 0.95 CI.

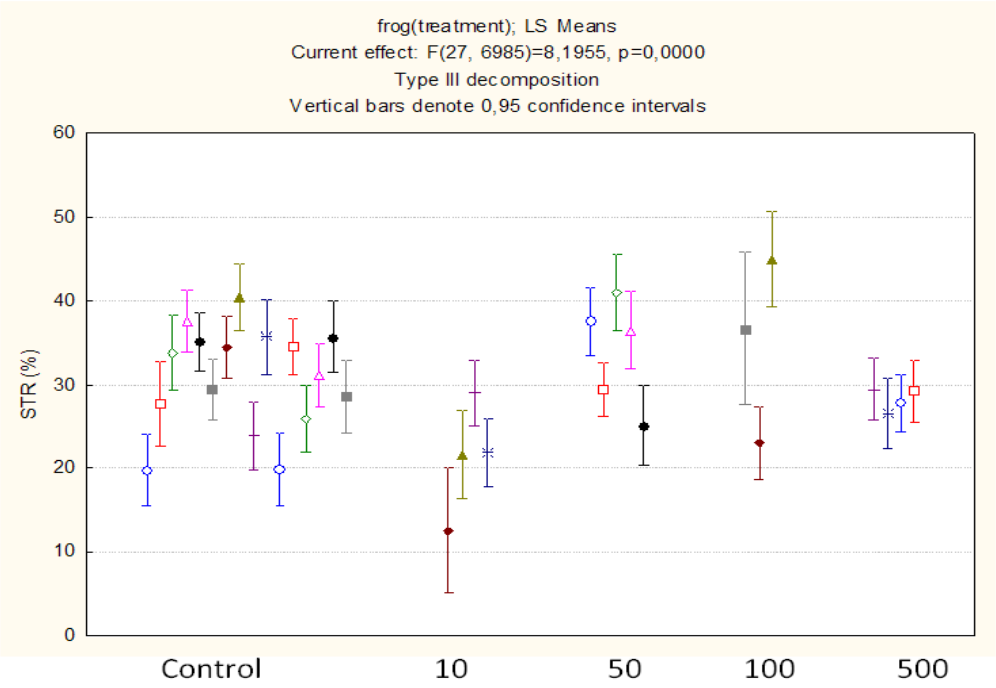

Figure 46. Mean Straightness (STR expressed in %) of sperm of sexually mature *Silurana tropicalis* males for each treatment group (nominal doses of EE_2 in ng/L). Frogs (from pair A and B reared at 25°C and 28°C) had been exposed to EE_2 during larval development. Number of spermatozoa analyzed per male varies between 200 and 400. Vertical bars represent 0.95 CI.

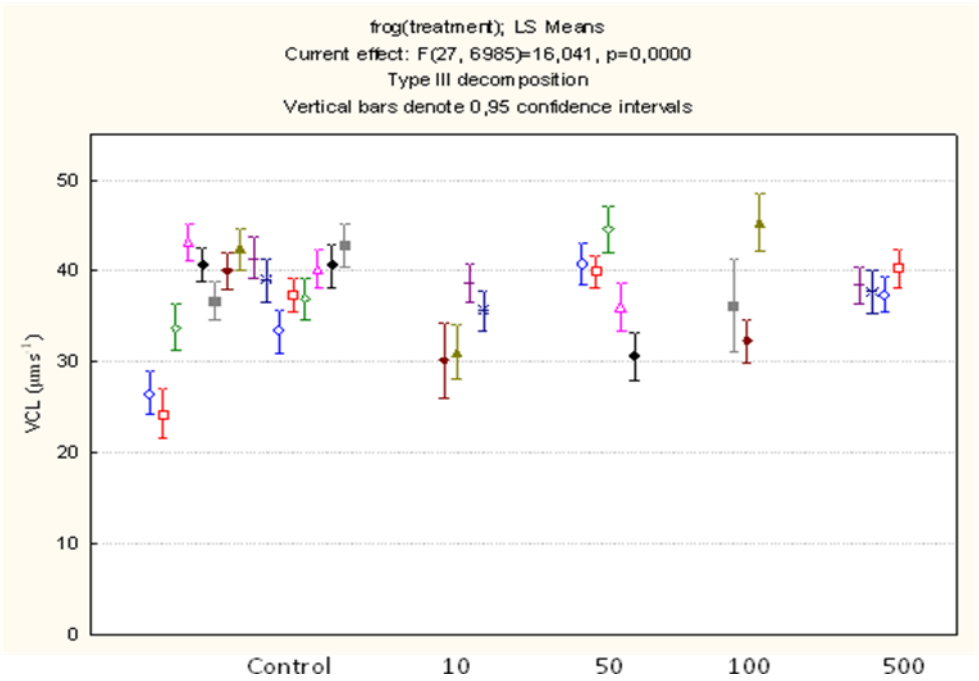

Figure 47. Mean curvilinear velocity (VCL in µm/sec) of sperm of sexually mature *Silurana tropicalis* males for each treatment group (nominal doses of EE_2 in ng/L). Frogs (from pair A and B reared at 25°C and 28°C) had been exposed to EE_2 during larval development. Number of spermatozoa analyzed per male varies between 200 and 400. Vertical bars represent 0.95 CI.

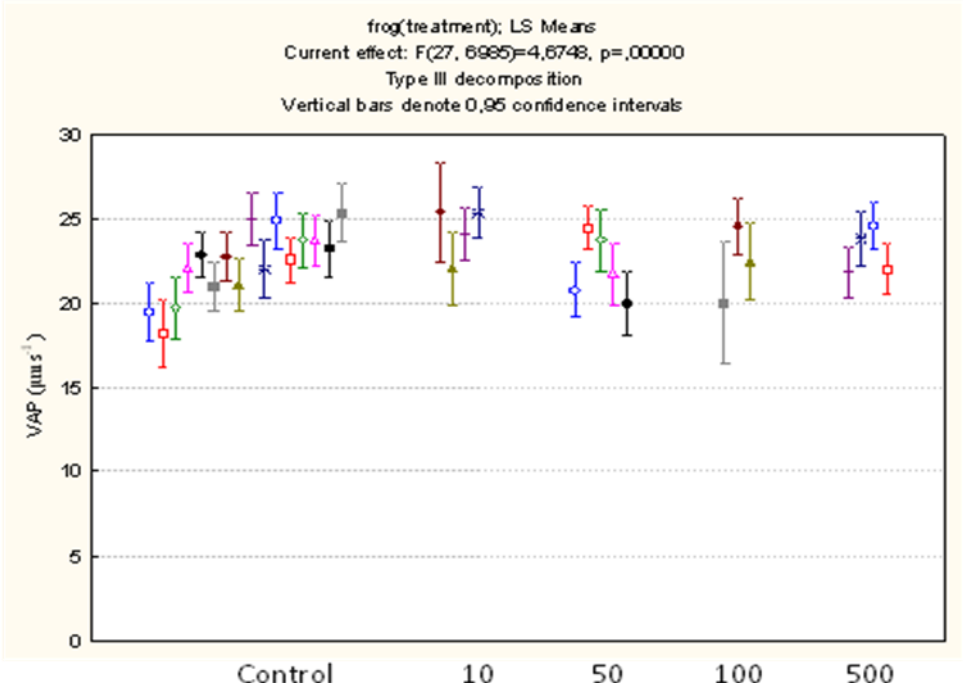

Figure 48. Mean average path velocity (VAP in µm/sec) of sperm of sexually mature *Silurana tropicalis* for each treatment group (nominal doses of EE_2 in ng/L). Frogs (from pair A and B reared at 25°C and 28°C) had been exposed to EE_2 during larval development. Number of spermatozoa analyzed per male varies between 200 and 400. Vertical bars represent ±0.95 CI.

	Group 1	Group 2
VCL (μms^{-1})	30.5 (0.24)	46.3 (0.3)
VSL (μms^{-1})	1.3 (0.17)	9.4 (0.15)
VAP (μms^{-1})	28.3 (0.2)	16.3 (0.16)
LIN (%)	6.4 (0.08)	19.5 (0.22)
STR (%)	6.8 (0.08)	57.5 (0.48)

Table 8. Sexually mature *Silurana tropicalis* sperm subpopulations characteristics. Subpopulations were established by cluster analysis (all sperm data from all males were pooled (all treatment groups, pair A and B)). Means curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN) and straightness (STR) for sperm group 1 and 2 (±SEM) .

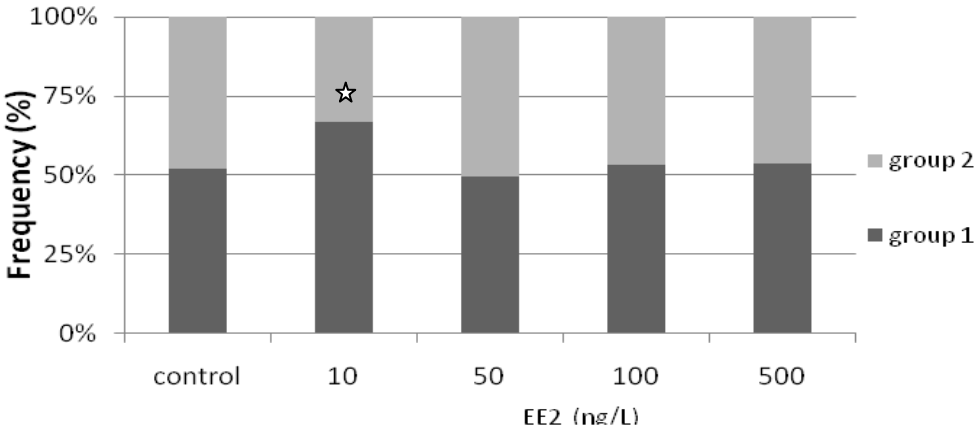

Figure 49. Frequency of *Silurana tropicalis* sperm belonging to group 1 and 2 for each treatment group (nominal doses of EE_2 in ng/L). Frogs (from pair A and B reared at both 25°C and 28°C) had been exposed to EE_2 during larval development. Star represents significant differences from the control group (Chi square, $p<0.05$).

3.2.3.6 Presence of female tissue in males

Histological analysis of the gonad revealed the incidence of regressed testicular oocytes (Reg-TO) in 96.2 % of the males (Figure 50) regardless of treatment. The presence of Reg-TO was even across the testes since no differences in the number of Reg-TO were found among the four sections taken per animal (Independent t test, $p>0.05$). There were no significant differences in the number of Reg-TO per sections among the different treatment groups (Kruskall-Wallis $p>0.05$). However, occurrence of Reg-TO was higher in replicates from pair A compared to pair B (Figure 51-52).

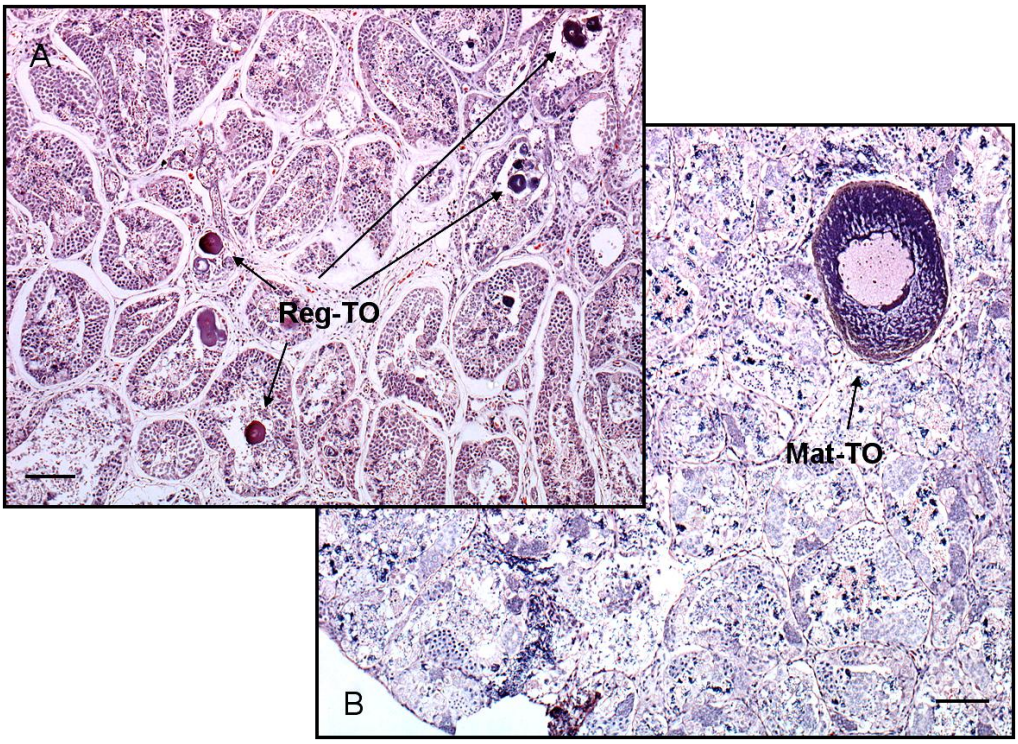

Figure 50. Histological section of sexually mature *Silurana tropicalis* testes which had been exposed to EE_2 during larval development (haematoxylin and eosin dies). A; Presence of Regressed testicular oocytes (Reg-TO). B; Occurrence of a matured testicular oocyte (Mat-TO). Bars represent 100µm.

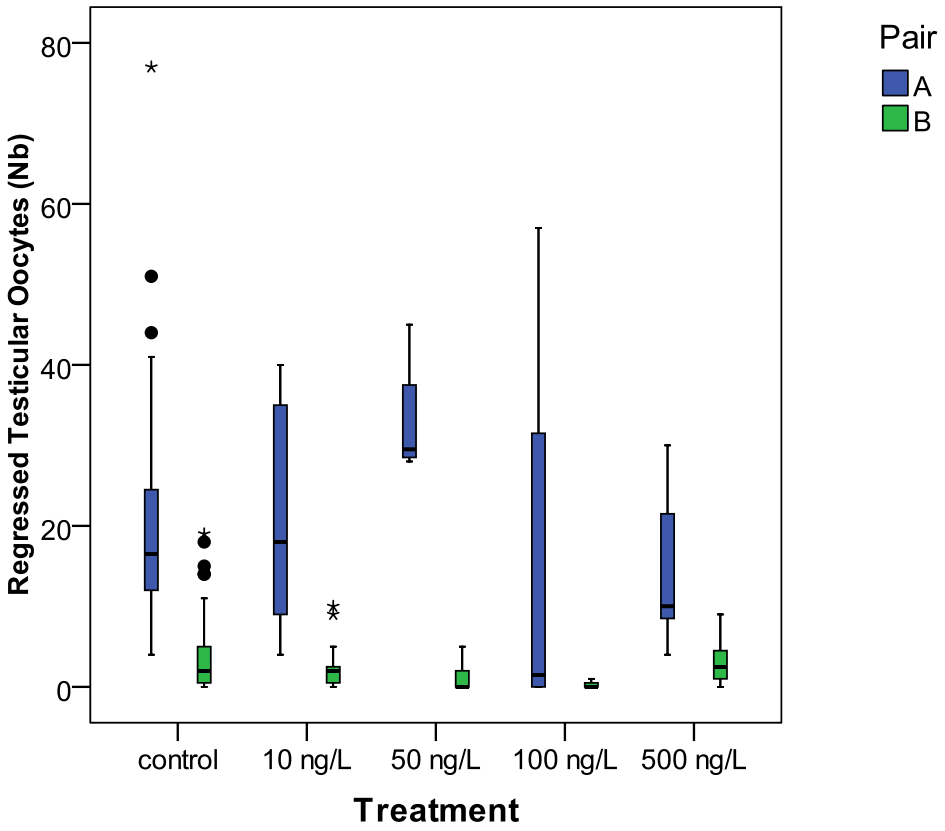

Figure 51. Number of regressed testicular oocytes in *Silurana tropicalis* testis sections in each pair replicate of each treatment group (nominal doses of EE_2 in ng/L). Animals were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Frogs had been exposed to EE_2 during larval development. The box represents 75th and 25th percentiles; the horizontal black line shows the median. Whiskers represent highest and lowest values. Stars and dots represent extreme values and outliers respectively.

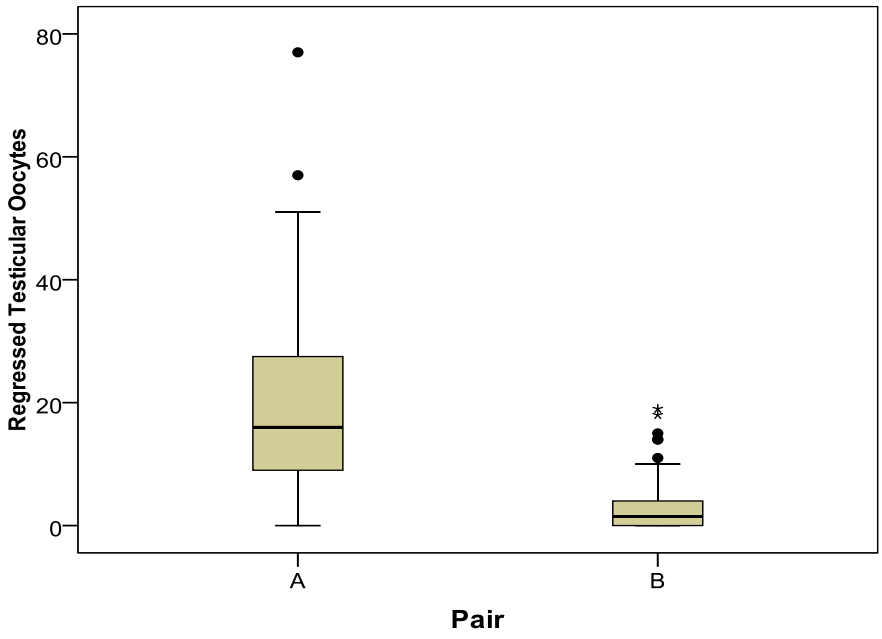

Figure 52. Number of Regressed testicular oocytes in *Silurana tropicalis* testis sections of pair A and B (data pooled for all treatment groups). Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Animals had been exposed to EE_2 during larval development. The box represents 75th and 25th percentiles; the horizontal black line shows the median. Whiskers represent highest and lowest values. Stars and dots represent extreme values and outliers respectively.

Some matured testicular oocytes (Mat-TO) were also observed in a few males, and two males had oviducts (Figure 53). All the male frogs exhibiting Mat-TO or/and oviducts had been exposed to EE_2 during larval growth (Figure 54). 66.7 % of the frogs exhibiting Mat-to and/or oviducts belonged to pair A (data not shown).

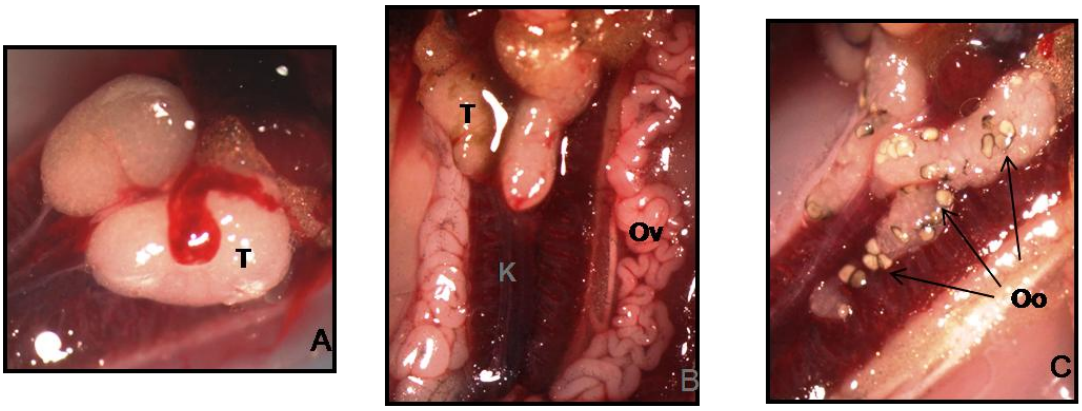

Figure 53. *Silurana tropicalis* male gonads. A; testis from a control frog. B; Intersex frog with 2 testis and 2 oviducts. The frog had been exposed to 10ng/L EE_2 (nominal dose) during larval development. C; Intersex frog with mature ovarian follicles on top of the testis (treatment group 500ng/L). T; testis. K; kidney. Ov; oviduct.Oo; oocyte.

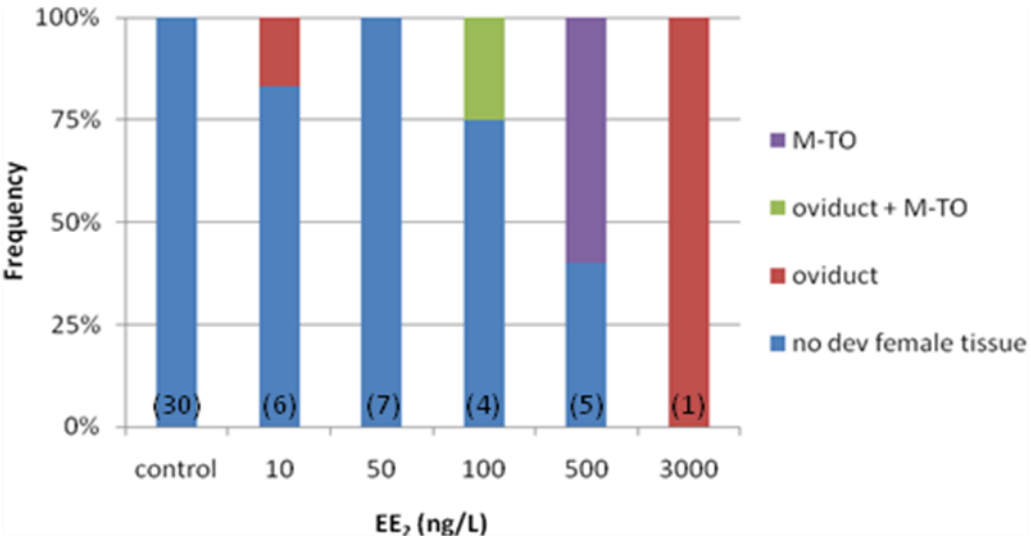

Figure 54. Frequency of *Silurana tropicalis* males with matured testicular oocytes (M-TO) or/and oviducts for each treatment group (nominal doses of EE_2 in ng/L).

Data represent pooled individuals from pair A and B reared at both 25°C and 28°C. Males had been exposed to EE_2 during larval development. No dev female tissue: males without M-TO or/and oviducts. Number in bracket represents the total number of males in each treatment group.

3.2.3.7 Correlations

Testosterone concentrations were negatively correlated with VSL ($p = 0.029$, $R^2 = 0.15$) and STR ($p = 0.044$, $R^2 = 0.13$) (Figure 56). The day of sampling was correlated with testosterone concentrations ($p = 0.016$, $R^2 = 0.11$), sperm density ($p = 0.000$, $R^2 = 0.36$), and VAP ($p = 0.018$, $R^2 = 0.17$). The number of Reg-TO was neither correlated with sperm motility (VCL, VSL, VAP, LIN, and STR), nor with testosterone concentrations in plasma, but was correlated with sperm viability ($p = 0.014$, $R^2 = 0.18$) (Figure 55). Sperm density was correlated with the number of keratinized epidermal hook on the wrist ($p = 0.005$, $R^2 = 0.23$) (Figure 57).

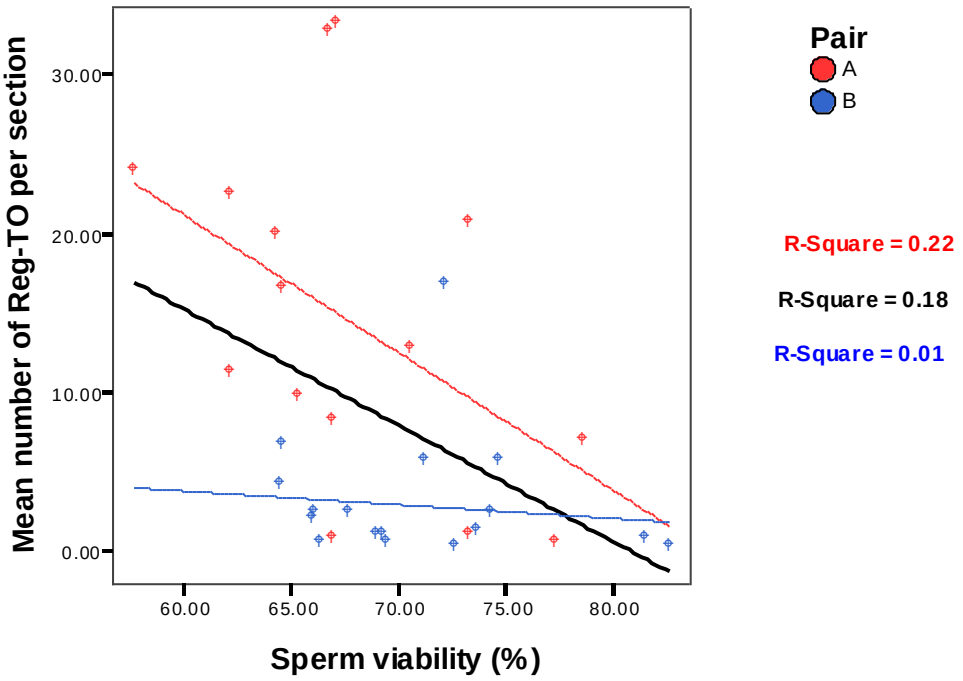

Figure 55. Correlation between sperm viability and the incidence of regressed testicular oocytes in *Silurana tropicalis* males. Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Lines represent linear regression for pair A (red), B (blue), and both combined (black).

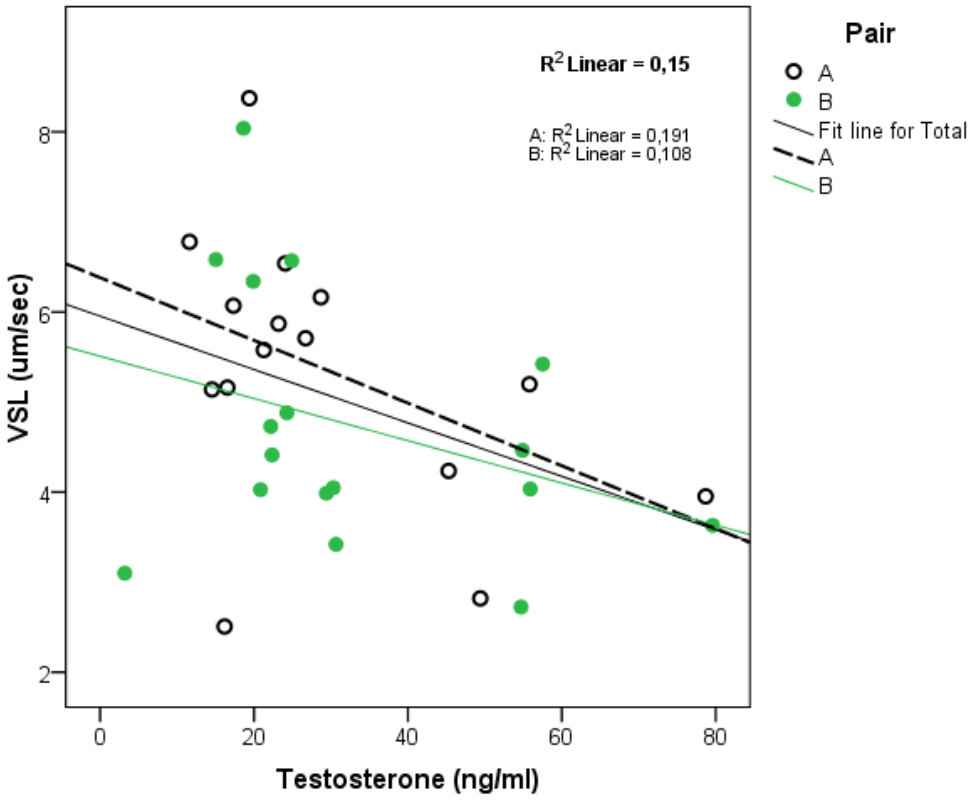

Figure 56. Correlations between testosterone plasma concentrations and sperm straight line velocity (VSL) of *Silurana tropicalis* males. Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Lines represent linear regression for pair A (dash), B (green), and both combined (black).

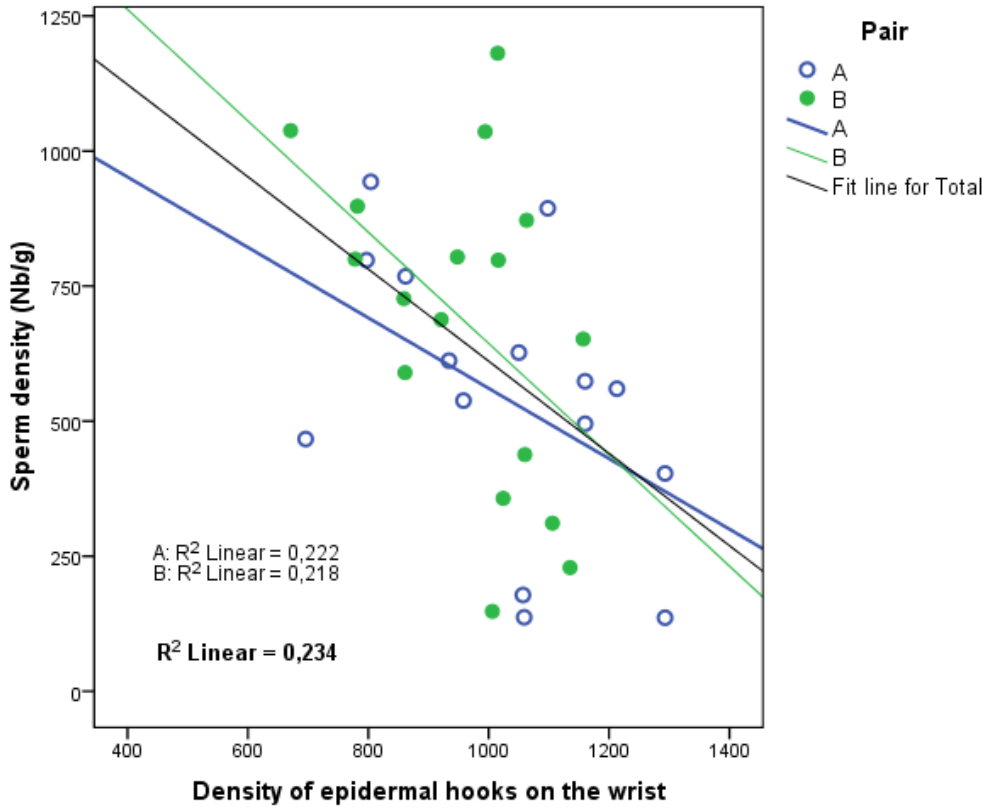

Figure 57. Correlation between sperm density and density of epidermal hooks on the wrist of *Silurana tropicalis* males. Frogs were reared at two temperatures (25°C or 28°C) during larval development and then maintained at 25°C post metamorphosis. Lines show linear regression for pair A (blue), pair B (green) and for total (black).

4 . Discussion

The aim of this study was to investigate the effects of larval exposure to a xenoestrogen on the adult reproductive potential in an amphibian partial life-cycle model. Indeed, despite the numerous studies available in the literature showing the impact of xenoestrogens on amphibian gonadal differentiation, very few studies have looked at the effects of xenoestrogens on the reproductive function of amphibian after exposure during gonadal differentiation. For this purpose, larvae of the emerging model amphibian *Silurana tropicalis* were exposed to EE_2 from NF stage 51-52 until completion of metamorphosis NF stage 66 and endpoints related to the reproductive function were examined on completion of metamorphosis and at sexual maturity.

The mortality rate was above 20% in the control group, which is slightly higher than mortality rates observed in previous studies (16% in (Gyllenhammar et al., 2009)). A lower density in the tanks would have possibly increased the survival rate.

Chemical analysis of the tanks water suggested a high contamination of the control group vessels by EE_2 (37.9 ng/L), although data on sex ratio and gonadal abnormalities suggest otherwise. In the control group, the sex ratio was similar to the expected 50:50 ratio, as seen in other studies (Grammer et al., 2005), but it was skewed toward females in the EE_2 treated groups, which is consistent with previous research (Pettersson & Berg, 2007). It can be hypothesized that a contamination occurred during the water sample preparations. But these findings clearly generate uncertainty about the EE_2 concentrations measured in the other treatment groups, and the concentrations that the larvae were exposed to during the larval phase. While this is problematic, the findings of this study could not therefore be used in a risk assessment of EE_2, the aim of the study was to generate a cohort of frogs exposed to

different concentrations of EE_2 during the larval development, manifest as aberrant gonadal differentiation, and follow this through to assess impacts on adult reproductive potential. In this respect, the study makes a valuable contribution to the development to the development of an amphibian partial life-cycle model for hazard characterization of suspected endocrine disrupters.

EE_2 affected both growth and development of the tadpoles, which is consistent with previous findings. Indeed, other experiments have showed that EE_2 can significantly delay metamorphosis of *Rana pipiens* and *Silurana tropicalis* larva (Hogan et al., 2008; Pettersson & Berg, 2007). Such observations confirm the existence of a cross-talk between thyroid and reproductive regulatory axes. In the present research, the effect of EE_2 on *Silurana tropicalis* development was more pronounced at higher water temperature, whereas at lower temperature, EE_2 had a bigger impact on the larval growth. A difference in response depending on water temperature can be explained by the link existing between TH kinetics and temperature. In *Rana cateisbana*, low temperature slows down T3 uptake in red blood cells (Murata & Yamauchi, 2005), which results in a slower development of the tadpoles. Also, larvae developing quickly tend to metamorphose at lower body weight. So, at low temperature, where TH kinetics/metabolism are reduced, there might be greater growth, which would make it easier to detect negative impact of EE_2, while the reduced rate of development would make it harder to differentiate impacts of EE_2 on TH-driven development. Conversely, at warmer temperature, it may be that development is faster at expense of growth, so growth inhibition from EE_2 is less obvious while development was more affected.

Differentiation of the Mullerian ducts seems to be a sensitive target to xenoestrogen in frogs since the percentage of female *Silurana tropicalis* displaying oviduct abnormalities increased in a concentration dependent manner, but no abnormalities were found in the female controls. Moreover, two males (displaying testis and nuptial pads) from the EE_2 treated groups developed oviducts. This type of response has already been observed in *Silurana tropicalis* (Berg, Gyllenhammar & Kvarnryd, 2009), but also in other taxa since in male fish, exposure to estrogenic compounds induces the gonadal ducts to grow ovarian-like attachments to the testis (Gimeno et al., 1996). Also, oviduct malformations have been observed in female mice exposed to DES in utero (Newbold, 2004), and in male and female birds after embryonic exposure to DES and EE_2 (Berg et al., 1999). Such abnormalities are likely to negatively affect the reproductive function of the frogs, especially in females, since oviducts are indispensable to coat oocytes with the different jelly layers, to transport them in the external environment, and indeed to enable subsequent fertilization (Newport, 1851).

In control males, the mean testosterone concentration was 29.2 ± 19.1 ng/ml (standard deviation). In control females, the mean testosterone concentration was 6.18 ± 3.7 ng/ml, and the mean estradiol concentration was 6.4 ± 3 ng/ml. Previous studies found that at 40 weeks after metamorphosis (beyond reproductive maturation), females and males have a mean of 5 ng/ml and 9 ng/ml plasma testosterone, respectively. In adult females, mean estradiol concentration was 2 ng/ml while estradiol was not detectable in males (Olmstead et al., 2009a). In the present study, estradiol plasma concentrations were lower in females whereas testosterone concentrations were similar in females and three times higher in males compared

with value reported by Olmstead *et al*. The frogs sampled in the present study were older, which could explain the differences in adult sex steroid titers in between this study and that of Olmstead *et al.* Hormone plasma concentrations vary greatly between individuals in *Silurana tropicalis*, as reported by Olmstead *et al*., which means that a large number of animals are required to see any treatment related effects. In the present study, the number of males in EE_2 treated frogs may have been too low to be able to see any treatment related effects. Negative correlations found between sperm VSL and testosterone concentrations suggest an increase in testosterone levels due to exposure to EDCs would negatively affects sperm swimming pattern. This finding is not consistent with previous research in fish where steroid levels were positively correlated with fecundity (Ankley et al., 2008). Correlations found between steroid concentrations and day of sampling suggest that the steroid profile of the frogs followed a cycle, and further research is needed to understand the impact of such cyclic pattern on analysis of the reproductive potential of amphibian held under laboratory conditions. On the other hand, sample size for each treatment group on each sampling day was very low, so an alternative explanation for these findings is that this is an artifact of sample size (i.e individual difference). Injection of gonadotropins before sampling could help standardize the method, but on the other hand, it can be argued that it could preclude any detection of impacts of test chemical exposure targeting the pituitary, hypothalamus or higher centres.

The initial objective of the assessment of the density of the epidermal hooks was to develop a non-invasive technique measuring the nuptial pads darkness that could be correlated with invasive endpoints such as testosterone concentrations in plasma

and/or sperm motility. Current research is trying to reduce, refine, and replace the use of animals in laboratory tests, and since the nuptial pads on the forelimbs are an external secondary sexual characteristic, the measurement of the density of the epidermal hooks could potentially be a non-invasive endpoint related to the reproductive function of amphibians. Breeding glands contain androgen receptors (Emerson et al., 1999), and adult male *Xenopus laevis* exposure to estrogenic or anti-androgenic compounds significantly decreases the glands epithelium height (van Wyk, Pool & Leslie, 2003b). In *Xenopus laevis*, exposure to atrazine during larval development significantly decreases the area of breeding glands (Hayes et al., 2010). In this experiment, EE_2 did not seem to have an effect on the epidermal hook density, although a correlation could be made with sperm density, but not with other endpoints. However, it can be argued that the method of the technique presents some weaknesses. Indeed, a 2-D picture was taken of a 3-D forelimb presenting a curvilinear shape, and the number of epidermal hooks was subsequently counted on a polygon of a known area. The accuracy of the calculations of the polygon's area can be doubted. Histological analysis of the nuptial pads and subsequent calculations of the breeding glands areas, or measurement of their epithelium heights, may have maybe been a better approach, but it was not envisaged since it is an invasive technique.

In *Silurana tropicalis* no significant differences were found in males exposed to EE_2 during larval development, in motility percentage and sperm concentrations in testis homogenates in the present study. These findings corroborate those of Gyllenhammar et al., 2009.

Sperm swimming velocity has been found to be a major determinant in fertilization success in many species including the red deer (*Cervusela phushispanicus*) (Malo et al., 2005), the domestic fowl (*Gallus domesticus*) (Froman et al., 1999), the sea urchin (*Lytechinus variegates*) (Levitan, 2000), the rainbow trout (*Oncorhyn chusmykiss*) (Geffen & Evans, 2000) and the Atlantic salmon (*Salmo salar*) (Gage et al., 2004). These studies reported that males with spermatozoa displaying a higher swimming velocity have higher fertilization rate. In amphibians, studies on sperm motility are scarce, especially studies using CASA. In *Xenopus laevis*, there is a positive association between sperm swimming speed and male fertility (Wolf & Hedrick, 1971). Research on *Crinia georginia* sperm swimming pattern found that males having sperm displaying lower VAP are advantaged when competing for fertilizations, although in a different experiment no correlations could be made between sperm VAP and the fertilization rates (Dziminski et al., 2009; Dziminski et al, 2010). The authors refer to sperm having a lower VAP as being slower, although VAP is not an appropriate variable to assess spermatozoa speed of progression, but instead indicates if the sperm is shaking, or swimming in circles. VSL is the best variable to assess sperm speed of progression, but more than this, using only one variable as Dziminski *et al*. 2009, did, does not provide much information on the sperm swimming patterns. In addition, these authors did not look for subpopulations although there are now evidences of the presence of sperm subpopulations in some species. Indeed, sperm subpopulations were found in golden hamsters, marmosets, boars, gazelles, stallions, and dogs (Quintero-Moreno et al., 2003), and also in fish (Martinez-Pastor et al., 2008). According to the findings from this study, there are two sperm subpopulations in *Silurana tropicalis* and *Xenopus laevis* (data on *Xenopus laevis* not shown).

Analysis of sperm swimming patterns in *Xenopus laevis* egg jelly coats revealed that sperm that are the most successful in penetrating the different layers of jelly exhibit sustained level of high motility and take direct routes, whereas sperm taking indirect routes (up to 50%), and described by the authors as "swimming circumferentially", are not so successful (Reinhart, Ridgway & Chandler, 1998). These unsuccessful sperm swimming in circles appear similar to the sperm of group 1 characterized in this study, displaying higher VAP, and lower VSL. Although the comparison between the two experiments seems inappropriate since the sperm swam in two media of different viscosity, there are evidences that *Xenopus laevis* sperm swim similarly in low salt buffer and in the egg jelly coat. Indeed Reinhart *et al*. found that *Xenopus laevis* sperm exhibit a similar ratio of rotational frequency to forward velocity in low salt buffer and in jelly (Reinhart, Ridgway & Chandler, 1998; Bernardini et al., 1988). On the other hand, the jelly layers surrounding *Xenopus laevis* eggs diffuse a small protein behaving as sperm chemoattractant (Al-Anzi & Chandler, 1998), and it would be interesting to measure sperm motility in media with and without jelly extracts to observe potential differences.

Overall, comparison of the sperm motility results gathered in the current study with findings by Reinhart *et al*. and Wolf *et al*. indicate that *Silurana tropicalis* sperm belonging to group 2 are more likely to be successful in fertilizing female eggs than sperm from group 1. Surprisingly, in the current study, the males that had been exposed to the lowest dose of EE_2 during larval development had a greater proportion of sperm from group 1 than the males from the controls and the other treatment groups. This finding is inconsistent with previous findings in mammals. In rats, neonatal exposure to DES induced a production of sperm at adulthood

displaying significantly lower LIN and STR at the highest dose, compared to the control group (Goyal et al., 2003). Considering the low numbers of male frogs in the treatment groups exposed to EE_2, and the high variability of sperm motility between individuals from similar treatment groups, it is difficult to conclude that the apparent lower quality of sperm from males belonging to the 10 ng/L group is the consequence of a low dose effect of EE_2 on sperm quality, or whether this finding represents uncontrolled experimental error. Further research is needed to investigate sperm motility of male frogs and the potential impact of pollutant on the latest. Nevertheless there is evidence that EDCs can affect sperm motility of externally fertilizing species since wild male Spottail Shiners (*Notropis hudsonius*) caught in rivers with high levels of xenoestrogens, with intersex gonads and exhibiting elevated expression of hepatic vitellogenin, had sperm with lower VAP, VCL, VSL, STR and LIN, but also lower sperm concentrations and a lower percentage of motile sperm compared to fish caught in a reference site (Aravindakshan et al., 2004).

To the author's knowledge, this research presents the first evidence that occurrence of regressed testicular oocyte in adult *Silurana tropicalis* is not linked with xenoestrogen exposure during larval development, but rather is a natural phenomenon linked to parental background. On the contrary, the occurrence of mature testicular oocytes is linked with xenoestrogen exposure in a concentration dependent manner.

The presence of regressed testicular oocytes in the control group of this study is consistent with the findings of DuPreez *et al*. on the natural incidence of testicular ovarian follicles in wild *Xenopus laevis* in South Africa (Du Preez et al., 2009). On the other hand, the present study in *Silurana tropicalis* shows large variation between

frogs originating from two different clutches with similar genetic background whereas Dupreez *et al*. study found that in wild adult *Xenopus laevis,* the occurrence of testicular oocytes varies greatly between different geographic populations. In the present study, the four adults used to generate larvae originated from the same batch, and although no phylogenetic analysis was performed, it is reasonable to assume the genetic similarity of their offspring. Nevertheless, the incidence of regressed testicular oocytes was not linked with xenoestrogens exposure.

Mature testicular oocytes and oviducts were only observed in males exposed to EE_2 during gonadal differentiation, corroborating the hypothesis of Storrs-Mendez *et al*. who suggested that a transitory phase of intersex might be common in amphibians during gonadal development, but that exposure to EDCs could render this transitory phase permanent. Although the incidence of regressed testicular oocytes does not seem to be related to xenoestrogen exposure, it was negatively correlated with sperm membrane integrity, indicating that males with a high number of regressed testicular oocytes produced spermatozoa with a lower viability.

The offspring of pair A had a higher mortality rate during the exposure study compared with pair B. In addition, at sexual maturity, males displayed a higher incidence of regressed testicular oocytes, a higher incidence of mature testicular oocytes and oviducts, and females belonging to pair A had more oviductal abnormalities than females from pair B. These observations suggest that the offspring from pair A were more sensitive to EE_2 exposure than the offspring of pair B, indicating there may be a high variability in response to xenostrogen exposure during sex differentiation depending on parental background in amphibians.

The impact of water temperature on the effect of EE_2 on the reproductive potential of frogs exposed to EE_2 during larval development was not assessed since two replicate tanks per treatment had to be pooled together due to lab space issues during the grow out phase. Also, there was no true replicate tank vessel for each treatment group during this exposure study, and this rendered the interpretation of the data difficult and has weakened the statistical power of the experimental design. Moreover, the accidental loss of sexually mature females from the treatment group 100ng/L from pair A seems to have affected the data and might have compromised the quality of data interpretation due to an imbalanced experimental design.

In retrospect, in similar experiment it would be best to sample individuals on same day as much as possible. Furthermore, this experiment provides useful information on the effect of xenoestrogens on the amphibian reproductive potential in *Silurana tropicalis* under laboratory conditions, but those results do not necessarily reflect what wild amphibian populations experience. Indeed, a similar experiment with a species displaying a different ovarian differentiation rate may have generated different results. In addition, some amphibian populations complete metamorphosis over several month (over-wintering), and their response to EDCs exposure during larval development might be very different from a laboratory model such as *Silurana tropicalis*. Furthermore, in the wild, amphibians are exposed to a mixture of chemicals, including EDCs displaying different modes of action.

Further work is needed to understand the impact of gonadal abnormalities on amphibian reproduction fitness. In the present study, the number of male frogs displaying mature testicular oocytes and/or oviducts was too low to establish any link with sperm quality or any other parameters linked to the reproductive function. A

similar study using all-ZZ tadpoles would provide a higher number of intersex animals enabling the assessment of the reproductive potential of male frogs with gonadal abnormalities. Moreover, there seems to be a gap of knowledge in amphibian sperm quality and particularly in the effect of EDCs on amphibian sperm quality. Similar experiments of this type are needed to understand the impact of EDCs on amphibian populations, using EDCs with different modes of action and also mixtures of chemicals potentially encountered by wild amphibian populations.

Overall, this study provides important information for future similar experiments screening for the effects of EDCs on the amphibian reproductive function. For example, it seems *Silurana tropicalis* gonadal differentiation is not completed by NF stage 66, and the sampling of the froglets several weeks after completion of metamorphosis would have provided a more accurate assessment of sex ratio and maybe also preliminary evidence of gonadal abnormalities. It must be noted that no gonadal abnormalities could be detected at NF stage 66, indicating that the percentages of gonadal abnormalities detected at completion of metamorphosis in previous studies, does not likely reflect the actual frequency of gonadal abnormalities that might have been manifest at sexual maturity. In addition, the present research highlighted the high variability between individuals in steroid plasma concentrations and in sperm quality in amphibian. Further research is needed to standardize a method when analyzing endpoints related to the reproductive function of sexually mature amphibian reared under laboratory conditions.

In summary, this research shows that exposure to xenoestrogens during larval development induces permanent gonadal abnormalities in *Silurana tropicalis* that persist at sexual maturity. In males, no direct link could be made between the

occurrence of gonadal abnormalities and the reproductive potential of the frogs, and further research is needed to investigate this hypothesis.

Appendix 1

Tissue dehydration

Solution	Immersion time /hours
70% IMS	3
90% IMS	2.5
95% IMS	1.5
100% IMS	1.5
100% IMS	1.5
100% IMS	1.5
100% IMS	1.5
Histoclear	1.5
Histoclear	1.5
Histoclear	1.5
Paraffin wax	1.25
Paraffin wax	1.25
Total	20

Staining process

Solution	Immersion time/seconds
Histoclear	900
100% IMS	120
90% IMS	120
70% IMS	120
Water	120
Haematoxylin	600
Water	600
Acid alcohol	20
Water	20
Saturated Li_2Co_3	20
Water	20
Eosin	40
Water	300
70% IMS	120
90% IMS	120
100% IMS	300
Histoclear	300
Total	3840

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