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Estrogenic Responses in Nile Crocodile (*Crocodylus niloticus*) Passively Exposed to Complex Mixture of Environmental Contaminants

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MSc in Biology
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Abstract

This study investigates biotransformation and endocrine disrupting responses in relation to chemical burden in liver and plasma of male and female Nile crocodiles (*Crocodylus niloticus*). Samples were collected from a commercial crocodile farm in Pretoria passively exposed to various anthropogenic aquatic pollutants.

Crocodile liver and plasma samples were analyzed for steroid concentration (testosterone, 11- ketotestosterone and estradiol) and various proteins (vitellogenin, zona pellucida protein, high density lipobinding protein and transthyretin), all parameters that previously have been identified as targets of endocrine disrupting chemicals in several species.

Analysis was performed by real time PCR and Principal components analysis (PCA) with Spearman's rank correlation coefficient. Groupings revealed significant correlating relationships (both negative and positive) between levels of biomarker responses and contaminants detected in individuals.

Overall, the data revealed production of the female oocyte protein vitellogenin in exposed male individuals, as well as high levels of estrogen and zona pellucida proteins, suggesting stress responses by exposure of endocrine disrupting contaminants in habitat.

Norwegian abstract

I denne oppgaven ble det sett nærmere på biotransformasjon- og endokrinforstyrrelser ved å analysere lever- og blodprøver hos Nilkrokodiller (*Crocodylus niloticus*) for ulike kjemikalier. Prøver ble hentet fra en kommersiell krokodilleoppdretter i Pretoria som bruker forurenset vann fra ulike antropogene kilder.

Lever- og plasmaprøver fra krokodillene ble analysert for steroider (testosteron, 11-ketotestosteron og østrogen) i tillegg til proteinene vitellogenin, zona pellucida, high density lipobinding protein og transthyretin, da de alle tidligere har blitt identifisert som relevante biomarkører for endokrinforstyrrende stoffer i flere arter.

Analysene ble gjennomført ved bruk av RT- PCR og prinsipalkomponentanalyse (PCA) med Spearman`s rangkorrelasjonskoeffisient. De ulike grupperingene avslørte korrelerende signifikante forhold (både negative og positive) mellom responser hos biomarkørene og de ulike stoffene funnet i individene. I generelle trekk viste de analyserte resultater produksjon av det østrogeninduserte proteinet vitellogenin, i tillegg til høye verdier av østrogen og zona pellucida proteiner hos eksponerte hanner. Dette indikerer stressresponser forårsaket av endokrinforstyrrende stoffer i habitat.

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Abbreviations

11-KT	11-ketotestosterone
AHR	Aryl hydrocarbon receptor
AR	Constitutively active receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
Atr	Atrazine
BCF	Bioconcentration factor
bp	base pairs
cDNA	complimentary DNA
Ct	Cycle treshold
CYP	Cytochrome P450
CYP11A	Cholesterol side chain cleavage enzyme
CYP19	Aromatase
DEPC	Diethylpyrocarbonate
dNTP	Deoxyribonucleotide triphosphate
E2	Estradiol / 17β-estradiol
EDC	Endocrine disrupting chemical
EIA	Enzyme immunoassay
ER	Estrogen receptor
ERE	Estrogen-responsive element
GnRH	Gonadotrophin-releasing hormone
GtH	Gonadotrophin
HPG	Hypothalamus-pituitary-gonad (in: HPG axis)
PCR	Polymerase chain reaction
PFAS	Perfluoroalkyl and polyfluoroalkyl substances

PFOS	Perfluorooctane sulfonate
POP	Persistent organic pollutant
ppm	Parts per million
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription polymerase chain reaction
T	Testosterone
TAE	Tris-acetate-EDTA
Tm	Melting temperature
TR	Thyroid hormone receptor
VTG	Vitellogenin
ZP	Zona pellucida protein

Background

Annual mortality events in Nile crocodiles (*Crocodylus niloticus*) were observed at the Olifants and lower Letaba River Gorge in Kruger National Park in South Africa from 2008 to 2012. The largest record of mortalities for Nile crocodiles in the Olifants River system was registered in 2008 with 170 reported incidents, followed by 28 cases in 2009 and further 30 in 2010. Many of the surviving crocodiles were lethargic (Ashton, 2010; Botha, 2010a). Researchers at the Kruger National Park suspected that increased anthropogenic aquatic pollution could be the reason for these mortalities. As a collaborative project between NTNU (Prof. Arukwe) and Pretoria University initiated in 2008, a crocodile farm with fertility problems was identified, using rearing water from a river recipient of sewage effluent. To further investigate the correlation between polluted water and contamination uptake in the Nile crocodile, water samples were collected over a 6 month period from the farm, as well as from waters outside of the farm for comparison. Tissue samples were collected during their routine slaughtering process.

Introduction

"In nature nothing exists alone."

- Rachel Carson, Silent Spring

Endocrine disrupting compounds (EDCs) interfere with natural metabolism and hormone biosynthesis, and alter vital regulations involving reproductive- and homeostatic control (Hernandez et al, 2013; Martenies & Perry, 2013). However, their routes of exposure and modes of action are still not fully revealed - especially within the groups of pesticides, the only class of chemicals with its main target to destroy living organisms.

Just after Second World War, the "organochloride pesticide revolution" saw its first light. During the following decades the usage of these persistent organic pollutants (POPs) evolved and increased worldwide (Heisey, 2007). However, since early 90s a decline in use and distribution has been observed for the more industrialized countries, mostly due to heavier restrictions on pesticide use.

The strict regulations of pollutants governed by EPA and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) in U.S.A and the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) established by the E.U in 2006, in addition to legislations through the Stockholm Convention from 2000, have greatly reduced the distribution and use of pollutants (Knaak et al, 2012; Vogt et al, 2012). The efficiency of most regulated chemical mixtures utilized has also increased, with fewer active ingredients while still obtain same degree of pest control (Klaassen, 2013). However, these regulations have not been successfully implemented into legislation of other high pesticide consuming countries, such as South Africa (Dabrowski et al, 2014b; London & Rother, 2000).

The current shortlist of pollutants regarded as representing a special threat to the public health by the United Nations Environment Program (UNEP), referred to as *the dirty dozen*, include dioxins, furans, polychlorinated biphenyls (PCB's), DDT, chlordane, heptachlor, hexachlorobenzene (HCB), toxaphene, aldrin, dieldrin, endrin, and mirex. These are banned in U.S and within the E.U for phase out (Kaiser & Enserink, 2000; United Nations Environmental Programme, 2001). Several of the "dirty dozen" are still

distributed in South Africa for social and economic reasons, such as DDT and chlordane. The continued use is based on their properties as effective pesticides for malaria mosquito and termite control, as they have still not developed resistance to it (Dabrowski et al, 2014b; Dalvie et al, 2009; van den Berg, 2009).

Most POPs include chemicals created intentionally, or as by-products of industrial activities. They are hydrophobic and lipophilic, which makes them not associated with aqueous phase, but usually partition into lipids and accumulate in organisms fatty tissue (Jones & de Voogt, 1999; Klaassen, 2013).

Persistency and movement of POPs are determined by their water solubility, the octanol/water partition coefficient (K_{ow}) and half-life in soil (DT_{50}). As many pollutants accumulate in soil many of them will reach surface water through runoff and leaching. Contamination of water by POPs are extensive, and in a study done by U.S Geological Survey (USGS) on major river basins across the country, they found that more than 90 % of water and fish samples from all streams contained one or several pesticides (Aktar et al, 2009; Kole et al, 2001).

The coherence between long-term elevated levels of POPs and decreased health in wildlife and humans has been documented in several studies, revealing implications such as increased mortality, reproductive failure and developmental abnormalities (Grant et al, 2013; Iguchi et al, 2006; Jørgensen, 2010). However, uptake and bioaccumulation of various pollutants varies in each species, and it is noted that especially reptilians has unique ways to handle uptake and biodegradation of xenochemicals (Sparling, 2010).

Reptilians have not previously been a field of scientific priority regarding exposure of pollutants and endocrine disruption, although new research within this field should be considered vital, as the global population of reptiles are declining and many under species are threatened. It should also be taken into consideration that reptilians, such as crocodilians, holds an important status as a bio indicator of aqueous pollution in several habitats (Böhm et al, 2013; Sparling, 2010).

The endocrine system of reptiles and mammals are delicate, and respond fast to slight changes of level of exposure to various emerging contaminants. Multiple substances may also act additively resulting in a biological relevant total concentration, also within less contaminated habitats. Accumulation of these substances has been

documented to affect multiple organisms both genotoxic, mutagenic and carcinogenic (Dabrowski et al, 2014a; van Dijck, 1986). Alterations of endocrine system receptors that governs important developmental- and cognitive cascades including modulation and regulation of early development, differentiation of tissues, reproductive success and behavior (Arukwe et al, 2012; Diamanti-Kandarakis et al, 2009; Louis J. Guillette, 2003; Norris, 2006; Sonne et al, 2015). It is therefore of great importance to gain further knowledge concerning these emerging, persistent substances.

Study outline

The aim of this study is to establish and pursue the biomarker route of steroids and key biomarker proteins, with environmental chemical analysis to discern cause- and effect relationships. This will be done by analyzing key parameters identified as targets of endocrine disrupting chemicals in several species, including steroids (testosterone, 11-keto testosterone and estradiol), vitellogenin and zona pellucida protein levels. Analyzed SPMD- water samples from the aquatic environment of animals will be investigated, as well as individual tissue contaminant levels in correlation with the various biomarker responses.

Our hypothesis is that there is a direct relationship between fertility problems registered within the Nile crocodile population and the source of rearing water. This may also be the explanation behind the unusual high mortality rates registered in the Olifants River system in Kruger National Park.

This is, to our knowledge, the first study to evaluate such effects in vivo from aquatic pollution and levels of steroid hormones, vitellogenin and zona pellucida protein in crocodilians. It may therefore contribute to valuable data and understanding for contaminant exposure for several aquatic species.

Nile crocodile as a model organism in aquatic toxicology

To further establish a cause- and effect route for polluted water and its endocrine effects on a high trophic level, it is plausible to assume that the Nile crocodile, as a reptilian apex predator can be of great usage for research and new information on distribution and implications of water pollution and its endocrine disrupting effects. Crocodilians are also accumulating pollutants both through diet and from its aquatic environment, and are quite widespread throughout various habitats and thus suitable for long- term bio monitoring. Their ability to display temperature dependent- sex determination may also be of great interest for further sex hormone studies. Numerous reports have documented that reptilians have similar or greater sensibility to contaminants than what is reported for birds and mammals (Crain & Guillette, 1998; Guillette et al, 2000; Newman, 2003; Sparling, 2010). Nile crocodiles can therefore be considered as a suitable model organism for providing relevant information on toxicological effects in aquatic biota.

Theory

The endocrine system

Stress caused by various pollutants has shown to affect the endocrine system and reproduction in various organisms, including crocodilians (Guillette et al, 2002; Milnes & Guillette, 2008; Sparling, 2010). Due to this sensitiveness to alterations by the sex steroid system, stress-induced alterations will affect normal sexual development, population level and fitness. How the endocrine functions and responds to external ques is therefore of vital ecological relevancy.

Normal endocrine function

Chemical regulators control every aspect of life in all vertebrates, including early development and gametogenesis, sexual differentiation, reproduction and behavior (Gleeson & Shalet, 2004; Norris, 2006; Olufsen & Arukwe, 2014). Because the endocrine system lacks the ability to detect environmental changes on its own, it is crucial that the nervous and endocrine system are tightly linked together, for maintenance of homeostasis and coordination of physiological processes such as growth and reproduction (Diamanti-Kandarakis et al, 2009; Norris, 2006). This communication is activated by the hypothalamus- pituitary (HP) axis, a dynamic endocrine system maintaining physiological conditions of reproduction by feedback mechanisms during exposure to stressors such as chemicals. The HP axis is an anatomical linkage between the hypothalamus and the pituitary gland consisting of the pituitary gland and neurosecretory neurons in the hypothalamus, projecting to the pituitary (Dang et al, 2015; Norris, 2006). It is strongly influenced by selective adaptations, and it has evolved independently in each species. Reptiles exhibit unique specializations of the female reproductive system for transferring nutrients from mother to offspring (Norris, 2006).

As the brain integrates internal and external cues to correctly regulate the incoming responses, the hypothalamic gonadotropin- releasing hormone (GnRH) activates the pituitary gland to secrete two separate gonadotropins (GtHs) in the bloodstream (just one in squamate reptiles), that are main modulators of reproduction by inducing and directing synthesis of sex steroid hormones (steroidogenesis) in the gonads (David J . Hoffman 2002; Levavi-Sivan et al, 2010; Norris, 2006). One of the gonadotropins, the follicle- stimulating hormone (FSH) regulates gamete production, while the luteinizing

hormone (LH) regulates gamete release. Reptiles, however, do not synthesize LH- like gonadotropin, but utilize FHS to regulate all gonadotropin- related functions. Both may also be regulating gonadal steroid secretion and synthesis of androgen (stimulated by LH) and its conversion to estrogen (stimulated by FSH) (David J . Hoffman 2002; Norris, 2006).

The principal androgens in vertebrates, including reptiles, are testosterone (T), 11-ketotestosterone (11- KT) and the estrogen17β- estradiol (E2) (Figure 1). Circulating steroid hormone levels are otherwise influenced by its synthesis, solubilisation, degradation, excretion and protection by specific transport proteins (Devlin & Nagahama, 2002; Norris, 2006).

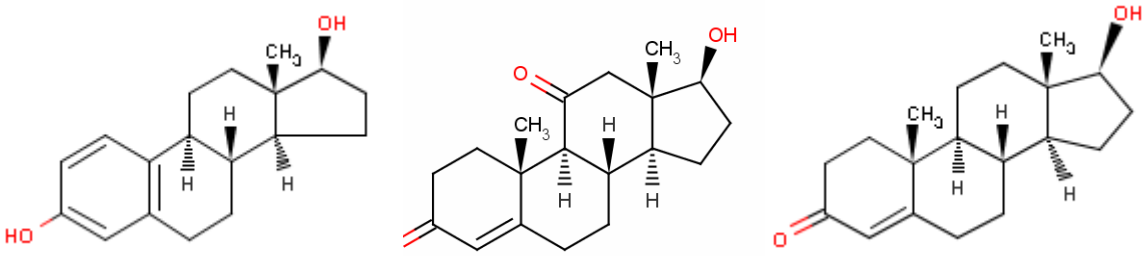


Figure 1. From left: 17beta estradiol, 11- keto testosterone and testosterone (National Center for Biotechnology Information, 2015).

Steroidogenesis and metabolism

The pathways of steroidogenesis plays an essential role in maintaining normal body functions as it involves regulations of sexual differentiation, reproduction, fertility, hypertension and physiological homeostasis (Miller & Auchus, 2011). Steroidogenesis are all the various processes by which cholesterol is converted to biologically active steroid hormones, occurring in specific tissues such as the gonads, adrenals, placenta and central nervous system. Still, the contribution of steroid synthesis is possibly species specific, and the overall pool of circulating steroids is not fully accounted for. The gonads are therefore considered as the major organ for steroidogenesis (Miller & Auchus, 2011; Young, 2005). Although it can be seen as adrenal, ovarian, placental or

other steroidogenic processes specifically linked to the glands, it may rather be a single process that is repeated in each gland with separate cell- type- specific variations (Klaassen, 2013; Miller & Auchus, 2011).

Steroid hormones are crucial for maintaining normal body function by mediating various physiological processes such as sexual differentiation, ion and carbohydrate homeostasis, immune system functioning, stress responses and reproduction. Classes of steroid hormones include adrenal glucocorticoids- and mineralocorticoids and sex steroids such as ovarian and placental progestogens, estrogens and testicular androgens. Sex steroids play an essential role in the reproductive system, involving development, behavior and sexual maturation regulation (Guerriero, 2009; Klaassen, 2013). The several pathways revolving the steroid biosynthesis are catalyzed by numerous steroidogenic enzymes- most of them are either from the cytochrome P450 (CYP) enzyme superfamily (type 1 in mitochrondria, type 2 in endoplasmic reticulum) or hydroxysteroid dehydrogenases (HSD), belonging to either the aldo- keto reductase or short- chain dehydrogenase/ reductase families (Miller & Auchus, 2011). E2 is synthesized from T by CYP aromatase (CYP19) catalyzation (Simpson et al, 2002).

Steroid hormones are inactivated and biotransformated mainly in liver, but also to some extent in kidneys and in the small intestines (Boelsterli, 2009; Devlin & Nagahama, 2002). The CYP450 superfamily are found in some prokaryotes and all eukaryotes, and essential in phase I active steroid hormones and xenobiotics are bioinactivated into hydroxylated metabolites, whereas the metabolites are further glucoronidated and sulfonated in phase II, ready for excretion (Boelsterli, 2009). Various CYPs mediate these xenobiotics, such as CYP2C19, CYP3A4/5 and CYP1A are hepatic expressed when exposure of endocrine disruptors (Hasselberg et al, 2005; Klaassen, 2013; Norris, 2006). CYP1A transcription are induced by the aryl hydrocarbon receptor (AHR), CYP2C by the constitutively active receptor (CAR), while CYP3A is regulated by the pregnane X receptor (PXP) (Boelsterli, 2009). Nuclear Receptors are divided in two superfamilies, where the first one includes the peroxisome proliferator- activated receptor (PPAR), the thyroid receptor (TR), the estrogen receptor (ER) and the androgen receptor (AR). The second family includes the aryl hydrocarbon receptor (AHR), an adverse receptor activated by numerous xenobiotics such as polychlorinated biphenyls, dioxins and polycyclic aromatic hydrocarbons (PAHs) (Figure 2) (Bersten et al, 2013). The AHR, upon ligand binding, forms a heterodimer with the AHR nuclear

translocator (ARNT) protein from the PAS family. This heterodimer binds to xenobiotic- responsive enchanters (XRE), where the main target genes include CYPs involved in xenobiotic metabolism (Boelsterli, 2009; Luecke et al, 2010). Steroid hormone receptors (also known as zinc-fingers) are also a large receptor family, that form dimers (hetero- or homodimers) (Boelsterli, 2009; Luecke et al, 2010).

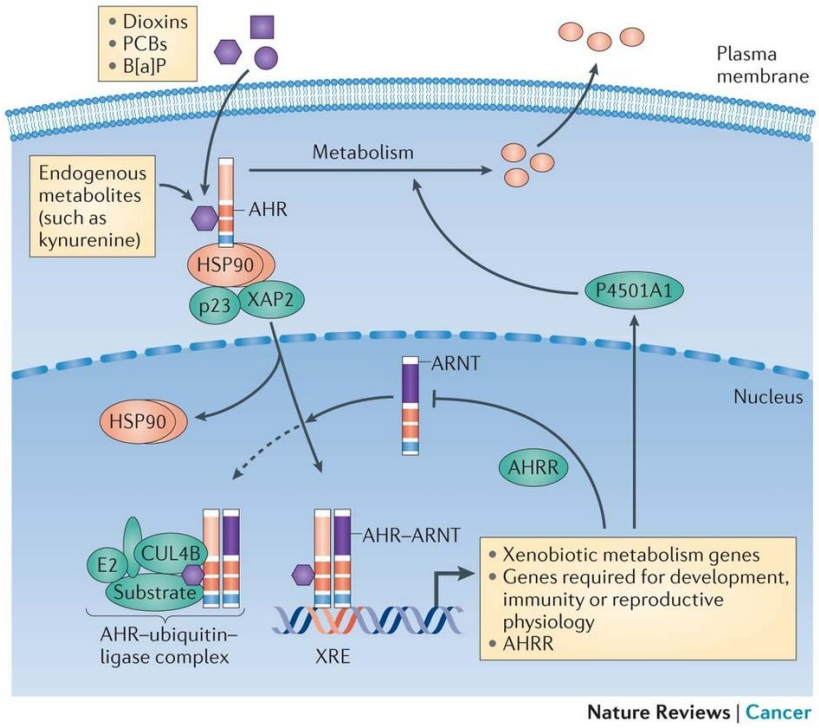


Figure 2. Various exogenous ligands (such as benzo[a]pyrene (B[a]P), 2,3,7,8-tetrachlorodibenzodioxin (TCDD), polychlorinated biphenyls (PCBs)) and endogenous ligands binding to the cytoplasmic aryl hydrocarbon receptor (AHR) (Bersten et al, 2013).

Xenoestrogens and the estrogen receptor

The estrogen receptor (ER) is the main mediator of 17β estradiol (E2) (Boelsterli, 2009). However, as the ER ligand cavity appears large in size for better binding with E2, it also allows binding of numerous xenobiotic- induced effects mimicking estrogenic or antiestrogenic action. These endocrine disruptors (ECDs) may bind to the ER and elicit an agonistic response. ECDs can be chemically unrelated ligands, such as various alkylphenols, phthalates, pesticides and other polychlorinated environmental chemicals (Guillette et al, 2002; Norris, 2006). Upon ligand binding, the ER forms heterodimers and the ligand- receptor complex binds to estrogen responsive elements

(ERE) at the DNA (Figure 3) (Boelsterli, 2009; Heldring et al, 2007; Klaassen, 2013). The ER has three isotypes, that all has been identified in vertebrates (also in reptiles/crocodilians) to have high affinity to estrogens: ERα, ERβ (also called ERβb or ERβ2) ERα and ERβ are primarily expressed in liver, and both found in steroidogenic tissue, such as the brain (Kuiper et al, 1997; Strobl-Mazzulla et al, 2008).

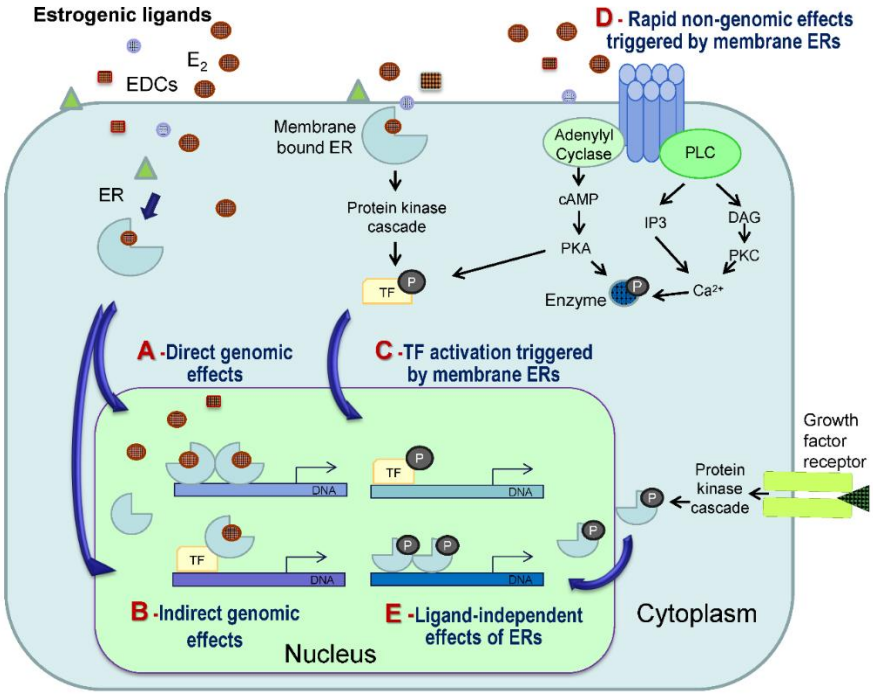


Figure 3. Scheme over the possible mechanisms involved in the cellular actions of natural estrogens (e.g., 17β-estradiol, E_2) and estrogenic endocrine disrupting compounds (EDCs) (Pinto et al, 2014).

Estrogen receptors in nonmammalian taxa, such as in reptiles may be more sensitive to some ECDs. Vonier et al (1996) found that alligator (*Alligator mississippiensis*) estrogen receptors could bind to atrazine (an herbicide), whereas mammalian receptors would not. In the same study atrazine and cyanazine also displaced E2 from the ERα in alligators, inhibiting 50 % of the estradiol from binding to the receptor. Interactions between some pesticides and ER may therefore be possible (Roberge et al, 2004; Sparling, 2010; Vonier et al, 1996).

E2 is mainly associated with female sexual development, reproduction responses and behavior in vertebrates. Maintenance and early increases of estradiol levels and aromatase activity, seem to correlate with sexual differentiation from undifferentiated gonads until development of female ovaries. It is the raising levels of E2 that drives the

development of oocytes, following meiosis and develop into mature eggs (oogenesis) (Grigg & Kirshner, 2015).

In males, environmental cues initiate reproduction by producing a crocodilian-equivalent GnRH by the hypothalamus. In vertebrates GnRH stimulates the pituitary to produce glycoprotein gonadotropins equivalent to Follicle Stimulating Hormones (FHS) and Luteinizing Hormone (LH). The LH stimulates androgen production by the male Leydig cells, the principal androgens in reptiles being testosterone, 11-ketotestosterone and dehydrotestosterone (DHT) (Licht et al, 1976; Moore et al, 2010; Norris, 2006; Sparling, 2010).

Testosterone can be locally converted to an E2 enzyme complex formed by the cytochrome 450 aromatase and NADPH cytochrome reductase that contributes to an additional important source of estrogen for the brain (McEwen, 2002; Strobl-Mazzulla et al, 2008). Growth of ovarian follicles in reptiles is stimulated by FHS, secreting estradiol (Figure 4). Circulating estradiol or estrogen mimics stimulates the production of vitellogenin (VTG) by the liver, a major supply of minerals to the oocytes. VTG can thereby be utilized as a biomarker for exposure to xenobiotic estrogens (Arukwe & Goksoyr, 2003; Grigg & Kirshner, 2015; Norris, 2006; Sparling, 2010).

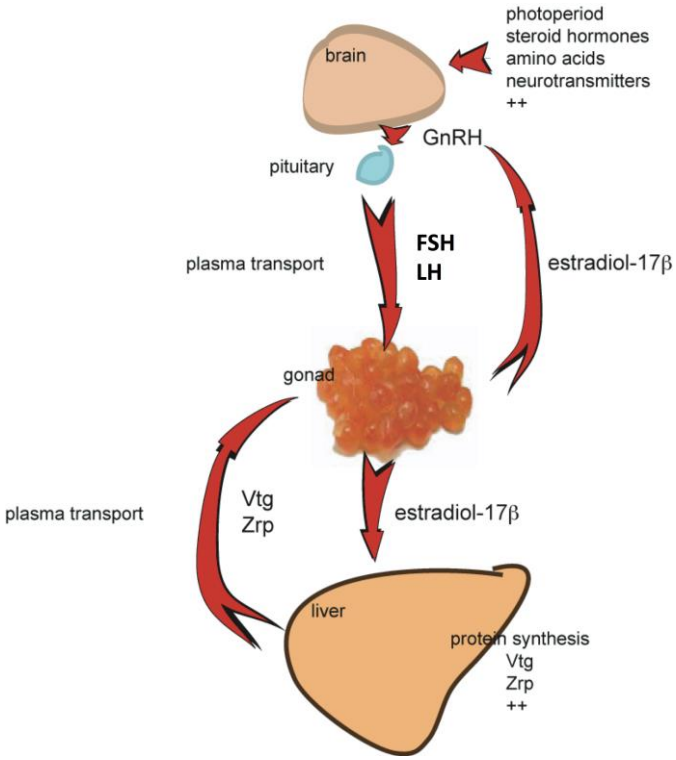


Figure 4. Schematic representation of the hypothalamus-pituitary-gonadal-liver (HPGL) axis during oogenic protein synthesis in female teleosts. The HPGL is regulated through the negative feedback mechanism by estradiol-17β (Arukwe & Goksoyr, 2003).

Endocrine disruption

Disruption of the sex steroid systems can permanently modify the organization and future function of the reproductive and endocrine system, by alterations of steroid controlled processes involving development and reproduction (Baker et al, 2013). Hormone mimicking may exhibit both agonistic and antagonistic effects in metabolism and secretion of endogenous hormones, including changes in the production and function of the various hormone receptors (Diamanti-Kandarakis et al, 2009; Louis J. Guillette, 2003). For instance changes in the expression or activity of cytochrome P450 (aromatase) will modulate estrogen levels, as the aromatase is regulated by phosphodiesterase (PDE) that converts cyclic adenosine monophosphate (cAMP) to 5 AMP. CAMP increases mRNA expression of aromatase, and thus estrogen levels as aromatase synthesizes estrogen production (Sparling, 2010). Pesticides, such as atrazine, can inhibit PDE, resulting in elevated concentrations of cAMP, and in turn increase expression of aromatase (Gibson et al, 1996; Parakh et al, 2006).

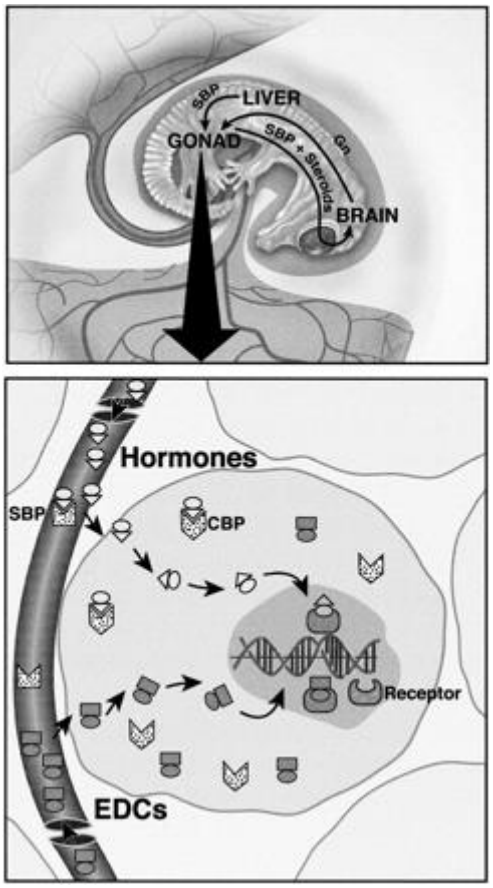


Figure 5: Target organs of endocrine disrupting compounds. Include a) brain, by production of hormones such as gonadotropins (Gn), b) liver, through altering hepatic degradation and the production of sex hormone binding proteins (SBP) and c) the gonard by alteration of the steroid production (Guillette et al, 2000).

Reptiles exposed to contaminants alter the endocrine system by receptor mediated, agonistic or antagonistic actions. Other changes include alterations in the enzyme action, such as in P450 aromatase activity, in hepatic biotransformation of androgens and alterations in gene expression (Louis J. Guillette, 2003).

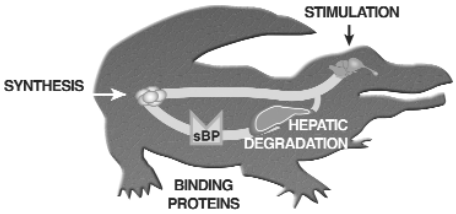
Although most pollutants, such as PAHs and phthalates binds to the Ah- receptor and ER, respectably, numerous pesticides may also bind to other receptors, such as the vertebrate neurotransmitter y- aminobutyric acid (GABA) receptor. ECDs can also bind to and alter modulations of other steroid receptors in plasma membranes and other signaling systems in the hypothalamus or pituitary (Norris, 2006).

Case studies of endocrine disrupting properties of pesticide exposure in reptiles

A number of industrial chemicals and pesticides have been shown to mimic naturally occurring steroids, altering reptilian organization or activation (Louis J. Guillette, 2003; Milnes et al, 2005; Sparling, 2010). Embryonic exposure causes organizational abnormalities by alterations of the chemical signals required for normal development, and thus disrupts the endocrine system (Louis J. Guillette, 2003). The effect of pesticide exposure on reptiles vary, affected by factors such as time of exposure (chronically or acutely), mixtures of contaminants and overall health of the individual (Sparling, 2010).

Guillette et al (1994) found that alligators (*alligator mississippiensis)* in Lake Apopka in Florida heavily contaminated with DDE, exhibited dramatically altered sex hormone levels and morphological alterations as compared with alligators in a control lake. Basal plasma testosterone levels were greatly decreased in alligators from the contaminated lake. Also, after stimulation with LH, that induces production of sex steroid hormones, estrogen levels increased, as expected in females, but also in males in the contaminated lake. This indicates an interference in the sexual differentiation and maturation in alligators by DDE (Boelsterli, 2009; Guillette et al, 1994).

- **Receptor Mediated**
  - **-Agonistic & Antagonistic**
    - *Estrogen receptor*
    - *Progesterone receptor*
- **Altered Enzyme Action**
  - *P450aromatase*
- **Altered Metabolism**
  - *Hepatic androgen*
- **Altered Hormone Availability**
- **Altered Gene Expression**
  - *P450aromatase*
  - *SF-1*



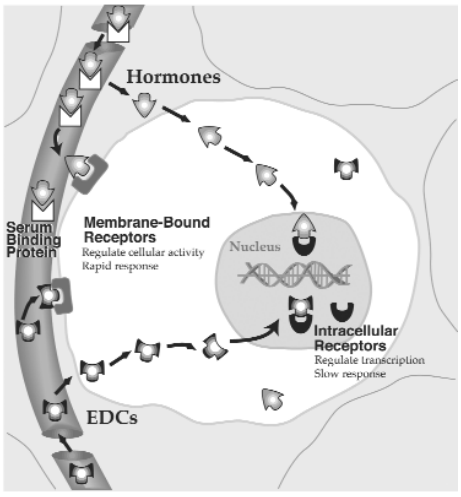


Figure 6: Effects of endocrine disrupting chemicals in reptiles at various levels of biological organization (Louis J. Guillette, 2003).

Further studies begun in the late 1980s and published in 2000 by same authors, revealed abnormalities in central and south Florida (USA) populations of the American alligator (*Alligator mississippiensis*) exposed to various contaminant mixtures used in modern agriculture such as insecticides, herbicides and fertilizers. Abnormalities included altered plasma sex steroid profiles, gonadal, genital, and immune tissue anatomy and hepatic steroid metabolism. Male alligators exposed *in ovo* to various pesticides exhibited significantly reduced plasma testosterone concentrations, aberrant testicular morphology and a small penis size. Females from contaminated locations showed significantly elevated plasma concentrations of estradiol as neonates, but reduced concentrations as subadults. They also exhibited a high frequency of polyovular follicles, an abnormality associated with high embryotic mortality and low fertility (Louis J. Guillette, 2003).

LaTorre et al (2013) exposed two groups of broad-snouted caiman (*Caiman latirostris*) to different doses of the insecticide Roundup for two months (11 or 21 mg/L; taking into account the concentration recommended for its application in the field) while one group was maintained as control. Roundup glyphosate- based formulation (RU) was used for parameter measurements of the immune system and growth responses in the caimans. The RU concentration was progressively decreased through the exposure period to simulate glyphosate degradation in water. Animals were measured and weighted at the beginning and end of the experiment, and blood samples were taken after exposure to determine total and differential white blood cell (WBC) counts, total protein concentration (TPC) and for protein electrophoresis. Results showed, compared to the control hosts, a decrease in white blood cell (WBC) counts, a higher percentage of heterophils, higher TPC and a negative effect on growth in the young caimans exposed to the Roundup formulation (Latorre et al, 2013).

Reptiles in an polluted environment

The Nile crocodile (*crocodylus niloticus*) as a large reptilian apex predator of freshwater systems throughout Africa, is exposed to contaminants at all life stages, considering their biological and ecological characteristics. Maternal transfer of both organic and inorganic contaminants has previously been described for reptiles, and eggs

are often used for analysis of maternal transfer burdens associated with developmental impacts *in ovo* resulting in reduced egg and embryo viability (Nagle et al, 2001; Rauschenberger et al, 2009; Sparling, 2010).

Reptiles are dependent on aquatic habitats to complete their early life stages and nest along rotting vegetation and wetlands close to surface waters, where eggs are buried in soils for incubation leading to a high exposure to contaminants in the surrounding matrix. The skin of most reptiles are considered rather impermeable, so it is not assumed that dermal uptake of chemicals are among the main routes of exposure for these organisms, but rather through the food web, with bio magnification increase the chemical potential of organisms with increasing organism trophic status, providing higher levels of contaminants accumulated in crocodilians (Grant et al, 2013; Jørgensen, 2010; Poletta et al, 2009; Sparling, 2010).

Although the overall interest in reptilian ecotoxicology has been low, their unique physiology has raised an increasing awareness, as they appear to accumulate and process chemicals in different ways than other vertebrates (Sparling, 2010). Reptiles, such as the Nile crocodile, are also important key stone species for aquatic biodiversity in many rivers and lakes (Ashton, 2010; Sparling, 2010). Today, the world wide population status among reptilians are severely declining. The World Conservation Union (IUCN) is a leading part in assessing the status of floral and faunal species in their Red List program. The assessment however, has not yet fully been reached for reptilians, and the conservation status of only 6 % of reptilians has been evaluated. In the first large scale study summarizing the global conservation status of reptiles, printed in journal of Biological Conservation, more than 200 experts assessed the extinction risk of 1,500 randomly selected reptilians, concluding that out of the 19% of reptiles threatened with extinction, 12% classified as Critically Endangered, 41% Endangered and 47% Vulnerable (Böhm et al, 2013; Sparling, 2010).

Water quality in South Africa and the Crocodylians of The Olifants River System

As South Africa is considered one of the largest importers of pesticides in the world, a high occurrence of emerging contaminants has been reported in various surface waters. Including waters utilized for human consumption (Manickum & John, 2014; Naidoo & Olaniran, 2014).

South Africa is a land with long dry periods and rainfall fluctuations, and is per 2014 considered as the 30th driest country in the world, thus highly dependent of surface water. 77 % of the total water used in South Africa today consist of surface water, while 9 % is groundwater and 14% is reused water from treated wastewater (Department of Water Affairs 2014; United Nations 2011). The few natural water resources make the reuse of treated effluents from wastewater treatment plants (WWTPs) an important way to obtain water income. Reused wastewater secure alternative water sources for landscape irrigation and industrial- and agricultural purposes, and the application of treated sludge (biosolid) to agricultural soils, has also expanded excessively. Both matrices contain significant amounts of emerging organic contaminants, including endocrine disruptors (Daso et al, 2012).

The extensive agriculture and industrialization also puts large pressure on the wastewater plants, which leads to higher levels of inadequately treated effluent water released into surrounding water sources. Today the estimated human annual growth rate for South Africa is considered high (1,2 %), with an urban population of 62 % (Annelie Roux et al, 2014; Naidoo & Olaniran, 2014; van Huyssteen et al, 2009).

In many self- sustaining communities living near to freshwater impoundments, surface water is often utilized for food preparations and drinking water, without proper purification. Aquatic species are also a vital part of the diet of rural populations in Africa. Therefore, tissue residues in the flesh may significantly contribute to the daily intake of contaminants in humans as well as the overall biota (Arukwe et al, 2015; Barnhoorn et al, 2015; Dabrowski et al, 2014b).

The Olifants River System

The Olifants River ("*the river of elephants*") consists of a network of rivers in north eastern South Africa, where the main parts of the basin lies in the Limpopo and Mpumalanga Provinces, joined by the Letaba River right before entering Mozambique (Figure 1).

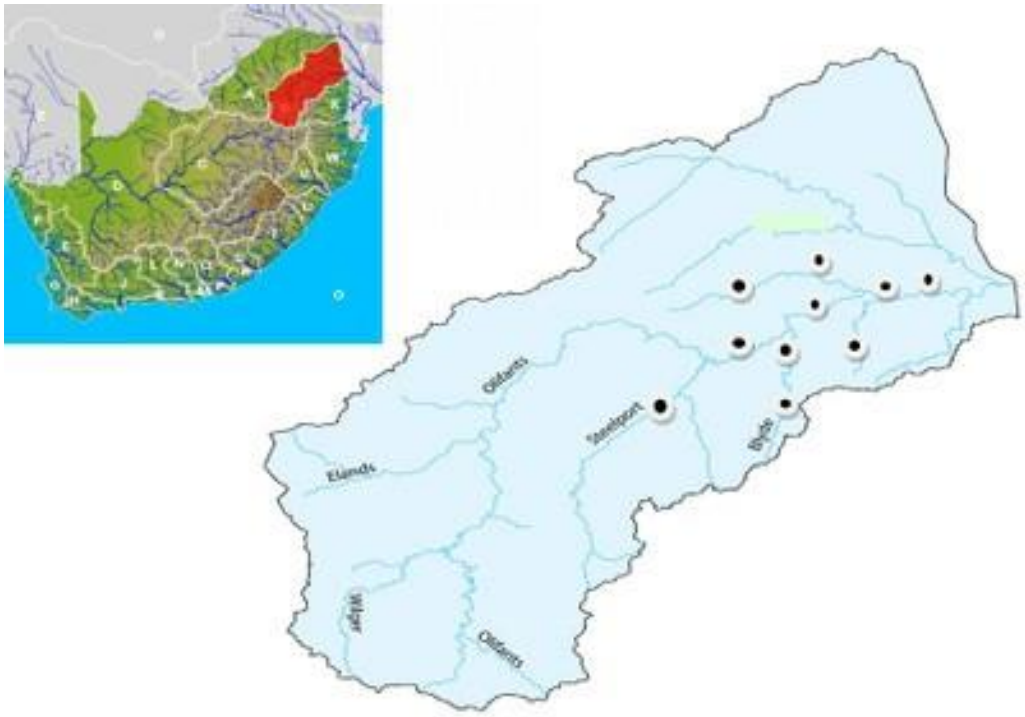


Figure 7: Location of Olifants River in South Africa (Swemmer & Mohlala, 2012).

The river is approximately 770 kilometer long with almost 4 million people living by its riverbed. The upper parts of the river are characterized by industrial large- scale coal mining, coal- fired power generation plants, agriculture and a diverse range of heavy and light industries (Ashton, 2010; de Lange et al., 2003). The middle reaches of the catchment contains large areas of irrigated agriculture as well as several platinum, chrome and vanadium mines, two ferrochrome refineries and various urban centers (Ashton, 2010). Therefore an increasing burden of contaminants has been found in rivers, from treated, partly treated and untreated effluents from mines, industries and sewage treatment plants, and the river has been described as one of the most polluted rivers of Southern Africa (Ashton, 2010; Botha, 2010b; Sparling, 2010).

The habitats of the Nile crocodile population in South Africa today, are mainly restricted to rivers and reservoirs along the middle and lower reaches, including the Incomati, Limpopo, Olifants, Maputo and Usutu basins, and lakes Kosi, Sibaya and St Lucia. However, the largest populations of crocodilians are within formally conserved areas in the National Kruger Park, regarded there as tourist attractions (Ashton, 2010; Joubert, 2007).

Materials and Methods

Chemicals and reagents

TRIzol reagent was purchased from Gibco-Invitrogen Life Technologies (Carlsbad, CA, USA). iScript TM cDNA synthesis kit, iTaq DNA polymerase, dNTP mix, iTaq TM Sybr® Green supermix with ROX were purchased from BioRad Laboratories (Hercules, CA, USA). Enzyme immune -assays for 17β estradiol (Cat. No. 582251), testosterone (Cat. No. 582701) and 11- ketotestosterone (Cat. No. 582751) was purchased from Cayman chemical company (Ann Arbor, MI, USA). All other chemicals were of highest commercially available grade.

Collection of samples and storage

Male and female (14 male, 15 female) Nile crocodile samples were sampled during periodic slaughtering at the farm abattoir at the Le Croc farm, a putative polluted farm in Pretoria (South Africa) in May and August 2010 and include gonads, kidney, liver, adipose tissues and blood. In this study liver and plasma samples were used. GPS location is: S 25 29.548, E 027 40.827, and water for the Le Croc farm were obtained directly from the Crocodile-West River. In addition, water samples were collected using semi- permeable membrane devises (SPMDs) from the recipient river of sewage effluents. Study animals were sampled at Le Croc farm, located downstream of the Hartbeespoort dam and the sewage treatment plant (STP) in Brits in South Africa. The Hartbeespoort dam is the primary recipient of all wastewater and sewage outflow from surrounding towns, including Johannesburg (Arukwe et al, 2015). The outflow water of the Hartbeespoort Dam is passing the crocodile farm on its eastern border, continuing to the Limpopo River. Study animals were approximately 2 years old, and total weight and length was not of significant difference between males and females. Crocodiles were given electro shock, and pithing was applied prior to decapitation. After collection of samples, they were labelled, snap-frozen in liquid nitrogen and transported to the laboratory at the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria at Onderstepoort, and subsequently shipped to Trondheim. Procedures and experiments conducted during sampling were done in accordance with laws and regulations controlling experiments with animals in South Africa. Because the crocodiles were not sacrificed for the main purpose of the present study, and tissue samples otherwise would have been gone to waste, the collection during the routine

slaughtering at Le Croc did not require approval from Institutional Animal Care and Use Committee (IACUC) or equivalent animal ethics committee. However, Le Croc farms operate in general under strict South African laws with a committee that governs guidelines for ethical care and sacrifice of the animals. All sampling procedures were also reviewed, or specifically approved for field study permit (Arukwe et al, 2015).

Figure 8. Map of the outflow water from the Hartbeespoort Dam through the Crocodile River-West in a northern direction, passing the sampling site (Le Croc farm), and then to the Limpopo River, at the border between South Africa, Botswana and Zimbabwe (Arukwe et al, 2015).

Experimental setup

Analysis of the semi permeable membrane devices (SPMD) was performed in collaboration with Dr. Monika Möder at Helmholtz center for Environmental Research UFZ, Department of Analytical Chemistry, Leipzig, Germany. Contaminant sampling in the aquatic environment was collected by using an Exposmeter Hydrophilic (EWH) and Lipophilic (EWL) for water samples based SPMD technology supplied by ExposMeter AB, (Trehorningen, Sweden). Polycyclic aliphatic hydrocarbons (PAHs) analysis was performed in collaboration with Prof. Francesco Regoli and his research group at Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy. Aliphatic hydrocarbons (C10-C40), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organo-halogenated pesticides (OCPs), chlorophenols, monoaromatic compounds (BTEX: benzene, toluene, ethylbenzene and xylene congeners), brominated flame retardants and trace metal were analyzed in crocodile liver by conventional procedures based on gas-chromatography with flame ionization detector, electron capture detector and mass detector, high performance liquid chromatography (HPLC) with diode array and fluorimetric detection, followed by atomic absorption spectrophotometry. For extended methods of chemical analyses and biomarker measurements see Appendix E.

Extraction of steroids from plasma

Tissue was homogenized in a 0.1 M sodium-phosphate-buffer (pH 7.42) in a volume ratio of 1:4 (400 μL buffer per 100 mg tissue), using a Glas-Col homogenator (GlasCol, Terre Haute, IN, USA) with a glass tube and a Teflon pistil. Homogenate was centrifuged (14,000 × g, 15 minutes, 4°C). Supernatant (100 μL from each sample) was transferred to 24 glass tubes for steroid hormone extraction with organic solvent.

The aqueous supernatant was thoroughly mixed with diethyl ether (4 mL) by quickly vortexing (15 seconds) and then frozen in liquid nitrogen for separation of the two phases, before the aqueous, steroid-containing ether phase was gently decanted into new glass tubes.

The process was repeated to ensure a high proportion of transfer of dissolved steroids. The walls inside glass tubes were washed twice, by adding more diethyl ether and thereafter left in a fumehood for evaporation of the ether and isolation of hormone raw material.

PBS fraction (100 µl) was subsequently added to the tubes and vortexed to dissolve all evaporated steroid containing extract on tube walls. Tubes with PBS fraction were then poured over into Eppendorf tubes and diluted (1:1) with EIA buffer.

Vortex mixing was used to aid the dissolution of the extracts. Purified and redissolved extracts were stored at 80°C until analysis.

Steroid hormone analysis (EIA)

Enzyme immunoassay protocol for estradiol, testosterone and 11- ketotestosterone was run according to the manufacturer`s instructions (Cayman Chemical Company) (Figure 5). All analyses were done at the same day to avoid repeated freezing and thawing of steroid extracts.

The 24 Eppendorf tubes with PBS fraction and EIA buffer were vortexed, and then 50 µl pipetted over to each well in a plate reader (96 wells), single replicas. 11-keto testosterone/ testosterone and estradiol AChE tracer and antiserum was made by mixing 6 ml with EIA buffer. Standard dilution series were expanded with two dilutions, and standards were added in two replicas in well plate. Two replicas of non-specific binding wells, blank wells and maximum binding wells were applied per well plate, including one well for total activity. Testosterone and estradiol were incubated on an orbital shaker for 1 and 2 hours, respectably. 5 µl tracer were added after washing plates (X5) followed by Ellman`s reagent (200 µl) in each well and then left in darkness for 60-90 minutes on an orbital shaker for color development. Changes in color were read repeatedly, starting when plates had developed for 60 minutes. 11-ketotestosterone kits were incubated at 4°C for 18 hours and developed for 90-120 minutes with repeated absorbance readings during the final development period. Absorbance was read at 405 nm using a Bio-Tek Synergy HT microplate reader (Bio-Tek instruments, Winooski, VT, USA).

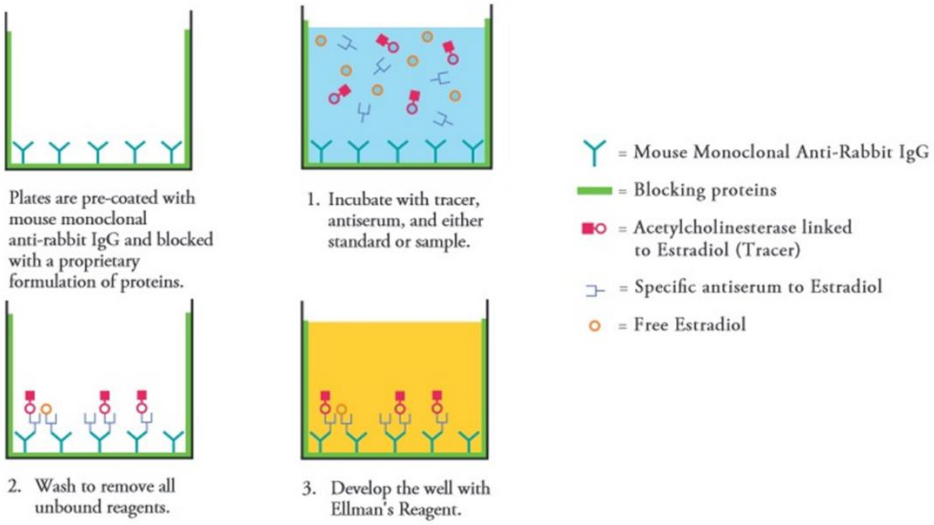


Figure 9. Steps in the performance of the enzyme-immunoassay (EIA) for steroid hormone assessments (Modified from Cayman EIA kit booklet).

Steroid concentrations in samples were quantified according to a standard curve fitted in Excel (1997) using a 4-parameter logistic fit plotting the % B/B0 (sample bound / maximum bound) versus log concentrations. Standard curves are included in Appendix A.

Gene expression analysis

Steroid/ xenobiotic- and estrogenic response related expressed genes were investigated in liver, which is a target organism for metabolism and estradiol activity. For measurements of gene expression levels, a relative quantification of messenger RNA (mRNA) was used for measurements by a two- step real-time reverse transcription polymerase chain reaction (real-time RT-PCR) assay. MRNA levels provide a "snapshot" of gene expression at the moment tissue was sampled (Nolan et al, 2006). Such gene expression analysis may reveal valuable information about toxic receptor-mediated mechanisms, such as endocrine disruption (Villeneuve et al, 2007).

RNA isolation of liver

When preforming a gene expression analysis it is crucial that high quality RNA is utilized, as quality of RNA will impact all downstream PCR reactions as well as the correct quantification basis (Yee et al, 2014). Therefore samples were kept in low temperatures (on ice) at all times and free from RNases. RNase inhibitors were added during the process to protect samples from base- and enzyme-catalyzed hydrolysis for

preservation of RNA integrity. Residues from biological extracts and reagents used during the isolation procedures, may cause impurities that interferes with downstream analyses and thereby interfere with correct quantification of RNA (Yee et al, 2014).

Total hepatic RNA extraction was preformed according to established procedures, and extraction was done by the guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987), as described by TRIzol manufacturer (Gibco-Invitrigen Life Technologies). The basic of this method is that RNA is separated from DNA after extraction with an acidic solution containing guanidium thiocyanate, sodium acetate, phenol and chloroform, followed by centrifugation. Total RNA will remain in the upper aqueous phase, while DNA and proteins remain in interphase or lower organic phase. Total RNA are then recovered by adding of isopropanol (Chomczynski & Sacchi, 2006).

The homogenization of RNA in TRIzol was performed using a Kinematica Polytron PT 3000 homogenizer (Kinematica AG, Markham, Ontario, Canada), approximately 1 mL TRIzol per 50-70 mg liver tissue. Homogenized samples were incubated in room temperature (5 minutes) and added chloroform (200 µL per mL TRIzol) for phase separation. Then samples were shaken hard (15 seconds), incubated again in room temperature (2-3 minutes) and centrifuged (12 000 x g 15 minutes, 4 ^{o} C) for phase separation.

Aqueous phase was collected by adding isopropanol (500 µL per mL TRIzol originally used), incubated in room temperature (10 minutes) and centrifuged (12,000 × g, 10 minutes, 4°C). RNA supernatant were removed and pellet washed in 75 % ethanol (1 mL per mL TRIzol), followed by vortexing (briefly) and centrifuged (7500 × g, 5 minutes, 4°C). Ethanol was discarded and RNA pellet was left to air dry before resuspending in DEPC (diethylpyrocarbonate)- treated Milli-Q water (150 µL) and incubated for 10 minutes (60 °C). Samples were thereafter stored at -80 °C awaiting further analysis.

RNA Quantification

Concentration and purity of RNA extracts were assessed by a NanoDrop® ND-2000c UV-visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). This is a spectrophotometric approach, where the analyzing of RNA quantity and quality are based on properties within the various molecules causing them to absorb

light at different wavelengths (Box et al, 2011; Desjardins & Conklin, 2010). Absorbance at 260 nm (A260) was used to determine nucleic acid concentration, by applying RNA (1 μL) onto the end of the fiber optic cable of the NanoDrop instrument. To assess purity that could be jeopardized by endogenous proteins and/or reagents, A260/A280 and A260/A230 ratios were used. These ratios should lie above threshold of $1{,}8 \geq 2.0$ nm (A260/A280) and approximately 1.8- 2.2 (A260/A230) (Box et al, 2011; Desjardins & Conklin, 2010).

Real- Time Reverse transcription PCR

To assess amount of particular gene transcripts, the reverse transcription of RNA into complimentary DNA (cDNA) can be utilized as template in PCR assays (RT- PCR). This can be carried out through two primary ways; either by including the RT step into the same tube as the PCR reaction (one step) or, by a two-step approach conducted/preformed in this experiment. This involves creating cDNA in a separate RT step before adding cDNA to the real time PCR. From this, a pool of cDNA can be generated and stored for a long time, and provides a highly defined DNA content for the synthetic oligo. However, it may also increase risk of contamination by increasing hands- on time, compared to the one- step approach (Bustin, 2000; Bustin & Mueller, 2005; Pfaffl, 2004). However, in the one step method gene specific primers are utilized, and thereby the whole procedure has to be repeated for each gene of interest, and fragile RNA has to be stored for future analysis. By a two-step method, all cDNA can be made in one reaction. This can later be used for real time PCR analysis, which is a huge advantage as cDNA is much more suitable for storage than RNA (Wacker & Godard, 2005).

cDNA synthesis

A complete conversion of all RNA to cDNA, and synthesizing cDNA for downstream real-time PCA, should result in a cDNA pool representing the original RNA profile. However, contaminating inhibitors in the RNA extracts might contribute to differential RT- PCR efficiencies between samples (Pfaffl, 2004). Also, yield and quality of cDNA can be highly variable, especially cDNA yield from sequences near the 5' end of partially degraded mRNAs are less degradable than from sequences near the poly-A tail. Therefore, it is important with reliable RT enzymes, primers and reaction mix composition, to achieve high quality results from downstream real-time PCA assays

(Pfaffl, 2004). For the iScript kit used in this experiment, iScript reverse transcriptase (RT) was used. This iScript contains a modified Moloney murine leukemia virus (MMLV)- derived RT with ribonuclease (RNase H) activity (BioRad), which lacks DNA endonuclease activity and has a lower activity of RNase H (Gerrard et al, 2012). The iScript reaction mix contains both oligo dT and random primers to obtain a maximum number of cDNA transcripts, as random hexamer primers anneal to all types of RNA (Jacob et al, 2013).

cDNA synthesis protocol

The synthesis was performed as described in the protocol of the manufacturer (Bio-Rad). The reaction mix and thermal parameters are presented in table 1 and 2, respectably. RNA extracts were diluted with DEPC- treated Milli- Q water to concentrations of 500-100 ng/ µL, and the master cycler was set with a heated lid (105°C). Replacing RNA with nuclease free water was done for negative control. PCR program on T100 Thermal cycler (Bio-Rad) used for generation of total cDNA. 1 µg total RNA was used to produce a total volume of 20 µL cDNA per sample. All RNA samples used for cDNA synthesis had an A260/A280 ratio within the range of 1.8-2, although they were generally detected at the lower range, indicating low traces of RNA. The obtained cDNA were afterwards stored at -20 °C for further use.

Table 1. Reaction mix with volume per reaction (µl).

Reaction mix with volume per reaction (µl)	
5 x iScript reaction mix	4
iScript reverse transcriptase	1
Nuclease free water	10
1 µl total RNA/Nuclease free water	5
Total	20

Table 2. Thermal parameters used for cDNA synthesis.

Steps	Temperature (°C)	Duration (minutes)
Primer annealing	25	5
First strand synthesis	42	30
Reaction termination	85	5
(Hold)	4	

Quantitative (real-time) Polymerase Chain Reaction

Real- time Polymerase chain reaction (real- time PCR) is used to quantify and amplify targeted DNA molecules. A specific primer pair for the selected DNA sequence is used for amplification. Also, the amplification of template cDNA can be monitored at each cycle of the PCR reaction by use of fluorescent labeling (fluorescent reporter probes or a nucleic acid stain) (Gibson et al, 1996). Here, the cycle threshold (Ct) will be registered, which is where the recorded fluorescent signal in the interpolated cycle for the first time is statistically significant above background fluorescence. Recorded Ct value can be converted into exact copy number by comparing against a generated standard curve with known concentrations, or relative amount of original templates in sample by comparing against standard curve with a known dilution factor (Bustin, 2000; Gibson et al, 1996). Nucleic acid stain iTaq TM Universal SYBR® Green supermix with ROX from Bio-rad was used for labeling and quantification in this experiment. SYBR green preferentially binds to double-stranded DNA, although it also to some extent can bind to single-stranded DNA and RNA. When SYBR green binds to double-stranded DNA it will emit a recognizable fluorescent signal, and will increase with increasing amount of product (Bustin, 2000). The efficiency and specificity of amounts of DNA of the real-time PCR reaction can be affected by primer design, the amplicon sequence and choice of PCR reaction mixture components (Bustin, 2000).

Primer optimization and design

It is essential with a reliable primer design, as the specificity of the real-time PCR with SYBR green rely on accurate amplification of the gene sequence of interest. SYBR green dye binds to double-stranded DNA and exhibit high fluorescence, while the emitted fluorescence in an unbound state is low (Bustin, 2000; Nolan et al, 2006) Because SYBR green binds to all double-stranded DNA, any double- stranded DNA will be amplified including wrongly amplified DNA sequences, primer- dimers and

genomic DNA. This can affect the result. To avoid this, one of the PCR primers should be able to span an intron; 5 or 6 bases of the 3' end attach to an exon. The rest of the primer should bind to the adjacent exon (Ginzinger, 2002). According to Bustin (2000) the optical primer length is 15-20 base pairs, the primer G/C content should be between 20- 70%, and primer pairs should aim for same melting temperature (Tm) around 58-60°C. Also, primers with only two G/Cs within the last five nucleotides at the 3'end are somewhat unstable and therefore make unspecific priming and primer-dimers less likely to occur (Bustin, 2000). Shorter amplicons (below 100 bp) are considered most optimal, as shorter amplicons are more prone to denature during the denaturation step of PCR, and by this amplify more efficiently. To ensure that the observed fluorescent signal produced during the real-time PCR are from the target products, a melting curve analysis was used. One single peak indicates one single PCR product. Concentrations of dideoxyribonucleotide triphosphates (dNTPs) and Mg2+ should also be optimized in PCR reaction mixture, for better PCR efficiency (Bustin, 2000). All primers were also tested for functionality and compatibility with PCR setting before use in real-time PCR. Primers that produced weak or multiple bands during primer testing, were further tested at different annealing temperatures. As some primers for Nile crocodile are not currently available, alternative primers from similar species were used. Primer sequences, annealing temperature and target genes are listed in Table 3, and performed primer tests with conventional PCR are listed in Appendix B.

Table 3. Primer pair sequences for genes of interest, with sequence reference and annealing temperature used for real-time RT-PCR

Target gene	Forward	Reverse primer	Annealing temp. (°C)
Vitellogenin (216 bp)	TACCCGAAGAAGACCATTCC	GGGTCTGCTGGAGTACCAAT	60
Zona pellucida (85 bp)	GTCTCTTCCACAGCCTGTCA	CTGCCCTGTCCCAAATAGAT	60
High-density lipoprotein binding protein (HDLBP) (145 bp)	GCTGAGGACTGCCAGTATGA	GCCACCATAATGCAATTCAG	60
Transthyretin (110 bp)	TGATTCCAAATGCCCACTTA	CTTGCCAGTCTCCATCTGAA	60

Protocol Real- Time Polymerase Chain Reaction

Quantitative real-time PCR was performed with iTaq TM Sybr® Green supermix with ROX, as described by the manufacturer (Bio-Rad), using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA) and MxProTM QPCR software. Reagents added in each well/reaction mixture used, thermal parameters and cDNA dilutions are listed in

Table 4 and 5. Negative controls lacking cDNA template were included to exclude external nucleic acid contamination.

Table 4. Thermal cycling program used for quantitative (real-time) PCR.

Step	Temperature (°C)	Duration
Initial polymerase activation	95	3 min
Polymerase Chain Reaction:		
Denaturation	95	30 s
Annealing	60	15 s
Extension	72	15 s
(40 cycles)		

Table 5. Reaction mixture components for quantitative real-time PCR.

Volume per reaction (µL)	
iTaq SYBR Green supermix with ROX	10.0
Autoclaved H2O	4.0
Forward primer	0.5
Reverse primer	0.5
cDNA template (dilution 1:6)	5.0
Total volume 20	

The iTaq DNA polymerase are used by iTaqTM Sybr® Green Supermix with ROX, with an antibody-mediated hot-start sequestering polymerase activity before the PCA denaturation step (BioRad). An optimized blend of dNTPs, MgC12, ROX and SYBR Green I dye was added into the supermix.

Real-time PCR data analysis

Results from real-time PCR were normalized against the total RNA utilized in RT-PCR. This is to ensure that measured gene expression from each sample is quantified on equal terms, as variations in starting material will lead to misinterpretation of target gene expression profiles, if not accounted for (Bustin, 2000). Normalization was done by a pre-made standard plot of Ct versus log copy number, derived from plasmid standards with established dilution factors for the analyzed genes.

Statistical analysis

Given homoscedasticity, all group means for males and females was tested by Student t-test in Microsoft Office Excel (2007) to detect significant differences between males and females. Chemical data together with biochemical and endocrine disruption parameter results were analyzed using Principal component analysis (PCA) with a significance level of $\alpha = 0.05$. The PCA calculations were produced using Umetrics SIMCA (version 14.0.0.1359), and graphically represented through Adobe Illustrator (CS6) software. The analysis was performed for chemical levels on all individuals, both females and males, with special regards to significant biomarker correlation.

Possibly correlated variables were evaluated using Spearman's rank correlation coefficient (Spearman's Rho) in XLSTAT (version 2015.4.01.20059). Pearson product-moment correlation coefficients (PCCs) were also calculated for the complete data set, as an addition to Spearman's Rho. However, as PCC is a parametric measure relying i.a. on the normality of the dataset, and being more sensitive to outliers, it was judged to be inferior to the more conservative Spearman's Rho. For extended data of PCA statistical analysis see Appendix E.

Standard curve for steroid concentrations in samples were quantified and fitted in Excel (1997), using a 4-parameter logistic fit plotting the% B/B0 (sample bound / maximum bound) versus log concentrations. Standard curves are included in Appendix A.

Results

Levels of contaminants measured by semi permeable membrane device in river, canal and dam at Le Croc farm

Water analysis from inside the farm, the canal and river showed a generally higher contaminantional burden within the dam, except for levels of propazin, terbutylazin, metolachlor and metoxuron, which were of highest values in the ingoing river (Figure 10). Especially high dam-levels of carbamazepine (713, 5 ng/sampler), desethylatrazin (699, 3 ng/sampler), caffeine (599, 2 ng/sampler), simazin (526, 9 ng/sampler) and atrazine (273, 4 ng/sampler) were detected. Carbamazepine (5H-Dibenz[b,f]azepin-5-carbamid) are mostly used as a neuroleptic drug, desethylatrazine **(**6-chloro-N-(propan-2-yl)-1,3,5-triazine-2,4-diamine) is a degradation product of the herbicide atrazine (1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine) and simazine (6-Chloro-*N*,*N*′-diethyl-1,3,5-triazine-2,4-diamine) is a herbicide often used in mixtures with atrazine.

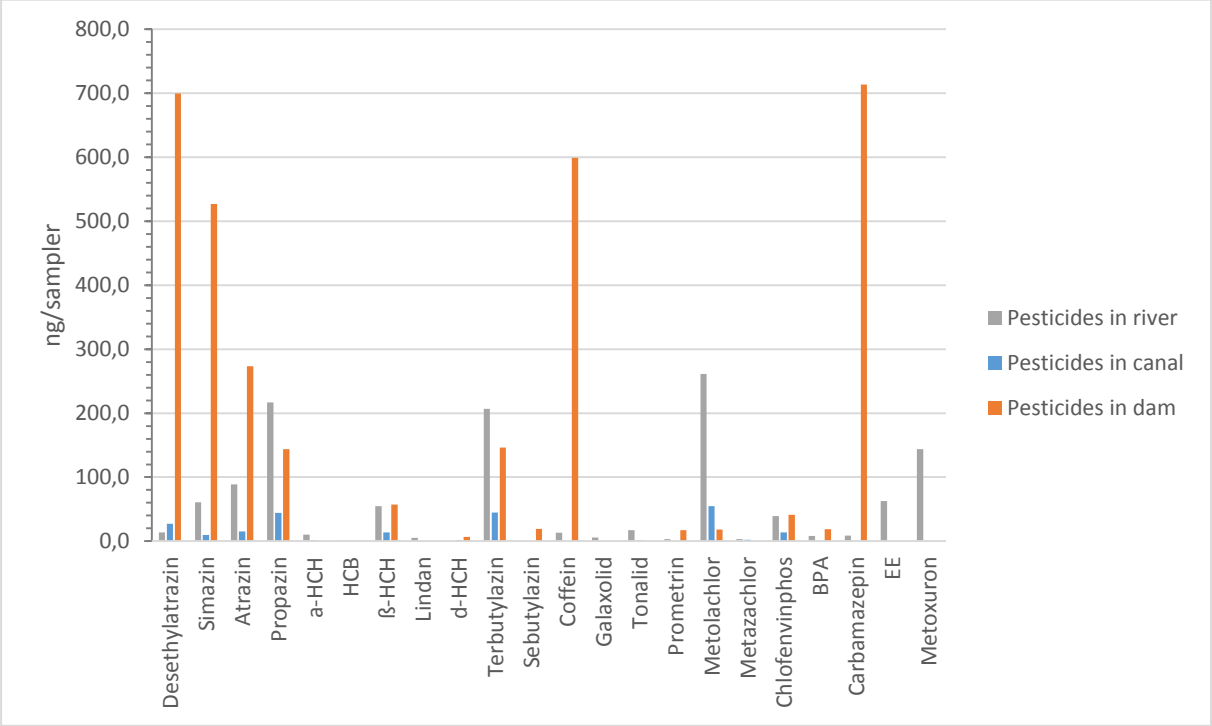


Figure 10. Levels of contaminants absorbed by SPMD in river, canal and dam nearby and inside Le Croc Crocodile farm, South Africa. Full datasheet in Appendix D.

Biomarker responses in females and males from RT- PCR analysis

For all results of biomarker analysis, see Appendix E.

Detectable transcripts of ZP were found in both male (5, 96 and female (5, 01) individuals at the farm, with a higher level in hepatic male tissues (Figure 11). No statistical differences were detected for female and male expression of ZP, according to student t-test (0, 67).

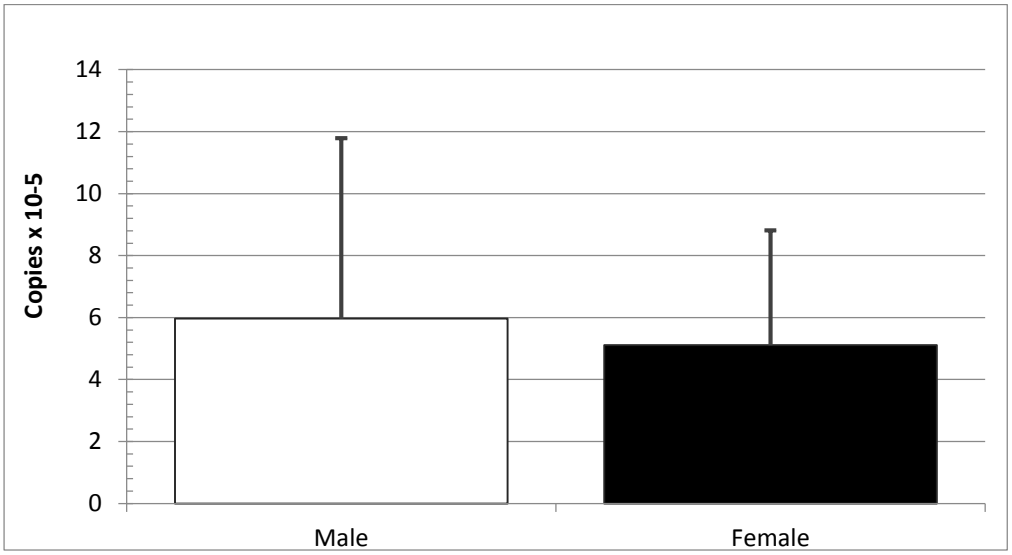


Figure 11. Transcript levels of zona pellucida (ZP) in liver.

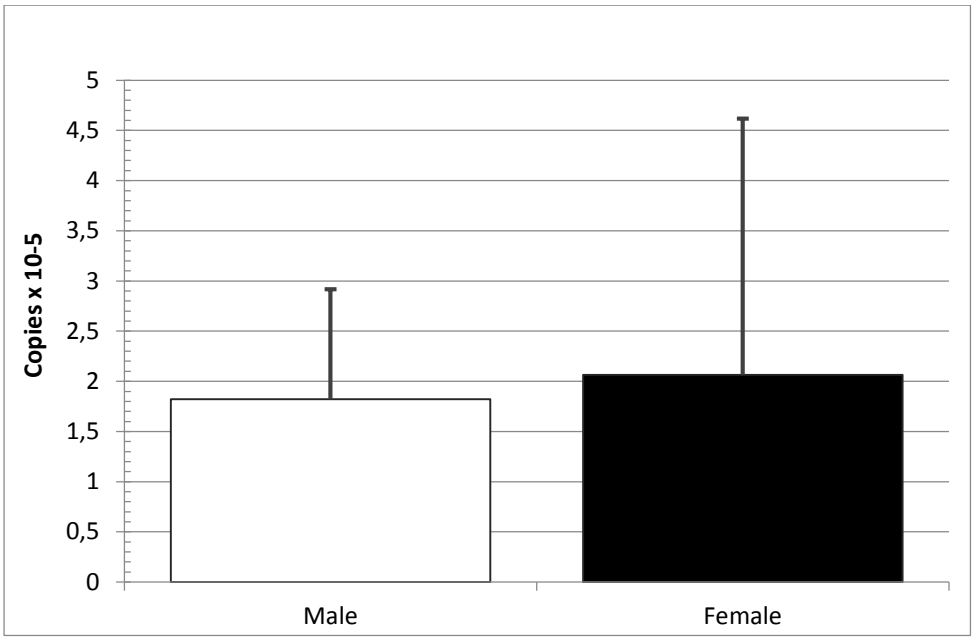


Figure 12. Transcript levels of high-density lipoprotein binding protein (HDLBP) in liver.

High density lipobinding protein was not found to be significant higher in either males or females, with a relative copy number of 1, 82 in males and 2, 06 in females. Student t- test value was estimated to 0, 78.

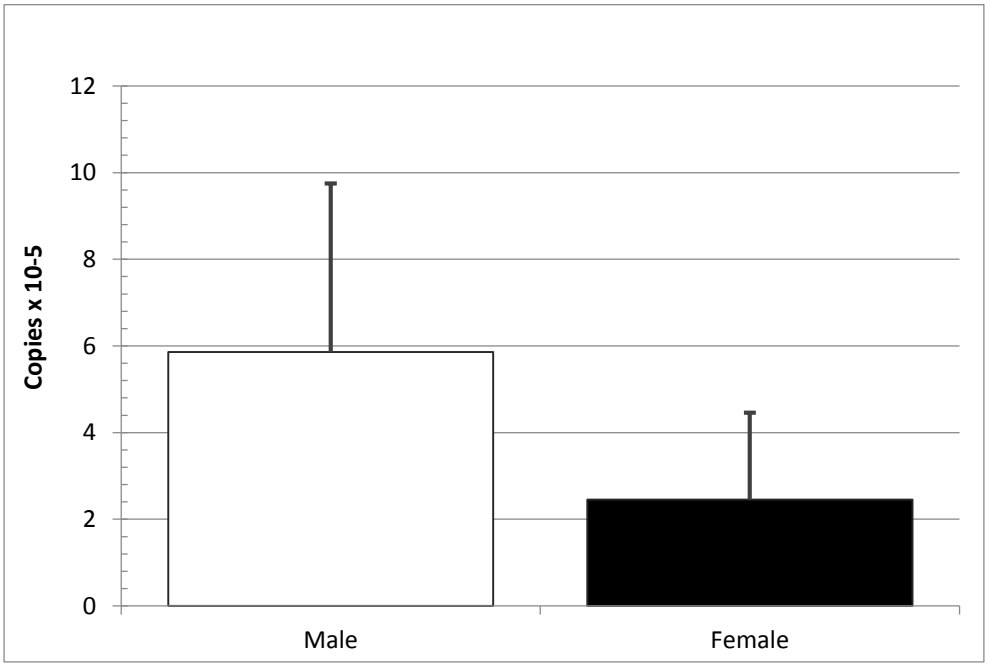


Figure 13. Transcript levels of transthyretin (TTR) in liver.

Transcript levels of TTR were generally higher in males (5, 86) than females (2, 72), with a statistically significant difference between the genders by student t-test (0, 04).

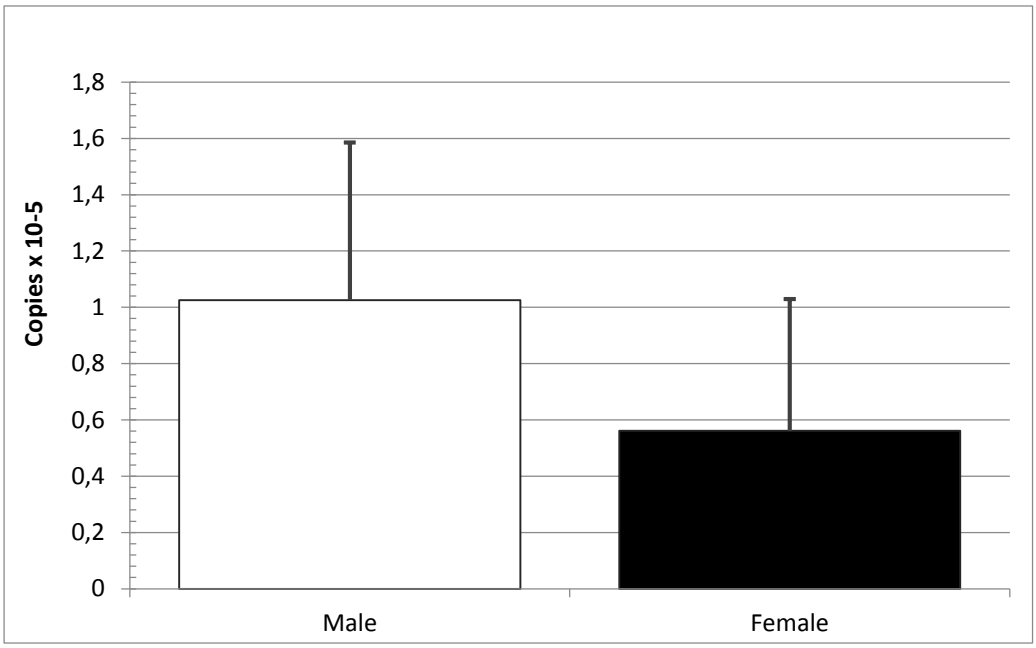


Figure 14. Transcript levels of vitellogenin (VTG) in liver.

Transcript levels for VTG was detectable in five male individuals with a mean of 1, 03, and 0, 69 in females. Student t- test showed that the levels of VTG in males were significantly different from female results (0, 29).

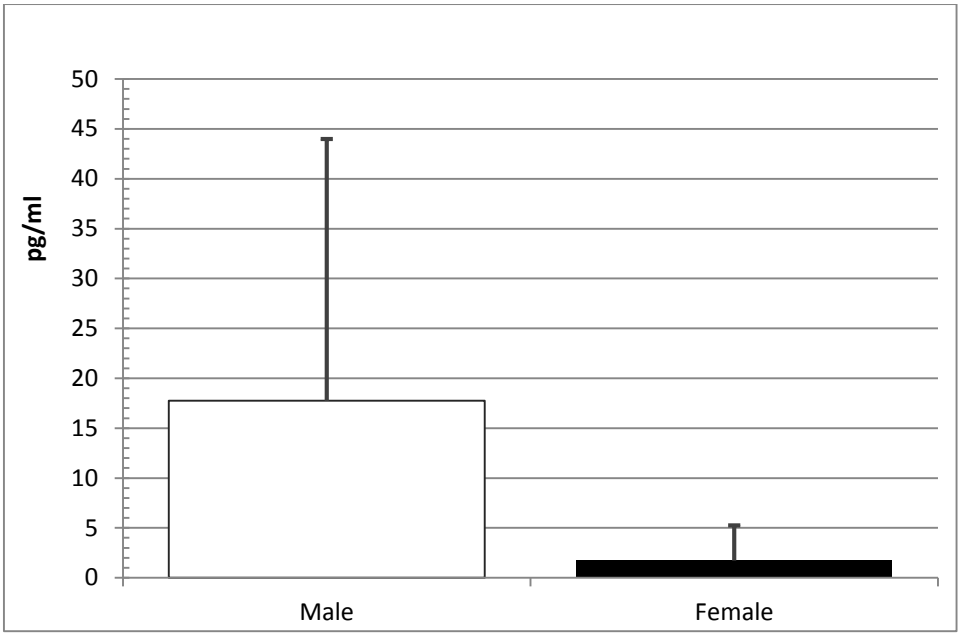


Figure 15. Levels of testosterone (T) in plasma (0, 04).

Higher levels of T were found in all males, with a mean of 27, 9 and 1, 73 in females. Student t-test confirmed statistical difference between genders with result of 0, 04.

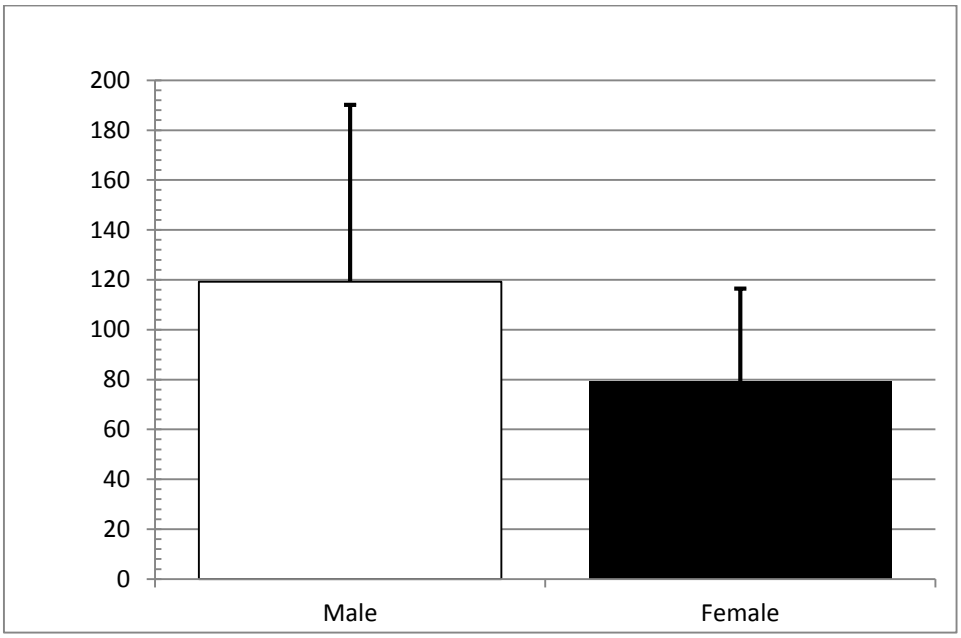


Figure 16. Levels of estradiol (E) in plasma (t-test: 0, 08).

Estradiol were found in both males (131, 21) and females (79, 125), with a significant difference of 0, 08 with student t-test analysis.

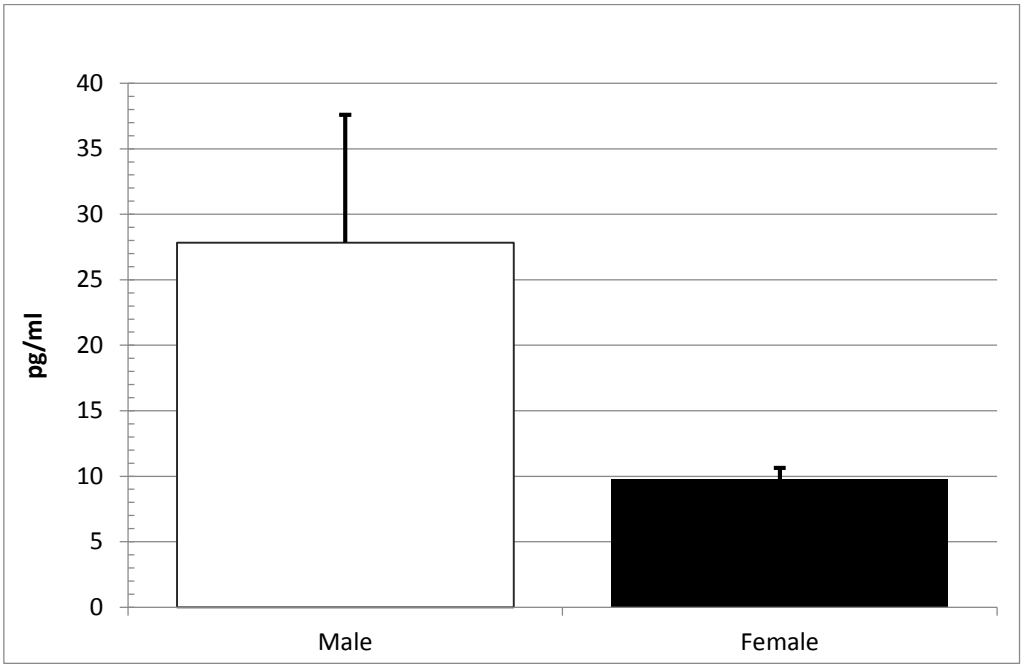
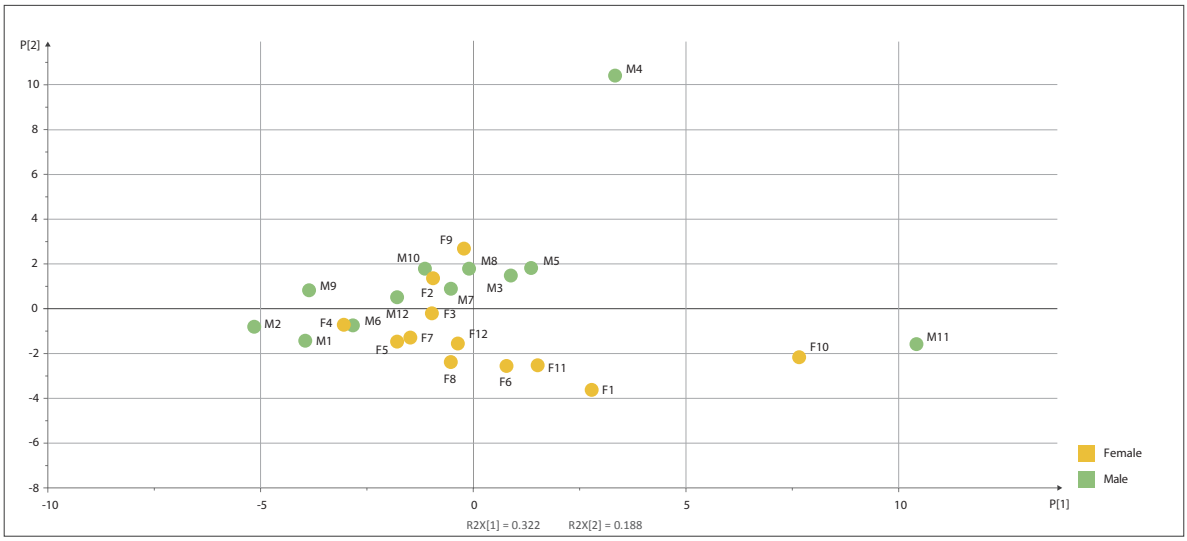


Figure 17. Levels of 11- ketotestosterone (11-KT) in plasma (t-test: 0, 10).

A significant difference between males and females was found in 11- KT analysis, with a t-test value of 0, 10 and a mean concentration of 27, 83 in males and 9, 8 in females.

Principal Component Analysis

Principal component analysis (PCA) was used to evaluate correlations between the various endpoints (i.e measured gene expressions and protein levels) to level of contamination burden in each individual. This was done by scattering individuals according to the different exposure groups. Patterns and trends were further studied, to investigate significant relations and not just comparing exposure group to mean values. Loading plots of PCA revealed significant groupings of individual contaminants, and several significant correlations with the selected biomarkers. Aliphatic hydrocarbons and PAHs showed significant negative relationships with endpoints in both male and females, and several trace metals showed a positive significant correlation with high density lipobinding protein (HDLBP). The significance of findings were evaluated statistically by Spearman`s rank correlation coefficient.



a)

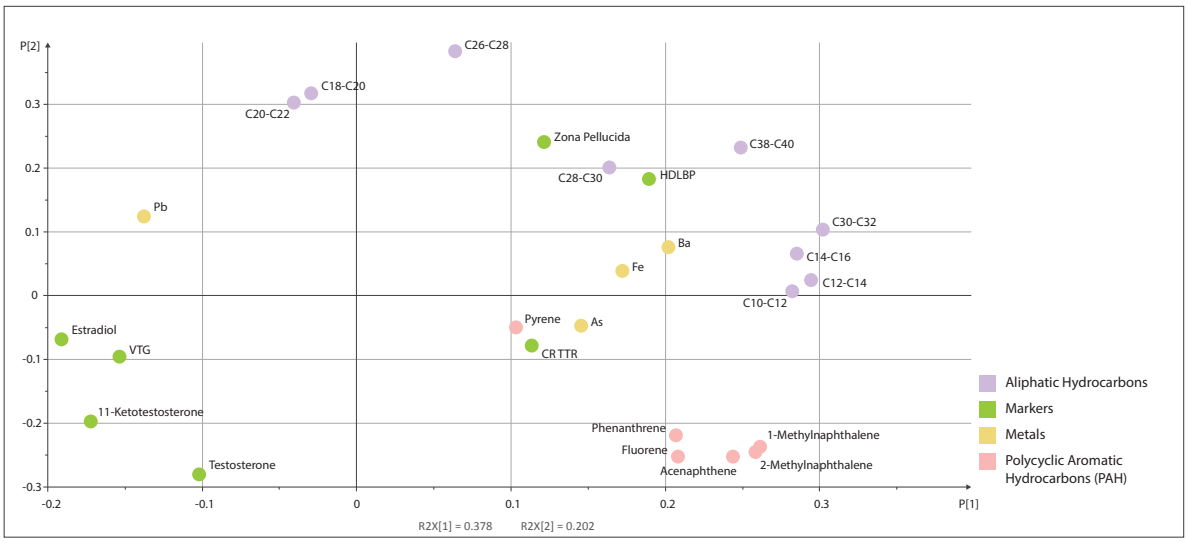
Figure 18. Principal component analysis (PCA) showing individual differences based on tissue contaminants, variable loadings and biological endpoints for all individuals.

b)

Figure 19. Principal component analysis (PCA) showing tissue contaminants and variable loadings in relation to biological endpoints for female individuals.

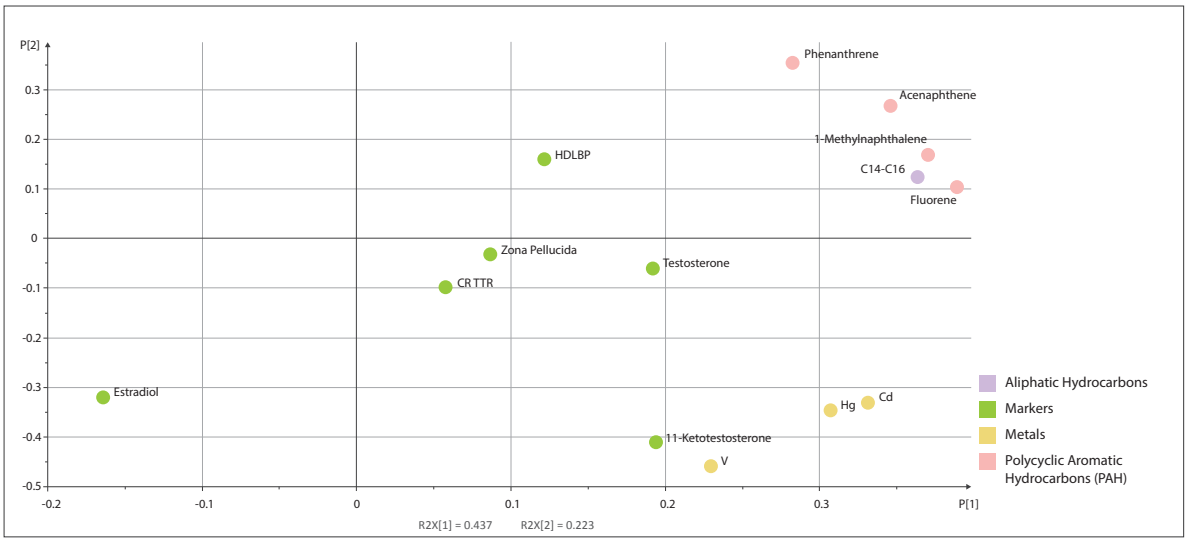
c)

Figure 20. Principal component analysis (PCA) showing tissue contaminants and variable loadings in relation to biological endpoints for male individuals.



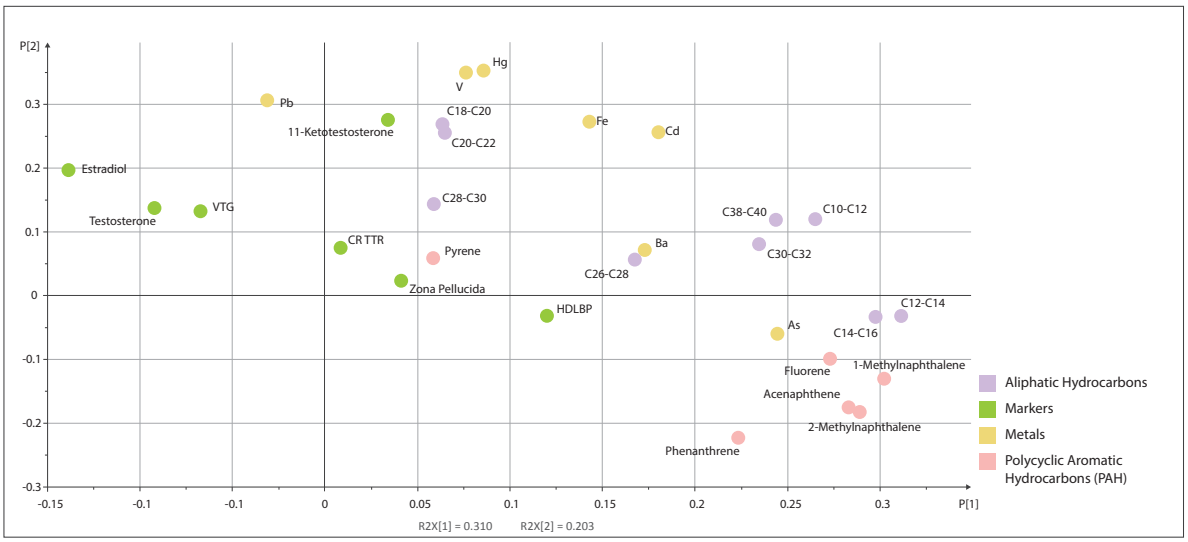
d)

Figure 21. Principal component analysis (PCA) showing tissue contaminants and variable loadings, with proven significant correlation (Spearman` Rho), in relation to biological endpoints for female individuals.



e)

Figure 22. Principal component analysis (PCA) showing tissue contaminants and variable loadings, with proven significant correlation (Spearman` Rho), in relation to biological endpoints for male individuals.



f)

Figure 23. Principal component analysis (PCA) showing tissue contaminants and variable loadings, with proven significant correlation (Spearman` Rho), in relation to biological endpoints for all individuals.

Spearman's rank correlation coefficient with P- values for all significant correlations

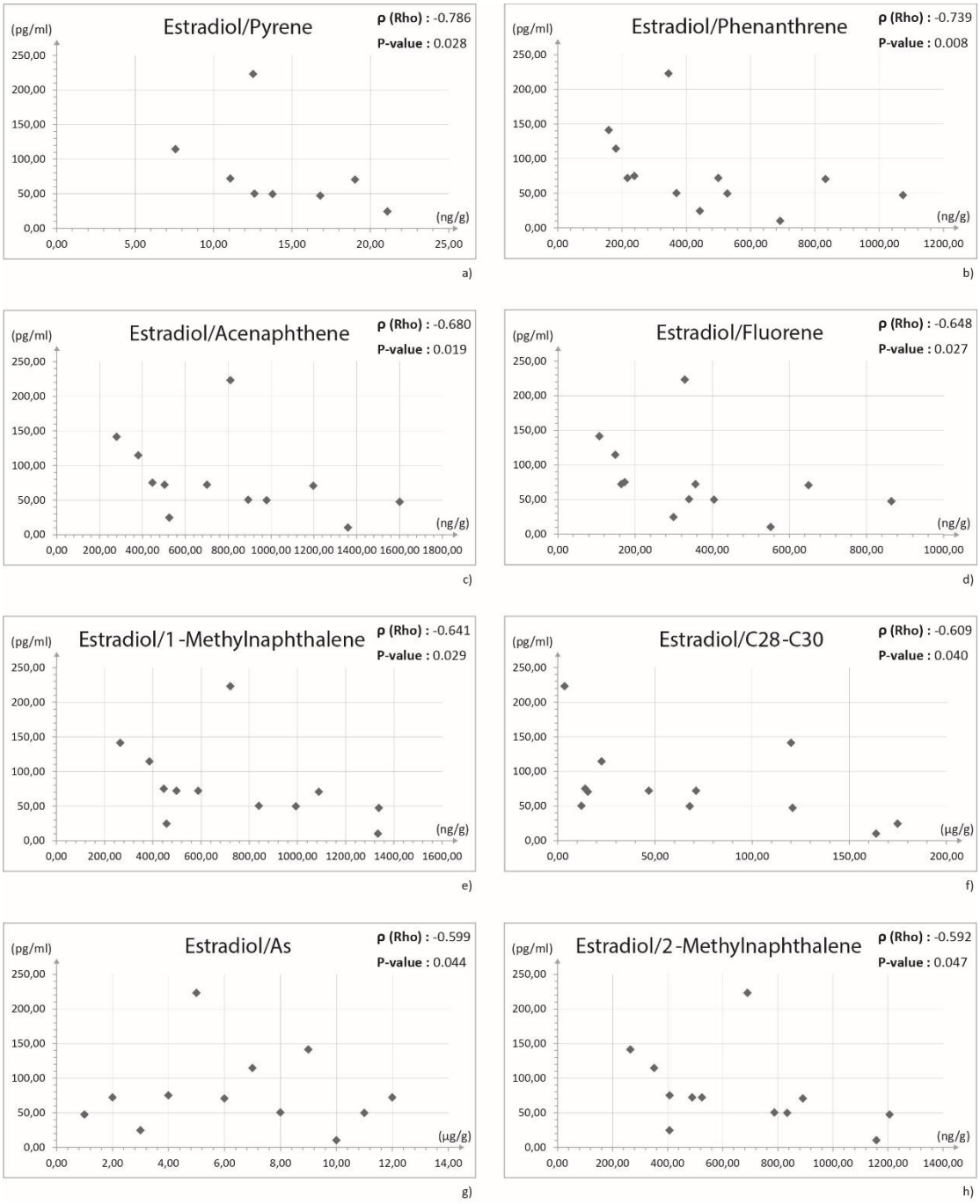


Figure 24 (a - h). Scatter plots showing compounds with significant statistical correlation with selected endpoints in females, performed by Spearman`s Rho.

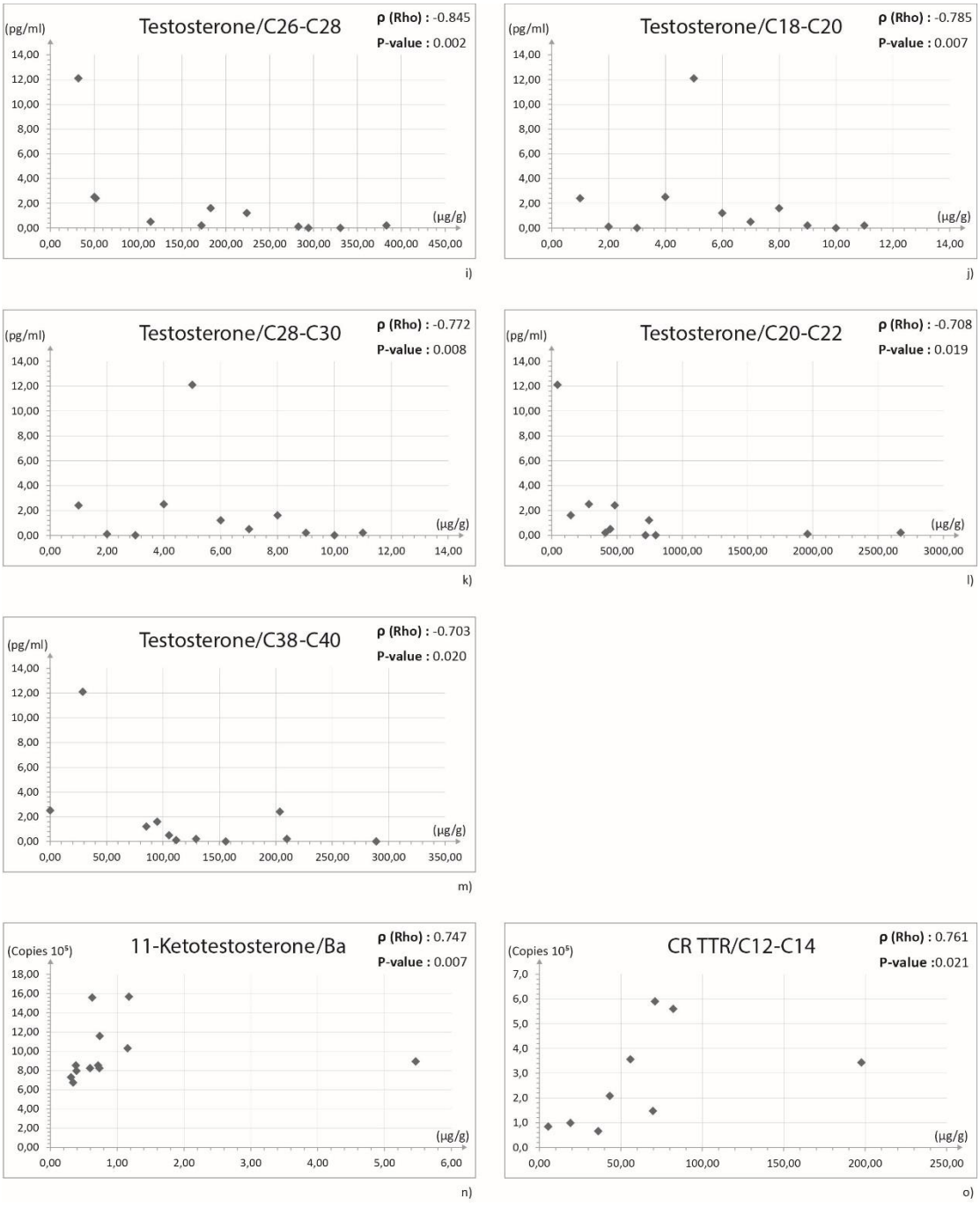


Figure 24 (i-o). Scatter plots showing compounds with significant statistical correlation with selected endpoints in females, performed by Spearman`s Rho.

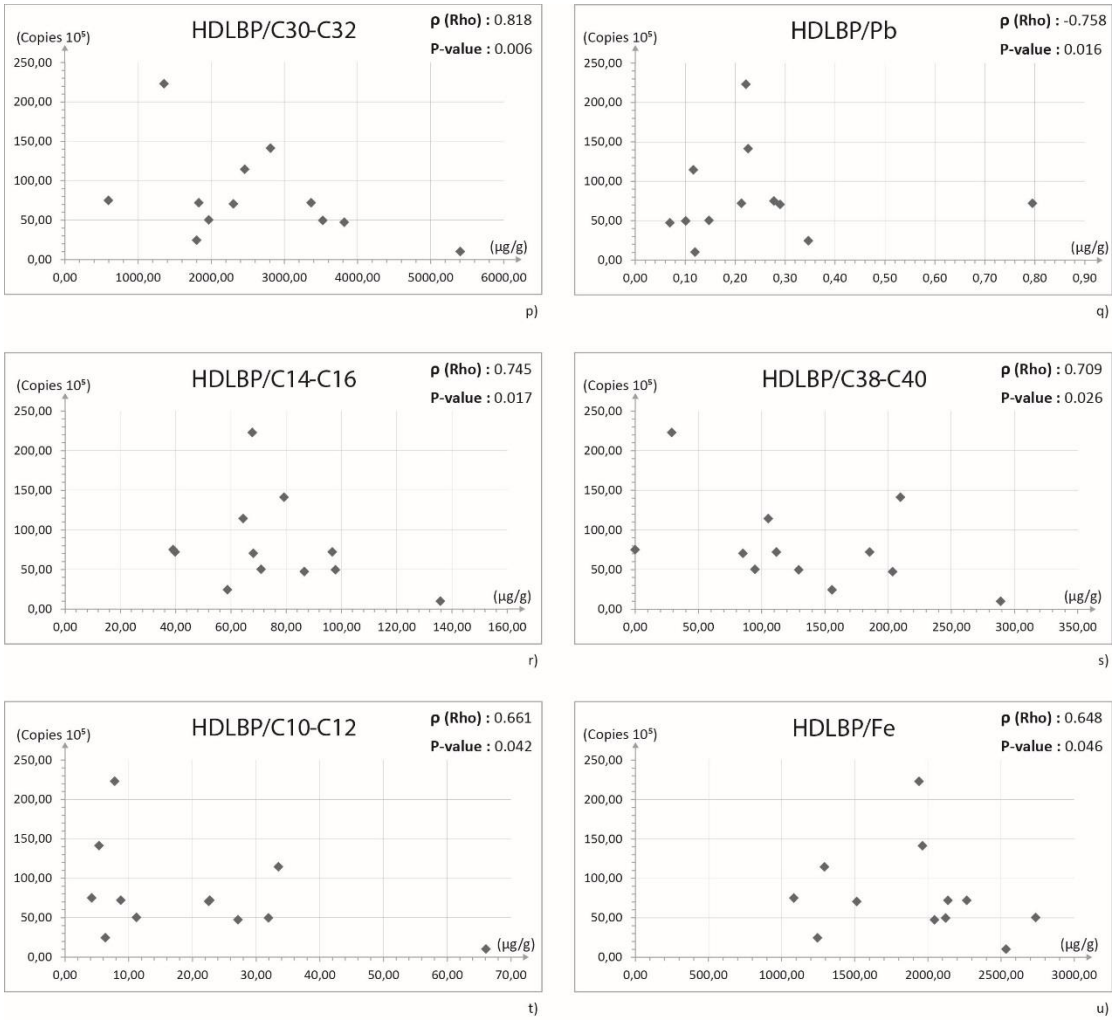


Figure 24 (o-u). Scatter plots showing compounds with significant statistical correlation with selected endpoints in females, performed by Spearman`s Rho.

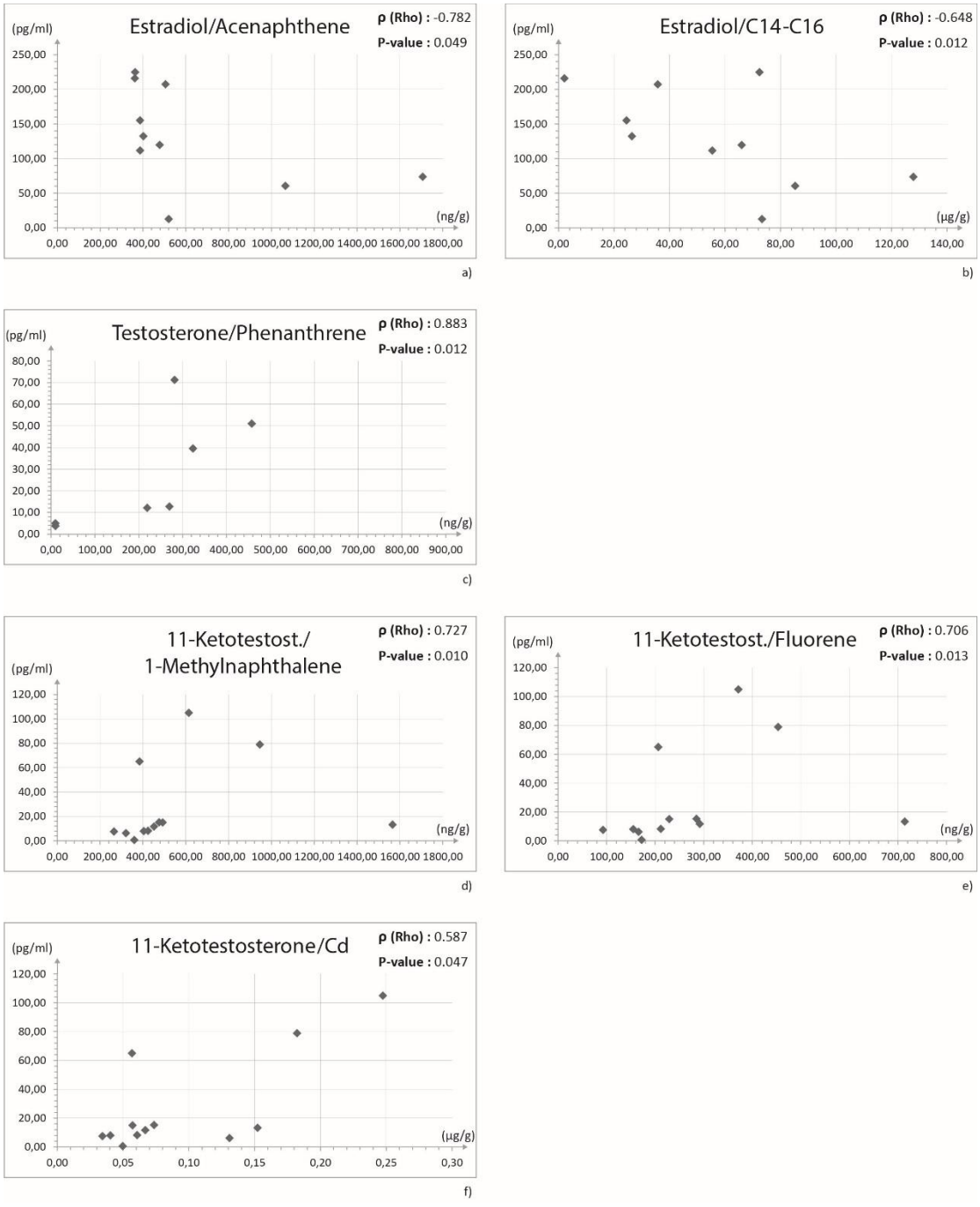


Figure 25 (a-f). Scatter plots showing compounds with significant statistical correlation with selected endpoints in males, performed by Spearman`s Rho.

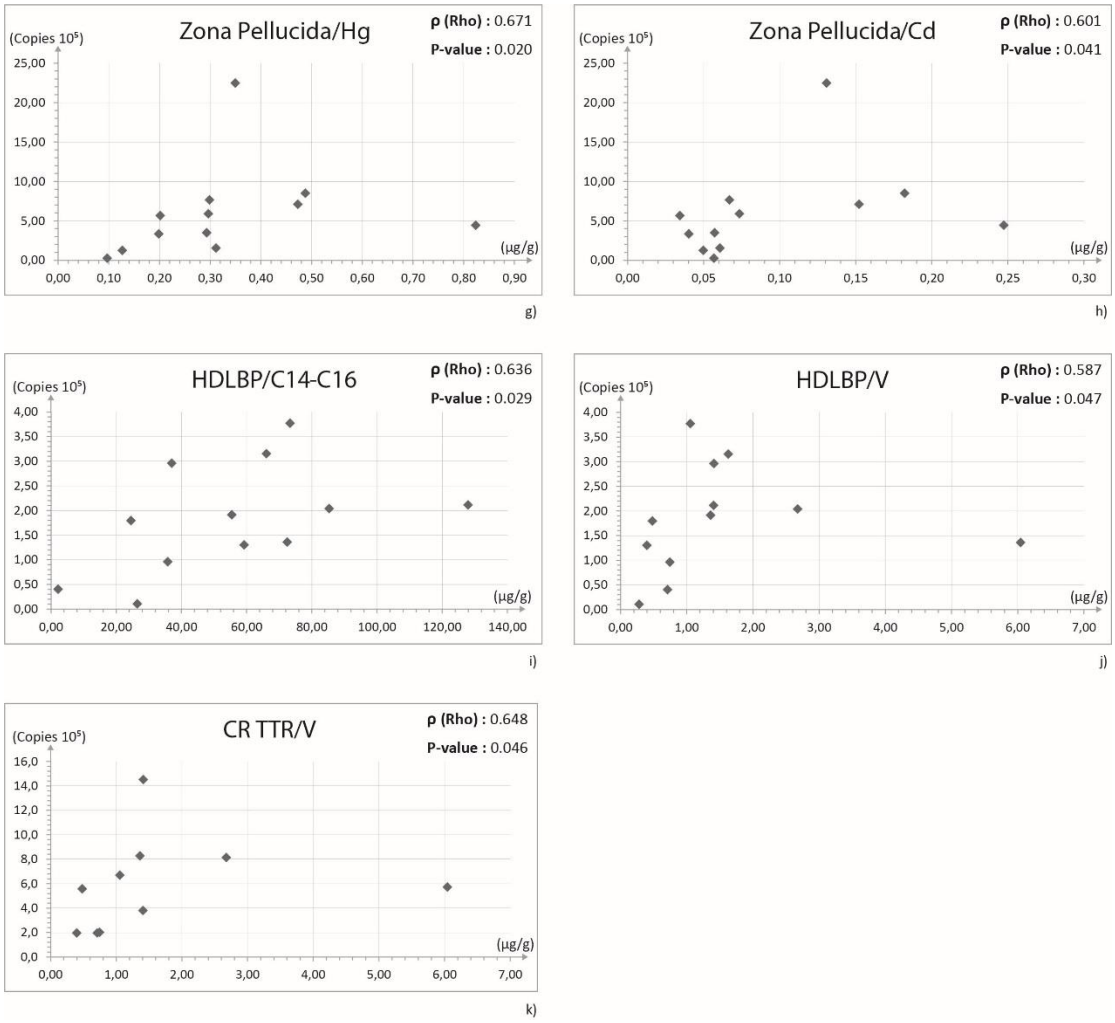


Figure 25 (g-k). Scatter plots showing compounds with significant statistical correlation with selected endpoints in males, performed by Spearman`s Rho.

Discussion

In this study, the biotransformation of endocrine disruption- related genes and proteins was investigated in liver and plasma of female and male Nile crocodiles, with varying burden of anthropogenic chemical contamination. Among the analyzed chemicals were organo-halogenated pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), benzene and aliphatic hydrocarbons.

The reptilian immune responses are very much affected by ecological factors, and in comparison with other vertebrate groups is the information available regarding ECD toxicity and reptiles scarce. Hematologic and plasma investigations are therefore important for providing relevant information within this field.

Analytical methods

During experiments and methods performed in the laboratory, it is vital that choices of experimental setup and methods are well-considered and reliable. Each step during the procedures may also introduce possible errors that may lead to wrong or inaccurate results. A thorough data analysis and evaluation therefore represent an important part of obtaining high quality results.

Steroid hormone assays

Antibodies used in the kits may display certain cross reactivity towards steroids and steroid metabolites (Cayman Chemical Company). This may interfere with the steroid hormone concentrations, as EIA assays are based upon highly specific antibody-antigen binding (Borrebaeck, 2000). However, cross reactivity are reported by the manufacturer as generally low, as both estradiol and androgens has been reported with a cross reactivity of below 0,5 % except for estradiol metabolite estrone (0,54%) (Krasowski et al, 2014). This is not expected to have a major effect on resulting steroid hormone data presented given the overall low concentration of sex steroid hormones detected in samples.

RNA assessment

Dissection of crocodile was performed under laboratory conditions, but not completely sterile. Therefore the animal body still contained enzymes such as RNases that may degrade RNA. To account for this problem, Trizol was added to the harvested organs, and snap-frozen in liquid nitrogen. RNA purity and integrity were inspected further by use of Nanodrop, and revealed that isolated RNA was of acceptable purity. Visual inspection of total RNA showed few signs if degradation, indicating that isolated RNA was a reliable template for generation of cDNA in RT- PCR.

Primers

Possible errors introduced at the cDNA synthesis stage, are likely to be amplified in downstream PCR steps, and thoughtful planning and execution are therefore vital for reliable results and a successful real-time PCR (Huggett et al, 2005). Specifity and accuracy of real-time PCR are also greatly dependent on well-designed primers, as they should be specific for the gene of interest with a high selectivity for cDNA. Primer-dimers or secondary intramolecular structures should also be avoided (Invitrogen, 2008). All primers were tested before RT- PCR procedures, and due to gene-specific high quality primers, it was only expected one amplification product at the end of each cycle. Primers with two peaks or none were excluded. Specificity of RT- PCR products were verified through a dissociation curve analysis each has individual properties such as GC content, size and melting point (Pfaffl, 2004). Primers that peaked at same melting point, indicating presence of single amplification product and were used in the RT- PCR analysis. Some of the amplification peaks were somewhat irregular, that may be induced by a low target sequence concentration.

Interpretation and normalization of RT- PCR results

Correct data analysis is of utter importance when interpretation of obtained results from RT- PCR. To adjust for variances in mRNA derived from the cDNA copies, the measured gene expression can be measured towards a reference gene, that are assumed to be constantly expressed regardless of tissue, experimental treatment or stage of development. This normalization will measure and quantify gene expressions in the various samples on equal basis (Huggett et al, 2005). These genes are known as housekeeping genes, and are involved in basic functions needed for sustainability in the cell. However, no genes have so far shown a completely unaltered expression levels

during experimental work, so it is considered that these normalization steps may cause more harm than benefits (Arukwe, 2006). Therefore this method has not been considered valid for this thesis. In addition, normalization against total RNA primarily measures ribosomal RNA (rRNA), transcribed by a certain polymerase and covers approximately 80% of the fraction. This can make the concentration of total RNA used for normalization slightly insensitive for measurements of mRNA (Bustin, 2000; Huggett et al, 2005). NanoDrop was instead used for RNA assessment. Contaminants present in the extracts may contribute to variations between samples, and may not be completely accurate. More advanced fluorometric assessments of RNA concentration may have accomplished more exact measurements for normalization.

Relative quantification

Presented real- time PCR data were quantified in a relative manner, by using the equation of a premade calibration curve, prepared from plasmid standards of target genes and proteins. Standards were not included in the real-time PCR runs during the various analyses of genes of interest. The relative copy number therefore assume equal efficiency during all real-time PCR reactions, both for used standards and genes of interest. Efficiency of PCR are determined and affected by several factors, such as specific primer design, amplicon sequence and size. Larger amplicons may bind a higher number of SYBR Green dye molecules, for instance, and thereby produce a stronger fluorescent signal per template. Also, each cycle with the same primer may vary slightly due to unspecific priming, primer dimers and differences in reaction concentrations (Bustin, 2000). However, as there is no need for an individual plasmid to each primer, this method is considered as easy and cost saving. And since the relative level of gene expression in the plasma- and liver samples were the interesting outcome of real-time PCR runs, this simplified approach to relative quantification was considered as sufficient. However, this may have the ability to affect the level of significance of measured gene expressions. Results of gene-expression levels should therefore be considered approximate estimates, not absolutes.

Variation and statistics

Variation in gene expression levels were high within the different groups, especially within the estrogen responsive genes. Certain individuals did also show deviant expression levels than other within the same group, indicating large individual

differences. This is however expected when performing an in vivo study (Arukwe et al, 2015). Experiment and analyses also consist of several steps that may contribute to an increased variance within samples. Interpretation of results has mainly been conducted by the resulting statistical significant differences, but trends and patterns were also considered. PCA were used for further evaluation of possible coherence between biological markers and contaminants.

Vitellogenin were not included in PCA analysis for males, due to few statistical significant data according to Spearman. There can be several reasons for this, but the samples may have felt below the detection limit due to low initial template concentration. A larger group size for this experiment in general would be beneficial, as this would contributed to better detecting statistically significant differences between the exposure groups in the real-time PCR assays, regardless of the high variability within each group.

Experimental validation

As this study did not have a control group for comparison of biomarker responses within the individuals, the outcomes of this study is heavily based on contamination and biomarker response correlations, as a reliable indicator for responses to contaminant exposure. Also, presence of protein and gene levels have been highlighted.

Levels of contaminants in the aquatic environment

High levels of various contaminants were found in the water of Le Croc farm, and all pollutants analyzed by SPMD showed higher levels in the dam, than in river and canal (Full datasheet in Appendix D). This was expected, as Le Croc farm used water from a rearing plant with sewage water. Several compounds with endocrine disrupting properties were detected in high levels, such as atrazine, desethylatrazine, simazine and caffeine. The major part of detected contaminants were various pesticides, indicating that a large part of the contaminant burden in farm comes from agricultural, anthropogenic activities.

Atrazine (Atr) is among the most common used herbicide worldwide, with desethylatrazine (DEA) as one of its major degradation products. Its mechanisms are based on inhibition of photosynthesis, and usually target and control annual grasses and broadleaf weeds in corn, mays and grain sorghum (Battaglin et al, 2009; Hayes et al, 2010; Krutz et al, 2003; Vandenberg et al, 2012). It is active in the parts per billion

range (ppb) (low-dose) and potent endocrine disrupting compound in multiple species, such as in fish (Hayes et al, 2010; Suzawa & Ingraham, 2008; Vandenberg et al, 2012), amphibians (Carr et al, 2003; Hayes et al, 2006), reptiles (Crain et al, 1997; Jørgensen, 2010; Rey et al, 2009; Sparling, 2010), rodents (Kucka et al, 2012; Rayner et al, 2005; Ueda et al, 2005) and human cell lines (Fan et al, 2007). Atr mechanism of action are dependent on its ability to bind to the ER receptor and disruptions of hypothalamic function (Figure 26) (The Australian Pesticides & Veterinary Medicines Authority, 2010). This is partly due to direct altering effects on the Gonadotropin-releasing hormone (GnRH), that has an essential role in providing reproductive tissues with adequate energy sources and release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Harrison et al, 2004). They also facilitate secretion of chorionic gonadotrophin (CG), which is needed to maintain corpora lutea function post ovum release and crucial for maintaining pregnancy. The major regulators of GnRh is estrogen and prolactin. It has been hypothesized that atrazine interferes and inhibits the release of CG levels, and if the CG levels are too low for corpora lutea maintenance, inadequate levels of progesterone and growth factors will be released. This will lead to a decreased ovarian function (Cooper et al, 2007; The Australian Pesticides & Veterinary Medicines Authority, 2010).

Many published articles have also indicated a possible association between atrazine and altered aromatase activity in vitro, although there are few relevant, available in vivo studies to confirm this. Aromatase, or cytochrome P450 from the CYP19 gene, catalyzes a key step in estrogen biosynthesis, by converting testosterone to estradiol, and thus obtain vital functions in reproductive success (Dang et al, 2015). By stimulation of aromatase activity, several studies has documented increasing estrogen levels in exposed individuals (Hayes et al, 2011; The Australian Pesticides & Veterinary Medicines Authority, 2010). This has documented to result in outcomes such as hermaphrodism, reducement of testicular volume and induced testicular oocytes in exposed male individuals in numerous studies (Du Preez et al, 2008; Hayes et al, 2011; Hayes et al, 2006; Tavera-Mendoza et al, 2002).

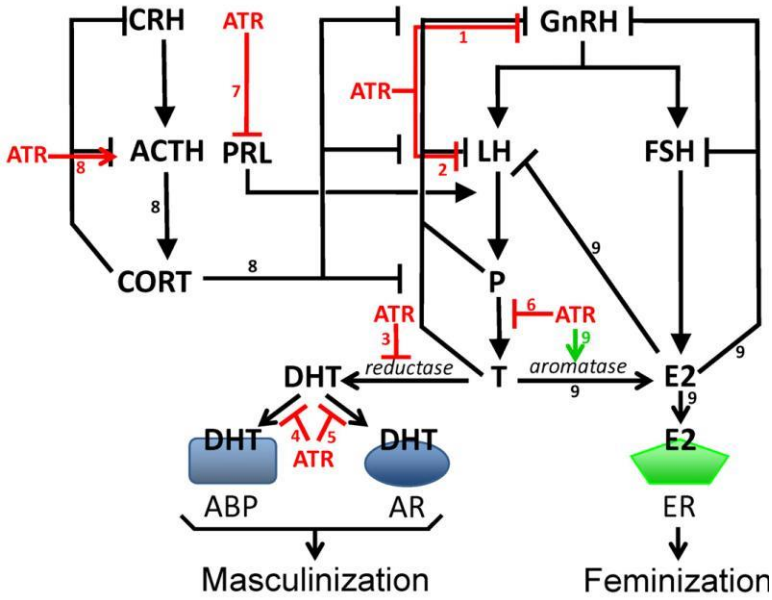


Figure 26. Several mode of action mechanisms has been identified for atrazine. Arrows indicate processes that are increased, and bars indicate inhibited processes. Red lines indicates demasculinizing pathways directly affected by Atr and green lines indicates feminizing pathways directly affected by Atr. ABP = androgen binding protein, ACTH = adrenocorticotropic hormone, AR = androgen receptor, ATR = atrazine, CORT = cortisol/corticosterone, CRH = corticotrophin-releasing hormone, DHT = dihydrotestosterone, FSH = follicle stimulating hormone, GnRH = gonadotropin stimulating hormone, LH = luteinizing hormone, P = progesterone, PRL = prolactin (Hayes et al, 2011).

High levels of caffeine were also found in the dam. Although it has not been considered as a potent pollutant in itself, it has recently been suggested as a reliable chemical indicator for pollution related to disposal of sewage in freshwater systems (Montagner et al, 2014; Spence, 2015). Montagner et al (2014) tested 96 surface water samples, and concluded that caffeine can be used to prioritize samples to be tested for estrogenic activity in water quality programs evaluating ECDs pollutants with endocrine disruptor activity (Daneshvar et al, 2012; Montagner et al, 2014). The high levels of caffeine, in addition to the high levels of other pollutants found in analysis, supports this hypothesis.

Correlation between contaminant and biomarkers levels biomarkers in female and male individuals

The various analyses did overall reveal highest significant correlations for aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) with several of the selected endpoints, but for PAHs and estradiol in particular. This suggests high potency of these substances. High correlations were also found for high density lipoprotein binding protein (HDLBP) and several heavy metals in males. Plasma E2 levels were significantly higher in males (131, 21 pg/ml)) than females (79, 13pg/ml), and levels of

the egg yolk precursor protein vitellogenin (VTG) and zona pellucida (ZP) were detected in male crocodiles, both strong indications of ECDs exposure in the aquatic habitat of the crocodilians.

Aliphatic hydrocarbons

Aliphatic hydrocarbons are mainly derivatives from various oil and fuel combustion activities (Guerin, 2015; Nikolopoulou et al, 2013). The highest correlations in Spearman`s Rho in females for aliphatic hydrocarbons were found for high- density lipoprotein binding proteins (HDLBP) with a positive correlation for several of the olefins including C10-C12 (0,661) and C14-C16 (0,745). A statistical significant positive coherence was also found in males for C14-C16 (0,636).

HDLBP, known as vigilin, contains 15 K-homology (KH) nucleic acid binding domains that specifically binds HDL molecules and function in the removal of excess cellular cholesterol (Goolsby & Shapiro, 2003; Krieger, 1999).

This is an interesting finding, as many of the dead individuals from the Olifants River suffered from pansteatitis, a lethal fat liver condition that is a nutritional disorder affecting various mammals and fish. It is characterized by inflammation and necrosis of adipose tissue in fat stores, kidneys, spleen and liver, which makes the affected fat deposits to harden, with a diffusely yellow- brown color instead of pale white (Lane et al, 2013).

The pathogenesis is still uncertain, but it is assumed that contributing factors revolving alterations in balance between uptake of dietary polyunsaturated fatty acids and deficiency of vitamin E and selenium. Vitamin E is especially important here, because it regulates oxidative damage due to polyunsaturated fatty acid peroxidation within the glutathione peroxidase and superoxide dismutase enzymes (Huchzermeyer, 2012; Lane et al, 2013). Alterations of high density lipoprotein (HDL) will also alter vitamin E, as this major lipophilic antioxidant is taken up by type II cells from HDL (Kolleck et al, 1999; Witt et al, 2000). The significant positive relationship between the aliphatic hydrocarbons and HDLBP may therefore reflect the high levels of cholesterol and inflammation found in carcasses.

The same period as the dead crocodilians appeared, several dead African sharptooth catfish (*Clarias gariepinus*) were found in the same basins, a vital part of the Nile crocodiles diet. They too were all suffered from pansteatitis (Huchzermeyer et al, 2013).

Catfish feed on the bottom of the river, accumulating high levels of pollutants and thus stores in fat tissue of top level organisms, suggesting pollution to be an important part of this condition that it yet to be fully understood.

HDLBP previously has been linked to stress- induced mechanism such as carsinogenesis and heterocromatin formation. In Yang (2014) they found that a knockdown of HDBP in human hepatocellular carcinomas (HCC) tumor cells, significantly inhibited their profilation, colony formation and migration. These results suggest that progressively upregulated vigilin also may function as a molecular risk marker for HCC development (Yang et al, 2014).

Estradiol levels in females showed a negative correlation with two of the aliphatic hydrocarbon olefins, (C28-C30 (-0,609) and C32-C34 (-0,525), suggesting that these aliphatic hydrocarbons readily taken up in the individuals may show an anti-estrogenic effect, also supported by previous studies (Oh et al, 2008). Aliphatic hydrocarbons are mediated though the AHR, and may exhibit both estrogenic and anti-estrogenic effect. However, does most of these hydrocarbon agonists mediate antiestrogenic effects, with shown abilities to conduct cross- talk through the AHR and ER signaling pathways (Mortensen & Arukwe, 2007; Yu et al, 2015). This include down-regulation of ER interference of ligand-activated binding of the ER to DNA, and thereby the metabolism of estradiol (Boelsterli, 2009). No correlation were found for aliphatic hydrocarbons and estradiol in males.

According to Spearman`s rank there was a negative correlation also between VTG and two of aliphatic hydrocarbons; C24-C26 (-0,595) and C26-C28 (-0,595) in females. As VTG measurements were not statistical valid for males, it is difficult to establish any clear trends effects in males. However, are VTG production in females dependent on adequate levels of estrogen to function in oocyte maturation and production of egg yolk proteins (Nagler et al, 2010). Low levels of estrogen were also found in females, and may therefore explain the low levels of VTG. Several hydrocarbons were also negatively correlated to testosterone levels in females: (C18-C20 (-0,785), C20-C22 (-0,708), C26-C28 (-0,845), C28-C30 (-0,772) and C38-C40 (-0,703). This may indicate hydrocarbon induced antiandrogen effects, that previously has been documented in studies (Kizu et al, 2003). The same trends were found for testosterone in males with C28-C30 (-0,571).

Spearman`s Rho also revealed a statistically high positive correlation between transthyretin (TTR) in females and olefins C12-C14 (0,767) and C22-C24 (0,667). In males, however, it showed a negative correlation (-0,624). TTR is a protein that binds and distributes thyroid hormones (THs), such as thyroxine (T4) and retinol. It is of vital importance in the thyroid system, secreted by the liver. Several studies has shown that chemicals, including PCBs, dioxins and pesticides can interact strongly with mammalian TTR, including a rise in plasma clearance rates of thyroid hormones with resulting hypothyroxinemia, caused by iodine deficiency, where the thyroid produces triiodothyronine instead of thyroxine (Brouwer et al, 1999; Ishihara et al, 2003). A large number of chemicals, including aliphatic hydrocarbons, has the ability to bind to TTR and thus interfering with its functions in the brain and neurobiological functions, by directly competing with THs for binding to the TTR. One role of TTR is delivery of thyroid hormones throughout the central nervous system (CNS) (Alshehri et al, 2015; Talsness, 2008; Zhang et al, 2013). Due to the significant binding affinity of TTR, the environmental contaminants or their metabolites displace thyroid hormones in vivo and alter TH homeostasis in plasma by an increased free concentration of THs. TTR has for this reason been marked as a molecular target for thyroid disruption (Yamauchi & Ishihara, 2009).

The correlation between expression of TTR and aliphatic hydrocarbon in both females and males may indicate some interaction with transthyretin receptors, but no clear trends were observed.

Benzene

No statistical correlation was found for benzene and the various endpoints in females. This was not expected as benzene is a major constituent of crude oil, and one of the most elementary petrochemicals (Kim et al, 2007). It is an aromatic hydrocarbon, and excessing both estrogenic and anti-estrogenic effects (Cavalieri & Rogan, 2014; Yang et al, 2011). Therefore, a negative or positive correlation was expected, but none were detected for neither female nor male individuals.

Halogenated Pesticides (PCP)

In males heptachlor epoxide were statistically negative correlated (-0,600) with estrogen, suggesting antiestrogenic properties. None correlation were found in females. Although HCPs are AHR agonists, they may exhibit both antiestrogenic and estrogenic

properties though ER- AHR crosstalk (Everett & Thompson, 2015; L'Héritier et al, 2014).

Metals

Multiple heavy metals, such as Cadmium (Cd), Mercury (Hg) and zinc (Zn) were detected in tissue, with both positive and negative significant correlation with several of selected endpoints in males and females, including HDLBP (both), VTG (females), ZP (males) and TTR (both). Interestingly, were vitellogenin in females negatively correlated with levels of Iron (Fe) in females (stat. sign. -0,667). This is reasonable, as VTG is a major contributor to iron delivery to developing ova, besides transthyretin. It covers for almost 30 % of the plasma iron concentration (Nys et al, 2011).

All metals of significance detected in males were found to be positively correlated with HBLBP: Cadmium (0,538), Copper (0,531), Iron (0,545), Mercury (0,559) and Zink (0,600). Cadmium (Cd), a non-essential heavy metal, is widely found in soil and reservoirs, both naturally and as a result of various anthropogenic emissions (Bhattacharyya, 2009; Tchounwou et al, 2012). Occupational exposure of Cd, are mostly from metallurgical and electroplating industries, while general population exposure is through ingestion of contaminated foods, such as meat, fruit and fish (Iavicoli et al, 2009). The metal is linked to numerous health problems including osteoporosis, lung cancer and leukemia (Park et al, 2012). Cadmium exposure also the reason behind the itai-itai ("it hurts, it hurts") disease that occurred in Minamata, Japan. Large amounts of Cd were released into rivers by and agricultural soil by mining companies, and accumulated in humans though food, especially rice, causing softening of the bones, kidney failure and death (Almeida & Stearns, 1998; Bhattacharyya, 2009).

Cd has not been regarded as estrogen disrupting compound, but has in several studies been linked to alter high density lipoprotein levels, and thus disturb the reverse transport of cholesterol (Turkcan et al, 2015). Skoczynska et al (2001) found that cadmium exposure impaired the mechanism of cholesterol transport in blood and induced vascular changes in rats (Skoczynska, 2001). The same trends of excess cholesterol by pansteatitis has been found in dead crocodiles in the Olifants River, and may indicate that heavy burdens of cadmium in water could be a contributing factor for pansteatitis.

Several of the other metals with significant correlation with biomarkers, was essential metals such as Copper (Cu), Iron (Fe) and Zink (Zn). Cu serves as an important co-

factor for oxidative stress related enzymes, such as catalase, superoxide dismutase and peroxidase, and is therefore an essential nutrient. But it also has the ability to vary between two different states; an oxidized state and a reduced state. This transition between states can however result in generation of superoxide and hydroxyl radicals, and cause oxidative stress in excessive exposure. Several of the other essential elements required for biological functioning may also produce cellular and tissue damage leading to adverse health effects, determined by level of dosage. There is a fine line between beneficial and toxic effects. The high up regulation of HDLBP indicate toxic effects of these metals (Tchounwou et al, 2012).

The role of heavy metals as ECDs however, is still a field that needs further investigation, as few reliable studies based on possible additive, synergistic or antagonistic effects on the endocrine system of mixtures of metals is conducted and/or is available. Most of the studies found regarding this field involves studies which exposure levels were particularly high, and thus do not reflect the long-term effects of metal mixtures based on low-level exposure (Iavicoli et al, 2009).

However, the general high levels of heavy metals found in crocodilian tissues, and the significant correlations with Spearmans Rho for several immunological relevant biomarkers, may provide vital information on the crocodilians nutritional and general health status, as biotransformation rates are based heavily on these factors (Kang et al, 2013; Rotter et al, 2015).

Polycyclic aromatic hydrocarbons (PAHs)

Most relevant findings from the statistical analysis in accordance of aquatic PAHs exposure was the strong negative correlation for estradiol levels found in both males and females, for phenanthrene (-0,571), acenaphtene (-0,782), pyrene (-0,800) in males and aceenaphtalene (-0,680), fluorene (-0,648), phenanthrene (-0,739) and methylnaphthalene (-0,592) in females. None were found to have positive correlations of the selected endpoints. Several of the PAHs correlated also both negative and positively to estrogen related biomarkers such as ZP and 11KT in females and males respectably, however no correlation was found in VTG expression for females.

Polycyclic aromatic hydrocarbons (PAHs) are common byproducts of incomplete combustion of fossil fuels, oil spills and industrial processes (Moraleda-Cibrián et al, 2015). Their alterational properties on the endocrine system are most likely due to their

ability to bind to ERα, and their structural similarities to estrogens as they have been documented inhibited with treatment of an ER antagonist (Lee et al, 2007; Moraleda-Cibrián et al, 2015). Several PAHs, and their derivatives, are known as mutagenic and carcinogenic compounds either individually or in a combination and linked to oxidative stress (Vandermeersch et al, 2015).

Estrogenic effects of contaminants in tissue and steroid levels

Vitellogenin (VTG) precursors provide the egg yolk proteins in females that are essential for nutrient supply during early development of oviparous vertebrates and invertebrates (Arukwe & Roe, 2008; Celander, 2011). In most mammals, the induction of VTG synthesis (vitellogenesis) is under control of the hepatic estrogen receptor (ERα), triggered by environmental cues and regulated by coordinated endocrine feedback loops in the HPGL axis (Bemanian et al, 2004).

Male levels of this protein in most vertebrates is usually low or absent. Induction of VTG synthesis in males via the activation of the ERα, is therefore a frequently used biomarker to assess exposure to estrogenic chemicals (Kime et al, 1999; Vasanth et al, 2015b). However, as the induction of ERα mediated VTG signaling in males is not a natural physiological response and will not reveal any information of the mechanisms behind the toxicity of estrogenic chemicals. Alterations in plasma VTG in females may provide more information behind the mechanisms involving endocrine regulation, as most chemicals that alter the conversion of testosterone to estrogen, does this by inhibition of aromatase (Vasanth et al, 2015a). Reduced levels of estradiol, and thereafter reduced VTG synthesis, which in return result in reduction of egg production and fecundity in females. This suggests further that reduced plasma VTG in females can influence population size (Celander, 2011; Miller et al, 2007).

PCA analysis conducted for females and males in this study, revealed both higher levels of estradiol and VTG in males, than in females. This strongly suggests uptake of xenoestrogens in the captivated crocodilians (Agbohessi et al, 2015). Results for estrogen levels in males were also significantly higher according to the student t- test (0,08), as well as t-test conducted for hepatic VTG (0,29).

In a study by Sole et al (2002) they investigated carps (*Cyprinus carpio*) in two rivers in Catalonia (Spain) both known to be polluted by estrogenic compounds. The estrogenity in the carp was measured as VTG presence in males and alterations in VTG

levels in females for a period of 6 months. They found presence of hepatic VTG in up to 54 % of the male fish analyzed and an overall higher expression of in males than females, in accordance with our results (Solé et al, 2002).

A common observation from both in vitro and in vivo studies has also documented pesticide interaction with the endocrine system by alteration in androgen synthesis and function in males (Louis J. Guillette, 2003). In Lavado et al (2004) did fish in a downstram river from a sewage treatment plant show reduced androgen plasma concentrations (t and DHT), as well as increased estrogen and vitellogenin levels (Lavado et al, 2004; Louis J. Guillette, 2003). The same modulations of androgen and estrogen were also found in female alligators (*Alligator mississippiensis),* and were detectable at hatching, and throughout juvenile and sub-adult life (Guillette et al, 2000).

However, the production of VTG have been proven to also have the ability to be regulated and possible mediated by the AHR through cross-talk (Bemanian et al, 2004; Mortensen & Arukwe, 2008a) Although the expression of VTG in cultivated salmon hepatocytes were strongly induced by 17β-estradiol (E2), the expression of VTG was disrupted by AHR mediated 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), while the CYP1A expression was enhanced. Effects of TCDD on VTG and CYP1A expression were annulled by α-naphthoflavone, an AHR antagonist. This shows mediated inhibitory effects on the expression of the VTG and ERα by the AHR. Run-off experiments also revealed that simultaneous exposure of the cells to TCDD and E2 strongly inhibited the initiation of transcription of VTG and ERα genes, suggesting that activation of AHR inhibit the transactivationality of ERα (Bemanian et al, 2004). Further, they suggested that activation of the ARH signaling led to a clear decrease in number of the nuclear ERα, and impairment of ERα `s ability to bind to target DNA sequence by activated AHR (Bemanian et al, 2004).

In a study from Mortensen et al (2008) the AHR agonist PCB126 were also found to modulate the ER mediated nonylphenol (NP), and produce estrogenic responses in primary culture of salmon hepatocytes (Mortensen & Arukwe, 2008b). There was particularly an increase of plasma VTG and ZR protein expression observed in salmon exposed to combined PCB77 and NP, compared to fish exposed to NP alone, showing estrogenic effects of PCB126 exposure. The same experimental data also showed that moderate levels of PCB126 produced increased mRNA transcripts for ERα, VTG and

ZR proteins. However, high concentrations of PCB126 decreased cellular VTG levels and suggests possible impairment of protein synthesis due to PCB126 toxicity (Mortensen & Arukwe, 2008b).

Levels of zona pellucida (ZP) were detected in all males (mean 5, 96 pg/ml), with a surprisingly higher mean level than found in females (5, 10 pg/ml). ZP is a structural extracellular egg coat protein that mediate sperm-oocyte binding, eggshell hardening and prevents polyspermy in vertebrates (Genovese et al, 2011; Wassarman & Litscher, 2012). The origin of ZP synthesis for crocodilians are still not fully known, but are in fish mainly carried out in the liver of adult females under estrogenic control (Arukwe & Goksøyr, 2003; Genovese et al, 2011). Zonagenesis precedes vitellogenesis, because the initial eggshell formation occurs before the active uptake of VTG (Celius & Walther, 1998). Most mammalian males do not produce ZP, but transcription of ZP and VTG genes will be induced by mediation of the ER by estrogens and xenoestrogens.

Males also exhibited higher levels of 11- ketotestosterone (11- KT) (9,79 pg/ml) than of testosterone (T) (27,87 pg/ml). 11-KT is the primary androgen in fish and to a certain extent reptilians, and differs from T by the addition of a ketone function at carbon 11. It is believed to reduce local levels of glucocorticoids in Leydig cells of the testes and ovaries, and may also have a role in stimulating folliculogenesis (Yazawa et al, 2008). 11- KT is the oxidized form of testosterone, and do not have the ability to be aromatized (by aromatase) into estradiol, androstenedione and estrone, as testosterone do (GUAL et al, 1962; Yazawa et al, 2008). As numerous xenoestrogens, such as PAHs can elicit estrogenic responses though the ER, this will also alterations of conversion levels of testosterone to estrogens (Ishikawa et al, 2006). The high levels 11 KT compared to T in males in this study may indicate that testosterone have been transformed into estrogens, that might also partly explain the level of VTG, ZP and E2 found in tissues of male crocodilians. Aliphatic hydrocarbons and polycyclic hydrocarbons both mediates through the AHR, but according to previous studies, they can interact with ERα and cause both estrogenic and antiestrogenic effects. This is in accordance with the results of the experiments conducted in this thesis, as several of the PAHs in crocodile tissue appear to have antiestrogenic effects. However, the males exhibit VTG and ZP synthesis, suggesting up-regulation of estrogens by contaminants in dam.

Estrogen levels and sex determination

In crocodilians, sex is not determined by a genetic mechanism alone, but also though the egg- incubating temperature. They have a certain thermosensitive period (TSP) where gender of the offspring is determined upon the temperature exposed. However, treating embryos with estrogen during the TSP, produces female offspring even at male incubation temperatures (Iguchi et al, 2006; Louis J. Guillette, 2003). Estrogens therefore has an important role of determining sex in all crocodilians.

Contributing factors affecting biomarker responses and toxicity of contaminants

Cocktail effects

When conducting a study based on several compounds in the environment, it is always important to consider the possible outcomes of mixtures working together. This can create various "cocktails" of substances with unknown properties (Celander, 2011).

For example may the induction of the hydrocarbon biomarker CYP1A be mediated by AHR, and the induction of VTG be mediated by activation of the ER be indirectly or directly affected by the presence of other classes of pollutants and exhibit cocktail effects (Celander, 2011). Substances that inhibits function of key metabolic enzymes involved in the elimination of AHR and ER agonists, may result in bioaccumulation of hydrocarbons and other estrogenic compounds. This can lead to an overestimation of the actual exposure level. Other cocktail effects may involve inhibiting of receptor cross-talk, resulting in decreased biomarker responses and thus an underestimation of the actual exposure (Celander, 2011).

Receptor down-regulation and desensitization

Receptors has the ability to desensitize, a process where the response to certain hormone decreases and not because the number of available receptor declines, but rather due to a biochemical inactivation of the receptor. This is a common mechanism that typically occurs when repeated or continuous exposure to ligand occurs (Vandenberg et al, 2012). Receptor desensibilitation has been observed for several hormones, such as glucagon, FSH, human chorionic gonadotropin and prostaglandins (Bohm et al, 1997; Vandenberg et al, 2012).

Binding of hormones to nuclear receptors, will most often ultimately lead to a change in transcription of target genes. When the receptor is bound by ligand, the response will increase and the relationship between the number of bound receptors and the hormone concentration will be nonlinear, including the relationship between number of bound receptors and the biological effect (Welshons et al, 2003). After binding of the nuclear receptor by the hormone and transcription of target genes, it is clear that the reaction eventually cease. That is, the bound receptor must eventually be inactivated to some extent, and thus ubiquitinated and degraded, normally conducted through the proteasome (Vandenberg et al, 2012; Welshons et al, 2003). However, the receptor degradation are dependent on the various hormones, and binding of estrogen, progesterone and androgens mediates the degradation of their receptors (Lange et al, 2000; Vandenberg et al, 2012). The number of inactivated and degraded receptors often correlate with higher hormone levels, leading to a strong down-regulation of receptor (Modrall et al, 2001). It is also found that signaling from one hormone receptor can influence protein levels of another receptor, as has been documented in ER signaling, that promotes degradation of the glucocorticoid receptor by increasing the expression of enzymes in the degradation proteasome pathway (Modrall et al, 2001). As individuals in dam has been exposed long-term to contaminants, this may have affected relevant receptors and should therefore be taken in consideration.

Exposure timing and seasonal variables

The growth of the individual crocodiles is an integrated response to numerous physiological processes that contribute to positive and negative factors, hence influencing various mechanisms needed for bodily homeostasis (Latorre et al, 2013). Juvenile crocodilians has a much higher growth rate, than older individuals, as growth and survival are closely linked together. Considerable scientific evidence points out that early life stages of oviparous organisms often exhibit a higher toxicological sensitivity to chemical contaminants than adult life stages (Latorre et al, 2013; Russell et al, 1999). EDC exposures during early development have organizational, permanent effects while exposure after puberty are mostly considered as activational, with abrogated effects when exposure declines (Latorre et al, 2013). In a study from Schonfelder et al (2004) they found that an adult uterus requires relatively large doses of BPA (parts per billion range), to induce alterations associated with uterotropic assay, whereas parts- per-trillion exposures during fetal period permanently altered the development of the uterus

(Markey et al, 2003; Markey et al, 2005; Schonfelder et al, 2004; Vandenberg et al, 2012). Tissue sampling from animals were in this study collected at juvenile stage, and may therefore display stronger contaminant induced biomarker responses that not reflect the sensitiveness and uptake rates at all ages.

Further research

Given the various observations reported in this thesis, several important points are necessary to further determine and establish contaminant exposure effects in key reptiles in aquatic freshwater ecosystems. For example use of other relevant biomarkers, such as aromatase and CYP enzymes, as they are essential biomarkers of estrogen- and oxidative stress responses. The toxicological mode of action for the occurring pansteatitis illness found in fish and crocodilians within the Olifants River, should also be further investigated as this condition most likely is a part of the explanation behind the mortalities of crocodilians and also linked to high exposure of contaminants in soil.

Summary and conclusion

Data obtained in this thesis display indications of altered endocrine functions, and a strong indication that the high occurrence of contaminants found in Le Croc dam has endocrine effects, as the egg-laying protein vitellogenin and zona pellucida were detected in male crocodilians. High, statistical relevant correlations were also found for heavy metals, aliphatic hydrocarbons and especially within the group of polycyclic hydrocarbons, suggesting endocrine disrupting properties.

As there are few previous studies documenting Nile crocodile transformation and endocrine disruption data, it is therefore difficult to prove any direct cause- and effects. This is also due to the complex interactions between contaminant levels and biotransformation.

But this study shows clear patterns and trends between tissue contaminant burden and biological responses that can be correlated to the health problems observed at Le Croc farm. It may therefore also be reasonable to assume together with previous reports that the heavy anthropogenic pollution of the Olifants River system has contributed to the deaths and health issues of the Nile crocodiles in Kruger National Park.

However, does not anthropogenic pollution restricts itself to the boarders of Kruger National Park. This study should therefore provide relevant, valuable data for general aquatic contaminant exposure of natural habitats.

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Appendix A: Enzyme immunoassay (EIA) standard curves

Enzyme immunoassay (EIA) standard curves EIA assays are competitive assays where concentration of a given substance are measured by its ability to displace enzyme-linked tracers. Thereby will an increased concentration of a given sample produce less color intensity during development of the wells with substrate, and by this lower absorbance readings. Maximum binding (B0) wells were used to measure the maximum amount of tracer that antibodies in the assay are able to bind in absence of sample substrate. Afterwards, the ratio of sample absorbance is compared to B0 for determination of sample concentration. This ratio are presented as %B/B0 (percent bound / maximum bound), and were calculated by:

Corrected maximum binding (corrected B0) by subtraction of average absorbance readings for non-specific binding (NSB) wells from average absorbance in maximum bound (B0) wells. %B/B0 were calculated for all samples and samples by subtracting average NSB absorbance from standard and sample absorbance, and then divide by corrected B0 and multiplying values by 100. Standard curves for wells were plotted as %B/B0 of standards versus log steroid concentration, using a 4- parameter logistic fit. Standard curves were fitted and concentration of each sample were calculated in Excel. The standard curves for individual EIA assays are presented in Figure A1-A3. Standard measurements are based on average of two replicates that were used in the analysis. However, a few replica measurements were removed as outliers.

Standard curves

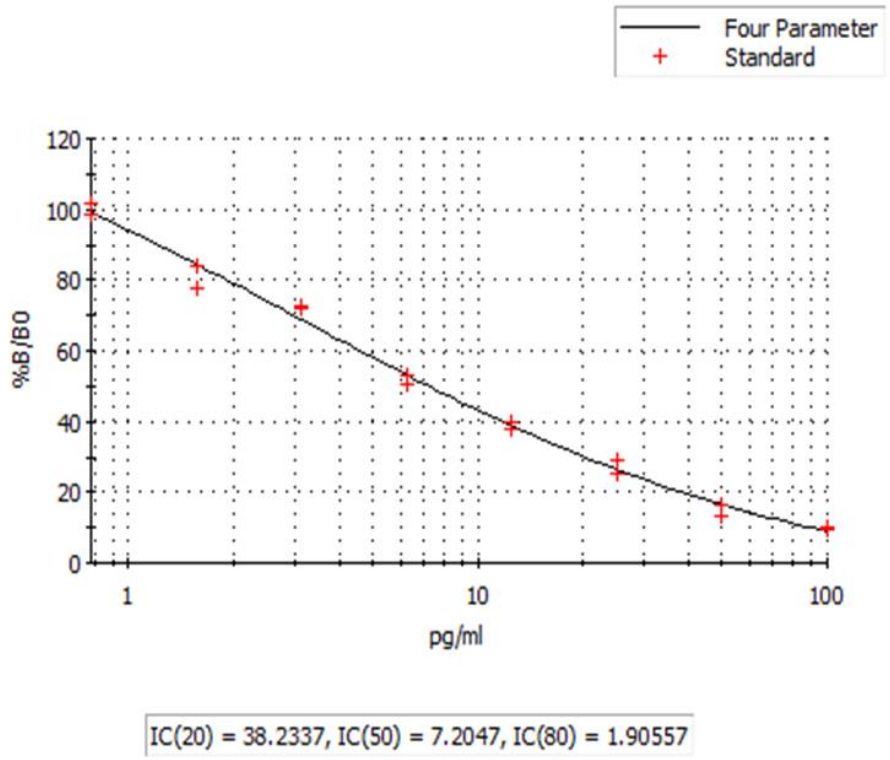


Figure A1: Standard curve for 11- ketotestosterone (11-KT)

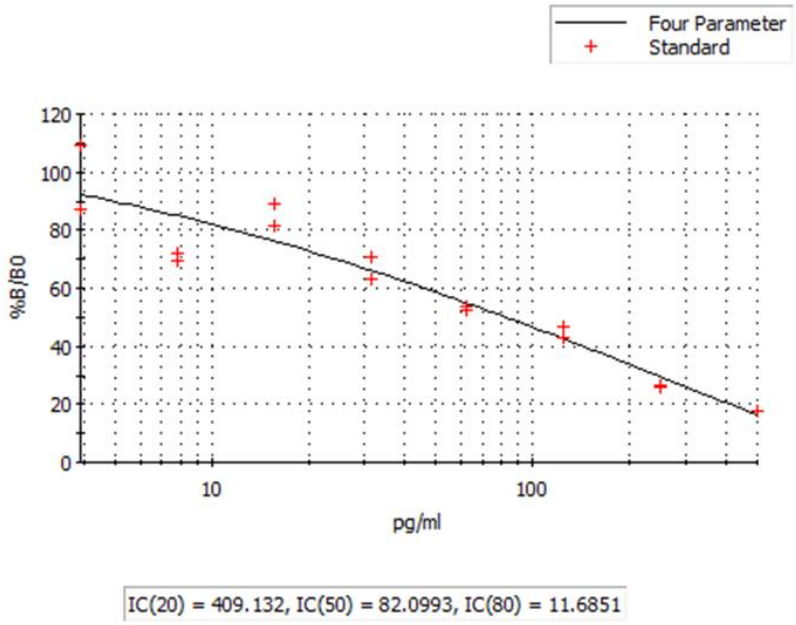


Figure A2: Standard curve for testosterone

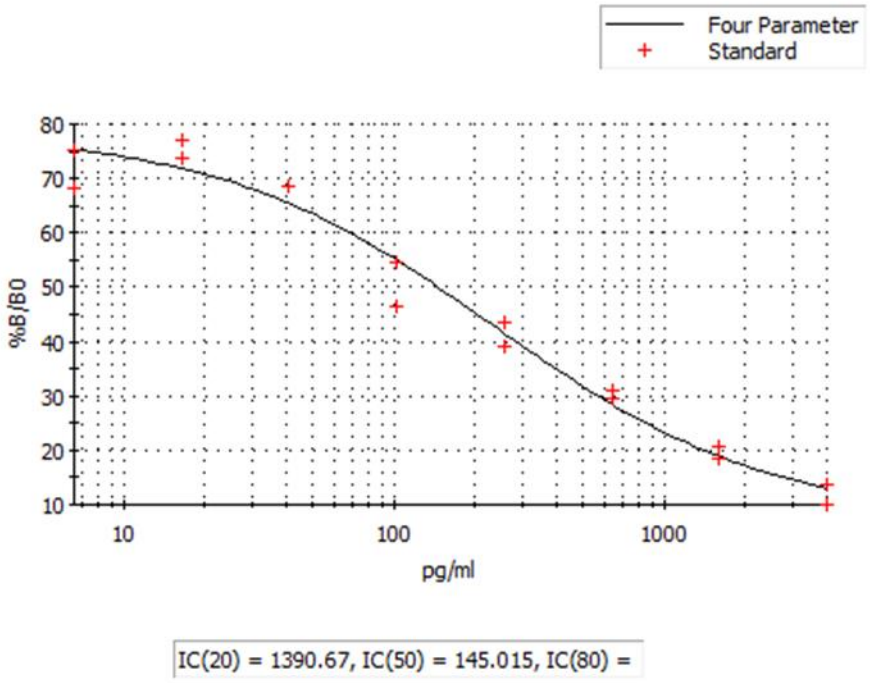
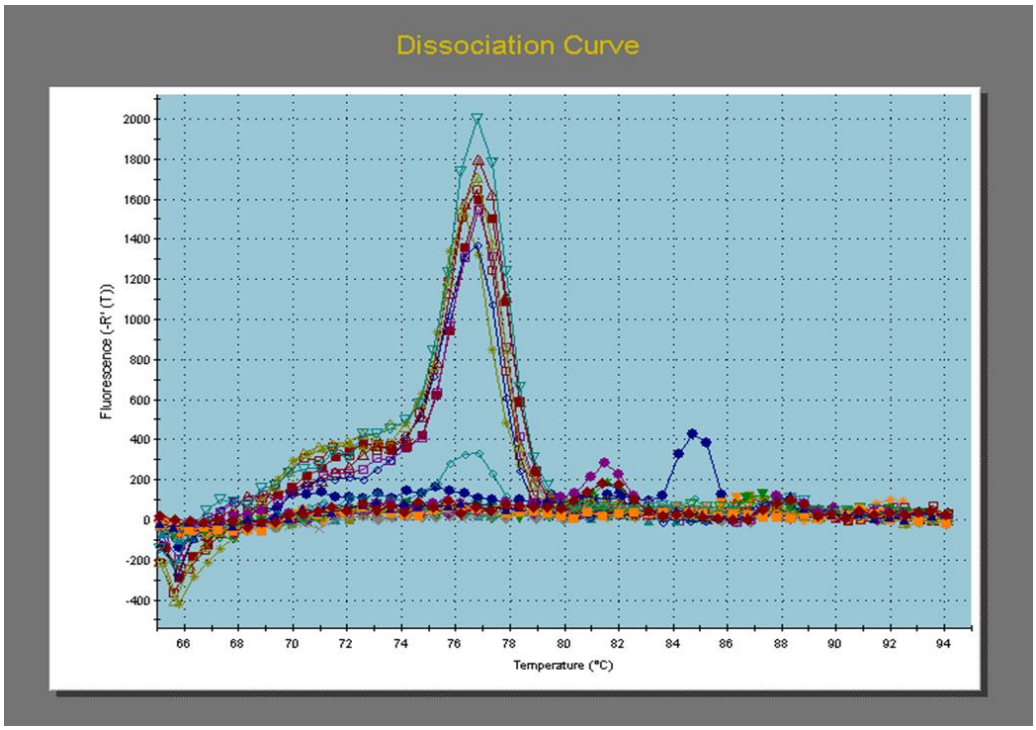


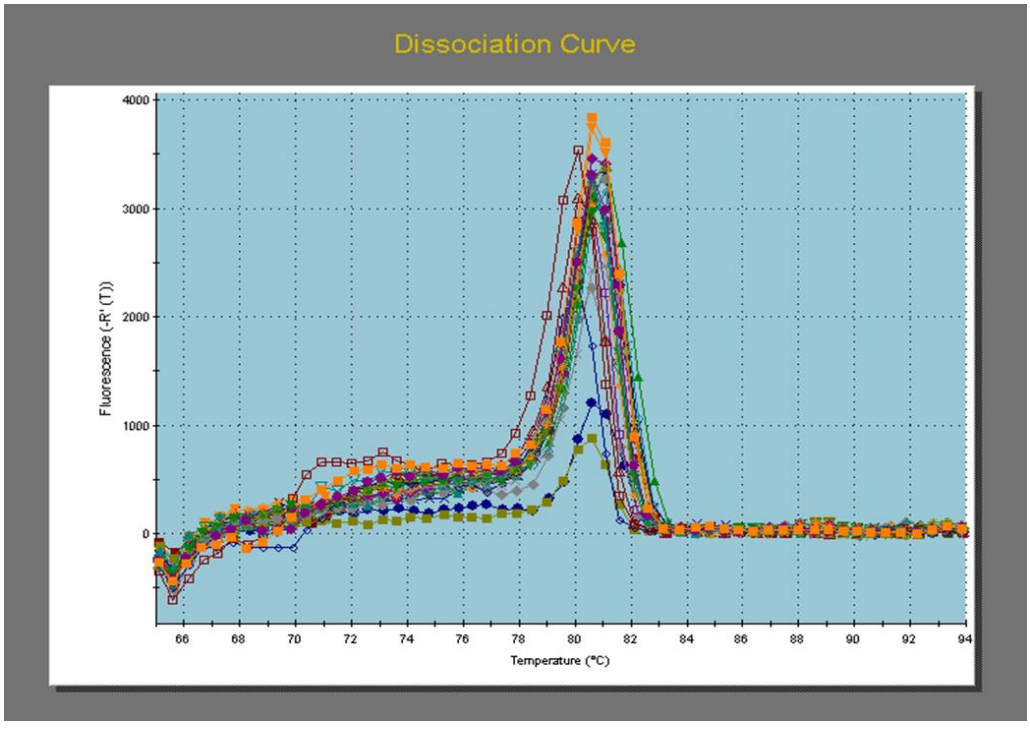
Figure A3: Standard curve for estradiol

All figures (steroids) are displayed as percent bound / maximum bound (%B/B0) versus log concentration. Steroid extract samples were distributed on two 96 well kits.

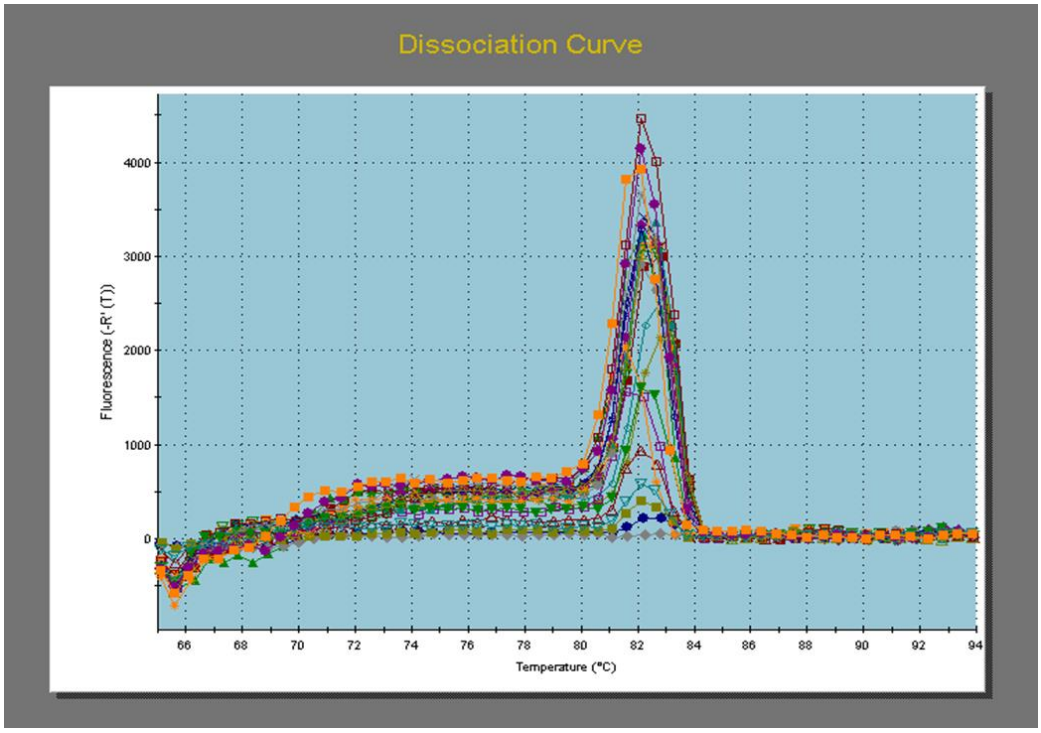
Appendix B: Melting curves of real-time RT-PCR



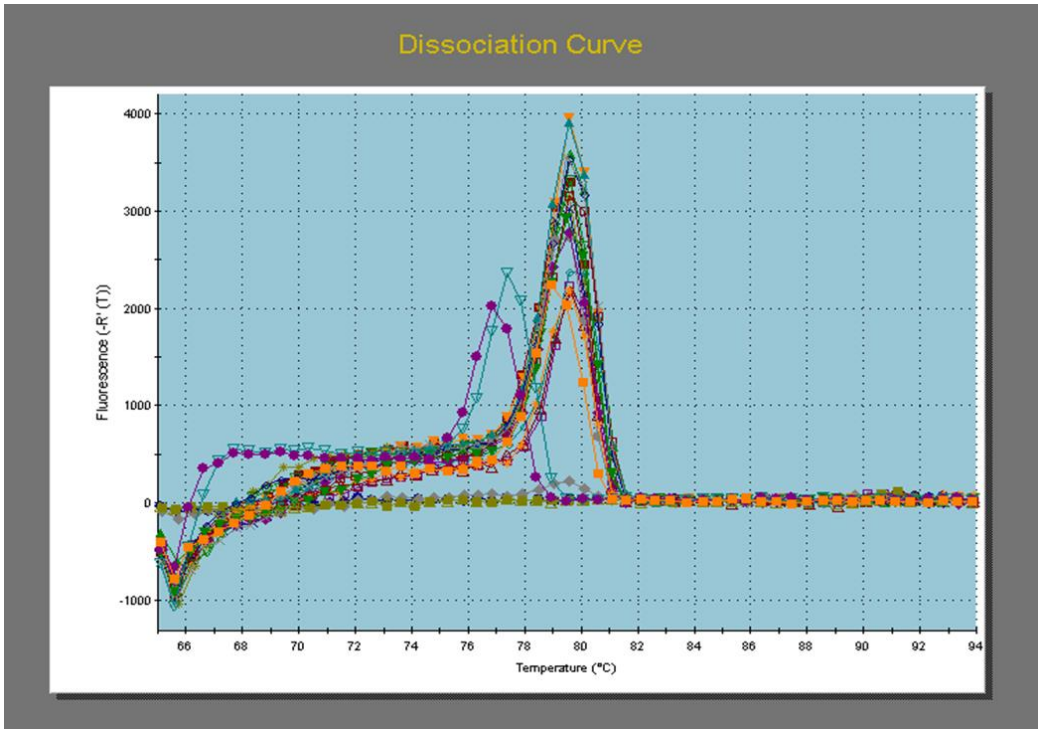
A5: Real-time PCR melting curve analysis for VTG



A6: Real-time PCR melting curve analysis for ZRP



A7: Real-time PCR melting curve analysis for HDLBP



A8: Real-time PCR melting curve analysis for TTR

Most of the curves revealed a single curve, and indicated presence of one main product. However, did some of the curves show a bulky and “left tail” indicating presence of smaller amounts of PCR products with lower Tm than the main product.

Appendix C: SPMD Datasheet

sampler name			Canal	Dam	River
sorbent amount		in mg	229	195	192
	instr LOD	precision			
	ng per 1 µl inj	% (n=3)	ng/sampler	ng/sampler	ng/sampler
Desethylatrazin			27,4	699,3	13,8
Simazin			9,7	526,9	60,6
Atrazin			15,5	273,4	88,5
Propazin			44,1	143,7	216,6
a-HCH			1,6	n.d.	10,4
HCB			0,1	0,2	0,4
ß-HCH			13,7	57,0	55,0
Lindan			0,0	n.d.	5,1
d-HCH			1,6	6,7	n.d.
Terbutylazin			44,7	146,3	207,0
Sebutylazin			n.d.	19,3	n.d.
Coffein			n.d.	599,2	13,1
Galaxolid			n.d.	n.d.	5,5
Tonalid			1,8	n.d.	17,1
Prometrin			n.d.	17,3	3,1
Metolachlor			54,7	18,0	261,5
Metazachlor			2,1	n.d.	3,1
Chlofenvinphos			13,8	41,5	39,4
BPA			n.d.	18,8	8,1
Carbamazepin			n.d.	713,5	8,8
EE			n.d.	n.d.	62,8
Metoxuron			n.d.	n.d.	143,9

Appendix D: Concentrations of steroids and proteins measured in individuals with student- t test

ESTRADIOL				
	Male ID	pg/ml	ID Female	pg/ml
			21910-2p	10
	8610-7p	215,8	21910-4p	72
	17811-4p	155	7610-3p	24,4
	17811-1p	111,4	8610-8p	70,5
	17811-2p	224,7	8610-9p	114,4
	17811-5p	119,6	7610-2p	72
	17811-9p	73,5	21910-3p	49,6
	17811-6p	12,4	7610-1p	47,2
	7610-5p	132,1	8610-10p	50,2
	17811-8p	207,2	7610-4p	75
	17811-3p	60,4	21910-1p	141,3
			7610-6p	222,9
SUM		1312,1		949,5
Mean		131,21		79,125
Std		70,886944		37,315503
Error				
T-test		0,08		Ulik

TESTOSTERONE				
	Male ID	pg/ml	ID Female	pg/ml
			21910-2p	0
	8610-7p	12,1	7610-2p	0,1
	17811-4p	3,7	7610-3p	0
	17811-1p	4,9	8610-8p	1,2
	17811-5p	12,7	8610-9p	0,5
	7610-5p	71,2	21910-3p	0,2
	17811-8p	39,5	7610-1p	2,4
	17811-3p	51	8610-10p	1,6
			7610-4p	2,5
			21910-1p	0,2
			7610-6p	12,1
SUM		195,1		20,8
Mean		27,871429		1,7333333
Std		26,262693		3,5132477
Error				
T-test		0,04		Ulik

11- KETOTESTOSTERONE				
	ID Female	pg/ml	Male ID	pg/ml
	LC21910-2p	8,9	LC17811-10p	7,5
	LC7610-2p	7,9	LC8610-7p	7,9
	LC7610-3p	8,2	LC17811-4p	0,6
	LC8610-8p	8,2	LC17811-7p	6,2
	LC8610-9p	8,5	LC17811-1p	11,6
	LC21910-4p	6,7	LC17811-2p	104,9
	LC21910-3p	7,3	LC17811-5p	8,2
	LC7610-1p	8,5	LC17811-9p	13,2
	LC8610-10p	11,6	LC17811-6p	15,1
	LC7610-4p	15,6	LC7610-5p	64,9
	LC21910-1p	10,3	LC17811-8p	15,0
	LC7610-6p	15,7	LC17811-3p	78,8
SUM		117,48073		334,01966
MEAN		9,7900604		27,834972
STDEV		3,0031333		34,539956
SEM		0,8669299		9,9708264
T-test		0,10		Ulik

ZONA PELLUCIDA				
	ID Female	Copies 10*5	Male ID	Copies 10*5
	LC7610-1L	0,5	LC17811-7L	22,5
	LC8610-9L	6,8	LC17811-8L	3,5
	LC7610-2L	1,1	LC17811-6L	5,9
	LC8610-10L	4,7	LC17811-9L	7,1
	LC7610-4L	3,9	LC17811-1L	7,6
	LC21910-4L	12,9	LC17811-5L	1,5
	LC7610-4L	3,7	LC17811-2L	4,4
	LC21910-1L	6,1	LC17811-10L	5,7
	LC7610-6L	2,6	LC7610-5L	0,3
	LC21910-2L	11,1	LC17811-3L	8,5
	LC21910-3L	3,6	LC17811-4L	1,2
	LC8610-8L	4,4	LC8610-7L	3,3
SUM		61,2984		71,5522
MEAN		5,1082		5,9626833
STDEV		3,7039201		5,823587
T-test		0,67		Lik

HDLBP				
	ID Female	Copies 10*5	Male ID	Copies 10*5
	LC7610-4L	0,4	LC17811-1L	1,9
	LC7610-4L	0,2	LC-17811-L	3,0
	LC8610-9L	1,8	LC17811-8L	1,0
	LC8610-10L	0,7	LC17811-9L	2,1
	LC21910-1L	1,7	LC17811-2L	1,4
	LC7610-6L	0,6	LC17811-10L	1,3
	LC21910-3L	1,8	LC7610-5L	0,1
	LC8610-8L	0,2	LC17811-3L	2,0
	LC21910-2L	5,8	LC17811-4L	1,8
	LC21910-4L	7,6	LC8610-7L	0,4
			LC17811-5L	3,2
			LC17811-6L	3,8
SUM		20,646645		21,857206
MEAN		2,0646645		1,8214338
STDEV		2,5540407		1,0956543
T-test		0,78		Lik

CR TTR				
	ID Female	Copies 10*5	Male ID	Copies 10*5
	LC8610-10L	3,6	LC17811-6L	6,7
	LC8610-9L	5,6	LC17811-9L	3,8
	LC7610-4L	0,8	LC17811-1L	8,3
	LC21910-1L	2,1	LC17811-2L	5,7
	LC7610-6L	0,7	LC17811-10L	2,0
	LC21910-2L	3,4	LC17811-8L	2,0
	LC7610-4L	1,0	LC17811-3L	8,1
	LC17811-6L	5,9	LC17811-4L	5,6
	LC21910-4L	1,5	LC8610-7L	2,0
			LC17811-7L	14,5
SUM		24,508744		5,8615116
MEAN		2,7231938		5,8615116
STDEV		2,0120935		3,8911641
T-test		0,04		Ulik

VTG				
	ID Female	Copies 10*5	Male ID	Copies 10*5
	LC21910-3L	0,2	LC17811-7L	0,1
	LC21910-4L	0,1	LC17811-8L	1,0
	LC7610-2L	1,0	LC17811-1L	1,3
	LC8610-10L	0,1	LC8610-7L	1,2
	LC7610-1L	1,1	LC17811-5L	1,6
	LC8610-9L	1,0		
	LC21910-1L	0,9		
	LC7610-6L	1,0		
SUM		5,4893		5,1274
MEAN		0,6861625		1,02548
STDEV		0,473543		0,5597029
T-test		0,29		Ulik

Appendix E: Spearman's rank correlation coefficient data

Female

Estradiol					**Testosterone**		
Compound	Rho	P-Value			Compound	Rho	P-Value
C10-C12	-0,445	0,150			C10-C12	-0,251	0,458
C12-C14	-0,385	0,218			C12-C14	-0,210	0,537
C14-C16	-0,420	0,176			C14-C16	-0,183	0,593
C16-C18	-0,207	0,520			C16-C18	-0,178	0,603
C18-C20	-0,207	0,520			**C18-C20**	**-0,785**	**0,007**
C20-C22	-0,214	0,506			**C20-C22**	**-0,708**	**0,019**
C22-C24	0,021	0,947			C22-C24	-0,489	0,131
C24-C26	-0,049	0,886			C24-C26	-0,557	0,080
C26-C28	-0,312	0,324			**C26-C28**	**-0,845**	**0,002**
C28-C30	**-0,609**	**0,040**			**C28-C30**	**-0,772**	**0,008**
C30-C32	-0,466	0,130			C30-C32	-0,370	0,265
C32-C34	-0,525	0,083			C32-C34	-0,374	0,258
C34-C36	-0,452	0,143			C34-C36	-0,603	0,055
C36-C38	-0,091	0,783			C36-C38	-0,237	0,483
C38-C40	-0,466	0,130			**C38-C40**	**-0,703**	**0,020**
Benzen	-0,245	0,443			Benzen	-0,438	0,180
Naphthalene	-0,494	0,106			Naphthalene	0,256	0,441
1-Methylnaphthalene	**-0,641**	**0,029**			1-Methylnaphthalene	0,059	0,859
2-Methylnaphthalene	**-0,592**	**0,047**			2-Methylnaphthalene	0,137	0,682
Acenaphthene	**-0,680**	**0,019**			Acenaphthene	0,059	0,859
Fluorene	**-0,648**	**0,027**			Fluorene	-0,009	0,989
Phenanthrene	**-0,739**	**0,008**			Phenanthrene	-0,155	0,652
Benzo[k]fluoranthene	-0,256	0,423			Benzo[k]fluoranthene	-0,598	0,057
As	**-0,599**	**0,044**			As	-0,119	0,733
Ba	0,140	0,659			Ba	-0,082	0,817
Cd	-0,088	0,791			Cd	-0,210	0,537
Cr	-0,550	0,068			Cr	0,037	0,913
Cu	0,074	0,817			Cu	-0,201	0,556
Fe	-0,333	0,291			Fe	-0,237	0,483
Hg	-0,091	0,783			Hg	-0,438	0,180
Ni	-0,375	0,231			Ni	-0,160	0,642
Pb	0,231	0,463			Pb	-0,233	0,492
V	0,161	0,611			V	-0,233	0,492
Zn	0,021	0,947			Zn	-0,507	0,116
Pyrene	**-0,786**	**0,028**			Pyrene	-0,393	0,396
Benzo[b]fluoranthene	0,241	0,467			Benzo[b]fluoranthene	-0,237	0,513
Mn	0,228	0,493			Mn	0,134	0,705

11-Ketotestosterone					**Zona Pellucida**		
Compound	Rho	P-Value			Compound	Rho	P-Value
C10-C12	-0,393	0,208			C10-C12	0,280	0,373
C12-C14	-0,284	0,371			C12-C14	0,322	0,303
C14-C16	-0,246	0,442			C14-C16	0,350	0,261
C16-C18	-0,007	0,991			C16-C18	0,350	0,261
C18-C20	-0,323	0,307			C18-C20	0,147	0,643
C20-C22	-0,372	0,235			C20-C22	0,021	0,947
C22-C24	-0,354	0,259			C22-C24	0,462	0,131
C24-C26	-0,291	0,359			C24-C26	0,329	0,292
C26-C28	-0,375	0,230			C26-C28	0,448	0,144
C28-C30	-0,418	0,179			C28-C30	0,035	0,912
C30-C32	-0,389	0,212			C30-C32	0,315	0,314
C32-C34	-0,509	0,095			C32-C34	0,455	0,137
C34-C36	-0,214	0,505			C34-C36	0,056	0,860
C36-C38	-0,074	0,825			C36-C38	0,420	0,173
C38-C40	-0,344	0,274			C38-C40	0,308	0,325
Benzen	-0,018	0,965			Benzen	-0,049	0,886
Naphthalene	0,140	0,658			Naphthalene	-0,077	0,817
1-Methylnaphthalene	-0,126	0,699			1-Methylnaphthalene	-0,301	0,342
2-Methylnaphthalene	-0,081	0,808			2-Methylnaphthalene	-0,308	0,331
Acenaphthene	-0,102	0,757			Acenaphthene	-0,343	0,276
Fluorene	-0,168	0,602			Fluorene	-0,469	0,127
Phenanthrene	-0,260	0,415			Phenanthrene	-0,462	0,134
Benzo[k]fluoranthene	-0,049	0,886			Benzo[k]fluoranthene	-0,210	0,514
As	-0,014	0,974			As	-0,063	0,852
Ba	**0,747**	**0,007**			Ba	0,294	0,348
Cd	0,365	0,240			Cd	0,294	0,348
Cr	0,281	0,371			Cr	-0,021	0,956
Cu	-0,365	0,244			Cu	0,140	0,659
Fe	-0,189	0,556			Fe	0,147	0,643
Hg	0,175	0,579			Hg	-0,231	0,471
Ni	0,200	0,527			Ni	0,245	0,437
Pb	-0,025	0,947			Pb	-0,098	0,766
V	0,249	0,428			V	-0,392	0,210
Zn	-0,147	0,650			Zn	0,035	0,912
Pyrene	-0,169	0,665			Pyrene	-0,500	0,216
Benzo[b]fluoranthene	0,005	0,989			Benzo[b]fluoranthene	-0,200	0,558
Mn	0,292	0,376			Mn	-0,100	0,776

HDLBP					**CR TTR**		
Compound	Rho	P-Value			Compound	Rho	P-Value
C10-C12	**0,661**	**0,042**			C10-C12	0,583	0,108
C12-C14	0,624	0,057			**C12-C14**	**0,767**	**0,021**
C14-C16	**0,745**	**0,017**			C14-C16	0,317	0,410
C16-C18	0,588	0,077			C16-C18	-0,383	0,313
C18-C20	0,139	0,694			C18-C20	0,333	0,385
C20-C22	-0,030	0,946			C20-C22	0,333	0,385
C22-C24	0,297	0,397			C22-C24	0,667	0,059
C24-C26	0,479	0,160			C24-C26	0,550	0,133
C26-C28	0,224	0,525			C26-C28	0,200	0,613
C28-C30	0,382	0,271			C28-C30	0,100	0,810
C30-C32	**0,818**	**0,006**			C30-C32	0,500	0,178
C32-C34	0,612	0,063			C32-C34	0,567	0,121
C34-C36	0,139	0,694			C34-C36	0,367	0,336
C36-C38	0,079	0,825			C36-C38	-0,083	0,843
C38-C40	**0,709**	**0,026**			C38-C40	0,233	0,552
Benzen	0,042	0,905			Benzen	-0,517	0,162
Naphthalene	0,261	0,459			Naphthalene	0,150	0,708
1-Methylnaphthalene	0,091	0,798			1-Methylnaphthalene	0,250	0,521
2-Methylnaphthalene	0,079	0,825			2-Methylnaphthalene	0,233	0,552
Acenaphthene	0,006	0,986			Acenaphthene	0,233	0,552
Fluorene	-0,188	0,608			Fluorene	0,250	0,521
Phenanthrene	-0,224	0,537			Phenanthrene	0,217	0,581
Benzo[k]fluoranthene	-0,055	0,892			Benzo[k]fluoranthene	-0,217	0,581
As	0,455	0,185			As	-0,350	0,336
Ba	-0,018	0,973			Ba	-0,183	0,644
Cd	0,467	0,172			Cd	0,133	0,744
Cr	0,200	0,572			Cr	0,300	0,437
Cu	0,200	0,572			Cu	0,467	0,213
Fe	**0,648**	**0,046**			Fe	0,283	0,463
Hg	-0,042	0,919			Hg	-0,600	0,097
Ni	0,164	0,644			Ni	0,250	0,521
Pb	**-0,758**	**0,016**			Pb	-0,333	0,385
V	-0,539	0,113			V	-0,483	0,194
Zn	-0,358	0,313			Zn	0,283	0,463
Pyrene	-0,643	0,139			Pyrene	-0,086	0,919
Benzo[b]fluoranthene	-0,433	0,250			Benzo[b]fluoranthene	-0,524	0,197
Mn	-0,017	0,982			Mn	-0,667	0,083

VTG		
Compound	Rho	P-Value
C10-C12	0,048	0,935
C12-C14	-0,071	0,882
C14-C16	-0,333	0,428
C16-C18	0,000	1,000
C18-C20	0,190	0,665
C20-C22	0,238	0,582
C22-C24	-0,405	0,327
C24-C26	-0,595	0,132
C26-C28	-0,595	0,132
C28-C30	0,238	0,582
C30-C32	-0,024	0,977
C32-C34	-0,143	0,752
C34-C36	-0,048	0,935
C36-C38	-0,238	0,582
C38-C40	0,000	1,000
Benzen	0,048	0,935
Naphthalene	0,048	0,935
1-Methylnaphthalene	0,143	0,752
2-Methylnaphthalene	0,143	0,752
Acenaphthene	0,143	0,752
Fluorene	0,190	0,665
Phenanthrene	0,190	0,665
Benzo[k]fluoranthene	0,095	0,840
As	0,095	0,840
Ba	0,214	0,619
Cd	-0,214	0,619
Cr	0,143	0,752
Cu	0,286	0,501
Fe	-0,667	0,083
Hg	0,095	0,840
Ni	0,452	0,267
Pb	-0,143	0,752
V	0,143	0,752
Zn	0,167	0,703
Pyrene	0,257	0,658
Benzo[b]fluoranthene	-0,095	0,840
Mn	-0,143	0,783

Male

Estradiol		
Compound	Rho	P-Value
C10-C12	0,212	0,548
C12-C14	0,018	0,959
C14-C16	**-0,648**	**0,049**
C16-C18	-0,430	0,218
C18-C20	-0,006	1,000
C20-C22	0,079	0,825
C22-C24	0,091	0,798
C24-C26	-0,164	0,657
C26-C28	-0,479	0,166
C28-C30	-0,006	1,000
C30-C32	-0,103	0,785
C32-C34	-0,176	0,632
C34-C36	-0,455	0,191
C36-C38	-0,297	0,407
C38-C40	-0,042	0,919
Benzen	-0,079	0,838
Naphthalene	0,030	0,932
1-Methylnaphthalene	-0,321	0,368
2-Methylnaphthalene	-0,588	0,080
Acenaphthene	**-0,782**	**0,012**
Fluorene	-0,479	0,166
Phenanthrene	-0,571	0,091
Benzo[b]fluoranthene	-0,248	0,492
As	-0,309	0,387
Ba	-0,188	0,608
Cd	-0,382	0,279
Cr	-0,285	0,427
Cu	0,055	0,878
Fe	-0,164	0,657
Hg	-0,212	0,560
Mn	0,042	0,905
Ni	-0,576	0,088
Pb	0,103	0,772
V	-0,188	0,608
Zn	-0,152	0,682
Pyrene	-0,800	0,133
Benzo[k]fluoranthene	-0,257	0,658
Benzo[a]pyrene	-0,029	1,000
Heptachlor epoxide	-0,600	0,350

Testosterone		
Compound	Rho	P-Value
C10-C12	0,179	0,713
C12-C14	-0,143	0,783
C14-C16	0,357	0,444
C16-C18	0,357	0,444
C18-C20	-0,250	0,595
C20-C22	-0,286	0,556
C22-C24	-0,464	0,302
C24-C26	-0,214	0,662
C26-C28	-0,429	0,354
C28-C30	-0,571	0,200
C30-C32	-0,071	0,906
C32-C34	-0,179	0,713
C34-C36	-0,429	0,354
C36-C38	-0,179	0,713
C38-C40	-0,321	0,498
Benzen	-0,036	0,963
Naphthalene	-0,107	0,840
1-Methylnaphthalene	0,357	0,444
2-Methylnaphthalene	0,143	0,783
Acenaphthene	0,679	0,110
Fluorene	0,321	0,498
Phenanthrene	**0,883**	**0,012**
Benzo[b]fluoranthene	0,000	1,000
As	0,143	0,783
Ba	0,071	0,906
Cd	0,286	0,556
Cr	0,321	0,498
Cu	-0,179	0,713
Fe	0,071	0,906
Hg	0,071	0,906
Mn	0,143	0,783
Ni	0,357	0,444
Pb	-0,071	0,906
V	0,071	0,906
Zn	0,036	0,963
Pyrene	0,500	1,000
Benzo[k]fluoranthene	0,200	0,917
Benzo[a]pyrene	-0,400	0,417
Heptachlor epoxide	0,400	0,750

11-Ketotestosterone					**Zona Pellucida**		
Compound	Rho	P-Value			Compound	Rho	P-Value
C10-C12	0,517	0,086			C10-C12	-0,378	0,227
C12-C14	0,427	0,165			C12-C14	0,112	0,724
C14-C16	0,497	0,101			C14-C16	0,559	0,061
C16-C18	0,336	0,281			C16-C18	0,462	0,131
C18-C20	0,175	0,581			C18-C20	0,399	0,197
C20-C22	0,126	0,692			C20-C22	0,273	0,385
C22-C24	0,056	0,860			C22-C24	0,007	0,982
C24-C26	-0,196	0,543			C24-C26	-0,014	0,974
C26-C28	-0,042	0,904			C26-C28	0,245	0,437
C28-C30	-0,364	0,246			C28-C30	-0,119	0,716
C30-C32	0,259	0,410			C30-C32	0,252	0,424
C32-C34	0,294	0,348			C32-C34	0,252	0,424
C34-C36	0,049	0,878			C34-C36	0,147	0,643
C36-C38	0,245	0,437			C36-C38	0,196	0,535
C38-C40	0,322	0,303			C38-C40	-0,070	0,834
Benzen	0,168	0,596			Benzen	0,322	0,303
Naphthalene	0,392	0,205			Naphthalene	0,133	0,675
1-Methylnaphthalene	**0,727**	**0,010**			1-Methylnaphthalene	0,280	0,373
2-Methylnaphthalene	0,259	0,410			2-Methylnaphthalene	0,245	0,437
Acenaphthene	0,545	0,069			Acenaphthene	0,105	0,741
Fluorene	**0,706**	**0,013**			Fluorene	0,371	0,232
Phenanthrene	0,473	0,121			Phenanthrene	0,168	0,595
Benzo[b]fluoranthene	0,434	0,158			Benzo[b]fluoranthene	0,147	0,643
As	0,259	0,410			As	0,343	0,271
Ba	0,357	0,251			Ba	0,490	0,107
Cd	**0,587**	**0,047**			**Cd**	**0,601**	**0,041**
Cr	0,336	0,281			Cr	-0,126	0,700
Cu	0,105	0,741			Cu	-0,175	0,588
Fe	0,469	0,124			Fe	0,245	0,437
Hg	0,406	0,189			**Hg**	**0,671**	**0,020**
Mn	0,329	0,292			Mn	0,524	0,082
Ni	0,336	0,281			Ni	-0,105	0,749
Pb	0,077	0,809			Pb	0,077	0,809
V	0,371	0,232			V	0,531	0,077
Zn	0,399	0,197			Zn	0,427	0,165
Pyrene	0,771	0,103			Pyrene	-0,086	0,919
Benzo[k]fluoranthene	-0,179	0,713			Benzo[k]fluoranthene	0,107	0,840
Benzo[a]pyrene	0,500	0,267			Benzo[a]pyrene	0,286	0,556
Heptachlor epoxide	0,571	0,200			Heptachlor epoxide	-0,286	0,556

HDLBP					**CR TTR**		
Compound	Rho	P-Value			Compound	Rho	P-Value
C10-C12	-0,147	0,651			C10-C12	-0,176	0,632
C12-C14	-0,021	0,956			C12-C14	0,067	0,851
C14-C16	**0,636**	**0,029**			C14-C16	0,297	0,397
C16-C18	0,364	0,241			C16-C18	0,067	0,851
C18-C20	0,084	0,792			C18-C20	0,042	0,905
C20-C22	0,056	0,860			C20-C22	0,042	0,905
C22-C24	-0,175	0,588			C22-C24	-0,479	0,166
C24-C26	0,217	0,492			C24-C26	-0,624	0,060
C26-C28	0,476	0,118			C26-C28	-0,152	0,682
C28-C30	0,014	0,965			C28-C30	-0,006	1,000
C30-C32	-0,126	0,700			C30-C32	-0,006	1,000
C32-C34	-0,028	0,939			C32-C34	-0,067	0,865
C34-C36	0,378	0,223			C34-C36	0,115	0,746
C36-C38	0,280	0,373			C36-C38	0,006	0,986
C38-C40	0,196	0,535			C38-C40	0,030	0,932
Benzen	0,434	0,158			Benzen	-0,079	0,838
Naphthalene	0,014	0,965			Naphthalene	-0,091	0,811
1-Methylnaphthalene	0,231	0,464			1-Methylnaphthalene	0,079	0,825
2-Methylnaphthalene	0,308	0,325			2-Methylnaphthalene	0,091	0,798
Acenaphthene	0,371	0,232			Acenaphthene	0,176	0,620
Fluorene	0,364	0,241			Fluorene	0,382	0,271
Phenanthrene	0,235	0,456			Phenanthrene	-0,128	0,732
Benzo[b]fluoranthene	0,070	0,826			Benzo[b]fluoranthene	-0,079	0,838
As	0,231	0,464			As	0,358	0,304
Ba	0,378	0,223			Ba	0,224	0,525
Cd	0,538	0,073			Cd	0,600	0,070
Cr	0,217	0,492			Cr	-0,115	0,759
Cu	0,531	0,077			Cu	0,127	0,720
Fe	0,545	0,069			Fe	0,297	0,397
Hg	0,559	0,061			Hg	0,552	0,100
Mn	0,322	0,303			Mn	0,612	0,063
Ni	0,336	0,281			Ni	0,152	0,669
Pb	0,196	0,535			Pb	0,139	0,694
V	**0,587**	**0,047**			**V**	**0,648**	**0,046**
Zn	0,559	0,061			Zn	0,503	0,138
Pyrene	0,143	0,803			Pyrene	-0,300	0,683
Benzo[k]fluoranthene	0,286	0,556			Benzo[k]fluoranthene	0,000	1,000
Benzo[a]pyrene	0,357	0,444			Benzo[a]pyrene	0,600	0,350
Heptachlor epoxide	0,464	0,302			Heptachlor epoxide	-0,257	0,658

Appendix F: Extended methods for chemical analyses and biomarker measurements

Semi permeable membrane device (SPMD) analysis

SPMD analysis was performed in collaboration with Dr. Monika Möder at Helmholtz center for Environmental Research UFZ, Department of Analytical Chemistry, Leipzig, Germany. Contaminant sampling in the aquatic environment was collected by using a Exposmeter Hydrophilic (EWH) and Lipophilic (EWL) for water samples based SPMD technology supplied by ExposMeter AB, (Trehorningen, Sweden) that were dismounted and separated in sorbent parts and the membrane. Materials were separately extracted and finally combined to analyzed extract. The EWH and EWL device series monitores lipophilic and hydrophilic contaminants with possible endocrine disrupting effects or acutely toxic in environment. Devices were assembled according to the manufacturer`s instructions and placed in 3 different locations: 1) In the Crocodile River that flows past Le Croc farm, 2) at the inflow area where the canal water enters the farm, and 3) in the crocodile breeder dam within Le Croc farm. The EWH and EWL samples were deployed in situ, and the contaminant concentration were measures in the SPMD for 28 days. By this, the samples were able to collect contaminant transport and episodic flow at the locations throughout the exposure period. After the end of the 28-days deployment period, the devices were removed, transformed to a laboratory and frozen at -80 °C for further analysis.

EWH and EWL Chemical analyses

The EWH and EWL membranes were air dried and extracted twice with methanol (5 mL) in the ultrasonic bath (Branson 5500) for 15 min. The unified extracts were evaporated using inert gas (TurboVap II, Zymark, Idstein, Germany) and reconstituted in *n*-hexane (1 mL), that afterwards was added to the extract of the sorbent material.

Extended methods for POCI analysis

The POCIS sampler were exposed over 30 days and removed from water on October 19th 2011. POCIS ET-100-628 hydrophilic PEST (ExpoMeter, Exposimeter AB,

Trehorningen, Sweden) were dismounted and separated in membrane and sorbent parts. Both materials were separately extracted and finally combined to one analyzed extract.

The membrane was air dried and extracted twice with methanol (5 mL) in a ultrasonic bath (Branson 5500) for 15 min. The unified extracts were evaporated using inert gas (TurboVap II, Zymark, Idstein, Germany) and reconstituted in n-hexane (1 mL) that was added later to the extract of the sorbent material.

The sorbent was filled in a glass cartridge (6 mL volume) as for SPE use. After drying the sorbent in a nitrogenstream, the elution was performed with methanol (12 mL) and ethylacetate/n-hexane (v/v , 1/1)(12 mL) (all solvents Sigma Aldrich, Fluka, HPLC- or GC-purity grade). Membrane material extract was combined with the sorbent extract, and the analysis was carried out from the solution (200 µL) after evaporation.

Gas chromatography- mass spectrometry (GC-MS) was applied at target and non-target screening mode using an Agilent 6890 GC coupled to an Agilent 5973N Mass spectrometer (Agilent Technologies, Waldbronn, Germany). One microliter of the extract was injected at 280 °C injector temperature. Helium serves as carrier gas at a flow of 1 mL/min. The substances were separated on a 30 m-long GC capillary (HP 5MS, 0.25 mm i.d., 0.25 µm film thickness, Agilent Technologies).The GC-oven was initially set on 60 °C , following a hold for 1 min and increased by 10 K/min to 280 °C hold for 15 min. The transfer line temperature was set at 280 °C. The mass spectrometer operated at electron ionization at 70 eV - electron energy. Full scan (non-target) analysis covered the mass range from 50 u to 500 u, and a blank analysis (solvent, instrument) was conducted after each sample. External standards (200 ng/mL each) were measured randomly between the samples of a batch. The calibration curves of the target analytes were linear in the range between LOD and 1 µg/mL.

Polycyclic aliphatic hydrocarbons (PAHs) analysis

PAH analysis was performed in collaboration with Prof. Francesco Regoli and his research group at Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy. Aliphatic hydrocarbons (C10-C40), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organo-halogenated pesticides (OCPs), chlorophenols, monoaromatic compounds (BTEX: benzene, toluene, ethylbenzene and xylene congeners), brominated flame retardants and trace metal were analyzed in crocodile liver by conventional procedures based on

gas-chromatography with flame ionization detector, electron capture detector and mass detector, high performance liquid chromatography (HPLC) with diode array and fluorimetric detection, followed by atomic absorption spectrophotometry (Bradford, 1976; Fattorini et al, 2008; Piva et al, 2011; Regoli et al, 2014).

For collection of trace metals tissues were dried in 60°C and digested under pressure with nitric acid and hydrogen peroxide (7:1) with microwave. Arsenic, cadmium, chromium, copper, iron, manganese, nickel, lead, vanadium, zinc were analyzed by atomic absorption spectrophotometry, with flame (Varian, SpectrAA 220FS) and flameless atomization (Varian SpectrAA 240Z). Mercury content was quantified by cold vapor atomic absorption spectrometry (Cetac QuickTrace M-6100 Mercury Analyzer) (Fattorini et al, 2008). Aliphatic hydrocarbons were extracted treating tissues (about 3 g, wet weight) with hexaneacetone (2:1) in a microwave (110°C for 25 min, 800 Watt) (Mars CEM, CEM Corporation, Matthews NC). After centrifugation, the supernatants were purified with solid-phase extraction (Phenomenex Strata-X, 500 mg × 6 mL plus Phenomenex Strata-FL, 1000 mg × 6 mL) and concentrated using a SpeedVac (RC1009; Jouan, Nantes, France) to dry. Samples were finally recovered with 1mL of pure, analytical GC grade *n*-hexane and analyzed with a gas chromatograph (Perkin Elmer) with an Elite-5 capillary column (30 m × 0.32 mm ID × 0.25 μm-df) and a flame ionization detector (Bradford, 1976).

The system was calibrated with an unsaturated pair *n*-alkane standard mixture according to EN ISO 9377-3 for quantitative determination (Fluka 68281). Analysis of polycyclic aromatic hydrocarbons (PAHs) were conducted by use of about 3 g (wet weight) of tissues, that first was extracted in 10 mL 0.5 M potassium hydroxide in methanol with microwave at 55°C for 20 min (800 Watt) (CEM, Mars System). After centrifugation, the methanolic solutions were concentrated using a SpeedVac and purified with solid-phase extraction (Octadecyl C18, 500 mg × 6 mL, Bakerbond). A final volume of 1 mL was recovered with pure HPLC gradient grade acetonitrile, and HPLC analyses were carried out in a water and acetonitrile gradient by fluorimetric and diode array detection. The PAHs were identified according to the retention times of an appropriate pure standards solution (EPA 610 Polynuclear Aromatic Hydrocarbons Mix), and classified as low molecular weight (LMW: naphthalene, acenaphthylene, 1-methyl naphthalene, 2-methyl naphthalene, acenaphthene, fluorene, phenanthrene, anthracene) or high molecular weight (HMW: fluoranthene, pyrene,

benzo(a)antrhacene, chrysene, 7,12-dimethyl benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, indeno(1,2,3,c,d)pyrene).

Polychlorinated biphenyls (PCBs), organo-halogenated pesticides (OCPs), chlorophenols, monoaromatic compounds (BTEX: benzene, toluene, ethylbenzene and xylene congeners) and brominated flame retardants (BFRs) have been determined according to EPA methods 1614, 3550B, 3665A, 3630C, 8020, 8081B, 8082A with slight modifications. 3 g of wet tissues were extracted with hexaneacetone (2:1) solutions in a microwave (110°C for 25 min, 800 Watt) (Mars CEM, CEM Corporation). After centrifugation, the supernatants were purified with solid-phase extraction (Phenomenex Strata-X, 500mg, 6mL and Phenomenex Strata-FL, 1000mg, 6 mL), using an automated SPE system (Gilson Aspec GX271, Gilson Middleton, WI, USA) and then concentrated using a SpeedVac (RC1009; Jouan, Nantes, France) to dry. Samples were recovered with analytical GC grade n-hexane (1 mL) and analyzed with a GC-MS system (Varian Saturn 2000 ion trap, Agilent Technologies, Santa Clara, CA, USA) using a GC capillary column Zebron (Zebron ZB-5MS, 30m, 0.25mmID, 0.25µ, Phenomenex), applying three different spit-splitless and oven temperature ramp methods for following pollutants: 1): BTEX (benzene, toluene, ethylbenzene and xylene congeners); 2): chlorophenols (2,4-dichlorophenol, 2,4,6-trichlorophenol, pentachlorophenol), OCPs (2,4-dichlorophenol, 2,4,6-trichlorophenol, pentachlorophenol, α-lindane, β-lindane, δ-lindane, γ-lindane, α-chlordane, γ-chlordane, 4,4'-DDD, 4,4'-DDE, 4,4'-DDT, aldrin, dichlorobenzidine, dieldrin, endrin, endrin aldehyde, endrin ketone, hexachlorobenzene, methoxychlor, endosulfan I, endosulfan II, endosulfan sulfate, heptachlor, heptachlor epoxide), chlorobiphenyls and PCBs congeners (2-chlorobiphenyl, 3-chlorobiphenyl, 4-chlorobiphenyl, PCB4, PCB8, PCB11, PCB16, PCB17, PCB18, PCB19, PCB28, PCB38, PCB44, PCB46, PCB52, PCB66, PCB77, PCB81, PCB101, PCB105, PCB118, PCB126, PCB128, PCB138, PCB153, PCB156, PCB169, PCB170, PCB172, PCB180, PCB182, PCB187, PCB192, PCB195, PCB203, PCB206 and PCB209); 3): BFRs, including hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA) and polybromodiphenylethers (PBDE28, PBDE47, PBDE100, PBDE99, PBDE154, PBDE153, PBDE183).

The multiple compounds were determined by the retention time of analytical standard solution mix (Supelco 47505-U, BTEX/MTBE; Supelco 43240-U, DM 471 Phenol mix; NIST 1493, PCBs congeners; Supelco 4-8862, Aroclor mix 2; Fluka 36989, PCBs congeners; Supelco 46845-U, Pesticide 8081; Supelco 4-0008, Hexachlorobenzene; Supelco 4-0026, 3,3'-dichlorobenzidine; AccuStandard M-1614-CSM, PBDEs congeners of primary interest; Fluka 11223, TBBPA; Aldrich 144762, HBCD) by comparing the mass spectra with the pure standard compounds, and verifying these by means of the NIST Database (NIST/EPA/NIH Mass Spectra Library version 2.0, National Institute for Standard and Technologies, NIST, Gaithersburg, MD, USA). For all of the chemical analyses, quality assurance and quality control were assured for all chemical analyses and monitored by processing blank and reference standard materials (NIST-2977, National Institute of Standards and Technology, USA). The resulting concentrations from these SRM analyses were all within the 95% confidence intervals of the certified values. The water content in tissues was determined for all the samples, and the concentrations were expressed as ng/g dry weight (dw) for PAHs, PCBs, OCPs, BFRs, while as μg/g dry weight (dw) for aliphatic hydrocarbons (C10-C40) and trace metals (Regoli et al, 2014).

Appendix G: Datasheet for contaminants in tissues

	Aliphatic hydrocarbons µg/g (dw)														BTEX ng/g (dw)
	C10-C12	C12-C14	C14-C16	C16-C18	C18-C20	C20-C22	C22-C24	C24-C26	C26-C28	C28-C30	C30-C32	C32-C34	C34-C36	C36-C38	C38-C40	Benzen
Female																
LC7610-1L	27,18	80,15	86,61	59,47	174,11	484,09	48,46	40,40	51,91	120,82	3820,99	336,77	71,39	75,75	203,64	3231,17
LC7610-2L	8,77	27,35	39,86	36,96	613,93	1999,39	153,47	303,92	282,80	71,17	1832,14	182,92	76,72	72,07	111,79	3291,1
LC7610-3L	6,36	19,11	58,80	53,16	274,40	797,63	34,32	122,50	330,72	174,83	1802,20	207,50	54,80	82,00	155,73	4188,1
LC7610-4L	4,19	5,46	39,13	54,37	115,92	285,79	29,20	66,45	50,44	14,17	595,33	35,01	16,87	315,63	0,00	4853,3
LC7610-6L	7,80	36,17	67,79	52,33	58,70	45,58	10,03	10,27	32,06	3,52	1355,19	92,46	26,45	8,30	28,99	2013,65
LC8610-8L	22,58	71,03	68,14	41,23	247,67	745,99	201,03	175,04	223,95	15,52	2305,39	329,84	146,39	100,93	85,37	2013,13
LC8610-9L	33,53	82,22	64,49	52,23	178,79	448,73	64,55	159,27	114,28	22,54	2458,97	265,01	25,00	73,67	105,40	2798,75
LC8610-10L	11,25	55,90	70,99	38,42	95,52	146,43	61,00	294,72	182,88	12,16	1968,17	184,51	29,99	46,25	94,86	1934,19
LC 21910-1L	5,37	43,25	79,25	71,75	863,99	2673,57	310,52	460,56	383,18	120,03	2813,12	236,62	220,63	114,96	209,93	3961,3
LC 21910-2L	66,10	197,79	135,87	82,93	294,46	719,52	109,06	198,43	294,35	163,80	5405,23	616,20	387,21	106,88	289,09	4008,44
LC 21910-3L	31,97	97,89	97,88	55,02	174,05	412,56	53,71	259,31	172,32	67,97	3527,06	324,45	52,69	82,33	129,44	3536,95
LC 21910-4L	22,74	69,69	96,74	66,97	168,25	352,96	65,45	179,42	281,98	46,89	3368,45	388,27	26,68	106,23	185,50	2968,88
Average	20,65	65,50	75,46	55,40	271,65	756,02	95,07	189,19	200,07	69,45	2604,35	266,63	94,57	98,75	133,31	3233,25
SEM	5,96	18,91	21,78	15,99	78,42	218,24	27,44	54,61	57,76	20,05	751,81	76,97	27,30	28,51	38,48	933,36
Male	C10-C12	C12-C14	C14-C16	C16-C18	C18-C20	C20-C22	C22-C24	C24-C26	C26-C28	C28-C30	C30-C32	C32-C34	C34-C36	C36-C38	C38-C40	Benzen
LC7610-5L	15,84	43,93	26,48	18,64	92,02	226,07	61,81	34,94	50,78	101,61	1940,07	129,26	21,35	24,13	120,85	1078,00
LC8610-7L	8,53	0,43	2,18	2,94	16,92	55,47	79,12	165,93	67,04	3,77	26,09	56,78	2,84	0,62	14,77	3442,5
LC17811-1L	7,88	45,13	55,48	64,29	977,62	2267,09	90,36	48,86	284,64	415,05	5872,59	895,78	553,98	212,74	333,54	2638,2
LC17811-2L	79,85	81,92	72,46	66,80	817,34	2139,31	173,78	95,41	187,41	146,05	3384,85	264,92	34,03	99,43	275,25	6904,6
LC17811-3L	8,63	47,55	85,31	80,27	136,81	201,68	18,69	50,29	89,23	8,93	3119,27	222,50	23,45	76,53	62,17	3296,49
LC17811-4L	26,31	50,57	24,55	17,41	160,32	456,47	84,90	121,67	106,10	215,91	2358,47	190,33	56,97	43,19	220,69	1733,32
LC17811-5L	7,97	7,60	66,06	72,70	117,63	282,41	42,90	145,66	289,84	197,53	1319,49	177,90	56,50	161,42	230,81	4333,3
LC17811-6L	14,36	26,97	73,35	44,70	382,43	1098,33	240,09	546,46	362,55	38,60	1737,32	184,02	73,17	44,11	156,98	5602,7
LC17811-7L	1,95	10,11	37,02	30,26	172,14	462,82	43,34	58,77	69,17	68,52	808,99	44,81	20,65	4,78	3,07	3507,4
LC17811-8L	10,91	47,23	35,81	51,58	1152,22	3184,23	473,53	175,50	251,25	172,53	4617,22	474,51	61,21	101,06	223,42	2729,9
LC17811-9L	72,16	204,43	127,93	84,67	556,16	1743,15	300,76	458,27	762,02	178,94	5346,60	617,18	193,45	327,22	517,02	4358,93
LC17811-10L	4,26	13,47	59,24	79,74	479,94	1356,93	290,47	539,29	530,46	217,45	4238,21	244,00	41,51	96,31	37,34	4487,7
Average	21,55	48,28	55,49	51,17	421,80	1122,83	158,31	203,42	254,21	147,07	2897,43	291,83	94,92	99,30	182,99	3676,08
SEM	6,22	13,94	16,02	14,77	121,76	324,13	45,70	58,72	73,38	42,46	836,42	84,24	27,40	28,66	52,83	1061,19

Chemical analysis (continued I)

PAH
ng/g (dw)

Female	Naphthalene	Ace-naphthylene	1-Methyl-naphthalene	2-Methyl-naphthalene	Ace-naphthene	Fluorene	Phen-anthrene	Anthracene	Fluor-anthene	Pyrene	Benzo(a)-anthracene	Chrysene	7,12-Di-methylbenzo-(a)anthracene	Benzo(b)-fluoranthene	Benzo(k)-fluoranthene	Benzo(a)-pyrene	Dibenzo(ah)-anthracene
LC7610-1L	1004,47		1338,13	1206,02	1601,37	864,85	1074,59			16,81				0,83	0,68		
LC7610-2L	380,734		588,635	525,036	701,542	356,907	499,631	16,700	404,507		178,899	241,881		73,554	41,434	16,273	
LC7610-3L	288,360		457,479	407,113	525,610	299,943	442,157		89,796	21,094				15,008	10,768	5,971	0,507
LC7610-4L	446,618	9,71137543	446,094	407,525	446,895	173,345	238,472					6,630		1,956	0,582		
LC7610-6L	750,62	52,88	722,06	690,25	811,18	329,03	345,03		15,88	12,53		7,88	15,34	15,97	5,65		
LC8610-8L	700,02		1088,85	891,27	1198,29	650,07	833,24		0,31	19,04		5,99		4,95	2,76		
LC8610-9L	435,04		386,22	351,26	380,90	149,11	181,10	8,26		7,57				0,65	0,35		
LC8610-10L	927,09		840,67	787,62	894,17	340,06	369,37			12,61		3,16		1,81	0,89		
LC 21910-1L	286,069		265,011	264,852	279,819	107,660	158,871							5,536	3,333		2,301
LC 21910-2L	1254,99		1334,15	1158,61	1359,89	551,74	692,14								25,59	0,67	1,32
LC 21910-3L	913,35	131,20	993,72	834,67	980,23	404,84	527,98			13,77				1,35	0,95		
LC 21910-4L	668,48		498,57	489,32	503,99	164,50	217,03		17,01	11,08		2,72		1,98	0,67		
Average	671,32	64,60	746,63	667,80	806,99	366,00	464,97	12,48	131,88	14,31		44,71		11,24	7,80	7,64	1,38
SEM	193,79	37,30	215,53	192,78	232,96	105,66	134,22	8,82	65,94	5,06		18,25		3,39	2,25	4,41	0,80

Male	Naphthalene	Ace-naphthylene	1-Methyl-naphthalene	2-Methyl-naphthalene	Ace-naphthene	Fluorene	Phen-anthrene	Anthracene	Fluor-anthene	Pyrene	Benzo(a)-anthracene	Chrysene	7,12-Di-methylbenzo-(a)anthracene	Benzo(b)-fluoranthene	Benzo(k)-fluoranthene	Benzo(a)-pyrene	Dibenzo(ah)-anthracene
LC7610-5L	261,76		384,47	304,11	401,77	206,34	281,80					2,44		1,56	1,36	0,32	0,28
LC8610-7L	344,017		404,335	337,930	362,251	154,983	219,423		38,552	8,655		6,257		2,333	1,258	0,563	
LC17811-1L	356,386		451,583	411,430	387,289	291,836	10						19,180	9,987			
LC17811-2L	763,306		614,063	253,984	363,654	371,333	16,807	96,967	17,213		0,582		15,777	8,965		5,932	
LC17811-3L	797,97		945,89	856,66	1065,68	453,30	457,57			12,02		2,91		0,50	0,41		
LC17811-4L	357,56		359,60	329,18	387,01	172,13	10,00		10,90		0,36		0,76	0,45			
LC17811-5L	316,776		423,683	385,959	478,255	211,505	269,892			8,461				0,981	1,105	0,299	
LC17811-6L	291,811		475,642	378,033	520,902	284,893	406,408	18,780	441,467	82,769	170,109	296,166		79,055	44,421	17,113	2,915
LC17811-7L	239,580		320,868	255,653	318,474	165,979	198,569		0,385	2,384	0,875	1,414		0,315			
LC17811-8L	370,877		491,901	421,392	505,589	229,010	323,620							6,123		0,040	7,412
LC17811-9L	1558,74		1565,87	1465,59	1707,50	713,82	829,37	1,84		26,98				31,39	29,55		33,55
LC17811-10L	272,294		265,171	245,937	238,983	92,569	109,651	4,983	0,609		1,276			3,010	1,879	0,226	0,505
Average	494,26		558,59	470,49	561,45	278,98	261,09	30,64	84,85	23,54	28,87	51,53	11,91	12,06	11,43	3,50	8,93
SEM																	

	Chloro-phenols		Halogenated pesticides (OCP) ng/g (dw)						Polybrominated diphenyl ethers (PBDE) ng/g (dw)					
Female	2,4,6 Trichloro Phenol	2,4-Dichloro Phenol	Endrin	4,4'-DDD	Endosulfan I	Endosulfan II	Heptachlor	Heptachlor epoxide	PBDE 28	PBDE 100	PBDE 99	PBDE 154	PBDE 153	PBDE 183
LC7610-1L												409,12		
LC7610-2L	83,16	96,90			154,82			128,07	41,72		9,16			
LC7610-3L				43,68	262,68		391,01			10,85				
LC7610-4L											9,68			
LC7610-6L					456,97	89,93		86,86				163,78		
LC8610-8L						84,13	143,97						42,68	
LC8610-9L														
LC8610-10L												33,47	34,04	
LC 21910-1L	93,48		4275,82								56,21			
LC 21910-2L										121,69		213,01	229,15	
LC 21910-3L														
LC 21910-4L												181,54		
Average	88,32		4275,82	43,68	291,49	87,03	267,49	107,47	41,72	66,27	25,02	200,18	101,95	0,00
SEM	62,45				168,29	61,54	189,14	75,99		46,86	14,44	89,52	58,86	0,00
Male	2,4,6 Trichloro Phenol	2,4-Dichloro Phenol	Endrin	4,4'-DDD	Endosulfan I	Endosulfan II	Heptachlor	Heptachlor epoxide	PBDE 28	PBDE 100	PBDE 99	PBDE 154	PBDE 153	PBDE 183
LC7610-5L												24,13		
LC8610-7L	628,03					1107,11								527,44
LC17811-1L	204,26				821,43		466,20	208,91						
LC17811-2L														
LC17811-3L						41,29		87,96				29,85	45,45	
LC17811-4L					17,74	20,95		62,95				313,66		
LC17811-5L								250,39						566,31
LC17811-6L	263,73							497,04				17,08		
LC17811-7L							223,81	35,48			8,37			638,22
LC17811-8L					1330,99					9,84	25,99			
LC17811-9L														
LC17811-10L								194,74						
Average			0,00	0,00	723,39	389,78	345,01	191,07	0,00	9,84	17,18	96,18	45,45	577,32
SEM			0,00	0,00	417,65	225,04	243,96	72,22	0,00		12,15	48,09		333,32

	µg/g (dw)											
Female	Arsene (As)	Barium (Ba)	Cadmium (Cd)	Chromium (Cr)	Copper (Cu)	Iron (Fe)	Mercury (Hg)	Manganese (Mn)	Nickel (Ni)	Lead (Pb)	Vanadium (V)	Zink (Zn)
LC7610-1L	0,20	0,38	0,08	0,42	20,35	2045,22	0,12	1,39	0,29	0,07	0,75	30,63
LC7610-2L	0,073	0,39	0,062	0,148	36,1	2265,8	0,188	0,756	0,068	0,796	1,13	43,60
LC7610-3L	0,194	0,74	0,081	0,344	15,7	1245,6	0,206	1,765	0,329	0,347	1,90	45,30
LC7610-4L	0,097	0,63	0,053	0,330	15,9	1083,8	0,206	1,905	0,107	0,278	0,95	30,70
LC7610-6L	0,15	1,18	0,08	0,35	18,83	1939,88	0,11	1,60	0,29	0,22	1,18	39,06
LC8610-8L	0,08	0,59	0,06	0,33	29,90	1513,85	0,08	0,77	0,24	0,29	0,86	41,01
LC8610-9L	0,11	0,72	0,08	0,36	20,96	1293,05	0,10	1,02	0,37	0,12	0,60	39,78
LC8610-10L	0,14	0,74	0,11	0,39	13,04	2737,68	0,11		0,24	0,15	0,77	37,51
LC 21910-1L	0,086	1,16	0,113	0,326	19,5	1963,2	0,616	2,895	0,154	0,226	1,68	37,70
LC 21910-2L	0,34	5,47	0,12	0,56	29,67	2534,44	0,18	0,78	0,55	0,12	0,80	38,89
LC 21910-3L	0,95	0,31	0,08	0,37	15,81	2122,30	0,13	2,43	0,22	0,10	0,88	32,26
LC 21910-4L	0,13	0,34	0,07	0,15	23,22	2137,19	0,10	1,06	0,18	0,21	0,57	31,45
Average	0,21	1,05	0,08	0,34	21,58	1906,83	0,18	1,49	0,25	0,24	1,01	37,32
SEM	0,06	0,30	0,02	0,10	6,23	550,45	0,05	0,45	0,07	0,07	0,29	10,77
Male	Arsene (As)	Barium (Ba)	Cadmium (Cd)	Chromium (Cr)	Copper (Cu)	Iron (Fe)	Mercury (Hg)	Manganese (Mn)	Nickel (Ni)	Lead (Pb)	Vanadium (V)	Zink (Zn)
LC7610-5L	0,13	0,30	0,06	0,39	14,11	485,94	0,10	0,89	0,20	0,03	0,28	23,61
LC8610-7L	0,064	0,36	0,040	0,246	15,9	913,8	0,199	1,522	0,080	0,168	0,71	35,49
LC17811-1L	0,189	0,79	0,067	0,243	10,3	1282,7	0,299	2,933	0,084	0,171	1,36	31,41
LC17811-2L	0,146	1,98	0,247	0,524	35,5	6331,5	0,824	4,239	0,098	1,187	6,04	115,81
LC17811-3L	0,21	1,31	0,18	0,53	18,65	2542,57	0,49	6,49	0,28	0,35	2,68	77,72
LC17811-4L	0,08	0,31	0,05	0,31	31,37	1510,44	0,13	1,41	0,16	0,31	0,48	23,88
LC17811-5L	0,167	1,07	0,061	0,681	41,2	1819,8	0,312	2,209	0,221	0,342	1,63	50,73
LC17811-6L	0,048	0,69	0,074	0,272	25,5	1603,7	0,296	1,944	0,141	0,283	1,06	40,26
LC17811-7L	0,130	0,58	0,131	0,194	16,4	1360,3	0,350	3,318	0,060	0,253	1,41	44,19
LC17811-8L	0,060	0,41	0,057	0,164	14,4	1516,9	0,293	2,965	0,056	0,179	0,74	31,21
LC17811-9L	1,68	0,89	0,15	0,70	24,86	1739,19	0,47	1,35	0,59	0,02	1,41	43,76
LC17811-10L	0,113	1,21	0,034	0,379	13,1	620,6	0,201	1,064	0,078	0,479	0,40	20,19
Average	0,25	0,82	0,10	0,39	21,77	1810,61	0,33	2,53	0,17	0,32	1,52	44,86
SEM	0,07	0,24	0,03	0,11	6,28	522,68	0,10	0,73	0,05	0,09	0,44	12,95