SÖDERTÖRN UNIVERSITY | STOCKHOLM
sh.se

Endocrine Disruption in Fish

Effects of 17α-ethinylestradiol exposure on non-reproductive behavior, fertility and brain and testis transcriptome

Tove Porseryd

SÖDERTÖRN DOCTORAL DISSERTATIONS

Endocrine Disruption in Fish

Effects of 17α-ethinylestradiol exposure on non-reproductive behavior, fertility and brain and testis transcriptome

Tove Porseryd

Subject: Environmental Science
Research Area: Environmental Studies
School: Natural Sciences, Technology and Environmental Studies

Södertörns högskola
(Södertörn University)
The Library
SE-141 89 Huddinge

www.sh.se/publications

Cover image: © Emil Sellström
Cover layout: Jonathan Robson
Graphic form: Per Lindblom & Jonathan Robson

Printed by Elanders, Stockholm 2018

Södertörn Doctoral Dissertations 155
ISSN 1652–7399

ISBN 978-91-88663-43-6 (print)
ISBN 978-91-88663-44-3 (digital)

Abstract
Aquatic environments are the end recipient for most anthropogenic chemical pollutants. One common chemical pollutant found in the aquatic environment is 17α-ethinylestradiol (EE_2), a synthetic estrogen used in contraceptive pills. EE_2 is found in sewage treatment plant effluents and surface waters in concentrations from non-detectable up to 300 ng/L. EE_2 has the ability to bioaccumulate and is more than 10 fold more potent in fish than the natural counterpart estradiol. Exposure has led to skewed sex ratios, decreased egg and sperm production, and altered reproductive behavior. The aim of this thesis was to investigate the effects of EE_2 exposure on non-reproductive behavior and fertility in fish. We found that zebra fish exposed to low concentrations of EE_2 during development showed increased anxiety-like behavior and decreased fertility that were persistent in adulthood, even after a long remediation period in clean water. The altered behavior and lowered fertility were accompanied by alterations in the testis and brain transcriptome of possible significance for the behavior and fertility effects. The zebrafish was also used in adult exposures of EE_2 and citalopram, alone and in combination to investigate if behavioral effects can be detected at very low concentrations, and if so, if the two compounds would interact and affect the behavioral outcome. Anxiety-like behavior was altered by EE_2 and the two compounds in combination affected the outcome of each other. Further, when developmental exposure of progeny to wild caught three spined stickleback was used as a link between laboratory fish and natural fish populations, EE_2 was found to decrease the anxiety-like behavior in the adult stickleback as well as cause ovotestis and intersex, feminization and sex reversal of genetic males. In conclusion, fertility and non-reproductive behaviors in the zebrafish and three spined stickleback are sensitive to EE_2 exposure and effects from developmental exposures seem to be persistent. Fertility and behavior are of high ecological significance for fish and alterations due to EE_2 exposure might have negative effects on population fitness. The persistent alterations in the transcriptome of the zebrafish testis and brain lead to generation of hypotheses of mechanisms involved in the behavior and reproductive phenotypes caused by developmental exposure to EE_2.

Keywords: 17α-ethinylestradiol, endocrine disrupting chemical, fish, behavior, fertility, transcriptome, RNA sequencing.

Till mamma och mormor

Sammanfattning
(Summary in Swedish)

Den akvatiska miljön är ofta särskilt utsatt för miljöföroreningar då de flesta ämnen förr eller senare sprids dit genom bland annat markavrinning, nedfall från luften och/eller från vattenreningsverk. Syntetiskt östrogen som används i p-piller, 17α-etinylestradiol (EE_2), sprider sig ofta till vattenmiljön från avloppsvatten, då det bara delvis renas bort i vattenreningsprocessen. EE_2 är ett hormonstörande ämne med förmågan att ansamlas i organismer och mer än tio gånger så verksamt i fisk som det naturliga hormonet estradiol (E_2). Man har tidigare sett att exponering för EE_2 även i de låga halter som påvisas vid vattenreningsverk bland annat har lett till minskade produktion av ägg och spermier samt förändrade parningsbeteenden.

I denna avhandling undersöks effekterna av EE_2 på fiskars icke-reproduktiva beteende och fertilitet. Dessa faktorer är av hög ekologisk relevans för fisk då förändringar på dessa kan ge negativa effekter på populationsnivå. Resultaten visar att zebrafisk som exponeras för låga halter av EE_2 under utvecklingen uppvisar ett mer ängsligt beteende och har lägre fertilitet när de blir vuxna även efter en lång återhämtningsperiod i rent vatten. Det förändrade beteendet uppvisas även hos avkomman till dessa fiskar. Förändringarna i beteende och fertilitet åtföljdes av förändringar i hjärnans och testikelvävnadens transkriptom, dvs. förändringar i vilka gener som uttrycks. I zebrafiskens testikelvävnad hittades bland annat en skillnad i uttryck av gener kopplade till könsdifferentiering och utveckling samt spermatogenesen, gener som kan ha betydelse för den nedsatta fertiliteten. I zebrafiskens hjärna hittades ingen skillnad i uttryck på gener direkt kopplade till stressaxeln men däremot på flera andra gener i nätverk som indirekt kan kopplas till det ängsliga beteendet som dygnsrytm och kolesterolsyntes. I naturen exponeras organismer ofta för en blandning av föroreningar. Därför undersöktes också effekter av EE_2 i kombination med citalopram, ett antidepressivt läkemedel som ofta påvisas i vattenmiljön, för att undersöka om förändringar i beteende kan påvisas även vid väldigt låga koncentrationer av varje ämne och, om så är fallet, vilka effekter en kombination av de två ämnena ger. Vuxna zebrafiskar uppvisade beteendeförändringar även vid dessa låga koncentrationer efter två veckors exponering och de två ämnena påverkade beteendet olika var för sig och i kombination. Vidare, användes vildfångad storspigg i ett exponeringsexperiment för att undersöka om resultaten från zebrafisken, som är en avlad laboratoriefisk, kan ses också i vilda fiskpopulationer. Även storspiggar som exponerades för EE_2 under utveckling fick ett modifierat beteende samt en skev könsfördelning som vuxna: fler honor fanns hos de stors-

piggar som exponerats och vidare undersökningar visade att dessa var genetiska hanar som utvecklats som honor.

Den här avhandlingen visar beständiga förändringar i zebrafiskens transkriptom i hjärna och testikelvävnad efter exponering för EE_2 i låga halter under utveckling. Både icke-reproduktiva beteenden och fertilitet visade sig känsliga för EE_2 i såväl zebrafisk som storspigg och effekterna verkar vara permanenta. Sammanfattningsvis har EE_2 effekter på ekologiskt relevanta faktorer hos fisk i de halter som förekommer i vattenmiljön och hormonstörande föroreningar som EE_2 kan ge långvariga effekter som syns senare i livet även vid låga koncentrationer.

Contents

List of papers

This thesis is based on the following papers referred to in the text by the Roman numerals (I–V):

Paper I: Volkova, K.*, Reyhanian Caspillo, N.*, Porseryd T., Hallgren S., Dinnétz, P., Porsch-Hällström, I., 2015. Developmental exposure of zebrafish (Danio rerio) to 17α-ethinylestradiol affects non-reproductive behavior and fertility as adults, and increases anxiety in unexposed progeny. *Hormones and Behavior*, *73*, pp.30–38. doi: 10.1016/j.yhbeh.2015.05.014

Paper II: Porseryd, T., Reyhanian Caspillo, N., Volkova, K., Elabbas, L., Källman, T., Dinnétz, P., Olsson, P-E., Porsch-Hällström, I., 2018. Testis transcriptome alterations in zebrafish (Danio rerio) with reduced fertility due to developmental exposure to 17a-ethinyl estradiol. *General and Comparative Endocrinology*, *262*, pp.44–58. doi: 10.1016/j.ygcen.2018.03.011

Paper III: Porseryd, T.*, Volkova, K.*, Reyhanian Caspillo, N., Källman, T., Dinnetz, P., Porsch-Hällström, I., 2017. Persistent Effects of Developmental Exposure to 17α-Ethinylestradiol on the Zebrafish (Danio rerio) Brain Transcriptome and Behavior. *Frontiers in Behavioral Neuroscience*, *11*, *69*. doi: 10.3389/fnbeh.2017.00069

Paper IV: Porseryd, T., Kellner, M., Reyhanian Caspillo, N., Volkova, K., Elabbas, L., Ullah, S., Olsén, H., Dinnétz, P., Porsch-Hällström, I., 2017. Combinatory effects of low concentrations of 17α-etinylestradiol and citalopram on non-reproductive behavior in adult zebrafish (Danio rerio) *Aquatic Toxicology*, *193*, pp.9–17. doi: 10.1016/j.aquatox.2017.10.001

Paper V: Porseryd T, Larsson J, Kellner M, Bollner T, Dinnétz P, and Porsch Hällström I. Altered non-reproductive behavior and feminization caused by developmental exposure to 17α-ethinylestradiol persist to adulthood in three-spined stickleback (*Gasterosteus aculeatus*). (In manuscript)

*Both are considered first authors

Contributions of the author to the different manuscripts

Paper I: Contributed to the fish experiment and reviewed drafts of the manuscript.

Paper II: Contributed to the fish experiment. Performed manual functional classifications and, statistical analyses, prepared figures and tables and completed the manuscript.

Paper III: Planned, designed, and conducted experiment, performed qPCR, prepared figures and tables and completed the manuscript.

Paper IV: Planned, designed, and conducted experiment and took part in water analyses. Performed statistical analyses, prepared figures and tables and wrote the manuscript.

Paper V: Planned, designed, and conducted experiment and took part in water analyses. Performed histological analyses, PCR and electrophoresis for sex determination. Performed statistical analyses, prepared figures and tables and wrote the manuscript.

Abbreviations

BPA	Bisphenol-A
DES	Diethylstilbestrol
Dpf	Days post fertilization
EDCs	Endocrine disrupting chemicals
E2	17β-estradiol
EE2	17α-ethinylestradiol
FDR	False discovery rate
GSI	Gonadosomatic index
HPA axis	Hypothalamic-pituitary-adrenal axis
HPG axis	Hypothalamic-pituitary-gonadal axis
HPI axis	Hypothalamic-pituitary-interrenal axis
HSI	Hepatosomatic index
LOEC	Lowest observed effect concentration
mRNA	Messenger RNA
miRNA	MicroRNA
ncRNA	Non-coding RNA
NT	Novel tank
PCR	Polymerase chain reaction
PPCPs	Pharmaceuticals and personal care products
PNEC	Predicted no effect concentration
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
SSRI	Selective serotonin reuptake inhibitors
STP	Sewage treatment plant
TBT	Tributyltin
Vtg	Vitellogenin

Introduction

Aquatic environments are the end recipients for many anthropogenic chemical pollutants and have been described as "the ultimate sink" for chemicals (Sumpter, 1998). The anthropogenic pollutants entering aquatic environments from, e.g. agriculture, industry and sewage treatment plants (STPs) include a wide variety of chemicals assumed to have endocrine disrupting activity (Kortenkamp et al., 2011). These chemicals are found in freshwater, estuarine and marine environments around the world, including the Baltic Sea (Ying et al., 2002, Nikolaou et al., 2007, Barbosa et al., 2016) placing the organisms therein at risk for exposure and potential negative effects. The negative effects could be especially detrimental in an environment such as the Baltic Sea. The Baltic Sea is a semi-enclosed sea with limited water exchange with the ocean and is considered one of the worlds most contaminated seas. Its drainage area is very large and inhabit approximately 85 million people (HELCOM, 2010). Anthropogenic pollution is a major threat to the Baltic Sea ecosystem (Lehtonen et al., 2017). The endocrine disrupting chemicals (EDCs) found in the environment include not only natural and synthetic hormones but also organic chemicals from the industries and agriculture; pesticides, plasticizers and surfactants (Porte et al., 2006). It is generally accepted that EDCs have impacted the reproduction and development of wildlife populations. International concerns have been raised (Colborn et al., 1993, Vos et al., 2000, Basrur, 2006, Bergman et al., 2012) and a debate concerning the magnitude and type of effects observed in wildlife as well as a possible intervention is ongoing.

Adverse effects of endocrine disrupting chemicals (EDCs) in the environment

EDCs are a diverse group of chemicals affecting all classes of vertebrates, and some invertebrates (Matthiessen, 2003). Most EDCs interfere with the endocrine system by directly mimicking or antagonizing hormone action on a hormone receptor level but they can also modulate the synthesis and metabolism of hormones (Mills and Chichester, 2005, Waring and Harris, 2005). Endocrine functions may be disturbed during sensitivity windows at specified periods during an organism's lifespan. In particular exposure during early life stages may cause alterations in developmental processes such as sexual differentiation and long-term effects such as higher susceptibility to chemical exposure later in life, intersex and sex reversal. Increased levels of intersex in individuals can cause skewed sex-ratios in the population (Kavlock et al., 1996, Arcand-Hoy and Benson, 1998) potentially leading to

population declines. Some of the best examples of endocrine disruption are found in aquatic environments (Tyler et al., 1998). One example is the high frequency of imposex, the masculinization of females, in aquatic gastropod mollusks causing worldwide population declines in the 1970s and 80s. The causative agent was shown to be tributyltin (TBT) a key component in antifouling paint that induced imposex through inference with androgen metabolism (Matthiessen and Gibbs, 1998). The gastropod population declines were partially reversed through banning the use of TBT in many countries (Harding et al., 1997). Due to their effects on endocrine functions, EDCs can be active at very low concentrations. Fish are considered especially vulnerable to EDCs due to their multiple uptake routes; directly from water via the gills and through the skin, drinking and the diet (Jobling et al., 1998, Vos et al., 2000, Hecker et al., 2002, Soffker and Tyler, 2012). For example roaches (*Rutilus rutilus*) living in rivers downstream STPs in the UK, showed reduced gonadal growth and a high incidence of intersex males, (Jobling et al., 1998); two of the investigated populations contained up to 100% intersex males. The feminization of the male roach was suggested to be caused by estrogens in sewage water.

17α-Ethinylestradiol

Estrogens are a group of female sex hormones essential for reproduction in both sexes in vertebrates, including humans. Estrogens are also involved in other physiological processes, e.g., growth, stress response and immunity (Sharpe, 1998, Enmark and Gustafsson, 1999). Several EDCs found in the environment are reported to have estrogenic effects (Shore et al., 1993, Arcand-Hoy and Benson, 1998). The compound 17α-ethinylestradiol (EE_2) is a synthetic estrogen commonly used as an active substance in oral contraceptives and hormonal replacement therapy in menopause. It is frequently found in waste water effluent and has been detected in a range of concentrations from ≤1 ng/L to 300 ng/L in sewage and surface waters (Ternes et al., 1999, Williams et al., 2003, Laurenson et al., 2014, Tiedeken et al., 2017). EE_2 bioaccumulates and is resistant to biodegradation (Aris et al., 2014). Exposure can affect factors such as egg viability, fertilization success, sex ratios and behavior in fish (Mills and Chichester, 2005, Xu et al., 2008, Aris et al., 2014). EE_2 is more than 10-fold more potent in fish than its natural counterpart, estradiol (Thorpe et al., 2003). In a whole lake experiment in Canada, EE_2 caused a near extinction of a population of fathead minnow (*Pimephales promelas*) when chronically exposed to environmentally relevant concentrations (5-6 ng/L). Fish showed intersex characteristics as well as failed reproduction (Kidd et al., 2007). However 4 years after the termination of exposure, the fathead minnow population had regained fertility and recovered to its original size (Blanchfield et al., 2015).

Effects on reproduction

Reproductive function in fish has been the main focus when the effects of EE_2 have been studied. Adult exposure has shown to reduce gonadosomatic indices (GSI), alter gonadal structure, disrupt follicular development and steroidogenesis, reduce fertility and alter genes involved in sex differentiation and steroidogenesis (Van den Belt et al., 2002, Segner et al., 2003, Versonnen and Janssen, 2004, Urbatzka et al., 2012, Reyhanian Caspillo et al., 2014). Exposure during adulthood seem to be reversible and several studies have shown that fish have been able to, at least partly recover from effects of exposure after remediation (Maack and Segner, 2004, Schäfers et al., 2007, Baumann et al., 2014). Morphological endpoints such as sex ratio, growth, the production of the egg yolk precursor protein vitellogenin (Vtg) in males and gonado-somatic indices are recovered after remediation (Hill Jr and Janz, 2003, Baumann et al., 2014). Changes in breeding success are more sensitive and remain, even after a long remediation (Baumann et al., 2014).

Effects on behavior

The effects of estrogens on sexual and reproductive behaviors have been widely studied; many of these studies are on fish (Espmark Wibe et al., 2002, Zala and Penn, 2004, Saaristo et al., 2010a, Soffker and Tyler, 2012, Lee et al., 2014). EE_2 exposure has caused increased territorial behavior in the breeding season in three-spined sticklebacks (*Gasterosteus aculeatus*) (Bell, 2001), altered nesting and courtship behavior as well as fanning of the eggs in sand gobies (*Pomatoschistus minutus*) (Saaristo et al., 2009, Saaristo et al., 2010b) and competition for spawning substrate in fathead minnow (Salierno and Kane, 2009). The effects on non-reproductive and non-sexual behavior have been less investigated, although changes in such behaviors can affect foraging, predator avoidance and reproduction opportunities and therefore have consequences for individual fitness and in the long run, populations and entire communities (Cohn and MacPhail, 1996, Clotfelter et al., 2004). Adult fish of several species have shown alterations in explorative, risky, social and aggressive behaviors when exposed to environmentally relevant concentrations of EE_2 during adulthood (Reyhanian et al., 2011, Hallgren et al., 2011, Bell, 2004, Filby et al., 2012, Chen and Hsieh, 2017, Dang et al., 2017). Earlier studies have shown that behavior can be more sensitive to EDCs than developmental and physiological characteristics (Clotfelter et al., 2004, Zala and Penn, 2004). However the opposite results have been obtained (Peakall, 1996). Hormones play an important role in animal behavior, and behavioral alterations might serve as additional biomarkers of EDC exposure and a link between physiology and ecology (Zala and Penn, 2004).

Developmental exposure

Fetal and embryonic development stages are sensitive periods, and exposure to estrogenic EDCs during narrow windows of development leads to irreversible changes. Many targets of the endocrine system are found in the brain. Reproductive behavior and mating choice are regulated by sex-differentiated imprinting of the hypothalamic regions by sex steroids, and are affected by EDCs (Gore and Patisaul, 2010). Estrogen is involved in many aspects of the development of the neuroendocrine system influencing both brain structure and behavior (Enmark and Gustafsson, 1999, Guillette and Gunderson, 2001). EE_2 exposure during early development has caused rats to show increased anxiety and social neophobia during adulthood (Dugard et al., 2001) and exposure to the estrogenic compound Bisphenol A (BPA) during early development has led to increased anxiety, impaired memory and altered social behavior in mice (Ryan and Vandenbergh, 2006, Wolstenholme et al., 2011b). Developmental studies on non-reproductive behaviors in fish are sparse but developmental exposure of zebrafish (*Danio rerio*) to BPA has caused learning deficits as adults (Saili et al., 2012) and guppy (*Poecilla reticulata*) exposed to EE_2 during development showed increased anxious behavior in adulthood (Volkova et al., 2012b). Reproductive behavior and gonad morphology were less sensitive to developmental treatment than non-reproductive behavior in developmentally exposed guppy (Volkova et al., 2012a). This result suggests that non-sexual behaviors are vulnerable to EDCs and underlines that behavior endpoints are candidates for environmental risk assessment together with reproductive and physiological endpoints.

Combinatory effects

In nature, fish are exposed to sewage treatment effluents that contain complex mixtures of different classes of chemicals. The components in these mixtures also vary in concentrations both temporally and spatially, making predictions of their effects complicated. In addition to the estrogens found in the environment, pharmaceuticals and personal care products (PPCPs) are a group of compounds also found frequently in the aquatic environment including sewage and surface water, sediment and fish (Nikolaou et al., 2007, Ebele et al., 2017). Pharmaceuticals are designed to alter physiological function in humans and many of the mechanisms through which pharmaceuticals act are conserved across animal phyla. A pharmaceutical designed to affect humans will thus likely affect wildlife when they are exposed in the environment. Zebrafish were predicted to have orthologs of 86% of 1318 analyzed human drug targets (Gunnarsson et al., 2008). Selective serotonin reuptake inhibitors (SSRI) are a group of antidepressant drugs ubiquitous in the aquatic environment (Vasskog et al., 2006, Corcoran et al., 2010). SSRIs have caused embryonic abnormalities in Japanese medaka (Foran et al., 2004), reduced clutch

size and ovarian estradiol levels in zebrafish (Lister et al., 2009) and caused anxiolytic effects on fish behavior from adult exposure (Valenti et al., 2009, Kellner et al., 2016). SSRI also reduces the levels of cortisol in fish (Lepage et al., 2005). Studies of combination exposures of pharmaceuticals with different modes of action in fish are sparse, and their combined effects are largely unknown.

Aim of the thesis

The overall aim of this thesis was to investigate the non-reproductive behavioral and reproductive effects of EE_2 in fish after adult and developmental exposure. The aquatic environment is particularly susceptible to endocrine disrupting pollutants such as EE_2, as it receives pollutants intentionally released into the environment through sewage effluents as well as non-intentional sources such as run off and spills. Fertility and behavior are of high ecological significance for fish, and their alteration due to EE_2 exposure may have negative effects on population fitness. To investigate this the specific aims were to:

i. Examine how developmental exposure to EE_2 affects fertility (paper I) and non-reproductive behavior (papers I and III) in zebrafish after a long remediation period to adulthood.

ii. Further investigate the mechanisms behind the effects on fertility and behavior by RNA sequencing of the testis (paper II) and brain (paper III) transcriptome.

iii. Study exposures of adult zebrafish to EE_2 and the antidepressant drug citalopram, alone and in combination, to investigate whether behavioral effects can be detected as a result of very low concentrations, and if so, whether the two compounds interact to affect the behavioral outcome (paper IV).

iv. Use wild caught stickleback to investigate if persistent effects on behavior can be discerned after developmental exposure to EE_2 against the background of genetic variation in natural populations. If so, if non-reproductive behavior might be useful to indicate disturbances due to developmental exposure in wild fish (paper V).

Methods

The zebrafish (*Danio rerio*)

The zebrafish is a small (3-5 cm), tropical, freshwater, naturally shoaling species native to the Indian subcontinent that is most commonly found in still standing water bodies and shallow ponds but is also found in rivers and streams (Spence et al., 2008). It was brought to the laboratory from a pet store in Oregon, USA, approximately 40 years ago and has since become a popular fish in genetic, developmental and toxicological studies for several reasons. It is easy to maintain in the laboratory, develops rapidly and is optically transparent during early development, abundantly produces offspring under laboratory conditions and can be stimulated to breed during the whole year (Westerfield, 2007). Zebra fish sex determination is elusive and complex. Recent studies suggest that the sex is determined by multiple genes and is secondarily influenced by epigenetic input of environmental factors (Nagabhushana and Mishra, 2016, Santos et al., 2017). Their relatively small size makes them easy to house and handle, and as the genome is fully sequenced (Howe et al., 2013a) the zebrafish is a good model organism for this project with studies of fertility, behavioral and molecular analysis.

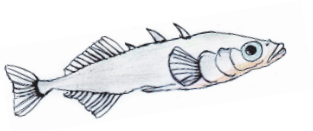
The zebrafish used in this thesis were of the wild type strain AB provided as eggs or adults by Karolinska Core facilities, Huddinge and Solna, Sweden. The AB strain has been bred for many generations and is considered suitable for behavioral studies (Guo, 2004)

The three-spined stickleback (*Gasterosteus aculeatus*)

The three-spined stickleback is found abundantly in marine, brackish and freshwater environments across the whole northern hemisphere and is common in the Baltic Sea. The stickleback is also relatively small, up to 10 cm in length, and a shoaling species. During non-breeding season, they form larger pelagic shoals, and during the breeding season, in the shallow waters, the males establish territories while females and non-reproductive males form groups (Ostlund-Nilsson et al., 2006). The sexual behavior of the stickleback is well characterized. This species breeds easily under laboratory conditions and is easy to maintain and physically robust, it

is thus tolerant to handling and transport, making the stickleback popular for use as a wild model species for studying behavior, physiology, evolution and ecology. The stickleback does not only offer a biomarker for ongoing estrogenic exposure, Vtg, as other fish species, it is also unique in offering a biomarker for environmental androgens (Katsiadaki et al., 2002). Spiggin is a protein complex normally produced in stickleback male kidneys during breeding season, is controlled by androgens and is used by the stickleback male to glue the nest (Jakobsson et al., 1999). The stickleback also offers a benefit in terms of a genetic sex that is possible to determine via relatively easy measures. In this project the Stickleback serves as a link between the established interbred zebrafish and wild fish populations.

The Sticklebacks used in this thesis were bred from parental fish caught in the Baltic Sea, at Askö on the East coast of Sweden or caught as adults in Skåne, in the South of Sweden.



Exposure

In paper I and III, zebrafish embryos were exposed to EE_2 1 to 80 days post fertilization (dpf). After exposure, zebrafish were allowed to remediate in clean water for 82 days (paper I) and 120 days (paper III) under normal maintenance conditions. The exposure protocols for the two experiments differed slightly. In paper I, the fertilized eggs were exposed in a semi-static system during the first 6 weeks and then moved to a flow-through system, where exposure continued. The fertilized eggs in paper III where exposed in a flow-through system from the beginning. In both papers I and III, the nominal concentrations of EE_2 were 0, 3 and 10 ng/L. However, in paper I, the concentrations measured during the exposure were 0, 1.2 and 1.6 ng/L, and the concentrations measured in paper III were 0, 2.74 and 7.34 ng/l. Raising of zebrafish larvae required an intense feeding schedule, resulting in a lipid rich layer on the aquaria walls. The lipophilic EE_2 most likely accumulated in the residue layer, contributing to the low measured concentrations. The experience gained in paper I led us to feed the larvae in paper III less abundantly, resulting in less residue as an explanation for the higher measured concentrations. In all the developmental exposures (both zebrafish and stickleback), the fish were exposed and kept in family groups. Each batch of eggs from one parental pair was divided, distributed into each treatment and held in family groups for the whole experiment. In paper IV, the combinatory effects of the SSRI citalopram and EE_2 were investigated. The nominal concentrations were 0.1 and 0.5 ng/L EE_2, 0.1 and 0.5 µg/L citalopram and combinations of these two chemicals, in high- and low-dose combinations. However, the water analysis revealed background levels of EE_2 in the water rendering higher nominal concentrations. The control

water contained 0.4 ng/L EE_2, and the measured exposure concentrations were 0.9 (nominal 0.1) and 1 ng/L EE_2 (nominal 0.5). The measured concentrations of citalopram were 0.1 (nominal 0.1) and 0.4 µg/L (nominal 0.5). In paper V sticklebacks were exposed to the nominal 0 and 20 ng/L EE_2. Concentration measurement surprisingly revealed 5 and 30 ng/L EE_2 in the 0 and 20 ng/L exposures, making use of historical controls from previous experiments necessary for comparisons. Measurements from earlier fish exposure used as a historical control showed no detectable traces of EE_2 (0 ng/L).

Chemical analysis of water samples

Water samples were collected during exposure in the different experiments (papers I, III, IV and V) at several occasions. The water samples were stored in darkness at -20° C before analyses were performed at Karolinska University Hospital laboratory. For papers I and III, EE_2-d4 was used as an internal standard with a detection limit of 0.5 ng/L EE_2, and for papers IV and V, EE_2-d6 was used with a detection limit of 0.2ng/L EE_2. The detection limit for citalopram was 0.1 ng/L. A 100 mL water sample spiked with internal standard was extracted on 100 mg Strata-X 33u Polymeric Reversed Phase cartridges after conditioning with MeOH. EE_2 and citalopram (for paper IV) were then eluted and content were analyzed using a Dionex Ultimate 3000 LC system (Thermo Fisher Scientific).

Fertility tests

In paper I the fertility of both male and female fish, developmentally exposed to EE_2 (F0) were tested for fertilization success. Fish from each exposure group (0, 1.2 and 1.6 ng/L) were mated with unexposed fish of the other sex. Each family was represented in each sex with 1-2 individuals, if possible. The fish were placed in breeding cages with 1-2 females and 1-2 males for 24 hours. The eggs were collected and counted, and the number of fertilized eggs, their hatching and the survival of larvae after 6 days were recorded.

Behavior studies

All behavioral tests were performed between 9 am and 1 pm to reduce variations in hormone levels and locomotor activity due to circadian rhythm. The fish were also filmed randomly between treatments, to make certain that all exposures were treated similarly during the whole time period. Studies on rodents and fish, two popular animal subjects in behavioral and neurological research, have shown that the handling of the animal in experiments not only affects the welfare of the animal but also influences anxiety-like behavior that can affect experimental outcomes (Pottinger and Calder, 1995, Hurst and West, 2010). We have experienced the

response to different environmental variables and designed our trials to minimize the influence on handling on the detectability of our behavioral phenotypes. For example, the two species reacted to human presence considerably different. The sticklebacks displayed higher sensitivity to visual cues of humans and experienced more stress from netting. The sticklebacks were moved to a smaller aquarium the night before filming with fewer fish to minimize the stress of the netting at the time of behavior trials. The zebrafish could, however, be handled directly from the aquaria they were kept in during the experiment. The behavioral tests were video recorded and later analyzed manually on screen.

Novel tank test

The novel tank (NT) test is a well-established test and one of the most popular behavior tests for anxiety-like behavior in fish (Maximino et al., 2010a, Stewart et al., 2012). The test makes use of the fact that when fish are individually introduced to a novel environment, they tend to dive to the bottom and spend the first period near or on the bottom. This behavior is a probably an adaptive response developed to avoid predators. Fish that take longer to move upwards and explore the environment tend to be in a more anxious state (Bencan et al., 2009, Egan et al., 2009). However, studies also suggest that bottom dwelling in this test may not reflect only anxiety but a combination of locomotor and motivational effects (Maximino et al., 2012).

The NT test in this thesis was performed in a 20×20×40 cm tank filled with 15 L of water for the zebrafish and for the stickleback, the tank was modified to a larger size, 24×25×49 cm, and filled with 30 L of water. The test aquaria had a compartment at the short end where a group of fish (4 stickleback or 5 zebrafish) was held hidden from the test fish for the following shoaling test. The compartments holding the fish groups were covered with a black sheet. The test aquaria had a vertical line and a horizontal midline drawn on the outside on the front (Fig. 1). The NT test started with the gentle release of the test fish into the aquaria and 5 minutes observation. The parameters that were measured were the time before first crossing the horizontal line, the number of crossings and the time spent in the upper half of the aquaria. The time spent freezing was also noted. Swimming activity, reflected by the number of times a fish crossed the vertical and horizontal lines in the grid, was recorded for 60 s; for paper I it was recorded 30 s after the NT test start, for paper IV 1 min into the NT session, and in paper III, the last minute of the test.

According to our observations the color of the tank bottom (black/white) plays a crucial role in how the fish performs in the test. The basal stress level also influences the performance. With the zebrafish a white bottom in the test tank suppressed the diving behavior; the fish tended to stay further above the bottom making measurements of the parameters challenging and hindering the ability to detect differences between the behavioral phenotypes. Due to the previous experience with zebrafish and a pilot study with the three-spined stickleback, a black tank bottom

was also used in their test tanks. The basal stress level increased with less gentle netting in both fish species. A fish handled less gentle displayed extensive freezing behavior and sticklebacks also displayed erratic swimming behavior up and down between freezing. Zebrafish raised on transparent shelves were less reactive in the NT test, and sticklebacks raised in large holding tanks were more reactive. The higher reactivity of the sticklebacks was likely partly explained by the slightly higher amount of stress when netting the fish, as a large tank allow more movement and could prolong the chase when netting. However, our experiences with the two fish species support the ideas that environmental factors are crucial and that the novelty stimulus of the NT test is conditional (Blaser and Rosemberg, 2012).

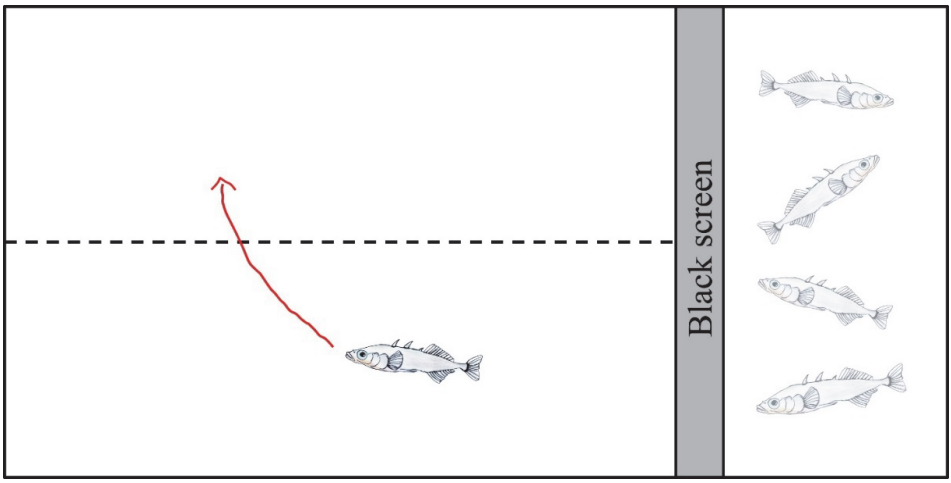


Fig.1. Experimental setup of the novel tank test (NT test) seen from the side.

Shoaling test

Both the stickleback and zebrafish are naturally shoaling species. Shoaling is a social aggregation behavior common among teleost fish that offers antipredator advantages and finding potential mates. However, the advantages of being able to exploit riskier feeding patches with a group are often outweighed by food competition. A further potential cost with shoaling is the increased frequencies of ectoparasites and disease. Although shoaling comes at costs, fish in shoals handle the stresses of a predator attack and a novel environment better in terms of returning to normal feeding behavior (Wright et al., 2006). Shoaling in fish has been quite studied but the underlying mechanisms and neurobiology are poorly understood. In our studies, we saw a difference in how the fish species reacted to the shoal when the shoal was revealed after the NT test. The sticklebacks were more often aggressive toward their conspecific fish, attacking and biting towards them on the glass screen and raising dorsal spines. Both the focal fish and fish from the shoal

displayed this behavior, and fish in the shoal occasionally intimidated the focal fish. The zebrafish did not display aggressive behavior toward conspecifics that could influence the shoaling results. The observations from these studies could indicate that this type of shoaling test design might be more suitable for fish that are more domesticated, such as the zebrafish, and not as suitable for wild fish without some alterations. Even if there was some stress related to the test, the shoaling tests were conducted subsequently to the NT test i.e., when the fish was acclimatized to the new environment, the group of fish was revealed by the removal of the black screen (Fig. 2). Observations started as soon as the focal fish made contact with the shoal. Observations were made for 5 minutes, and the parameters that were observed were the number of times the fish crossed the vertical line away from the shoal, latency to cross this line and the time spent away from the shoal.

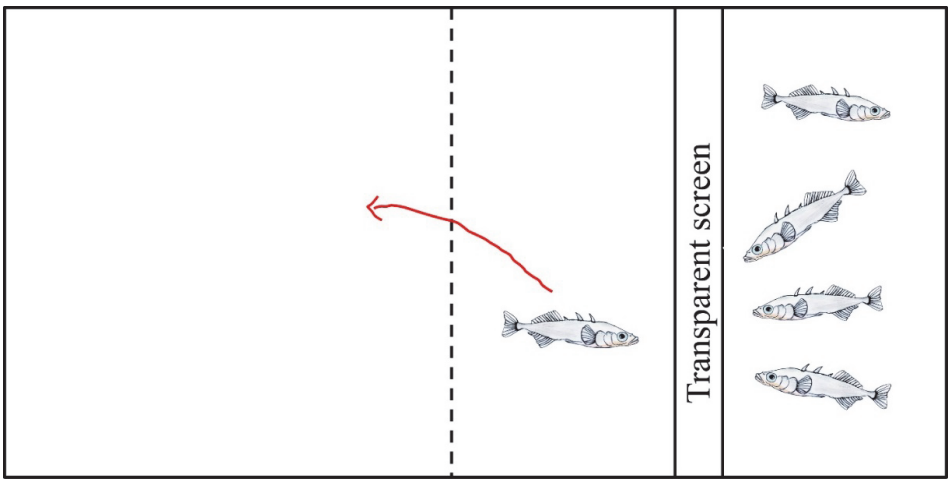


Fig. 2. Experimental setup of the shoaling test seen from the side.

Scototaxis test, dark/light preference

The scototaxis test is driven by an approach-avoidance conflict (explore/protection) in the fish (Maximino et al., 2010b). Several fish species, including the zebrafish, have a preference for the dark compartment as adults (Guo, 2004, Maximino et al., 2007, Maximino et al., 2010b, Thompson et al., 2016). This test creates a conflict between the fishes need to explore to find resources and the unpleasant experience of the bright environment in the white compartment. The white bright area is considered a risky environment to the fish due to the contrast between the fish and the white area, reducing the background camouflage and thus making the fish more vulnerable for predators. The scototaxis test is sometimes suggested as a better alternative to the NT test, as this test does not allow for habituation as the novel tank allows. The bottom dwelling in the NT test seems to be driven by novelty,

which does not seem to be the case in the scototaxis test (Blaser and Rosemberg, 2012, Maximino et al., 2012, Thompson et al., 2016). The scototaxis test was performed in a 20×20×40 cm (zebrafish) 25×24×49 cm (stickleback) aquarium filled with preheated tap water to a 10 cm, or 20 cm level respectively. The tank was divided into one dark and one white side with the bottom and all sides covered with white or black plastic sheets (Fig. 3). Two central transparent sliding doors constituted a central compartment of 5 cm (zebrafish) or 10 cm (stickleback). A fish was placed in the central compartment and allowed to habituate for 5 minutes. After the habituation period, the doors were raised, and the fish was registered from above for 5 minutes. The parameters observed were latency to the white half, the total entries into the white half and the total time in the white compartment.

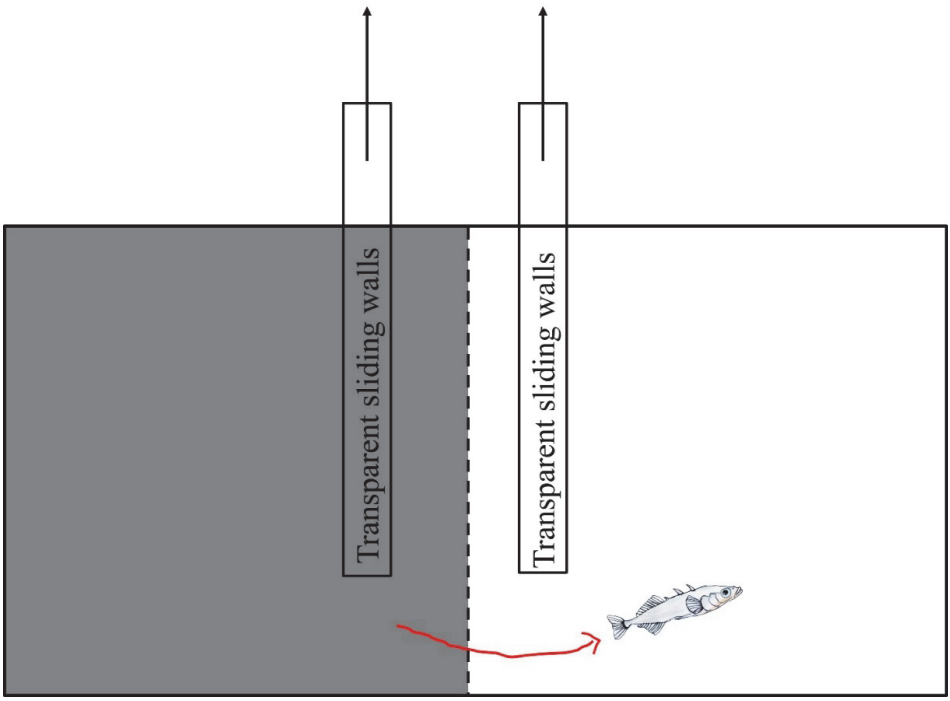


Fig. 3. Experimental setup of the scototaxis test seen from the side.

Real-Time Quantitative PCR

Real-time quantitative PCR (qPCR) works in the same manner as PCR but in contrast to conventional PCR, qPCR uses a fluorescent dye that binds to the produced doublestranded DNA allowing its detection and measurement as the reaction progresses. The fluorescent molecules bind to the amplified DNA and signal an increase in the amplified DNA concentration by an proportional increase in the

fluorescent signal (Bustin, 2002). RNA was extracted from tissue homogenized in TriReagent (0.8 ml/sample, Sigma-Aldrich) according to the manufacturer's instructions. The RNA was quantified and its quality was determined by the 269/280 nm absorption ratio measured with a NanoDrop ND-1000 spectrophotometer. cDNA was obtained by reverse transcription. For more details of each experiment, the readers are directed to the individual papers. SYBR® Green dye (BIO RAD) was used and a CFX96 Touch™ Real-Time PCR System optical reaction module (BIO RAD). The reference genes Elf1α (paper II), 18S rRNA (paper I), and a combination of the two (paper III) were used. The two reference genes are considered suitable for zebrafish tissue analysis (Tang et al., 2007). All samples were run in triplicates. In paper I, qPCR was used to measure hepatic Vtg expression to verify that the fish had been completely remediated after EE_2 exposure. The qPCR method was used to verify differentially expressed genes found in the RNA-Seq analysis in papers II and III.

RNA sequencing

RNA-Seq is an emerging tool for the characterization and quantification of the entire transcriptome. It is sensitive and can measure the quantity of RNA in a sample at a given time. It can also be used with any species and is not limited by previous knowledge of the genome, as it can detect transcripts even though the corresponding genomic sequence is not known (Wang et al., 2009). Based on the lower fertility recorded in the male zebrafish in paper I, RNA from testis tissue was sequenced to search for possible mechanisms behind the lowered fertility. Four biological replicates from each treatment (0, 1.2 and 1.6 ng/L) were sequenced. Two families were represented in all three treatments, and the other families were found in one or two treatment groups. In paper III, the whole-brain transcriptome was analyzed to search for mechanisms behind the persistent behavioral effects from developmental exposure. The samples for analysis were chosen based only on the results from the NT test, as the other behavioral tests were not fully analyzed at the time for sequencing. Hence, brains from males exposed to 2.74 ng/L and females exposed to 7.31 ng/L EE_2 were sequenced as the strongest behavioral effects were seen in those exposures in the NT test. Three biological replicates were used from each treatment group and sex. The females were represented by the same families in the two treatments, however, the males were not and thus introduced additional variation to the analysis. RNA-Seq was performed at Genome Infrastructure, SciLife/Uppsala Genome Center, Sweden according to current procedures of purification, quantification and sequencing and 40 million reads were used to increase detection capacity. The bioinformatics was performed by BILS (Bioinformatics Infrastructure for Life Sciences, Uppsala, Sweden). Quality assessment reads were then mapped to version 9 of the zebrafish genome assembly, Zv9 (Howe et al., 2013b). The mapped reads were converted into count data. Only genes with

expression higher than 1 count per million in at least 3 of the sequence libraries were used for differential gene expression analysis. Estimated p-values with false discovery rate (FDR) correction was used to correct for multiple testing, and genes with adjusted p-values <0.05 were regarded as significantly differentially expressed. The functional classifications of genes and the predictions of the biological gene function were performed manually and based on the gene ontology (GO) terms for zebrafish, rodents and humans. An orthologue search was conducted in Ensembl and GO terms were found in Zfin (for zebrafish), Entrez (for humans and rodent orthologs), NGNC (human orthologs) and MGI (mouse orthologs) databases.

Morphological measurements

In papers III and V, somatic indices were measured. Livers and gonads were excised and weighed. The hepatosomatic index was calculated as HSI = (liver weight / body weight) × 100, and the gonadosomatic index was calculated as GSI = (gonad weight / body weight) ×100. In paper V, a subset of the gonads was collected for histological analyses. Gonads were fixed in Bouin's solution for 48 hours at 4 °C before transfer to 70% ethanol. The samples were then dehydrated stepwise in increasing concentrations of ethanol, cleared in Histo-clear II and embedded in paraffin. The paraffin embedded gonads were cut into 5 µm sections and stained with eosin and hematoxylin before being examined under light microscopy.

Sex determination

In paper V, the genetic sex determination of stickleback was conducted. Fin clippings were sampled for genetic analyses. A multimarker assay was used, where the genetic sex is scored by multiple markers on agarose gel, that has been proven reliable for sex identification in sticklebacks (Toli et al., 2016). Total genomic DNA was extracted from a small clipping of fin as described by Laird et al (Laird et al., 1991) and PCR reactions as described by Toli et al., 2016 (Toli et al., 2016) were performed to amplify three loci, *Idh*, *Gasm6* and *Stn190*, situated in the recombination-suppressed region in the sex chromosome. When PCR products were electrophoresed together with a DNA ladder on 2% agarose gel, the products produce one band in females and two bands in males. The method for sex determination is described in more detail in paper V.

Statistical analysis

Behavioral parameters, relative gene expression data from qPCR and morphological data were analyzed with linear mixed models using family or aquaria as random effects to handle the pseudo replication due to dependencies within families and treatment aquaria. Treatment, sex and treatment × sex were used as fixed factors in the models. When response variables were measurements of time(s), relative gene

expression and morphology a Gaussian distribution of residuals was applied, and if needed, the data were log- or square-root transformed to improve homoscedasticity. For count data, a Poisson distribution was applied, and a generalized linear mixed model was used. The estimates from all mixed models were significance tested with likelihood-ratio tests. In paper II, the qPCR data were analyzed with a Welch Two Sample t-test assuming unequal variances. Fertilization and sex ratio were tested with Chi-square tests.

Ethical considerations

Fish welfare was a priority and all experiments and handling of the animals were performed according to the Swedish Animal Care legislation and approved by the Southern Stockholm Animal Research Ethics committee (*Stockholm Södra Djurförsöksetiska nämnd*, Dnr: 1742/42/2008, Dnr: 1556/42/2011, Dnr: 806/3.1.1/2014, and Dnr: S39-13).

Summary of papers

I. Developmental exposure of zebrafish (*Danio rerio*) to 17α-ethinylestradiol affects non-reproductive behavior and fertility as adults, and increases anxiety in unexposed progeny.

This study examined if developmental exposure of zebrafish to EE_2 gave persistent effects on their fertility and behavior and if the behavioral effects were transmitted to the unexposed progeny. The fish were exposed to 0, 1.2 and 1.6 ng/L measured concentrations of EE_2 from 1 dpf to 80 dpf and allowed to recover for 82 days in clean water. After the fish recovered to adulthood their non-reproductive behaviors were examined in the NT test and a shoaling test, and their fertilization success and hepatic Vtg expression were analyzed. We found persistent changes in zebrafish non-reproductive behavior and fertility as an effect of exposure to EE_2 during development. The results show increased shoaling intensity and increased anxiety-like behaviors in both female and male fish. No alterations in Vtg expression or sex ratio were found, but fertilization success had decreased in both males and females, suggesting that effects of developmental exposure to EE_2 are irreversible to some extent. In addition, adult progeny (F1) of the fish treated with 1.2 ng/L showed increased anxiety-like behaviors in the NT test and scototaxis test than the progeny of fish from unexposed parents. No effects on shoaling were found.

II. Testis transcriptome alterations in zebrafish (*Danio rerio*) with reduced fertility due to developmental exposure to 17α-ethinylestradiol.

To search for the underlying mechanisms of the lowered fertility in paper I, transcriptome analysis was performed by RNA-Seq. Testes from male zebrafish with reported lowered fertility due to exposure to EE_2 during development were used. RNA-Seq analysis revealed the differential expression of 249 and 16 genes after exposure to 1.2 and 1.6 ng/L EE_2, respectively. The expression of 11 genes was altered by both exposures and in the same direction. Three genes were connected to the GO term response to estrogen in the 1.2 ng/L exposure group and no gene was connected to this term in the 1.6 ng/L exposure group, supporting the idea that direct effects on estrogen target genes are remediated after recovery of exposure. However, genes and pathways previously shown to be upregulated in fish testis by the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), for example, genes coding for inhibin subunits, cytochrome c oxidase

subunits, keratin, and insulin growth factor binding protein were differentially expressed due to developmental treatment with EE_2. The coding sequences most affected could be categorized to have putative functions in cell signaling, proteolysis, protein metabolic transport and lipid metabolic processes. Several homeobox transcription factors involved in development and differentiation showed increased expression in response to EE_2. Among them was *meis1b*, which has recently been shown to be regulated by estrogen and encodes a protein that contributes to Hox activity during development. Differential expression of genes related to cell death, differentiation and proliferation was observed. In addition, several genes related to steroid synthesis, testis development and function were differentially expressed. Several genes associated with spermatogenesis in zebrafish and/or mouse were differentially expressed. For example, *Inhbab*, involved in testis development in mouse, and *cxcl12a* and *foxc1b,*which are associated with germ cell migration in zebrafish respective mouse were upregulated. Upregulation of *ptgs2a* (COX 2), was also observed. The gene *ptgs2a* is suggested to take an active part in sex determination in zebrafish and could represent a sign of demasculinization due to developmental exposure. Further, differences in the expression of non-coding sequences was observed, several of which were differentially expressed miRNA that might affect testis gene expression at the post-transcriptional level.

III. Persistent effects of developmental exposure to 17α-ethinylestradiol on the zebrafish (*Danio rerio*) brain transcriptome and behavior.

The aim of this study was to verify the persistent effects on non-reproductive behavior in zebrafish exposed to EE_2 during development and further investigate potential mechanisms behind the behavioral effects. Zebrafish embryos were exposed from 1 dpf until 80 dpf to 0, 2.14 and 7.34 ng/L measured concentrations of EE_2. After exposure ceased, the animals were allowed to recover for 120 days in clean water. After remediation, the adult fish were tested for changes in non-reproductive behavior, and their whole-brain transcriptome was analyzed with RNA-Seq. The results show increased anxiety-like behaviors in the NT test and scototaxis test as a result of EE_2 exposure during development. Female zebrafish tended to stay closer to the shoal, shown as decreased number of transitions away from the shoal. RNA-Seq revealed the differential expression of 33 genes in male brains and 63 in female brains as a result of the exposure. Only one gene, *Sv2b*, coding for a synaptic vesicle protein, was affected by EE_2 in both sexes, demonstrating marked differences in the differential expression between females and males. In males, functional analyses showed that EE_2 had significant effects on pathways related to cholesterol biosynthesis, the immune response, synaptic proteins, DNA repair and heme biosynthesis/degradation. In the female brains, EE_2 altered the expression of genes in pathways connected to circadian rhythm, the immune response, lipid metabolism, heme biosynthesis and degradation, the

cytoskeleton and motor proteins. No differentially expressed genes were directly connected with the stress axis, but the pathways of cholesterol biosynthesis and circadian rhythm were possibly involved in the anxious phenotype. Cholesterol and circadian rhythm pathways have previously been shown to be related to stress (Wong et al., 2015, Rey et al., 2013) and affected by estrogens. In addition, several miRNAs were differentially expressed in the female brains.

IV. Combinatory effects of low concentrations of 17α-ethinylestradiol and citalopram on non-reproductive behavior in adult zebrafish (*danio rerio*).

Organisms in the aquatic environment are often exposed to a complex mixture of contaminants. In this paper, we explore the combination effect of low concentrations of EE_2 and the SSRI citalopram. Citalopram is a prescription antidepressant drug commonly found in sewage and surface waters that has previously been shown to cause anxiolytic behavior in fish. Chemical analysis revealed that the control water contained 0.4 ng/L EE_2 resulting in exposure concentrations of 0.9 ng/L (nominal 0.1) and 1 ng/L (nominal 0.5). The citalopram exposure concentrations were 0.1 μg/L (nominal 0.1) and 0.4 μg/L (nominal 0.5). The behavior tests showed that these chemicals had a more prominent effect on males and there was some inconsistency between the different tests. Both exposures of EE_2 resulted in increased anxiety-like behaviors in males in the scototaxis test. Citalopram alone (0.1 and 0.5 ng/L) had little effect, however, in the combined treatment groups with EE_2 and citalopram, the effects of EE_2 on anxiety-like behavior was abolished. Surprisingly, in the NT test, lower anxiety occurred after EE_2 exposure as well as citalopram exposure than after the control and combination exposures. This study shows that EE_2 affects behavior in zebrafish males at very low concentrations and that citalopram affects this response despite marginal effects of its own at these low levels. This study is an initial effort to understand the complex responses of the organisms in the aquatic environment to mixtures of pollutants.

V. Altered non-reproductive behavior and feminization caused by developmental exposure to 17α-ethinylestradiol persists to adulthood in three-spined stickleback (*Gasterosteus aculeatus*).

In this study, wild-caught three-spined sticklebacks (*Gasterosteus aculeatus*) were allowed to mate, and the fertilized embryos were exposed to nominal 0 and 20 ng/L EE_2 from fertilization to 7 weeks post-hatch. After 8 months of remediation in tap water, three non-reproductive behaviors, not previously analyzed in developmentally EE_2-exposed fish of this species or other wild caught fish, were evaluated. Chemical analysis surprisingly revealed that the concentrations of EE_2 were 5 and 30 ng/L in the nominal 0 and 20 ng/L samples, making use of historical controls from previous experiments necessary for comparisons. The fish exposed during

development showed significant reductions in anxiety in the scototaxis and NT tests by means of more visits to the white and upper halves of the aquaria, respectively. In the scototaxis test, fish also took longer to first enter to the white compartment, and spent more total time in white than unexposed fish. In the NT test, developmental exposure decreased the time spent freezing. In the shoaling test, EE_2 exposure did not affect any behavior parameters compared to the control group but exposure to 30 ng/L EE_2 altered the time to leave the shoal group after establishing contact and altered the number of transitions away from the shoal compared to 5 ng/L. A skewed sex ratio toward females and intersex was observed in the group of fish exposed to 30 ng/L EE_2 but not those exposed to 5 ng/L EE_2. Induction of ovotestis as well as total feminization of genetic males were observed in the group exposed to 30 ng/L. In conclusion, EE_2 exposure of three-spined sticklebacks during development in resulted in persistent effects on anxiety and shoaling, behaviors of significance for fitness in wild fish populations. Additionally, a skewed sex ratio occurred after exposure to 30 ng/L EE_2, but not 5 ng/L EE_2. As 5 ng/L EE_2 had effects on non-reproductive behavior but not on feminization, non-reproductive behavior may also be a sensitive parameter for endocrine disruption in this species.

Discussion

This thesis shows that exposure to EE_2 has both non-reproductive behavioral and reproductive effects on fish. Behavioral deviations occur after both adult short-term exposure (paper IV) and developmental exposure, including after a long remediation period (papers I, III and V). In both adult and developmental exposures, an anxiogenic response was displayed in the scototaxis test by the zebrafish. Developmental exposure to EE_2 increased anxiety-like behavior of zebrafish in scototaxis and NT tests and increased shoal cohesion (paper I and III). It also resulted in persistent decrease in fertilization success (paper I). The different behavior tests show some contradicting results in the short-term adult exposure, e.g., EE_2 exposure reduced anxiety-like behavior in the NT test but increased anxiety-like behavior in the scototaxis test. The sticklebacks also showed altered behavior due to developmental exposure as well as a skewed sex ratio in the higher exposure. RNA sequencing analysis of zebrafish testis (paper II) and brain (paper III) was used to explore the underlying transcriptomic patterns of the deviations in behavior and fertilization success and resulted in identifying several interesting pathways to further investigate in the search for the mechanisms behind the persistent effects of developmental exposure to EE_2.

Persistent changes in non-reproductive behavior due to developmental exposure of zebrafish to EE_2

Persistent changes in non-reproductive behavior due to exposure to EE_2 during development in zebrafish were found. The non-reproductive behavior included anxiety-like behaviors in the NT and scototaxis tests, and increased shoaling intensity in both female and male fish. Exposure to anxiogenic substances promote diving and immobility in the NT test, cortisol levels are increased during the session and anxiolytic substances like buspirone and diazepam have been shown to increase the time spent in the top zone (Kysil et al., 2017). The scototaxis test has previously also been validated by experiments with anxiogenic substances, which increased the time fish spent in the dark zone, whereas anxiolytics decreased the time in the dark zone (Maximino et al., 2011). Cortisol levels are also increased in the scototaxis test, but not as much as in the NT test (Kysil et al., 2017). Although both tests are sensitive to anxiogenic and anxiolytic drugs, the two tests do not render identical responses to different substances suggesting that the two tests may measure different aspects or levels of anxiety and reflect slightly different motivational processes. They should be regarded as complementary rather than interchangeable

(Blaser and Rosemberg, 2012, Maximino et al., 2012, Thompson et al., 2016). The exact stimuli of the NT and the scototaxis tests are unknown and the behavioral effects might include risk taking and/or other unknown behavioral parameters. Shoaling is a complex behavior of group dynamics and social interaction thought to be directly related to predator avoidance. However, the shoaling behavior test is less validated by pharmacological measures than the NT and the scototaxis tests (Maximino et al., 2010a) and the results from such validations are contradictory. Acute stress has been shown to increase shoaling intensity in sticklebacks (Wotton, 1984) and the substance MK-801, a NMDA-receptor antagonist, that usually causes an anxiolytic response causes zebrafish to increase shoaling, while ethanol reduces shoaling (Maximino et al., 2010a). Fish exposed to EDCs have shown a varying response in shoal cohesion with differences also between females and males. Sticklebacks exposed to Butyl benzyl phtalate (BBP) as adults showed an increased shoal cohesion (Wibe et al., 2002). Stickleback females exposed to EE_2 as adults showed a decreased shoal cohesion although no effect on shoal cohesion was seen in males (Dzieweczynski and Greaney, 2017). In contrast, guppies exposed to EE_2 showed an increased shoal cohesion in females and a decrease in the males (Heintz et al., 2015) highlighting the complexity of the response in shoaling behavior.

To further investigate the potential reasons behind the alterations in non-reproductive behavior, we analyzed the brain transcriptome of zebrafish developmentally exposed to EE_2 (paper III). The RNA-Seq of the zebrafish brain revealed differential gene expression between fish exposed to EE_2 during development and control fish. Only one gene whose expression was affected by EE_2 was shared among the differentially expressed genes in the both sexes suggesting that the behavioral phenotype was due to different mechanisms in males and females. Anxiety-like behavior is often associated with a physiological stress response, and acute adult exposure to EE_2 in fish has previously been shown to affect such stress pathways (Harding et al., 2013). No effects on genes directly associated with the stress axis, i.e., hypothalamic-pituitary-interrenal axis (HPI axis), (the analogue to the hypothalamic-pituitary-adrenal axis (HPA axis) in mammals), were observed that could explain the anxious phenotype, but as we did not measure stress axis endpoints like cortisol or the adrenocorticotropic hormone (ACTH) levels, the assumption that the behavior measured reflects stress should be used with some caution. Differential expressions of genes that could indirectly affect the stress response were, however, observed. In males, one of the major affected putative pathways was cholesterol biosynthesis, which was previously linked to stress response in the mammalian brain (Hu et al., 2014, Sun et al., 2015). The genes that were upregulated in the present study were down-regulated in adult fluoxetine-exposed fish brain that was accompanied by anxiolytic behavior (Wong et al., 2013). The same genes in the cholesterol biosynthesis pathway were later also shown to be expressed more in fish brain from a zebrafish strain with high stress sensitivity than in brain from a fish strain with low stress sensitivity (Wong et al., 2015) In females,

one of the top putative pathways was that of circadian rhythm. In zebrafish, circadian rhythm genes differ between fish with different sensitivities to stress (Rey et al., 2013). Several studies in rodents have found alterations in genes associated with circadian rhythm after estrogen exposure (Nakamura et al., 2008, Krizo and Mintz, 2014) and the altered genes related to the circadian rhythm in females might be connected to the observed anxiety-like phenotypes. Analyses of the brain transcriptome in rare minnows exposed to TBT revealed genes in the circadian pathway to be down-regulated in the females but not in the males (Zhang et al., 2018). In addition, genes with functions in the synapse and axon myelination in the brain were differentially expressed, suggesting that pronounced alterations in nerve signaling might remain in the adult fish after exposure during development.

Persistent changes in reproductive endpoints due to developmental exposure to EE_2 in the zebrafish

Decreased fertilization success occurred in both male and female zebrafish (paper I), supporting that effects of developmental exposure on reproductive endpoints are irreversible at least to some extent. The impaired fertility is in accordance with earlier findings that reproductive endpoints are sensitive to developmental EE_2 exposure at similar concentrations in zebrafish (Hill Jr and Janz, 2003, Xu et al., 2008). The mechanisms behind the impairment of reproductive endpoints by developmental EE_2 exposure are still largely unknown. To further understand the mechanism behind the deceased fertilization success we conducted a RNA-Seq analysis of the testis transcriptome of males showing decreased fertility after developmental exposure to EE_2 (paper II). Estrogens have an important role in the developing testis (O'Donnell et al., 2001) and in spermatogenesis (Carreau et al., 2011), and estrogen receptors are abundantly present in fish gonads in both males and females (Filby and Tyler, 2005). In accordance with this, genes coding for several homeobox transcription factors involved in development and differentiation were differentially expressed in the testis. Genes connected to spermatogenesis and testis development and function were also differentially expressed in the testis of zebrafish developmentally exposed to EE_2 of possible significance for the reduced fertility. Exposure to EE_2 during adulthood has previously been shown to affect genes related to development and male sex determination and differentiation in fish testis (Filby et al., 2007, Reyhanian Caspillo et al., 2014, Feswick et al., 2016, Nikoleris et al., 2016), and a few of these genes were also differentially expressed in the developmentally exposed males in paper II supporting that EE_2 exposure affected sex determination. Few genes found to be significantly differentially expressed in the testis of developmentally exposed fish compared to the control fish were directly related to estrogen response. Many of the effects of estrogen on the gonads are however indirect, mediated by the hypothalamus-pituitary-gonadal (HPG) axis via the gonadotropins released from the pituitary gland. Gonadotropins are in-

volved in the regulation of gametogenesis and steroidogenesis (Evans et al., 2013). Although no genes connected to the HPG axis were found to be differentially expressed in the brain (paper III), the expression of a few testicular gonadotropin target genes was persistently altered by EE_2, suggesting that the HPG axis could have been involved in the organizational effects of EE_2 exposure during development in the testis, although it no longer affected the brain after termination of exposure. Exposure to EE_2 during adulthood has led to alterations in gene expression in the HPG axis in both brain and testis of the pejerrey fish (*Odontesthes bonariensis*) (Garriz et al., 2017) and exposure to diethylstilbestrol (DES), another synthetic estrogen, has caused alterations in the HPG axis of yellow juvenile catfish (*Pelteobagrus fulvidraco*) (Liu et al., 2018) as well as in the adult zebrafish in addition to disrupting meiosis and apoptosis in the testis (Yin et al., 2017).

Effects on unexposed progeny and transgenerational effects

In paper I the persistent anxiogenic effects on behavior were transmitted to the unexposed progeny (F1) of the developmentally EE_2-exposed zebrafish. Although not representing a true transgenerational effect, this result suggests that such effects might be found. Transgenerational effects, resulting from heritable epigenetic changes, occur when the effects are transmitted to progeny that were not exposed to the environmental factor that caused the effect. For a true transgenerational effect, at least an F2 generation, i.e., a generation not exposed as germ cells, would need to be observed. Transgenerational effects of male rat fertility induced by EDCs have been found with concomitant alterations in testis transcriptome and methylome (Anway et al., 2008, Salian et al., 2009, Wolstenholme et al., 2011a, Guerrero-Bosagna et al., 2012, Manikkam et al., 2012, Prados et al., 2015). We have shown that the anxiogenic behavioral effects of EE_2 exposure during development in guppy were transmitted transgenerationally to two consecutive unexposed generations (Volkova et al., 2015). Transgenerational effects on fertility after EE_2 exposure during development has also been shown in medaka (*Oryzias latipes*), whose F2 generation had a lower fertilization rate (Bhandari et al., 2015).

Although epigenetic effects such as DNA methylation and histone modifications have not been studied in this thesis, EE_2 exposure during development resulted in differential expression of several miRNA in both testis and brain. In general, miRNAs are involved in epigenetic regulation of gene expression and gene silencing (He and Hannon, 2004, Morales et al., 2017) suggesting that miRNA-mediated post-transcriptional effects on gene expression may contribute to the fertility and behavioral alterations of the zebrafish in papers I and III. Epigenetic alterations are tightly associated with impaired male spermatogenesis and infertility in mammals (Rajender et al., 2011) and it has been suggested that epigenetic factors can influence sex determination in zebrafish (Nagabhushana and Mishra, 2016). Epigenetic mechanisms are also involved in sex differentiation in the brain (McCarthy and

Nugent, 2015). Several miRNAs were differentially expressed in the testis and the brain. In the brain, the miRNAs were all novel and not further studied, but their presence was intriguing and might be important for estrogen effects on the brain. In the testis, the expression of several known miRNAs was enhanced. Among them was *mir214,* which alters the expression of genes in the Hedgehog and dispatched 2 signaling pathways during zebrafish development (Flynt et al., 2007, Li et al., 2008). Estrogen exposure during adulthood has caused changes in miRNA expression in zebrafish liver; vitellogenin 3, *vtg3*, has been identified as a target gene for *mir122*, and estrogen receptor 1*, esr1*, is a target gene for *mir214* (Cohen and Smith, 2014). Both of these miRNAs were affected in the testis in paper II. It is possible that these alterations in miRNAs might affect fertility by post-transcriptional effects on testis gene expression. These observations warrant further experiments on the level of protein expression of miRNA target genes to clarify whether miRNA contributes to alterations in fertility and/or behavior. Further studies evaluating and elaborating the descriptive results in this thesis are needed, but many alterations were observed in both the brain and testis transcriptomes that might be related to the observed phenotypes and can be used to generate hypotheses for future studies.

Persistent changes due to developmental exposure to EE_2 in the stickleback

EE_2 exposure during development in the three-spined stickleback also resulted in persistent effects on non-reproductive behaviors as well as a skewed sex ratio toward females (Paper V). The stickleback were tested with the same behavioral tests as the zebrafish, i.e., the NT, the scototaxis and the shoaling tests, but in contrast to the zebrafish, the stickleback displayed an anxiolytic behavior response in the NT and the scototaxis tests. The results are puzzling but contradictory results in behavior response to developmental exposure to EE_2 between the two species of fish could be explained by species differences, timing and/or dose differences For example, similar contradictory results have previously been reported in the stickleback when exposed to citalopram; where adult acute exposure gave an anxiolytic response in the stickleback exposed to citalopram (Kellner et al., 2016), and developmental exposure gave an anxiogenic response after remediation to adulthood (Kellner et al., 2017). In addition, a possible explanation is the presence of non-monotonic dose response curves. Behavior responses in a non-monotonic dose curve have been observed in zebra fish after adult exposure to EE_2 where fish exposed to 25 ng/L EE_2 showed a decrease in anxiety-like behavior while fish exposed to 5 ng/L showed an increase in anxiety-like behavior (Reyhanian et al., 2011). It cannot be excluded that an anxiogenic response would be observed below a dose of 5 ng/L in the present study.

In the shoaling test, however, the behavior of sticklebacks exposed to EE_2 did not differ from that of the control group, but the fish exposed to 30 ng/L EE_2 showed

significantly fewer transitions away from the shoal than the fish exposed to 5 ng/L EE_2 and took longer time to leave the shoal than 5 ng/L group suggesting closer shoal cohesion in the higher exposure group. A skewed sex ratio occurred after exposure to the high concentration 30 ng/L, but not to the lower 5 ng/L (paper V). The skewed sex ratio toward feminization was explained by feminization of genetic male fish, as revealed by further histological and genetic sex examinations. Both the shift in behavior and sex ratio are of significance for fitness in wild fish populations. As the effects on behavior but not those on feminization were observed also after exposure to the environmental relevant concentration 5 ng/L, non-reproductive behavior might be a sensitive parameter of endocrine disruption also in this species.

The results in this thesis suggest that zebrafish behavior is more sensitive than the stickleback when these fish are exposed to EE_2. In the zebrafish, clear anxiogenic effects was observed at approximately 1 ng/L EE_2 while in the stickleback, 5 ng/L EE_2 affected anxiety behavior less pronounced, although comparisons might not be clearly defined. The lab strain of zebrafish was more genetically homogenous than the wild-caught stickleback so it was expected that the stickleback will be more robust and show a higher statistical dispersion, which makes detecting such alterations more difficult. The sensitivity to behavioral effects of pharmaceuticals can differ between species (Palace et al., 2009, Brodin et al., 2014) and should be considered during risk assessment together with timing, dose and exposures to additional chemicals.

Due to factors out of our control, EE_2 was present in the water of the control group in the exposure experiment for the sticklebacks. To account for this contamination a historical control was applied. However, since this study is the first to show developmental effects of EE_2 on non-reproductive behavior in wild fish the results are still new and important.

The unexpected detection of measurable levels of EE_2 in the control water in paper V was an unfortunate weakness. We have utilized the tap water in the fish facility at Södertörn University for many years without experiencing a single sample with a detectable level of EE_2 above 0.5 ng/L, which was the detection level in the analysis protocol used before 2014. When an improved detection limit of 0.2 ng/L was used, water samples from paper IV in fall 2014 revealed 0.45 ng/L EE_2 in control water. However, paper V performed in fall 2015 showed a surprisingly high level of 5 ng/L EE_2 in control water. After finding the high level of EE_2 in the control water we made additional measurements of the tap water at the department to rule out contamination from human mistakes or other explanations. The tap water measurements showed that the levels of EE_2 in samples taken in April and May 2017 ranged from undetectable to 5.5 ng/L. The time period when the exposure of sticklebacks in paper V was conducted was rainy and at least one of the STPs upstream of Huddinge's water source reported several overflows during the period. These situations are difficult to predict during long term developmental exposures. Knowledge of exposure water concentrations is of high importance for experimental diligence.

A future alternative, especially for conducting very low concentration exposure experiments, could be to use reconstituted water or to additionally treat the water. However, this approach only applies to semi-static or static experiments, as flow-through systems demand very large quantities of water. The high concentrations of EE_2 in the tap water underlines the importance of the efficient removal of EDCs in STPs as drinking water EE_2 exposures at such high levels cannot be regarded as acceptable. To ensure safe environments, reducing and minimizing the amounts of EDCs reaching the aquatic environment is crucial. There are techniques for improving the waste water treatment, reducing estrogenic chemicals more efficiently (Yang et al., 2017) but they are rarely used due to their high costs. The current removal efficiency of EE_2 in STPs is approximately 50-70 %, it varies greatly within and between STPs, (Comber et al., 2018) and is not sufficient in removing EE_2 and other pharmaceuticals (Verlicchi et al., 2012).

Adult exposure and combination effects

The effects of environmental contaminants have mainly been determined in single substance experiments under laboratory conditions. In paper IV, we made a contribution to the sparse but growing literature of effects of mixtures of pollutants by investigating the effects in a study of a combination exposure of EE_2 and the SSRI citalopram.

We found the expected anxiogenic response to exposure to EE_2 in concentrations as low as 0.9 ng/L in zebrafish males in the scototaxis test. In the NT test, however, the results indicate anxiolytic behavior. EE_2 alone had no effect on shoaling behavior. The divergent results may be due to the influence of non-monotonic dose responses, i.e., the dose-response curve changes direction within the range of doses examined, creating U or inverted-U curves. Even small fluctuations in the concentrations could cause different responses. Non-monotonous dose response curves for EDCs are not uncommon (Vandenberg et al., 2012, Lagarde et al., 2015) and the behavioral results could be a reflection of such pattern. Citalopram caused the expected response in males that was anxiolytic compared to the response of the controls in the NT test. In the scototaxis test, the behavior of the fish exposed to citalopram did not differ from that of the control but was more anxiolytic compared to the behavior of the EE_2 exposed fish. Although citalopram caused few effects on its own it counteracted the anxiogenic effects of EE_2 in the scototaxis test. Combination exposure however resulted in an anxiogenic response in the NT test. The effects of SSRI in fish have shown complex relations with the timing of endpoint measurements, as shown in a review of McDonald, 2017. The anxiolytic effects of SSRI on fish were of a much higher degree when studies with an exposure shorter than 21 days are excluded. The anxiolytic effects of acute exposures (shorter than 21 days) to SSRI were suggested to be masked by compensatory mechanisms (McDonald, 2017). In paper IV of this thesis , we used an exposure period of 14

days, which could have affected the response in the behavior test. A recent study (Simmons et al., 2017) conducted with caged adult goldfish outside of waste water treatment plants, finding fifteen PPCPs in the fish after 21 days of exposure, revealed the fish to show less anxious behavior in an activity and startle response assay. Stickleback males exposed to STP effluent for three weeks also showed reduced courtship behavior and built fewer nests (Sebire et al., 2011) and male fathead minnows exposed to effluents for the same time were less successful in competition for nests (Garcia-Reyero et al., 2011). Behavioral effects in the fathead minnow were further examined with a gene expression study and compared to alterations in fish exposed only to estrogens or androgens. Although some of the expression patterns from STP effluent exposed fish were shared with the two estrogen and androgen groups, the overall gene expression pattern was unique (Garcia-Reyero et al., 2011) underscoring the complexity of the mixtures in the effluents and the difficulty to interpret results from such. A meta-analysis of short-term adult exposure of fish have shown that eight EDCs give a rapid response in different endpoints (Ankley and Villeneuve, 2015), that significant changes in biological responses were a function of dose and time and that non-monotonic dose-response relationships that are present as compensatory responses are ubiquitous within the HPG axis after chemical exposure. The combination effects were not straight forward, but mixtures are likely to have complex effects on living organisms and many influencing factors complicate the ecotoxicological evaluation of pharmaceutical cocktails.

Conclusions

To conclude, the results in this thesis show that fertility and non-reproductive behaviors in the zebrafish and three spined stickleback were sensitive to EE_2 exposure. The effects of developmental exposure on behavior and fertility were persistent after remediation to adulthood. We also found persistent alterations in the transcriptome of the zebrafish testis and brain, giving first insights into possible mechanisms behind the effects of developmental exposure on behavior and fertility. The insights from these explorative studies of the transcriptome generate new hypotheses for further studies exploring the persistent effects of developmental exposure to EE_2. The persistence implies that the effects should be detectable in field studies, and behavioral studies may thus represent an important component in the risk evaluation of EDCs.

References

ANKLEY, G. T. & VILLENEUVE, D. L. 2015. Temporal Changes in Biological Responses and Uncertainty in Assessing Risks of Endocrine-Disrupting Chemicals: Insights from Intensive Time-Course Studies with Fish. *Toxicological Sciences,* 144**,** 259–275.

ANWAY, M. D., REKOW, S. S. & SKINNER, M. K. 2008. Transgenerational epigenetic programming of the embryonic testis transcriptome. *Genomics,* 91**,** 30–40.

ARCAND-HOY, L. D. & BENSON, W. H. 1998. Fish reproduction: An ecologically relevant indicator of endocrine disruption. *Environmental Toxicology and Chemistry,* 17**,** 49–57.

ARIS, A. Z., SHAMSUDDIN, A. S. & PRAVEENA, S. M. 2014. Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. *Environment International,* 69**,** 104–119.

BARBOSA, M. O., MOREIRA, N. F. F., RIBEIRO, A. R., PEREIRA, M. F. R. & SILVA, A. M. T. 2016. Occurrence and removal of organic micropollutants: An overview of the watch list of EU Decision 2015/495. *Water Research,* 94**,** 257–279.

BASRUR, P. K. 2006. Disrupted sex differentiation and feminization of man and domestic animals. *Environmental Research,* 100**,** 18–38.

BAUMANN, L., KNÖRR, S., KEITER, S., REHBERGER, K., VOLZ, S., SCHILLER, V., FENSKE, M., HOLBECH, H., SEGNER, H. & BRAUNBECK, T. 2014. Reversibility of endocrine disruption in zebrafish (Danio rerio) after discontinued exposure to the estrogen 17α-ethinylestradiol. *Toxicology and Applied Pharmacology,* 278**,** 230–237.

BELL, A. M. 2001. Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined stickleback, Gasterosteus aculeatus. *Animal Behaviour,* 62**,** 775–780.

BELL, A. M. 2004. An endocrine disrupter increases growth and risky behavior in three-spined stickleback (Gasterosteus aculeatus). *Hormones and Behavior,* 45**,** 108–114.

BENCAN, Z., SLEDGE, D. & LEVIN, E. D. 2009. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. *Pharmacology Biochemistry and Behavior,* 94**,** 75–80.

BERGMAN, Å., HEINDEL, J. J., JOBLING, S., KIDD, K. A. & ZOELLER, R. T. 2012. *State of the Science of Endocrine Disrupting Chemicals – 2012*.

BHANDARI, R. K., VOM SAAL, F. S. & TILLITT, D. E. 2015. Transgenerational effects from early developmental exposures to bisphenol A or 17α-ethinylestradiol in medaka, Oryzias latipes. *Scientific Reports,* 5**,** 9303.

BLANCHFIELD, P., KIDD, K., DOCKER, M., PALACE, V., PARK, B. & POSTMA, L. 2015. Recovery of a Wild Fish Population from Whole-lake Additions of a Synthetic Estrogen. *Environmental Science & Technology*.

BLASER, R. E. & ROSEMBERG, D. B. 2012. Measures of Anxiety in Zebrafish (*Danio rerio)*: Dissociation of Black/White Preference and Novel Tank Test. *PLoS ONE,* 7**,** e36931.

BRODIN, T., PIOVANO, S., FICK, J., KLAMINDER, J., HEYNEN, M. & JONSSON, M. 2014. Ecological effects of pharmaceuticals in aquatic systems—impacts through behavioural alterations. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 369.

BUSTIN, S. A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology,* 29.

CARREAU, S., BOURAIMA-LELONG, H. & DELALANDE, C. 2011. Estrogens – new players in spermatogenesis. *Reproductive Biology,* 11**,** 174–193.

CHEN, T.-H. & HSIEH, C.-Y. 2017. Fighting Nemo: Effect of 17α-ethinylestradiol (EE2) on aggressive behavior and social hierarchy of the false clown anemonefish Amphiprion ocellaris. *Marine Pollution Bulletin,* 124**,** 760–766.

CLOTFELTER, E. D., BELL, A. M. & LEVERING, K. R. 2004. The role of animal behaviour in the study of endocrine-disrupting chemicals. *Animal Behaviour,* 68**,** 665–676.

COHEN, A. & SMITH, Y. 2014. Estrogen regulation of microRNAs, target genes, and microRNA expression associated with vitellogenesis in the zebrafish. *Zebrafish,* 11**,** 462–78.

COHN, J. & MACPHAIL, R. C. 1996. Ethological and experimental approaches to behavior analysis: Implications for ecotoxicology. *Environmental Health Perspectives,* 104**,** 299–305.

COLBORN, T., SAAL, F. S. V. & SOTO, A. M. 1993. Developmental Effects of Endocrine-Disrupting Chemicals in Wildlife and Humans. *Environ Health Perspect.,* 101**,** 378–84.

COMBER, S., GARDNER, M., SÖRME, P., LEVERETT, D. & ELLOR, B. 2018. Active pharmaceutical ingredients entering the aquatic environment from wastewater treatment works: A cause for concern? *Science of The Total Environment,* 613–614**,** 538–547.

CORCORAN, J., WINTER, M. J. & TYLER, C. R. 2010. Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. *Crit Rev Toxicol,* 40**,** 287–304.

DANG, H. M., INAGAKI, Y., YAMAUCHI, Y., KURIHARA, T., VO, C. H. & SAKAKIBARA, Y. 2017. Acute Exposure to 17α-Ethinylestradiol Alters Aggressive Behavior of Mosquitofish (Gambusia affinis) Toward Japanese Medaka (Oryzias latipes). *Bulletin of Environmental Contamination and Toxicology,* 98**,** 643–648.

DUGARD, M. L., TREMBLAY-LEVEAU, H., MELLIER, D. & CASTON, J. 2001. Prenatal exposure to ethinylestradiol elicits behavioral abnormalities in the rat. *Developmental Brain Research,* 129**,** 189–199.

DZIEWECZYNSKI, T. L. & GREANEY, N. E. 2017. Sex and dose-dependent effects of an estrogen mimic on boldness in threespine stickleback, Gasterosteus aculeatus, from an anadromous population. *Journal of Experimental Marine Biology and Ecology,* 497**,** 78–85.

EBELE, A. J., ABOU-ELWAFA ABDALLAH, M. & HARRAD, S. 2017. Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. *Emerging Contaminants,* 3**,** 1–16.

EGAN, R. J., BERGNER, C. L., HART, P. C., CACHAT, J. M., CANAVELLO, P. R., ELEGANTE, M. F., ELKHAYAT, S. I., BARTELS, B. K., TIEN, A. K., TIEN, D. H., MOHNOT, S., BEESON, E., GLASGOW, E., AMRI, H., ZUKOWSKA, Z. & KALUEFF,

A. V. 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research,* 205**,** 38–44.

ENMARK, E. & GUSTAFSSON, J. A. 1999. Oestrogen receptors - an overview. *Journal of Internal Medicine,* 246**,** 133–138.

ESPMARK WIBE, A., ROSENQVIST, G. & JENSSEN, B. M. 2002. Disruption of Male Reproductive Behavior in Threespine Stickleback *Gasterosteus aculeatus* Exposed to 17β-Estradiol. *Environmental Research,* 90**,** 136–141.

EVANS, D. H., CLAIBORNE, J. B. & CURRIE, S. 2013. *The Physiology of Fishes, Fourth Edition*, Taylor & Francis.

FESWICK, A., LOUGHERY, J. R., ISAACS, M. A., MUNKITTRICK, K. R. & MARTYNIUK, C. J. 2016. Molecular initiating events of the intersex phenotype: Low-dose exposure to 17α-ethinylestradiol rapidly regulates molecular networks associated with gonad differentiation in the adult fathead minnow testis. *Aquatic Toxicology,* 181**,** 46–56.

FILBY, A. L., PAULL, G. C., SEARLE, F., ORTIZ-ZARRAGOITIA, M. & TYLER, C. R. 2012. Environmental Estrogen-Induced Alterations of Male Aggression and Dominance Hierarchies in Fish: A Mechanistic Analysis. *Environmental Science & Technology,* 46**,** 3472–3479.

FILBY, A. L., THORPE, K. L., MAACK, G. & TYLER, C. R. 2007. Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. *Aquatic Toxicology,* 81**,** 219–231.

FILBY, A. L. & TYLER, C. R. 2005. Molecular characterization of estrogen receptors 1, 2a and 2b and their tissue and ontogenic expression profiles in fathead minnow (Pimephales promelas). *Biology of Reproduction,* 73.

FLYNT, A. S., LI, N., THATCHER, E. J., SOLNICA-KREZEL, L. & PATTON, J. G. 2007. Zebrafish miR-214 modulates Hedgehog signaling to specify muscle cell fate. *Nat Genet,* 39**,** 259–63.

FORAN, C. M., WESTON, J., SLATTERY, M., BROOKS, B. W. & HUGGETT, D. B. 2004. Reproductive assessment of Japanese medaka (Oryzias latipes) following a four-week fluoxetine (SSRI) exposure. *Archives of Environmental Contamination and Toxicology,* 46**,** 511–517.

GARCIA-REYERO, N., LAVELLE, C. M., ESCALON, B. L., MARTINOVIĆ, D., KROLL, K. J., SORENSEN, P. W. & DENSLOW, N. D. 2011. Behavioral and genomic impacts of a wastewater effluent on the fathead minnow. *Aquatic Toxicology,* 101**,** 38–48.

GARRIZ, A., DEL FRESNO, P. S. & MIRANDA, L. A. 2017. Exposure to E2 and EE2 environmental concentrations affect different components of the Brain-Pituitary-Gonadal axis in pejerrey fish (Odontesthes bonariensis). *Ecotoxicol Environ Saf,* 144**,** 45–53.

GORE, A. C. & PATISAUL, H. B. 2010. Neuroendocrine disruption: Historical roots, current progress, questions for the future. *Frontiers in Neuroendocrinology,* 31**,** 395–399.

GUERRERO-BOSAGNA, C., COVERT, T. R., HAQUE, M. M., SETTLES, M., NILSSON, E. E., ANWAY, M. D. & SKINNER, M. K. 2012. Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. *Reproductive Toxicology,* 34**,** 694–707.

GUILLETTE, L. J. & GUNDERSON, M. P. 2001. Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. *Reproduction,* 122**,** 857–864.

GUNNARSSON, L., JAUHIAINEN, A., KRISTIANSSON, E., NERMAN, O. & LARSSON, D. G. J. 2008. Evolutionary Conservation of Human Drug Targets in Organisms used for Environmental Risk Assessments. *Environmental Science & Technology,* 42**,** 5807–5813.

GUO, S. 2004. Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish? *Genes, Brain and Behavior,* 3**,** 63–74.

HALLGREN, S., VOLKOVA, K., REYHANIAN, N., OLSÉN, H. & PORSCH HÄLLSTRÖM, I. 2011. Anxiogenic behaviour induced by 17α-ethynylestradiol in male guppies (*Poecilia reticulata*). *Fish Physiology and Biochemistry,* 37**,** 911–918.

HARDING, L. B., SCHULTZ, I. R., GOETZ, G. W., LUCKENBACH, J. A., YOUNG, G., GOETZ, F. W. & SWANSON, P. 2013. High-throughput sequencing and pathway analysis reveal alteration of the pituitary transcriptome by 17α-ethynylestradiol (EE2) in female coho salmon, Oncorhynchus kisutch. *Aquatic Toxicology,* 142–143**,** 146–163.

HARDING, M. J. C., RODGER, G. K., DAVIES, I. M. & MOORE, J. J. 1997. Partial recovery of the Dogwhelk (Nucella lapillus) in Sullom Voe, Shetland from tributyltin contamination. *Marine Environmental Research,* 44**,** 285–304.

HE, L. & HANNON, G. J. 2004. MicroRNAs: small RNAs with a big role in gene regulation. *Nature Reviews Genetics,* 5**,** 522.

HECKER, M., TYLER, C. R., HOFFMANN, M., MADDIX, S. & KARBE, L. 2002. Plasma biomarkers in fish provide evidence for endocrine modulation in the Elbe River, Germany. *Environmental Science & Technology,* 36**,** 2311–2321.

HEINTZ, M. M., BRANDER, S. M. & WHITE, J. W. 2015. Endocrine Disrupting Compounds Alter Risk-Taking Behavior in Guppies (Poecilia reticulata). *Ethology,* 121**,** 480–491.

HELCOM 2010. Hazardous substances in the Baltic Sea – An integrated thematic assessment of hazardous substances in the Baltic Sea. Balt. Sea Environ. Proc. No. 120B.

HILL JR, R. L. & JANZ, D. M. 2003. Developmental estrogenic exposure in zebrafish (Danio rerio): I. Effects on sex ratio and breeding success. *Aquatic Toxicology,* 63**,** 417–429.

HOWE, D. G., BRADFORD, Y. M., CONLIN, T., EAGLE, A. E., FASHENA, D., FRAZER, K., KNIGHT, J., MANI, P., MARTIN, R., MOXON, S. A., PADDOCK, H., PICH, C., RAMACHANDRAN, S., RUEF, B. J., RUZICKA, L., SCHAPER, K., SHAO, X., SINGER, A., SPRUNGER, B., VAN SLYKE, C. E. & WESTERFIELD, M. 2013a. ZFIN, the Zebrafish Model Organism Database: increased support for mutants and transgenics. *Nucleic Acids Res,* 41**,** D854–60.

HOWE, K., CLARK, M. D., TORROJA, C. F., TORRANCE, J., BERTHELOT, C., MUFFATO, M., COLLINS, J. E., HUMPHRAY, S., MCLAREN, K., MATTHEWS, L., MCLAREN, S., SEALY, I., CACCAMO, M., CHURCHER, C., SCOTT, C., BARRETT, J. C., KOCH, R., RAUCH, G.-J., WHITE, S., CHOW, W., KILIAN, B., QUINTAIS, L. T., GUERRA-ASSUNÇÃO, J. A., ZHOU, Y., GU, Y., YEN, J., VOGEL, J.-H., EYRE, T., REDMOND, S., BANERJEE, R., CHI, J., FU, B., LANGLEY, E., MAGUIRE, S. F., LAIRD, G. K., LLOYD, D., KENYON, E., DONALDSON, S., SEHRA, H., ALMEIDA-KING, J., LOVELAND, J., TREVANION, S., JONES, M., QUAIL, M., WILLEY, D.,

HUNT, A., BURTON, J., SIMS, S., MCLAY, K., PLUMB, B., DAVIS, J., CLEE, C., OLIVER, K., CLARK, R., RIDDLE, C., ELLIOTT, D., THREADGOLD, G., HARDEN, G., WARE, D., BEGUM, S., MORTIMORE, B., KERRY, G., HEATH, P., PHILLIMORE, B., TRACEY, A., CORBY, N., DUNN, M., JOHNSON, C., WOOD, J., CLARK, S., PELAN, S., GRIFFITHS, G., SMITH, M., GLITHERO, R., HOWDEN, P., BARKER, N., LLOYD, C., STEVENS, C., HARLEY, J., HOLT, K., PANAGIOTIDIS, G., LOVELL, J., BEASLEY, H., HENDERSON, C., GORDON, D., AUGER, K., WRIGHT, D., COLLINS, J., RAISEN, C., DYER, L., LEUNG, K., ROBERTSON, L., AMBRIDGE, K., LEONGAMORNLERT, D., MCGUIRE, S., GILDERTHORP, R., GRIFFITHS, C., MANTHRAVADI, D., NICHOL, S., BARKER, G., et al. 2013b. The zebrafish reference genome sequence and its relationship to the human genome. *Nature,* 496**,** 498.

HU, X., WANG, T., LUO, J., LIANG, S., LI, W., WU, X., JIN, F. & WANG, L. 2014. Age-dependent effect of high cholesterol diets on anxiety-like behavior in elevated plus maze test in rats. *Behavioral and Brain Functions* 10**,** 30.

HURST, J. L. & WEST, R. S. 2010. Taming anxiety in laboratory mice. *Nature Methods,* 7**,** 825.

JAKOBSSON, S., BORG, B., HAUX, C. & HYLLNER, S. J. 1999. An 11-ketotestosterone induced kidney-secreted protein: the nest building glue from male three-spined stickleback, Gasterosteus aculeatus. *Fish Physiology and Biochemistry,* 20**,** 79–85.

JOBLING, S., NOLAN, M., TYLER, C. R., BRIGHTY, G. & SUMPTER, J. P. 1998. Widespread sexual disruption in wild fish. *Environmental Science & Technology,* 32**,** 2498–2506.

KATSIADAKI, I., SCOTT, A. P., HURST, M. R., MATTHIESSEN, P. & MAYER, I. 2002. Detection of environmental androgens: A novel method based on enzyme-linked immunosorbent assay of spiggin, the stickleback (Gasterosteus aculeatus) glue protein. *Environmental Toxicology and Chemistry,* 21**,** 1946–1954.

KAVLOCK, R. J., DASTON, G. P., DEROSA, C., FENNERCRISP, P., GRAY, L. E., KAATTARI, S., LUCIER, G., LUSTER, M., MAC, M. J., MACZKA, C., MILLER, R., MOORE, J., ROLLAND, R., SCOTT, G., SHEEHAN, D. M., SINKS, T. & TILSON, H. A. 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the US EPA-sponsored workshop. *Environmental Health Perspectives,* 104**,** 715–740.

KELLNER, M., PORSERYD, T., HALLGREN, S., PORSCH-HÄLLSTRÖM, I., HANSEN, S. H. & OLSÉN, K. H. 2016. Waterborne citalopram has anxiolytic effects and increases locomotor activity in the three-spine stickleback (Gasterosteus aculeatus). *Aquatic Toxicology,* 173**,** 19–28.

KELLNER, M., PORSERYD, T., PORSCH-HALLSTROM, I., BORG, B., ROUFIDOU, C. & OLSEN, K. H. 2017. Developmental exposure to the SSRI citalopram causes long-lasting behavioural effects in the three-spined stickleback (Gasterosteus aculeatus). *Ecotoxicology*.

KIDD, K. A., BLANCHFIELD, P. J., MILLS, K. H., PALACE, V. P., EVANS, R. E., LAZORCHAK, J. M. & FLICK, R. W. 2007. Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 8897–8901.

KORTENKAMP, A., MARTIN, O., FAUST, M., EVANS, R., MCKINLAY, R., ORTON, F. & ROSIVATZ, E. 2011. State of the art assessment of endocrine disrupters: Final report. European Comission, DG Environment, Brussels, Belgium.

KRIZO, J. A. & MINTZ, E. M. 2014. Sex Differences in Behavioral Circadian Rhythms in Laboratory Rodents. *Frontiers in Endocrinology,* 5.

KYSIL, E. V., MESHALKINA, D. A., FRICK, E. E., ECHEVARRIA, D. J., ROSEMBERG, D. B., MAXIMINO, C., LIMA, M. G., ABREU, M. S., GIACOMINI, A. C., BARCELLOS, L. J. G., SONG, C. & KALUEFF, A. V. 2017. Comparative Analyses of Zebrafish Anxiety-Like Behavior Using Conflict-Based Novelty Tests. *Zebrafish,* 14**,** 197–208.

LAGARDE, F., BEAUSOLEIL, C., BELCHER, S. M., BELZUNCES, L. P., EMOND, C., GUERBET, M. & ROUSSELLE, C. 2015. Non-monotonic dose-response relationships and endocrine disruptors: a qualitative method of assessment. *Environmental Health,* 14**,** 13.

LAIRD, P. W., ZIJDERVELD, A., LINDERS, K., RUDNICKI, M. A., JAENISCH, R. & BERNS, A. 1991. Simplified mammalian DNA isolation procedure. *Nucleic Acids Res,* 19**,** 4293.

LAURENSON, J. P., BLOOM, R. A., PAGE, S. & SADRIEH, N. 2014. Ethinyl Estradiol and Other Human Pharmaceutical Estrogens in the Aquatic Environment: A Review of Recent Risk Assessment Data. *The AAPS Journal,* 16**,** 299–310.

LEE, P.-Y., LIN, C.-Y. & CHEN, T.-H. 2014. Environmentally relevant exposure of 17α-ethinylestradiol impairs spawning and reproductive behavior in the brackish medaka Oryzias melastigma. *Marine Pollution Bulletin,* 85**,** 338–343.

LEHTONEN, K. K., BIGNERT, A., BRADSHAW, C., BROEG, K. & SCHIEDEK, D. 2017. Chemical pollution and ecotoxicology. *In:* SNOEIJS-LEIJONMALM, P., SCHUBERT, H. & RADZIEJEWSKA, T. (eds.) *Biological Oceanography of the Baltic Sea.* Dordrecht: Springer Netherlands.

LEPAGE, O., LARSON, E. T., MAYER, I. & WINBERG, S. 2005. Serotonin, but not melatonin, plays a role in shaping dominant–subordinate relationships and aggression in rainbow trout. *Hormones and Behavior,* 48**,** 233–242.

LI, N., FLYNT, A. S., KIM, H. R., SOLNICA-KREZEL, L. & PATTON, J. G. 2008. Dispatched Homolog 2 is targeted by miR-214 through a combination of three weak microRNA recognition sites. *Nucleic Acids Research,* 36**,** 4277–4285.

LISTER, A., REGAN, C., VAN ZWOL, J. & VAN DER KRAAK, G. 2009. Inhibition of egg production in zebrafish by fluoxetine and municipal effluents: A mechanistic evaluation. *Aquatic Toxicology,* 95**,** 320–329.

LIU, Z. H., CHEN, Q. L., CHEN, Q., LI, F. & LI, Y. W. 2018. Diethylstilbestrol arrested spermatogenesis and somatic growth in the juveniles of yellow catfish (Pelteobagrus fulvidraco), a fish with sexual dimorphic growth. *Fish Physiol Biochem.*

MAACK, G. & SEGNER, H. 2004. Life-stage-dependent sensitivity of zebrafish (Danio rerio) to estrogen exposure. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology,* 139**,** 47–55.

MANIKKAM, M., TRACEY, R., GUERRERO-BOSAGNA, C. & SKINNER, M. K. 2012. Dioxin (TCDD) Induces Epigenetic Transgenerational Inheritance of Adult Onset Disease and Sperm Epimutations. *PLoS ONE,* 7**,** e46249.

MATTHIESSEN, P. 2003. Endocrine disruption in marine fish. *Pure and Applied Chemistry,* 75**,** 2249–2261.

MATTHIESSEN, P. & GIBBS, P. E. 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. *Environmental Toxicology and Chemistry,* 17**,** 37–43.

MAXIMINO, C., BENZECRY, R., MATOS OLIVEIRA, K. R., DE JESUS OLIVEIRA BATISTA, E., HERCULANO, A. M., BROOCK ROSEMBERG, D., DE OLIVEIRA, D. L. & BLASER, R. 2012. A comparison of the light/dark and novel tank tests in zebrafish. *Behaviour,* 149**,** 1099–1123.

MAXIMINO, C., DA SILVA, A. W. B., GOUVEIA JR, A. & HERCULANO, A. M. 2011. Pharmacological analysis of zebrafish (Danio rerio) scototaxis. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 35**,** 624–631.

MAXIMINO, C., DE BRITO, T. M., BATISTA, A. W. D., HERCULANO, A. M., MORATO, S. & GOUVEIA, A. 2010a. Measuring anxiety in zebrafish: A critical review. *Behavioural Brain Research,* 214**,** 157–171.

MAXIMINO, C., MARQUES DE BRITO, T., DIAS, C. A. G. D. M., GOUVEIA, A. & MORATO, S. 2010b. Scototaxis as anxiety-like behavior in fish. *Nat. Protocols,* 5**,** 209–216.

MAXIMINO, C., MARQUES DE BRITO, T., DIAS DE MORAES, F., CORTES DE OLIVIERA, F. V., BATISTA TACCOLINI, I., MENDES PEREIRA, P., COLMANETTA, R., LOZANO, R., ANTÔNIO GAZOLLA, R., TENÓRIO, R., INAH TAVARES DE LACERDA, R., TERUME KOSHIKENE RODRIGUES, S., ANTONIO ASSIS PONTES, A., FERREIRA ROMÃO, C., MIRANDA PRADO, V. & GOUVEIA JR, A. 2007. A Comparative Analysis of the Prefernece for Dark Environments in Five Teleosts. *International Journal of Comparative Psychology***,** 351–367.

MCCARTHY, M. M. & NUGENT, B. M. 2015. At the frontier of epigenetics of brain sex differences. *Frontiers in Behavioral Neuroscience,* 9**,** 221.

MCDONALD, M. D. 2017. An AOP analysis of selective serotonin reuptake inhibitors (SSRIs) for fish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 197**,** 19–31.

MILLS, L. J. & CHICHESTER, C. 2005. Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Science of The Total Environment,* 343**,** 1–34.

MORALES, S., MONZO, M. & NAVARRO, A. 2017. Epigenetic regulation mechanisms of microRNA expression. *Biomol Concepts,* 8**,** 203–212.

NAGABHUSHANA, A. & MISHRA, R. K. 2016. Finding clues to the riddle of sex determination in zebrafish. *Journal of Biosciences,* 41**,** 145–155.

NAKAMURA, T. J., SELLIX, M. T., MENAKER, M. & BLOCK, G. D. 2008. Estrogen directly modulates circadian rhythms of PER2 expression in the uterus. *Am J Physiol Endocrinol Metab,* 295**,** E1025–31.

NIKOLAOU, A., MERIC, S. & FATTA, D. 2007. Occurrence patterns of pharmaceuticals in water and wastewater environments. *Analytical and Bioanalytical Chemistry,* 387**,** 1225–1234.

NIKOLERIS, L., HULTIN, C. L., HALLGREN, P. & HANSSON, M. C. 2016. 17α-Ethinylestradiol (EE2) treatment of wild roach (Rutilus rutilus) during early life development disrupts expression of genes directly involved in the feedback cycle of estrogen. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 180**,** 56–64.

O'DONNELL, L., ROBERTSON, K. M., JONES, M. E. & SIMPSON, E. R. 2001. Estrogen and Spermatogenesis. *Endocr Rev,* 22**,** 289–318.

OSTLUND-NILSSON, S., MAYER, I. & HUNTINGFORD, F. A. 2006. *Biology of the Three-Spined Stickleback*, CRC Press.

PALACE, V. P., EVANS, R. E., WAUTIER, K. G., MILLS, K. H., BLANCHFIELD, P. J., PARK, B. J., BARON, C. L. & KIDD, K. A. 2009. Interspecies differences in biochemical, histopathological, and population responses in four wild fish species exposed to ethynylestradiol added to a whole lakeThis paper is part of the series "Forty Years of Aquatic Research at the Experimental Lakes Area". *Canadian Journal of Fisheries and Aquatic Sciences,* 66**,** 1920–1935.

PEAKALL, D. B. 1996. Disrupted patterns of behavior in natural populations as an index of ecotoxicity. *Environmental Health Perspectives,* 104**,** 331–335.

PORTE, C., JANER, G., LORUSSO, L. C., ORTIZ-ZARRAGOITIA, M., CAJARAVILLE, M. P., FOSSI, M. C. & CANESI, L. 2006. Endocrine disruptors in marine organisms: Approaches and perspectives. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology,* 143**,** 303–315.

POTTINGER, T. G. & CALDER, G. M. 1995. Physiological stress in fish during toxicological procedures: A potentially confounding factor. *Environmental Toxicology and Water Quality,* 10**,** 135–146.

PRADOS, J., STENZ, L., SOMM, E., STOUDER, C., DAYER, A. & PAOLONI-GIACOBINO, A. 2015. Prenatal Exposure to DEHP Affects Spermatogenesis and Sperm DNA Methylation in a Strain-Dependent Manner. *PLoS ONE,* 10**,** e0132136.

RAJENDER, S., AVERY, K. & AGARWAL, A. 2011. Epigenetics, spermatogenesis and male infertility. *Mutation Research/Reviews in Mutation Research,* 727**,** 62–71.

REY, S., BOLTANA, S., VARGAS, R., ROHER, N. & MACKENZIE, S. 2013. Combining animal personalities with transcriptomics resolves individual variation within a wild-type zebrafish population and identifies underpinning molecular differences in brain function. *Mol Ecol,* 22**,** 6100–15.

REYHANIAN CASPILLO, N., VOLKOVA, K., HALLGREN, S., OLSSON, P.-E. & PORSCH-HÄLLSTRÖM, I. 2014. Short-term treatment of adult male zebrafish (Danio Rerio) with 17α-ethinyl estradiol affects the transcription of genes involved in development and male sex differentiation. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 164**,** 35–42.

REYHANIAN, N., VOLKOVA, K., HALLGREN, S., BOLLNER, T., OLSSON, P.-E., OLSÉN, H. & HÄLLSTRÖM, I. P. 2011. 17α-Ethinyl estradiol affects anxiety and shoaling behavior in adult male zebra fish (Danio rerio). *Aquatic Toxicology,* 105**,** 41–48.

RYAN, B. C. & VANDENBERGH, J. G. 2006. Developmental exposure to environmental estrogens alters anxiety and spatial memory in female mice. *Hormones and Behavior,* 50**,** 85–93.

SAARISTO, M., CRAFT, J. A., LEHTONEN, K. K. & LINDSTROM, K. 2009. Sand goby (Pomatoschistus minutus) males exposed to an endocrine disrupting chemical fail in nest and mate competition. *Hormones and Behavior,* 56**,** 315–321.

SAARISTO, M., CRAFT, J. A., LEHTONEN, K. K. & LINDSTROM, K. 2010a. An endocrine disrupting chemical changes courtship and parental care in the sand goby. *Aquatic Toxicology,* 97**,** 285–292.

SAARISTO, M., CRAFT, J. A., LEHTONEN, K. K. & LINDSTRÖM, K. 2010b. Exposure to 17α-ethinyl estradiol impairs courtship and aggressive behaviour of male sand gobies (*Pomatoschistus minutus*). *Chemosphere,* 79**,** 541–546.

SAILI, K. S., CORVI, M. M., WEBER, D. N., PATEL, A. U., DAS, S. R., PRZYBYLA, J., ANDERSON, K. A. & TANGUAY, R. L. 2012. Neurodevelopmental low-dose bisphenol A exposure leads to early life-stage hyperactivity and learning deficits in adult zebrafish. *Toxicology,* 291**,** 83–92.

SALIAN, S., DOSHI, T. & VANAGE, G. 2009. Perinatal exposure of rats to Bisphenol A affects the fertility of male offspring. *Life Sciences,* 85**,** 742–752.

SALIERNO, J. D. & KANE, A. S. 2009. 17α-Ethinylestradiol alters reproductive behaviors, circulating hormones, and sexual morphology in male fathead minnows (*Pimephales promelas*). *Environmental Toxicology and Chemistry,* 28**,** 953–961.

SANTOS, D., LUZIO, A. & COIMBRA, A. M. 2017. Zebrafish sex differentiation and gonad development: A review on the impact of environmental factors. *Aquatic Toxicology,* 191**,** 141–163.

SCHÄFERS, C., TEIGELER, M., WENZEL, A., MAACK, G., FENSKE, M. & SEGNER, H. 2007. Concentration- and Time-dependent Effects of the Synthetic Estrogen, 17α-ethinylestradiol, on Reproductive Capabilities of the Zebrafish, Danio rerio. *Journal of Toxicology and Environmental Health, Part A,* 70**,** 768–779.

SEBIRE, M., KATSIADAKI, I., TAYLOR, N. G. H., MAACK, G. & TYLER, C. R. 2011. Short-term exposure to a treated sewage effluent alters reproductive behaviour in the three-spined stickleback (Gasterosteus aculeatus). *Aquatic Toxicology,* 105**,** 78–88.

SEGNER, H., CAROLL, K., FENSKE, M., JANSSEN, C. R., MAACK, G., PASCOE, D., SCHAFERS, C., VANDENBERGH, G. F., WATTS, M. & WENZEL, A. 2003. Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. *Ecotoxicology and Environmental Safety,* 54**,** 302–314.

SHARPE, R. M. 1998. The Roles of Oestrogen in the Male. *Trends in Endocrinology & Metabolism,* 9**,** 371–377.

SHORE, L. S., GUREVITZ, M. & SHEMESH, M. 1993. ESTROGEN AS AN ENVIRONMENTAL-POLLUTANT. *Bulletin of Environmental Contamination and Toxicology,* 51**,** 361–366.

SIMMONS, D. B. D., MCCALLUM, E. S., BALSHINE, S., CHANDRAMOULI, B., COSGROVE, J. & SHERRY, J. P. 2017. Reduced anxiety is associated with the accumulation of six serotonin reuptake inhibitors in wastewater treatment effluent exposed goldfish Carassius auratus. *Scientific Reports,* 7**,** 17001.

SOFFKER, M. & TYLER, C. R. 2012. Endocrine disrupting chemicals and sexual behaviors in fish – a critical review on effects and possible consequences. *Critical Reviews in Toxicology,* 42**,** 653–668.

STEWART, A., GAIKWAD, S., KYZAR, E., GREEN, J., ROTH, A. & KALUEFF, A. V. 2012. Modeling anxiety using adult zebrafish: A conceptual review. *Neuropharmacology,* 62**,** 135–143.

SUMPTER, J. P. 1998. Xenoendocrine disrupters - environmental impacts. *Toxicology Letters,* 103**,** 337–342.

SUN, S., YANG, S., MAO, Y., JIA, X. & ZHANG, Z. 2015. Reduced cholesterol is associated with the depressive-like behavior in rats through modulation of the brain 5-HT1A receptor. *Lipids in Health and Disease* 14**,** 22–33.

TANG, R., DODD, A., LAI, D., MCNABB, W. C. & LOVE, D. R. 2007. Validation of Zebrafish (Danio rerio) Reference Genes for Quantitative Real-time QPCR Normalization. *Acta Biochim Biophys Sin,* 39.

TERNES, T. A., KRECKEL, P. & MUELLER, J. 1999. Behaviour and occurrence of estrogens in municipal sewage treatment plants – II. Aerobic batch experiments with activated sludge (vol 225, pg 91, 1999). *Science of the Total Environment,* 228**,** 89–89.

THOMPSON, R. R. J., PAUL, E. S., RADFORD, A. N., PURSER, J. & MENDL, M. 2016. Routine handling methods affect behaviour of three-spined sticklebacks in a novel test of anxiety. *Behavioural Brain Research,* 306**,** 26–35.

THORPE, K. L., CUMMINGS, R. I., HUTCHINSON, T. H., SCHOLZE, M., BRIGHTY, G., SUMPTER, J. P. & TYLER, C. R. 2003. Relative Potencies and Combination Effects of Steroidal Estrogens in Fish. *Environmental Science & Technology,* 37**,** 1142–1149.

TIEDEKEN, E. J., TAHAR, A., MCHUGH, B. & ROWAN, N. J. 2017. Monitoring, sources, receptors, and control measures for three European Union watch list substances of emerging concern in receiving waters – A 20year systematic review. *Science of The Total Environment,* 574**,** 1140–1163.

TOLI, E. A., CALBOLI, F. C. F., SHIKANO, T. & MERILÄ, J. 2016. A universal and reliable assay for molecular sex identification of three-spined sticklebacks (Gasterosteus aculeatus). *Molecular Ecology Resources,* 16**,** 1389–1400.

TYLER, C. R., JOBLING, S. & SUMPTER, J. P. 1998. Endocrine disruption in wildlife: A critical review of the evidence. *Critical Reviews in Toxicology,* 28**,** 319–361.

URBATZKA, R., ROCHA, E., REIS, B., CRUZEIRO, C., MONTEIRO, R. A. & ROCHA, M. J. 2012. Effects of ethinylestradiol and of an environmentally relevant mixture of xenoestrogens on steroidogenic gene expression and specific transcription factors in zebrafish. *Environ Pollut,* 164**,** 28–35.

VALENTI, T. W., JR., PEREZ-HURTADO, P., CHAMBLISS, C. K. & BROOKS, B. W. 2009. Aquatic toxicity of sertraline to Pimephales promelas at environmentally relevant surface water pH. *Environ Toxicol Chem,* 28**,** 2685–94.

VAN DEN BELT, K., WESTER, P. W., VAN DER VEN, L. T., VERHEYEN, R. & WITTERS, H. 2002. Effects of ethynylestradiol on the reproductive physiology in zebrafish (Danio rerio): time dependency and reversibility. *Environ Toxicol Chem,* 21**,** 767–75.

VANDENBERG, L. N., COLBORN, T., HAYES, T. B., HEINDEL, J. J., JACOBS, D. R., LEE, D.-H., SHIODA, T., SOTO, A. M., VOM SAAL, F. S., WELSHONS, W. V., ZOELLER, R. T. & MYERS, J. P. 2012. Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses. *Endocrine Reviews,* 33**,** 378–455.

WANG, Z., GERSTEIN, M. & SNYDER, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews. Genetics,* 10**,** 57–63.

WARING, R. H. & HARRIS, R. M. 2005. Endocrine disrupters: A human risk? *Molecular and Cellular Endocrinology,* 244**,** 2–9.

VASSKOG, T., BERGER, U., SAMUELSEN, P.-J., KALLENBORN, R. & JENSEN, E. 2006. Selective serotonin reuptake inhibitors in sewage influents and effluents from Tromsø, Norway. *Journal of Chromatography A,* 1115**,** 187–195.

VERLICCHI, P., AL AUKIDY, M. & ZAMBELLO, E. 2012. Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment—A review. *Science of The Total Environment,* 429**,** 123–155.

VERSONNEN, B. J. & JANSSEN, C. R. 2004. Xenoestrogenic effects of ethinylestradiol in zebrafish (Danio rerio). *Environmental Toxicology,* 19**,** 198–206.

WESTERFIELD, M. 2007. *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio Rerio),* Eugene, University of Oregon Press.

WIBE, A. E., BILLING, A., ROSENQVIST, G. & JENSSEN, B. M. 2002. Butyl benzyl phthalate affects shoaling behavior and bottom-dwelling behavior in threespine stickleback. *Environmental Research,* 89**,** 180–187.

WILLIAMS, R. J., JOHNSON, A. C., SMITH, J. J. L. & KANDA, R. 2003. Steroid estrogens profiles along river stretches arising from sewage treatment works discharges. *Environmental Science & Technology,* 37**,** 1744–1750.

VOLKOVA, K., REYHANIAN CASPILLO, N., PORSERYD, T., HALLGREN, S., DINNETZ, P., OLSÉN, H. & PORSCH HÄLLSTRÖM, I. 2015. Transgenerational effects of 17α-ethinyl estradiol on anxiety behavior in the guppy, Poecilia reticulata. *General and Comparative Endocrinology,* 223**,** 66–72.

VOLKOVA, K., REYHANIAN, N., KOT-WASIK, A., OLSEN, H., PORSCH-HALLSTROM, I. & HALLGREN, S. 2012a. Brain circuit imprints of developmental 17 alpha-Ethinylestradiol exposure in guppies (Poecilia reticulata): Persistent effects on anxiety but not on reproductive behaviour. *General and Comparative Endocrinology,* 178**,** 282–290.

VOLKOVA, K., REYHANIAN, N., KOT-WASIK, A., OLSÉN, H., PORSCH-HÄLLSTRÖM, I. & HALLGREN, S. 2012b. Brain circuit imprints of developmental 17α-Ethinylestradiol exposure in guppies (Poecilia reticulata): Persistent effects on anxiety but not on reproductive behaviour. *General and Comparative Endocrinology,* 178**,** 282–290.

WOLSTENHOLME, J. T., RISSMAN, E. F. & CONNELLY, J. J. 2011a. The role of Bisphenol A in shaping the brain, epigenome and behavior. *Hormones and Behavior,* 59**,** 296–305.

WOLSTENHOLME, J. T., TAYLOR, J. A., SHETTY, S. R. J., EDWARDS, M., CONNELLY, J. J. & RISSMAN, E. F. 2011b. Gestational Exposure to Low Dose Bisphenol A Alters Social Behavior in Juvenile Mice. *PLOS ONE,* 6**,** e25448.

WONG, R. Y., LAMM, M. S. & GODWIN, J. 2015. Characterizing the neurotranscriptomic states in alternative stress coping styles. *BMC Genomics,* 16**,** 425.

WONG, R. Y., OXENDINE, S. E. & GODWIN, J. 2013. Behavioral and neurogenomic transcriptome changes in wild-derived zebrafish with fluoxetine treatment. *BMC Genomics,* 14**,** 348.

VOS, J. G., DYBING, E., GREIM, H. A., LADEFOGED, O., LAMBRE, C., TARAZONA, J. V., BRANDT, I. & VETHAAK, A. D. 2000. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. *Critical Reviews in Toxicology,* 30**,** 71–133.

WOTTON, R. J. 1984. *A functional Biology of Sticklebacks*, University of California Press.

WRIGHT, D., WARD, A. J. W., CROFT, D. P. & KRAUSE, J. 2006. Social Organization, Grouping, and Domestication in Fish. *Zebrafish,* 3**,** 141–155.

XU, H., YANG, J., WANG, Y., JIANG, Q., CHEN, H. & SONG, H. 2008. Exposure to 17α-ethynylestradiol impairs reproductive functions of both male and female zebrafish (Danio rerio). *Aquatic Toxicology,* 88**,** 1–8.

YANG, Y., OK, Y. S., KIM, K.-H., KWON, E. E. & TSANG, Y. F. 2017. Occurrences and removal of pharmaceuticals and personal care products (PPCPs) in drinking water and water/sewage treatment plants: A review. *Science of The Total Environment,* 596–597**,** 303–320.

YIN, P., LI, Y. W., CHEN, Q. L. & LIU, Z. H. 2017. Diethylstilbestrol, flutamide and their combination impaired the spermatogenesis of male adult zebrafish through disrupting HPG axis, meiosis and apoptosis. *Aquat Toxicol,* 185**,** 129–137.

YING, G. G., KOOKANA, R. S. & RU, Y. J. 2002. Occurrence and fate of hormone steroids in the environment. *Environment International,* 28**,** 545–551.

ZALA, S. M. & PENN, D. J. 2004. Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. *Animal Behaviour,* 68**,** 649–664.

ZHANG, J. L., LIU, M., ZHANG, C. N., LI, E. C., FAN, M. Z. & HUANG, M. X. 2018. Transcriptomic analyses of tributyltin-induced sexual dimorphisms in rare minnow (Gobiocypris rarus) brains. *Ecotoxicol Environ Saf,* 156**,** 18–24.

Tack

Under dessa roliga, lärorika men även slitiga år så har jag haft turen att träffa, hänga med, jobba med och få stöd av så många fantastiska, smarta och bra personer som jag skulle vilja tacka.

Först och främst skulle jag vilja tacka **Inger** för att du alltid har tagit dig tid för att hjälpa till, läsa texter och bolla idéer. Du har låtit mig göra min grej men alltid haft en plan för allt. Din hängivenhet och kunskap är fantastisk och jag är oändligt tacksam att jag fick dig som handledare. **Josefine Dr Josè Larsson**. Du har stöttat, inte bara genom att vara den fantastiskt fina vän du är utan också hjälpt och handlett mig med allt från att extrahera RNA och DNA, PCR -felsökning, beställa primers, odla artemia och mata fiskar, R-script, köra geler, räkna på spädningserier, bioinformatik, gulla med fiskbebisar och så mycket mera. Alltså tack! **Kristina** och **Nasim**, tack för att ni lärde mig allt om zebrafisk (och mycket annat)! Det var ett sant nöje att få komma in i ert projekt som lite lagom förvirrad grön biolog. **Andrea**, våra kaffestunder och pratstunder får alltid allt att kännas mycket bättre och det är fint att ha dig som hejaklack, varm famn och bollplank! **Linn**, tack för fina pratstunder nere i källaren, det bästa med att hänga med fiskarna var att det slutade i häng med dig. På något magiskt sätt har jag kunnat hitta rätt blanketter utan att ringa dig men det har inte varit lätt ;). **Patrik**, tack för all hjälp med R och allmänna statistikproblem och för ditt tålamod när jag kommer med samma fråga för sjätte (eller tionde) gången. Får man verkligen köra ett post-hoc test när modellen inte är signifikant? Och hur många familjer behövs det egentligen om man ska ha som random factor i en mixad modell? **Anders**, aka Mr Gadget, tack för alla testakvarier, apparatur, slangar och kemikalier du lyckats trolla fram när jag behövt det. **Martin**, tack för spiggsamarbetet och intressanta diskussioner bland akvarier och mikroskop. **Stefan** och **Håkan** mina två biträdande handledare, tack! **Lubna**, tack för all ovärderlig hjälp med fiskarna. **Tomas B**, tack för hjälp med histologi samt läsning inför halvtid och slutseminarium. **Mathilde,** för hjälp med texter, engagemang och pepp. All the other clever PhD students at Södertörn I´ve had the pleasure to work with/around: **Christian**, **Elise**, **Emma L**, **Erika**, **Falkje**, **Igne**, **John**, **Juliana**, **Kajsa-Stina**, **Lena**, **Natalya**, **Natasja**, **Naveed**, **Oksana**, **Olena**, **Oskar, Pernilla, Petter**, **Pernilla**, **Ralph**, **Sara**, **Sophie**, **Tiina**. Bästa! Alla andra på Södertörn, kollegor, kompisar, administrativa, seniora, studierektorer, osv ni är också alla så bra och hjälpsamma. Tack! SU-gänget, gamla, mellan och nya, för inspiration och skoj; **Sissel**, **Tove (T2)**, **A-K**, **Maria**, **Stina** och **Lina**. Biologer ftw! **Emil Sellström**, tack för det fina omslaget! **Bertil Borg**, tack för allt du lärt mig om

spigg, för spiggar och spiggägg samt för trevligt sällskap i fält. **Emma J**, även om du inte varit på plats i person så har du ju varit med på chatten varje dag och förgyllt dagarna. **Helena**, det var i Honduras för 16 år sen det började. Tack för att du lockade ner mig under ytan! **Fina familj och vänner, tack alla! Malin**, **Carro**, **Bea, Joanna**, **Henke**, **Markus**, **Linn** och **Asim**, **Josè** och **Stefan**, **Emma** och **Fredrik**, **Jos** och **Nebeyu**, **Lina** och **Dulla,** och alla finaste bästa kidsen som redan fanns, som kom under tiden och som snart kommer, för alla roliga stunder som fått mig att glömma bort att jag egentligen har hundra saker som jag inte gjort som skulle ha varit gjorde igår. **Pelle P**, du vet att du alltid har varit en förebild och en trygghet, ovärderligt i tider som dessa. Och för hjälp med förskole-hämtningar. TACK. Och **Simone**, det är fint att du och din härliga familj kom in i mitt liv, med värme och stöd! **Satchi Blåbär Porseryd** också såklart <3. **Kristina, Martin, Naima** och **Pelle**, våra mysiga fredagsmiddagar/söndagshäng har varit bästa moroten för arbetsveckan! **Kerstin** och **Bertil**, tack för att ni är så fina farföräldrar till Alfred och svärföräldrar till mig, för all hjälp med barnvaktande och logistik som har underlättat så oändligt mycket för att få denna avhandling klar med hälsan i behåll. Tack **Peder** och **Alfred**, mina bästa här i livet. Peder, för att du är det bästa stöd man kan önska. Alfred, för att du är det finaste jag vet och min drivkraft för att göra det bästa jag kan. Att jag har er gör att allt annat kan misslyckas men att jag ändå har lyckats.

Till de som jag glömt: förlåt och tack såklart!

Funding

I would like to acknowledge The Foundation for Baltic and East European Studies and the Stockholm County Council for funding and BILS (Bioinformatics Infrastructure for Life Sciences, Uppsala, Sweden) for bioinformatics support.

Södertörn Doctoral Dissertations

1. Jolanta Aidukaite, *The Emergence of the Post-Socialist Welfare State: The case of the Baltic States: Estonia, Latvia and Lithuania*, 2004
2. Xavier Fraudet, *Politique étrangère française en mer Baltique (1871–1914): de l'exclusion à l'affirmation*, 2005
3. Piotr Wawrzeniuk, *Confessional Civilising in Ukraine: The Bishop Iosyf Shumliansky and the Introduction of Reforms in the Diocese of Lviv 1668–1708*, 2005
4. Andrej Kotljarchuk, *In the Shadows of Poland and Russia: The Grand Duchy of Lithuania and Sweden in the European Crisis of the mid-17th Century*, 2006
5. Håkan Blomqvist, *Nation, ras och civilisation i svensk arbetarrörelse före nazismen*, 2006
6. Karin S Lindelöf, *Om vi nu ska bli som Europa: Könsskapande och normalitet bland unga kvinnor i transitionens Polen*, 2006
7. Andrew Stickley. *On Interpersonal Violence in Russia in the Present and the Past: A Sociological Study*, 2006
8. Arne Ek, *Att konstruera en uppslutning kring den enda vägen: Om folkrörelsers modernisering i skuggan av det Österuropeiska systemskiftet*, 2006
9. Agnes Ers, *I mänsklighetens namn: En etnologisk studie av ett svenskt biståndsprojekt i Rumänien*, 2006
10. Johnny Rodin, *Rethinking Russian Federalism: The Politics of Intergovernmental Relations and Federal Reforms at the Turn of the Millennium*, 2006
11. Kristian Petrov, *Tillbaka till framtiden: Modernitet, postmodernitet och generationsidentitet i Gorbačevs glasnost' och perestrojka*, 2006
12. Sophie Söderholm Werkö, *Patient patients?: Achieving Patient Empowerment through Active Participation, Increased Knowledge and Organisation*, 2008
13. Peter Bötker, *Leviatan i arkipelagen: Staten, förvaltningen och samhället. Fallet Estland*, 2007
14. Matilda Dahl, *States under scrutiny: International organizations, transformation and the construction of progress*, 2007
15. Margrethe B. Søvik, *Support, resistance and pragmatism: An examination of motivation in language policy in Kharkiv*, Ukraine, 2007
16. Yulia Gradskova, *Soviet People with female Bodies: Performing beauty and maternity in Soviet Russia in the mid 1930–1960s*, 2007
17. Renata Ingbrant, *From Her Point of View: Woman's Anti-World in the Poetry of Anna Świrszczyńska*, 2007
18. Johan Eellend, *Cultivating the Rural Citizen: Modernity, Agrarianism and Citizenship in Late Tsarist Estonia*, 2007
19. Petra Garberding, *Musik och politik i skuggan av nazismen: Kurt Atterberg och de svensk-tyska musikrelationerna*, 2007

20. Aleksei Semenenko, *Hamlet the Sign: Russian Translations of Hamlet and Literary Canon Formation*, 2007
21. Vytautas Petronis, *Constructing Lithuania: Ethnic Mapping in the Tsarist Russia, ca. 1800–1914*, 2007
22. Akvile Motiejunaite, *Female employment, gender roles, and attitudes: the Baltic countries in a broader context*, 2008
23. Tove Lindén, *Explaining Civil Society Core Activism in Post-Soviet Latvia*, 2008
24. Pelle Åberg, *Translating Popular Education: Civil Society Cooperation between Sweden and Estonia*, 2008
25. Anders Nordström, *The Interactive Dynamics of Regulation: Exploring the Council of Europe's monitoring of Ukraine*, 2008
26. Fredrik Doeser, *In Search of Security After the Collapse of the Soviet Union: Foreign Policy Change in Denmark, Finland and Sweden, 1988–1993*, 2008
27. Zhanna Kravchenko. *Family (versus) Policy: Combining Work and Care in Russia and Sweden*, 2008
28. Rein Jüriado, *Learning within and between public-private partnerships*, 2008
29. Elin Boalt, *Ecology and evolution of tolerance in two cruciferous species*, 2008
30. Lars Forsberg, *Genetic Aspects of Sexual Selection and Mate Choice in Salmonids*, 2008
31. Eglė Rindzevičiūtė, *Constructing Soviet Cultural Policy: Cybernetics and Governance in Lithuania after World War II*, 2008
32. Joakim Philipson, *The Purpose of Evolution: 'struggle for existence' in the Russian-Jewish press 1860–1900*, 2008
33. Sofie Bedford, *Islamic activism in Azerbaijan: Repression and mobilization in a post-Soviet context*, 2009
34. Tommy Larsson Segerlind, *Team Entrepreneurship: A process analysis of the venture team and the venture team roles in relation to the innovation process*, 2009
35. Jenny Svensson, *The Regulation of Rule-Following: Imitation and Soft Regulation in the European Union*, 2009
36. Stefan Hallgren, *Brain Aromatase in the guppy, Poecilia reticulate: Distribution, control and role in behavior*, 2009
37. Karin Ellencrona, *Functional characterization of interactions between the flavivirus NS5 protein and PDZ proteins of the mammalian host,* 2009
38. Makiko Kanematsu, *Saga och verklighet: Barnboksproduktion i det postsovjetiska Lettland,* 2009
39. Daniel Lindvall, *The Limits of the European Vision in Bosnia and Herzegovina: An Analysis of the Police Reform Negotiations,* 2009
40. Charlotta Hillerdal, *People in Between – Ethnicity and Material Identity: A New Approach to Deconstructed Concepts*, 2009
41. Jonna Bornemark, *Kunskapens gräns – gränsens vetande,* 2009
42. Adolphine G. Kateka, *Co-Management Challenges in the Lake Victoria Fisheries: A Context Approach*, 2010

43. René León Rosales, *Vid framtidens hitersta gräns: Om pojkar och elevpositioner i en multietnisk skola*, 2010
44. Simon Larsson, *Intelligensaristokrater och arkivmartyrer: Normerna för vetenskaplig skicklighet i svensk historieforskning 1900–1945*, 2010
45. Håkan Lättman, *Studies on spatial and temporal distributions of epiphytic lichens*, 2010
46. Alia Jaensson, *Pheromonal mediated behaviour and endocrine response in salmonids: The impact of cypermethrin, copper, and glyphosate*, 2010
47. Michael Wigerius, *Roles of mammalian Scribble in polarity signaling, virus offense and cell-fate determination,* 2010
48. Anna Hedtjärn Wester, *Män i kostym: Prinsar, konstnärer och tegelbärare vid sekelskiftet 1900*, 2010
49. Magnus Linnarsson, *Postgång på växlande villkor: Det svenska postväsendets organisation under stormaktstiden*, 2010
50. Barbara Kunz, *Kind words, cruise missiles and everything in between: A neoclassical realist study of the use of power resources in U.S. policies towards Poland, Ukraine and Belarus 1989–2008*, 2010
51. Anders Bartonek, *Philosophie im Konjunktiv: Nichtidentität als Ort der Möglichkeit des Utopischen in der negativen Dialektik Theodor W. Adornos,* 2010
52. Carl Cederberg, *Resaying the Human: Levinas Beyond Humanism and Antihumanism*, 2010
53. Johanna Ringarp, *Professionens problematik: Lärarkårens kommunalisering och välfärdsstatens förvandling*, 2011
54. Sofi Gerber, *Öst är Väst men Väst är bäst: Östtysk identitetsformering i det förenade Tyskland*, 2011
55. Susanna Sjödin Lindenskoug, *Manlighetens bortre gräns: Tidelagsrättegångar i Livland åren 1685–1709*, 2011
56. Dominika Polanska, *The emergence of enclaves of wealth and poverty: A sociological study of residential differentiation in post-communist Poland*, 2011
57. Christina Douglas, *Kärlek per korrespondens: Två förlovade par under andra hälften av 1800-talet*, 2011
58. Fred Saunders, *The Politics of People – Not just Mangroves and Monkeys: A study of the theory and practice of community-based management of natural resources in Zanzibar*, 2011
59. Anna Rosengren, *Åldrandet och språket: En språkhistorisk analys av hög ålder och åldrande i Sverige cirka 1875–1975*, 2011
60. Emelie Lilliefeldt, *European Party Politics and Gender: Configuring Gender-Balanced Parliamentary Presence*, 2011
61. Ola Svenonius, *Sensitising Urban Transport Security: Surveillance and Policing in Berlin, Stockholm, and Warsaw*, 2011
62. Andreas Johansson, *Dissenting Democrats: Nation and Democracy in the Republic of Moldova*, 2011
63. Wessam Melik, *Molecular characterization of the Tick-borne encephalitis virus: Environments and replication*, 2012
64. Steffen Werther, *SS-Vision und Grenzland-Realität: Vom Umgang dänischer und „volksdeutscher" Nationalsozialisten in Sønderjylland mit der „großgermanischen" Ideologie der SS*, 2012
65. Peter Jakobsson, *Öppenhetsindustrin*, 2012

66. Kristin Ilves, *Seaward Landward: Investigations on the archaeological source value of the landing site category in the Baltic Sea region*, 2012
67. Anne Kaun, *Civic Experiences and Public Connection: Media and Young People in Estonia*, 2012
68. Anna Tessmann, *On the Good Faith: A Fourfold Discursive Construction of Zoroastripanism in Contemporary Russia*, 2012
69. Jonas Lindström, *Drömmen om den nya staden: stadsförnyelse i det postsovjetisk Riga,* 2012
70. Maria Wolrath Söderberg, *Topos som meningsskapare: retorikens topiska perspektiv på tänkande och lärande genom argumentation*, 2012
71. Linus Andersson, *Alternativ television: former av kritik i konstnärlig TV-produktion,* 2012
72. Håkan Lättman, *Studies on spatial and temporal distributions of epiphytic lichens*, 2012
73. Fredrik Stiernstedt, Mediearbete i mediehuset: produktion i förändring på MTG-radio, 2013
74. Jessica Moberg, *Piety, Intimacy and Mobility: A Case Study of Charismatic Christianity in Present-day Stockholm*, 2013
75. Elisabeth Hemby, *Historiemåleri och bilder av vardag: Tatjana Nazarenkos konstnärskap i 1970-talets Sovjet*, 2013
76. Tanya Jukkala, *Suicide in Russia: A macro-sociological study*, 2013
77. Maria Nyman, *Resandets gränser: svenska resenärers skildringar av Ryssland under 1700-talet*, 2013
78. Beate Feldmann Eellend, *Visionära planer och vardagliga praktiker: postmilitära landskap i Östersjöområdet,* 2013
79. Emma Lind, *Genetic response to pollution in sticklebacks: natural selection in the wild*, 2013
80. Anne Ross Solberg, *The Mahdi wears Armani: An analysis of the Harun Yahya enterprise,* 2013
81. Nikolay Zakharov, *Attaining Whiteness: A Sociological Study of Race and Racialization in Russia,* 2013
82. Anna Kharkina, *From Kinship to Global Brand: the Discourse on Culture in Nordic Cooperation after World War II*, 2013
83. Florence Fröhlig, *A painful legacy of World War II: Nazi forced enlistment: Alsatian/Mosellan Prisoners of war and the Soviet Prison Camp of Tambov*, 2013
84. Oskar Henriksson, *Genetic connectivity of fish in the Western Indian Ocean*, 2013
85. Hans Geir Aasmundsen, *Pentecostalism, Globalisation and Society in Contemporary Argentina*, 2013
86. Anna McWilliams, *An Archaeology of the Iron Curtain: Material and Metaphor*, 2013
87. Anna Danielsson, *On the power of informal economies and the informal economies of power: rethinking informality, resilience and violence in Kosovo*, 2014
88. Carina Guyard, *Kommunikationsarbete på distans*, 2014
89. Sofia Norling, *Mot "väst": om vetenskap, politik och transformation i Polen 1989–2011*, 2014
90. Markus Huss, *Motståndets akustik: språk och (o)ljud hos Peter Weiss 1946–1960,* 2014
91. Ann-Christin Randahl, *Strategiska skribenter: skrivprocesser i fysik och svenska*, 2014
92. Péter Balogh, *Perpetual borders: German-Polish cross-border contacts in the Szczecin area*, 2014
93. Erika Lundell, *Förkroppsligad fiktion och fiktionaliserade kroppar: levande rollspel i Östersjö-regionen*, 2014

94. Henriette Cederlöf, *Alien Places in Late Soviet Science Fiction: The "Unexpected Encounters" of Arkady and Boris Strugatsky as Novels and Films*, 2014
95. Niklas Eriksson, *Urbanism Under Sail: An archaeology of fluit ships in early modern everyday life*, 2014
96. Signe Opermann, *Generational Use of News Media in Estonia: Media Access, Spatial Orientations and Discursive Characteristics of the News Media*, 2014
97. Liudmila Voronova, *Gendering in political journalism: A comparative study of Russia and Sweden,* 2014
98. Ekaterina Kalinina, *Mediated Post-Soviet Nostalgia*, 2014
99. Anders E. B. Blomqvist, *Economic Natonalizing in the Ethnic Borderlands of Hungary and Romania: Inclusion, Exclusion and Annihilation in Szatmár/Satu-Mare, 1867–1944,* 2014
100. Ann-Judith Rabenschlag, *Völkerfreundschaft nach Bedarf: Ausländische Arbeitskräfte in der Wahrnehmung von Staat und Bevölkerung der DDR*, 2014
101. Yuliya Yurchuck, *Ukrainian Nationalists and the Ukrainian Insurgent Army in Post-Soviet Ukraine*, 2014
102. Hanna Sofia Rehnberg, *Organisationer berättar: narrativitet som resurs i strategisk kommunikation*, 2014
103. Jaakko Turunen, *Semiotics of Politics: Dialogicality of Parliamentary Talk*, 2015
104. Iveta Jurkane-Hobein, *I Imagine You Here Now: Relationship Maintenance Strategies in Long-Distance Intimate Relationships*, 2015
105. Katharina Wesolowski*, Maybe baby? Reproductive behaviour, fertility intentions, and family policies in post-communist countries, with a special focus on Ukraine*, 2015
106. Ann af Burén, *Living Simultaneity: On religion among semi-secular Swedes*, 2015
107. Larissa Mickwitz, *En reformerad lärare: konstruktionen av en professionell och betygssättande lärare i skolpolitik och skolpraktik,* 2015
108. Daniel Wojahn, *Språkaktivism: diskussioner om feministiska språkförändringar i Sverige från 1960-talet till 2015*, 2015
109. Hélène Edberg, *Kreativt skrivande för kritiskt tänkande: en fallstudie av studenters arbete med kritisk metareflektion*, 2015
110. Kristina Volkova, *Fishy Behavior: Persistent effects of early-life exposure to 17α-ethinylestradiol*, 2015
111. Björn Sjöstrand, *Att tänka det tekniska: en studie i Derridas teknikfilosofi,* 2015
112. Håkan Forsberg, *Kampen om eleverna: gymnasiefältet och skolmarknadens framväxt i Stockholm, 1987–2011*, 2015
113. Johan Stake, *Essays on quality evaluation and bidding behavior in public procurement auctions*, 2015
114. Martin Gunnarson, *Please Be Patient: A Cultural Phenomenological Study of Haemodialysis and Kidney Transplantation Care*, 2016
115. Nasim Reyhanian Caspillo, *Studies of alterations in behavior and fertility in ethinyl estradiol-exposed zebrafish and search for related biomarkers,* 2016
116. Pernilla Andersson, *The Responsible Business Person: Studies of Business Education for Sustainability,* 2016

117. Kim Silow Kallenberg, *Gränsland: svensk ungdomsvård mellan vård och straff,* 2016
118. Sari Vuorenpää, *Literacitet genom interaction*, 2016
119. Francesco Zavatti, *Writing History in a Propaganda Institute: Political Power and Network Dynamics in Communist Romania*, 2016
120. Cecilia Annell, *Begärets politiska potential: Feministiska motståndsstrategier i Elin Wägners 'Pennskaftet', Gabriele Reuters 'Aus guter Familie', Hilma Angered-Strandbergs 'Lydia Vik' och Grete Meisel-Hess 'Die Intellektuellen',* 2016
121. Marco Nase, *Academics and Politics: Northern European Area Studies at Greifswald University, 1917–1992,* 2016
122. Jenni Rinne, *Searching for Authentic Living Through Native Faith – The Maausk movement in Estonia*, 2016
123. Petra Werner, *Ett medialt museum: lärandets estetik i svensk television 1956–1969*, 2016
124. Ramona Rat, *Un-common Sociality: Thinking sociality with Levinas*, 2016
125. Petter Thureborn, *Microbial ecosystem functions along the steep oxygen gradient of the Landsort Deep, Baltic Sea*, 2016
126. Kajsa-Stina Benulic, *A Beef with Meat Media and audience framings of environmentally unsustainable production and consumption*, 2016
127. Naveed Asghar, *Ticks and Tick-borne Encephalitis Virus – From nature to infection*, 2016
128. Linn Rabe, *Participation and legitimacy: Actor involvement for nature conservation*, 2017
129. Maryam Adjam, *Minnesspår: hågkomstens rum och rörelse i skuggan av en flykt*, 2017
130. Kim West, *The Exhibitionary Complex: Exhibition, Apparatus and Media from Kulturhuset to the Centre Pompidou, 1963–1977*, 2017
131. Ekaterina Tarasova*, Anti-nuclear Movements in Discursive and Political Contexts: Between expert voices and local protests, 2017*
132. Sanja Obrenović Johansson*, Från kombifeminism till rörelse: Kvinnlig serbisk organisering i förändring, 2017*
133. Michał Salamonik, *In Their Majesties' Service: The Career of Francesco De Gratta (1613–1676) as a Royal Servant and Trader in Gdańsk,* 2017
134. Jenny Ingridsdotter, *The Promises of the Free World: Postsocialist Experience in Argentina and the Making of Migrants, Race, and Coloniality*, 2017
135. Julia Malitska, *Negotiating Imperial Rule: Colonists and Marriage in the Nineteenth century Black Sea Steppe,* 2017
136. Natalya Yakusheva, *Parks, Policies and People: Nature Conservation Governance in Post-Socialist EU Countries,* 2017
137. Martin Kellner, *Selective Serotonin Re-uptake Inhibitors in the Environment: Effects of Citalopram on Fish Behaviour,* 2017
138. Krystof Kasprzak, *Vara – Framträdande – Värld: Fenomenets negativitet hos Martin Heidegger, Jan Patočka och Eugen Fink*, 2017
139. Alberto Frigo, *Life-stowing from a Digital Media Perspective: Past, Present and Future*, 2017
140. Maarja Saar, *The Answers You Seek Will Never Be Found At Home: Reflexivity, biographical narratives and lifestyle migration among highly-skilled Estonians*, 2017

141. Anh Mai, *Organizing for Efficiency: Essay on merger policies, independence of authorities, and technology diffusion*, 2017
142. Gustav Strandberg, *Politikens omskakning: Negativitet, samexistens och frihet i Jan Patočkas tänkande*, 2017
143. Lovisa Andén, *Litteratur och erfarenhet i Merleau-Pontys läsning av Proust, Valéry och Stendhal*, 2017
144. Fredrik Bertilsson, *Frihetstida policyskapande: uppfostringskommissionen och de akademiska konstitutionerna 1738–1766*, 2017
145. Börjeson, Natasja, *Toxic Textiles – towards responsibility in complex supply chains*, 2017
146. Julia Velkova, *Media Technologies in the Making – User-Driven Software and Infrastructures for computer Graphics Production*, 2017
147. Karin Jonsson, *Fångna i begreppen? Revolution, tid och politik i svensk socialistisk press 1917–1924*, 2017
148. Josefine Larsson, *Genetic Aspects of Environmental Disturbances in Marine Ecosystems – Studies of the Blue Mussel in the Baltic Sea*, 2017
149. Roman Horbyk, *Mediated Europes – Discourse and Power in Ukraine, Russia and Poland during Euromaidan*, 2017
150. Nadezda Petrusenko, *Creating the Revolutionary Heroines: The Case of Female Terrorists of the PSR (Russia, Beginning of the 20th Century)*, 2017
151. Rahel Kuflu, *Bröder emellan: Identitetsformering i det koloniserade Eritrea*, 2018
152. Karin Edberg, *Energilandskap i förändring: Inramningar av kontroversiella lokaliseringar på norra Gotland*, 2018
153. Rebecka Thor, *Beyond the Witness: Holocaust Representation and the Testimony of Images - Three films by Yael Hersonski, Harun Farocki, and Eyal Sivan*, 2018
154. Maria Lönn, *Bruten vithet: Om den ryska femininitetens sinnliga och temporala villkor*, 2018
155. Tove Porseryd, *Endocrine Disruption in Fish: Effects of 17α-ethinylestradiol exposure on non-reproductive behavior, fertility and brain and testis transcriptome*, 2018

Aquatic environments can be described as the ultimate sink: they are the end recipient for most anthropogenic chemical pollutants. Contributors to anthropogenic pollutants in the environment are agriculture, industries and sewage treatment plants, and include a wide variety of chemicals assumed to disrupt the endocrine system.

Endocrine disrupting chemicals (EDCs) can be active at very low concentrations and are found in freshwater, estuarine and marine environments around the world, putting aquatic organisms at risk for exposure and negative effects. Fish are considered especially vulnerable as they have multiple uptake routes: through the gills, food and contact with water and sediment. One of the EDCs found frequently in sewage effluents is 17α-etinylestradiol (EE2), used as the main component in contraceptive pills. EE2 is a synthetic estrogen with a very high potency and considered one of the most concerning EDCs in the environment.

This thesis describes the effects of EE2 exposure on fish during development and adulthood, spanning from altered behavior and lowered fertility to persistent changes in the transcriptome. Alterations due to EE2 exposure may thus have negative effects on fish populations, making these aspects of high ecological relevance.

Tove Porseryd has a Master of Science in Biology from Stockholm University. She carries out research within the field of environmental science using a multidisciplinary approach. This is her doctoral thesis.

Environmental Science, School of Natural Sciences, Technology and Environmental Studies

ISBN 978-91-88663-43-6 (print) / 978-91-88663-44-3 (digital)
Södertörn University | Library, SE-141 89 Huddinge | publications@sh.se