Endocrine Disruption in Fish
Effects of 17\(\alpha\)-ethinylestradiol exposure on non-reproductive behavior, fertility and brain and testis transcriptome

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Abstract
Aquatic environments are the end recipient for most anthropogenic chemical pollutants. One common chemical pollutant found in the aquatic environment is 17α-ethinylestradiol (EE2), a synthetic estrogen used in contraceptive pills. EE2 is found in sewage treatment plant effluents and surface waters in concentrations from nondetectable up to 300 ng/L. EE2 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 EE2 exposure on non-reproductive behavior and fertility in fish. We found that zebra fish exposed to low concentrations of EE2 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 EE2 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 EE2 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, EE2 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 EE2 exposure and effects from developmental exposures seem to be persistent. Fertility and behavior are of high ecological significance for fish and alterations due to EE2 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 EE2.

Keywords: 17α-ethinylestradiol, endocrine disrupting chemical, fish, behavior, fertility, transcriptome, RNA sequencing.
Till mamma och mormor
Den akvatiska miljön är ofta särskilt utsatt för miljöförreningar då de flesta ämnen förr eller senare sprids dit genom bland annat markavrinning, nedfall från luften och/eller från vattenrenningsverk. Syntetisk östrogen som används i p-piller, 17α-ethylstenestradiol (EE2), sprider sig ofta till vattenmiljön från avloppsvatten, då det bara delvis renas bort i vattenrenningsprocessen. EE2 är ett hormonstörande ämne med förmågan att ansamlas i organismer och mer än tio gånger så verksamt i fisk som det naturliga hormonet estradiol (E2). Man har tidigare sett att exponering för EE2 även i de låga halter som påvisas vid vattenrenningsverk bland annat har lett till minskade produktion av ägg och spermier samt förändrade parningsbeteenden.

I denna avhandling undersöks effekterna av EE2 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 EE2 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 uttrycktes. I zebrafiskens testikelvävnad hittades bland annat en skillnad i uttryck av gener kopplade till könsdifferentiering och utveckling samt spermatogén, gener som kan ha betydelse för den nedsatta fertiliteten. I zebrafiskens hjärna hittades ingen skillnad i uttryck på gener direktt kopplade till stressaxeln men däremot på flera andra gener i nätverk som indirekt kan kopplas till det ängsliga beteendet som dygnrhytm och kolesterol syntes. I naturen exponeras organismer ofta för en blandning av föroreningar. Därför undersöktes också effekter av EE2 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 zebrafisken uppgav 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 vindfångad 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 EE2 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 EE₂ i låga halter under utveckling. Både icke-reproduktiva beteenden och fertilitet visade sig känsliga för EE₂ i såväl zebrafisk som storspigg och effekterna verkar vara permanenta. Sammanfattningsvis har EE₂ effekter på ekologiskt relevanta faktorer hos fisk i de halter som förekommer i vattenmiljön och hormonstörande föroreningar som EE₂ kan ge långvariga effekter som syns senare i livet även vid låga koncentrationer.
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This thesis is based on the following papers referred to in the text by the Roman numerals (I–V):


**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

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

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 (Salerno 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 neuro-endocrine system influencing both brain structure and behavior (Enmark and Gustafsson, 1999, Guillette and Gunderson, 2001). EE₂ 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 (Poecilia reticulata) exposed to EE₂ 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). Zebrafish 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
METHODS

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, EE2-d4 was used as an internal standard with a detection limit of 0.5 ng/L EE2, and for papers IV and V, EE2-d6 was used with a detection limit of 0.2 ng/L EE2. 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. EE2 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 EE2 (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
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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).

![Experimental setup of the novel tank test (NT test) seen from the side.](image)

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 Rosenberg, 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 Elflα (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 EE2 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 EE2 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, \textit{Idh}, \textit{Gasm6} and \textit{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 Djurförsökssetiska 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 EE₂ 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 EE₂ 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 EE₂ 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 EE₂ 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 EE₂ 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 EE₂, 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 EE2. 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 EE2. 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 ccl12a 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 EE2 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 EE2. 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 EE2 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 EE2 in both sexes, demonstrating marked differences in the differential expression between females and males. In males, functional analyses showed that EE2 had significant effects on pathways related to cholesterol biosynthesis, the immune response, synaptic proteins, DNA repair and heme biosynthesis/degradation. In the female brains, EE2 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 EE2 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 EE2 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 EE2 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 EE2 and citalopram, the effects of EE2 on anxiety-like behavior was abolished. Surprisingly, in the NT test, lower anxiety occurred after EE2 exposure as well as citalopram exposure than after the control and combination exposures. This study shows that EE2 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 EE2 from fertilization to 7 weeks post-hatch. After 8 months of remediation in tap water, three non-reproductive behaviors, not previously analyzed in developmentally EE2-exposed fish of this species or other wild caught fish, were evaluated. Chemical analysis surprisingly revealed that the concentrations of EE2 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, EE2 exposure did not affect any behavior parameters compared to the control group but exposure to 30 ng/L EE2 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 EE2 but not those exposed to 5 ng/L EE2. Induction of ovotestis as well as total feminization of genetic males were observed in the group exposed to 30 ng/L. In conclusion, EE2 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 EE2, but not 5 ng/L EE2. As 5 ng/L EE2 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 Rosenberg, 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 phthalate (BBP) as adults showed an increased shoal cohesion (Wibe et al., 2002). Stickleback females exposed to EE2 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 EE2 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 EE2 (paper III). The RNA-Seq of the zebrafish brain revealed differential gene expression between fish exposed to EE2 during development and control fish. Only one gene whose expression was affected by EE2 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 EE2 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 EE2 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 EE2 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 EE2 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 EE2 (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 EE2 of possible significance for the reduced fertility. Exposure to EE2 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 EE2 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-
involved 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 EE2, suggesting that the HPG axis could have been involved in the organizational effects of EE2 exposure during development in the testis, although it no longer affected the brain after termination of exposure. Exposure to EE2 during adulthood has led to alterations in gene expression in the HPG axis in both brain and testis of the pejerrey fish \textit{(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 \textit{(Peltobagrus 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 EE2-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 EE2 exposure during development in guppy were transmitted transgenerationally to two consecutive unexposed generations (Volkova et al., 2015). Transgenerational effects on fertility after EE2 exposure during development has also been shown in medaka \textit{(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, EE2 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 EE2 in the stickleback**

EE2 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 EE2 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 EE2 where fish exposed to 25 ng/L EE2 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 EE2 did not differ from that of the control group, but the fish exposed to 30 ng/L EE2 showed
significantly fewer transitions away from the shoal than the fish exposed to 5 ng/L EE2 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 EE2. In the zebrafish, clear anxiogenic effects was observed at approximately 1 ng/L EE2 while in the stickleback, 5 ng/L EE2 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, EE2 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 EE2 on non-reproductive behavior in wild fish the results are still new and important.

The unexpected detection of measurable levels of EE2 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 EE2 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 EE2 in control water. However, paper V performed in fall 2015 showed a surprisingly high level of 5 ng/L EE2 in control water. After finding the high level of EE2 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 EE2 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 EE₂ in the tap water underlines the importance of the efficient removal of EDCs in STPs as drinking water EE₂ 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 EE₂ in STPs is approximately 50-70 %, it varies greatly within and between STPs, (Comber et al., 2018) and is not sufficient in removing EE₂ 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 EE₂ and the SSRI citalopram.

We found the expected anxiogenic response to exposure to EE₂ 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. EE₂ 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 EE₂ exposed fish. Although citalopram caused few effects on its own it counteracted the anxiogenic effects of EE₂ 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 straightforward, 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 EE2 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 EE2. 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.


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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-ethinylestradiol (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.