The impact of 17α-ethinylestradiol on roach (*Rutilus rutilus*) growth

Sara Sol Windahl

Uppsala BioCenter

Department of Microbiology
Faculty of Natural Resources
& Agriculture Sciences
Swedish University of
Agricultural Sciences

Uppsala

Independent project 2011:3

ISSN 1101-8151
ISRN SLU-MIKRO-EX–11/3–SE

The impact of 17α-ethinylestradiol on roach (*Rutilus rutilus*) growth

Sara Sol Windahl

Supervisors: Matilda Olstorpe, SLU
Department of Microbiology
Per Hallgren, Lund University
Department of Ecology

Examiner: Volkmar Passoth, SLU
Department of Microbiology

Keywords: *estrogen, roach, R. rutilus, mört, hormone, EDC, synthetic estrogen & 17α-ethinylestradiol*

EX0418 Independent degree project in Biology
Bachelor C, 15 HEC

Uppsala BioCenter

Department of Microbiology
Faculty of Natural Resources
& Agriculture Sciences
Swedish University of
Agricultural Sciences

Uppsala

Independent project 2011:3

ISSN 1101-8151
ISRN SLU-MIKRO-EX–11/3–SE

Abstract

17α-ethinylestradiol (EE_2), found in pharmaceuticals is regularly consumed and secreted mainly from women. Consequently, a significant level of this endocrine disrupting chemical is continuously discharged into the aquatic environment. EE_2 is the most potent known estrogen discharged, it can bioaccumulate in animals and persist in nature relatively long. Laboratory research indicates decreased population growth in fish exposed to EE_2 and this may consequently change the time of fecundity, bias the sex ratio and decrease the survival rate in young fish. In this laboratory experiment the growth (length and weight) of roach was analyzed. Roach was exposed to different concentrations of EE_2 (0 ng/l (control), 0.5 ng/l, 5.0 ng/l and 50.0 ng/l) for 75 days in their egg, larvae and juvenile phase. In the final result the mortality was higher among the estrogen treated roach. The weight and length was significantly increased among the estrogen treated roach (50.0 ng/l), but the condition factor was higher for the control. The high growth rate indicates a physiological response caused by the higher food supply among EE_2 treated roach. The high mortality among the smallest EE_2 treated roach probably influenced the final composition of EE_2 treated roach, which were significantly larger. This is enhanced by the lower condition factor among the estrogen treated roach, which may indicate a depressed health due to EE_2.

Table of Contents

1 Introduction

There are numerous molecules dispersed into the environment that affect the hormone systems in organisms negatively, these exogenous substances are so called endocrine disrupting chemicals (EDCs) (Jobling & Tyler, 2006). Researchers have shown disturbances caused by EDCs in different organism, from fish and amphibians to mammals (Sumpter & Jobling, 1995; Jobling & Tyler, 2006). Decades ago changes in roach (*Rutilus rutilus* or Swe. mört) were revealed, where intersex roach (i.e. one individual with both oocyte and testes tissue) were discovered in U.K. rivers downstream municipal sewage treatment works (STWs) (Jobling *et al.* 1998). At some locations 100 % of the male roach were intersex, at lowly contaminated sites usually no intersex roach occurs (Jobling *et al.* 1998; Geraudie *et al.*, 2009). Laboratory research has revealed numerous estrogenic EDCs in the environment and abnormalities caused by these estrogens have been discovered in a variety species. (Jobling *et al.* 1998; Bjerregaard *et al*., 2006; U.S. EPA. 2010). This has given estrogens a huge research interest and studies confirm that estrogen exposure of roach can generate impaired functions e.g. delayed sexual maturity, reduced fecundity and multiple persistent disorders (Rodgers-Gray *et al*., 2001; Jobling & Tyler, 2006; Schäfers *et al*., 2007). A long-term study during seven years, when 17α-ethinylestradiol (EE_2) was added regularly to a lake in Canada showed that the whole fathead minnow (*Pimephales promelas*) population nearly collapsed (Kidd *et al*., 2007).

Research shows that EE_2 depress the growth of tilapia (*Oreochromis niloticus*) larvae, which is likely relevant for other fish species (Shved *et al*., 2007 & 2008). Altered fish size have showed to change the timing of fecundity, sex ratio of the population and the survival rate in young fish, thus the changed growth may cause large disturbances for the specie and consequently the ecosystem, (Shved *et al*., 2007; Paull *et al*., 2008).
This report is a literature study combined with a practical part, focusing on how EE_2 affects roach growth and the vital consequences of altered juvenile roach growth.

1.1 Roach (*Rutilus rutilus*)

Roach is one of the most common Swedish fish species and it is found in a variety of habitats throughout Europe (Curry-Lindahl, 1985; Paull *et al*., 2008). It belongs to the *Cyprinidae* family living in fresh and brackish water (Paull *et al*., 2008). Roach growth depends much on environmental factors mediated through the endocrine system, for instance food intake, food availability, age, temperature and pollutants (Beckman *et al*. 2004; Kime, 1998; Krause *et al*., 1998; Cragg-Hine & Jones 1969). Pollutants can affect growth indirectly by contaminating the food resources and in various manners by direct changing e.g. the pituitary GH secretion, the foraging behavior and the thyroid hormones (Kime, 1998). The growth is distributed between weight and length and involves a variety of factors e.g. individual variation, age, sexual maturity, season, feeding and sex (Okgerman *et al*., 2009). The condition of the fish can be evaluate by means of the weight (W) and length (L) using Fulton's formula ($Cf = W \cdot 100 / L^3$), generally the higher value/condition factor (Cf) the better condition of the fish (Bagenal & Tesh, 1978). Roach has also a seasonal growth cycle and the resources are distributed between gonad and somatic growth (Kime, 1998). The somatic growth is important for the survival i.e. as the young fish enlarge the survival chances raise, furthermore the juvenile size and condition in the end of the first season is vital during the winter (Kime, 1998; Pauly, 1980) In mammals the growth terminates with sexual maturation, but in fish the growth is maintained during the entire life (Kime, 1998). Female roach have faster growth than males and reach a larger ultimate size (Cowx 1988; Cragg-Hine & Jones 1969). Although, a clearer depressed growth in males is not seen until after four years according to Cowx (1988) and Cragg-Hine & Jones (1969).

Roach sexually matures around two to five years of age, relatively late compared to other fish species (Curry-Lindahl, 1985; Paull, 2008). Studies show a linkage between the somatic growth and the timing of sexual development i.e. there is a positive correlation between growth and early maturity (Shelton *et al*., 1995; Paull *et al*., 2008). Faster growth is more related to earlier maturation than age, though male roach can reproduce at smaller size than female (Paull *et al*., 2008 and 2009). However, the growth and maturation much depends on environmental factors and there is a significant genetic variation in age and size at sexual maturation between populations, as a result of environmental adaptation (Curry-Lindahl, 1985; Svedäng, 1993; Paull *et al*., 2008). A study by Paull *et al*. (2009) showed a sex bias towards females among faster growing roaches during early life. This indicates that the growth during the first month may be important in the sex differentiation and successively the sex ratio in the population (Paull *et al*. 2009).

1.2 Estrogens

Environmental estrogens are the collection names for natural or synthetic substances with endogenous estrogen effects e.g. the endogenous estrogen steroids; 17β-estradiol (E_2), estriol and estron, the synthetic ethinylestradiol steroid, the man-made xeno-estrogenes and the plant phyto-estrogens (Larsson *et al*., 1999; Routledge *et al*., 1999).

Endogenous estrogens exist in all vertebrates and the molecular structures do not differ between species (Läkemedelsverket). The most important is E_2, but all have hormonal functions. They are involved in numerous regulatory steps controlling reproduction and female secondary sex characters (Läkemedelsverket; Berg *et al*., 2007; Wasserman, 2008). The endogenous estrogens are lipophilic steroid molecules that regulate gene transcription through intracellular receptors in target cells throughout the organism (Berg *et al*., 2007; Wasserman, 2008). They are mostly produced in the gonads, derived from the precursor cholesterol with progesterone as an intermediate in the process (Wasserman, 2008; Berg *et al*., 2007). There are at least two known estrogen receptors in fish, the α-receptor (ERα) and β -receptor (ERβ), and EE_2 binds to both with high affinity (Denny *et al*., 2005). The receptors and the endogenous estrogens also exist in males, since they as well involve e.g. the immune system, central nerve system and lungs. The receptors production are increased by estrogens, thereby the cell sensitivity and the biological response to estrogens raise (Pakdel *et al*., 1991; Shved *et al*., 2008). The main function of estrogens in maturing female fish is to stimulate liver cells to produce vitellogenin (an egg yolk protein, important as food reserve for the developing embryo) (Kime, 1998). Normally it is the endogenous estrogens that stimulate the estrogen receptors, which in turn induce the vitellogenin synthesis in female fish (Kime, 1998). This protein synthesis has turned into an applicable biomarker to determine if a substance has an estrogenic effect, as the estrogenicity is in relation to the vitellogenin production in male and juvenile fish (Kime, 1998; Jobling *et al*. 1998; Jobling *et al*. 2002). *In vivo* the vitellogenin produced is measured in male and juvenile fish which normally have a scanty production of vitellogenin (Jobling *et al*. 1998; Geraudie *et al*., 2009). An elevated level of vitellogenin in male fish is the most common observation due to EDCs in the wild (Purdom *et al*., 1994; Bjerregaard *et al*., 2006).

In vitro and *in vivo* studies have shown that there are many other substances with estrogenic activity, generally by binding to the estrogen receptor and give the same response as the estrogens. **Phytoestrogens** are compounds from plants similar in structure to estrogen which induce estrogenic ef-

fect. Other examples are **xenoestrogens,** industrial chemicals, inorganic or organic compounds with estrogenicity e.g. nonylphenol, some pesticides, biphenolic chemicals and alkylphenols. (Sumpter & Jobling, 1995; Sumpter, 1998; Jobling *et al.* 1998; Routledge *et al.*, 1999; Naturvårdsverket, 2008)

1.3 The synthetic estrogen 17α-ethinylestradiol (EE_2)

The pharmaceutical EE_2, designed to serve in the same manner as the endogenous E_2, is the active substance added to e.g. combined oral contraceptive pills and estrogen patches/rings used during menopause (Läkemedelsverket). In all examined vertebrate species EE_2 have showed to be particularly potent (Läkemedelsverket). As many other pharmaceuticals are EE_2 produced sufficient stable to ensure its biological effect in the body, which also causes EE_2 to be more persistent in the environment (Läkemedelsverket). The degradation of EE_2 in the environment can take time and almost completely stand still in a sterilized environmental without microorganisms (Jürgens *et al.*, 2002). According to a research by Jürgens *et al.* (2002) the half-life of EE_2 was 17 days, several times longer compared to E_2 with 1-2 days. EE_2 is designed to be metabolized slower than E_2 in the body, resulting in slower clearance. High intake can lead to elevated bioconcentration (Kime, 1998; Läkemedelsverket). This is due to an ethynyl group in EE_2 preventing the metabolic conversion to estrone (Kime, 1998). Later investigation has shown that EE_2 can bioaccumulate in fish, revealed by Larsson *et al.* (1999) in rainbow trout downstream STW.

The level of estrogenic activity induced in an organism differs massively among the environmental estrogens. EE_2 has the highest known estrogenic effect and thereby the greatest efficiency to induce vitellogenesis, followed by E_2 (Larsson *et al.*, 1999). Researches confirm that EE_2 can cause biological effects in the low ng/l range compared to xenoestrogens that are weak estrogens with biological effects often in higher concentrations (Purdom *et al.*, 1994; Sumpter & Jobling, 1995). EE_2 is one of many EDC with estrogenic effect in nature and the mixtures of substances have additive effect and maybe also synergistic (Brian *et al.*, 2005). The highest concentration in this experiment was 50.0 ng/l of EE_2, which may not be considered as an environmentally relevant concentration. Occasionally, this and higher EE_2-concentrations have been measured in some locations, but according to different research groups the average levels of EE_2 in the undiluted wastewater are generally between 1 and 3 ng/l (Heberer, 2002). However, these low concentrations are enough to generate effects and 4 ng EE_2/l have been shown to feminize entire roach populations (Purdom *et al.*, 1994; Heberer, 2002; Lange *et al.*, 2008).

1.4 Sewage treatment work (STW)

Numerous pollutants from domestic, industry, and/or agricultural sites reach the STW were they are degraded and/or eliminated (Pillon *et al*. 2005). However many molecules are not degraded or simply partly degraded in the treatment processes and eventually reach the aquatic environment (Routledge *et al*., 1999; Pillon *et al*., 2005). Effluents from STWs are considered a main source of estrogens in the environment (Pillon *et al*., 2005). Experiments show a positive correlation between the exposure to STWs discharges and the plasma vitellogenin concentration in male roach, and in turn the vitellogenin concentration is in proportion to the quantity of female tissue in the gonads of intersex male fish (Rodgers-Gray, 2001; Jobling *et al*., 2002).

Roach and other aquatic organisms are the first to be exposed to pollutants such as estrogens in the STW effluents (Läkemedelsverket). The amount of estrogen in the wastewater may seem to be low with the consideration of dilution in the STW and later as it is discharged into more water. However, the fundamental difference between fish and terrestrial species is that fish breathe through water (Läkemedelsverket). The fish gills serves as a direct contact with a large amount of water and also to the contaminants within (Läkemedelsverket). The wastewater includes natural and synthetic estrogens, regularly and mainly excreted by women. Moreover, a significant amount of estrogens originate from farm animals (Routledge *et al*., 1999; Läkemedelsverket). Estrogen steroids have low water solubility and in order to facilitate the secretion from the body the molecules are conjugated to a water-soluble group in the liver (Routledge *et al*., 1999; Ternes *et al*., 1999). The steroid estrogens are mainly excreted via the urine but also in feces, they are inactive when linked to the water-soluble group and have no hormonal effect on fish (Larsson *et al*., 1999; Routledge *et al*., 1999; Ternes *et al*., 1999; Läkemedelsverket). However microorganisms can cleave the conjugate and thereby reactivate the hormone, which most likely occurs during the transport through the sewer and STW (Larsson *et al*., 1999; Routledge *et al*., 1999; Jürgens *et al*., 2002).

The purification efficiency in STWs concerns the environmental impact. The purification efficiency in STWs differ significantly, a range from 100% to almost nothing of the EE_2 in the effluent remains (Johnson & Sumpter, 2001). The estrogen degradation or removal can be increased in a variety of methods, STW with an activated sludge process or a prolonged treatment time increasing the biodegradation of estrogen (Johnson & Sumpter, 2001). There are other methods, e.g. nanofiltration and ozone treatment that also have shown to improve the effluent quality regarding EE_2 levels (von Gunten *et al*., 2005; Bodzek & Dudziak, 2006).

In Sweden the amount of EE_2 prescribed is relatively diminutive and the estrogen problem is much greater at other location around the world (Läkemedelsverket). The EE_2 discharges in Sweden are small due to few inhabitants together with high water expenditure in relation to many other countries (Naturvårdsverket, 2008). However, even these small amounts have shown to have negative effect.

1.5 Early life estrogen exposure

In this experiment roach in the developing phase during early life was exposed to EE_2 and this reflects how it can befall in nature. The EE_2 concentration can locally reach high levels in the environment, since the discharge of estrogens occurs continuously at same locations (Naturvårdsverket, 2008). As many exposed populations of roach move around during life (e.g. to find food) the estrogenic exposure level may differ during life and this may contribute to diminutive effects. However, during spawning numerous roach populations migrate to rivers and streams whereas STW discharge may flow forth (Curry-Lindahl, 1985). This may initiate estrogenic exposure for the eggs and newly hatched larvae i.e. during the sensitive developing phase in early life, which has shown to end in persistent disorders (Rodgers-Gray, 2001).

1.6 Aim of the Project

The main purpose of this project was to investigate the impact of the synthetic estrogen, 17α-ethinylestradiol (EE_2) on roach growth i.e. weight and length. In order to perform the experiment, roach was exposed to different concentrations of EE_2 during 75 days in their egg, larvae and juvenile phase. Three measuring points with three weeks intervals were completed. This project was made as a side part of an experiment where the estrogen receptor productions in roach and the behaviour were studied after EE_2 exposure.

2 Material & Method

2.1 Preparation of roach experiment

Eight aquariums à 48 liters of tap water were prepared (**Fig. 1**). Different concentrations (0 ng/l (control), 0.5 ng/l, 5.0 ng/l and 50.0 ng/l) of EE_2 (Sigma-Aldrich, ≥98 purity) were established with two randomly placed replicates for each concentration. The aquariums were equipped with filters and circulating water systems. Water pumps were installed in barrels with 50 liters of water and the water flow through the circulating system was regulated to about 25 l/hr in each aquarium. The replicates i.e. aquariums with the same EE_2 concentrations were interconnected by the circulating system through the barrels (**Fig. 1**). Black plastic was placed on the side of each aquarium to avoid interaction between the roach in the different aquariums (**Fig. 1**). To prevent the roach from escape, the aquariums were covered with plastic net. In order to maintain protection and supervision of the roach eggs and later small larvae, net-cages were placed in each aquarium. When the roach were about 60 days past first day of exposure (FDE) they were released from the net-cages to the entire aquariums.

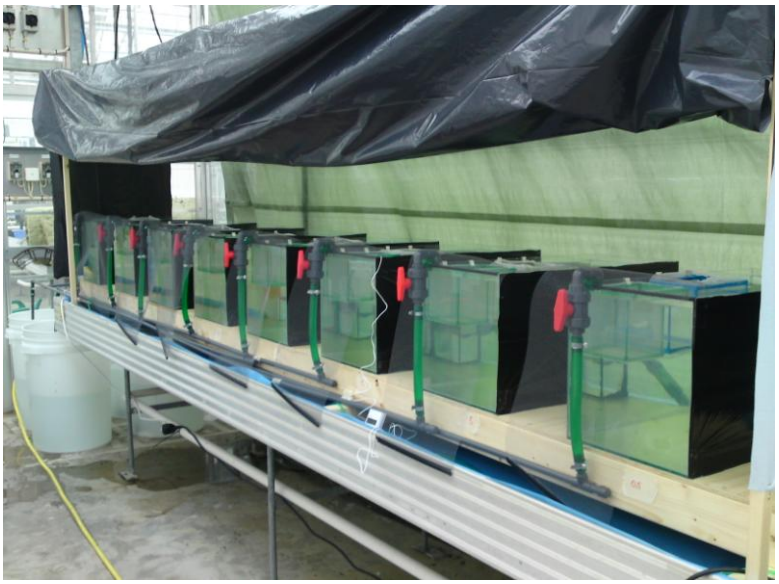

Figure 1. The arrangement of roach experiment. Photograph of eight aquariums with black plastic, net-cages within and circulating water system. The circulating system connected the aquariums with the barrels to the left in the picture. The aquariums were numbered one to eight from left to right. (Photo: Sara Windahl)

2.2 Performance of roach experiment

Initially EE_2 concentrations (0 ng / l, 0.5 ng / l, 5.0 ng / l and 50.0 ng / l) were established in the aquariums and their interconnected barrels. To maintain the initial concentration new EE_2 /DMSO solution was added with a predicted half life of 4 days. The water in the barrels was changed every week with air enriched tap water, and EE_2 was added to retain the concentrations. Roach eggs were gathered on fifth of May 2010 in the Björkaån stream (55˚39'N, 13˚38'E). The eggs were placed in aerated stream water over night and then positioned in the aquariums. The average weight per roach egg was calculated by dividing the weight of 100 counted eggs. A high margin was taken when a total of 8.0 g eggs were placed in each aquarium, corresponding to an amount of about 160 eggs. Continuous exposure to EE_2 for a total of 75 days started when the eggs were placed in the net-cages (i.e. sixth of May was the FDE). The experiment was completed in a greenhouse during spring and summertime at water temperatures between 13 ºC and 34 ºC.

Fungus spread in the aquariums and to avoid further fungus growth on the roach the water in the systems was removed a couple of weeks after FDE. The aquariums were disinfected with ethanol (95%) and 300 liters of water was run through each aquarium before fresh water was added. To generate thicker mucus layer of the fish and in that way get more fungal resistant roach, two salt tablets (AXAL®Pro) was added to each water barrel i.e. one tab/aquarium. Finally a phytoplankton suspension (0, 1% of the total volume) was added to depress the fungus by natural competition.

2.3 Feeding of the roach

In the region of seven days after the eggs had been placed in the net-cages they began to hatch and the feeding begun. From each net-cage 50 larvae were detained for further examination, completed after fungal cleaning. In the beginning of the experiment the larvae were mostly fed on regularly caught zooplankton (e.g. rotifers, copepods, nauplii and daphnia), as roach are dependent on zooplankton during early life stages. The zooplanktons were stored in open barrels of 400 liters and phytoplankton was added as their food supply. The roach were fed with zooplankton once a day until 25 days past FDE, when the food supply was increased to twice a day. Plankton from the barrels was filtered through filters of different dimensions (50 µm, 100 µm and 240 µm). The first days the plankton caught in the 50 µm filter net were used as food and as the roach grew they were fed with progressively larger zooplankton. As additional food scaled brine shrimp (*Artemia sp.*) eggs were added to the

mixture of zooplankton. Towards the end the food supply progressively went over to exclusively cultivated daphnia and freshly hatched brine shrimp until the end of the experiment.

2.4 Weight and length measurements

The weight and length measurements were performed with three weeks intervals and the first measurement point was at 33 days post FDE. In the first and second sampling, two to five roach were randomly picked within the replicates for total body length and weight measurements. The weight was determined with a sensitive balance after removing the majority of water. The length was analyzed by Image-J 1.43u[1] after photographs were taken (**Fig. 2**). In the third and last measurement all remaining roach was measured. After each measurement the remaining individuals in the aquariums were counted for survival analyze. In order to evaluate the relationship between length and weight, by means of EE_2 effects on the condition, the condition factor (Cf) was calculated for each roach. Accomplished by using the weight (W) in gram and the length (L) in cm according to Fulton's formula: $Cf = W \cdot 100 / L^3$.

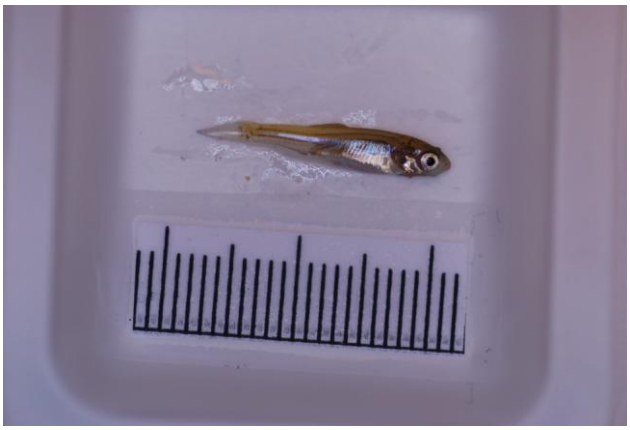

Figure 2. Photographed roach exposed to 50 ng EE_2/l at the second measurement point, use for length measurement with the Image-J program. (Photo: Sara Windahl)

2.5 Statistics

Significant differences in length, weight and Cf between control and EE_2 exposed roach was determined. Since the mortality was high among the estrogen treated roach only the 0.0 ng EE_2/l (control) and 50.0 ng EE_2/l replicates were compared in the analysis and at the last measuring point (75 days post FDE), whereas all remaining fish was measured. Length, weight and condition factor was analyzed by MANOVA when appropriate. To determine significant differences in length, weight and Cf between control and EE_2 exposed roach a two sample t-test was performed and in order to perform the analysis the statistic program PASW statistics 18[2] was utilized.

1 National Institutes of Health, USA. Available from: http://rsb.info.nih.gov/ij
2IBM Company Headquarters, USA, Available from: http://www.spss.com/?source=homepage&hpzone=nav_bar

3 Results

3.1 Hatched eggs and survival

In most net-cages the majority of eggs were hatched. The mortality of the newly hatched roach was elevated in the beginning and at the first measurement point a decrease of roach was observed in all net-cages (**Tab. 1**). The highest mortality was among roach in aquarium (from left 3 and 8) corresponding to exposure of 5.0 ng/l respective 0.5 ng/l EE_2. In general the EE_2 exposed roach had a higher mortality compared to the control. After the first measurement barely any roach died and a more stabilized overall survival rate was continuous throughout the rest of the experiment.

Table 1. *The decrease of fish after 50 roach had been retained, calculated before the measurement points; 33 days, 54 days and 75 days post FDE, (the collected measured roach at the measuring points are not included and - denotes the empty aquariums).*

Concentrations EE_2 (ng/l)	Decrease before 33 days	Decrease before 54 days	Decrease before 75 days
50.0	35	37	38
0.5	39	40	41
5.0	48	-	-
Control	33	33	34
50.0	39	40	40
Control	29	31	32
5.0	43	43	-
0.5	50	-	-

3.2 Weight and length measurements

The total body length and weight were measured at three occasions, at 33 days, 54 days and 75 days post FDE. The average length and weight for the EE_2 exposed roach were higher compared to the control in nearly all of the measurements (**Tab. 2**). The greatest diversity in size, both in length and weight was among the roach in the control, i.e. the longest respective shortest and the heaviest respec-

tive lightest roach were found among the untreated roach. In contrast to the largest roach found in the control, half the roach in the control were smaller in size than the smallest EE_2 treated roach.
To verify if the length was in relation to the weight the Cf was calculated for each roach. The Cf increased in relation to the progressively growing roach. At the last measurement the Cfs were between 0.97-1.09 for the treated roach, which was an enhancement of 60-63 % from the first measurement point. At each time of measurement the Cfs differed slightly among the various treatments (**Tab.2**).

Table 2. *The average weight (AW), length (AL) and Cf (ACF) at three measuring points, 33, 54 and 75 days post FDE of roach eggs and roach exposed to different concentrations of EE_2 (0 ng/l (control), 0.5 ng/l, 5.0 ng/l and 50.0 ng/l).*

Concentrations (ng/l)	AW 33 d (g)	AW 54 d (g)	AW 75 d (g)	AL 33 d (mm)	AL 54 d (mm)	AL 75 d (mm)	ACF 33 d	ACF 54
50.0	0.0017	0.0270	0.29	7.8	15.9	30.7	0.35	0.58
5.0	0.0028	0.0510		8.8	20.0		0.40	0.60
0.5	0.0031	0.0312	0.36	9.1	17.6	31.9	0.40	0.55
0.0	0.0029	0.0214	0.22	8.9	15.4	27.4	0.40	0.49

The average weight and length, for roach exposed to 50 ng/l of EE_2 at 75 days post FDE were a few grams heavier and a few millimeters longer compared to the control (**Tab. 3**). The average Cfs for the control replicates was slightly higher than the EE_2 treated replicates (**Tab. 3**).

Table 3. *Effect of EE_2 on average weight, length and Cf at 75 days post FDE of roach exposed to 50 ng/l and 0 ng/l (control) of EE_2.*

Replicates	Concentration EE_2 (ng/l)	Weight (g)	Length (mm)	Condition Factor
1	50	0.28	30.2	0.98
2	50	0.30	31.1	0.96
1	0	0.22	27.8	0.99
2	0	0.22	27.0	1.04

3.3 Statistics

The two sample t-test confirmed a significant increase in total body length and weight among the roach exposed to EE_2 (50.0 ng/l) compared to the control (**Tab. 4** & **Fig. 3**). The Cf of the control roach was higher than for the EE_2 exposed, although without any significance (**Tab. 3**, **Tab. 4** & **Fig. 3**).

Table 4. *Tests of between-subjects effects. The F-factor and significance for EE_2 exposed roach (50.0 ng/l) compared to control. The asterisk indicate significant difference (*P < 0.05).*

Variables	F	Significance
Weight	29.229	0.033*
Length	30.344	0.031*
Condition Factor	3.734	0.193

Weight (a), length (b) and Cf (c) at 75 days post FDE

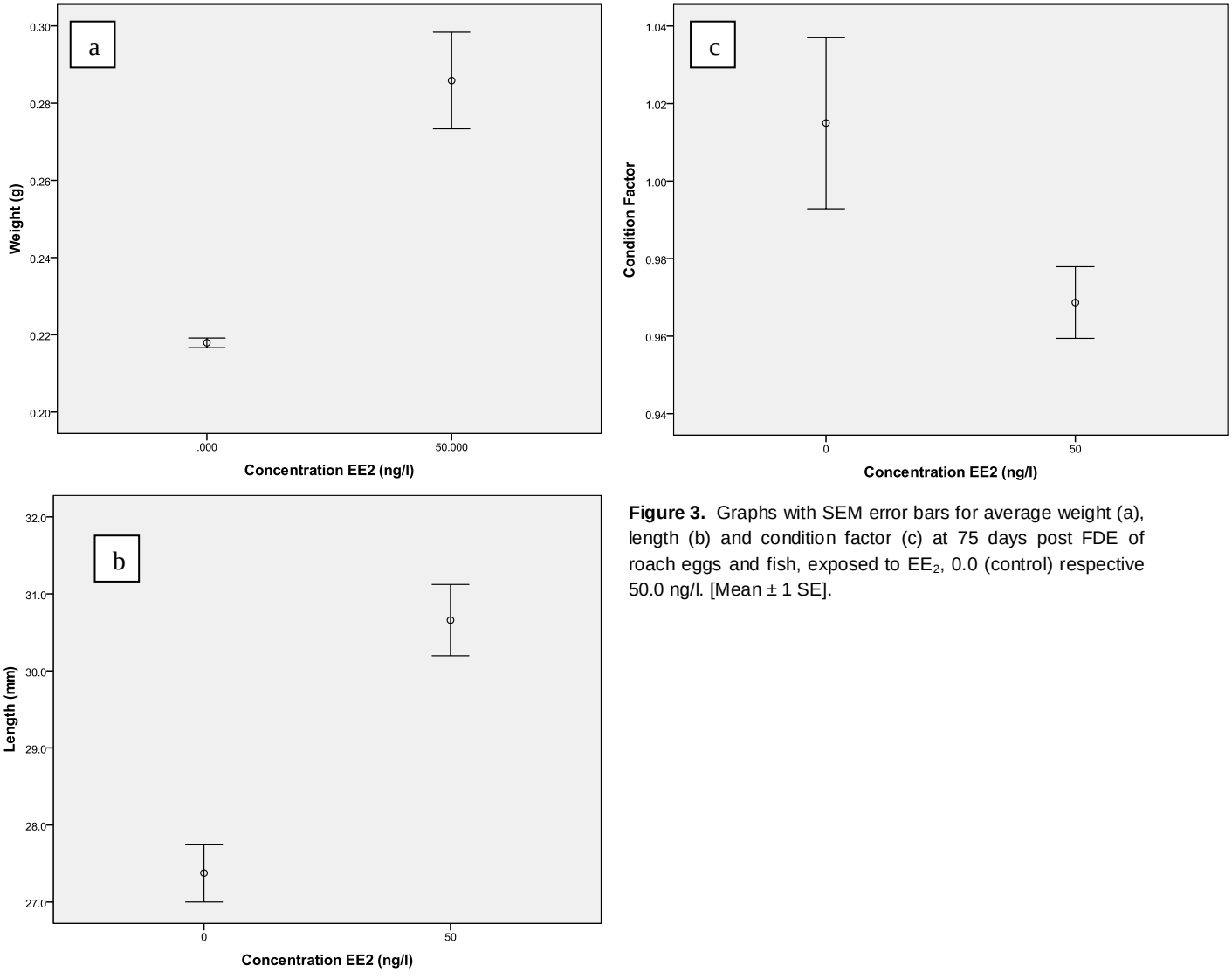

Figure 3. Graphs with SEM error bars for average weight (a), length (b) and condition factor (c) at 75 days post FDE of roach eggs and fish, exposed to EE_2, 0.0 (control) respective 50.0 ng/l. [Mean ± 1 SE].

4 Discussion

4.1 Hatched eggs and survival

In most net-cages the majority of eggs were hatched, but the amount of newly hatched larvae varied between the aquariums. Earlier experiments reveal decreased hatch frequency among estrogen exposed fish eggs (Versonnen & Janssen, 2004). Contradictory, within the two control replicates a lower amount of larvae were sighted compared to the EE_2 treated. This may have been a coincidence, since the eggs might have been damaged in different degrees during the handling and some may not been fertilized. Also the exact amount of eggs was not known as vegetation and small stones were stuck to the clusters of eggs during scaling. All this contributed to the total measured weight and consequently variation in the amount of positioned eggs per aquarium.

The mortality was elevated in the beginning of the experiment and at the first measurement point a decrease of larvae was observed in all net-cages. After the first measurement barely any larvae died and a more stabilized overall survival rate was continued throughout the rest of the experiment. The high mortality during the first days after hatching was perhaps due to natural causes. Most larvae died as they got stuck in the net-cages, between the net and the frame. The fungus obviously contributed to the high mortality rate, since many individuals died when it expanded and finally grew on some of the roach. A white fungus was observed in all net-cages after some days, particularly on the egg remains, which might have delayed the growth if it had been removed. The fungus spread and a film was noticed at the walls of the net-cages. After additionally time, the water was white, dully and the aquarium waters had to be cleaned. The fungus was not totally extincted after cleaning, the growth continued and about one centimeter long fungal hoops could be noticed from some of the fish gills. In general the EE_2 exposed roach had a higher mortality and the highest was among roach in aquarium 3 (5.0 ng EE_2/l) and 8 (0.5 ng EE_2/l), where the long fungus hoops also occurred more frequently. The fungus grew well in the aquariums, presumably since the arranged environment was “too clean” and with relatively few organisms competing with the fungus (as bacteria or algae). The addition of salt

and algae reduced the continued fungal growth and possibly assisted with the clearly decreasing mortality with time.

Since the smallest sized roach in the end were simply found in the control it might have been a selection among the EE_2 treated (i.e. survival of the fittest). Roach are sensitive the first period in life and when they grow the mortality decrease. It is likely that the combination of being small together with estrogen exposure reduce the survival chances, since estrogens also are involved in the immune system. Research reveals that EE_2 exposure may decrease the infection immunity and this appear to be a reasonable explanation to the higher mortality among EE_2 treated roach in this experiment (Shved *et al.*, 2009). It may also have influenced the fact that half the unexposed roach were smaller than the smallest EE_2 exposed. Explicitly the small control roach had a better immunity against the fungus than the small estrogen treated roach and thereby better survival chances. This is also supported by the lower Cf among the EE_2 treated roach, which may indicate a lower health.

4.2 Weight and length measurements

An obvious increase of total body length and weight was observed between the three measurement points for all concentrations of EE_2 and the control. The two sample t-test confirmed a significant increase in total body length and weight at the last measurement point among the larvae exposed to EE_2 (50.0 ng/l) compared to the control, contradictory to the expected results. The roach were meant to be fed with the same amount of food per roach, since the food supply has a major effect on roach growth (Krause *et al.*, 1998). However, a lot of roach died and the food supply was not regulated to the amount of roach. As more roach died in the estrogen treated aquariums the remaining roach had a higher food supply per roach compared to the control. This gave most certainly a misleading result in terms of estrogen effect on growth. It had been preferable to correlate the amount of food to the number of roach in each aquarium, since this had most likely presented another outcome.

Meanwhile the roach grew, an increasing diversity in fish size could be observed and it appeared to be in relation to the amount of roach in each aquariums. The size diversity could be observed especially among the control replicates in the end of the experiment, where the amount of roach was highest. This also indicates that the control was not fed in excess because with more roach the competition for food increases and probably the smallest roach did not get the same amount of food in relation to de greater ones during the experiment.

4.3 Condition Factor

The Cf usually increases with age and size, which was confirmed in this experiment as the Cf increased in relation to the roach growth (an enhancement of 60-63 % between the first and last measurement points). However, this conflict with the lower Cf among the EE_2 treated roach that were larger and obtained more food compared to the control. The Cf of the control larvae was higher than for the EE_2 exposed at the last measuring point, although without any significance. This indicates that the relation between the length and weight was not as good in the EE_2 treated population compared to the control, in spite of the food excess. As contaminants sometimes have been shown to alter the length and thus to change the relation of length and weight, this outcome can be a result of a toxic response due to estrogen exposure. If the experiment had lasted for a longer time, perhaps the increased exposure time might had given a significant difference in Cf between control and EE_2 treated roach.

4.4 Conclusion

In conclusion the significant differences of weight and length in this study suggest a physiological response caused by the higher food supply among EE_2 treated roach. When analyzing the estrogen effect on roach growth, same food supply and equal food consumption in all treatments is central. The high mortality among the smallest EE_2 treated roach was probably due to the combination of EE_2 exposure and decreased survival chances among small fish. The increased mortality observed in the beginning of the experiment, especially among the EE_2 treated roach occurred when the environment in the aquariums was harsher due to fungus and water changing. This demanded higher pathogen defense and survival requirements. Furthermore, the Cf was lower within the EE_2 treatments, which might indicate unhealthier roach among the EE_2 exposed. In conclusion EE_2 may affect roach negatively by depressing the roach immunity, resulting in high mortality among the smallest EE_2 treated roach. However, further investigation is needed to confirm the results.

5 Acknowledgement

This work was performed as a side part of a scientific research project by Per Hallgren and Lina Nikoleris at Lund University. I like to thank Lund University and my second supervisor, Per Hallgren for all the support and help in Lund. I also like to thank my supervisor, Matilda Olstorpe and examiner, Volkmar Passoth at SLU in Uppsala, I am grateful for all the assistance and guidance in the writing part.

6 References

Beckman, B. R., Shimizu, M., Gadberry, B. A., Cooper, K. A. (2004) Response of the somatotropic axis of juvenile coho salmon to alterations in plane of nutrition with an analysis of the relationships among growth rate and circulating IGF-I and 41 kDa IGFBP. *General and Comparative Endocrinology* 135:334–344

Bagenal, B.T. & Tesh, F.W. (1978) Methods for the Assessment of Fish Production in Fresh Waters. IBP Handbook No.3. *Blackwell scientific publications* 101–136

Berg, J. M., Tymoczko, J. L., Stryer, L. (2007) *Biochemistry*. 6. ed. New York : W. H. Freeman and Co. ISBN 0-7167-8724-5

Bjerregaard, L. B., Korsgaarda, B., Bjerregaard, P. (2006) Intersex in wild roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams. *Ecotoxicology and Environmental Safety* 64:321–328

Bodzek, M. & Dudziak, M. (2006) Removal of Natural Estrogens and Synthetic Compounds Considered to be Endocrine Disrupting Substances (EDs) by Coagulation and Nanofiltration. *Polish Journal of Environmental Studies* 15:35–40

Brian, J. V., Harris, C.A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Runnalls, T., Bonfà, A., Marcomini, A., Sumpter, J. P. (2005) Accurate Prediction of the Response of Freshwater Fish to a Mixture of Estrogenic Chemicals. *Environmental health perspectives* 113:721–8

Cragg-Hine, D. & Jones, J. W. (1969) The growth of dace *Leuciscus leuciscus* (L.), roach *Rutilus rutilus* (L.) and chub *Squalius cephalus* (L.) in Willow Brook, Northamptonshire. *Journal of Fish Biology* 1:59–82

Cowx, I. G. (1988) Distribution and variation in the growth of roach, *Rutilus rutilus* (L.), and dace, Leuciscus leuciscus (L.), in a river catchment in south-west England. *Journal of Fish Biology* 33:59–72

Curry-Lindahl, K. (1985) *Våra fiskar. Havs- och sötvattensfiskar i Norden och övriga Europa. Stockholm*. P.A. Norstedts & Söners förlag. ISBN 91-1-844202-1

Denny, J. S., Tapper, M. A., Schmieder, P. K., Hornung, M. W., Jensen, K. M., Ankley, G. T., Henry, T. R. (2005) Comparison of relative binding affinities of endocrine active compounds to fathead minnow and rainbow trout estrogen receptors. *Environmental Toxicology and Chemistry* 24:2948–2953

Geraudie, P., Gerbron, M., Hill, E., Minier, C. (2009) Roach (*Rutilus rutilus*) reproductive cycle: a study of biochemical and histological parameters in a low contaminated site. *Fish Physiology and Biochemistry* DOI 10.1007/s10695-009-9351-5

Jobling, S., Nolan, M., Tyler, C. R., Brighty, G., Sumpter, J. P. (1998) Widespread sexual disruption in wild fish. *Environmental Science and Technology* 32:2498–2506

Jobling, S., Beresford, N., Nolan, M., Rodgers-Gray, T., Brighty, G. C., Sumpter, J. P., Tyler, C. R. (2002) Altered sexual maturation and gamete production in wild roach (Rutilus rutilus) living in rivers that receive treated sewage effluents. *Biology of Reproduction* 66:272–281

Jobling, S. & Tyler, C. R. (2006) Introduction: The Ecological Relevance of Chemically Induced Endocrine Disruption in Wildlife. *Environmental Health Perspectives* 114:7–8

Johnson, A. C. & Sumpter, J. P. (2001) Removal of endocrine-disrupting chemicals in activated sludge treatment works. *Environmental Science and Technology* 35:4697–4703

Jürgens, M. D., Holthaus, K. I. E., Johnson, A. C., Smith, J. J. L., Hetheridge, M., Williams, R. J. (2002) The potential for estradiol and ethinylestradiol degradation in English rivers. *Environmental Toxicology and Chemistry* 21:480–488

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* 104:8897–8901

Kime, E. D. (1998) *Endocrine disruption in fish*. Norwell, Massachusetts, Kluwer Academic Publisher

Krause, J., Staaks, G., Mehner, T. (1998) Habitat choice in shoals of roach as a function of water temperature and feeding rate. *Journal of Fish Biology* 53:377–386

Lange, A., Katsu, Y., Ichikawa, R., Paull, G. C., Coe, T. C., Chidgey, L. L., Coe, T. S., Iguchi, T., Tyler. C. R. (2008) Altered sexual development in roach (*Rutilus rutilus*) exposed to environmental concentrations of the pharmaceutical 17α-ethinylestradiol and associated expression dynamics of aromatases and estrogen receptors. *Toxicological Sciences* 106:113–123

Larsson, D. G. J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A. H., Olsson, P. E., Förlin, L. (1999) Ethinyloestradiol - an undesired fish contraceptive? *Aquatic Toxicology* 45:91–97

Läkemedelsverket. Home page. [online] (2003-01-07) Available from: http://www.lakemedelsverket.se/Alla-nyheter/NYHETER---2003/Potentiella-miljorisker-med-EVRA-och-NuvaRing/ [2010-08-07]

Okgerman, H., Oral, M., Yigit, S. (2009) Biological Aspects of *Rutilus rutilus* (Roach) in Sapanca Lake (Turkey). *Journal of Animal and Veterinary Advances* 8:441–446

Pakdel, F., Feon, S., Gac, F. L., Menn, F. L., and Valotaire, Y. (1991) In vivo estrogen induction of hepatic estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout. *Molecular and Cellular Endocrinology* 75:205–212

Paull, G. C., Lange, A., Henshaw, A.C., Tyler, C.R. (2008) Ontogeny of Sexual Development in the Roach (*Rutilus rutilus*) and Its Interrelationships with Growth and Age. *Journal of Morphology* 269:884–895

Paull, G. C., Filby, A. L., Tyler, C. R. (2009) Growth rate during early life affects sexual differentiation in roach (*Rutilus rutilus*). *Environmental Biology of Fishes* 85:277–284

Pauly, D. (1980) On the interrelationships between natural mortality, growth parameters, and mean environmental temperature in 175 fish stocks. *Journal du Conseil International pour l'Exploration de la Mer* 39:175–192

Pillon, A., Boussioux, A-M., Escande, A., Aït-Aïssa, S., Gomez, E., Fenet, H., Ruff, M., Moras, D., Vignon, F., Duchesne, M-J., Casellas, C., Nicolas J-C., Balaguer, P. (2005) Binding of Estrogenic Compounds to Recombinant Estrogen Receptor-α: Application to Environmental Analysis. *Environmental Health Perspectives* 113:278–284

Purdom, C. E., Hardiman, P. A., Bye, V. J., Eno, N. C., Tyler, C. R., Sumpter, J. P., (1994) Estrogenic effects of effluents from sewage treatment works. *Chemistry and Ecology* 8:275–285

Rodgers-Gray, T. P., Jobling, S., Kelly, C., Morris, S., Brighty, G., Waldock, M. J., Sumpter, J. P., Tyler, C. R. (2001) Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. *Environmental Science and Technology* 35:462–470

Routledge, E. J., Waldock, M., Sumpter J. P. (1999) Response to Comment on "Identification of Estrogenic Chemicals in STW Effluent. 1. Chemical Fractionation and in Vitro Biological Screening". *Environmental Science & Technology* 33:371

Schäfers, C., Teigeler, M., Wenzel, A., Maack, G., Fenske, M., Segner, H. (2007) Concentration- and time-dependent effects of the synthetic estrogen, 17alphaethinylestradiol, on reproductive capabilities of the zebrafish, *Danio rerio*. *Journal of Toxicology and Environmental Health* 70:768–779

Shelton, W. L., Wanniasingham, V., Hiott, A. E. (1995) Ovarian differentiation in common carp (*Cyprinus carpio*) in relation to growth rate. *Aquaculture* 137:203–211

Shved, N., Berishvili, G., D'Cotta, H., Baroiller, J-F., Segner, H., Eppler, E., Reinecke, M. (2007) Ethinylestradiol differentially interferes with IGF-I in liver and extrahepatic sites during development of male and female bony fish. *Journal of Endocrinology* 195:513–523

Shved, N., Berishvili, G., Baroiller, J-F., Segner, H., Reinecke, M. (2008) Environmentally relevant concentrations of 17alpha-ethinylestradiol (EE_2) interfere with the growth hormone (GH)/insulin-like growth factor (IGF)-I system in developing bony fish. *Toxicological Sciences* 106:93–102

Shved, N., Berishvili, G., Häusermann, E., D'Cotta, H., Baroiller, J-F., Eppler, E. (2009) Challenge with 17a-ethinylestradiol (EE2) during early development persistently impairs growth, differentiation, and local expression of IGF-I and IGF-II in immune organs of tilapia. *Fish & Shellfish Immunology* 26:524–530

Sumpter, J. P. & Jobling, S. (1995) Vitellogenesis as a Biomarker for Estrogenic Contamination of the Aquatic Environment. *Environmental Health Perspectives* 103:173–178

Sumpter, J. P. (1998) Xenoendocrine disrupters – environmental impacts. *Toxicology Letters* 102–103:337–342

Svedäng, H. (1993) *Mörtens (Rutilus rutilus) livshistoria En litteratursammanställning*. Sweden. Öregrund: Fiskeriverket Kustlaboratoriet. Kustrapport 1993:11. ISSN 1102–5670

et al

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. *The Science of the Total Environment* 225:91–99

U.S. EPA. [online] (2010) U.S. Environmental Protection Agency, Las Vegas (CG Daughton and MST Scuderi) Available from: http://www.epa.gov/ppcp/lit.html

Versonnen, B. J. & Janssen, C. R. (2004) Xenoestrogenic Effects of Ethinylestradiol in Zebrafish (*Danio rerio*). *Environmental Toxicology* 19:198–206

von Gunten, U., Huber, M. M., Göbel, A., Joss, A., Hermann, N., Löffler, D., McArdell, C. S., Ried, A., Siegrist, H., Ternes, T. A., (2005) Oxidation of pharmaceuticals during ozonation of municipal wastewater effluents: a pilot study. *Environmental Science and Technology* 39:4290–4299

Wasserman, S.A. (2008) Hormones and the Endocrine System. In: Campbell, N. A., Reece, J. B., *Biology*. 8. Ed. pp. 975–996. San Francisco: Pearson Education