Effects of 17α-ethinyl estradiol on the mating system of the sand goby (*Pomatoschistus minutus*)

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Academic dissertation

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“Anything is possible, if you wish hard enough”

(Barrien, J.M., 1904. Peter Pan)

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I. Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K. An endocrine disrupting chemical affects sand goby male courtship and parental care. – Submitted revised manuscript to Aquatic Toxicology.

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III. Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2009. Sand goby (*Pomatoschistus minutus*) males exposed to an endocrine disrupting chemical fail in nest and mate competition. – Hormones and Behavior. 56, 315–321.

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THE AUTHOR'S CONTRIBUTIONS TO THE ARTICLES

I. Lindström designed the study together with Lehtonen and Saaristo. Saaristo was responsible for data collection, behavioural experiments, and data analyses. Molecular analysis (Vtg- and Zrp- mRNA expression) were conducted by Saaristo. Saaristo prepared water samples for LC-MS and Björk ran the samples. Saaristo was responsible for the manuscript preparation and Lehtonen, Lindström and Craft contributed with comments.

II. Saaristo designed the study together with Lindström. Saaristo was responsible for data collection, behavioural experiment, molecular and data analyses. Saaristo prepared water samples for LC-MS and Björk ran the samples. Saaristo was responsible for the manuscript preparation and Lehtonen, Lindström and Craft contributed with comments.

III. Saaristo designed the study and was responsible for the data collection, behavioural experiment, molecular and data analyses. Saaristo prepared water samples for LC-MS and Björk ran the samples. Saaristo was responsible for the manuscript preparation and Lehtonen, Lindström and Craft contributed with comments.

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ABBREVATIONS

EDC	Endocrine disrupting chemical
Xenoestrogens	Industrially made or natural compounds that have estrogenic effects
EE2	17α-ethinyl estradiol
Vtg	Vitellogenin protein
Zrp	Zona radiata protein
LC-MS	Liquid chromatography in tandem with mass spectrometry

ABSTRACT

In aquatic environments, endocrine disrupting chemicals (EDCs) that interfere with the endocrinology of males and females form a threat to the maintenance of populations. EDCs are a diverse group of natural and manmade chemicals that already at very low concentrations (at nanogram levels) can have severe effects on reproduction by individuals, e.g. complete sex reversal, feminisation of males, impaired reproduction even resulting in near extinction of populations. With regard to fish, despite the extensive literature on physiological effects of EDCs, very little is known about potential population-level effects.

In this thesis, I examined how 17α-ethinyl estradiol (EE2), a synthetic estrogen used in oral contraceptive pills, affects the reproductive behaviour of a marine fish, the sand goby (*Pomatoschistus minutus*). The aims were fourfold. First, I investigated how exposure to EE2 affects courtship and parental care of sand goby males. Secondly, I looked at effects on the mating system and sexual selection. In the third study, I observed the effects of exposure in a social context where exposed males had to compete with non-exposed males for resources and mates. Finally, I studied the effects of exposure on male-male competition and male aggressive behaviour.

This work revealed that EE2 exposure impairs the ability of males to acquire and defend a nest, as well as diminishes the attractiveness of males to females by decreasing their courtship and aggressive behaviour. These effects are harmful for a male whose reproductive success is determined by the ability to compete for limited resources and to attract mates. Furthermore, this thesis showed that selection on male size was relaxed after EE2 exposure and male size had a smaller effect on mating success. These effects can be of a profound nature as they interfere with sexual selection, and may in the long run lead to the loss of traits maintained through sexual selection.

The thesis shows that an exposure to environmentally relevant levels of EE2 clearly reduces the chances of individuals to reproduce successfully. Furthermore, it strongly suggests that several types of biomarkers should be used to detect and assess the effects of EDC exposure because severe behavioural effects can sometimes be seen before effects are detectable at the molecular or morphometric level. Behavioural assays should be considered an important complementary tool for the standard ecotoxicological assays because observed behavioural changes have direct and negative effects on fitness, while the connection between changes in molecular expression and fitness may be less obvious.

1. INTRODUCTION

Endocrine disrupting chemicals (EDCs) are a large group of natural and man-made compounds, which disturb the endocrinology of organisms. EDCs interfere with endocrinology by stimulating or inhibiting the system, affecting the synthesis, storage, release, transport, clearance, and receptor recognition or binding for one or more hormones (Crisp et al. 1998). EDCs exert effects at very low concentrations (nanogram per liter) and they have been detected in sewage treatment plant effluents and surface waters around the world (Johnson and Sumpter 2001; Vigano et al. 2006; Thorpe et al. 2009). Sources of these compounds include pharmaceuticals and chemicals discharged into wastewater by households, run-off from agriculture and industrial effluents. There has been considerable progress in research on the effects of EDCs during the last two decades, and studies have shown that these compounds do effectively interfere with the reproduction of aquatic and terrestrial organisms (reviewed by Scott and Sloman 2004; Waring and Harris 2005). What is still unclear for many species is whether these compounds disturb reproductive success and thereby the sustainability of populations. Ecotoxicology is thus faced with the challenge to determine what the consequences of EDCs are at the population level.

1.1. Tools for studying the effects of EDC contamination

Effects of EDCs on organisms have mostly been studied using physiological and molecular endpoints. One of the most documented biomarkers of estrogen activity by chemical exposure is the induction of vitellogenin (Vtg), a precursor of egg yolk protein, vitellin (Vn). Vitellin provides energy reserves for developing embryos (Matozzo et al. 2008). Another common biomarker of estrogen activity by chemical exposure is the zona radiata protein (Zrp), which forms the inner layer of the egg envelope (Oppen-Berntsen et al. 1992). The egg envelope prevents polyspermy during fertilization and provides protection against mechanical disturbances for the developing embryo (Hyllner et al. 1994). Males also carry the genes for Vtg and Zrp and are able to produce these proteins if these genes are activated. Therefore, expression of Vtg and Zrp are quantifiable responses of exposure to exogenous estrogenic EDCs and widely used as sensitive biomarkers in both monitoring and laboratory

testing (Jobling and Sumpter 1993; Arukwe and Goksoyr 1998; Arukwe et al. 2000; Christiansen et al. 2000; Kwak et al. 2001; Boon et al. 2002; Robinson et al. 2003).

Somatic indices are also widely used markers in pollution studies. These indices are calculated as tissue mass (g) / body mass (g) * 100. Tissue somatic indices measure the general condition of fish (West 1990) and are commonly used in fisheries studies. The hepatosomatic index (HSI) is a good predictor of adverse health in fish (Adams and McLean 1985) and often correlates with the degree of pollution. In female fish, the gonadosomatic index (GSI) reflects sexual maturity. In males, the sperm duct gland somatic index (SDGSI) has been used as a measure of sexual maturity and reproductive stage (Robinson et al. 2003; 2007). The sensitivity of somatic indices to contaminants is dependent on the species, reproductive season and exposure concentration (Jobling et al. 1996; Christensen et al. 1999), and therefore these are usually used only as complementary tools in EDC studies.

1.2. Behaviour: a promising biomarker of EDC exposure

EDCs can act as hormone agonists or antagonists. Since sex steroids influence the control of sexual and agonistic behaviour, reproductive behaviour provides a complex measure of EDC effects. Moreover, an animal can adjust to changes in the environment with its behaviour, and therefore behaviour potentially provides a sensitive early warning signal for the presence of EDCs. Recent studies applying behavioural ecology to ecotoxicology are summarized in Table 1.

1.2.1. Why use behaviour in EDC studies?

In 1958, a study was published showing that DDT causes abnormal changes in nesting and courtship behaviour of Florida Bald Eagles (Broley 1958). These behavioural changes did not convince the scientific community of the dangers of DDT, and it was only the unexpected deaths of wildlife, e.g. Beluga whales (De Guise et al. 1995) 30 years later, which awakened researchers. This example illustrates that EDCs have profound effects on animal reproductive behaviour, and behaviour is a good early warning signal of contaminants in the environment. Since many targets of the endocrine system are in the central nervous system, the effects of EDCs on behaviour are often direct (Clotfelter et al. 2004; Ottinger et al. 2008). Hormones also affect behaviour indirectly, e.g. through metabolism (Zala and Penn 2004). Short-term toxicity assays generally ignore the fact that if an animals' normal behaviour is altered, it may be unable to function in

Table 1. List of recent behavioural ecotoxicological fish studies, which demonstrate effects of estrogenic EDCs on behaviour and reproductive success at environmentally relevant exposure concentrations.

Species	Exposure compound	Concen.	Exposure duration	Behavioural responses	Effects on reproductive success	Reference
Atlantic salmon (*Salmo salar*)	E2[1]	8-16 ng L^{-1}	26 d	E2: Fish migrated at lower frequency	E2 & OP-10: Early exposure of salmon parr affected negatively the transformation several months later	Bangsgaard et al. 2006
	OP[2]	a) 4.5-6.5 µg L^{-1} b) 10-30 µg L^{-1}	26 d	OP-10: Fish migrated at lower frequency		
Fathead minnow (*Pimephales promelas*)	EE2[3]	2, 8 ng L^{-1}	27 d	Impaired male's ability to compete and acquire territories Decreased aggressiveness		Majewski et al. 2002
	E2, MT[4]	E2: 50 ng L^{-1} MT: EEQ 44 ng L^{-1}	21 d	E2: Reduced levels of agonistic behaviour, less able to acquire nests MT: Increases agonistic behaviour	E2: Exposed males suffered nearly total reproductive failure under compatitive scenario MT: Outcompeted control males	Martinovic et al. 2007
Goldfish (*Carassius auratus)*	E2	0, 1, 10, 100 µg/g food 0, 1, 10 µg L^{-1} water	24-28 d (during spawning period)	10 µg/g: Following behaviour decreased, frequency of pushing decreased 100 µg/g: Decreased pushing and frequency of courting 1, 10 µg/L: Decreased pushing activity and frequency of courting	10, 100 µg/g : Milt production decreased All food and water treatments: Sexual activity was dramatically decreased in both treatments Significantly less males with tubercles in all treatments	Bjerselius et al. 2001
Guppy (*Poecilia reticulata*)	E2	10 µg L^{-1}	28 d	Used less time to sigmoid display, and repeated it seldom		Bayley, et al. 1999
	OP	150 µg L^{-1}	28 d	Used less time to sigmoid display, and repeated it seldom		
	EE2	10.5, 44.4, 112 ng L^{-1}	From birth to adulthood	10.5 ng: Decreased duration of sigmoid displays 112 ng: Frequency of sigmoid displays decreased	112 ng: Decreased sexual coloration, sperm count and highly feminized 112 ng: Almost total elimination of reproduction when they had to compete for mates with unexposed males	Kristensen et al. 2005
Japanese medaka (*Oryzias latipes*)	E2	3, 30 µg/g body weight daily	14 d	Frequency of circular swimming increased Frequencies of following, dancing, floating and crossing decreased significantly in both treatments	Fecundity decreased, exposed males spawned fewer eggs in both treatment groups Fertility was not affected	Oshima et al. 2003

Species	Exposure compound	Concen.	Exposure duration	Behavioural responses	Effects on reproductive success	Reference
Mosquitofish (*Gambusia holbrooki*)	E2	20, 100, 500 ng L^{-1}	84 d	Decreased number of approaches and copulation attempts	100, 500 ng: Fertilized significantly fewer females than the control males	Doyle and Lim 2005
Sand goby (*Pomatoschistus minutus*)	SE[5]	0.03 and 0.3 % v/v	7 mo	SE: No effects	SE 0.3% v/v: Reduced population output	Robinson et al. 2003
	EE2	6 ng L^{-1}		EE2: Significantly fewer males nested	EE2: Significantly fewer pairings produced eggs Fertile egg production was reduced by 90% Delayed and inhibited development of nuptial coloration	
	E2	16, 97, 669 ng L^{-1}	8 mo	97 ng: Delayed spawning	97 ng: Inhibited male sexual maturation, produced fertile eggs at slower rate, delayed spawning 669 ng: Increased mortality, caused almost total lack of reproductive activity, both sexes failed to mature	Robinson et al. 2007
Three-spined stickleback (*Gasterosteus aculeatus*)	EE2	100 ng L^{-1}	Lifetime exposure	Risky behaviour increases: fish were active and foraging under predation risk	Accelerated growth in early life Greater mortality	Bell, 2004
	EE2	10-50 ng L^{-1}	8-32 d	Decreased courtship behaviour Decreased aggressive behaviour		Bell, 2001
	EE2	1.75, 27.7 ng L^{-1}	4-wk posthatch	1.75, 27.7 ng L^{-1}: Fewer nests built, fewer eggs deposited per nest	Occurrence of ovotestis Less intense nuptial coloration	Maunder et al. 2007
Zebra fish (*Danio rerio*)	EE2	0.05, 0.5, 5 ng L^{-1}	4 mo	0.05 ng: Fewer visits to the spawning area 5 ng: Fewer visits to spawning area, shorter distance swum in spawning area All: Decreased aggressive behaviour	0.05 ng: Feminised with development of urogenital papillae, change in body colour 0.5 ng: Sex ratio was altered 59% were males (control 69%)	Larsen et al. 2008
	EE2	0, 0.5, 5, 50 ng L^{-1}	48 h	0.5 ng: Frequency of courtship-specific behavior decreased in dominant males 50 ng: Social dominance reversal in 50% of the fish, 50% of dominant males relinquished paternal dominance	5ng: 5% of the males developed into males, males were unable to induce spawning Shift in individual social status and reproductive success in male zebrafish.	Colman et al. 2009

[1]E2: 17β-estradiol; [2]OP: 4-*tert*-octylphenol; [3]EE2: 17α-ethinyl estradiol; [4]MT: methyltestosterone; [5]SE: sewage effluent

an ecological context. Thus, behaviour can provide direct information both at the individual and at the population level, and therefore be used as a comprehensive, sensitive and non-invasive measure of exposure to EDCs.

Fish with complex reproductive behaviours are particularly at risk to EDCs, as complex behaviours require more coordination (Bruton 1995). The most intricate reproductive behaviours may be found among fishes with parental care. Among teleost families, 21% exhibit parental care (Gross and Sargent 1985) and most of these fish are small in size and breed in freshwater, estuaries and coastal marine environments, where the risk of EDC contamination is the highest. In theory, any external force that alters the behaviour of individuals away from what is optimal under natural and sexual selection could lead to reduced population sizes (Andersson 1994). These effects may be far from straightforward, depending on, for example, the nature of density dependence of behaviours in the population. Small variations in offspring mortality can have a major impact on recruitment (Hounde 1987), and behavioural changes, such as reckless braveness and offspring defence, can cause greater mortality among adults (Matta et al. 2001; Bell 2004). So far, behavioural ecotoxicology studies have been conducted on mammals, birds, fish, rodents and amphibians (reviewed by Zala and Penn 2004). Only a few studies have been done on invertebrates, possibly because of the more limited knowledge of their endocrinology.

1.2.2. What are the potential disadvantages of using behavioural assays?

Behavioural experiments can be time and space consuming. Moreover, their repeatability may suffer because human observers usually have to be involved to quantify the behaviours. However, new automated monitoring techniques that rely on computer analysis may reduce observer bias in the future.

It has been claimed that behaviour is too difficult to measure and highly variable. These claims reveal one of the biggest problems, which is the design and statistical analysis of rigorous behavioural experiments. Especially in the early ecotoxicological works, studies using behavioural traits often lacked adequate sample sizes and replication (reviewed by Jones and Reynolds 1997). Many behavioural studies on fish have to be performed in individual aquaria. These aquaria will differ from each other in many more respects than just the presence or absence of the pollutant of concern. Such factors include nutrients, light intensities, noise and other uncontrolled factors that should be taken into account in the experimental set-up. In order to control for these ‘random’ factors it is

necessary to have adequate replication to detect exposure effects. Furthermore, not all behaviours are affected and hence, if the behavioural assay is limited, there is a risk that the study reports negative effects (Zala and Penn 2004). Therefore, a good observation protocol with enough behavioural resolution is required. Using behaviour as a biomarker also requires substantial cross-disciplinary knowledge to apply ecology and behavioural ecology together with ecotoxicology.

Behavioural experiments conducted in laboratory conditions, however, often lack ecological realism. Altered behaviour after exposure to EDCs in the laboratory does not implicitly mean impaired behaviour in the wild. Animals in the wild may be able to avoid the exposure or develop tolerance to EDCs, and therefore escape the negative effects (Zala and Penn 2004). Even though a direct extrapolation of laboratory results to the field is not easy, behavioural assays still potentially provide a better understanding of reproductive success and sustainability of tested fish species than solely molecular tools.

1.3. Model compound of estrogenic EDCs: 17α-ethinyl estradiol

Pharmaceutical 17α-ethinyl estradiol (EE2) is used in oral contraceptives and has been detected in ecologically relevant concentrations (< 1 to 15 ng L^{-1}) from sewage effluents, surface waters, and activated and digested sludge (Baronti et al. 2000; Muller et al. 2008). Over 99% of the estrogenic activity in sewage effluents and surface waters is attributable to the presence of 17β-estradiol (E2) and EE2 due to insufficient removal during wastewater treatment (reviewed by Clouzot et al. 2008). EE2 can cause sex reversal (Lange et al. 2009) and collapse fish populations at concentrations as low as a few ng L^{-1} (Kidd et al. 2007), which makes EE2 the EDC compound of the greatest concern. EE2 is engineered from the natural hormone E2 by attaching an ethinyl group to C-17. This additional ethinyl group is responsible for EE2's high resistance to biodegradation (Clouzot et al. 2008). Since the natural and synthetic steroid estrogens are mostly excreted as a variety of largely inactive glucuronide, sulphate or sulpho-glucuronide conjugates, it was not expected that the discharge of these compounds would give rise to endocrine disruption in marine and freshwater organisms (Andreolini et al. 1987). According to a large number of studies, EE2 is first metabolized into a biologically inactive form by the hydroxylation of an aromatic ring (Clouzot et al. 2008). This is followed by conjugation with sulphate or glucuronide, which are excreted via bile, at positions C-3 and/or C-17. The natural intestinal flora then unconjugates these conjugated estrogens before they are

excreted from the bowel. Therefore, EE2 is in active form when entering the sewer (Lombardi et al. 1978; Panter et al. 1999; Lai et al. 2002; D'Ascenzo et al. 2003; Johnson and Williams 2004; Clouzot et al. 2008).

EE2 has a relatively high octanol–water partition coefficient (log K_{ow} 4.15; solubility 4.8 mg L^{-1}; Jürgens et al. 1999) indicating a tendency to adsorb to organic material and accumulate in biota or sediments. EE2 exhibits much lower aerobic biodegradation than E2: the half life of EE2 is 17 d, as opposed to 1.2 d for E2 (Jürgens et al. 2002). On sunny spring days, EE2 degradation can be enhanced by photolysis. Photolysis can reduce EE2's half-life from 20 to 1.5 days (Segmuller et al. 2000; Zuo et al. 2006). This is due to EE2's phenolic functional group, which is susceptible to photodegradation (Zuo and Jones 1997; Pozdnyakov et al. 2004). In this respect, photolysis represents a sink for EE2 in natural surface seawater (Zuo et al. 2006).

1.4. The model species

I used the sand goby (*Pomatoschistus minutus*), a small marine fish, as a model species for examining the effects of EE2 on reproductive behaviour and mating system (Fig. 1). The sand goby has a wide distribution from the Baltic Sea to the Mediterranean Sea, and is present in high numbers, which facilitates reasonable sample sizes. It

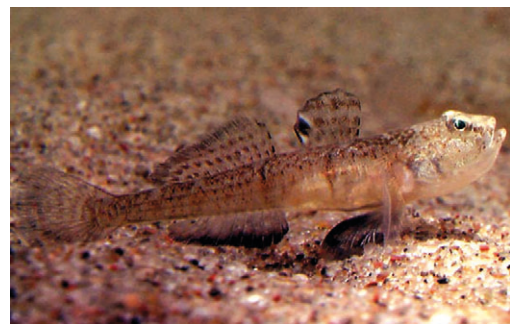

Figure 1. Male sand goby (*Pomatoschistus minutus*).

feeds on zooplankton and benthic invertebrates and has a one-year life cycle (Healey 1971). The sand goby has a resource-defence mating system and male parental care. In early summer it comes to shallow sandy bays to breed. The male builds a nest by excavating a suitable substrate, such as a rock or a mussel shell, and covers it by piling sand on top of it. The male then attracts females by active courtship and tends the eggs until they hatch. Females attach their eggs in a single layer to the ceiling of the nest and leave the nest right after spawning. A male's nest usually contains eggs from several females (Jones et al. 2001), and males and females may spawn repeatedly with multiple partners (Lindström 1988; 1992). Recently, the sand goby has been used in EDC studies in the United Kingdom (EDMAR-project, Matthiessen et al. 2002).

The sand goby is a suitable model for behavioural studies. Its reproductive behaviour is well documented (e.g. Healey 1971; Lindström 1998; 1992; Järvenpää and Lindström 2004; Lehtonen et al. 2007; Lindström

et al. 2007), and the species shows its natural behavioural repertoire in captivity. Females base their mate choice on several cues, including male size, courtship display, parental care, and nest size and quality. Male size can be a sign of genetic quality (Berglund et al. 1996), and large males may be more efficient in caring for the eggs and defending the offspring (Andersson 1994). Large males are more successful in male-male competition, but not necessarily favoured by females (Forsgren 1997). Sand goby females prefer males that court intensively (Forsgren 1997) and show a high level of parental care (Lindström et al. 2006), i.e. females use parental care behaviour directly in mate choice (Lindström et al. 2006). Moreover, females can base their mate preferences on a combination of male body size and nest size (Lehtonen et al. 2007). The nest, built by the male, is an important resource because the female deposits her eggs there and it is crucial for egg development. For a female, the size and location of the nest are signs of male quality, as building big nests demands more energy, while the ability to find and defend a good location could decrease predation pressure on the nest (Kvarnemo et al. 1998; Hansell 2000; Östlund-Nilsson 2000).

1.4.1. Female mate choice

Mate choice is dependent on the environmental and social context, and varies among females (Qvarnström 2001). Environmental factors, such as predation pressure can cause females to give up their preference for colourful males (Forsgren 1992). Moreover, water turbidity can lead to a more random distribution of mating success, resulting in a breakdown of sexual selection (Järvenpää and Lindström 2004). Social factors, like intrasexual competition, may prevent females from mating with their preferred male (Jennions and Petrie 1997; Wong and Candolin 2005). A male's attractiveness is also related to his genotypic quality in the present environment (Qvarnström 2001). Since female choice is, in addition to environmental and social factors, also dependent on the female's own properties, the same male does not gain the highest mating success everywhere. A female's body condition, for example, determines the distance a female is ready to travel to choose a mate, and therefore her mate sampling strategy. Furthermore, females vary in their ability to discriminate among male signals (Jennions et al. 1997). Females also favour genetically dissimilar or compatible males (Widemo and Saether 1999; Mays and Hill 2004), and differ in the attention or weight they give to different mate qualities (Candolin 2003). Therefore, males can be equally attractive in different ways (Jennions and Petrie 1997).

1.5. Aims of the study

My general aim in this work was to investigate how EE2 affects the mating system of sand gobies. The mating system could be described as the distribution of mating success among individuals of the same sex. Because of this, the mating system will set limits on the opportunity for selection. I examined a number of behavioural mechanisms that might be the reasons for a potential disruption of the mating system. Successful reproduction requires that a male sand goby has a nest. After a male has obtained a nest, he needs to attract females. Competing with other males over nest sites and females constitutes a major part of sexual selection in my study population. Therefore, I designed experiments that would test how EE2-exposed males would perform in these tasks, in competition with other males, both EE2-exposed and non-exposed.

The more specific aims were:

1. To assess how EE2 exposure affects the mating system of the sand goby (**II**).

2. To examine how EE2 exposure affects aggression and male-male competition for nests (**IV, III**).

3. To evaluate effects of EE2 exposure on male courtship behaviour (**I, III**).

4. To examine female mate choice, when given the opportunity to choose between an exposed and a control male (**III**).

5. To compare parental behaviour of exposed and non-exposed males (**I**).

6. To compare the performance of traditional molecular biomarkers with that of behavioural measurements (**I-IV**).

In this work, I have only evaluated how exposure of males changes the reproductive behaviour. It is clear that, in the future, it will be equally important to assess the effects of EDCs on female behaviour.

2. MATERIALS AND METHODS

This study consists of four series of experiments performed at the Tvärminne Zoological Station, University of Helsinki during 2005-2008.

2.1. Exposure of fish to EE2 (I-IV)

The setup for exposing the fish to EE2 is illustrated in Fig. 2. This set-up was used in all four experiments except that the exposure concentrations differed for each experiment. All studies were carried out during May - July, which corresponds to the main breeding season of sand gobies in the Northern Baltic. The fish used in the experiments were caught using a hand trawl from a nearby natural breeding site. Only males were exposed to EE2. Before males were introduced into the exposure tanks they were individually marked (not in study **I**) using injected elastomeric colours. The males were then randomly divided among different exposure tanks (80 x 80 x 40 cm) and three tanks were assigned to each exposure treatment. All tanks were provided with a flow-through of fresh seawater. EE2 was dissolved in 2-propanol (< 0.002% v/v) in study **I**, and in acetone in studies **II-IV**. Acetone was evaporated with a light stream of nitrogen before adding the water. Thus, the stock solution in studies **II-IV** did not contain any solvent. The water to the

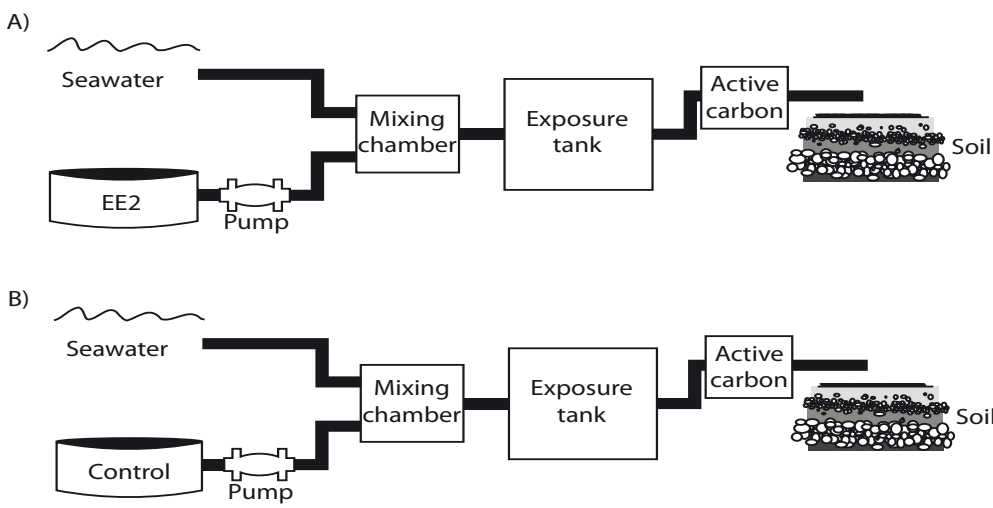

Figure 2. Illustration of the design used in the exposures of male sand goby. (A) 17α-ethinyl estradiol (EE2) and (B) control (in study **I** also a solvent control).

exposure tanks was led through a mixing tank into which EE2 was pumped from a stock solution using peristaltic pumps (Fig. 2). From there the water was channelled into the exposure tanks using silicon tubing. The flow of water (9.6 L/h) was kept similar for all tanks using flow meters equipped with adjustable valves. Before males were introduced to the behavioural experiments they were exposed for 1 to 4 weeks. The first subset of fish was taken after 8 days (except study **I**) of exposure and the following subsets every third or fourth day depending on the duration of the behavioural assays. During the exposure, the fish were fed twice a day with live *Mysis* spp. and frozen chironomid larvae.

2.2. Behavioural experiments (I-IV)

Behavioural experiments were conducted in aquaria with flow-through seawater (except study **I**) to prevent any EE2 effects on non-exposed females. Beforehand, each male was weighed and its length measured (to the nearest mm). Large halved clay flowerpots (Ø 10 cm) were used as artificial nest sites in all experiments. A piece of transparent film cut to fit the dimensions of the nest was placed inside each nest. When spawning, the female attaches the eggs to this film, which can be removed and photographed to count the number of eggs. The state of nest building was checked every morning in all experiments. Fish behaviour was video recorded in studies **I**, **III** and **IV**. In each study, the experimental set-up was designed according to the goals in question. Afterwards, I analyzed the behaviour from the videotapes using a simple event recorder program written specifically for this purpose. The program calculates duration (s) and frequency of the behaviours. Fish were not fed during the behavioural experiments.

2.2.1. Courtship and parental care (I)

The aim of this study was to examine the effects of EE2 on male courtship and parental care in the simplest possible scenario. Study **I** was the first experiment conducted, and without previous experience with EE2 exposures and their effects on sand goby I used a slightly higher concentration of EE2 than in all subsequent studies to have a higher certainty of obtaining responses in reproductive behaviour. Furthermore, I wanted to minimize the time males were without exposure, and therefore continued the EE2-exposure treatment during the behavioural experiments. However, to avoid any EE2 effects on females, no exposure was applied when the females were free swimming in the behaviour experiment tank. Instead, the water was replaced by clean seawater for these periods.

2.2.2. Mating system (II)

The purpose of this experiment was to test how EE2 affects the distribution of mating success among males, i.e. the opportunity for selection. For this, I set up tanks (80 x 80 x 40 cm), each one provided with four similar-sized nest sites. Four randomly selected males, all from the same treatment (either high EE2, low EE2, or control), were introduced into each tank and allowed to occupy and build nests. There was no EE2 exposure during the experiment, and male behaviour was not recorded.

After males had been allowed to build nests for 24 hrs, the first sexually mature female was added and after that a new female was added at every 12 hrs until four females had been introduced. Before adding the next female, the identity of each nest owner male and the presence of eggs were checked. If a nest contained eggs, the transparent film lining the inside of the nest was carefully removed and photographed. The same lining was then carefully returned back into the nest. Twelve hours after the last female had been added (i.e. 60 hrs after the replicate started), and while all four females still were in the tanks, the males were caught and taken into the laboratory for tissue sampling.

2.2.3. Nest competition and female choice (III)

The previous mating system study (**II**) revealed that male body size was a less important determinant of male mating success in EE2-exposed sand gobies as compared to control animals. To understand further why this was the case, I investigated the effects of EE2 exposure on male ability to compete for nest sites and mates. The nest competition experiment consisted of two stages. In Stage 1, the two males competed over an empty nest site, and in Stage 2, the nest holder (winner of Stage 1) was challenged by a new competitor. Male-male interactions were not video recorded during nest competition experiment.

The female choice experiment was done in aquaria that were divided into three compartments by transparent dividers (see Figure in Study **III**). A nest site was placed in each end compartment of the tank. One male exposed to EE2 was put into a randomly chosen end compartment and another non-exposed control male into the other end compartment. As in the nest competition experiment, males were size matched. Male and female courtship behaviour was recorded for 20 minutes, and then the dividers were removed and the female was released to spawn. The presence of eggs in the nest was used as an indication of the female's final choice and the egg masses were photographed.

2.2.4. Male-male competition (IV)

After observing that EE2-males lost in the competition for nest sites (**III**), I needed to know why. Did EE2 affect male agonistic behaviour? Did it make them less aggressive? Earlier studies supported the latter, that exposure to estrogenic EDC decreases the aggressiveness of male fish (Bell 2001; Majewski et al. 2002).

To persuade the males really to defend their nests, I let them spawn with several females. For this study, there was only one nest owning male in each aquarium and males were size-matched between treatments. Male courtship behaviour was video recorded for 20 minutes. After a male had received eggs, three rival males were introduced into the aquarium. This was to mimic the natural nest-constrained habitat of the Northern Baltic. The rival males were all from the control treatment and bigger than the nest-owning male. The nest owner's behaviour was video recorded for 20 minutes.

2.3. Morphometric measurements (I-IV)

The nuptial colouration of each experimental male was scored according to the following: (1) very weak colouration, (2) anal and ventral fins light blue, and (3) anal and ventral fins dark blue with a bright blue spot on the first dorsal fin. Male body mass and total length were measured as well as the mass of several organs and the length of the urogenital papilla (UGP). Gonadosomatic index (GSI), hepatosomatic index (HSI) and sperm-duct gland somatic index (SDGSI) were calculated for each male.

2.4. Vtg and Zrp mRNA expression (I-IV)

Vtg and Zrp were determined from total RNA on slot blots hybridized with [^{32}P]-labelled cDNA fragments and subsequent quantification by phosphor imager using the method described previously (Kirby et al. 2003). In study **III**, only Zrp mRNA expression was determined.

2.5. Analytical chemistry (I-IV)

The EE2 concentration in exposure tanks was measured weekly with liquid chromatograph-mass spectrometer (LC-MS; HS 1100-Waters Quattro II) using monitoring techniques adapted from tandem-spectrophotometric reactions (MRM). A one-litre sample was taken from each EE2-exposure tank and from one control tank every week. External (**I-IV**) and internal (**II, III, IV**) standards were used for the analysis, and with each batch of samples, four-point calibration was conducted.

3. RESULTS AND DISCUSSION

3.1. Reproductive behaviour (I, III, IV)

In study **I**, I assessed how exposure to EE2 affected male courtship and parental care behaviour in the simplest possible situation. During the courtship phase, i.e. before males had received eggs, courtship fanning among control males was positively related to male size (**I**) (Fig. 3). In exposed males, there was no relationship between male body size and fanning rate (**I**) (Fig. 3). Females normally show a preference for larger males (Forsgren 1992), and for males that fan more (Lindström et al. 2006). Hence, a female that would mate with a large male would also mate with a male that fans the most. According to study **I**, under EE2 exposure, a female choosing the exposed males that fan the most would not be mating with the largest males. Indeed, in the study **II**, where I examined the effects of EE2 exposure to the mating system, the male size was observed to be less important determinant for female choice when she could only choose among exposed males. The size difference among mated and unmated males was significantly smaller in EE2 tanks compared to control tanks (**II**). Studies (**I, II**) provide further support that courtship fanning is an important mate choice cue, and that females do not always favour the largest males.

Increased courtship fanning was observed in study **I**, but not in studies **III** and **IV**. This is most likely due to the quite high exposure concentration (41ng L^{-1}) used in study **I**, and that the males were exposed during the first days of the behavioural observations. It is possible that increased fanning represents a toxicity effect. Such an effect could be a sign of a breakdown of behavioural control mechanisms, to which small males responded more strongly than larger males. Furthermore, increased courtship fanning could be an adaptive response to increase current reproductive success. In three-spined sticklebacks, females use the red nuptial colouration of males as a mate choice cue. Redness is an honest signal of male quality as only males in good condition are able to maintain high intensity red colour (Milinski and Bakker 1990). However, some males in very poor condition increase the intensity of the nuptial colouration possibly as a “terminal fitness investment” (Candolin 1999). If exposure to the high level of EE2 was associated with a lower life expectancy, then

the increased fanning of small males could have similarly represented an increased investment in current reproduction, i.e. a terminal fitness investment.

In sand goby, EE2 exposure decreased significantly some elements of courtship in studies **III** and **IV** (study **IV**, Fig. 4). Such behaviours were close swim, where the male courts the female at a very close distance, and leading behaviour, where the male leads the female to his nest. These findings are consistent with several previous

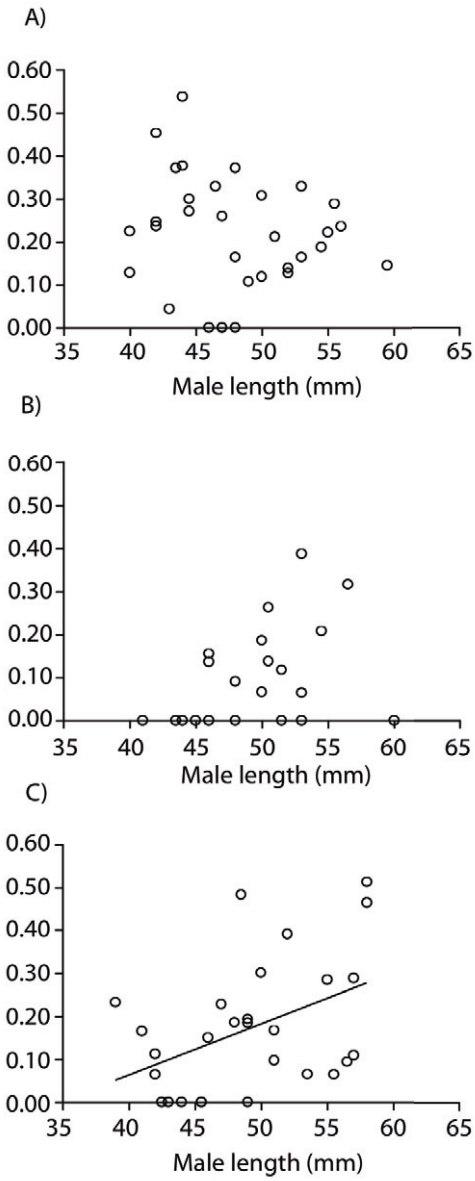

Figure 3. The proportion of time (%) sand goby males displayed courtship fanning in comparison to his body size in different treatments (n = 27 in each group). (A) 17α-ethinyl estradiol (41 ng L^{-1} EE2), (B) solvent (< 0.002% v/v 2-propanol), (C) seawater control. The regression line represents the only significant relationship.

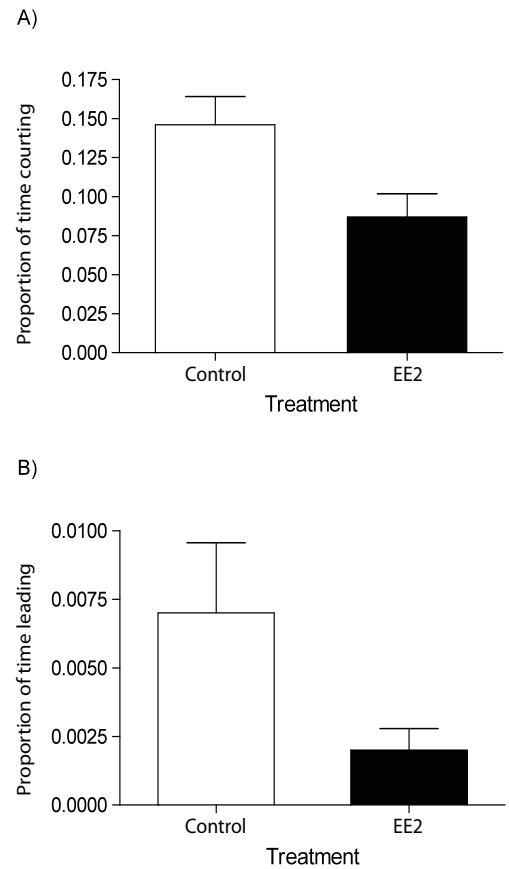

Figure 4. The proportion of time (%) sand goby males spent in (A) courtship and (B) leading female to his nest during the female choice experiment. White bars represent control males (n = 40), exposed to untreated seawater, and black bars males exposed to 4 ng L^{-1} of 17α-ethinyl estradiol (n = 40). Error bars represent one s.e.m.

fish studies conducted at environmentally relevant exposure concentrations (Bayley et al. 1999; Bjerselius et al. 2001; Oshima et al. 2003; Doyle and Lim, 2005; Larsen et al. 2008) (Table 1). Sand goby females prefer males that court intensively (Forsgren 1997), and therefore EE2-induced changes in male performance are likely to decrease the attractiveness of males to females. This could potentially greatly reduce the chances of an individual male reproducing successfully.

Exposure to EE2 increased the amount of time males spent egg fanning during the parental care phase (**I**). Interestingly, egg fanning was not related to the body size of the exposed male. Egg fanning is thought to oxygenate the eggs and possibly remove harmful debris and waste products from the surface of the eggs. In most cave nesting species, fanning is crucial for the survival of the eggs (Clutton-Brock 1991). Egg fanning is costly (Coleman and Fischer 1991; Lindström and Hellström 1993; Lissåker et al. 2003). Hence, it is likely that the increased egg fanning of EE2-exposed males increased their parental care costs. That could lead to a shortened reproductive season and/or decreased male survival. However, at the same time, egg survival may be improved due to increased egg fanning, which will be a clear benefit to both males and females.

The relationship between courtship and parental care behaviour and blood estrogen level is in general unclear. It is affirmed that male fish have high androgen levels during courtship (Knapp et al. 1999; Pankhurst et al. 1999; Rodgers et al. 2006) and during parental care blood androgen levels are often down regulated (Wingfield et al. 1990; Oliveira et al. 2002; Hirschenhauser and Oliveira 2006). The regulatory relationship of hormones and reproductive behaviours is, however, highly variable among species, and thus seems to be specific to the mating system (Ros et al. 2004; Magee et al. 2006). It has been suggested that exogenous estrogen down-regulates endogenous androgen production in males (Borg 1994; Bell 2001), leading to reductions in behaviours that are related to the levels of plasma androgens. This could explain the decreased egg fanning among larger exposed males in study **I**. Furthermore, decreased courtship behaviours observed in studies **III** and **IV** support the androgen-driven down-regulation hypothesis as well. However, without hormonal measurements, I am unable to assess this in detail.

3.2. Nest building (I-V)

I recorded the state of nest building in all studies (**I-IV**). The nest is an important resource for a male because the female deposits her eggs there. Moreover, females prefer males with large and well-constructed nests (Forsgren

1997; Kvarnemo et al. 1998). A large nest can hold eggs from several females.

During the behavioural experiments, all nests were checked every morning and their construction level recorded. Study **IV** revealed a difference between the exposed and control males, as EE2-exposed males took more time to build a nest. Only 46% of the exposed males had built a nest within the first 12 hours, whereas most of the control males (69%) had done so (**IV**). However, the total number of nests built during the experiment did not differ significantly between the treatments (Control = 43 nests, EE2 = 40 nests). Hence, exposed males were somewhat slower to build nests than control males. This is in accordance with earlier studies on three-spined sticklebacks (Wibe et al. 2002; Brian et al. 2006).

Overall, slowness in nest building can be costly for a sand goby male, because, e.g., in the present study area nest sites are scarce and the few nest sites are quickly colonized (Forsgren et al. 1996). Moreover, late nesters attract fewer or no females (Mori 1993), which, via a smaller number of built nests, is likely to impair a population's overall reproductive success.

3.3. Competition over nest sites and mates (III, IV)

After observing that EE2 exposure impairs courtship behaviour of sand goby, I was interested in knowing how the compound affects a male's ability to compete for nest sites and mates. Furthermore, if male competitiveness is weakened, could it be related to decreased aggressiveness? I conducted two studies where it was first looked at how exposed males handle competition over an empty nest site and mates (**III**), and then, how exposed males defended their nest after they had already received eggs (**IV**). Additionally, I observed the aggressive behaviour of EE2-exposed males in a male-male competition context (**IV**).

Study **IV** revealed, in conjunction with previous studies (Bell 2001; Majewski et al. 2002), that EE2-treated males were less aggressive against rivals than control males. EE2-treated males spent significantly less time displaying their operculum (erecting the gill covers) than control males and repeated operculum displays less frequently than control males (**IV**) (Fig. 5). Operculum display has been shown to correlate with an elevation in energy metabolism (Castro et al. 2006). Hence, the display might be used as a signal of energy expenditure in communication (Castro et al. 2006) and thus settle the conflict between males without an escalated fight. Ritualized agonistic displays are typically costly to produce and therefore honest signals of male readiness and ability to fight (Zahavi 1977; Grafen 1990; Castro et al. 2006). Honest information about the rival's

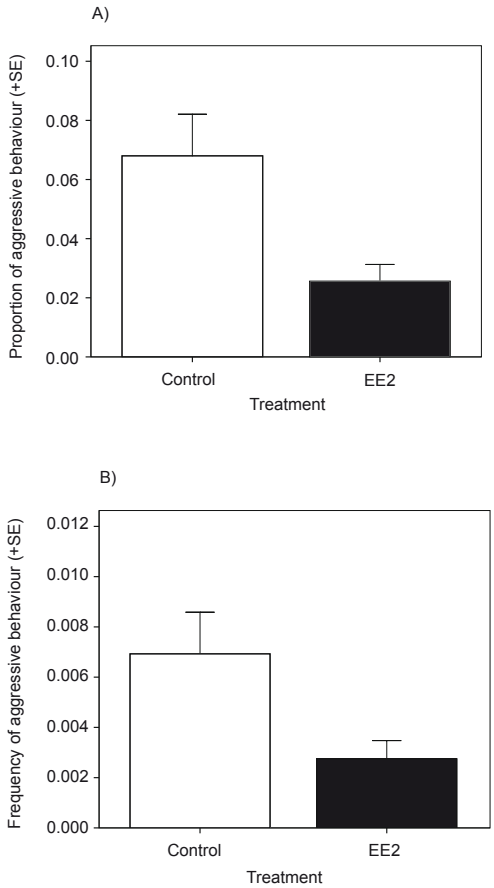

Figure 5. (A) The proportion of time (%) sand goby males spent giving operculum displays and (B) frequency (times per second) of this aggressive behaviour during the experiment. White bars represent control males (n = 39) exposed to untreated seawater and black bars represent males exposed to 8 ng L^{-1} of 17α-ethinyl estradiol (EE2) (n = 39). Error bars represent one s.e.m.

competitive ability helps a male to adjust its behaviour to match expected costs and benefits (Beaugrand 1997; Lopez and Martin 2001). The decrease in aggressive displays by EE2-exposed males can be an honest signal of fighting ability only if they truly are weaker fighters. If these males are not weaker but still display at a lower level, they may receive more attacks from rival males. These displays are likely to escalate into fights, which might involve injuries and increase predation risk (Hurd 1997; Briffa and Sneddon 2007).

EE2-exposed males were unsuccessful at competing for empty nest sites (**III**). They were able to occupy the offered nest site in only 25% of the cases, when competing with a similar-sized control male (Fig. 6) (**III**). When the resulting nest holder was challenged with a new same-size male from the opposite treatment, all control males kept their nests, but only half of the EE2-treated males could keep theirs (Fig. 6) (**III**). Exposed males were clearly unable to acquire or defend nest sites.

When males were allowed to mate and defend eggs before facing challenges to their ownership, the same number of control and EE2-treated males lost their nest to rival males

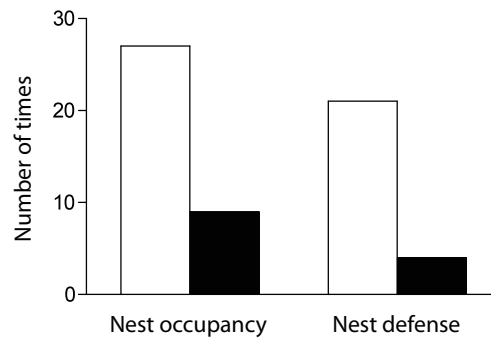

Figure 6. Number of times a nest site was occupied or taken over by male sand gobies exposed to clean seawater (white bars) or to 4 ng L^{-1} of 17α-ethinyl estradiol (black bars) in competition for a nest site. 'Nest occupancy' refers to the situation where the two males competed for an empty nest site (Stage 1) (n = 36 ,total number of individuals). 'Nest defense' refers to the situation where the winner of Stage 1 was defending his nest site against a new male from the opposite treatment (Stage 2) (n = 31, total number of individuals).

(**IV**). A nest with eggs has more fitness value for its owner than an empty nest, while at the same time its value for an intruder has not changed. Because of the higher fitness value one expects that the males should increase their defence of the nest (Sargent and Gross 1993). This difference in fitness value could well explain the higher success of EE2-exposed males in study **IV**.

The result that EE2-exposed males of the sand goby were successfully defending the nests against rivals (**IV**) is still puzzling because exposed males were less aggressive than controls (**IV**). Aggressive behaviour is often related to territory and nest ownership (Whoriskey and FitzGerald 1994). How did the exposed males still manage to defend their nests? One explanation is that I missed some communication between the nest holder and rival males while observing the behaviours. Odours might have played a role here, as fish release hormones as pheromones (Stacey and Sorensen 1991) and it is possible that also exogenous estrogenic exposure affects the chemical communication of exposed individuals. Furthermore, rival males probably had different motivational statuses and adjusted their aggressive behaviour according to behaviour of the nest owner and the other rival males. Information about the rival's competitive ability helps a male adjust its behaviour to match expected costs and benefits (Beaugrand 1997; Lopez and Martin 2001). Thus, in the situation where three rival males were in the same aquarium, taking over the nest from the weaker nest holder male may not have been worth a battle because the new owner would have had to fight against the two other equally competitive males.

Female sand gobies do not seem to favour dominant males and dominant males have been shown to be poor fathers (Forsgren 1997), even if they acquire and defend the highest quality nests (Lindström 1992). This could favour EE2-exposed males if females pay attention to male dominance and agonistic traits. However, in study **III**, females preferred to mate with control males, which were successful in male-male competition and presumably more aggressive during male-male interaction. This suggests that females are not trying to avoid aggressive or dominant males per se but rather that they are paying attention to courtship traits. In addition, it suggests that courtship traits are not correlated with male dominance, but this needs further investigation.

3.4. Mating system (II)

To understand what would be the consequences of impaired reproductive behaviour on mate choice and sexual selection, I conducted a mating system study, which was an attempt to come a step closer to understanding the population-level interaction in presence of

EE2 contamination.

This study showed that male body size was a less important determinant of male mating success in EE2-exposed sand gobies as compared to control animals (**II**). Mated males were larger and selection on male body length was more intense in control than in EE2 tanks (**II**) (Fig. 7). The sand goby exhibits a polygynous mating system in which male mating success is typically skewed towards the largest males. This leads to strong sexual selection for increased male size.

There are two potential explanations for the change in sexual selection that was observed. Firstly, females may have been constrained in their ability to distinguish among male quality if males did not perform adequately. I do not think females changed their preferences because females were unexposed to EE2 and there was no EE2 exposure in the experimental tanks. Indeed, a change in mate choice could be due to impaired courtship behaviour of exposed males (**I, III, IV**). As a result, males may have appeared as a grey mass, where they all behaved the same, making it difficult for females to distinguish among males. Also, chemical cues might have been diminished, as shown by a study on red-spotted newts (*Notophthalmus viridescens*) where exposure to the insecticide endosulfan impaired the pheromonal system, leading to disrupted mate choice and decreased mating success (Park et al. 2001).

The second potential explanation is that exposure to EE2 changed male dominance relations and bigger males no longer had an advantage. EE2 exposure decreased the aggressive be-

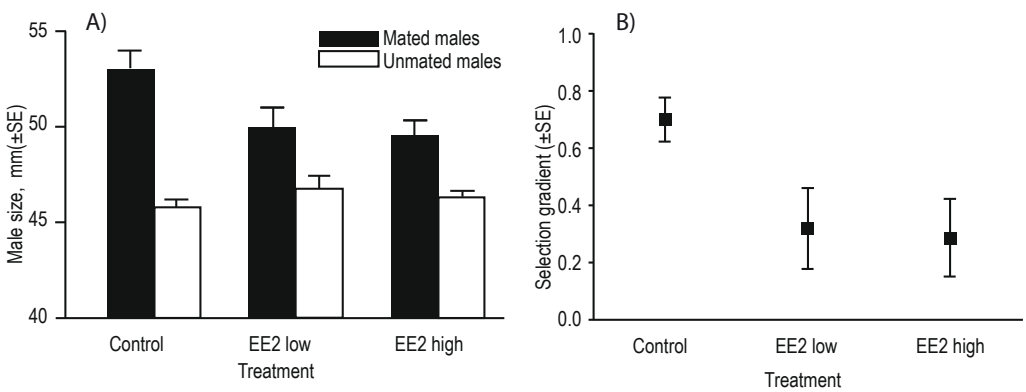

Figure 7. (A) Size of mated and unmated males in three 17α-ethinyl estradiol (EE2) exposure treatments. Black bars represent the mean total body length of mated males (n = 12 trials) and white bars for unmated males (n = 12 trials). (B) Sexual selection gradient on male total body length in three EE2 exposure treatments. Standardized selection gradients are calculated as the regression coefficient between a male's relative mating success and his relative total body length. Relative mating success is based on the number of eggs a male received divided by the mean number of eggs received by males in that particular experimental tank. Similarly, relative total body length is calculated as a male's total body length divided by the mean total body length of all males in that particular experimental tank. The three treatments are: Control = males exposed to untreated seawater (n = 15 trials), Low = males exposed to 5 ng L^{-1} EE2 (n = 15 trials) and High = males exposed to 24 ng L^{-1} of EE2 (n = 15 trials). Error bars represent one s.e.m.

haviour of males (**IV**), and this could lead to a situation where all males had equal chances to obtain nest sites and mates. Equal chances could result in a situation where the owner of the largest nest in the most valuable location was no longer the strongest male. Decreased aggression could also lead to less interference among males during mate attraction, hence reducing the advantages of more dominant males.

As shown by the study **III**, females prefer control males to exposed males as mating partners. This was most likely due to impaired courtship behaviour of exposed males and their weakened ability to defend and acquire a nest site (**III**). However, increased courtship fanning (**I**) and decreased aggressiveness (**IV**) instead might even improve EE2-exposed males' mating success. Females favour males that fan more during courtship (Lindström et al. 2006) but do not dominate the population because dominant males have been shown to be poor fathers (Forsgren 1997). The dilemma, however, is how a less dominant male acquires a large nest in a good location? For a male, a large nest is important as the number of eggs that can fit into a nest is limited by its size. Sand goby females also prefer large nests, even if the nest is guarded by a small male (Lehtonen et al. 2007), except under conditions of intense male-male competition when male body size again becomes important (Lehtonen and Lindström 2009). Thus, when female choice is affected by both environmental and social factors, it becomes exceedingly difficult to predict how contamination by xenoestrogens will affect the mating system. Study **II** demonstrated that EE2 contamination resulted in a relaxation of sexual selection, which in the long run may lead to changes in the genetic structure of the population.

3.5. Molecular biomarkers: Vtg and Zrp (I-IV)

To ensure that EE2 exposure had a physiological effect on the male sand gobies, I used Vtg and Zrp mRNA expressions as biomarkers of exposure. The expression of both of these markers was measured in studies **I, II** and **IV**, but only Zrp was measured in study **III**. I used mRNA expression instead of detection of proteins by ELISA because no antibodies for Vtg and Zrp proteins in the sand goby were available.

Exposure to EE2 at environmentally relevant concentrations (< 5ng L^{-1}) did not induce Vtg and Zrp mRNA expression (**II, III**) (study **II**: Fig. 8). Vtg and Zrp mRNA revealed EE2 exposure only if the exposure concentration was over 5 ng L^{-1} (**I, II, IV**). Exposure duration in all studies (**I-IV**) was from 1 to 4 weeks, but males had spent at least two days in clean water during the behavioural assays before they were sampled for the expression studies. This may have decreased the induction of Vtg and Zrp mRNA. However, because the half-life

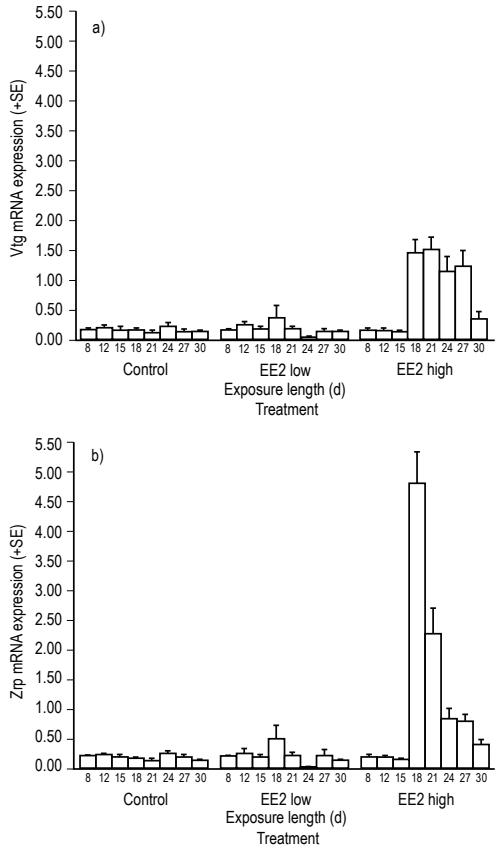

Figure 8. Effects on (A) male hepatic vitellogenin and (B) male zona radiata protein mRNA expression in different treatments. Sand goby males were exposed from 8 to 30 days to 17α-ethinyl estradiol (EE2) or untreated seawater. Bars represent mean expression level (arbitrary units) + standard error of mean. The three treatments are: Control = males exposed to untreated seawater (n = 62), Low = males exposed to 5 ng L^{-1} (n = 61) and High = males exposed to 24 ng L^{-1}- EE2 (n = 62).

of Vtg mRNA is 4.4 days in sand gobies (Craft et al. 2004), it should still have been able to see differences between exposed and control males.

A clear dependence of exposure length on expression levels of Vtg and Zrp mRNA was found in studies **II** and **IV**. Moreover, there was a significant interaction between exposure treatment and exposure time for both Vtg and Zrp mRNA levels (**II**). There was an initial increase and then a decrease in the expression levels, while the control treatment showed nearly no expression at all and no time effect. The appearance of both transcripts followed a delay (Craft et al. 2004) before reaching a maximum and then declining as observed in earlier studies (Robinson et al. 2003; Brown et al. 2004). Such time dependence could be due to physiological acclimation e.g. increased biotransformation capacity or elimination of xenoestrogens with time (Walker et al. 2006). The important point is, in any case, that it is not the total length of exposure that seems to affect the expression of molecular biomarkers but the particular timing when the biomarkers are assayed. If the expression levels of both markers for the shortest and longest exposure times are compared, there is no difference between the low EE2 exposure and the control. Thus, if only these subsets of samples had been analyzed, the effect of EE2 would have been missed.

3.6. Was behaviour a sensitive biomarker of EE2 exposure?

This work showed that behaviour is a sensitive biomarker of EE2 exposure when studying effects at environmentally relevant concentrations. Reproductive behaviour and mating system responded to low concentrations of EE2 (**II**, **III**), while molecular markers

showed no signs of exposure. This work highlights the importance of using several biomarkers when studying effects of EDC contamination. Furthermore, results of this work underline that the absence of hepatic responses, measured as two specific mRNA expression biomarkers, does not mean the absence of significant reproductive effects.

Behavioural assays, however, do have their disadvantages. They demand a lot of space, working hours, and money to be successful. Such resources are not often available for routine administrative use and this is likely to limit the use of behavioural assays. Behavioural assays are probably most useful when done on locally relevant species. Hence fish should be wild caught. I used sand gobies that were collected from the wild and fish might have been exposed to xenoestrogens during their earlier life. Background contamination is likely to vary between the years and be under the detection levels of currently used biomarkers, but still affecting the fish. However, this applies to molecular biomarkers as well as to behavioural assays.

The consequences of impaired behaviour on survival or reproduction are difficult to interpret because plasticity of behavioural patterns may alleviate the net harm of contamination (Dell'Omo 2002). However, since behavioural plasticity is based on the ability of a single genotype to produce an alternative behaviour, it may vary between individuals (West-Eberhard 1989). Some individuals may thus be unable to adapt their behaviour to contaminant-induced changes in their environment (Dell'Omo 2002) and suffer from diminished reproductive success.

I conclude that although behavioural assays will probably never replace the more-used ecotoxicity assays and standardized tests, they should be considered as important complementary tools in ecotoxicological studies and especially in assessments of the biological impacts that contamination may have.

4. FUTURE PERSPECTIVES AND NEW CHALLENGES

There is a growing need to study sublethal effects of pollutants and understand the consequences of exposure at the population level. Research should move from examining reproductive behaviour to studying reproductive success (hatching rate, egg survival, fecundity), and get closer to assessing the effects on future generations. A step even further is to study population genetic responses. Male body size, for example, often has a genetic basis and changes in mating behaviour in response to environmental contaminants may have genetic consequences for the population (Scott and Sloman 2004). Moreover, if only a short part of a life cycle is studied, some effects caused by pollutants might be overlooked. Exposure at the adult stage to low levels of perchlorate, for example, caused no differences in the behaviour or reproductive output of three-spined stickleback, but their offspring, exposed throughout development, had poorer swimming skills and impaired reproductive behaviour (Bernhardt and Hippel 2008). Future studies should further explore the links between alterations in reproductive behaviour, reproductive output, and the mechanisms that govern population dynamics. Indeed, the implications of these interactions for population survival need to be addressed more.

The next step in behavioural ecotoxicology is to design a behavioural assay for toxicity testing for monitoring purposes. It could be based on courtship, parental care or agonistic behaviour. The assay should be a simple, focused method with good repeatability, and taking a reasonably short time to perform (see Table 2). For example, the nest competition experiment used in Study III could be such an assay.

Assessing the risks of EDCs to aquatic organisms is a challenge. EDCs can act additively at concentrations that by themselves are harmless but in mixtures can be more toxic than the individual compounds (Thorpe et al. 2005). EDCs may have non-classical dose-response relationships, i.e. low doses cause stimulatory responses, but higher doses inhibitory responses (Calabrese, 2005). Their action might have delayed effects as exposures received during early life stages may become evident only at adulthood. Hence, EDCs should be assessed at ecologically relevant concentrations and mixtures using different species at different life stages and, if possible, under natural conditions. In addition, post-regulatory monitoring should

not be forgotten because there probably will always be chemicals that slip through the risk assessment and cause significant environmental damage. This list does sound like a mission impossible. Comprehensive tests covering also behavioural responses will be costly, but the price of underestimating the effects and consequences of EDCs for terrestrial and aquatic organisms will be much higher.

Table 2. Behavioural assays for toxicity testing and monitoring of estrogenic actions in fish.

Behavioural assay	Tanks / treatment	Fish / tank	Exposure ng/L	Exposure period*	Set-up time	Trial time	Video recording
Resource competition	20	2 males	1, 5, 10	15d	2-3h	48h	no
Courtship: leading	20	1 male 1 female	1, 5, 10	15d	12h[1]	10min	Yes
Parental care: fanning	20	1 male	1, 5, 10	15d	24h[2]	10min	Yes

*Set of samples in days 7, 11 and 15

[1] Time for nest building (12h)

[2] Time for nest building and courtship to receive eggs

5. CONCLUSIONS

This thesis demonstrates that exposure to environmentally relevant concentrations of EE2, an estrogenic EDC, greatly impair the reproductive behaviour of male sand gobies. EE2 exposure reduced the ability of males to acquire and defend a nest, as well as decreased the attractiveness of males to females by decreasing courtship and aggressive behaviour. Furthermore, sexual selection on male size was relaxed after EE2 exposure, suggesting that females were unable to distinguish the differences among males. These effects are harmful for a male whose reproductive success is determined by the ability to compete for limited resources and to attract mates.

This work highlights the fact that behaviour can be at least as sensitive marker of EE2 exposure as current molecular tools. The observed behavioural changes have direct and negative effects on fitness, while the connection between molecular expression and fitness may be less obvious. This thesis demonstrates that severe behavioural effects can sometimes be seen before effects are detectable at the molecular or morphometric level. “The absence of evidence is not evidence of absence” (Zala and Penn 2004); thus several types of biomarkers should be used because the lack of responses in one or two markers does not mean the absence of significant effects elsewhere.

6. ACKNOWLEDGEMENTS

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