Hormones and Behavior 55 (2009) 530–537

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Disruption of sexual selection in sand gobies (*Pomatoschistus minutus*) by 17α-ethinyl estradiol, an endocrine disruptor

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ARTICLE INFO

Article history:
Received 5 December 2008
Revised 21 January 2009
Accepted 21 January 2009
Available online 30 January 2009

Keywords:
Endocrine disruptor
Sexual selection
Reproductive behavior
Sand goby
Pomatoschistus
Molecular biomarkers

ABSTRACT

In aquatic environments, endocrine disrupting chemicals (EDCs) that interfere with the reproductive physiology of males form a threat to the reproduction of populations. This is often manifested as decreased sexual performance or sterility among males. We show that exposure to EDCs can directly affect the mating system of a marine fish, the sand goby (*Pomatoschistus minutus*). We exposed males for 1 to 4 weeks to two different concentrations (5 ng L^{-1} and 24 ng L^{-1}) of 17α-ethinyl estradiol (EE2); a synthetic compound mimicking estrogen and a water control. The sand goby exhibits a polygynous mating system, in which male mating success is typically skewed towards the largest males, resulting in strong sexual selection for increased male size. Our experiment shows that when males have been exposed to EE2, male size has a smaller effect on mating success, resulting in weaker sexual selection on male size as compared to the control. There was an interaction between treatment and exposure time on the expression of vitellogenin and zona radiata protein mRNAs. Males exposed to high EE2 reached much higher expression levels than males exposed to low EE2. Of the somatic markers, the hepatosomatic index was lower in males exposed to high EE2 than in the low EE2 and control males. Our results suggest that exposure to EDCs can have effects on the mating system before physiological changes are observable. These effects can be of profound nature as they interfere with sexual selection, and may in the long run lead to the loss of traits maintained through sexual selection.

Introduction

Research on endocrine disrupting chemicals (EDCs) and their effects on wildlife at the molecular and physiological level has increased considerably during the last decades (Sumpter and Jobling, 1995; Desbrow et al., 1998; Kramer et al., 1998; Andersson et al., 2007). However, EDCs are also known to result in behavioral changes and it has recently been suggested that more research should focus on these effects (Clotfelter et al., 2004). With regard to marine and freshwater organisms, few studies have investigated the effects of EDCs on reproductive behavior with only a limited number of studies on fish (Bayley et al., 1999; Bjerselius et al., 2001; Wibe et al., 2002; Ros et al., 2004; Martinovic et al., 2007). Obviously, behavioral studies are more challenging to carry out and interpret than classical toxicity tests, but a single behavioral parameter is in many ways much more comprehensive than a physiological or biochemical parameter considering its ecological relevance (Jones and Reynolds, 1997). In addition, a change in the reproductive behavior of an organism is a good signal of very early-stage alterations in molecular traits (Smith and Logan, 1997). An alteration in reproductive behavior is believed to be of particular ecological relevance since this, in most cases, will result in direct effects on reproductive success (Wibe et al., 2002).

Reproductive behavior is usually hormonally regulated and disruptions in this regulation may lead to severe behavioral changes that disrupt intra- and intersexual communication. As this communication breaks down, one may expect that it changes the species' mating system, which can be defined as the distribution of mating success among individuals, and hence the operation of sexual selection (Reynolds, 1996). Eventually these disturbances may lead to a breakdown of sexual selection mechanisms (Andersson, 1994), which can have profound future consequences for populations, communities and the entire local ecosystem (Jones and Reynolds, 1997; Seehausen et al., 1997).

In the present study, we used the sand goby (*Pomatoschistus minutus*) as a model organism to test how EDCs affect the mating system of a marine fish species. The sand goby is a small (42–58 mm in length) marine fish with a one-year life cycle (Healey, 1971). It feeds on zooplankton and benthic invertebrates and occurs over a wide salinity and temperature range. The sand goby has a resource-defense

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doi:10.1016/j.yhbeh.2009.01.006

mating system and male parental care: the male builds a nest under a suitable substrate, attracts females by courtship and tends the eggs until they hatch. A male's nest usually contains eggs from several females (Jones et al., 2001) and females can spawn with several males over the season. Nest availability and size determines the distribution of male mating success and sexual selection in the sand goby (Forsgren et al., 1996; Lindström and Seppä, 1996). In addition to male–male competition (Lindström, 1988, 1992), female mate preference is an important process determining male mating success. Female sand gobies show mate preferences based on a number of male traits such as size, color and behavior (Forsgren, 1992; Lindström et al., 2006).

To study the effects of estrogenic compounds on the mating system of sand goby we chose the pharmaceutical 17α-ethinyl estradiol (EE2) as our model compound. EE2 is used in oral contraceptives and it is more persistent in the environment than natural steroids (Jürgens et al., 1999; Young et al., 2004). EE2 exhibits much lower aerobic biodegradation than E2: the half life of EE2 is 17 d, as opposed to 1.2 d for E2 in the River Thames in spring 2000 (Jürgens et al., 2002). It has been shown to induce vitellogenin (Vtg) production at concentrations as low as 0.1 ng L^{-1} (Purdom et al., 1994) and cause collapse of wild fathead minnow (*Pimephales promelas*) populations after 2-year chronic 5 ng L^{-1} exposure (Kidd et al., 2007). Prolonged exposure to EE2 (6 months at 6 ng L^{-1}) under laboratory conditions also resulted in significant reduction of reproductive success in sand goby (Robinson et al., 2003). EE2 has been detected in ecologically relevant concentrations (<5 ng to 15 ng L^{-1}) from sewage effluents, surface and river waters, and activated and digested sludge (Baronti et al., 2000; Johnson and Sumpter, 2001; Muller et al., 2008).

To ensure that EE2 exposure was having a physiological response on the fish, two molecular biomarkers were used in this study. Vitellogenin (Vtg) is an egg yolk precursor protein (Mommsen and Walsh, 1988) and the classical biomarker for estrogenic exposure in male fish. Because males express estrogen receptors in liver, exposed animals produce Vtg which can be conveniently detected in sand goby by the appearance of Vtg mRNA (Kirby et al., 2003; Robinson et al., 2003). Zona radiata protein (Zrp) is a protein which forms the inner core of the eggshell (Oppen-Berntsen et al., 1992) and like Vtg is produced in the liver of male fish exposed to estrogens and can also be detected at the mRNA level (Robinson et al., 2003). Vtg and Zrp have been widely used as sensitive biomarkers of exposure to EDCs in both monitoring and laboratory testing (Arukwe and Goksoyr, 1998; Christiansen et al., 2000; Arukwe et al., 2000; Kwak et al., 2001; Boon et al., 2002; Robinson et al., 2003), but it has been suggested that Zrp may have greater sensitivity in some species (Arukwe et al., 2000).

We exposed sand goby males to two concentrations of EE2 (intended concentrations of 25 ng and 50 ng L^{-1}, but when measured from the seawater in the exposure tanks 5 and 24 ng L^{-1}) and then allowed four males at a time to compete for matings in experimental pools. The aims of this study were (1) to test if exposure to EE2 affects the mating system of the sand goby, and (2) to compare two ways of detecting EE2 exposure: molecular biomarkers (Vtg and Zrp) and behavioral studies. We expected EE2 exposure to change the mating system of the sand goby, because in another study (Saaristo et al. in preparation) we found that exposed males were less competitive and less preferred by females than control males.

Material and methods

Study site and exposure setup

The study was carried out at the Tvärminne Zoological Station, southern Finland, during May–July 2006, which corresponds to the main breeding season of sand gobies in the Northern Baltic. The fishes used in the experiments were caught at a nearby natural breeding site using a hand trawl, brought back to the lab and sorted into males and females. Only males were exposed to EE2. Before the males used in the experiments were introduced into the exposure tanks they were individually marked using injected elastomeric colors (Northwest Inc). Before marking they were anaesthetized in benzocaine. The males were then randomly divided among 6 different exposure tanks (80×80×40 cm) and two tanks were assigned to each of three different exposure treatments: 1) high concentration of EE2 (actual 24, intended 50 ng L^{-1}), 2) low concentration EE2 (actual 5, intended 25 ng L^{-1}) and 3) water control (no exposure to EE2). All tanks were provided with a flow-through of fresh seawater. The water to the exposure tanks was led through a mixing tank into which EE2 was pumped from stock solution using peristaltic pumps (Watson Marlow). From there the water was channeled into the exposure tanks using silicon tubing. The flow of water was kept similar for all tanks using flow meters (Kyrömäki) equipped with adjustable valves. The males used in the experiment were exposed for 1 to 4 weeks before introducing them to the mating system experiment. The first subset of fish was taken after 8 days of exposure and the following subsets every fourth day until the day 31. During the exposure the fishes were fed *ad libitum* with live *Mysis* spp. and frozen chironomid larvae.

Mating system experiment

The design follows a previous study (Lindström and Seppä, 1996) in its general layout. The experiment was carried out in clean seawater in order to prevent any EE2 effects on females. The experimental pools (80×80×40 cm) were equipped with a continuous flow-through of seawater. Each pool was provided with four clay flowerpots (10 cm in diameter) as nest sites. The inside of the each flowerpot was lined with a transparent film cut to fit the nest dimensions. Female sand gobies attach their eggs to the inner, upper surface of the nest in a single layer when spawning. The film can then be removed and photographed in order to count the number of eggs. After the nest sites were added to each pool, four randomly selected males were introduced into each and they were allowed to occupy and build nests

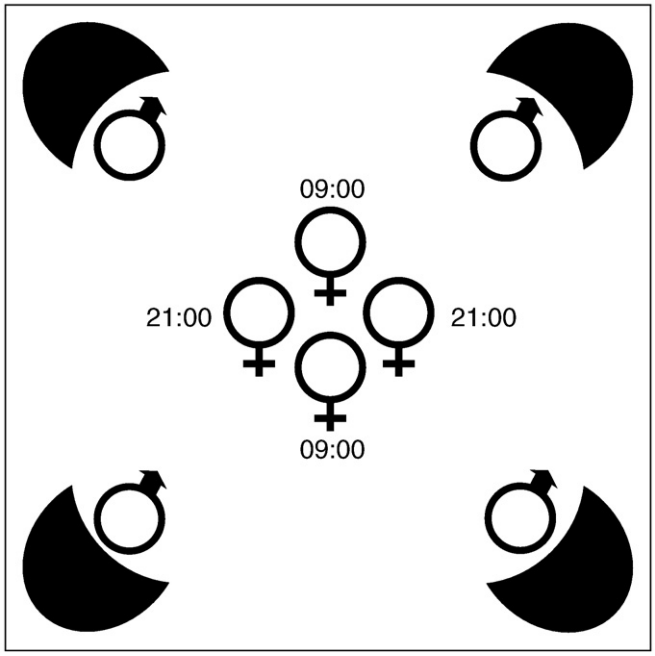

Fig. 1. Design for mating system experiment. Each pool had four nest sites (similar in size), and each nest had a transparent film lining inside. Four males of different size, but from same treatment, were placed to the pool in the evening. After 24 h of nest building a sexually mature female was added every 12 h until 4 females had been introduced.

(Fig. 1). Beforehand, each male was weighed and its length measured (to the nearest mm). As males were randomly selected there were no differences in male size among treatments (ANOVA, $F_{2,\ 189} = 0.288$, $p = 0.750$). Two replicates of each treatment were run simultaneously, and this was repeated eight times, producing a total sample size of 48 (16 replicates per treatment).

After the males had been allowed 24 h for nest building, the first sexually mature female was added and after that a new female was added at every 12 h 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 using a digital camera (CANON Powershot G5). The lining was then carefully returned back into the flowerpot allowing the eggs to be counted later. Twelve hours after the last female had been added (i.e. 60 h after the replicate started), and all four females still were in the pools, the males were caught and taken into the lab. The nuptial coloration of each male was scored according to the following: (1) very weak coloration, (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. Males were then anesthetized using benzocaine. The weight and length of the individual were measured as well as the length of the urogenital papilla (UGP). The fish was killed by cutting the spinal cord, and the liver, gonads and accessory glands were excised and weighed. The liver was snap-frozen in liquid nitrogen and stored in −80 °C. Gonadosomatic index (GSI), hepatosomatic index (HSI) and sperm-duct gland somatic index (SDGSI) were calculated as follows: tissue weight / total weight × 100.

Measures of sexual selection

We used four different measures of sexual selection. First, as a measure of the selection differential the size of mated and unmated males were compared. Second, the size of the male receiving the first mating was used as a measure of female mate preference (Järvenpää and Lindström, 2004). Third, as a measure of opportunity for selection, the coefficient of variation (CV) was calculated in mating success, measured as the number of eggs a male received within each replicate (Forsgren et al., 1996; Lindström and Seppä, 1996). Fourth, the selection gradient on male size (Arnold and Wade, 1984a) was calculated. The selection gradient is the slope of the regression of relative male mating success on relative male size. The former is obtained as the number of eggs male guards divided by the mean number of eggs guarded by all the males in that particular pool. Similarly, relative male size is a male's body length divided by the mean body length of all males in that particular pool.

Measurement of actual EE2 concentrations in exposure tanks

The concentration of EE2 in the exposure tanks was measured by liquid chromatograph-mass spectrometer (LC–MS; HS 1100-Waters Quattro II) using monitoring techniques adapted from tandem-spectrophotometric reactions (MRM). A one-liter sample was taken from each EE2-exposure tank and from one control tank every week, acidified to pH 2 with formic acid. Furthermore, four control samples were prepared from the stock solution (3, 10, 25 and 50 ng L^{-1}). The samples were vacuum-filtered using glass fiber filters (GF/C, 1.2 μm, Whatman) and cleaned up using the solid-phase extraction (SPE) method. The one-liter samples were loaded on Oasis HLB cartridges (5 ml) preconditioned with 10 ml of methanol followed by 10 ml of Milli-Q (MQ) water. After loading, the cartridges were washed with 10 ml of MQ water. The samples were dried using a vacuum pump for one hour and then eluted with 8 ml of ethyl acetate, which was then evaporated with nitrogen flow. To the pure sample 250 μl of methanol and 50 μl of MQ were added to prepare the sample for liquid chromatograph–mass spectrometer (LC–MS) analysis.

The LC–MS was operated in the negative electro spray ionization mode using multiple reactions monitoring (MRM). EE2 was fractionated from the matrix with liquid chromatograph, ionized by atmospheric pressure chemical ionization (APCI) and analyzed following two reactions of the molecule-ion of the proton-EE2 ($[M+H]^+$; m/z 279): m/z 279>159 (0.2 s) and m/z 279>133 (0.2 s). External standards were used for the analysis, and with each batch of samples four-point calibration curves were made from the 4 control samples.

The concentrations of EE2 in the exposure tanks were found to be lower than intended: mean EE2 in the lower exposure concentration was 5 ng L^{-1} (SD = 2.3, $n = 4$) rather than the intended 25 ng L^{-1}, while the higher exposure concentration was 24 ng L^{-1} (SD = 1.7, $n = 4$) rather than the intended 50 ng L^{-1}. The concentration of EE2 in the control tanks was below detection limit (<1 ng L^{-1}) throughout the exposure period.

Vtg and Zrp mRNA expression

Vtg and Zrp was determined in total RNA on slot blots hybridised with [32P]-labelled cDNA fragments and subsequent quantization by phosphor imager using the method described previously (Kirby et al., 2003).

Total RNA isolation and isolating Vtg and Zrp-fragments from the total RNA

Total RNA was isolated from the liver of sand goby liver (5–30 mg tissue) using the NucleoSpin RNA II kit (Marchery-Nagel, Germany). Tissue was added to the kit lysis buffer and homogenised in Lysing Matrix D (Bio101, UK) in the FastPrep FP120 (Bio101/Thermo, UK) for two bursts of 20 s each at setting four. RNA isolation then followed the manufacturer's protocol. RNA concentration and quality were determined by spectroscopy using the Nanodrop ND-1000. Concentrations varied between 100 and 950 ng μL^{-1} and 260/280 ratios were >1.8.

Application of RNA samples to nylon membrane

RNA samples (10 μg for the majority of samples and 5 μg for the rest) in 50 μl water were prepared for application to a nylon membrane (Hybond N, Amersham Biosciences, UK) by addition of an equal volume of denaturing buffer (34.5 μl formamide, 12.1 μl formaldehyde and 3.4 μl 20× SSC). In addition to test samples, reference samples (range 0.4–4 μg) of total RNA isolated from the livers of sand goby exposed to EE2 (200 ng L for 7 days) were applied to each membrane. Samples were heated at 68 °C for 15 min and then quenched on ice prior to membrane (Hybond-N, Amersham Biosciences) loading under vacuum with a slot blot apparatus (BioRad, UK). Wells were washed twice (100 μl) with 10× SSC, before removing the membrane and filters (Bio-Rad, Filter paper 60) from the apparatus. Membrane was air dried and UV cross-linked (Amersham RPN 2500/2501) at 70 000 μJ cm^{-2} for 5 min.

Probe labelling and membrane hybridisation

Fragments of Vtg cDNA (101 bp, GenBank AJ416329) or Zrp cDNA (506 bp, GenBank AJ416330) were used for probe preparation. The fragments were generated by PCR from recombinant plasmids using probe specific primers and the resulting products gel purified. The probes were labelled with [32P]-dCTP using Random Primers DNA Labelling system (Invitrogen, UK). cDNA (25 ng) was heat denatured (65 °C for 5 min) and then cooled on ice. A reaction mixture was prepared following the kit protocol and included [32P]-dCTP (50 μCi) and reaction was initiated by addition of 1 μl of Klenow Fragment. Reaction continued for one hour at 25 °C and then terminated by addition of 5 μl of Stop Buffer. Unincorporated radioactivity was removed by probe purification with ProbeQuant columns (G-50 Micro Columns, Amersham Biosciences).

Membranes were prehybridised overnight at 42 °C with 50% prehybridisation buffer (2.5 ml of 20× SSPE, 0.5 mL 10% SDS, 5 ml formamide, 1 ml 50× Denhardt's solution, 100 μl of salmon sperm DNA

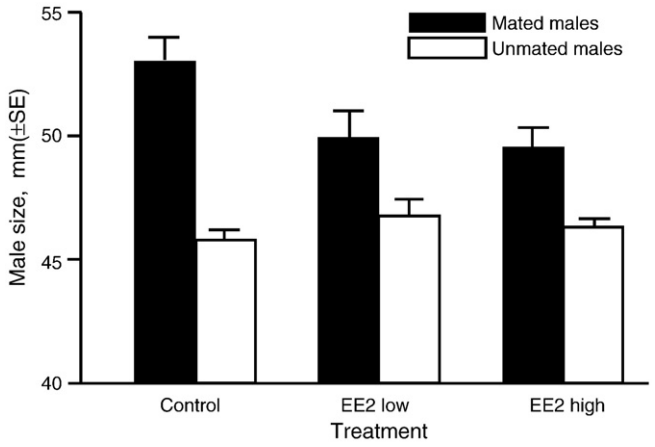

Fig. 2. Size of mated and unmated males in three EE2 exposure treatments. White bars represent the mean total body length of mated males and black bars for un-mated males. Error bars represent one s.e.m. The three treatments are: Control = males exposed to untreated seawater, Low = males exposed to 5 ng L^{-1} and High = males exposed to 24 ng $L^{-1}$of 17α-ethinyl estradiol (EE2).

(10 mg/ml) and 950 µl of water). Prehybridisation buffer was replaced with hybridisation buffer (2.5 ml of 20× SSPE, 0.5 ml 10% SDS, 5 ml formamide, 0.2 ml 50× Denhardt's solution, 100 µl salmon sperm DNA (10 mg/ml) and 2 ml of dextran sulphate). The labelled probe was heat denatured (95 °C for 5 min) immediately prior to addition to the hybridisation bottles and left to hybridise overnight at 42 °C. Membranes were washed three times (five minutes each wash) in 50 ml 20× SSPE, 5 ml 10% SDS and 445 ml of ultrapure water at room temperature and then three times more (15 min each wash) in 2.5 ml 20× SSPE, 5 ml of 10% SDS and 492.5 ml of ultrapure water at + 50 °C.

After the washes the filters were removed from the bottles, wrapped in plastic wrap and placed against phosphor imaging screens prior to data capture with an FX-scanner (Bio-Rad, UK). Slot intensities were quantified with Quantity One software (BioRad, UK) using the values from the reference RNA slots to construct a calibration curve. The software calculated the best fit to the reference values and from this values for test samples were calculated. The final values are a mean of duplicate determinations for each sample on separate membranes. Initial validation checks, where known quantities of the reference DNA were used as test samples, showed that the calibration curve accurately predicted the values of the test samples (within ±2%) and that variance between replicate samples was less than ±2.5%).

The same filters were used to quantify Zrp-expression. After scanning, the Vtg-labelled filters were stripped using hot SDS 0.1% (w/v), prehybridised and rehybridised but with the [32P]-labelled Zrp probe.

Statistical treatment of data

Parametric statistical methods were applied whenever the data fulfilled the requirements of these analyses (Meddis, 1984). In our analyses of sexual selection (see above) we used ANOVA and repeated measures ANOVA. Initially both treatment and exposure time were included in the models. However, in none of these did exposure time or the interaction between treatment and exposure time have a significant effect on the response variables. Therefore exposure time was excluded from all final models and only the effect of treatment was included.

Vtg and Zrp did not conform to a normal distribution and we were unable to render them normal with transformation. Therefore we analyzed the effect of treatment and exposure time on these variables using the Scheirer–Ray–Hare extension of the Kruskal–Wallis tests (Sokal and Rohlf, 1995). Other non-parametric data were tested using the Kruskal–Wallis test with the Mann–Whitney test as a post hoc-test (somatic indices). All statistical analyses were performed using SPSS 12.0 software.

Ethical note

All fish were fed during the exposure period and females immediately returned to their natural habitat after the trials. We minimized the stress to animals during the experiment with gentle handling and keeping the pool conditions close to natural conditions. Males were anesthetized before elastomer marking and before dissection. The study was approved and permit granted by the Finnish National Board for Laboratory Animals.

Results

Mating system experiment

We found that male body size was a less important determinant of male mating success in exposed sand gobies compared to control animals. This is demonstrated by two results. Firstly, mated males were on average larger than non-mated males in both treatments (repeated measures ANOVA, within subject's test comparing mated and non-mated males, $F_{1,\ 24} = 60.82$, $n = 24$, $p < 0.001$, Fig. 2). However, the difference between mated and unmated males was bigger in the control than in the EE2 treatments (within subjects test for interaction between male mating status and water treatment, $F_{2,\ 24} = 5.13$, $n = 24$, $p = 0.014$, Fig. 2). Secondly, the first male to receive a mating was on average smaller in pools with exposed males, than in control pools with non-exposed males (ANOVA, $F_{2,\ 45} = 3.53$, $n = 45$, $p = 0.038$).

The study population shows sexual size dimorphism possibly as a result of more intense selection on increased male size (Forsgren et al., 1996). Therefore, we measured the opportunity for selection on male size, but did not find any effect of EE2 exposure (ANOVA, $F_{2,\ 45} = 0.85$, $n = 45$, $p = 0.434$). Selection gradient on male body length was, however, most intense in the control and weakest for the fish exposed to the high level of EE2 (ANOVA, $F_{2,\ 45} = 3.61$, $n = 45$, $p = 0.035$, Fig. 3).

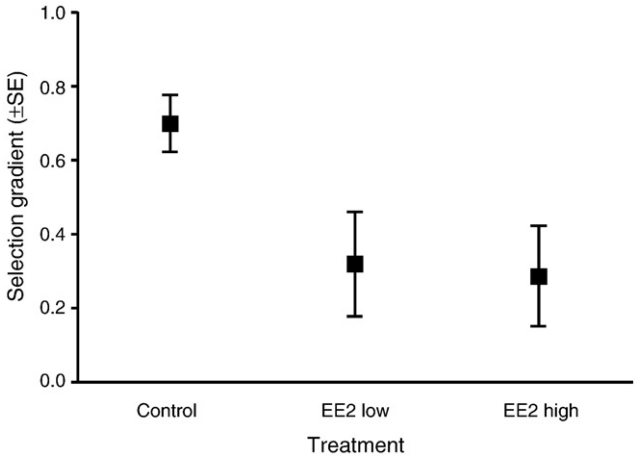

Fig. 3. Sexual selection gradient on male total body length in three endocrine disruptor exposure treatments. Standardized selection gradients (Arnold and Wade, 1984b) are calculated as the regression coefficient between a male's relative mating success and his relative total body length. Error bars are ± s.e.m. Relative mating success is based on the number of eggs a male received divided by the average number of eggs received by males in that particular experimental pool. 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 pool. The three treatments are: Control = males exposed to untreated seawater, Low = males exposed to 5 ng L^{-1} and High = males exposed to 24 ng L^{-1} of 17α-ethinyl estradiol (EE2).

Vtg and Zrp-mRNA expression

There was a highly significant interaction between treatment and exposure time both for Vtg-mRNA levels (Scheirer–Ray–Hare, interaction effect, $H = 50.026$, $df = 14$, $p < 0.001$; $n = 185$) and Zrp-mRNA levels ($H = 48.486$, $df = 14$, $p < 0.001$; $n = 185$). This interaction arises, because in both the low and high EE2 treatments, there is an initial increase and then a decrease in the expression levels, while the control treatment shows nearly no expression at all and no time effect. Males exposed to high EE2 reach much higher expression levels than males exposed to low EE2 (Figs. 4a and b).

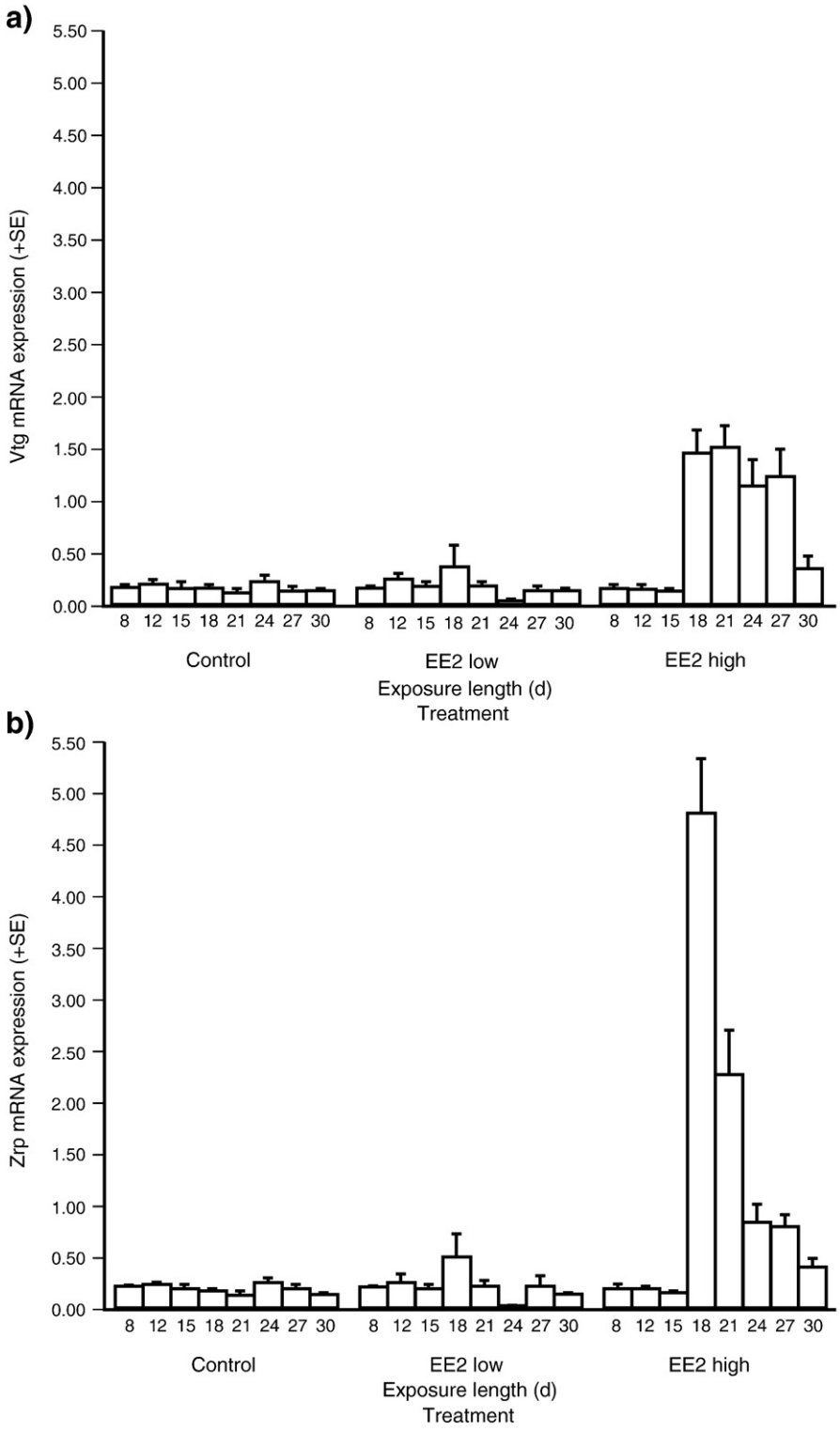

Fig. 4. Effects on (a) male hepatic vitellogenin and (b) male zona radiata protein mRNA expression in different treatments. Sand goby males were exposed from 8 to 30 days to 17α-ethinyl estradiol. Bars represent (a) Vtg: mean expression level + standard error of mean, (b) Zrp: mean expression level + standard error of mean. The three treatments are: Control = males exposed to untreated seawater, Low = males exposed to 5 ng L^{-1} and High = males exposed to 24 ng $L^{-1}$of 17α-ethinyl estradiol (EE2).

Table 1
Calculated somatic indices: hepatosomatic index (HSI), gonadosomatic index (GSI) and sperm-duct gland somatic index (SDGSI), and nuptial coloration of sand goby males in different treatments

Treatment	*N*	HSI	GSI	SDGSI	Nuptial coloration[a]
Control	64	2.14 ± 0.633	1.23 ± 0.313	1.22 ± 0.532	2.25 ± 0.685
EE2 low	61	2.01 ± 0.588	1.06 ± 0.499	1.22 ± 0.593	2.29 ± 0.507
EE2 high	60	1.84 ± 0.67***	1.16 ± 0.747	1.22 ± 0.503	2.21 ± 0.555

Numbers presented are mean values ± standard deviation. Mean values that are significantly different from control are indicated as ***$p<0.001$.
Treatments: EE2 low = 8 ng L^{-1}, EE2 high = 22 ng L^{-1}, control = seawater.
[a]Nuptial coloration scored according to the following: 1 — very weak coloration, 2 — anal and ventral fins light blue, 3 — anal and ventral fins dark blue with bright blue spot on the first dorsal fin.

Somatic indices, nuptial coloration and UGP

HSI was the only somatic index that differed between the treatments: males exposed to high EE2 had significantly lower values of HSI compared to control males (post hoc-test: Mann–Whitney $Z = -2.935$, $p = 0.003$, $n = 124$). HSI values on males exposed to low-EE2 did not diverge statistically from control males (post hoc-test: Mann–Whitney $Z = -0.998$: $n = 125$, $p = 0.318$). All treatments showed the same trend with time: the length of exposure had no effect on HSI values in any of the treatments (Kruskal–Wallis $H = 12.972$, $df = 7$, $p = 0.073$, $n = 185$). The other somatic and morphometric indices (GSI, SDGSI, UGP and nuptial coloration) showed no clear concentration- or time-dependent effects during the experiment (Table 1).

Discussion

Mating system experiment

In the present study, we are among the first to show that endocrine disruptors can change the mating system of a fish species. In the sand goby, large males have a two fold reproductive advantage over small males; they are superior in competition for nest sites (Lindström, 1992) and they are preferred by females (Forsgren, 1992). Previous studies using similar setups, as the current study, have shown that large males are favored by conditions where males can capitalize on their large size (Grant et al., 1995; Lindström and Seppä, 1996). Our most notable finding is that sexual selection on male size is greatly relaxed among EE2 exposed males even in a situation where all males had been treated similarly. Hence, this was not a result of control males being superior competitors to exposed males. Consequently it would be wrong to assume that population level processes would continue in an unchanged way if all individuals were similarly treated. This finding is of special importance in assessing the impacts of pollution in natural populations.

There are two potential explanations for the change in sexual selection that we observed. Firstly, females may have been constrained in their ability to distinguish among male quality, if males did not perform adequately. We do not think that females changed their preferences, when choosing among exposed males. This is because females were unexposed and there was no exposure in the experimental pools. In the three-spined stickleback, *Gasterosteus aculeatus*, males exposed to EDCs exhibited lowered courtship activity (Bell, 2001). If this was the case also in the sand gobies, it could make it more difficult for females to distinguish among males. An earlier study on sand gobies has also shown that exposure to EDCs may weaken male nuptial coloration (Robinson et al., 2003), possibly further contributing to the extinguishment of quality signaling among males. The second potential explanation is that exposure to EE2 changed male dominance behavior and bigger males no longer had an advantage. Agonistic behavior is related to androgen level and there are several reports that affirm a positive correlation between number of agonistic interactions and higher levels of androgens in teleosts (Oliveira et al., 2001; Ros et al., 2004). Androgenic hormones are suppressed by estrogens (Borg, 1994). It has been suggested that estradiol causes males to down-regulate androgen production and reduce behaviors related to androgen, which is supported by a number of empirical studies (Bell, 2001; Martinovic et al., 2007). It should be noted that these two explanations are by no means mutually exclusive. Instead, it is likely that both operate at the same time. Testing this requires experiments that specifically tests for possible changes in mate choice and male–male competition.

This study clearly demonstrates that looking at a species' mating system can be more sensitive in detecting EDC effects than some traditional biomarkers. There were distinct differences in the mating system between control and EE2-treatments, while the expression of Vtg- and Zrp-mRNA had increased significantly only in males treated with high levels of EE2. Previous studies on juvenile carp (*Cyprinus carpio*) and fathead minnows (*Pimephales promelas*), have demonstrated that exposure to xenoestrogens reduced reproductive success without an induced Vtg production (Gimeno et al., 1998; Sohoni et al., 2001). Therefore, one should be cautious while interpreting the absence of Vtg and/or Zrp induction: it does not explicitly mean the absence of significant reproductive effects.

Vtg- and Zrp-mRNA expression

Both molecular biomarkers (Vtg and Zrp) showed clear time dependence, when males were exposed to high EE2. This was also seen in males exposed to low EE2 where expression levels increased after exposure day 18. The appearance of both transcripts followed a typical delay (Craft et al., 2004) before reaching a maxima and then declining as observed previously (Robinson et al., 2003, Brown et al., 2004). Why we see such time dependence is currently unknown. The important point is 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 we compare the expression levels of both markers for the shortest and longest exposure times we find no difference between the low EE2 exposure and the control. Thus, if we had only analyzed these subsets of samples, we would have concluded that molecular biomarkers are not sensitive in detecting EE2 exposure effects at low exposure levels. An earlier study by Gimeno et al. (1998) reported that when juvenile male carps were exposed to 90 μg L^{-1} 4-tert-pentylphenol, males did not show significantly elevated Vtg expression, although 100% of the test individuals (XY male) exhibited female reproductive tract formation. In addition, work by Peters et al. (2007) showed that exposure to 10 ng L^{-1} EE2 for 28-days did not induce vitellogenin production in mummichog males (*Fundulus heteroclitus*). A possibility is that the timing for the biomarker assays in these studies did not match the maximum expressions of the markers.

It is clear from our study that males exposed to high EE2 express more Vtg- and Zrp-mRNA than males exposed to low EE2. However, our low EE2 level is more close to concentrations detected in nature. As shown by our results, transcripts from males exposed to the low EE2 did not differ greatly from control males, and it is less clear how well the Vtg- and Zrp-mRNA expression could have revealed such exposure.

Somatic indices, nuptial coloration and UGP

Somatic indices such as HIS, GSI and SDGSI measure the overall condition of fish (West, 1990). Especially HSI is a good predictor of adverse health in fish (Adams and McLean, 1985). In the present study high exposure to EE2 decreased the HSI levels of males compared to controls. This is opposite to previous studies where HSI was significantly higher in rainbow trout males exposed to estradiol in lab experiments (Herman and Kincaid, 1988), as well as rainbow trout

caged downstream of a sewage treatment plant's final effluent discharge (Sheahan et al., 2002), and in rare minnow (*Gobiocypris rarus*) after exposure to 25 ng L^{-1} for 28-days (Zha et al., 2007). In this study, the low EE2 exposure did not increase the expression of vitellogenin, which could be one explanation for the absent increase in HSI: males were not actively producing vitellogenin. The HSI index seems to increase in a dose-dependent manner along with vitellogenesis (Medda et al., 1980; Christensen et al., 1999): accelerated vitellogenin production increases liver size. It remains to be further investigated why males exposed to high EE2 showed decreased levels of HSI in this study.

EE2 exposure did not have any effects on GSI; control males had similar index values as EE2-treated males. This is unlike many previous studies, which have demonstrated a reduction in the GSI index (Harries et al., 2000; Scholz and Gutzeit, 2000; Van den Belt et al., 2002). The absence of effects on GSI could be due to the timing of the exposure if inhibitory effects of exposure are weakened or absent once the gonads are fully developed (Christensen et al., 1999). Another explanation for the lack of effects result could be the short exposure period, but this is unlikely as many studies on several species report a decrease in GSI after exposures to EE2 that are shorter than used in our study (Harries et al., 2000; Van den Belt et al., 2002; Zha et al., 2007; Peters et al., 2007).

In conclusion, the exposure of sand goby males to EE2 resulted in a disruption of sexual selection. Our experiment showed that in pools with exposed males, the size difference between mated and unmated males was smaller than in control pools, resulting in a weaker selection gradient on male body size. Because only males were exposed in the present study we conclude that this was due to changes in male behavior, not in female behavior. In the long run this could lead to a weakening or complete loss of secondary sexual characters. Endocrine disruptors may therefore be an important anthropogenic factor that can change the overall output of a population, as well as the quality of reproducing individuals. Finally it should be noted that when our study males were introduced to the exposure treatment they were already sexually mature. In addition, exposure to the endocrine disruptor chemical lasted for a maximum of only 4 weeks. This time period is at most less than half the duration of the sand goby-breeding season, more typically only a third of it. Since sand gobies migrate out to sea during winter, they might be able to avoid contaminated near shore areas for most of the year, including periods of gonadal maturation that may be even more sensitive to EDC exposure. However, as our study shows, there is still plenty of opportunity for the population to be affected by endocrine disrupting chemicals, as breeding takes place in shallow, high-risk areas and exposure times need not be long.

Acknowledgments

We thank Tvärminne Zoological Station for providing excellent facilities for the fieldwork and M. Brown at the Glasgow Caledonian University for help in the laboratory. This research work was funded by Onni Talas Foundation.

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