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An endocrine disrupting chemical changes courtship and parental care in the sand goby

Minna Saaristo[a,*], John A. Craft[b], Kari K. Lehtonen[c], Kai Lindström[d]

[a] Department of Bio- and Environmental Sciences, University of Helsinki, P.O. Box 65, FI-00014 Helsinki, Finland
[b] Biological and Biomedical Sciences, Glasgow Caledonian University, Cowcaddens Road, Glasgow G4 0BA, Scotland, UK
[c] Finnish Environment Institute, Marine Research Centre, P.O. Box 140, FI-00251 Helsinki, Finland
[d] Environmental and Marine Biology, Åbo Akademi University, FI-20520 Turku, Finland

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ABSTRACT

Endocrine disrupting chemicals (EDCs) are a diverse group of compounds that can mimic, block or modulate the synthesis of natural hormones. They are known to cause impairment of reproduction of aquatic organisms at very low concentrations. The aim of this study was to examine how exposure from 10 to 31 days to 17α-ethinyl estradiol (EE2, 41 ng L^{-1}) affects the courtship and parental care behaviour of male sand gobies (*Pomatoschistus minutus*). The sand goby exhibits a polygynous mating system, where males compete for females and provide paternal care. First, male courtship performance towards a stimulus female was recorded with video camera. Secondly, after the male had received eggs his parental care behaviour was video recorded. In addition to behavioural endpoints, we measured the expression of hepatic vitellogenin (Vtg) and zona radiata protein (Zrp) mRNA, as well as common somatic indices. Our study shows that exposure to EE2 affected male fanning behaviour during both courtship and parental care. Interestingly, small exposed males increased their courtship fanning to similar levels as larger control males. However, during parental care egg fanning was not related to male size, and all exposed males fanned more than control males. The EE2-exposure induced Vtg and Zrp mRNA expression in males and decreased hepatosomatic index (HSI), and increased gonadosomatic index (GSI). Females prefer males that fan more, which will favour the small EDC exposed males. This may lead to mating that favours males that are not strong enough to tend the eggs until they hatch, thus decreasing the reproductive success of individuals.

1. Introduction

Endocrine disrupting chemicals (EDCs) are a diverse group of compounds from persistent organic pollutants (DDT, PCB), dioxins, polychlorinated biphenyls, pesticides, phenols, and phthalates to natural and synthetic hormones (Caserta et al., 2008). EDCs may mimic, block or modulate hormone synthesis, with many acting as agonists of estrogen receptors or antagonizing androgen receptors (Scippo et al., 2004). Additionally, they can also act through non-genomic mechanisms altering steroid synthesis and metabolism (Waring and Harris, 2005; Tabb and Blumberg, 2006). Most known EDCs originate from paper, plastic and pharmaceutical industries, agriculture and households. They occur in the aquatic environment at very low concentrations (ng L^{-1}), and often in mixtures, which can be more harmful than individual compounds (Aerni et al., 2004; Brian et al., 2007).

The use of behaviour as a tool to measure effects of EDC exposure is fairly recent (Jones and Reynolds, 1997; Clotfelter et al., 2004; Zala and Penn, 2004). Even though it has been shown to be a sensitive marker of EDC exposure (Smith and Logan, 1997; Scott and Sloman, 2004) and provides a starting point for evaluating population level consequences, it is still quite rarely used in ecotoxicological studies. This may be due to behavioural experiments being time-consuming when the behaviour is assessed by a human observer, and difficulties with repetition. However, changes in behaviour bring us direct information on the consequences that exposure may have on individual fitness. Moreover, relaying on single molecular, biochemical or cellular biomarker responses alone can underestimate the impact of EDCs on aquatic organisms (Thorpe et al., 2009).

Reproductive systems are especially sensitive to EDCs and reproduction is closely connected to individual fitness. The reproductive behaviour of fish exhibiting parental care, like the sand goby (*Pomatoschistus minutus*), is therefore a good subject for studying potential effects of EDCs. Courtship behaviour often consists of several individual behaviours, and active courtship is

* Corresponding author. Tel.: +358 9 191 57826.
E-mail address: Minna.Saaristo@helsinki.fi (M. Saaristo).

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energetically demanding (Andersson, 1994; Lindström, 1998) and therefore potentially serving as an honest signal of male condition (Zahavi, 1975). Parental care behaviour protects and promotes the development of offspring (Keenleyside, 1979; Smith and Wootton, 1995). In many fish, it includes (1) egg fanning, where the parent moves fresh water over the eggs with his pectoral fins, (2) cleaning the nest from fungi and other harmful substances, (3) removing diseased eggs, and (4) protecting the nest against predators. Parental care has its costs since it can reduce the survival of a parent by increasing its exposure to predators (Svensson, 1988) or deplete its energy reserves to an extent that the parent is more susceptible to disease or starvation (Smith and Wootton, 1995). Therefore, parental care can reduce the chances for additional matings, although among fish with paternal care most male parents continue to court females while still caring for previous broods (Gross and Sargent, 1985).

The sand goby is a small marine fish with a wide geographic distribution. It has a one-year life cycle and mating system with resource-defence and paternal care (Healey, 1971). Sand gobies live a major part of their life in shallow, sandy shores and feed on benthic invertebrates and zooplankton. They also breed in shallow water, and the male builds a nest under a shell or rock, attracts females with courtship and tends the eggs until they hatch. Females base their mate choice on male body size, colouration, courtship display, parental care, and nest size and quality. Males that court intensively (Forsgren, 1997) and show high level of parental care are particularly favoured (Lindström et al., 2006). A male usually receives eggs from several females (Jones et al., 2001) and females prefer males who already have eggs in their nest (Forsgren et al., 1996).

Our model compound representing EDCs was the synthetic pharmaceutical 17α-ethinyl estradiol (EE2). EE2 is used in oral contraceptives and has been detected in ecologically relevant concentrations (<1 to 15 ng L^{-1}) in sewage effluents worldwide (Baronti et al., 2000; Johnson and Sumpter, 2001; Onda et al., 2002; Muller et al., 2008). EE2 was chosen because it is more persistent in the environment than natural steroids (Schweinfurth et al., 1996; Jürgens et al., 2002; Clouzot et al., 2008), has a high tendency for bioaccumulation (Lai et al., 2000, 2002), and can cause 35–50% of the surface water estrogenicity (Cargouet et al., 2004). A concentration as low as of 1 ng L^{-1} of EE2 can impair reproductive success of fish (Parrott and Blunt, 2005; Schäfers et al., 2007; Länge et al., 2009). Because sand gobies spend such a major part of their life in shallow water areas, it is likely that they will encounter EDCs during their lifetime. To confirm that EE2 exposure caused a physiological response in the experimental fish, two "classical" molecular biomarkers for estrogenic exposure were used in this study. Vitellogenin (Vtg) is the egg yolk precursor protein (Mommsen and Walsh, 1988) and *zona radiata* protein (Zrp) forms the inner core of the egg envelope (Oppen-Berntsen et al., 1992). Males have estrogen receptors, and therefore exposure to estrogenic EDCs can be detected as production of Vtg and Zrp by the liver.

Our aim was to examine the effects of short-term EDC exposure on courtship and parental care behaviour of sand goby males. In addition, we compared the performance of traditional biomarkers to our behavioural measurements for assessing EDC exposure.

2. Material and methods

2.1. Exposure setup

The study was carried out at the Tvärminne Zoological Station, southern Finland, during May–July 2005. The fish were caught with a hand trawl from their natural breeding area. Males were randomly placed into nine flow-through exposure tanks (80 cm × 80 cm × 40 cm), each receiving 45 males. Three tanks were assigned to each treatment level: (1) EE2 exposure, with the measured concentration 41 ng L^{-1} (SD = 24.4 ng L^{-1}, n = 12), (2) solvent control (2-propanol, <0.002%, v/v), and (3) natural seawater control. A stock solution of EE2 was prepared every second day by dissolving EE2 (Sigma–Aldrich) in 2-propanol (Merck), and added to flow-through mixing chambers using peristaltic pumps (Watson Marlow). From the mixing chambers, water was channelled into the exposure tanks using silicon tubing and flow rate was set to 9.6 L h^{-1} using glass flow meters equipped with adjustable valves (Kyrömäki, Finland). The water temperature ranged between 11 and 19 °C, salinity 5.2–6.6‰ and pH 7.7–8.3 following natural variation in the sea. During the exposure fish were fed *ad libitum* with live *Mysis* spp. and frozen chironomid larvae.

Males were exposed from 10 to 31 days before they were either used in behavioural experiments and dissected for Vtg and Zrp mRNA analysis or only dissected. A set of samples (EE2: n = 6, solvent: n = 6, seawater control: n = 6) was dissected every 5th day from the exposure tanks to follow the effect of EE2 exposure duration on Vtg and Zrp mRNA expression. The first behavioural experiments were performed after 10 days of exposure, and thereafter a new set of behavioural experiments was started every 4th day, always using new fish. This gave us a total sample size of 223 males that had gone through the behavioural tests and another sample of 81 males that only had gone through the exposure treatment.

2.2. Behavioural experiments

We used 30 individual tanks (35 cm × 40 cm × 16 cm) for the behavioural experiments, 10 tanks for each treatment. These tanks had no flow-through of seawater. Each tank had a 3-cm layer of fine sand on the bottom and a large halved clay flowerpot (10 cm in diameter) that served as a nest site. Inside the flowerpot, a transparent film lined the ceiling of the pot. The film served as a substrate for the eggs laid by the female. It could be then removed and returned to the nest, making it possible to photograph and count the number of eggs. The water temperature in each tank was checked daily.

2.2.1. Nest building

A total of 30 males (10 from each treatment group) were randomly picked from the exposure tanks, and measured for total length (to the nearest mm) and body mass (to the nearest g). One male was placed in each behavioural tank, and males were left to build a nest overnight. During nest building, the tanks were exposed according to the treatment from which the male originated. The mean measured concentration in the EE2 treatment was 50 ng EE2 L^{-1}, (SD = 18.4 ng L^{-1}, n = 20). In the other two treatments (solvent and seawater controls), EE2 was below the detection limit (<1 ng L^{-1}). Males build their nests by excavating the inside and piling sand on top of the nest substrate. As a measure of nest building we used the amount of sand piled on the nest. This was scored as follows: (1) no sand had been piled on the nest; (2) some sand was piled to the top and front of the nest, but the clay pot was still visible; (3) the male had totally hidden the clay pot by piling plenty of sand to the top of the nest. In most cases, the entrance to the nest was as small as the male's head.

2.2.2. Courtship behaviour

Male courtship involves a repertoire of behaviours, which he uses to attract the female's attention and to lead her into his nest to spawn. An important part of this repertoire is courtship fanning, a behaviour where the male moves his pectoral fins back and forth to create a flow of water. This behaviour is usually used to fan fresh water over the developing eggs, but is frequently used among fish even when there are no eggs in the nest. This behaviour was first described for the three-spined stickleback by Sevenster (1961). It is important to note the difference between fanning during courtship