Marine Environmental Research 113 (2016) 80–87

Contents lists available at ScienceDirect

Marine Environmental Research

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

Severe malformations of eelpout (*Zoarces viviparus*) fry are induced by maternal estrogenic exposure during early embryogenesis

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ARTICLE INFO

Article history:
Received 2 September 2015
Received in revised form
5 November 2015
Accepted 9 November 2015
Available online 14 November 2015

Keywords:
Eelpout
Malformation
Embryos
Teratogenicity
Embryonic development
Blenny
Endocrine disruption

ABSTRACT

Pregnant eelpout were exposed via the water to known endocrine disrupting compounds (EDCs) to clarify if EDCs could be causing the increased eelpout fry malformation frequencies observed in coastal areas receiving high anthropogenic input. The presence of a teratogenic window for estrogen-induced malformations was also investigated by starting the exposure at different times during eelpout pregnancy.

Both 17α-ethinylestradiol (EE2) (17.8 ng/L) and pyrene (0.5 μg/L) significantly increased fry malformation frequency whereas 4-*t*-octylphenol (4-*t*-OP) up to 14.3 μg/L did not. Vitellogenin was significantly induced by EE2 (5.7 and 17.8 ng/L) but not by 4-*t*-OP and pyrene. A critical period for estrogen-induced fry malformations was identified and closed between 14 and 22 days post fertilization (dpf). Exposure to 17β-estradiol (E2) between 0 and 14 dpf caused severe malformations and severity increased the closer exposure start was to fertilization, whereas malformations were absent by exposure starting later than 14 dpf. Data on ovarian fluid volume and larval length supported the suggested teratogenic window. Larval mortality also increased when exposure started right after fertilization.

1. Introduction

The eelpout (*Zoarces viviparus*) is a benthic live-breeder and a common inhabitant of Northern European marine coastal areas. Fertilization takes place in September and approximately five months later each female releases up to 400 larvae. The eelpout is viviparous and has a prevailingly stationary behavior (Kinitz et al., 2013), and hence it is possible to relate changes in their physiological conditions, reproduction, and fry development to local environmental conditions. The frequency of malformed eelpout fry has been observed to increase in marine areas receiving high anthropogenic inputs (Gercken et al., 2006; Strand et al., 2004). Developing embryos are more sensitive to exogenous hormones than the adult (Bern, 1992), and intersex is also observed in male eelpout living in areas where increased fry malformation frequencies have been observed (Gercken and Sordyl, 2002). Therefore it has been suggested that endocrine disrupting compounds (EDCs) could be a contributing factor to the increase in fry malformations in certain areas.

17β-estradiol (E2) is the primary female sex hormone in vertebrates and plays an important role in fish sexual development and regulation of yolk protein (vitellogenin) synthesis. However, non-reproductive tissues and processes are also influenced by E2 e.g. heart development (Allgood et al., 2013), bone formation (Gao et al., 2013), and development of cartilage (chondrogenesis) (Fushimi et al., 2009), and the synthetic estrogen 17-α-ethinylestradiol (EE2) has been shown to influence the embryo development in oviparous fish (Santos et al., 2014).

Polyaromatic hydrocarbons (PAH) such as pyrene are formed by incomplete combustion of organic compounds, fossil fuels, oil and wood and enter the aquatic environment by runoff and atmospheric deposition. Benzo(a)pyrene or fluoranthene (Le Bihanic et al., 2014) (additional references in Corrales et al. (2014)) induced larval abnormalities in rainbow trout (*Oncorhynchus mykiss*) and Brande-Lavridsen et al. (2013) found indications that pyrene injections of pregnant eelpout could induce fry malformations. Alkylphenol polyethoxylates are used as detergents and constituents of paints, cosmetics and pesticides and they are degraded into alkylphenols such as nonylphenol (NP) and octylphenol (OP) (reviewed by Sharma et al. (2009)). Alkylphenols are stable and accumulate in sediments and filter-feeding organisms such as bivalves. In marine molluscs NP and OP have been detected

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http://dx.doi.org/10.1016/j.marenvres.2015.11.007
0141-1136/

in concentrations up to thousands of ng/g tissue but normally concentrations are in the low ng/g range (reviewed by David et al. (2009)). Eelpout are likely to be exposed to alkylphenols via water and food as they are bottom-dwelling and feed on benthic crustaceans, worms and bivalves. Laboratory experiments showed that alkylphenols feminize male fish (Gimeno et al., 1998; Gray and Metcalfe, 1997) and impair embryo development in oviparous fish (Chaube et al., 2013).

By conducting a small-scale pilot study it became clear that maternal exposure to E2 and 4-*tert*-octylphenol (4-*t*-OP) during certain periods of pregnancy impairs larval growth and increases the malformation frequency (data not published), and later we showed that E2 (≥53.6 ng/L) causes severe malformations in eelpout fry (Morthorst et al., 2014). It remains unknown if environmental xenoestrogens have similar properties and if the severity of the effects depends on the timing of the exposure. When pregnant eelpout were exposed to EDCs several weeks after fertilization the malformation frequency was not increased (Rasmussen et al., 2002) but sensitive windows for xenoestrogens and E2 exposure have been suggested for *Xenopus laevis* embryos (Nishimura et al., 1997). Knowledge about sensitivity and responsiveness are important as eelpout are used as biomonitoring organisms in several environmental programs and both the regional Oslo–Paris Convention (OSPAR, 2007) and the Helsinki Commission (HELCOM, 2008) have suggested using eelpout as sentinel species for monitoring effects of toxic compounds in marine environments.

In the present experiments we investigate if maternal exposure to common environmental EDCs induces malformations in eelpout fry and if the malformations occur only by exposure during a temporally narrow, sensitive window.

The goals were (1) to clarify if waterborne exposure to known EDCs could lead to abnormal embryo development and (2) to determine the duration of the terotogenic window for E2-induced fry malformations as this compound is known to cause malformations in eelpout fry.

2. Materials and methods

2.1. Animals

Ovulation and fertilization takes place in late summer and early autumn but may shift a few weeks from year to year. Beginning in mid-August the reproductive status of wild females was monitored weekly by spot checks of the ovaries. When fertilized eggs were observed in the majority of the females, the collection of experimental animals was initiated. Feral eelpout (*Z. viviparus*) were caught in seines in the coastal areas around the island of Birkholm (54′56″N, 10′31″E), Denmark during August and September 2012 and 2013 and transported by car to the Marine Biological Research Centre in Kerteminde, Denmark. The fish were transported for approximately 1 h in an aerated tank (500 L) with seawater. The eelpout from this area have previously been used for experiments and elevated frequencies of fry malformations have not been observed. After arrival at the Research Centre the fish were sexed immediately and 10 or 11 females were randomly distributed in each experimental tank (Table 1). Acclimatization was not allowed because the assumed sensitive window for exposure begins right after fertilization. It should be noted that the term 'days post fertilization' (dpf) used throughout the article may be a slightly inaccurate term, since the fish were caught wild and it is not possible to catch the females on the exact day of fertilization. However, a fixed overall term is necessary to be able to compare the exposure groups in the 2013 experiment to each other.

2.2. Exposure

Both experiments were set up according to Morthorst et al. (2014) and with triplicates of each exposure concentration. In 2012 the fish were put in tanks over a 4-day period (September 11 to 14) due to low catchment success on some days. Four fish were put in all tanks on the first and second day and three fish on the fourth day. In 2013 the exposures were started between September 14 and October 6 to study the sensitive window for teratogenic effects. An overview of the experimental details including experimental timing and setup is presented in Table 1. Briefly, twenty-four polyethylene tanks were set up in a flow-through system with a water exchange of 200 L per day and each tank was provided with two air stones and two circulation pumps. The seawater was pumped directly from the mouth of Kerteminde Fjord, which is a small fjord with high water exchange and low anthropogenic input and it is connected to the Great Belt in the Baltic Sea. To provide hiding opportunities and shade during light hours pieces of drainpipe were put in the tanks and the tanks were partly covered by black plastic. Primary stock solutions of 17β-estradiol (CAS 50-28-2), 17α-ethinylestradiol (CAS 57-63-6), pyrene (CAS 129-00-0) and 4-*tert*-octylphenol (CAS 140-66-9) were prepared in 100% isopropanol and kept at 5 °C. The secondary stock solutions that were used to feed the exposure tanks were prepared in 10% isopropanol (CAS no 67-63-0) and 90% ASTM type 1a water. The secondary stock solutions were renewed twice per week and the final solvent concentration in the exposure tanks was <0.01%. All chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany).

Water samples were collected regularly from the exposure tanks and frozen at −20 °C for subsequent chemical analysis (Table 2). The concentration of stock solutions was monitored regularly during the experimental period. Temperature, salinity and oxygen saturation were measured regularly in the header tanks but only weekly in each individual exposure tank because the fish are very sensitive to movement and sound disturbance.

2.3. Pilot studies – early observations and range finding

In a pilot study (2010) we observed severe malformations in fry of mothers (n = 4–6 females per group) exposed to E2 (nominal 500 ng/L) and 4-*t*-OP (nominal 100 μg/L and actual 31 μg/L) from fertilization and 29 days onwards. The maximum amount of isopropanol in the tanks was 0.036%. Both larvae weight and length were significantly reduced and the larvae had various types of malformations. The malformation frequency in the E2 group was 99% and 91% in the 4-*t*-OP group compared to 23% in the solvent control.

To avoid the risk of toxic effects of the solvent it was decided to reduce the solvent concentration according to OECD test guideline recommendations in subsequent experiments.

As the toxicity of pyrene in fish and especially viviparous fish has not been investigated a pilot study was set up. In August 12 fish of mixed sex were exposed to a nominal pyrene concentration of 5 μg/L. After one week of exposure the mortality was 42% and after 15 days it was 75%; therefore, the nominal exposure concentration was reduced to 500 ng/L in the subsequent experiment. The exposure setup and conditions in the pilot studies were as described previously.

2.4. Sampling procedure

The sampling procedures and the calculations of biometric indices are described in detail in Morthorst et al. (2014). The somatic weight (somatic weight (g) = total body weight (g) − ovary