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Uptake of 17β-estradiol and biomarker responses in brown trout (*Salmo trutta*) exposed to pulses

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ABSTRACT

In streams, chemicals such as 17β-estradiol (E2) are likely to occur in pulses. We investigated uptake and biomarker responses in juvenile brown trout (*Salmo trutta*) of 3- or 6-h pulses of concentrations up to 370 ng E2 L^{-1}. Uptake by the fish was estimated from disappearance of E2 from tank water. A single 6-h pulse of 370 ng E2 L^{-1} increased the plasma vitellogenin concentration, liver Erα- and vitellogenin-mRNA. Exposure to 150–160 ng E2 L^{-1} for 6 h increased vitellogenin in one experiment but not in another. Two 6-h pulses had a larger effect one pulse. Brown trout in the size range 24–74 g took up E2 linearly with time and exposure concentration with a concentration ratio rate of 20.2 h^{-1}. In conclusion, the threshold for induction of estrogenic effects in juvenile brown trout at short term pulse exposure appears to be in the range 150–200 ng E2 L^{-1}.

1. Introduction

Estrogens discharged from wastewater treatment plants (WWTP) may cause feminisation of male fish in contaminated rivers and estuaries (Sumpter, 2005; Desforges et al., 2010). Estrone (E1), 17β-estradiol (E2) and ethinylestradiol (EE2) are the three estrogenic species known to contribute most of the estrogenicity in wastewater from WWTPs (Desbrow et al., 1998).

Recent investigations have shown that WWTPs are not the only sources for addition of estrogens to the aquatic environment: Discharges of estrogens from scattered houses in the open land (Stuer-Lauridsen et al., 2006), leaching from farmland treated with liquid manure (Dyer et al., 2001; Stuer-Lauridsen et al., 2005; Matthiessen et al., 2006; Hildebrand et al., 2006; Kjaer et al., 2007) and discharges from dairy farms (Matthiessen et al., 2006) may contribute to the estrogenic load in streams. Discharges from simple septic tanks in the open land may have estrogenic activities as high as 400 ng 17β-estradiol equivalents (EEQ) L^{-1} (Stuer-Lauridsen et al., 2005, 2006) and Kjaer et al. (2007) observed that precipitation incidents caused E1 and E2 to leach into drainage water for as long as three months after application of manure to farmland. This type of leaching and discharges from houses in the open land are likely to result in pulsed rather than constant exposure of the fish inhabiting the streams – especially in headwater streams with limited dilution capacity. Yet most of our knowledge about concentration–response relationships and thresholds for estrogenic responses in various fish species has been obtained in laboratory studies with constant exposure regimes. Therefore, the question is: What does an EC50-value (15 ng E2 L^{-1}) for induction of vitellogenin in juvenile brown trout over 8 days constant exposure (Bjerregaard et al., 2008) tell us about the potential impacts of exposure to shorter pulses of e.g. 400 ng EEQ L^{-1}?

Induction of the synthesis of the yolk-precursor protein vitellogenin in juvenile and male fish is the most well established biomarker of estrogenic contamination of the aquatic environment (Sumpter and Jobling, 1995; Harries et al., 1997), but other sensitive and more rapidly responding molecular biomarkers of estrogenic activity include hepatic transcript levels of vitellogenin and the hepatic estrogen receptor α, as these are known to represent estrogen-responsive genes (Pakdel et al., 1991; Burki et al., 2006).

Several E2-injection experiments have demonstrated the effect of acute estrogen exposure. Most of these have used relatively high E2 doses (0.1–5 mg kg^{-1}; rainbow trout (*Oncorhynchus mykiss*) (Leguellec et al., 1988; Pakdel et al., 1991; Donohoe and Curtis, 1996; Arukwe et al., 2001), fathead minnow (*Pimephales promelas*) (Korte et al., 2000), sheepshead minnow (*Cyprinodon variegatus*) and brown trout (Sherry et al., 1999)). The data from these experiments are, however, difficult to extrapolate to a natural scenario, where pulse exposure to lower and waterborne concentrations often occurs. Only a few studies have investigated the lower threshold (dosage and duration) for waterborne estrogenic exposure. Yamaguchi et al. (2005) water-exposed Japanese medaka

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(*Oryzias latipes*) to E2 for 8 h and reported LOEC of 0.1 μg E2 L^{-1} for vitellogenin-mRNA induction and <0.1 μg E2 L^{-1} for ERα-mRNA induction. Panter et al. (2000) exposed fathead minnow to intermittent 1–3 d exposures of E2 over a period of 120 days and plasma vitellogenin concentrations were elevated to approximately the same degree as those in chronic exposure experiments using the same concentration. Thus, the response was higher than in continuous exposure to the equivalent time-weighted average concentration, suggesting a "memory-effect" of previous exposures.

The brown trout is widely distributed in headwater streams in Europe and has been used as a monitoring organism for endocrine disruption (Korner et al., 2005; Vermeirssen et al., 2005; Burki et al., 2006). There are reports of elevated plasma concentrations of vitellogenin in male brown trout from a number of streams (Bjerregaard et al., 2006, 2008; Kelly et al., 2010).

The purpose of the present investigation was to determine E2-uptake and use different molecular biomarkers (ERα-mRNA, vitellogenin-mRNA, vitellogenin protein) to investigate the threshold for the estrogenic effect in brown trout upon pulsed exposure to 17β-estradiol.

2. Materials and methods

2.1. Experimental animals

Sexually immature brown trout (*Salmo trutta*) were obtained during 2006 from Funen Salmon Fishing, Elsesminde, Denmark, where they had been reared from hatch. The juvenile fish were the offspring of parent brown trout caught during the previous year in Funen in streams on their way back to breed.

At Elsesminde the juvenile fish had been kept in 16 m^3 tanks with approximately one water exchange per day at 7–9 °C and ambient light:dark regime. The water supply consisted of 99.9% re-circulated, filtered groundwater and 1‰ groundwater. The fish were brought up on commercial trout food (Aller Mølle, Brande, Denmark).

In the laboratory, the fish were acclimatized (12.5–14 °C) in flow-through systems of 55 or 112.5 L stainless steel tanks for 4–30 days. During the acclimatization and experimental periods the fish were held at a 12:12 light:dark cycle and they were fed commercial (Aller Aqua, Brande, Denmark) trout feed (0.5–1% body weight/day).

2.2. Experimental set-up

During experiments, the fish were held in the stainless steel tanks (55 or 112.5 L) with flows of tap water (groundwater) between 130 and 173 L per 24 h. The fish were exposed to pulses of E2 (Sigma–Aldrich, Brøndby, Denmark) by being transferred by netting to steel exposure tanks with the desired concentration of E2 for a period of 3 or 6 h (Table 1). In experiment 1, a static system was used (3 h exposure) and flow-through systems were used in experiment 2–6 (6 h exposure). In the flow-through system, administration of water and E2 was controlled by two peristaltic pumps (Ole Dich Instrument Makers, Copenhagen, Denmark). E2 was dissolved in 96% ethanol before administration. During the experiment, tap water and stock solution were mixed to the desired concentration before entering the steel tanks. Submerged circulation pumps positioned in the tanks were used to ensure complete and uniform dispersal of the test compounds. Only the ethanol was added to control tanks.

To characterise actual exposure concentrations and to follow the removal of E2 from the water phase during the exposure, water samples for quantification of E2 were taken during the pulse exposures as indicated in Table 1.

2.3. Experiments

The brown trout were subjected to various types (duration, exposure concentrations, single/repeated pulses) of pulsed exposure to E2 as presented in Table 1.

After the pulse exposure, the fish were transferred by netting to flow-through tanks with uncontaminated water and blood samples were taken from anaesthetized (50–100 mg MS-222 L^{-1} [ethyl 3-aminobenzoate methanesulfonate salt, Sigma–Aldrich, Brøndby, Denmark]) fish during the days after the exposure as indicated in Table 1. In experiment 3, the liver was removed for analysis of ERα-mRNA and vitellogenin-mRNA.

2.4. Analytical procedures

2.4.1. Vitellogenin analysis

Vitellogenin was measured in either plasma samples (Exp. 1–4 and 6) or whole body homogenates (Exp. 5). Blood was collected from the caudal vessels from MS-222 anaesthetized fish by means of a heparinised (5000 IU ml^{-1}) syringe, transferred to heparinised Eppendorf tubes on ice, centrifuged (3 min, 20,000 rpm, 4 °C) and the supernatant was stored at −80 °C in aliquots until measurement.

Whole body homogenates were prepared by first anaesthetising and killing the fish with an overdose of buffered MS-222. After being weighed, fish were placed separately in microcentrifuge tubes on ice, crushed with a small plastic pistil and immediately mixed with two times the body weight of ice cold homogenisation buffer (50 mM Tris–HCl, pH 7.4; 1% protease inhibitor cocktail (Sigma–Aldrich, MO, USA)). The homogenates were centrifuged (20 min, 50,000 g, 4 °C) and the supernatants were stored at −80 °C in aliquots until measurement. A homologous, direct non-competitive sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) as described by Bjerregaard et al. (2006) was used to quantify the vitellogenin concentration in plasma or whole body homogenates.

2.4.2. RT Q-PCR

Livers removed from the decapitated fish were immediately frozen in liquid nitrogen and stored at −80 °C until RNA isolation. Total RNA was isolated by the Trizol method according to the manufacturer's manual (Invitrogen, Carlsbad, CA,

Table 1
Salmo trutta. Experimental conditions. Actual concentrations and fish weights are given as mean ± Standard error of mean.

Exp	[E2] (ng L^{-1})		Num-ber of tanks	Fish pr. tank	Exposure		Weight (g)	Month	Age	Sampling		Temp. °C
	Nomi-nal	Actual			Day in experiment	Duration (h)				Water (hours in pulse)	Vitellogenin (days)	
1	0	<1	1	12	0	3	53.0 ± 3.0	Feb	1	0, 1, 2, 3	3[a], 6[a]	12.5
	25	24 ± 2	1	12	0	3	63.4 ± 5.0					
	50	48 ± 4	1	12	0	3	61.9 ± 4.5					
	100	87 ± 9	1	12	0	3	61.0 ± 4.5					
	200	182 ± 18	1	12	0	3	60.9 ± 4.8					
2	0	<1	2	13	0	6	44.6 ± 3.4	Mar	1	0, 3, 6	2, 4, 6	12.5
	200	156 ± 11	5	13–15	0	6	60.0 ± 2.7					
3	0	<1	2	16	0	6	49.3 ± 3.2	May	1+	0, 3, 6	1, 2, 4, 6	12.5
	400	370 ± 5	4	10–11	0	6	53.6 ± 2.9					
4	0	<1	2	10	0	6	74.2 ± 6.8	Jul	1+	0, 3, 6	1, 4	15.5
	200	159 ± 6	2	10	0	6	70.3 ± 5.4					
5	0	<1	2	25→15	0, 2	6	1.80 ± 0.07	Aug	0+	0, 3, 6	4, 6	14
	200	206 ± 2	2	30→20	0, 2	6	1.8 ± 0.1					
6	0	<1	1	50→35→20	0, 2, 4	6	28.6 ± 1.1	Dec	0+	0, 3, 6	4, 6, 8	12.5
	100	64 ± 6	1	50→35→20	0, 2, 4	6	23.6 ± 0.8					

[a] Repeated sampling from the same fish.