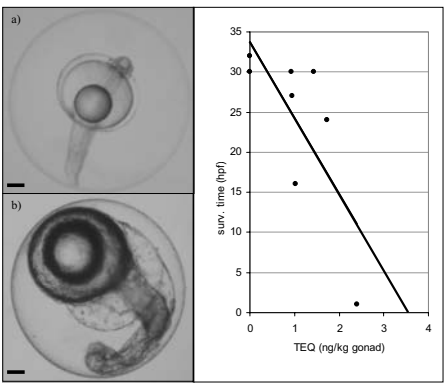

Figure 2 a) Healthy European eel embryo at 30-35 hpf with heartbeat and yolk sac with large fat droplet, b) Larger embryo of an unhealthy batch at identical time of development displaying yolksac oedema, deformed head region and absence of heartbeat. Scale bars represent 100 µm. c) Negative correlation between total TEQ values (ng/kg gonad) and embryo survival time (hours post fertilisation) of fertilised eggs of 8 hormone induced, stripped females.

Chapter 6

Are dioxin-like contaminants responsible for the eel (*Anguilla anguilla*) drama?

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ABSTRACT

Eel populations world-wide are dangerously close to collapse. Our study is the first to show that current levels of dioxin-like contaminants are strong candidates, because of their devastating effects on development and survival of eel embryos. Female and male silver eels were artificially stimulated to maturation and reproduction by treatment with carp pituitary extracts and hCG respectively. During maturation of female European silver eels about 60 g fat per kg eel is incorporated in the oocytes. Together with the fat however, persistent organic pollutants such as dioxin-like polychlorinated biphenyls (PCBs) are incorporated too. The total dioxin-like toxic potency of the individual gonad batches was determined as TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxine) – equivalents (TEQs), using an in vitro reporter gene assay. The observed differences in development and survival showed a significant negative correlation with the TEQ levels in the gonads, already at levels far below the maximal allowable level for fish consumption i.e. 4 ng TEQ/kg fish. The clear inverse relationship between the TEQ-level and the survival period of the fertilised eggs strongly suggest that the current levels of dioxin-like compounds seriously impair the reproduction of the European eel. The peak of the environmental levels of dioxin-like PCBs and the decline of eel coincide world-wide, further suggesting that, in addition to other threats, these contaminants contributed significantly to the current collapse of eel populations.

INTRODUCTION

Eel populations world-wide are dangerously close to collapse. The numbers of glass eels caught have declined by 99% since the early 80s (Anonymous 2003). Several anthropogenic factors implicated in the decline of European eel (*Anguilla anguilla*) are assumed to act before or during the eels' oceanic phase. Migrating (silver) eels do not feed 'en route' and are totally dependent on their fat stores to fuel migration and gonad development. With fat consumption however, internal concentrations of lipophilic pollutants rise, thus increasing the risk for toxic effects. Eels often reside in contaminated sediments and accumulate high levels of especially polychlorinated biphenyls (PCBs, van Leeuwen et al. 2002). These compounds have been shown to have adverse effects on fertility in fish (Stouthart et al. 1998) and amphibians (Gutleb et al. 1999) but also to disrupt mammalian oocyte maturation and follicle physiology in every species studied (Pocar et al. 2003). These effects are at least partially mediated via interaction with the aryl hydrocarbon receptor (AhR), which after binding is translocated to the cell's nucleus. There it interacts with dioxin response elements and disturbs physiological and developmental processes (Safe 1994).

In a recent review, Robinet & Feunteun (2002) stated that ecotoxicological studies on the reproduction capacity of contaminated eels were not available. Until now, it was not possible to study the effects of contaminants on fertility and embryonic development since artificial reproduction has been unsuccessful. Recently however, we have been able to fertilise eggs and follow embryonic development (Palstra et al. 2005). In these and subsequent trials, large differences were observed with respect to development in fertilised egg batches. We hypothesised that this was caused by maternal dioxin-like contaminants

deposited in the egg yolk. Therefore we measured in this study the levels of dioxin-like compounds in muscle and gonad tissues from these eels and correlated their distribution to embryonic development.

MATERIAL & METHODS

Twenty-five female (500-1700g) and fifty male silver eels (100-150g) were caught in the wild during their seaward migration. All males and most females (n=18) were caught in Lake Grevelingen (Bout, Bruinisse, The Netherlands) and 7 females were caught in River Loire (France) in the fall (October-November) of 2001, 2002 and 2003. After transport to our laboratory, twelve females were immediately sacrificed as control animals. The remaining females were injected weekly with Carp Pituitary Extract (CPE: 20-mg/kg) and were not fed during the experimental period. Ovulation could be induced by injecting female eels with 17,20β-dihydroxy-4-pregnen-3-one (2-mg/kg) after which they were hand-stripped. Males were injected weekly with Human Chorionic Gonadotropin (125 IU/male) for at least 7 weeks, when sperm was collected to fertilise the eggs. Fertilised eggs were reared in artificial seawater at 20 °C as long as development proceeded. The protocol is described in detail by Palstra et al. (2005). Stripped females were sacrificed to determine age, based on otolith rings (Svedäng et al. 1998), and Gonadosomatic Index (GSI; relative gonadal weight). Lipid extraction of muscle and gonad tissue was performed as previously described by Murk et al. (1998).

Dioxin-like compounds, expressed as TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxine) – equivalents (TEQs), were determined by using a reporter gene assay DR-CALUX (Aarts et al. 1995). This assay is based on rat-hepatoma cells (H4IIE) stably transfected with a plasmid carrying the luciferase gene of fireflies (*Photinus pyralis*) as a reporter gene. The obtained TEQ-value is specific for dioxin-like compounds including PCBs as well as polyhalogenated dibenzo-dioxins/-furans (PCDD/Fs). However, dioxin-like PCBs are predominant in aquatic ecosystems (Murk et al. 1998; de Boer et al. 1994). In eel, PCBs constitute at least 86% of the total TEQ (de Vries 2002).

Normality of data distribution was tested with a Kolmogorov-Smirnov test. For comparison between muscle and gonad samples within groups, one-tailed paired t-tests were performed. For comparison between control and hormone-treated groups, one-tailed univariate analyses of covariance (ANCOVA) was performed. Bodyweight was used as cofactor for differences in fat percentage. Fat percentage was used as cofactor for differences in TEQ level. For correlation analyses, one-tailed Pearson tests were performed for control and hormone-treated groups. Significant correlations with start parameters were analysed with ANCOVA to estimate the determinant. All tests were performed using SPSS 10.0 for Windows. Results were calculated and plotted as means ± SD.

RESULTS

Control and hormone-treated females were of similar size and age (Table 1), between 6 and 25 years old. Females of the control group had a GSI of 1.4 ± 0.3 (Fig. 1).

Upon hormone-treatment all females fully matured resulting in a significant increase of the GSIs to 27-51 after 12-25 weekly CPE injections (Fig. 1). After stripping the total number of eggs varied between 0.8-1.7 million eggs.

Table 1 Parameters of artificially matured female silver eels and controls (means ± SD). Eel were treated 12-25 weeks with carp pituitary extracts; condition factor expressed as W/L^3; age was estimated by otolithometry; toxicity equivalent to dioxin (TCDD) in ng TEQ/kg fat was determined by the DR-Calux method.

		control	hormone-treated
body length (cm)		77±8	80±5
body weight (g)		911±249	1095±250
condition factor		0.20±0.02	0.21±0.02
age (est. years)		14±5	11±4
TEQ (ng/kg fat)	muscle	4.9±5.4	8.7±7.8
	gonad	5.6±4.7	8.1±7.1

Fat percentages in muscle tissue were about 20% both in hormone-treated and control females (range 9 to 35%; Fig. 1). The total fat in gonads of hormone-treated females (58 ± 21 g per kg eel) was 12 times higher than in gonads of control females (5 ± 1 g per kg eel; Fig. 1). Positive correlations with the amount of incorporated fat in the gonads in hormone-treated females were found with age (Pearson; n=13; P=0.012; r=0.619), bodyweight (Pearson; n=13; P=0.026; r=0.549) and condition factor (Pearson; n=13; P=0.006; r=0.668). ANCOVA showed that age (ANCOVA; n=13; P=0.02) was the significant determinant for the amount of incorporated fat. A negative correlation (Pearson; n=13; P=0.036; r=-0.514) was found between age and the amount of fat in muscles in hormone-treated females.

Although the total fat content in the gonads of hormone-treated females was significantly increased (Fig. 1), relative fat levels in the gonads were significantly lower (ANCOVA; n=25; P<0.001). In the mature oocytes of hormone treated females large accumulation of proteic stores (vitellus) had occurred while in the immature oocyte mostly lipid inclusions were found. The TEQ-levels on a fat basis in muscle and gonads tend to be higher in hormone-treated eels (Table 1). TEQ-levels in the muscle were 1.8 times higher, but not significantly different (ANCOVA; n=25; P=0.08). TEQ-levels in the gonads were 1.4 times higher (ANCOVA; n=25; P=0.05) with fat percentage as significant cofactor (ANCOVA; n=25; P=0.008). Average total TEQ-levels in both muscle and gonads of Lake Grevelingen silver eels in this study were slightly lower than the 12 ng TEQ/kg eel measured in eels of the nearby Volkerak in 2001 by van Leeuwen et al. (2002).

During the first hours post fertilisation (hpf), eggs from 8 out of 13 hormone-treated females showed cleavage up to the eight-cell stage. Seven of these 8 batches showed a continued development till 15 hpf but died thereafter. One of these 7 batches resulted in about 1500 embryos at 30 hpf, that however all showed serious oedema of the yolksac; a deformed head region (Fig. 2) and absence of a heartbeat. These embryos died at 43 hpf in

contrast to healthy embryos of another batch, which reached a pre-hatching stage at 100 hpf. A negative correlation (Pearson; n=8; P=0.019; r=-0.736) was found between embryonic survival time from different batches and the corresponding TEQ levels expressed as ng/kg gonad (Fig. 2).

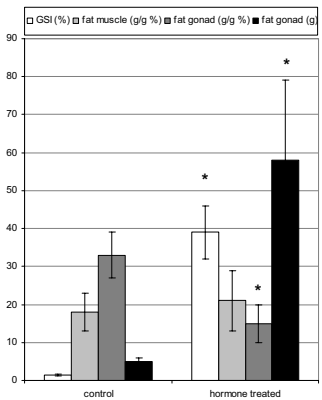

Figure 1 GSI (relative gonad weight), relative fat content in muscle and gonad (g fat/g tissue *100%) and total fat in gonad (g/kg eel) in control and hormone-treated eels. Highly significant differences (P<0.001) between groups are indicated with an asterisk.

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DISCUSSION

The fat levels in the untreated silver eels in this study are similar to those found in ecological surveys (Svedäng and Wickström 1997). However, the absolute fat content in the gonads was 12 times higher in hormone-treated females showing an extensive fat incorporation in the oocytes. The total fat content of these gonads was between 33 and 103 g per kg eel. For an average 1-kg silver eels with a fat reserve of 200 g this means that 17 to 52% of the total fat reserve is transported into the gonads. The amount of fat transported to the gonads was found positively related to the age of the eel. This suggests an increased capacity of older eels to incorporate more fat from the muscle into the eggs. As egg quality depends heavily on incorporation of reserves, this increased capacity of older eels suggests a higher reproduction potency.

The embryonic malformations observed in our study (Fig. 2) are typical for PCB-exposed eggs such as observed in pike *Esox lucius* (Helder 1980), carp *Cyprinus carpio* (Stouthart et al. 1998), lake trout *Salvelinus namaycush* (Walker et al. 1994) and rainbow trout *Oncorhynchus mykiss* (Walker and Peterson 1991). Similar symptoms are described for fish-eating birds and are known as GLEMEDS or 'Great Lakes embryo mortality, edema and deformities syndrome' (Gilbertson et al. 1991). The observed correlation between embryo survival time and TEQ levels in the gonads implies TEQ-induced teratogenic effects. The disrupting effects occurred at levels below 4 ng TEQ/kg gonad, below the EU eel consumption standard. Since July 2002 this is set at 4 ng TEQ/kg filet, thus far based only on dioxins and furanes (Anonymous 2001). Total TEQ levels in wild eels from all Dutch locations are in the range between 1 and 61 ng TEQ/kg eel. In the same study TEQ levels from 10 locations in 5 other European countries were found between 0 and 20 ng TEQ/kg eel (van Leeuwen et al. 2002). Only eels from Sardinia (Italy) and some locations in Ireland had TEQ levels below the limit of detection. Therefore most of the TEQ-levels in wild eel are above the levels severely impairing recruitment in our study.

In addition, migrating silver eels will use at least 60g fat/kg eel (40% of the total fat reserves) for their spawning migration (van Ginneken and van den Thillart 2000). This means, considering a biological half-live of PCBs between 1 to 4 years (de Boer et al. 1994), an increase in the concentration of the dioxin-like compounds with at least 40%. So, the TEQ values in gonads of the eels spawning in the Sargasso Sea will be even higher than those in the gonads of the artificially spawned eels in this study.

Our study suggests that current gonadal levels of dioxin-like contaminants, including PCBs, in eels from most European locations impair normal embryonic development. This conclusion is further strengthened by the fact that the emission of PCBs in the environment (van Leeuwen and Hermens 1995) coincides with the decline of eel populations (Anonymous 2003). Therefore we consider it likely that dioxin-like PCBs contributed to the current collapse of the European eel populations.

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