RECENT ADVANCES IN THE STUDY OF
FISH EGGS AND LARVAE
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Reproductive fitness of lake trout (*Salvelinus namaycush*) exposed to environmentally relevant concentrations of the potent estrogen ethynylestradiol (EE2) in a whole lake exposure experiment

JULIETA WERNER [1], VINCE P. PALACE [1, 2], KERRY G. WAUTIER [2], KENNETH H. MILLS [2], SANDRA M. CHALANCHUK [2] AND KAREN A. KIDD [2]

[1] University of Manitoba, Department of Zoology, Winnipeg, Canada.
[2] Department of Fisheries and Oceans, Freshwater Institute, Winnipeg, Canada.

SUMMARY: The synthetic estrogen, ethynylestradiol (EE2), has been identified in many aquatic environments. EE2 induces biochemical and physiological effects in exposed fish, but linkages to widespread reproductive dysfunction in populations have not been established. Mortality in early life stages has only been documented at relatively high concentrations, above those found in the environment. To examine the potential effects of environmentally relevant concentrations of EE2, reproductive endpoints were examined in lake trout (*Salvelinus namaycush*) captured from a lake experimentally treated with ~5 ng/L EE2. Monitoring began two years prior to EE2 additions in the lake and for 3 years of additions. A nearby lake in which no EE2 was added was used as a reference. Eggs from fish in each lake were fertilised with milt from the same fish stocks. Fertilization and hatch, mortality, deformities, and size of the fry at swim up were not negatively affected by EE2 exposure. While our earlier studies have reported impaired reproductive success in small-bodied fish exposed to EE2 in the same system, lake trout appear to be less affected at the biochemical level and no impacts were determined in other reproductive and population level impacts.

Keywords: reproduction, environmental estrogen, deformities, fertilization.

RESUMEN: CAPACIDAD REPRODUCTIVA DE LA TRUCHA DE LAGO (*SALVELINUS NAMAYCUSH*) EXPUESTA A CONCENTRACIONES AMBIENTALES RELEVANTES DEL POTENTE ESTRÓGENO ETILESTRADIOL (EEZ) EN UN EXPERIMENTO DE EXPOSICIÓN EN UN LAGO. – El estrógeno sintético, etinilestradiol (EE2), ha sido encontrado en varios sistemas acuáticos. Se ha demostrado que los peces expuestos a este compuesto sufren cambios bioquímicos y fisiológicos, pero el impacto en las funciones reproductivas de las poblaciones no ha sido examinado. Para examinar el efecto de concentraciones relevantes del EE2, examinamos los marcadores reproductivos en la trucha de lago (*Salvelinus namaycush*) expuesta a ~ 5 ng/L EE2. El EE2 fue adicionado a un lago cuyo monitoreo se llevo a cabo 2 años antes y tres años después de la adición del compuesto. Un lago sin contaminar fue utilizado como referencia. Huevos obtenidos de las truchas de ambos lagos fueron fertilizados con semen de machos del lago correspondiente. Los porcentajes de fertilización, eclosión, mortalidad y deformidades, así como la talla de los alevines no fueron afectados por la exposición al EE2. Anteriormente, en este mismo lago, habíamos demostrado que el éxito reproductivo en los peces de pequeña talla estaba significativamente afectado, sin embargo la trucha de lago no parece ser afectada a nivel bioquímico y no se produjeron impactos, en términos reproductivos o a nivel de poblaciones.

Palabras clave: reproducción, estrogeno ambiental, deformidades, fertilización.

INTRODUCTION

Over the past two decades, considerable effort has been expended to quantify the potential effects of contaminants with the ability to mimic, alter or inhibit the action of endogenous hormones in exposed wildlife (Jobling *et al*., 1998; Larsson *et al*., 1999; Folmar *et al*., 2001). In aquatic systems, one of the

most relevant "endocrine disruptors" is ethynylestradiol (EE2), the main component of birth control pills (Larsson *et al.*, 1999; Korsgaard *et al.*, 2002). EE2 has been quantified downstream of sewage treatment plants in Europe, the United Kingdom, Brazil and Canada (Desbrow *et al.*, 1998; Routledge *et al.*, 1998; Ternes *et al.*, 1999) at concentrations as high as 47 ng/l. However, typical concentrations in STP effluents range between 1 and 10 ng/l.

Several diagnostic indicators of exposure have been developed that are reliable for confirming the presence of environmental estrogens as well as for confirming exposure to these compounds in aquatic organisms. These include production of the egg yolk protein vitellogenin (VTG) (Arcand-Hoy and Benson, 1998; Larsson *et al.*, 1999; Folmar *et al.*, 2001; Palace *et al.*, 2002; Van den Belt *et al.*, 2003) in male fish and the development of ova-testis (i.e. intersex) (Jobling *et al.* 1998). However, there have been few examinations of the links between biochemical indicators measured in fish and recruitment in those populations. Some studies have shown that EE2 exposure can reduce the number of eggs a female produces and lower the hatching success of those eggs. However, these effects have only been observed at high concentrations relative to environmental levels (Van den Belt *et al.*, 2001; Zillioux *et al.*, 2001; Schultz *et al.*, 2003) and primarily in small-bodied fish with relatively short reproductive cycles (Scholz and Gutzeit, 2000; Lange *et al.*, 2001; Gonzales-Doncel *et al.*, 2003; Seki *et al.*, 2003; Nakayama *et al.*, 2004). Here we describe a detailed examination of reproductive endpoints in lake trout (*Salvelinus namaycush*), a large bodied fish with a relatively long reproductive cycle exposed to ~5 ng/l EE2. The lake trout in this study were obtained from a whole lake experimentally treated with EE2 in northwestern Ontario, Canada. Percent fertilisation, mortality, deformities, and growth indicators measured at the swim up stage are reported over a 3-year period during EE2 addition and for 2 years prior to the EE2 treatment in fish from the experimental lake and from a nearby unmanipulated, reference lake.

MATERIALS AND METHODS

Chemicals and EE2 lake exposure

17α-ethynylestradiol was generously provided by Schering AG, Berlin, Germany. Details regarding the procedures for adding EE2 to Lake 260 and analysis of the compound in the water-column have been previously described (Palace *et al.*, 2002). Briefly, EE2 was dissolved in distilled glass grade methanol (Caledon Laboratories, Georgetown, ON, Canada) and pumped into the propeller wash of a boat as it was driven in transects across the lake. EE2 was added three times per week at a daily rate of between 100 and 450 mg/day, depending on the previously measured concentration. Continuous addition was based on previous enclosure experiments that determined the half-life of EE2 in the water column of this lake to be 12 d (K.A. Kidd, unpublished data). A total of 43.3, 44.2, and 38.7 g of EE2 were added in the three respective addition years of this study.

Collection of gametes and fertilisation

Lake trout were collected from spawning shoals in Lakes 260 (EE2 addition lake: Area 34 ha, Max depth 14.4 m, Volume 17.64 10^5 m^3) and 442 (reference lake: Area 8 ha, Max depth 17 m, Volume 17.54 10^5 m^3) using small mesh gillnets that were emptied every 10 to 15 minutes. Collections from 1999 and 2000 in Lake 260 were obtained prior to additions of the EE2. EE2 additions began in spring 2001 and continued throughout the ice free season (May-October) each year until 2003. Male and female lake trout used were anaesthetised with MS-222 (0.4 mg/l). After fin movement ceased (<2 min), fish were blotted dry to avoid any contact of the eggs or milt with water. Eggs and milt were then obtained by gently massaging the abdominal region. If eggs from a given female became contaminated with water or urine, the entire batch was discarded and not used for these studies. Eggs from each female were collected into a clean, dry graduated cup to determine expressed egg volume to the nearest 5 ml and then gently poured into a separate plastic bag in which the headspace was filled with 100% oxygen. Bags were sealed and placed on their sides so that the eggs were distributed in a monolayer. Bags were transported on ice to the laboratory at the Freshwater Institute in Winnipeg approximately 4 h away from the site of collection. Milt was also kept in oxygenated plastic bags on ice and was from a pool of several males (3-5). Upon arrival at the lab, the eggs from each female were dry-fertilised in stainless steel bowls by adding 100 μl milt per 100 ml or fraction thereof of eggs and stirring with a sterilised goose feather. The eggs were then allowed to water harden for 5 min by

adding enough dechlorinated Winnipeg city tap water (8ºC) to just cover the eggs. After water hardening, the fertilised eggs were randomly assigned to compartments of a Heath type hatchery trays (Marisource, Milton WA) and incubated at 8±1ºC; dissolved oxygen in the water was always >90%. Mortalities were recorded daily and dead eggs and fry were preserved in Davidson's fixative [95% ethyl alcohol:formalin:glacial acetic acid:distilled water:glycerine (30:20:10:30:10)]. Percent mortality was calculated based on the number of fertilised eggs that died during the incubation period to swim-up compared to the total number of eggs that were fertilised. All fry were sacrificed at swim up when yolk reabsorption was almost complete (Mean=707±31 Temperature Units) by first anaesthetising them in MS-222 (0.4 g/l) until movement ceased and then preserving them in Davidson fixative. Time to hatch, percent fertilisation, percent mortality, weight and length of the fry at swim up were all recorded. Percent fertilisation was calculated by subtracting the number of eggs that did not show any signs of fertilisation or the eggs that died before 14 days after fertilisation from the total number of eggs.

Spinal and cranial deformities were also evaluated in each fry using previously described methods (Holm *et al*., 2002). Briefly, three different categories of deformities were recorded in the fry: cranial deformities included reduction or absence of the jaws as well as asymmetry of the cranium, skeletal deformities included kyphosis (convex curvature of the thoracic region of the spine), lordosis (concave curvature of the lumbar region of the spine) and scoliosis (lateral curvature of the spine), and bifurcation of the spine (Fig. 3). The presence of oedema in the region of the yolk sac was also recorded.

Statistical analysis

Data are expressed as mean ± SEM. Percent fertilisation, hatch, deformity and mortality data were arcsin transformed and analysed for normality and homogeneity of variance. A one-way ANOVA was performed on the data followed by Dunnett's test, and a p value of <0.05 was regarded as significant. When the data failed normality and homogeneity of variance, an ANOVA on ranks was performed followed by Dunn's test. The same procedures were used to analyse the data for condition factor, length and weight of the adult females and length and weight of the fry at the swim up stage.

RESULTS

Adult somatic parameters

There were no differences in length or weight of the females obtained from the lake treated with EE2 compared to the reference lake, and there were also no differences when compared with collections obtained from the same lake in each of the 5 study years (Table 1). There was a significant difference in the length of the females obtained in 1999 from Lake 442 when compared through the years. No significant differences in female length were found in Lake 260. Although there were some significant differences in condition factor (K=weight/(length $X10^5)^3$) between the two lakes when compared within the same year (i.e. in 2002, K in Lake 442 was significantly higher and in 2003 K in Lake 260 was significantly higher ($P<0.05$)), there were no consistent differences related to EE2 exposure.

TABLE 1. – Length, weight, condition and expressed egg volume from adult female lake trout captured in Lakes 442 and 260 from 1999 to 2003. A = significantly different from Lake 442 within the same year. B = significantly different from 2003 in the same lake. C = Significantly different from 2000 to 2003 in the same lake. D = Significantly different from 2001 in the same lake. Data are expressed as Mean ± standard error, $p<0.05$.

	Lake	1999	2000	2001	2002	2003
Length (cm)	442	43.18±0.28B	41.98±0.77	40.86±0.51	41.38±0.18	40.85±0.56
	260	44.33±1.23	43.14±0.39	42.8±0.71	42.38±0.94	42.03±0.58
Weight (g)	442	768±10.15	896±64.31	738±22.67	738±26.80	810±37.18
	260	753±63.38	846±30.21	805±27.43	823±41.55	778±17.01
Condition Factor (K)	442	0.95±0.02C	1.20±0.50	1.15±0.01	1.08±0.07	1.05±0.01
	260	0.86±0.07D	1.06±0.08A	1.15±0.08	1.10±0.07	1.18±0.05A
Egg Volume (ml/g Bdwt)	442	0.078±0.014	0.088 ± 0.012	0.089 ± 0.011	0.071 ± 0.012	0.088 ± 0.005
	260	0.087±0.008	0.066 ± 0.008	0.094 ± 0.014	0.079 ± 0.006	0.090 ± 0.009

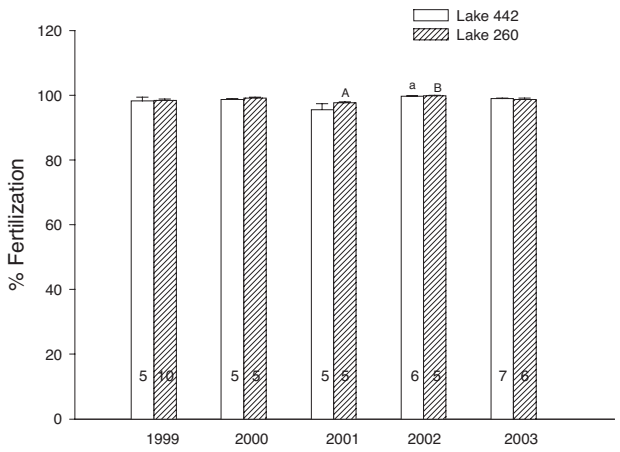

FIG. 1. – Percent Fertilisation. A = Significantly different from Lake 260 in 2000, P<0.05. B = Significantly different from Lake 260 in 1999, 2000, 2001 and 2003, P<0.05. a = Significantly different from Lake 442 in 2001, P<0.05. Numbers within the bars represent number of females. Data are expressed as mean with standard error.

Fertilisation success

Fertilisation success in lake trout eggs from Lake 260 was significantly different in 2000 compared to eggs obtained from the same lake in 2001 and 2002 (Fig. 1). In Lake 442, percent fertilisation was significantly different between 2001 and 2002. There were no significant differences in fertilisation success between the two study lakes for any of the 5 years.

Hatching success

In total from the two study lakes, 94% of the fertilised eggs hatched. Hatching success was lower in both lakes in 2001 than in 1999, but there were no other differences between the remaining years. Hatching success was significantly different between the two lakes in 2000, 2001 and 2003, where lower hatching success in Lake 442 was observed in all three years when compared to Lake 260 (Fig. 2).

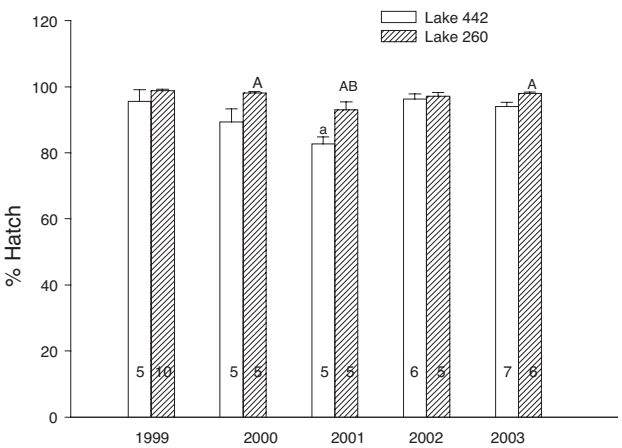

FIG. 2. – Percent Hatch. A = Significantly different from Lake 442 within the same year, P<0.05. B = Significantly different from Lake 260 in 1999, P<0.05. a = Significantly different from Lake 442 in 1999, P<0.05. Numbers within the bars represent number of females. Data are expressed mean with standard error.

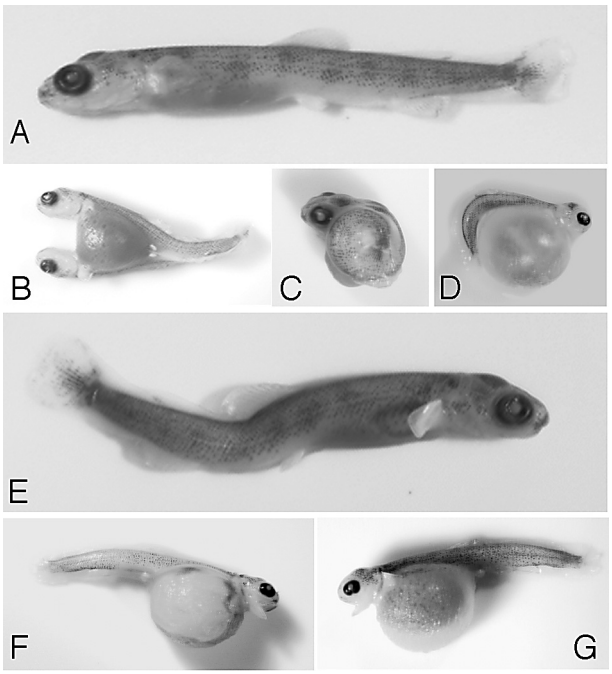

FIG. 3. – Lake trout fry from Lake 442 showing the most commonly observed deformities. A is a normal fry, B is a fry with a bifurcated spine. C, lateral spinal curvature; D, shortened embryo with dorso ventral spinal curvature; E, distal lateral curvature of the spine; F, haemorrhage in the yolk sac; G, deformed lower jaw.

Deformities

Edema in the region of the yolk sac was often associated with spinal curvatures and shortened jaws (Fig. 3). Edematous fry were usually shorter and had utilised less yolk than more typical fry. Percent deformities found in 1999 were different from those of other years for both of the study lakes (Fig. 4). Higher rates of deformities were found in fry from Lake 442 than in those from Lake 260 in 2001 and 2003.

Mortalities

Mortality was less than 40 percent during the 5-year study period (Fig. 5). There was consistently less mortality in eggs/fry from Lake 260 than in those from Lake 442. These differences were significant in 1999, 2000 and 2001.

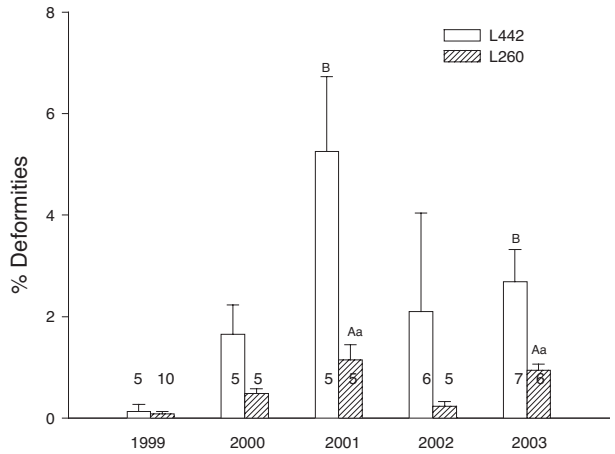

FIG. 4. – Percent Deformities. A = Significantly different from Lake 442 within the same year, P<0.05. B = Significantly different from Lake 260 in 1999, P<0.05. a = Significantly different from Lake 442 in 1999, P<0.05. Numbers within the bars represent number of females. Data are expressed as mean with standard error.

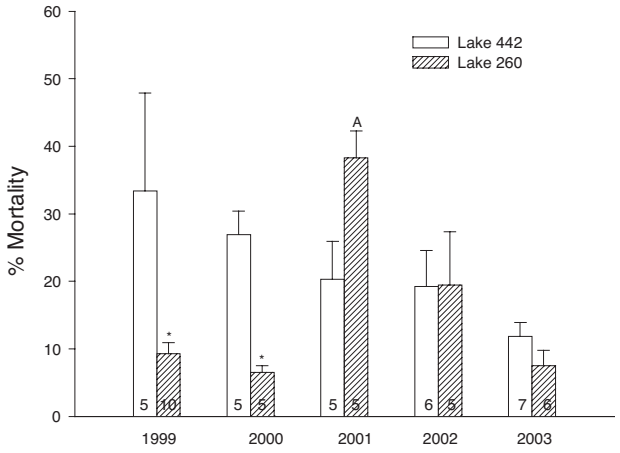

FIG. 5. – Percent Mortality. A = Significantly different from Lake 442 within the same year, P<0.05. Numbers within the bars represent number of females. Data are expressed as mean with standard error.

Length and weight of fry at swim up stage

Through the 5-year period, fry from Lake 442 varied significantly in body length, whereas fry from Lake 260 showed a more consistent size throughout the 5-year study period (Fig. 6). Fry from Lake 260 were significantly longer than fry from Lake 442 within the same year in all years with the exception of 2001. The only significant difference in weight within a lake was in Lake 260, where fry in 2000 were heavier than those obtained in 1999, 2001 and 2002 (Fig. 7). Fry from Lake 260 were significantly heavier than fry from Lake 442 in 1999, 2000 and 2003.

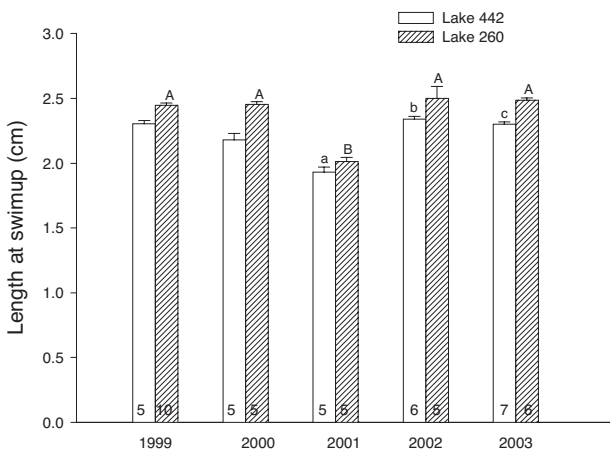

FIG. 6. – Length of fry at the swim up stage. A = Significantly different from Lake 442 within the same year, P<0.05 B = Significantly different from Lake 260 in 1999, 2000, 2002 and 2003, P<0.05. a = Significantly different from Lake 442 in 1999 and 2000, p<0.05. b = Significantly different from Lake 442 in 2000 and 2001, P<0.05. c = Significantly different from Lake 442 in 2001, P<0.05. Numbers within the bars represent number of females. Data are expressed as mean with standard error.

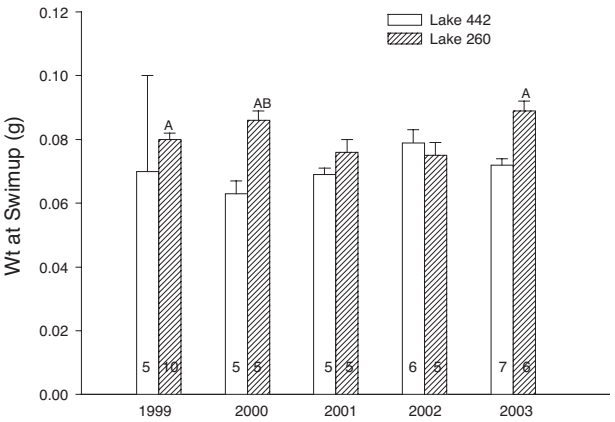

FIG. 7. – Weight of fry at the swim up stage. A = Significantly different from Lake 442 within the same year, P<0.05. B = Significantly different from Lake 260 in 1999, 2001 and 2002, P<0.05. Numbers within the bars represent number of females. Data are expressed as mean with standard error.

DISCUSSION

We have previously reported biochemical and population level effects in adult small-bodied fish exposed to EE2 from this whole-lake experiment (Palace *et al.*, 2002, 2006). While we have also identified significant induction of VTG in adult lake trout from this experiment (data not shown), the results presented in Table 1 suggest that condition and fecundity of the females has not yet been affected in this species. The absence of an effect from prolonged exposure to EE2 is an important finding in light of previous results from other species. For

example, in female zebrafish, (*Danio rerio*) reproductive success can be impaired by estrogen exposure through reduced egg production (Orn *et al.*, 2000; Van den Belt *et al.*, 2001). Non-spawning female zebrafish also show significantly smaller ovaries that lack mature oocytes when exposed to EE2 at concentrations of 10 ng/L for a period of 3 weeks (Van den Belt *et al.*, 2001). Lower egg production has also been reported in fathead minnow (*Pimephales promelas*) (Schweinfurth *et al.*, 1996; Parrott and Blunt, 2005), sheepshead minnow (*Cyprinodon variegatus*) (Shoida and Wakabayashi, 2000) and medaka (*Oryzias latipes*) (Zillioux *et al.*, 2001) when they are exposed to EE2 at concentrations that range from 3 to 27 ng/L.

Reproductive success of adult male fish can also be affected by exposure to EE2, but similar to our findings in females, we found no impairment in the ability of milt from lake trout exposed to EE2 to fertilise eggs (Fig. 1). It has already been established that male fish exposed to estrogenic compounds produce high levels of VTG and may also exhibit the presence of ovarian tissue within the testes structure (ova-testis or intersex) in their gonads (Schwaiger *et al.*, 2002; Seki *et al.*, 2003). The presence of this ovarian tissue mixed with testicular tissue in genetically male fish can affect sperm density, sperm motility and duration of sperm motility (Bachmann-Christiansen, 2002; Jobling *et al.*, 2002). In fact, fertilisation success was below 70% in male zebrafish exposed to EE2 at concentrations of 5-25 ng/L, and at 10-25 ng/L EE2 these fish showed a significant reduction in GSI (Van den Belt *et al.*, 2001). No spermatogenesis was observed in male medaka exposed to concentrations of EE2 that were higher than 25 ng/L. Parrott and Blunt (2005) showed reduced ability of male fathead minnows to fertilise eggs even when fish were exposed to less than 1 ng/L of EE2.

The absence of overt effects on the quality of gametes from lake trout in this study may, in part, be related to differences among fish species in their sensitivities to EE2. We have reported that VTG was induced to a far greater extent in fathead minnows (*Pimephales promelas*) than in pearl dace (*Margariscus margarita*) (Palace *et al.*, 2002; Palace *et al.*, 2006), which were in turn induced more than lake trout or white suckers (*Catostomus commersoni*) (unpubl. data). As a biochemical indicator, the sensitivity of VTG induction appears to be the same as the appearance of disturbances at higher levels of organisation, i.e. histopathological tissue lesions and population declines. Specifically, histopathological lesions in kidney and inhibited testicular development were observed in fathead minnows (*P. promelas*) during the first year of the experiment and declining population numbers were identified in the second year of EE2 exposure within the lake (unpublished observations). Pearl dace (*M. margarita*) had the next highest VTG induction levels, but similar histopathological lesions did not appear until the second and third year of exposure and some evidence of population level decline appeared after 3 years of exposure (Palace *et al.*, 2006). Lake trout (*S. namaycush*) and white sucker (*C. commersoni*) from the lake have shown lesser inductions of VTG, an absence of histopathological lesions and no evidence of population decline to this point.

Whereas the reproductive potential of adult lake trout from this study appear not to have been affected as yet by EE2 exposure, it is the early life stages of fish that are generally considered to be the most sensitive to exposure to endocrine active compounds. This is because sex determination takes place within an early developmental window (Piferrer, 2001). Sharpe and Skakkebaek (1993) have further proposed that exposure of males (animals and humans) to estrogens or to chemicals that mimic estrogens during a critical period of their lifetime can disrupt normal development. Mortalities and deformities were not elevated by exposure of adult females or males to EE2 in this study. In fact, mortality and deformities were marginally higher in the reference lake than in the EE2-treated lake. These differences probably reflect environmental factors rather than a beneficial effect of EE2 exposure. For example, it is known that survival rates decrease and embryo malformation rates increase with an increase in post-ovulatory oocyte ageing (Aegerter and Jalabert, 2004). It should be noted that the timing for spawning of eggs from broodstock in each of the lakes was determined by periodic examination of the adult fish caught in trap nets in each lake. When ripe and running fish were observed, a sub-sample of the next available catch was recruited for these studies. Both lakes were sampled at the same time, so it is possible that the fish from Lake 442 began spawning at an earlier date than those from Lake 260. This would have made it more likely that the eggs and milt obtained from fish in Lake 442 were past their optimal sampling period.

Obtaining eggs and milt during their optimal ripened period is particularly important in the case of salmonids, in which mature oocytes are expelled from the gonad proper at ovulation into the coelomic cavity. They remain immersed in ovarian fluid until spawning is triggered by environmental and social stimuli (Aegerter and Jalabert, 2004). After ovulation, the proportion of fertilisable oocytes progressively decreases at a rate which depends on donor females and external factors such as temperature. This post-ovulatory ageing period can last from several days to a few weeks, during which modifications progressively occur affecting egg viability (Kjorsvik *et al*., 1990), egg shape (Sakai *et al*., 1975) and egg biochemical composition (Lahnsteiner, 2000) as well as damage to germ cells (Knorr and Braunbeck, 2002).

Post-ovulatory oocyte ageing in *Cyprinus carpio* has been associated with a decrease in oocyte ATP concentration, leading to an irreversible shortage of available metabolic energy that could alter cytoskeletal organisation (Boulekbache *et al*., 1989). When female rainbow trout were held at 12ºC, survival rates at eyeing, hatch and swim-up stages remained constant in eggs sampled at 0 and 7 days post-ovulation, and significantly decreased thereafter (Aegerter and Jalabert, 2004). The percentage of malformed alevins increased significantly after only 1 week of post-ovulatory oocyte ageing at 12ºC. It remained constant between 7 and 14 days post-ovulation, and decreased significantly thereafter (Aegerter and Jalabert, 2004). The rate of abnormal embryonic development increases significantly with the retention time of the eggs in the ovarian cavity following ovulation (Linhart and Billard, 1995). An increase in egg mortality, infertility, and malformations has also been observed in eggs from female Atlantic salmon (*Salmo salar*) obtained a number of days after ovulation (De Gaudemar and Beall, 1998). Malformations in European catfish (*Silurus glanis* L.) eggs stripped 6 h after the first spawning showed three types of abnormalities, including strong curvature of the spinal column, deformed tail and pericardial oedema (Varkonyi *et al*., 1998).

Declining quality of fertilised eggs after the optimal period of spawning is probably a reflection of egg quality and not a result of compromised sperm quality. Yamazaki and Arai (1982) found no significant increase in abnormal salmon embryos from eggs fertilised with sperm obtained 5-28 days after spermiation in masu salmon (*Oncorhynchus masou*). This suggests that the source of the aberrations is in the female gamete and not in the ability of the sperm to fertilise the eggs.

In conclusion, we have shown that after three years of exposure to environmentally relevant concentrations of the estrogenic contaminant, EE2, lake trout exhibited no detrimental effects in terms of the ability of their eggs to be fertilised or of their sperm to fertilise eggs. Additionally, weight and size, as well as the rates of deformities, were not negatively affected in fry derived from EE2 exposed adults. These findings are in contrast with our earlier work, in which we have described impaired reproductive success in small-bodied fish exposed to EE2 in the same experimentally treated lake. It appears that the sensitivity of reproductive endpoints among fish species within this system closely resembles the sensitivity of disturbances at higher levels of biological organisation, i.e. population decline.

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