Der Einfluß von hormonell aktiven Substanzen aus der Umwelt (endocrine disrupters) auf die Reproduktion von Fischen (ENDOREP)

ABSCHLUSSBERICHT

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Zusammenfassung

Für die erfolgreiche Fortpflanzung von Fischen ist der ungestörte Ablauf einer Reihe biologischer Prozesse notwendig, die unter hormoneller Kontrolle stehen. Hormonell wirksame Stoffe aus der Umwelt können diese Prozesse stören oder unterbrechen. Im durchgeführten Projekt wurde der Einfluss von drei hormonell wirksamen Stoffen aus der Umwelt, von 4-Nonylphenol, Bisphenol A und ß-Östradiol auf die Fortpflanzung der Salmonidae (Regenbogenforelle, Bachforelle, Äsche) untersucht. In Laboruntersuchungen wurde bestimmt, welche Phasen der Fortpflanzung von hormonell aktiven Substanzen aus der Umwelt beeinflusst werden und bei welchen minimalen Konzentrationen diese Effekte auftreten.

Es wurden umweltrelevante Konzentrationen von 4-Nonylphenol, Bisphenol A und ß-Östradiol getestet, die in österreichischen Gewässern vorkommen, sowie jene Konzentrationen, die entsprechend dem bisherigen Wissenstand keinen Einfluss auf die Reproduktion von Fischen haben sollten (Unbedenklichkeitskonzentrationen).

Eine negative Beeinflussung der Fortpflanzung wurde bereits bei den bisher als unbedenklich erachteten Konzentrationen von Bisphenol A (1.7 µg l^{-1}), ß-Östradiol (1 ng l^{-1}) und 4-Nonylphenol (130 ng l^{-1}) festgestellt. Bei diesen Konzentrationen trat eine Verringerung der Samenqualität (4-Nonylphenol, Bisphenol A, ß-Östradiol), eine Störung des zeitlichen Ablaufes der Gametenreifung (Bisphenol-A, ß-Östradiol) und eine Verringerung des Wachstums der Larven und Jungfische (4-Nonylphenol, Bisphenol A, ß-Östradiol) auf. Es ist sehr wahrscheinlich, dass diese negativen Effekte den natürlichen Fortpflanzungserfolg der Salmonidae entscheidend verringern. Die beschriebenen Versuche wurden über eine begrenzte Zeitspanne von 2 – 4 Monaten und in speziellen Lebensabschnitten der Fische durchgeführt. Es ist zu erwarten, dass die Unbedenklichkeitskonzentrationen noch bedeutend niedriger liegen, wenn die Fische während ihres gesamten Lebenszyklus hormonell aktiven Substanzen aus der Umwelt ausgesetzt sind.

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Abschlussbericht

Im durchgeführten Projekt wurde untersucht, welche Auswirkungen die hormonell wirksamen Substanzen aus der Umwelt 4-Nonylphenol, Bisphenol A und ß-Östradiol auf die Fortpflanzung bei Salmoniden (Regenbogenforelle - *Oncorhynchus mykiss*, Bachforelle - *Salmo trutta f. fario*, Äsche - *Thymallus thymallus*) haben. Im Detail wurde der Einfluss auf die Samenqualität (produzierte Samenmenge, Spermiendichte, Spermienmotilität und Spermienfertilität) und Eiqualität (produzierte Anzahl von Eiern, Eigewicht, Quellungsrate, Befruchtungsfähigkeit), auf den Zeitpunkt der Reife, auf den Befruchtungsvorgang und auf die Embryonal- und Larvalentwicklung (Überlebensrate, Wachstum) untersucht.

Um diese Versuche durchführen zu können, wurde eine geeignete Versuchsanlage aufgebaut, in der die Versuchsfische, die befruchteten Eier und die Larven im Durchfluss über Zeiträume von 2 – 4 Monaten den aufgelisteten Schadstoffen ausgesetzt werden konnten. Die aufgebaute Versuchsanlage hat 4 Testeinheiten. Jede dieser Testeinheiten weist eine exakte und konstante Quellwasserversorgung auf und die Testsubstanzen werden mittels Dosierpumpen zugesetzt. Die exakte Anordnung dieses Systems ist in den beiliegenden Publikationen Lahnsteiner et al. (2005a) und Lahnsteiner et al. (2005b) im Detail beschrieben. Diese Versuchsanlage ist auch dazu geeignet, den Einfluss anderer Umweltgifte auf Fische, und Fischeier und Fischlarven zu testen.

Die Testkonzentrationen betrugen für 4-Nonylphenol 100, 250 und 750 ng/l, für Bisphenol A 1,7, 2,4 und 5,0 µg/l und für ß-Östradiol 0,5, 1,0, und 2,0 ng/l. Dies sind umweltrelevante Konzentrationen, die in österreichischen Gewässern vorkommen, sowie die Konzentrationen, die entsprechend dem bisherigen Wissenstand keinen Einfluss auf die Reproduktion bei Fischen haben sollten (Unbedenklichkeitskonzentrationen; 330 ng/l für 4-Nonylphenol, 1,7 µg/l für Bisphenol A und 1 ng/l für ß-Östradiol).

1. Einfluss von hormonell aktiven Substanzen aus der Umwelt auf Reifung und Qualität der Gameten

Um den Einfluss von Bisphenol A auf die Reifung und auf die Qualität von Samen und Eiern zu untersuchen, wurden männliche und weibliche Bachforellen während der Vorlaich- und Laichzeit Bisphenol A Konzentrationen von 1,7, 2,4 und 5,0 µg/l ausgesetzt. Die Details dieser Studie sind der beigelegten Publikation Lahnsteiner et al. (2005b) zu entnehmen. In Bachforellen, die Bisphenol A Konzentrationen von 1,7 und 2,4 µg/l ausgesetzt waren, war im Vergleich zur Kontrolle die Samenqualität am Beginn der Laichzeit und in der Mitte der Laichzeit niedriger als in der Kontrolle. Diese Bachforellen produzierten nur am

Ende der Laichzeit und mit einer Verzögerung von etwa 4 Wochen hochqualitativen Samen. Bisphenol A Konzentrationen von 5,0 µg/l hemmten die Samenproduktion, da nur ein sehr geringer Prozentsatz der Fische geringe Samenmengen von sehr schlechter Qualität produzierte. Der Prozentsatz der weiblichen Fische mit reifen Eiern war in den Kontrollfischen und in den Fischen, die Bisphenol A Konzentrationen von 1,7 und 2,4 µg/l ausgesetzt worden waren, gleich hoch. Jedoch fand die Reifung der Eier bei Bisphenol A Konzentrationen von 1,7 µg/l Bisphenol A ungefähr 2 Wochen später als bei den Kontrollfischen statt, bei Bisphenol A Konzentrationen von 2,4 µg/l ungefähr 3 Wochen später. Es konnte kein Einfluss auf die Eiqualität festgestellt werden. Bei Bisphenol A Konzentrationen von 5,0 µg/l war die Reifung der Eier gehemmt.

Der Einfluss von ß-Östradiol auf die Reifung und Qualität der Gameten wurde in der Regenbogenforelle und in der Äsche untersucht. Die ausführliche Beschreibung dieses Experiments ist der beigelegten Publikation Lahnsteiner et al. (2005c) zu entnehmen. Bei Regenbogenforellen, die während der Laichzeit ß-Östradiolkonzentrationen von $\geq$ 1 ng/l ausgesetzt waren, zeigten sich nach 35 Tagen Exposition negative Effekte, da das Samenvolumen, das pro Fisch produziert wurde, die Samendichte und die Samenfertilität erniedrigt waren. Ebenso war bei Äschen, die während der Vorlaichzeit ß-Östradiolkonzentrationen von $\geq$ 1.0 ng/l ausgesetzt waren, das Samenvolumen und die Spermienmotilität erniedrigt, was wiederum erniedrigte Fertilität zur Folge hatte. Der Zeitpunkt der Samenreifung wurde nicht beeinflusst. Wurden reife, weibliche Regenbogenforellen ß-Östradiolkonzentrationen von 0,5 – 2 ng/l ausgesetzt und die Eier portionsweise in 1-wöchigem Intervall abgestreift, veränderte sich die Eiqualität auf die gleiche Weise wie in der Kontrolle. Daher beeinflusste ß-Östradiol die Überreifung der Eier nicht. Wurden weibliche Äschen während der Vorlaichzeit 1,0 ng/l ß-Östradiol ausgesetzt, fand die Reifung der Eier 2 Wochen früher statt als in der Kontrolle, die Reifung der Eier war also beschleunigt.

Um den Einfluss von 4-Nonylphenol auf die Fortpflanzung der Salmonidae zu untersuchen, wurden Regenbogenforellen während der Laichzeit 4-Nonylphenolkonzentrationen von 100 - 750 ng/l ausgesetzt. Die Durchführung dieses Versuchs sowie die exakten Ergebnisse sind der beigelegten Publikation Lahnsteiner et al. (2005a) zu entnehmen. Bei einer Konzentration von 750 ng/l stellten die Fische die Samenproduktion vollständig ein. Bei Konzentrationen von 280 ng/l und 130 ng/l war die Samenproduktion im Vergleich zur Kontrolle signifikant reduziert. Spermiendichte, Spermienmotilität und Spermienfertilität wurden nicht beeinflusst. Der Einfluss auf die Eier

wurde nicht untersucht.

2. Einfluss von endokrin aktiven Substanzen aus der Umwelt auf den Befruchtungsprozess

Da Salmonidae äußere Befruchtung haben, könnte der Befruchtungsprozess von Umweltgiften negativ beeinflusst werden. In der Regenbogenforelle, Bachforelle und Äsche hatten Bisphenol A, 4-Nonylphenol und ß-Östradiol in den getesteten Konzentrationen keinen Einfluss auf den Befruchtungsvorgang. Die Daten für 4-Nonylphenol wurden in der Publikation Lahnsteiner et al. (2005a) veröffentlicht. Wurde die Befruchtung der Eier in mit Bisphenol A, 4-Nonylphenol oder ß-Östradiol belastetem Wasser durchgeführt, unterschied sich die Befruchtungsrate nicht von der Kontrolle. Wurde die Spermienmotilität in mit Bisphenol A, 4-Nonylphenol oder ß-Östradiol belastetem Wasser aktiviert, zeigten sich in den Motilitätsparametern keine Unterschiede zur Kontrolle. Bisphenol A, 4-Nonylphenol und ß-Östradiol hatte auch keinen Einfluss auf die Befruchtungsfähigkeit der Eier.

3. Einfluss von endokrin aktiven Substanzen aus der Umwelt auf die Entwicklung der Embryonen und Larven

Wie in den beigelegten Publikationen Lahnsteiner et al. (2005a) und Lahnsteiner et al. (2005d) dargestellt, hatten von den getesteten hormonell aktiven Substanzen aus der Umwelt nur 280 ng/l und 750 ng/l Nonylphenol einen Einfluss auf die Überlebensrate der Embryonen und Larven von Regenbogenforelle, Äsche, Bachforelle und Renke. Bei diesen 4-Nonylphenolkonzentrationen war der Prozentsatz der Embryonen im Augenpunktsstadium geringfügig aber signifikant um 2 - 4% reduziert. Bedeutend empfindlicher reagierten die Larven auf 4-Nonylphenol. Bei 4-Nonylphenolkonzentrationen von 750 ng/l überlebten nur 23,8 ± 1,2% der Larven bis zum Ende des Dottersackstadiums, bei 280 ng/l 53,7 ± 8,2%, während bei 4-Nonylphenolkonzentrationen von 130 ng/l 73,8 ± 1,5% überlebten und in der Kontrolle 70,9 ± 1,8%. Bisphenol A und ß-Östradiol hatten keinen statistisch signifikanten Einfluss auf die Überlebensrate der Embryonen und Larven.

Das Wachstum der Larven wurde von den getesteten hormonell aktiven Substanzen aus der Umwelt signifikant beeinflusst. Die exakten Ergebnisse sind der Publikation Lahnsteiner et al. (2005d) zu entnehmen. Wurden die Embryonen und Larven der Äsche und Renke in mit 4,5 µg/l Bisphenol A belastetem Wasser aufgezogen, betrug das Gewicht der Fische nach 60 – 68 d (Stadium der Metamorphose zu Jungfischen) nur 35% der Kontrolle. In mit 0,13 µg/l 4-Nonylphenol und 1,5 ng/l ß-Östradiol belastetem Wasser betrug das Gewicht

der Jungfische 50-70% der Kontrolle. Auch die Länge der Fische war signifikant reduziert.

Zusammenfassend kann ausgesagt werden, dass hormonell aktive Substanzen aus der Umwelt folgenden Einfluss auf die Fortpflanzung der Salmoniden haben: Verringerung der Samenqualität (4-Nonylphenol, Bisphenol A, ß-Östradiol), Desynchronisation der Gametenreifung (Bisphenol A, ß-Östradiol) und Reduktion des Wachstums der Larven und Jungfische (4-Nonylphenol, Bisphenol A, ß-Östradiol). Es ist wahrscheinlich, dass dies den natürlichen Fortpflanzungserfolg der Salmonidae entscheidend verringert. Die geringsten Konzentrationen, bei denen diese Effekte beobachtet wurden (LOEC – lowest observed effect concentrations) sind 1,7 µg/l für Bisphenol A, 1 ng/l für ß-Östradiol und 130 ng/l für 4-Nonylphenol. Dies sind Konzentrationen, die bisher als Unbedenklichkeitskonzentrationen betrachtet wurden. Die beschriebenen Versuche wurden über eine begrenzte Zeitspanne von 2 bis 4 Monaten und in speziellen Lebensabschnitten der Fische durchgeführt. Es ist zu erwarten, dass die Unbedenklichkeitskonzentrationen noch bedeutend niedriger liegen, wenn die Fische während ihres gesamten Lebenszyklus hormonell aktiven Substanzen aus der Umwelt ausgesetzt sind.

Projektrelevante Publikationen

Lahnsteiner F., Berger, B., Grubinger, F., Weismann, T., 2005a. The effect of 4-nonylphenol on semen quality, viability of gametes, fertilization success, and embryo and larvae survival in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 71, 297 - 306.

Lahnsteiner F., Berger B., Kletzl M., Weismann, T., 2005b. Effect of bisphenol A on maturation and quality of semen and eggs in the brown trout, *Salmo trutta f. fario.* Aquatic Toxicology 75, 213-224.

Lahnsteiner F., Berger B., Kletzl M., T., 2005c. Effect of ß-estradiol on gamete quality and time point of maturation in the Salmonidae as indicated by laboratory experiments. Environmental Pollution, in Druck.

Lahnsteiner F., 2005d. Reduced somatic growth of salmonid larvae exposed to 4-nonylphenol, bisphenol A, and ß-estradiol. Journal of Fish Biology, eingereicht.

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The effect of 4-nonylphenol on semen quality, viability of gametes, fertilization success, and embryo and larvae survival in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The present study investigated in vivo and in vitro effects of environmental relevant concentrations of 4-nonylphenol (100–750 ng l^{-1}) on the reproduction of rainbow trout (*Oncorhynchus mykiss*). To determine the effect of 4-nonylphenol on semen quality rainbow trout were exposed to three concentrations of 4-nonylphenol in a flow-through system during the spawning period (60 days). At an estimated 4-nonylphenol concentration of 750 ng l^{-1} semen production was completely inhibited, at 280 and 130 ng l^{-1} the semen production was significantly reduced in comparison to the control. Sperm density, sperm motility and sperm fertility were not affected.

Also the development of embryos and larvae at the end of yolk sac stage was affected by 4-nonylphenol. At estimated 4-nonylphenol exposure levels of 280 and 750 ng l^{-1} the percentage of eyed stage embryos was slightly but significantly lower (2–4%) than at 130 ng l^{-1} 4-nonylphenol and in the control. At 4-nonylphenol concentrations of 750 ng l^{-1} only $23.8 \pm 1.2\%$ of the larvae survived to the end of the yolk sac stage, at 280 ng l^{-1} $53.7 \pm 8.2\%$, at 130 ng l^{-1} $73.8 \pm 1.5\%$, and in the control $70.9 \pm 1.8\%$.

Sperm motility was not affected by 4-nonylphenol as sperm motility rate, swimming velocity, swimming pattern and motility duration were similar in water and in water containing of 100, 250, or 750 ng l^{-1} 4-nonylphenol. Incubation of eggs in physiological saline solution containing of 100, 250, or 750 ng l^{-1} 4-nonylphenol did not change their fertilizability in comparison to the control. Therefore, 4-nonylphenol did not affect the egg viability. Also the fertilization process (sperm egg contact) was not influenced by 4-nonylphenol as the fertilization rate (percentage of hatched larvae) was similar to the control when eggs were fertilized in water containing of 100, 250, or 750 ng l^{-1} 4-nonylphenol.

Keywords: 4-Nonylphenol; Rainbow trout; *Oncorhynchus mykiss*; Spermatozoa; Eggs; Embryos; Larvae; Motility; Fertility

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1. Introduction

4-Nonylphenol, a degradation product of 4-nonylphenol ethoxylates is estrogenic in aquatic animals (see reviews of Servos, 1999; Segner et al., 2003). In fish, it changes the concentrations of sex hormones (*Catostomus commersoni*—McMaster et al., 1991; Munkittrick et al., 1994) and increases the levels of plasma vitellogenin and of zona radiata proteins (*Leuciscus cephalus*—Flammarion et al., 2000; different species—Kime et al., 1999). It inhibits spermiogenesis, induces the development of ovotestes in males, alters the gonadosomatic indices (*Oncorhynchus mykiss*—Jobling et al., 1996; *Oncorhynchus mykiss* and *Rutilius rutilus*—Routledge et al., 1998; *Rivulus marmoratus*—Tanaka and Grizzle, 2003) and leads to feminisation of juvenile stages (*Cyprinus carpio*—Gimeno et al., 1997, 1998).

The effect of 4-nonylphenol on many other parameters in the reproduction of fish remains still unknown. The final maturation of spermatozoa in the testicular main ducts and spermatic ducts (Billard, 1986; Loir et al., 1990) and the production of seminal fluid which is necessary to maintain the viability of mature spermatozoa (Lahnsteiner et al., 1999) are under hormonal control (Billard et al., 1978; Tanimoto and Morisawa, 1988; Marshall et al., 1989). Low and environmental relevant concentrations of 4-nonylphenol might affect the maturation of spermatozoa and the functionality of the efferent duct system and subsequently the quality of semen (i.e. motility, fertility, density).

The effect of 4-nonylphenol on the sperm and egg viability is unknown, too. Gametes of most teleost fish are released into water for fertilization. During fertilization the gametes are very sensitive as they cannot compensate for suboptimal environmental conditions (Lahnsteiner et al., 1999; Lahnsteiner, 2002). 4-Nonylphenol might effect the spermatozoal motility or fertility, the egg viability or the process of sperm egg contact.

Also the influence of environmental relevant concentrations of 4-nonylphenol on the fertilized eggs, the developing embryos and the hatched larvae is unclear. Only one study has been conducted until now using high 4-nonylphenol concentrations up to 114 $\mu g\,l^{-1}$ (Brooke, 1993). 4-Nonylphenol might enter the perivitelline space and the egg internal due to water influx during hardening and affect the embryogenesis. The freshly hatched larvae are generally very sensitive to environmental parameters (Blaxter, 1988).

Therefore, the present study investigates the effect of environmental relevant concentrations of 4-nonylphenol on the above-described reproductive parameters in rainbow trout (*Oncorhynchus mykiss*). Rainbow trout was used as only few data are available for the Salmonidae, and as they represent a commercial important family of fish reacting sensitively to environmental parameters. Three 4-nonylphenol concentrations were selected basing on the occurrence in Austrian water systems (measured range: 0–900 $ng\,l^{-1}$) and on federal regulations, i.e. the predicted non-effect concentration of 300 $ng\,l^{-1}$ (Paumann and Vetter, 2003), and lower (100 $ng\,l^{-1}$) and higher concentrations (750 $ng\,l^{-1}$).

2. Materials and methods

2.1. Experimental design

All experiments were conducted in the hatchery of Kreuzstein in Sankt Gilgen, Upper Austria, with rainbow trout (*Oncorhynchus mykiss*) and in full compliance with the Austrian Federal law for animal care (GZ 68.210/58-Br GT/2003) A stock solution of 4-nonylphenol was prepared by dissolving 0.5 g 4-nonylphenol in 100 ml DMSO. Required 4-nonylphenol concentrations were obtained by diluting the stock solutions with well water.

For in vivo exposure of fish and eggs to 4-nonylphenol a flow-through system was used which is shown in Fig. 1. The system was adjusted to obtain final 4-nonylphenol concentrations of 100, 300 and 750 $ng\,l^{-1}$ and equilibrated for 1 week to reach an equilibrium between potential 4-nonylphenol adsorption on equipment and concentrations in water. The water supply, the rate of 4-nonylphenol injection and the estimated 4-nonylphenol exposure levels are shown in Table 1. Egg incubators were supplied with the effluent water of the fish tanks. As under such incubation conditions the risk of fungus infections is high eggs were regularly (LOEC: 280 $ng\,l^{-1}$) disinfected with 4% formaldehyde.

Nonylpenol concentrations were not measured but calculated based on the flow rate of uncontaminated well water and on the injection rate of 4-nonylphenol.

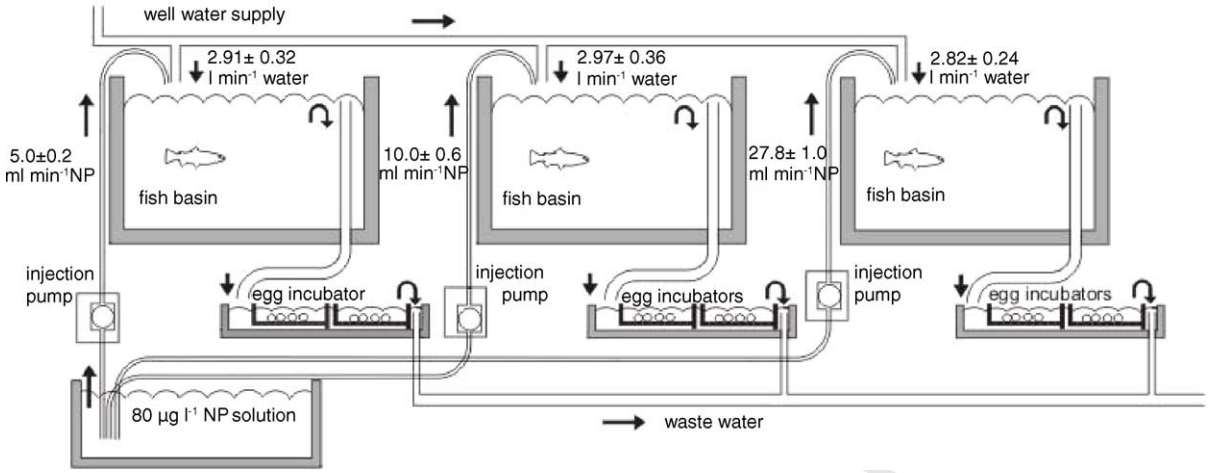

Fig. 1. Flow-through system used to expose male rainbow trouts and rainbow trout eggs and larvae to 4-nonylphenol. Volume of fish tanks: $6\,m^{-3}$. Arrows indicate direction of water flow. The effluent water drained off from the tanks was directed into trays containing egg incubators.

For closed, circular systems it has been reported that the actual concentration of 4-nonylphenol might be much lower than the planned one due to adsorption by organisms, tank walls, tubings and other equipment (Gray and Metcalfe, 1997; Tanaka and Grizzle, 2003). For the present experiments the estimated 4-nonylphenol exposure levels are considered to represent the actual ones because of the following reasons: 4-nonylphenol was continuously added to the water and therefore adsorption problems during the experiment could be neglected. The system was equilibrated for 1 week to reach equilibrium between potential 4-nonylphenol adsorption on equipment and concentrations in water. Day per day fluctuations in the estimated 4-nonylphenol exposure levels were caused by variations in the supply with fresh water and the 4-nonylphenol injection rate. Therefore, the fresh water supply and 4-nonylphenol injection rates were controlled daily and readjusted when necessary. Variations were taken in account in the calculations.

The criteria for assessment of semen quality were the semen volume, the sperm density, the sperm motility as assessed by computer assisted cell motility analysis, and the sperm fertility. Semen volume was measured in graduated reaction vials to the nearest 0.1 ml. Sperm density was determined with the spectrophotometric method of Ciereszko and Dabrowski (1993). The method was standardised by counting sperm concentrations using a standard curve in a Burker Türk counting chamber. Sperm motility was determined with computer assisted cell motility analysis at $4 \pm 1\,^{\circ}C$ (Lahnsteiner et al., 1999). A volume of 100 μl sperm motility activating solution was added into the Makler investigation chamber and 2 μl semen was added and mixed. The chamber was closed with a coverslip, the sample was transferred into an inverse phase

Table 1
Water supply and 4-nonylphenol injection rate (expressed as mean ± S.D.) and estimated 4-nonylphenol exposure levels in the flow-through system used for the in vivo experiments

	Tank 1	Tank 2	Tank 3	Tank 4 (control)
Well water flow through ($l\,min^{-1}$)	2.91 ± 0.32	2.97 ± 0.36	2.92 ± 0.24	2.95 ± 0.27
4-Nonylphenol injection[a] (ml/min^{-1})	5.00 ± 0.25	10.41 ± 0.60	27.83 ± 1.02	27.90 ± 1.12
Estimated 4-nonylphenol exposure levels ($ng\,l^{-1}$)	130	280	750	–
Estimated DMSO exposure levels ($\mu g\,l^{-1}$)	130	270	700	715

[a] 4-Nonylphenol concentration in injection solution: $80\,\mu g\,l^{-1}$.

contrast microscope coupled with a video camera (20-fold magnification) and the motility was recorded on videotapes until it had ended (about 45 s). The following sperm motility parameters were measured 10 ± 2 s after activation in a Stroemberg Mika cell motility analysis program: % immotile (velocity $< 5\ \mu m\ s^{-1}$), % locally motile (velocity of 5–20 $\mu m\ s^{-1}$), % motile (velocity $< 20\ \mu m\ s^{-1}$), % linear motile (linearity index ≥ 0.9), % non linear motile (linearity index < 0.9), average path swimming velocity of the motile spermatozoa ($\mu m\ s^{-1}$). The linearity index (LI) was calculated on base of the swimming path as LI = SL/AL, where SL represents the straight line swimming path between the measuring points and AL the actual swimming path between the measuring points.

To test the sperm fertility eggs were stripped from two females, pooled, and divided in subsamples of 200 ± 10 eggs. Semen collected from the experimental fish was prediluted in sperm motility inhibiting saline solution (103 mmol l^{-1} NaCl, 40 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 0.8 mmol l^{-1} $MgSO_2$, 20 mmol l^{-1} Tris, pH 7.8—Lahnsteiner et al., 1999) in a ratio of 1:3 (semen:saline). Eggs were fertilized using 5 μl prediluted semen and 6 ml of 4 °C well water (sperm to egg ratio 55,000:1 to 65,000:1) and incubated in flow incubators. After 30 days the percentage of embryos in the eyed stage was evaluated.

2.2. In vivo effects

To determine the influence of 4-nonylphenol on semen quality male +2 year rainbow trout (total length: 20–35 cm) were exposed to 4-nonylphenol during the spermiation period (=sperm production period [beginning of December–end of January]). Spermiation started about 1 week before the onset of the experiment (last week of November) and was terminated about 1 week later (first week of February). Before the onset of the experiment rainbow trout considered as potential experimental fish were stripped and checked on semen quality. Fish with unsuitable semen quality (i.e. fish giving < 0.5 ml semen or semen with a motility < 25%) were rejected from the experiments. Fish with suitable semen quality were divided into three categories: Fish with low (25–50% motile spermatozoa), with medium (50–75% motile spermatozoa) and with high semen quality (75–100% motile spermatozoa). Using these fish four experimental groups were formed each consisting of 10 males with equal numbers of low, medium and high semen quality. Also the fish density (kg/m^3) was approximately similar in the four tanks. Fish groups 1–3 were exposed to the three 4-nonylphenol concentrations (Fig. 1; Table 1), the control group received an adequate concentration of DMSO which was the carrier for 4-nonylphenol (Fig. 1; Table 1). The water influx and the 4-nonylphenol injection were checked daily. After 30 and 60 days the fish were stripped and the semen quality was determined.

To determine the influence of 4-nonylphenol on egg and larvae development eggs from two rainbow trout were used. Each egg batch was fertilized with semen at condition of sperm saturation using uncontaminated well water as fertilization solution. After 30 s, each egg sample was divided in four subsamples of 2000 eggs which were separately placed into the egg incubators within 3 min after fertilization. After 30 days the percentage of embryos in the eyed stage was evaluated. Thereafter, the dead eggs were removed and the viable eggs were further incubated for determination of the percentage of hatched larvae and the percentage of larvae in the end of the yolk sac stage.

2.3. In vitro exposures

To determine the influence of 4-nonylphenol on sperm motility three semen samples were stripped from untreated broodfish. From each sample one subsample was activated in uncontaminated well water and the other subsamples in well water containing 100, 300 or 750 ng l^{-1} 4-nonylphenol.

To determine the influence of 4-nonylphenol on egg viability three egg batches were used. From each sample a subsample of 200 ± 10 eggs was incubated in physiological saline solution (control) or in physiological saline solution containing 4-nonylphenol in concentrations of 100, 300, or 750 ng l^{-1} at 4 °C for 10 min. The physiological saline solution consisted of 125 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 1.5 mmol l^{-1} $CaCl_2$, 0.8 mmol l^{-1} $MgSO_4$, and 20 mmol l^{-1} Tris, pH 8.5 and prevented the egg activation at 4 °C for at least 20 min (Lahnsteiner, 2002). After incubation was terminated the incubation solution was drained away, replaced by fresh saline solution and eggs were fertilized at conditions of sperm saturation.

To investigate the influence of 4-nonylphenol on sperm egg contact three semen and three egg sam-

ples were used. From each egg sample subsamples of 200 ± 10 eggs were taken, placed in a beaker containing 6 ml uncontaminated well water or well water containing 100, 300 or 750 ng l^{-1} 4-nonylphenol. Immediately thereafter the eggs were fertilized with 5, 10 or 20 μl semen prediluted at a ratio of 1:3 in sperm motility inhibiting saline solution (egg batch 1 × semen 1, . . ., egg batch 3 × semen 3) at sperm to egg ratios of 75,000:1 to 375,000:1. After 2 min the eggs were washed and incubated in uncontaminated well water until they reached the hatching stage.

2.4. Statistics

For statistical analysis relative abundances were transformed by angular transformation (arcsin $\sqrt{P}$). To determine if the experimental treatments resulted in significant different results analysis of variance (ANOVA) was used. In experiments where semen samples in different times were obtained from the same fish repeated measure one-way ANOVA was used whereby time was included as repeated measure variable. The Waller Duncan posthoc test was used as a multiple comparison test to determine which treatments differed significantly. For pair wise comparison of mean values (data reported in Table 3) Dunetts's T3 posthoc test was used.

3. Results

3.1. In vivo effects

The mean flow-through rate of well water, the mean 4-nonylphenol injection rate, and the estimated 4-nonylphenol concentrations are shown in Table 1. The semen volume obtained per male decreased slightly and non-significantly for rainbow trout from the control group (Fig. 2a). For rainbow trout exposed to estimated 4-nonylphenol concentrations of 130, 280 and 750 ng l^{-1} the semen volume decreased significantly (Fig. 2a).

The sperm density of rainbow trouts from the control group and of rainbow trout exposed to estimated 4-nonylphenol concentrations of 130 and 280 ng l^{-1} decreased slightly and non-significantly during the experiment (Fig. 2b).

The semen fertility of rainbow trout exposed to the three 4-nonylphenol concentration for 30 or 60 days was not significantly different from the fertility of control semen (Fig. 2c). The percentage of locally motile (Fig. 2d) and of motile spermatozoa (Fig. 2e) and the swimming velocity (Fig. 2f) were not affected by 4-nonylphenol, too. The sperm swimming pattern was not influenced by 4-nonylphenol but it changed during the course of the experiment. At the onset of the experiment the circular motion was the main motility pattern, after 30 and 60 days the linear motion. This was similar for rainbow trout from the control group and for rainbow trout exposed to 4-nonylphenol. The sperm swimming pattern of the control group is shown in Fig. 3.

When eggs were hatched at estimated 4-nonylphenol exposure levels of 280 and 750 ng l^{-1} the percentage of eyed stage embryos was slightly but significantly lower than at an estimated 4-nonylphenol concentration of 130 ng l^{-1} and in the control (Table 2). The percentage of yolk sac larvae was significantly decreased at 4-nonylphenol concentrations of 750 ng l^{-1} (for 67.0%) and 280 ng l^{-1} (for 35.6%) (Table 2). The decrease was modest (21.7%) and similar to the control (23.7%) at a 4-nonylphenol concentrations of 130 ng l^{-1} (Table 2).

Table 2
Effect of 4-nonylphenol on the development of rainbow trout embryos and larvae

	Control	Estimated 4-nonylphenol exposure levels		
	0	130 ng l^{-1}	280 ng l^{-1}	750 ng l^{-1}
Eyed stage embryos (%)	94.6 ± 0.8 a	95.5 ± 1.6 a	89.3 ± 7.3 b	90.8 ± 0.4 b
Hatched larvae (%)	74.8 ± 2.7 c	74.0 ± 1.4 c	58.3 ± 9.8 e	33.6 ± 1.6 f
Yolk sac stage larvae (%)	70.9 ± 1.8 d	73.8 ± 1.5 d	53.7 ± 8.2 e	23.8 ± 1.2 g

Eggs were incubated in the described flow-through system at 6 °C. Percentage of eyed stage embryos was determined 30 days after fertilization, percentage of hatched larvae after 45 days, and percentage of larvae in the end of yolk sac stage after 60 days. Values are mean ± S.D., $n = 3$. Values with different letters are significantly different, $P \leq 0.05$.

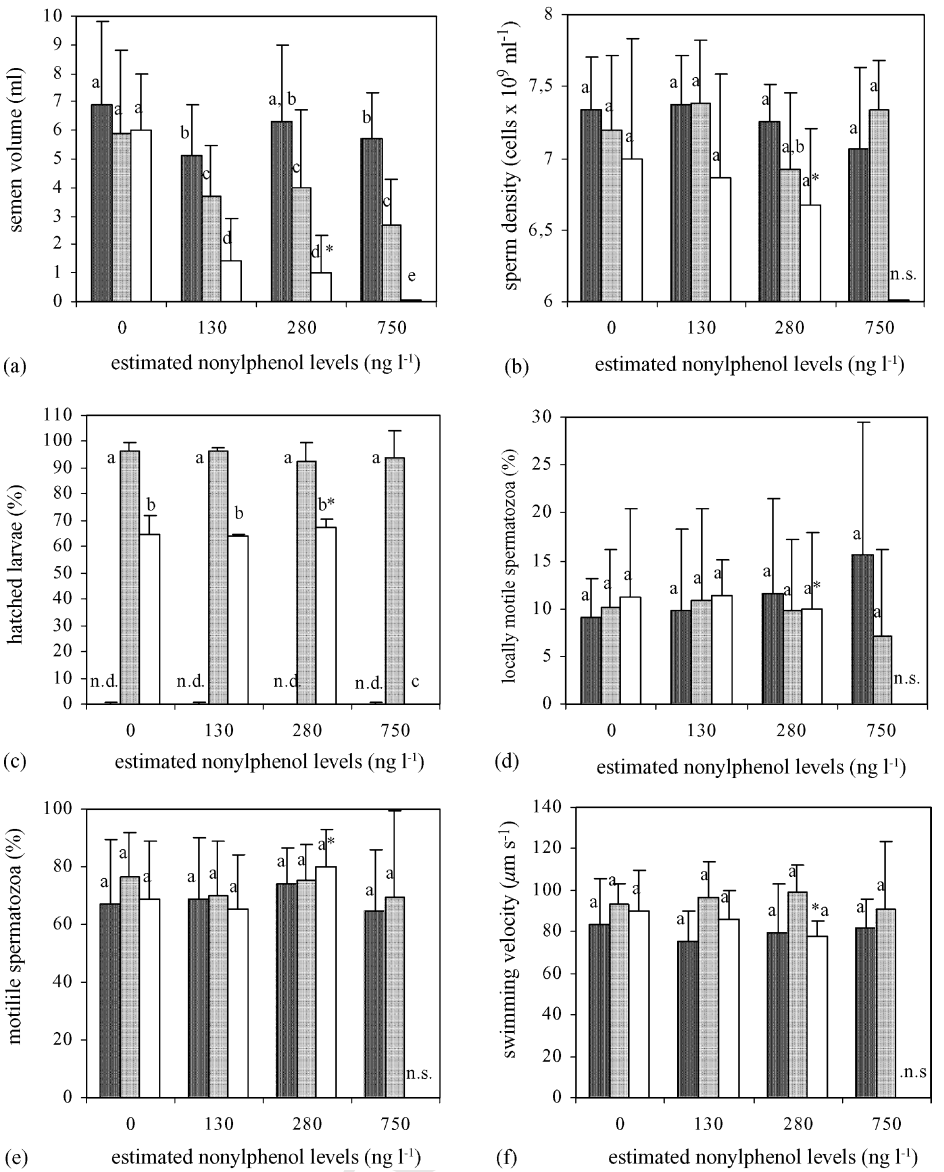

Fig. 2. Effect of 4-nonylphenol on semen quality in rainbow trout. Fishes were exposed to 4-nonylphenol during the spawning period and semen was sampled before the onset of the experiment and after 30 and 60 days. Motility was measured 10 ± 2 s after activation. Values are mean ± S.D., $n = 10$, for values superscripted with *, $n = 5$. Values with different letters are significantly different, $P \leq 0.05$. n.d.—not determined, n.s.—no samples. (■) 0 days, (▨) 30 days, (□) 60 days. (a) Effect on semen volume. (b) Effect on sperm density. (c) Effect on sperm fertility. (d) Effect on the percentage of locally motile spermatozoa. (e) Effect on the percentage of motile spermatozoa. (f) Effect on the average path sperm swimming velocity.

3.2. *In vitro exposures*

When sperm motility was activated in distilled water or in distilled water containing 100, 300, or 750 ng l^{-1} 4-nonylphenol the motility parameters 10 s after activation were similar (Table 3, data for 100 and 300 ng l^{-1} 4-nonylphenol not shown). Also the motility duration was similar (Table 3).

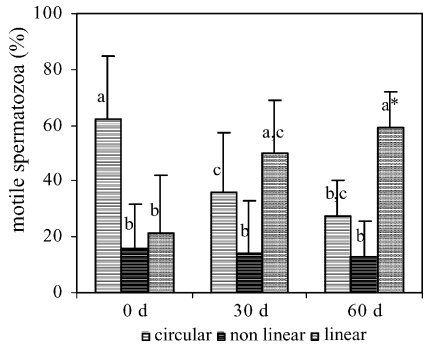

Fig. 3. Changes in sperm motility pattern of the control group during the experiment. The changes were similar for the control group and for the 4-nonylphenol exposed groups. Semen was sampled before the onset of the experiment and after 30 and 60 days. Motility was measured 10 ± 2 s after activation. Values are mean ± S.D., $n = 10$. Values with different letters are significantly different, $P \leq 0.05$.

When unfertilized eggs were incubated for 10 min in 4-nonylphenol containing physiological saline solution and then fertilized in non contaminated water the percentage of hatched embryos was similar high as for eggs incubated in physiological saline solution without 4-nonylphenol (Table 4).

When eggs were fertilized in water containing 100, 300, or 750 ng l^{-1} 4-nonylphenol the percentage of hatched embryos was similar high as in the control (uncontaminated water) at all tested sperm to egg ratios (Table 5).

Table 3
Motility behaviour of rainbow trout spermatozoa (10 ± 2 s after activation) in the presence of 4-nonylphenol

	4-Nonylphenol concentration	
	0 ng l^{-1}	750 ng l^{-1}
Immotile (%)	20.1 ± 5.1 a	25.9 ± 6.9 a
Locally motile (%)	13.8 ± 1.3 a	8.1 ± 2.5 a
Motile (%)	66.1 ± 5.9 a	66.0 ± 4.7 a
Circular motile (%)	58.4 ± 9.7 a	56.6 ± 15.8 a
Non linear motile (%)	11.7 ± 4.7 a	12.2 ± 10.7
Linear motile (%)	29.9 ± 7.7 a	31.2 ± 7.8 a
Swimming velocity (μm/s)	91.4 ± 20.8 a	100.0 ± 19.1 a
Motility duration[a] (s)	25 ± 5 a	25 ± 5 a

Semen was stripped from untreated rainbow trouts and activated in well water containing 750 ng l^{-1} 4-nonylphenol. Values are mean ± S.D., $n = 3$. Values within a row with different letters are significantly different, $P \leq 0.05$.

[a] Until ≥90% of spermatozoa stopped progressive movement.

Table 4
Effect of 4-nonylphenol (NP) on egg fertilizability in rainbow trout

Incubation conditions	Eyed stage embryos (%)
Physiological saline	97.5 ± 1.0 a
Hundred nanogram per liter NP in physiological saline	98.8 ± 0.1 a
Three hundred nanogram per liter NP in physiological saline	92.1 ± 7.7 a
Seven hundred and fifty nanogram per liter NP in physiological saline	92.8 ± 2.3 a

Eggs (200 ± 10) were incubated in 4-nonylphenol containing physiological saline solution for 10 min, rinsed in pure physiological saline solution and fertilized at sperm to egg ratios of $>5 \times 10^6$ spermatozoa/egg. Values are mean ± S.D., $n = 3$. Values with different letters are significantly different, $P \leq 0.05$.

4. Discussion

The present study demonstrated that estimated 4-nonylphenol exposure levels of ≥130 ng l^{-1} decreased the semen quantity and exposure levels of ≥280 ng l^{-1} the percentage of eggs surviving to the eyed stage and to the yolk sac larvae in rainbow trout. Sperm density, sperm motility, sperm fertility, egg viability (fertilizability), and fertilization process (sperm egg contact) were not affected. Therefore, estimated 4-nonylphenol exposure levels in the range and below the range of the predicted non-effect concentration (not uniformly regulated for EC, for Austria: 330 ng l^{-1}—Paumann and Vetter, 2003) significantly influenced the reproduction of rainbow trout. The lowest observed effect concentration (LOEC) on reproduction of rainbow trout was an estimated exposure level of 130 ng l^{-1}. This 4-nonylphenol concentration was also environmental relevant as 0–900 ng l^{-1} have been measured in natural Austrian water systems (Paumann and Vetter, 2003) and still higher concentrations in other European and North American waters (Talmage, 1994; Bennie, 1999; Blackburn et al., 1999). As the observed effects were time dependent much lower 4-nonylphenol concentrations may be effective in natural water systems where organisms are exposed during their whole life cycle. Schwaiger et al. (2002) exposed rainbow trouts to 1–10 μg l^{-1} 4-nonylphenol for 4 months prior to spawning, and observed increased levels of plasma vitellogenin in males.

Table 5
Influence of 4-nonylphenol on the fertilization process (sperm egg contact)

Sperm:egg ratio	Hatched larvae (%) after fertilization in			
	0 ng l^{-1} NP	100 ng l^{-1} NP	300 ng l^{-1} NP	750 ng l^{-1} NP
(3.75×10^5):1	86.8	97.4	97.5	96.8
(1.87×10^5):1	94.3	85.8	95.8	94.0
(0.75×10^5):1	95.7	92.9	97.9	94.9
Mean ± S.D.	92.3 ± 4.7 a	92.0 ± 5.8 a	97.0 ± 1.1 a	95.2 ± 1.4 a

Eggs (200 ± 10) were fertilized in 4-nonylphenol containing water using the indicated sperm to egg ratios. After 5 min the eggs were rinsed and incubated in uncontaminated water for hatching. Mean values ($n = 3$) with different letters are significantly different, $P \leq 0.05$.

In the in vivo exposure experiments with male rainbow trout the sperm density and the sperm swimming pattern changed in the control group and in the 4-nonylphenol exposed groups in a similar way. Therefore, these changes were not induced by 4-nonylphenol but depended on the reproductive cycle and maturity state of the fish (Billard et al., 1978). The decrease in sperm density at the end of the spawning period is conform to earlier studies on the Salmonidae and is due to an activity decrease in spermiogenetic processes (Billard, 1986). The changes in motility pattern from circular in the beginning of spawning to linear in the middle and in the end of spawning might depend on the cell internal calcium levels which effect the motility pattern of trout sperm (Boitano and Omoto, 1992; Cosson et al., 1999). They might be indicative for the maturation stage of spermatozoa (Cosson et al., 1999).

Estimated 4-nonylphenol exposure levels $\geq$130 ng l^{-1} (LOEC: 130 ng l^{-1}) decreased the semen volume. 4-Nonylphenol has estrogenic activity decreasing or disrupting the synthesis of androgens and subsequently inhibiting the maturation of the testis (Segner et al., 2003). Hence, the observed effect on semen quantity is due to inhibition of spermiogenesis. Histopathological investigations confirmed this hypothesis as testes of fish exposed to estimated 4-nonylphenol concentrations of 750 ng l^{-1} were immature or in a stage of early spermatogenesis (unpublished data). Similar results and moreover the development of ovotestes was also observed in other studies (Jobling et al., 1996; Gimeno et al., 1998; Flammarion et al., 2000). 4-Nonylphenol had no effect on the sperm density, sperm motility, and sperm fertility (no observed effect concentration [NOEC]: 130 ng l^{-1}). This indicates that the spermatozoa had full and normal functionality and that 4-nonylphenol did not interfere with the differentiation and maturation processes of spermatozoa in the germinal cysts of the Sertoli cells and in the spermatic ducts. The blood testis border (Billard, 1986) and blood spermatic duct border (Marshall et al., 1989) may protect the developing germ cells from 4-nonylphenol.

The in vivo exposure experiments of fertilized eggs and larvae demonstrated that during embryonic development the eggs were relatively insensitive to the tested concentrations of 4-nonylphenol as the percentage of eyed stage embryos differed only slightly from the control (2–4%) (LOEC: 280 ng l^{-1}). Probably 4-nonylphenol could not enter the egg internal during water hardening and during embryonic development. Generally, the water-hardened eggs have three effective permeability barriers, the egg shell, the perivitelline space, and the oolemma (Alderdice, 1988). The protection by the three permeability barriers was lost after hatching. It is obvious that 4-nonylphenol was taken up by the larvae as estimated exposure levels of $\geq$280 ng l^{-1} (LOEC: 280 ng l^{-1}) were toxic and caused a severe decrease in the percentage of viable larvae. Schwaiger et al. (2002) observed decreased larvae mortality at $\geq$1 μg 4-nonylphenol. As demonstrated in other studies 4-nonylphenol does not affect exclusively the reproduction. In *Salmo salar* balance between levels of thyroid hormone, growth hormone, cortisol, insuline like growth factor-I and sex steroids is necessary for smoltification and osmoregulation and may be disturbed by xenoestrogen (Moore et al., 2003). Effects on differentiation processes and on the functionality of organ systems are suggested as responsible for high larvae mortality also in the present study.

Immediately after their release into water the gametes of teleost fish are very sensible to environmental influences. Spermatozoa have no ability to compen-

sate for environmental changes and external substances may easy penetrate the sperm membrane (Lahnsteiner et al., 1999). Between the freshly released eggs and the environment water fluxes occur until an osmotic equilibrium is reached, a process which is termed water hardening (Alderdice, 1988) and which persists 2 h (Lahnsteiner, 2000). During this process xenoestrogens may enter the egg internal, too. As in the in vitro exposure experiments the sperm motility, the egg viability, and the percentage of eggs developing to eyed stage embryos were not affected by 4-nonylphenol in concentrations $\geq 130\ ng\ l^{-1}$ an influence on sperm motility, sperm fertility or egg fertilizability could be excluded (NOEC: $130\ ng\ l^{-1}$). Sperm motility was probably not effected as the exposition to 4-nonylphenol was very short. For the eggs insensitivity to 4-nonylphenol might be explained by the low permeability of the oolemma (Alderdice, 1988).

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Effect of bisphenol A on maturation and quality of semen and eggs in the brown trout, *Salmo trutta f. fario*

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Abstract

In the present study male and female brown trout (*Salmo trutta f. fario*) were exposed to environmentally relevant concentrations of bisphenol A (1.75, 2.40, 5.00 $\mu g\,l^{-1}$) during the late prespawning and spawning period and the effect of this contaminant on maturation, quantity and quality of semen and eggs was investigated.

In males exposed to estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$ semen quality was lower than in the control in the beginning of spawning (reduced sperm density, motility rate, and swimming velocity) and in the middle of spawning (reduced swimming velocity, at 2.40 $\mu g\,l^{-1}$ BPA also reduced sperm motility rate). Therefore, production of high quality semen was restricted to the end of the spawning season and delayed for approximately 4 weeks in comparison to the control. At BPA exposure levels of 5.00 $\mu g\,l^{-1}$ only one of eight males gave semen of low quality (reduced semen mass, motility rate, and swimming velocity).

The percentage of ovulated females was similar for the control group and the groups exposed to estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$, whereas at 5.00 $\mu g\,l^{-1}$ BPA females did not ovulate during the investigation. While brown trout of the control group ovulated between the 28 October and 12 November, brown trout exposed to estimated BPA concentrations of 1.75 $\mu g\,l^{-1}$ BPA ovulated approximately 2 weeks later and brown trout exposed to 2.40 $\mu g\,l^{-1}$ BPA approximately 3 weeks later. Therefore, the tested BPA concentrations affected the percentage of ovulated females and the time point of ovulation. No effect was observed on the quality of eggs (egg mass, percentile mass increase during hardening, egg fertility).

Keywords: Bisphenol A; Brown trout; *Salmo trutta f. fario*; Spermatozoa; Eggs; Motility; Fertility

1. Introduction

Bisphenol-A (BPA) is a synthetic chemical used in the production of epoxy resins and polycarbonate plastics. Sources of environmental releases are epoxy man-

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ufacturing facilities. Recent research showed that BPA has estrogenic potency and is therefore an endocrine disrupter (Toppari et al., 1995). In fish, it changes the levels of sex hormones (*Chasmichthys dolichognathus*, Baek et al., 2003), increases the levels of plasma vitellogenin (*Rhynchocypris oxycephalus*, Park et al., 2003a; *Phoxinus oxycephalus*, Park et al., 2003b; *Fundulus heteroclitus*, Pait and Nelson, 2003) and of zona radiata proteins (*Oryzias latipes*, Lee et al., 2002), alters the fecundity offish (*O. latipes*, Kang et al., 2002; *Pimephales promelas*, Sohoni et al., 2001), changes the testis structure (*Poecilia reticulata*, Kinnberg and Toft, 2003) and affects the egg and larval development (*Salmo salar m. Sebago*, Honkanen et al., 2001; *O. latipes*, Na et al., 2000; *O. latipes*, Yokota et al., 2000; *O. latipes*, Pastva et al., 2001).

Effects of endocrine disrupting chemicals on fish are evaluated by gross gonad morphology and histology (development of ovotestes), and induction of vitellogenin and choriogenin production in males (Kime et al., 1999; Jobling et al., 2003). Based on the BPA levels which increased vitellogenin levels in male rainbow trout (70 $\mu g\,l^{-1}$ BPA, Lindholst et al., 2000) a predicted non-effect concentration (PNEC) of 64 $\mu g\,l^{-1}$ was defined (Staples et al., 2000). The described risk assessment is quick, reliable and easily comparable between different labs. However, changes in gonad morphology, histology and vitellogenin levels indicate biological endpoints where sex reversion already occurs. At lower pollutant concentrations alterations in reproduction could occur which are not detectable with the described methods. The maturation of spermatozoa and eggs could be disturbed resulting in reduced gamete quantity or quality, or in desynchronization of reproduction. Such changes could drastically reduce the reproductive potential of wild populations. However, data evaluating these points are very limited. Only Haubruge et al. (2000) described that sperm densities in guppies (*Poecilia reticulata*) are decreased after exposure to low levels of BPA.

Therefore, in the present study brown trout (*S. trutta f. fario*) were exposed to environmentally relevant concentrations of BPA (1.75, 2.40, 5.00 $\mu g\,l^{-1}$) during the late prespawning and spawning season to determine the effect on the final maturation processes of gametes (time point of spawning, quality and quantity of gametes). The tested BPA concentrations were selected on the basis of their occurrence in Austrian water systems and on the PNEC defined for Austria (1.6 $\mu g\,l^{-1}$) (Paumann and Vetter, 2003). Brown trout were used as a model as only very few data are available on the species and as they represent recreationally important fish populations in many parts of the world. The criteria for assessment of semen quality were the mass of produced semen, the sperm density, the sperm motility as assessed by computer assisted cell motility analysis, and the sperm fertility. The criteria for assessment of egg quality were the number of produced eggs, the egg mass, the mass increase during hardening and the egg fertility.

2. Materials and methods

2.1. Experimental design

All experiments were conducted in the hatchery of Kreuzstein in Sankt Gilgen, Upper Austria, with brown trout (*S. trutta f. fario*) and in compliance with the Austrian Federal law for animal care (GZ 68.210/58-Br GT/2003). Fish derived from a wild population in a mountain area of Salzburg (Blühnbach) with water system containing no endocrine disrupting substances (unpublished data). Fish were caught in the end of August by electroshocking and then transported to the fish farm Kreuzstein. There they were acclimated for 2 weeks before they were used for the experiments.

In order to expose fish to BPA, a flow through system was used which has been described previously (Lahnsteiner et al., 2005) (Fig. 1). Briefly, the system consisted of four 0.5 m^3 tanks. The tanks were supplied with well water of 6 °C and an oxygen content of >90% saturation. BPA was added by means of an injection pump. Consequently, the BPA concentrations were adjusted by changing the injection rates. In comparison to the previously described system (Lahnsteiner et al., 2005) the flow through system used in the present experiments was modified to increase its accuracy. Well water was supplied via a storage reservoir in a height of 1.5 m above the tanks where after the water flow was regulated by reduction pieces (diameter reduction from 30 to 6 mm) (Fig. 1). This set up reduced variations in well water flow rates to <2.0%. The injection pumps were precise, having variations of <0.5%. Final BPA concentrations were calculated based on the flow rate

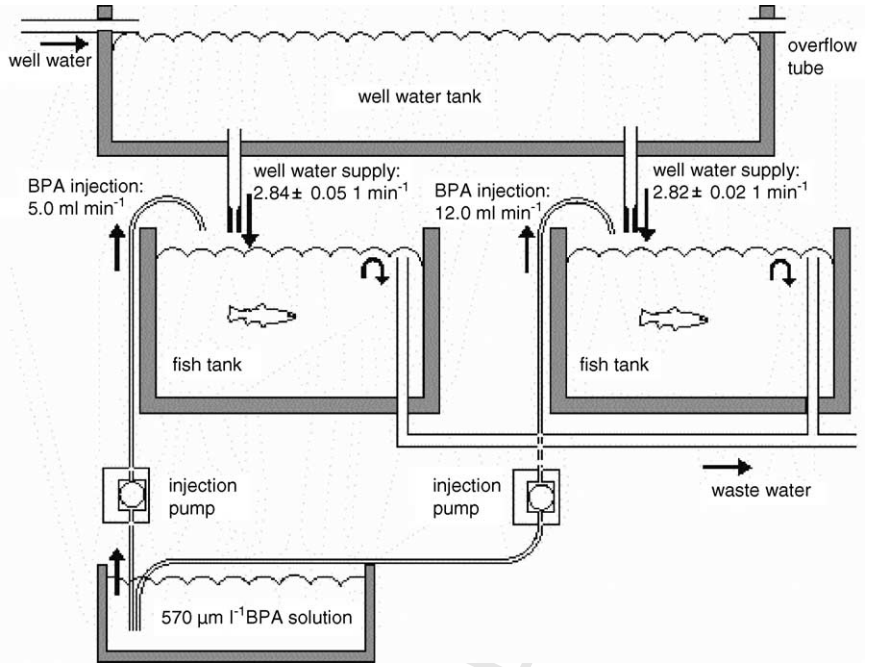

Fig. 1. Scheme of the flow through system used for exposure of brown trout to bisphenol A (BPA). Tanks had a volume of 0.5 m^{-3} and well water supply was adjusted by reduction pieces which reduced the tube diameter from 30 to 6 mm. The legend is relevant for tanks with final BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$.

of uncontaminated well water and on the injection rate of BPA.

A stock solution of BPA was prepared by dissolving 1.0 g BPA in 100 ml DMSO. The stock solution was diluted to 570 $\mu g\,l^{-1}$ with well water. In the experiment estimated final BPA concentrations of 1.5, 2.4 and 5 $\mu g\,l^{-1}$ were used. To obtain these concentrations the well water flow through rate and the BPA injection rate were adjusted in the following way—tank 1: well water flow through rate: $2.84 \pm 0.05\,l\,min^{-1}$, BPA injection rate: $5.00 \pm 0.01\,ml\,min^{-1}$, resulting estimated BPA exposure level 1.5 $\mu g\,l^{-1}$, and resulting estimated DMSO concentration 100 $\mu g\,l^{-1}$; tank 2: well water flow through rate: $2.82 \pm 0.02\,l\,min^{-1}$, BPA injection rate: $12.00 \pm 0.02\,ml\,min^{-1}$, estimated BPA exposure level: 2.5 $\mu g\,l^{-1}$, and estimated DMSO concentration: 245 $\mu g\,l^{-1}$, tank 3: well water flow through rate: $2.73 \pm 0.04\,l\,min^{-1}$, BPA injection rate: $26.00 \pm 0.01\,ml\,min^{-1}$, estimated BPA exposure level: 5.0 $\mu g\,l^{-1}$, and estimated DMSO concentration: 550 $\mu g\,l^{-1}$. Tank 4 served as control. The well water flow through rate was $2.95 \pm 0.04\,l\,min^{-1}$, and instead of BPA an aqueous DMSO solution was injected at a rate of $26.00 \pm 0.01\,ml\,min^{-1}$ to obtain estimated DMSO concentrations of 510 $\mu g\,l^{-1}$. This DMSO concentration was similar to the highest concentration used in the experiment.

Before the onset of the experiment the system was equilibrated for 1 week to reach equilibrium between potential BPA adsorption on equipment and concentrations in water. In the beginning of the experiment the system was controlled daily on well water flow rates and injection rates. As the system was constant further controls were performed in 1-week intervals.

2.2. Experiments

In the wild, spawning of the investigated brown trout population takes place in the second half of November. To determine the influence of BPA on semen and egg maturation ±3 year brown trout (total length: 15–20 cm) were exposed to BPA during the late prespawning period and during the spawning period (3 September–14 December). Four experimental

groups were formed each consisting of 10 males and 6 females. Also the fish density (approximately 4 kg m^{-3}) was approximately similar in the four tanks. Fish groups 1–3 were exposed to the described BPA concentrations, group 4 served as control. Fish were fed two times per week with small cyprinids and had a natural photoperiod. Starting on 5 October fish were examined in 1 week intervals if they gave already semen and eggs. Semen of all males was stripped in the beginning (10 October), middle (28 October) and end (17 November) of spawning to examine semen quality. When females gave eggs they were completely stripped out, the time point of egg collection was recorded and then eggs were processed for quality determination.

2.3. Determination of gamete quality

The mass of produced semen was weighed using an analytical balance. Sperm density was determined spectrophotometrically (Ciereszko and Dabrowski, 1993) at 405 nm after 100-fold semen dilution in 4% formaldehyde. The method was standardised by counting sperm concentrations in a Burker Türk counting chamber.

Sperm motility was determined with computer assisted cell motility analysis at 4 ± 1 °C (Lahnsteiner et al., 1999). A volume of 100 μl sperm motility activating solution was added into the Makler investigation chamber and 2 μl semen was added and mixed. The chamber was closed with a coverslip, the sample was transferred into an inverse phase contrast microscope coupled with a video camera (20-fold magnification) and the motility was recorded on videotapes until it had ended (about 45 s). The following sperm motility parameters were measured 10 ± 2 s after activation in a Stroemberg Mika cell motility analysis program: % immotile (velocity < 5 μm s^{-1}), % locally motile (velocity of 5–20 μm s^{-1}), % motile (velocity > 20 μm s^{-1}), % linear motile (linearity index ≥ 0.9), % non-linear motile (linearity index < 0.9), average path swimming velocity of the motile spermatozoa (μm s^{-1}). The linearity index (LI) was calculated on base of the swimming path as LI = SL/AL, where SL represents the straight line swimming path between the measuring points and AL the actual swimming path between the measuring points.

The sperm fertility was tested for semen samples collected on the third sampling date (17 November). Eggs were stripped from four additional females which had not been involved in the experiment, pooled, and divided in subsamples of 200 ± 10 eggs. Semen collected from the experimental fish was prediluted in sperm motility immobilizing saline solution (103 mmol l^{-1} NaCl, 40 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 0.8 mmol l^{-1} $MgSO_2$, 20 mmol l^{-1} Tris, pH 7.8, Lahnsteiner et al., 1999) in a ratio of 1:5 (semen:saline). Eggs were fertilized using sperm to egg ratios sensitive to detect differences in semen quality (2.5 μl prediluted semen = sperm to egg ratio 45,000:1–60,000:1) (Lahnsteiner et al., 1998). Six milliliters of 6 °C well water was added, after 1 min the eggs were rinsed and incubated in flow incubators supplied with 6 °C well water. The percentage of embryos in the eyed stage was evaluated after 35 days.

To determine the number of produced eggs ovarian fluid was drained off from the egg batches. Then each egg batch was weighed. A 1–2 g subsample was taken and weighed with an analytical balance to the nearest 0.1 mg. The total number of eggs in the subsample was counted. Based on the mass of the subsample and the number of eggs in the subsample the egg mass and the number of produced eggs were calculated.

For determination of the egg mass increase during water hardening (which is a quality marker for eggs of Salmonidae, Lahnsteiner and Patzner, 2000) 10 eggs were randomly selected and remaining ovarian fluid was drained off with absorbent paper and weighed to the nearest 0.1 mg (wet weight of an unhardened egg). Then the eggs were placed in a 25 ml volume glass beaker, which was filled with well water. The eggs were incubated for exactly 2 h at a temperature of 6 °C. Thereafter the eggs were collected, water was drained off, remaining droplets sucked off with adsorbent paper and the eggs were re-weighed (wet mass of a water hardened egg). The parameter mass increase during water hardening was calculated as percentage in wet mass.

To determine the egg viability 200 ± 10 eggs were fertilized at conditions of sperm saturation (40 μl undiluted semen, sperm to egg ratio = 500,000–600,000:1) as standardized in previous studies for Salmonidae (Lahnsteiner, 2000; Lahnsteiner and Patzner, 2001). As the dates of egg sampling differed different semen samples had to be used for fertilization. To exclude

that differences in semen quality might influence the results semen pools consisting out of three samples were used which were stripped from untreated brown trout and had motility rates ≥70%. Six milliliters of 6 °C well water was used as fertilization solution. Eggs were incubated in flow incubators as described and the eyed stage rate was determined.

2.4. Statistics

For statistical analysis relative abundances were transformed by angular transformation ($\arcsin\sqrt{P}$). In the experiment where the effect of BPA on semen quality during the spawning season was investigated ANOVA was used with the influence of spawning season and the influence of treatment procedure as independent variables and the semen parameters as dependent variables. To determine which treatments differed in dependence of the spawning season and in dependence of the treatment procedure Tukey's *b* post hoc test was used as a multiple comparison test. Also for determination of the effect of BPA exposure on semen fertility and egg parameters (egg volume, eggs produced per female, egg mass, mass increase during hardening, egg viability) ANOVA was used with treatment procedure as independent variable and the above mentioned semen or egg parameters as dependent variables (post hoc test: Tukey's *b*).

3. Results

3.1. Effect on semen

In the control group and at estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$ a similar number of males (6–8) gave semen during the spawning season (Table 1), at estimated BPA concentrations of 5.00 $\mu g\,l^{-1}$ only one male (Table 1). The semen mass obtained per male was constant for brown trouts of the control group during the spawning season (Table 1). The semen mass from brown trout exposed to 1.75 and 2.40 $\mu g\,l^{-1}$ BPA did not differ significantly from the control group during the spawning season (Table 1). At

Table 1
Effect of bisphenol A (BPA) on semen mass in brown trout

Estimated BPA concentration ($\mu g\,l^{-1}$)	Semen mass (g)		
	Beginning of spawning (10 October)	Middle of spawning (28 October)	End of spawning (17 November)
0.00	0.51 ± 0.33 a (7)	0.49 ± 0.24 a (8)	0.46 ± 0.17 a (7)
1.75	0.56 ± 0.18 a (8)	0.59 ± 0.32 a (8)	0.54 ± 0.35 a (6)
2.40	0.60 ± 0.12 a (6)	0.53 ± 0.22 a (8)	0.65 ± 0.30 a (6)
5.00	0.05 (1)	0.02 (1)	0.09 (1)

Fish were exposed to BPA during late prespawning and during spawning period and semen was sampled three times as indicated. Values are mean ± S.D., values in parenthesis indicate the number of males giving semen. Values followed by the same letter are not significantly different, $P > 0.05$. No statistical tests were performed for 5.00 $\mu g\,l^{-1}$ BPA as only one sample was available per sampling date.

Table 2
Effect of bisphenol A (BPA) on sperm density in brown trout

Estimated BPA concentration ($\mu g\,l^{-1}$)	Sperm density ($\times 10^{10}$ spermatozoa ml^{-1})		
	Beginning of spawning (10 October)	Middle of spawning (28 October)	End of spawning (17 November)
0.00	2.91 ± 0.03 a (7)	2.51 ± 0.14 b (8)	2.33 ± 0.09 c (7)
1.75	2.66 ± 0.26 b (8)	2.60 ± 0.10 b (8)	2.41 ± 0.27 b,c (6)
2.40	2.68 ± 0.03 b (6)	2.58 ± 0.10 b (8)	2.42 ± 0.13 c (6)
5.00	2.62 (1)	2.52 (1)	2.54 (1)

Fish were exposed to BPA during late prespawning and during spawning period and semen was sampled three times as indicated. Values are mean ± S.D., values in parenthesis indicate the number of males giving semen. Values followed by the same letter are not significantly different, $P > 0.05$. No statistical tests were performed for 5.00 $\mu g\,l^{-1}$ BPA as only one sample was available per sampling date.

Table 3
Effect of bisphenol A (BPA) on the sperm motility rate in brown trout

Estimated BPA concentration ($\mu g\,l^{-1}$)	Motility rate (%)		
	Beginning of spawning (10 October)	Middle of spawning (28 October)	End of spawning (17 November)
0.00	83.5 ± 24.6 a (7)	88.1 ± 14.6 a (8)	85.8 ± 13.4 a (7)
1.75	32.6 ± 19.2 b (8)	73.4 ± 17.6 a (8)	69.8 ± 18.5 a (6)
2.40	39.8 ± 196.1 b (6)	35.8 ± 25.1 b (8)	80.7 ± 12.1 a (6)
5.00	4.0 (1)	0.9 (1)	3.5 (1)

Fish were exposed to BPA during late prespawning and during spawning period and semen was sampled three times as indicated. Values are mean ± S.D., values in parenthesis indicate the number of males giving semen. Values followed by the same letter are not significantly different, $P > 0.05$. No statistical tests were performed for 5.00 $\mu g\,l^{-1}$ BPA as only one sample was available per sampling date.

Table 4
Effect of bisphenol A (BPA) on the average path swimming velocity in brown trout

Estimated BPA concentration ($\mu g\,l^{-1}$)	Average path swimming velocity ($\mu l\,s^{-1}$)		
	Beginning of spawning (10 October)	Middle of spawning (28 October)	End of spawning (17 November)
0.00	96.7 ± 17.6 a (7)	107.2 ± 19.3 a (8)	106.2 ± 14.0 a (7)
1.75	62.0 ± 17.6 b (8)	74.9 ± 12.6 b (8)	111.1 ± 10.9 a (6)
2.40	74.9 ± 9.2 b (6)	74.7 ± 14.3 b (8)	92.4 ± 21.6 a,b (6)
5.00	32.5	49.0	41.0

Fish were exposed to BPA during late prespawning and during spawning period and semen was sampled three times as indicated. Values are mean ± S.D., values in parenthesis indicate the number of males giving semen. Values followed by the same letter are not significantly different, $P > 0.05$. No statistical tests were performed for 5.00 $\mu g\,l^{-1}$ BPA as only one sample was available per sampling date.

estimated BPA concentrations of 5.00 $\mu g\,l^{-1}$ the semen mass was very small (Table 1).

In the control group sperm density was highest in the beginning of spawning (10 October) and decreased constantly during the spawning season (Table 2). At estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$ sperm density was significantly lower than in the control group in the beginning of spawning (10 October) (Table 2). In the middle (28 October) and in the end of spawning (17 November) the sperm density of fish exposed to estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$ was similar to the control (Table 2). For brown trout exposed to estimated BPA concentrations of 5.00 $\mu g\,l^{-1}$ only one sample could be obtained per sampling date. The sperm density was in a similar range as for brown trout exposed to lower BPA concentrations (Table 2).

The rate of locally motile spermatozoa was constant in the control group during the whole spawning season. It was 8.6 ± 11.1% in the beginning of spawning, 9.1 ± 9.3% in the middle of spawning, and 5.2 ± 6.7% in the end of spawning. For brown trout exposed to estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$ the rate of locally motile spermatozoa was not significantly different from the control group (data not shown). For the semen sample obtained from brown trout exposed to estimated BPA concentrations of 5.00 $\mu g\,l^{-1}$, the rate of locally motile spermatozoa was <5%.

The rate of motile spermatozoa and the sperm swimming velocity were high and constant in the control group during spawning (Tables 3 and 4). In the group exposed to estimated BPA concentrations of 1.75 $\mu g\,l^{-1}$ the motility rate was significantly lower than in the control in the beginning of spawning (Table 3), and the swimming velocity in the beginning and in the middle of spawning (Table 4). In the group exposed to estimated BPA concentrations of 2.40 $\mu g\,l^{-1}$, the motility rate and swimming velocity were significantly lower than in the control in the beginning and in the middle of spawning (Tables 3 and 4). The semen samples obtained from the group exposed to estimated BPA concentrations of 5.00 $\mu g\,l^{-1}$ BPA had

Table 5
Effect of bisphenol A (BPA) on the sperm swimming pattern in brown trout

	Sperm swimming pattern (%)		
	Beginning of spawning (10 October)	Middle of spawning (28 October)	End of spawning (17 November)
Control (estimated BPA concentration of 0.00 μg l^{-1})			
Circular (%)	55.2 ± 10.1 a (7)	35.6 ± 1.56 b (8)	19.8 ± 14.3 c (7)
Non-linear (%)	20.2 ± 10.3 c (7)	11.7 ± 1.8 c (8)	14.9 ± 8.5 c (7)
Linear (%)	24.6 ± 12.5 b,c (7)	52.7 ± 1.3 a (8)	65.3 ± 20.1 a (7)
Estimated BPA concentration of 1.76 μg l^{-1}			
Circular (%)	55.9 ± 22.5 a (8)	43.1 ± 7.8 d (8)	25.6 ± 11.4 b,c (6)
Non-linear (%)	14.4 ± 18.2 c (8)	22.7 ± 9.1 c (8)	10.2 ± 10.6 c (6)
Linear (%)	29.7 ± 23.8 b,c (8)	34.2 ± 11.1 b,d (8)	64.3 ± 13.8 a (6)
Estimated BPA concentration of 2.40 μg l^{-1}			
Circular (%)	46.7 ± 24.1 a,d (6)	45.3 ± 11.0 d (8)	39.5 ± 21.6 b,d (6)
Non-linear (%)	27.3 ± 20.3 b,c (6)	27.0 ± 7.6 b,c (8)	19.8 ± 5.4 c (6)
Linear (%)	26.0 ± 10.0 b,c (6)	27.7 ± 11.6 b,c (8)	40.7 ± 18.6 b,d (6)

Fish were exposed to BPA during late prespawning and during spawning period. Values are mean ± S.D., values in parenthesis indicate the number of males giving semen. Values followed by the same letter are not significantly different, $P > 0.05$. In the only semen sample obtained from the group exposed to 5.00 μg l^{-1} BPA the motility pattern was variable during the course of the spawning season as only <5% spermatozoa were motile.

a very low motility of <5% during the whole spawning period (Tables 3 and 4).

In the control group, the rate of circularly motile spermatozoa was significantly higher than the rate of linearly motile spermatozoa in the beginning of spawning and therefore circular motion was the main motility pattern (Table 5). In the middle and in the end of the spawning season linear motion was the main motility pattern as the rate of linearly motile spermatozoa was significantly higher than the rate of circularly motile ones (Table 5). The rate of non-linearly swimming spermatozoa was low and constant (Table 5). In the group exposed to estimated BPA concentrations of 1.75 μg l^{-1} the motility pattern was not significantly different from the control in the beginning of spawning and in the end of spawning. However, in the middle of spawning in the group exposed to estimated BPA concentrations of 1.75 μg l^{-1}, circular motility was the main motility pattern, in the control group linear motility (Table 5). In the group exposed to estimated BPA concentrations of 2.40 μg l^{-1} the motility pattern was similar to the control group only in the beginning of spawning. In the middle of spawning in the group exposed to estimated BPA concentrations of 2.40 μg l^{-1}, circular motility was the main motility pattern, in the control group linear motility (Table 5). In the end of spawning in the group exposed to estimated BPA concentrations of 2.40 μg l^{-1} the rate of circularly motile and linearly motile spermatozoa was similar high while in the control group linear motility was the main motility pattern (Table 6). The rate of non-linearly swimming spermatozoa was low, constant, and

Table 6
Influence of bisphenol A (BPA) on the time-point of ovulation in brown trout

BPA concentration (μg l^{-1})	Number of ovulated females			Total
	28 October–12 November	13 –28 November	29 November–14 December	
0.00	4	0	0	4 (67%)
1.75	0	2	1	3 (50%)
2.40	0	0	4	4 (67%)
5.00	0	0	0	0 (0%)

Fish were exposed to BPA during late prespawning and during spawning period. Starting on 5 October the fish were examined in 1-week intervals if they already gave eggs. In each experimental group six females were used. Numbers are total abundances of females giving eggs in the indicated time interval.

similar to the control in the groups exposed to estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$ (Table 6). In the semen sample obtained from fish exposed to estimated BPA concentrations of 5.00 $\mu g\,l^{-1}$, the motility pattern was variable throughout the spawning season (data not shown).

The semen fertility was tested in the end of spawning (17 November). It was not different between the control group (65.5 ± 27.3%, $n = 7$), the group exposed to 1.75 $\mu g\,l^{-1}$ BPA (72.6 ± 20.7%, $n = 6$) and the group exposed to 2.40 $\mu g\,l^{-1}$ BPA (76.3 ± 8.1%, $n = 6$). The only semen sample obtained from the group exposed to 5.00 $\mu g\,l^{-1}$ BPA had a fertility of 28.0%.

3.2. Effect on eggs

In the control group 67% of the females gave eggs (Table 6). At estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$ 50 and 67% of the females gave eggs, respectively (Table 6). Contrary, at estimated BPA concentrations of 5.00 $\mu g\,l^{-1}$ BPA no females gave eggs (0%) (Table 6). Also the time period in which females ovulated differed. In the control group females ovulated from the 28.10–20.11, while ovulation was delayed after BPA exposure. In the group exposed to estimated BPA concentrations of 1.75 $\mu g\,l^{-1}$ females ovulated from 21 November to 6 December, in the group exposed to 2.40 $\mu g\,l^{-1}$ from 29 November to 13 December (Table 6).

The total mass of eggs and the total number of eggs produced per female, the egg mass, the egg mass increase during hardening and the egg viability did not differ between the control group and the groups exposed to 1.75 or 2.40 $\mu g\,l^{-1}$ BPA (Table 7).

4. Discussion

The mean reported bisphenol A water concentrations from 21 European and 13 United States studies are 0.016 and 0.5 $\mu g\,l^{-1}$, respectively (Kolpin et al., 2002). Concentrations in receiving waters near manufacturing facilities reach high values from 8 $\mu g\,l^{-1}$ (Staples et al., 2000) to 21 $\mu g\,l^{-1}$ (Belfroid et al., 2002). In Austria, 0–0.6 $\mu g\,l^{-1}$ BPA have been measured in surface water, 0–0.9 $\mu g\,l^{-1}$ in groundwater (Paumann and Vetter, 2003). The BPA concentrations tested in the present study (estimated to 1.75, 2.40, 5.00 $\mu g\,l^{-1}$) are therefore in the upper range of environmentally relevant concentrations. All tested BPA concentrations significantly influenced the sperm and egg production in brown trout indicating a lowest observed effect concentration (LOEC) of 1.75 $\mu g\,l^{-1}$. As the exposure time was limited to 2 month in the present study the LOEC might be lower for wild populations exposed to BPA during the whole life cycle. Contrary, based on vitellogenin synthesis in rainbow trout the PNEC was estimated to be as high as 64 $\mu g\,l^{-1}$ (Lindholst et al., 2000; Staples et al., 2000). In the fathead minnow (*P. promelas*) BPA induced vitellogenin synthesis in males at concentrations ≥160 $\mu g\,l^{-1}$ whereby the effect depended on the exposure time (Sohoni et al., 2001), in the medaka (*O. latipes*) at 3120 $\mu g\,l^{-1}$ (Kang et al., 2002).

4.1. Effect on semen

Low sperm density, motility rate and swimming velocity are indicative for low semen quality (=fertility) in the Salmonidae (Lahnsteiner et al., 1998), while no

Table 7
Effect of bisphenol A on egg production and egg quality in brown trout

	Estimated bisphenol A concentrations			
	0.00 $\mu g\,l^{-1}$	1.75 $\mu g\,l^{-1}$	2.40 $\mu g\,l^{-1}$	5.00 $\mu g\,l^{-1}$
Number of mature females	4	3	3	0
Egg volume (g)	17.3 ± 4.5 a	18.7 ± 0.7 a	12.8 ± 9.3 a	0.0 ± 0.0 c
Eggs produced per female	330 ± 85 a	350 ± 45 a	210 ± 95 b	0.0 ± 0.0 c
Egg mass (mg)	53 ± 6 a	54 ± 5 a	51 ± 4 a	–
Mass increase during hardening (%)	121 ± 3 a	117 ± 4 a	119 ± 3 a	–
Egg viability (%)	94.7 ± 1.9 a	89.3 ± 14.5 a	91.1 ± 8.3 b	–

Fish were exposed to BPA during late prespawning and during spawning period. Values are mean ± S.D., values followed by the same letter are not significantly different, $P > 0.05$.

correlation could be detected between the sperm swimming pattern and semen quality until now (Lahnsteiner et al., 1998). The sperm swimming pattern depends on the cell internal calcium levels (Boitano and Omoto, 1992; Cosson et al., 1999) and circular motility may be indicative for immaturity of spermatozoa as it is often found in the beginning of the spawning season just when males start to give semen (Billard, 1986).

In the present study, in brown trout of the control group the semen mass, the sperm motility rate, and the sperm swimming velocity were constant and the sperm density decreased throughout the spawning season. The main motility pattern changed from circular in the beginning of spawning to linear in the middle and in the end of spawning. The described semen characteristics are typical for Salmonidae throughout the spawning season (Billard, 1986; Lahnsteiner et al., 2005) and indicate that sperm quality is constant with exception of the parameter sperm density.

The present data demonstrate that exposure of brown trout to BPA negatively affected the semen quality. In the groups exposed to estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$ semen quality was lower than in the control in the beginning of spawning (reduced sperm density, motility rate, and swimming velocity) and in the middle of spawning (reduced swimming velocity, reduced sperm motility rate [only at 2.40 $\mu g\,l^{-1}$ BPA]). These data clearly demonstrate that for brown trout exposed to estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$ production of high quality semen was restricted to the end of the spawning season (delayed for approximately 4 weeks in comparison to the control). In the control the sperm swimming pattern changed from circular to linear in the middle of spawning. Contrary, in the group exposed to estimated BPA concentrations of 1.75 $\mu g\,l^{-1}$ it changed to linear in the end of spawning. At estimated BPA concentrations of 2.40 $\mu g\,l^{-1}$ circular motility remained the main swimming pattern throughout the whole spawning season. As linear motility may be indicative for full maturity of spermatozoa (see above) these results could support the hypothesis that estimated BPA concentrations of 1.75–2.40 $\mu g\,l^{-1}$ delay sperm maturation.

In the Salmonidae, sperm motility rate and sperm swimming velocity are correlated with sperm fertility, and therefore semen with a high percentage of motile spermatozoa has also a high fertility (Lahnsteiner et al., 1998). Under the influence of BPA other parameters than sperm motility may be disturbed, too (e.g. parameters influencing the sperm egg contact as composition of the plasma membrane, DNA, or sperm metabolism). However, this was not the case as there were no differences in sperm fertility between the control group and the groups exposed to estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$ BPA when tested in the end of the spawning season, when fish had comparable motility parameters and sperm densities.

When brown trout are exposed to BPA 1 month before the beginning of spawning the testes contain testicular spermatozoa within the germinal cysts (Billard, 1987). Therefore, estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$ probably influenced the sperm final maturation processes in the testes and in the spermatic ducts. The final maturation processes of spermatozoa in the testicular main ducts and spermatic ducts (Billard, 1986; Loir et al., 1990) and the synthesis of seminal fluid which is necessary to maintain the viability of mature spermatozoa (Lahnsteiner et al., 1999) are under hormonal control (Tanimoto and Morisawa, 1988; Marshall et al., 1989; Estay et al., 1998).

At estimated BPA exposure levels of 5.00 $\mu g\,l^{-1}$ only one of eight males gave small semen masses. The semen quality (sperm motility rate, swimming velocity, fertility) was low in comparison to the control during the whole spawning period whereby the reduced fertility must be considered to be due to the low motility rate. Unpublished investigations of the gonad morphology revealed that brown trout exposed to 5.00 $\mu g\,l^{-1}$ BPA had normal testes of mature appearance. Fish might have produced semen later when the experiment had already been terminated. As no semen could be stripped spermatozoa were not released out of the germinal cysts and therefore estimated BPA concentrations of 5.00 $\mu g\,l^{-1}$ probably affected the final stages of spermiogenesis. Generally spermiogenesis is under hormonal control, whereby androgens regulate the differentiation of spermatozoa and 17α,20β-dihydroxy-4-pregnene-3-one (17,20P) regulates the semen production and seminal fluid synthesis (Estay et al., 1998).

The here described effects of BPA on brown trout semen production are different from those observed with nonylphenol (130–750 $ng\,l^{-1}$) in rainbow trout (Lahnsteiner et al., 2005). While BPA influenced the time point when high quality semen was produced nonylphenol affected the semen quantity in a time-

and dose-dependent manner (Lahnsteiner et al., 2005). These results give first indications that BPA and 4-nonylphenol have different effects on semen production in the Salmonidae which might be related to differences in binding efficiency to the estrogen receptors and/or differences in antagonistic actions to male sex hormones.

4.2. Effect on eggs

While for hatchery reared Salmonidae the percentage of ovulated females is generally 80–90% (Lahnsteiner, 2000) for the brown trout used in the experiment it was only 50–70%. As the used fish derived from a wild population they were probably not fully adapted to hatchery conditions. The present results indicate that the tested concentrations of BPA affected the percentage of ovulated females and the time point of ovulation but not the quantity and quality of eggs. The percentage of ovulated females was similar for the control group and the groups exposed to estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$, whereas at estimated BPA concentrations of 5.00 $\mu g\,l^{-1}$ females did not ovulate during the investigation. While brown trout of the control group ovulated between the 28 October and 12 November which is within the natural spawning period of the wild population (unpublished data), brown trout exposed to estimated BPA concentrations of 1.75 $\mu g\,l^{-1}$ BPA ovulated approximately 2 weeks later (22 November–6 December) and brown trout exposed to estimated BPA concentrations of 2.40 $\mu g\,l^{-1}$ BPA approximately 3 weeks later (29 November–13 December). Therefore, ovulation was delayed under the influence of BPA in a similar fashion as the production of high quality semen in males. Morphologically, the ovaries from brown trout exposed to 5.00 $\mu g\,l^{-1}$ BPA were of mature appearance and contained preovulatory follicles but no ovulated eggs (unpublished data). Therefore, females exposed to 5.00 $\mu g\,l^{-1}$ BPA might have produced mature gametes at a later time point when the experiment was already terminated.

Egg quality did not differ between the control group and the groups exposed to estimated BPA concentrations of 1.75 and 2.40 $\mu g\,l^{-1}$ as the egg volume and egg number produced per female, the egg mass, the percentile egg mass increase during hardening (an egg quality marker in the Salmonidae, Lahnsteiner and Patzner, 2000), and the egg viability were not different.

In the Salmonidae, serum levels of estradiol and testosterone increase during oogenesis to reach peak levels at the beginning of ovulation (Donaldson and Hunter, 1983; Nagahama, 1994). During final oocyte maturation and ovulation, plasma estradiol and testosterone levels decrease, whereas plasma progestogen levels increase (Donaldson and Hunter, 1983). A sharp peak of prostaglandins occurs prior to spontaneous or induced ovulation (Donaldson and Hunter, 1983; Nagahama, 1994). As females produced preovulatory follicles at all tested BPA concentrations it is concluded that BPA did not affect oogenesis but the process of ovulation. 17α-Ethinylestradiol, another endocrine disruptor, stimulated egg production at low doses and inhibited egg production at higher doses in the fathead minnow (*P. promelas*) (Jobling et al., 2003).

In the wild, delayed spawning delays the larval development. A critical period for larvae is when they absorb yolk and begin to feed (Milinski and Parker, 1991; Murdoch, 1994). If larvae development is delayed larvae may hatch when no food is available in the region or first feeding phase may temporally mismatch with food availability (Milinski and Parker, 1991; Murdoch, 1994). Mismatch between the beginning of the feeding phase and food availability leads to high larval mortality (Milinski and Parker, 1991; Murdoch, 1994). Delayed development (smaller size, different ontogeny and behaviour) may make the larvae available as prey in higher extent or for other predators, too (Olson et al., 1995).

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Reduced somatic growth of salmonid larvae exposed to 4-nonylphenol, bisphenol A, and ß-estradiol

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Abstract

Grayling (*Thymallus thymallus*) and white fish (*Coregonus sp.*) were exposed to 4-nonylphenol (0.13 µg l^{-1}), bisphenol A (4.5 µg l^{-1}) and ß-estradiol (1.5 ng l^{-1}) from the fertilized egg stage to the stage of metamorphosis to young fish (60-68d). The 3 tested endocrine disruptor had no definitive effect on the survival of embryos and larvae. However, somatic growth was significantly affected. After 60 - 68 d exposure to bisphenol A fish were smallest as their weight was only circa 35% of the control. After exposure to ß-estradiol and 4-nonylphenol the weight was circa 50 - 70% of the control. Also the total length of the fish was significantly decreased.

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Introduction

In teleost fish the embryo and larval stage are sensitive phases of the life cycle which are affected by environmental pollutants (Schulte and Nagel, 1994; Friccius et al., 1995). Until now only little is known about the influence of endocrine disruptors (4-nonylphenol, bisphenol A and ß-estradiol) on viability, differentiation, and growth of embryos and larvae. 4-nonylphenol at estimated concentrations of $\geq$ 280 ± 40 ng l^{-1} significantly decreases the

larvae viability in rainbow trout (*Oncorhychus mykiss*) (Lahnsteiner et al. 2004). Bisphenol A negatively affects the egg and larval development of *Salmo salar m. sebago* (Honkanen et al., 2001). In zebrafish (*Brachydanio rerio*) in a full life cycle test > 1.67 ng l^{-1} 17α-ethinyl ß-estradiol reduces the growth rate of fish (Segner et al., 2003). ß-estradiol disturbs the smoltification of Atlantic salmon (*Salmo salar*) larvae (Madsen et al., 2004).

In the present preliminary study the effect of 4-nonylphenol (0.13 µg l^{-1}), bisphenol A (4.5 µg l^{-1}) and ß-estradiol (1.5 ng l^{-1}) on somatic growth of embryos and larvae is studied in two salmonid species, the grayling (*Thymallus thymallus*) and the white fish (*Coregonus sp.*). The investigated concentrations of endocrine disruptors are predicted non effect concentrations (PNEC) on embryo and larvae viability in the Salmonidae which have been determined in preliminary experiments.

Material and methods

All experiments were conducted in the hatchery of Kreuzstein in Sankt Gilgen, Upper Austria, with whitefish, *Coregonus sp*., and grayling, *Thymallus thymallus* and in compliance with the Austrian Federal law for animal care. To expose eggs and larvae to the different types of endocrine disruptors a flow through system was used which has been described previously (Lahnsteiner et al., 2005) (Fig. 1). Briefly, the system consisted of four 0.5 m^3 tanks. The tanks were supplied with well water with an oxygen content of > 90% saturation. The effluent water drained off from the 4 tanks was directed into 4 separate trays containing the egg incubators. Fertilized eggs were placed in the egg incubators until the end of the yolk sac stage. Then the larvae were collected and placed in the tanks for feeding.

The required concentrations of 4-nonylphenol, bisphenol A, and ß-estradiole were added to the well water by means of injection pumps whereby the concentrations were adjusted by changing the injection rates. Final concentrations of endocrine disruptors were calculated based on the flow rate of uncontaminated well water and on the injection rate of endocrine disruptors.

Stock solutions of bisphenol A (2.5 g l^{-1}), ß-estradiole (0.015 g l^{-1}), and 4-nonylphenol (0.5 g l^{-1}) were prepared by dissolving the chemicals in dimethylsulfoxide (DMSO). The stock solutions were diluted with well water, whereby bisphenol A was diluted to 2840 µg l^{-1}, ß-estradiole to 1 µg l^{-1}, and 4-nonylphenol to 83 µg l^{-1}. The diluted solutions were injected to

the well water using an injection rate of 10 ml min^{-1}. The resulting estimated final concentrations were 4.5 µg/l for bisphenol A (well water flow through rate: 5.95 l min^{-1}), 1.5 ng/l for ß-estradiole (well water flow through rate: 6.45 l min^{-1}) and 0.13 µg/l for 4-nonylphenol (well water flow through rate: 6.30 l min^{-1}). Tank 4 served as control. The well water flow through rate was 5.95 l min^{-1}, and instead of endocrine disruptors an aqueous DMSO solution was injected to obtain estimated concentrations similar to those in the experiments.

During their spawning time (December for *Coregonus sp*. and April for *Thymallus thymallus*) semen and eggs were stripped from the broodfish. In each species eggs from 3 to 4 individuals were pooled and fertilized with semen at conditions of sperm saturation. Then the fertilized egg batches were divided in 4 subsamples. In *Thymallus thymallus* each subsample had a weight of 5 g (circa 500 eggs), in *Coregonus* sp. 7.5 g (circa 500 eggs). In each species three subsamples were exposed to 4-nonylphenol, bispenol A, and ß-estradiol, respectively. The fourth subsample served as control. *Coregonus sp.* eggs and larvae were incubated at 8-10°C in flow through incubator, *Thymallus thymallus* eggs and larvae at 10-12°C. After 20 d incubation in *Coregonus sp.* and after 14 d incubation in *Thymallus thymallus* the percentage of eyed stage embryos was calculated in relation to the total number of incubated eggs. After 22 d and 28 d for *Thymallus thymallus* and *Coregonus sp*., respectively, the percentage of larvae being in the end of the yolk sac stage was calculated. Then the larvae were removed from the incubators and placed in the 5 m^3 tanks. There they were fed twice a day with plankton using a procedure routinely applied for feeding of fish larvae in the fish farm. Natural plankton was fished off from lake Mondsee using plankton nets with a mesh size of 200 µm. The plankton was re-diluted in well water and 2 l diluted plankton (density circa 30,000 - 40,000 animals per l) was added to the tanks every morning and every afternoon. After 60 d in *Thymallus thymallus* and after 68 d in *Coregonus* sp. the percentage of young fish survival in relation to the total number of incubated eggs was calculated once more. Then 100 fish were killed using an over dose of MS222. Thereafter they were fixed in 4% neutral formaldehyde and the length of each fish was measured in a stereomicroscope. However, this parameter was difficult to measure as many larvae were not straight but bent when killed by MS 222. Therefore, also the weight of each fish was measured whereby the larvae were placed on a filter paper to remove adhering water and weighed to the nearest 0.1 mg using an analytical balance.

For statistical analysis relative abundances were transformed by angular transformation ($\arcsin\sqrt{P}$). To determine the effect of treatments on embryo and larvae

survival and on larvae weight and total length ANOVA was used with treatment procedure as independent variable and the above mentioned parameters as dependent variables (posthoc test: Tukey´s- b).

Results

In *Thymallus thymallus* the survival rates did not differ from the control after exposure to bisphenol A (estimated concentrations: 4.5 µg l^{-1}), ß-estradiole (1.5 ng l^{-1}), and 4-nonylphenol (0.13 µg l^{-1}) (Figure 1). Embryo survival to the eyed stage was 81.0 - 86.2 %, larvae survival to the end of the yolk sac stage 58.3 - 63.2 %, and young fish survival to the end of the experiment (young fish stage) 54.8 - 58.3 % (Figure 1). In *Coregonus* sp. embryo survival to the eyed stage was similar in the control and in all treatments (65.1 - 71.2%) (Figure 1). Larvae survival to the end of the yolk sac stage was 63.0 - 69.7% in the control and after exposure to bisphenol A and 4-nonylphenol, but only 66.0 % after exposure to ß-estradiole (Figure 1). Young fish survival to the end of the experiment was similar in the control, and after exposure to bisphenol A and 4-nonylphenol (46.0 - 47.0%), but decreased to 34 % after exposure to ß-estradiole (Figure 1).

Fig. 1a. Percentage of survival to the eyed embryo stage (20 d), yolk sac stage (28 d), and stage of metamorphosis to young fish (68 d) in *Coregonus* sp.

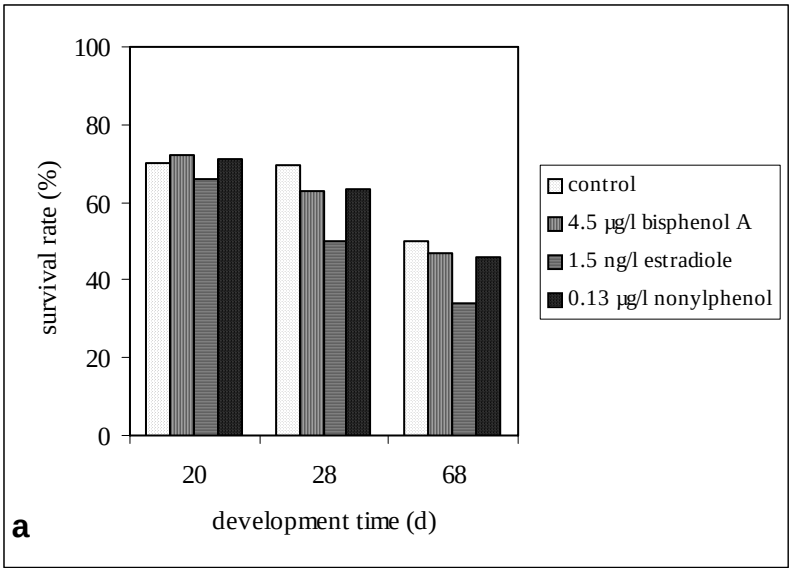

Fig. 1b. Percentage of survival to the eyed embryo stage (14 d), yolk sac stage (22 d), and stage of metamorphosis to young fish (60 d) in *Thymallus thymallus.*

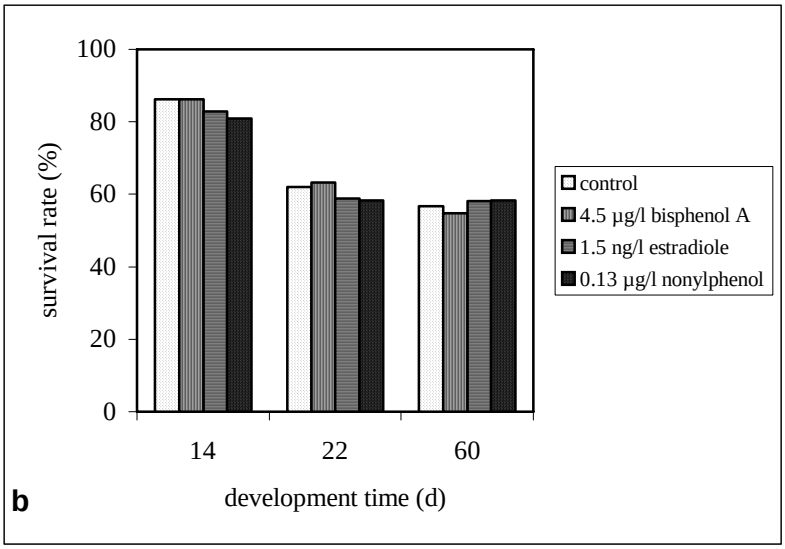

Table 1. Influence of bisphenol A, ß-estradiole, and 4-nonylphenol on somatic growth of larvae and young fish of *Thymallus thymallus* and *Coregonus* sp.. Both species were exposed to endocrine disruptors from the fertilized egg stage to the stage of metamorphosis to young fish (60 d at 10-12°C in *Thymallus thymallus*, 68 d at 8-10°C in *Coregonus* sp.). Values are mean ± standard deviation, n = 100 for each treatment.

	control	**bisphenol A** ($4.5\ \mu g\ l^{-1}$)	**estradiole** ($1.5\ ng\ l^{-1}$)	**4-nonylphenol** ($0.13\ \mu g\ l^{-1}$)
Thymallus thymallus				
weight, mg	118.2 ± 19.5^{a}	42.4 ± 6.9^{b}	79.5 ± 14.0^{c}	60.3 ± 14.3^{c}
total length, mm	28.2 ± 2.2^{a}	18.8 ± 1.2^{b}	22.3 ± 1.2^{c}	$20.9 \pm 2.2^{b,c}$
Coregonus sp.				
weight, mg	206.4 ± 39.2^{a}	71.6 ± 27.0^{b}	112.1 ± 2.2^{c}	101.9 ± 16.9^{d}
total length, mm	28.1 ± 1.9^{a}	18.7 ± 1.3^{b}	25.0 ± 1.4^{c}	20.7 ± 2.3^{d}

When *Thymallus thymallus* and *Coregonus* sp. were exposed to bisphenol A (estimated concentrations: 4.5 µg l^{-1}), ß-estradiole (1.5 ng l^{-1}), and 4-nonylphenol (0.13 ng l^{-1}) from the fertilized egg stage to the stage of metamorphosis to young fish the weight and the total length of the young fish was significantly lower than in the control, whereby both parameters were lowest after exposure to bisphenol A (Table 1).

Discussion

The present results demonstrate that the tested concentrations of bisphenol A and 4-nonylphenol did not affect the viability of embryos and larvae in *Thymallus thymallus* as the percentage of survival to the eyed stage, to the end of the yolk sac stage and to the young fish stage were similar to the control. In the white fish, *Coregonus sp.*, the percentage of survival to the end of the yolk sac stage and to the stage of metamorphosis to young fish were lower than in the control indicating that ß-estradiole may be toxic at the tested concentration. However, more experiments are necessary for statistical confirmation of these preliminary results.

The present results indicate very clearly that the somatic growth of larvae is decreased at the tested concentrations of endocrine disruptors in both investigated species. After exposure to 4.5 µg l^{-1} bisphenol A fish were smallest as their weight was only circa 35% of the control. In larvae exposed to 1.5 ng l^{-1} ß-estradiole and 0.13 µg l^{-1} 4-nonylphenol the weight was circa 50 - 70% of the control. Also the length of the fish was significantly decreased after exposure to the 3 types of endocrine disruptors. Reduced growth may result from imbalance of hormone systems or endocrine disruptors may be toxic at sublethal level. More detailed studies are necessary on this topic. In adult male fathead minnow (*Pimephales promelas*) somatic growth was reduced after exposure to $\geq$640 µg l^{-1} bisphenol A for $\geq$71 days (Sohoni et al. 2001). In zebrafish (*Brachydanio rerio*) in a full life cycle test > 1.67 ng l^{-1} 17α-ethinyl ß-estradiol reduced the growth rate, too (Segner et al., 2003).

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Effect of ß-estradiol on gamete quality and time point of maturation in the Salmonidae as indicated by laboratory experiments

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Abstract

In the present study the effect of environmental relevant concentrations of ß-estradiol on gamete quality and gamete maturation in rainbow trout (*Oncorhynchus mykiss*) and grayling (*Thymallus thymallus*) was investigated. When male rainbow trout were exposed to $\geq$ 1 ng l^{-1} ß-estradiol for 35 d during the spawning season the semen volume obtained per male was significantly reduced, after 50 d also the sperm density and the sperm fertility. When male grayling were exposed to 1.0 ng l^{-1} ß-estradiol for 50 d during the prespawning season a similar number of males gave semen as in the control. However, the volume of semen produced per male was decreased. Also the percentage of motile spermatozoa and their sperm swimming velocity was decreased while the percentage of locally motile spermatozoa was increased.

When female rainbow trout were exposed to 0.5 – 2 ng l^{-1} ß-estradiol and eggs were stripped in portions in 1 week intervals the egg viability changed in a similar way as in the control indicating that egg overripening processes were not influenced by ß-estradiol. When female grayling were exposed to 1.0 ng l^{-1} ß-estradiol during the prespawning time ovulation occurred earlier than in the control group. Therefore, lowest observed effect concentration in the Salmonidae was 1 ng/l.

Keywords: ß-estradiol; Endocrine disruptors; Spermatozoa; Eggs; Gamete quality; Rainbow trout; Grayling.

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1. Introduction

Beside of industrial chemicals like bisphenol A, octylphenol, or nonylphenol there exists another group of endocrine disruptors which are natural and synthetic estrogens (Bennie 1999, Segner et. al., 2003). They derive from drugs and medication (antibaby pill, hormone replacement therapies) and are mainly 17α-ethynylestradiol, 17α–estradiol, estrone, and estriol (Belfroid et al., 1999; Jobling et al., 2003; Paumann and Vetter 2003). Except for 17α - estradiol, which is mainly disposed via solid waste, estrogens get into the waste-water (Belfroid et al., 1999; Paumann and Vetter 2003) where they can influence the reproduction of aquatic organisms (Jobling et al., 2003). As estrogens are naturally occurring hormones their functions and mechanisms are well understood in aquatic organisms especially in teleost fish where hormone treatments play an important role in aquaculture (sex reversal, artificial gamete maturation) (Donaldson and Hunter 1983; Devlin and Nagahama 2003). In teleosts estrogens (estradiol) are responsible for oocyte growth and maturation in the follicles (Peter and Yu, 1997). Together with testosterone and progesteron they play also an important role during final oocyte maturation and ovulation (Peter and Yu 1997; Kime 1993). In males ß-estradiol plays a role during spermatogenesis in controlling the early mitotic proliferation phase of germ cells (Scott and Sumpter 1989; Campbell et al., 2003; Miura and Miura 2003). Administration of high dosages of ß-estradiol to male fish leads to the regression of testicular tissue and to the development of secondary ovaries (sex reversal) (Devlin and Nagahama, 2003).

For teleost fish also endocrine disrupting effects of estrogens have been studied in several species. In zebrafish (*Brachydanio rerio*) in a full life cycle test > 1.67 ng l^{-1} 17α-ethinyl ß-estradiol induced vitellogenin production, reduced the egg number produced per female, the fertilization success and the growth rate of fish (Segner et al., 2003). In partial life cycle tests effects were found only at higher concentrations (Segner et al., 2003). In the fathead minnow (*Pimephales promelas*) 0.1 ng l^{-1} 17α-ethinyl ß-estradiol induced vitellogenesis after an exposure time of 3 weeks (Jobling et al., 2003). It led to a dose dependent increase in the mean number of spawned eggs at 0.1 - 1 ng l^{-1} and at higher concentrations it decreased the number of spawned eggs (Jobling et al., 2003). In *Fundulus heteroclitus* ß-estradiol (injection of 0.5 mg/kg body weight) induced vitellogenin synthesis whereby the results were comparable with rainbow trout (Pait and Nelson, 2003). Schultz et al. (2003) exposed male rainbow trout to 10, 100, and 1000 ng l^{-1} 17α-ethinyl ß-estradiol for 62 d during prespawning. Thousand ng l^{-1} was lethal to fish, 10 – 100 ng l^{-1} reduced semen

fertility for approximately 50%. Also smoltification of Atlantic salmon (*Salmo salar*) larvae was disturbed by ß-estradiol (Madsen et al., 2004).

Based on the above summarized knowledge severe effects of estrogens on the male reproductive potential could be demonstrated. However, with exception of the study of Schulz et al. (2003) almost nothing is known about the influence of environmental relevant concentrations of estrogens on spermiogenesis and consecutively on the quality of semen in fish. It is also unclear whether estrogens affect the egg overripening processes and therefore the period during which spawning of high quality eggs is possible. This problem is of particular importance in species which have reproductive cycles and where all eggs mature simultaneously. Finally it is unknown whether the duration of gamete maturation is influenced, too, which could lead to desynchronization of spawning between males and females or to changed spawning times.

These problems are investigated in the Salmonidae which are commercially important fish in many parts of the world and particularly sensitive to environmental pollutants. To study the effect of ß-estradiol on semen quality and on egg overripening processes rainbow trout (*Oncorhynchus mykiss*) are exposed to three environmental relevant ß-estradiol concentrations (0.5, 1.0, 2.0 ng l^{-1}) during spawning. To investigate whether the duration of spermiogenesis and oogenesis and subsequently the time point of gamete maturation and spawning is changed under the influence of ß-estradiol grayling (*Thymallus thymallus*) are exposed to this compound during the prespawning season.

Material and methods

2.1. Experimental design

All experiments were conducted in the hatchery of Kreuzstein in Sankt Gilgen, Upper Austria in compliance with the Austrian Federal law for animal care (GZ 68.210/58-Br GT/2003). Rainbow trout (*Oncorhynchus mykiss*) and grayling (*Thymallus thymallus*) were obtained from commercial broodstocks of fish farmers in Upper Austria. A stock solution of ß-estradiol was prepared by dissolving 0.15 g ß-estradiol in 100 ml DMSO. Required ß-estradiol concentrations were obtained by diluting the stock solutions with well water.

For *in vivo* exposure of fish to ß-estradiol a flow through system was used which has been described recently (Lahnsteiner et al., 2005). The system consisted of 4 0.5 m^3 tanks, 1 control tank and 3 experimental tanks. The tanks were supplied with 6°C well water for rainbow trout and 8°C well water for grayling. Oxygen content was > 90%. ß-estradiol was

added with an injection pump whereby the concentrations were adjusted via the injection rates. The system was equilibrated for 1 week before the experiment was started. Fresh water supply and ß-estradiol injection rates were controlled regularly and readjusted when necessary. ß-estradiol concentrations were calculated based on the flow rate of uncontaminated well water and on the injection rate of ß-estradiol. Two experiments were conducted. In experiment 1 rainbow trout were exposed to 0.5, 1.0, and 2.0 ng l^{-1}ß-estradiol during the spawning period to investigate its influence on semen quality and on egg overripening processes. In experiment 2 grayling were exposed to 1.0 ng l^{-1} ß-estradiol during the prespawning period to determine its effect on the duration and time point of gamete maturation.

Experiment 1: Male +2 year rainbow trout (total length: 20 – 35 cm) and female +3 year rainbow trout (30 – 45 cm) were exposed to ß-estradiol during their spawning period from the beginning of December to the middle of January. For males the spawning period was defined as the sperm production period, for females as the period from the time point of ovulation until eggs were overmatured. Sperm production started about 1 week before the onset of the experiment, ovulation occurred at the onset of the experiment or several days ($\leq$ 5d) thereafter. Before the onset of the experiment those male rainbow trout which were considered as potential experimental fish were stripped and checked on semen quality. Fish giving < 0.5 ml semen or having semen with a motility < 50% were rejected from the experiments. Females were only used when having ovulated within 5d after the onset of the experiment. Soft pressure on the abdomen of the fish was used to control maturity, i.e. if eggs could be already stripped. The selected fish were used to form four experimental groups each consisting of 10 males and 3 females. Also fish size and fish weight was approximately similar in the four tanks. Fish groups 1, 2, and 3 were exposed to the three ß-estradiol concentrations (0.5, 1.0, 2.0 ng/l), the control group received an adequate concentration of DMSO which was the carrier for ß-estradiol. Males were stripped at the beginning of the experiment and after 35 and 50 days exposure. In semen samples collected in the beginning of the experiment and after 35 days exposure the semen volume, sperm density and sperm motility were determined, in semen samples collected after 50 days exposure the semen volume, sperm density and sperm fertility were determined. In females egg portions of about 20-30 g were stripped in 1 week intervals and the egg fertility was determined for each sample.

Experiment 2: To determine the effect of ß-estradiol on spermiogenesis and oogenesis +3 year grayling (total length: 15 - 20 cm) were used. They were exposed to ß-estradiol during the

prespawning and spawning period, i.e. from the beginning of February to the middle of April. Males and females were distinguished by the shape of the dorsal fin and two experimental groups were formed each consisting of 6 males and 6 females. Fish group 1 was exposed to 1.0 ng l^{-1} estradiol, fish group 2 served as control. Fish were fed 2 times per week with pellets or small cyprinids and had a natural photoperiod. After 35 d grayling were stripped in 1 week intervals to determine it they gave already semen and eggs. Onset of spermiation and time point of ovulation were recorded and the quantity and quality of gametes were measured. When females had ovulated all eggs were stripped, and the amount and viability were determined as described below. Quantitative (semen volume) and qualitative investigations (sperm motility) on semen samples were performed after an exposure time of 50 d.

2.2. Determination of gamete quality

Semen volume was determined gravimetrically to the nearest 0.01 g. Sperm density was determined spectrophotometrically at 450 nm. The method was standardised by sperm counts in a Burker Türk counting chamber. Sperm motility was determined with computer assisted cell motility analysis at 4 ± 1°C (Lahnsteiner et al., 1999). Hundred µl sperm motility activating solution was added into the Makler investigation chamber and 2 µl semen was added and mixed. The chamber was closed with a coverslip, the sample was transferred into an inverse phase contrast microscope coupled with a video camera (20-fold magnification) and the motility was recorded on videotapes until it had ended (about 45 sec). The following sperm motility parameters were measured 10 ± 2 sec after activation in a Stroemberg Mika cell motility analysis program: % immotile (velocity < 5 µm s^{-1}), % locally motile (velocity of 5-20 µm s^{-1}), % motile (velocity < 20 µm s^{-1}), % linear motile (linearity index $\geq$ 0.9), % non linear motile (linearity index < 0.9), average path swimming velocity of the motile spermatozoa (µm s^{-1}). The linearity index (LI) was calculated on base of the swimming path as LI = SL/AL, where SL represents the straight line swimming path between the measuring points and AL the actual swimming path between the measuring points.

To test the sperm fertility eggs were stripped from two females, pooled, and divided in subsamples of 200 ± 10 eggs. Semen collected from the experimental fish was prediluted in sperm motility inhibiting saline solution (103 mmol l^{-1} NaCl, 40 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 0.8 mmol l^{-1} $MgSO_2$, 20 mmol l^{-1} tris, pH 7.8 - Lahnsteiner et al., 1999) in a ratio of 1 : 3 (semen : saline). Eggs were fertilized using 5 µl prediluted semen and 6 ml of 6°C well water (sperm to egg ratio 55,000 : 1 to 65,000 : 1) and incubated in flow incubators. After 30 days the percentage of embryos in the eyed stage was evaluated.

The number of eggs produced per female was determined based on the total weight of the stripped eggs and on the individual egg weight after ovarian fluid had been drained off.

To determine the egg viability 200 ± 10 eggs were fertilized at conditions of sperm saturation (40 µl undiluted semen, sperm to egg ratio = 500,000 – 600,000 : 1). Six ml of 6°C well water was used as fertilization solution. Eggs were incubated in flow incubators as described and the eyed stage rate was determined.

2.3. Statistics

For statistical analysis relative abundances were transformed by angular transformation (arcsin√P). To determine if the experimental treatments resulted in significant different results analysis of variance (ANOVA) was used. In experiments where semen samples in different times were obtained from the same fish repeated measure one way ANOVA was used whereby time was included as repeated measure variable. The Waller Duncan posthoc test was used as a multiple comparison test to determine which treatments differed significantly. For pair wise comparison of mean values Dunetts`s T3 posthoc test was used.

3. Results

When male rainbow trout were exposed to ß-estradiol during the spawning period semen parameters changed in the following way: After an exposure time of $\geq$ 35days the semen volume obtained per male was significantly lower in rainbow trout exposed to 1 or 2 ng l^{-1} ß-estradiol than in the control group and in the group exposed to 0.5 ng l^{-1} ß-estradiol (Fig. 1a). After an exposure time of 50 d also the sperm density was significantly lower in the groups exposed to 1 or 2 ng l^{-1} ß-estradiol than in the control group and in the group exposed to 0.5 ng l^{-1} ß-estradiol (Fig. 1b). The semen fertility was only investigated after 50 days exposure. In the groups exposed to 1.0 and 2.0 ng l^{-1} ß-estradiol the semen fertility was significantly reduced in comparison to the control group and to the group exposed to 0.5 ng l^{-1} ß-estradiol (Fig. 1f).

The rate of immotile, locally motile (Fig. 1c), and motile (Fig. 1d) spermatozoa and the sperm swimming velocity (Fig. 1e) were not affected by a 35 d exposure period to the three ß-estradiol concentrations. The sperm swimming pattern changed. In the control group the main sperm swimming pattern was circular in the beginning of the experiment and changed to linear after 35 d. At the three ß-estradiol exposure levels the main motility pattern was circular in the beginning of the experiment, too, but did not change to linear (Table 1).

Table 1. Influence of ß-estradiol on the sperm motility pattern in rainbow trout (*Oncorhynchus mykiss*). Rainbow trout were exposed to ß-estradiol for 35 d during the spawning season. Values are mean ± standard deviation (n = 10). Values superscripted by the same letter are not statistical significantly different. (P >0.05).

duration	0.5 ng/l	1.0 ng/l	2.0 ng/l	0.0 ng/l
0 day exposure				
circular, %	45.7 ± 9.1 [a]	44.2 ± 15.6 [a]	50.4 ± 22.0 [a]	50.8 ± 11.8 [a]
non linear, %	24.4 ± 13.5 [b]	25.3 ± 9.0 [b]	22.3 ± 15.9 [b]	21.6 ± 12.8 [b]
linear, %	29.9 ± 11.1 [b]	30.5 ± 18.9 [b]	27.3 ± 23.4 [b]	27.6 ± 15.4 [b]
35 days exposure				
circular, %	44.9 ± 16.6 [a]	39.6 ± 21.6 [a]	45.1 ± 15.1 [a]	17.2 ± 4.2 [b]
non linear, %	18.6 ± 7.8 [b]	19.7 ± 9.7 [b]	19.1 ± 5.5 [b]	22.6 ± 13.4 [b]
linear, %	36.5 ± 20.0 [a]	40.7 ± 29.7 [a]	35.8 ± 16.8 [a]	60.2 ± 9.3 [c]

Table 2. Influence of ß-estradiol on overripening of eggs of the rainbow trout (*Oncorhynchus mykiss*). Female rainbow trout were exposed to ß-estradiol within 5 d after ovulation, portions of eggs were stripped in 7 day intervals and their viability (percentage of eggs developing to eyed stage embryos) was determined. Numbers in parenthesis are samples numbers. For treatments resulting in more than 1 sample values are mean ± standard deviation and values superscripted by the same letter are not significantly different (P>0.05). No statistical tests were performed when only 1 sample was available.

exposure time	egg viability (% eggs developing to eyed stage embryos)			
	Control	0.5 ng l^{-1} estradiol	1.0 ng l^{-1} estradiol	2.0 ng l^{-1} estradiol
7 d	76.0 ± 9.5 [a] (3)	90.5 ± 10.6 [a] (3)	86.0 ± 5.2 (3)	78.4 ± 26.6 [a] (3)
14 d	88.8 ± 10.1 [a] (3)	77.5 ± 30.3 [a] (3)	95.2 ± 4.5 [a] (3)	75.7 ± 3.8 [a] (3)
21 d	83.4 ± 9.2 [a] (3)	29.5 ± 51.2 [b] (2)	98.9 ± 0.5 [a] (3)	90.9 ± 10.5 [a] (3)
28 d	85.9 ± 14.3 [a] (3)	2.4 ± 4.3 [b] (2)	91.1 ± 5.0 [a] (3)	79.6 ± 23.9 [a] (3)
35 d	45.0 ± 24.0 [b] (3)	5.0 (1)	50.5 ± 28.3 [b] (2)	32.5 ± 34.8 [b] (3)

Table 3. Influence of estadiole on semen quality in the grayling, *Thymallus thymallus*. Grayling were exposed to 1.0 ng l^{-1} ß-estradiol during the prespawning season for 50 d. Values are mean ± standard deviation, n = 6. Values superscripted by the same letter are not significantly different (P>0.05).

parameter	control	1 ng l^{-1} estradiol
semen volume, g	0.25 ± 0.10 [a]	0.12 ± 0.03 [b]
immotile, %	29.3 ± 25.9 [a]	23.8 ± 1.9 [a]
locally motile, %	3.8 ± 14.0 [a]	29.8 ± 13.2 [b]
motile, %	73.5 ± 9.6 [a]	46.2 ± 13.21 [b]
circular motile, %	37.9 ± 8.1 [a]	53.7 ± 12.8 [b]
non linear motile, %	41.7 ± 9.6 [a]	42.9 ± 12.1 [a]
linear motile,%	21.3 ± 1.6 [a]	3.3 ± 4.2 [b]
swimming velocity, µm s^{-1}	104.6 ± 12.7 [a]	78.3 ± 8.6 [a]

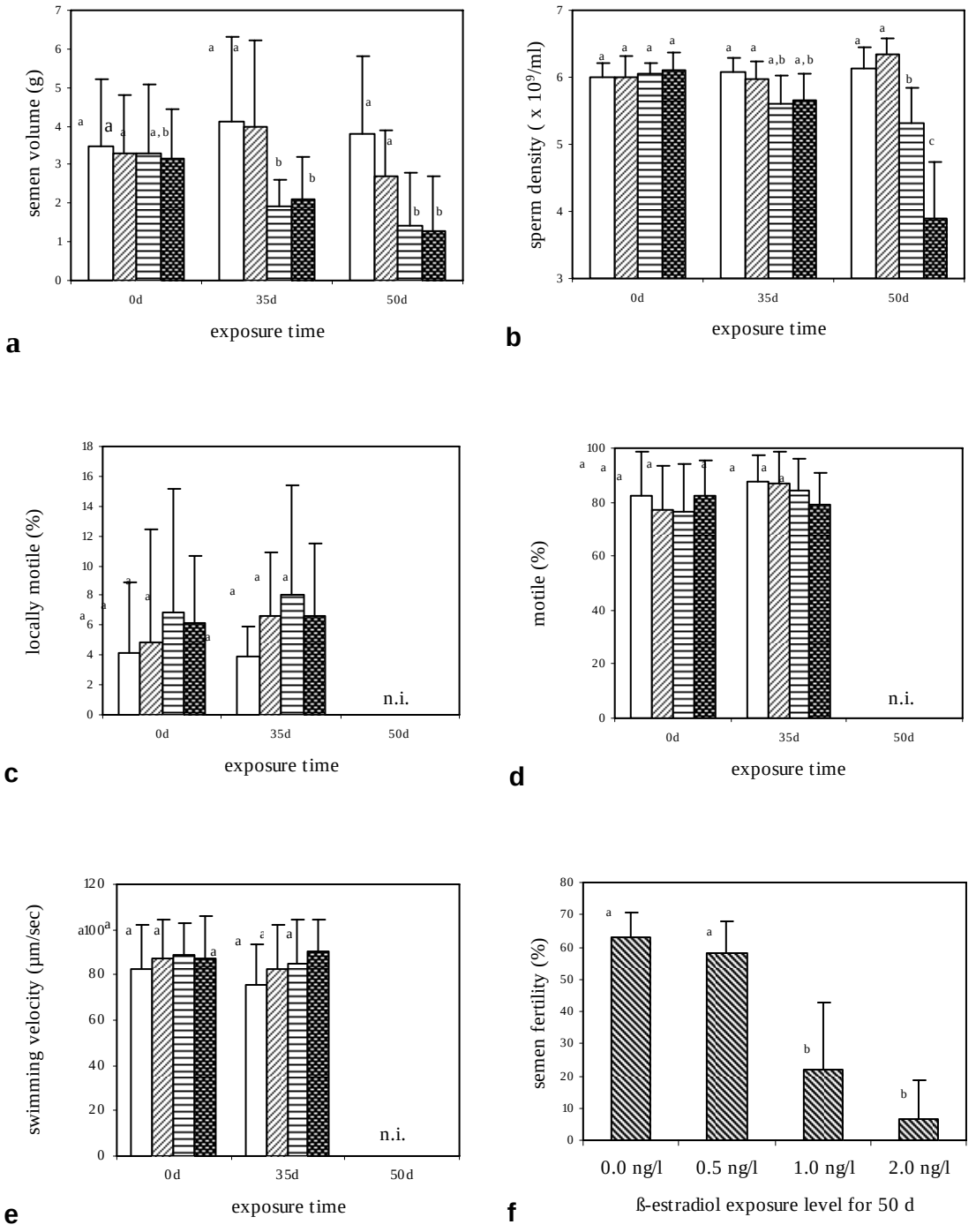
When female rainbow trout within 5 days after ovulation were exposed to ß-estradiol and eggs were stripped in portions in 7 day intervals no differences in egg fertility were observed between the control group and the groups exposed to 1.0 and 2.0 ng l^{-1} ß-estradiol (Table 2). In these groups egg fertility remained constant for a period of 28 d. Thereafter it decreased and became very variable (Table 2). In the group exposed to 0.5 ng l^{-1} ß-estradiol the egg quality was low and variable throughout the experiment (Table 2).

When male grayling were exposed to 1.0 ng l^{-1} ß-estradiol during the prespawning period the time point of spermiation (when males started to give semen) and the number of males giving semen was not different from the control (Fig. 2a). The semen volume obtained per male was significantly lower in the group exposed to 1.0 ng l^{-1} ß-estradiol than in the control group (Table 3). Also sperm motility parameters changed. In grayling exposed to 1.0 ng l^{-1} ß-estradiol the percentage of locally motile spermatozoa was increased and the percentage of motile spermatozoa was decreased (Table 3). The average path swimming velocity was decreased, too (Table 3). Also the sperm swimming pattern changed. In grayling exposed to 1.0 ng l^{-1} ß-estradiol the percentage of linear motile spermatozoa was decreased and the percentage of circular motile spermatozoa was increased in comparison to the control (Table 3). The percentage of non linear spermatozoa was similar as in the control (Table 3).
When female grayling were exposed to 1.0 ng l^{-1} ß-estradiol during the prespawning season all females had already ovulated 35 d after the onset of the experiment (Fig. 2b).

Figure 1. Influence of ß-estradiol on semen quality (semen volume, sperm density, sperm motility parameters, fertility [% eggs developing to eyed stage embryos]) in rainbow trout (*Oncorhynchus mykiss*). Rainbow trout were exposed to ß-estradiol during spawning. n.i. – non investigated.

0.0 ng l^{-1} , 0.5 ng l^{-1}, 1.0 ng l^{-1}, 2.0 ng l^{-1} ß-estradiol

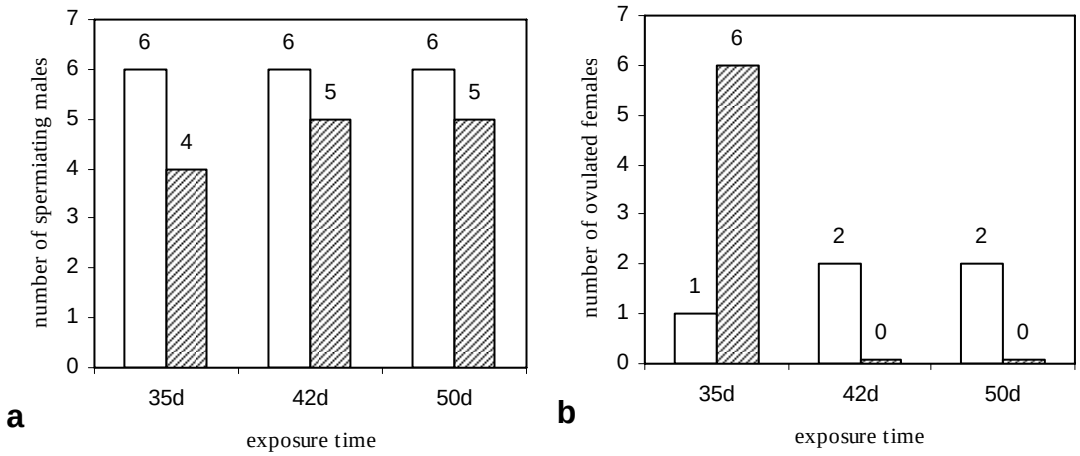
Vales are mean ± standard deviation (n = 10). Values superscripted by the same letter are not statistical significantly different. (P >0.05).



In the control group ovulation occurred 35-50 d after the onset of the experiment (Fig. 2b). The quantity of the produced eggs did not differ. It was 14.3 ± 8.6 g (1020 ± 615 eggs) in grayling exposed to 1.0 ng l^{-1} ß-estradiol and 12.6 ± 4.3 g in the control (905 ± 310 eggs). Egg

fertility was highly variable (1.0 ng l^{-1} estradiol: 40.5 ± 45.5 %, control: 52.0 ± 48.3 %) and therefore no statistical differences could be found between the two treatments.

Figure 2. Influence of 1.0 ng l^{-1} ß-estadiol on the time point of spawning in the grayling, *Thymallus thymallus*. Grayling were exposed to ß-estradiol during prespawning. After 35 d fish were examined in 1 week intervals on the onset of spermiation and the time point of ovulation. Values above bars indicate the number of mature fish. 0.0 ng l^{-1} , 1.0 ng l^{-1} ß-estradiol.



Discussion

The present study demonstrates that the tested ß-estradiol concentrations affected reproductive parameters in males and females of rainbow trout and grayling. For both species the lowest observed effect concentration (LOEC) was 1.0 ng l^{-1} estradiol. ß-estradiol concentrations $\geq$ 1 ng l^{-1} significantly reduced the semen volume obtained per male. This was similar for rainbow trout exposed to ß-estradiol during the spawning season and for grayling exposed to ß-estradiol during the prespawning season. For rainbow trout exposed for 50 d to $\geq$ 1 ng l^{-1} ß-estradiol also the sperm density was decreased while this parameter was not investigated in the grayling. These results probably indicate a reduction in semen production due to partial inhibition of spermiogenesis. Schultz et al. (2003) exposed rainbow trout for 62 d to 10 – 100 ng l^{-1} 17α-ethinyl ß-estradiol during the prespawning period and in contrast to the present study an increase in sperm density was observed. This discrepancy cannot be explained presently. It might depend on different types of hormones tested. In the present study exposure of rainbow trout to $\geq$ 1 ng l^{-1} ß-estradiol for 50 d affected also the semen fertility very negatively. Presently it is not known whether the loss in semen fertility was associated with a decrease in sperm motility as motility was analyzed only after 35 d exposure. As in the grayling sperm motility parameters were negatively affected by 50 d to ß-

estradiol during the prespawning season an effect via motility is likely. In this species the percentage of motile spermatozoa and the swimming velocity was decreased while the percentage of locally motile spermatozoa was increased indicating that semen contained only slowly swimming spermatozoa. Generally, low swimming velocities are correlated with low semen quality (Lahnsteiner et al., 1998) as they reduce the chance of spermatozoa to reach the micropyle. The observed decrease in semen fertility establishes earlier data of Schultz et al. (2003). In this study semen fertility of rainbow trout exposed to 17α-ethinyl ß-estradiol (10 – 100 ng l^{-1}) during the prespawning period for 62 d was reduced for about 50%. Spermiogenesis and maturation of male fish was not inhibited or delayed by ß-estradiol as in grayling exposed to 1.0 ng l^{-1} ß-estradiol during the prespawning season a similar number of males gave semen as in the control and the onset of spawning was similar. These results indicate that ß-estradiol concentrations $\geq$ 1.0 ng l^{-1} decrease the semen quality of Salmonidae whereby due to the combined negative effect on semen volume, sperm density, sperm motility and sperm fertility a severe reduction of reproductive capacity must be expected.

When female rainbow trout within 5 d after ovulation were exposed to ß-estradiol and eggs were stripped in 1 week intervals the egg viability changed in a similar way as in the control indicating that egg overripening processes were not influenced by estradiol. The variability in egg viability of the group exposed to 0.5 ng l^{-1} is considered to be due to low egg quality of fish involved in the experiment. Generally, in the Salmonidae the ovulated eggs are released into the coelomic cavity and there their viability decreases due to degenerative processes (Lahnsteiner, 2000). Therefore overripening processes limit the time span during which high quality eggs can be spawned. When female grayling were exposed to 1.0 ng l^{-1} ß-estradiol during the prespawning time ovulation occurred earlier and all females ovulated in a shorter time span than in the control group. This is demonstrated by the result that all females exposed to 1.0 ng l^{-1} ß-estradiol had ovulated 36 d after the start of the experiment while control fish ovulated in a period from 36 to 50 d after the onset of the experiment. Under ecological aspects seasonally earlier spawning times could theoretically lead to a temporal mismatch between larval food requirements and food availability and subsequently to high larvae mortality due to starvation (Milinski and Parker, 1991; Murdoch, 1994). Stimulation of egg production by low doses of estrogens was also reported in an earlier studies on the fathead minnow (*Pimephales promelas*) (Jobling et al., 2003).

From the described laboratory data (semen quality decrease, acceleration of oogenesis) it can be concluded that ß-estradiol concentrations $\geq$1.0 ng l^{-1} affect also the natural reproduction in the Salmonidae. Concentrations up to 88 ng l^{-1} in sewage effluent and up to

15.5 ng l^{-1} in surface water have been reported (Routledge et al., 1998; Lisette-Bachmann et al., 2002). LOECs for vitellogenin induction and development of intersex fish are 5 - 10 ng l^{-1} (Young et al., 2002). ß-estradiol concentrations of 1 ng l^{-1} are considered as predicted non effect concentration (PNEC) for Austrian water systems (Paumann and Vetter, 2003) which according to the present results should be corrected. The LOEC for 17α -ethinyl ß-estradiol defined in a full life cycle test in zebrafish (*Brachydanio rerio*) was > 1.67 ng l^{-1} (Segner et al., 2003). In other studies for 17α -ethinyl ß-estradiol vitellogenin induction and development of intersex has been reported at still lower concentrations of 0.1 - 0.6 ng l^{-1} (Young et al., 2002; Lisette-Bachmann et al., 2002).

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