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The Ultraviolet Filter 3-Benzylidene Camphor Adversely Affects Reproduction in Fathead Minnow (*Pimephales promelas*)

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The ultraviolet (UV) filter 3-benzylidene camphor (3BC) is used in personal care products and in a number of materials for UV protection. 3BC has been shown *in vitro* and *in vivo* in fish to be estrogenic, but possible effects on fertility and reproduction are unknown. In this study we evaluate whether 3BC affects reproduction of fish *Pimephales promelas*. After a preexposure period of 21 days, reproductively mature fathead minnows were exposed to increasing concentrations of 3BC for 21 days in a static-renewal procedure. Actual 3BC concentrations decreased to 23% of initial levels and median concentrations were 0.5, 3, 33, 74, and 285 μg/l. 3BC affected reproduction in a dose-dependent manner with weak effects on fecundity at 3 μg/l, a significant decrease at 74 μg/l, and a cessation of reproduction at 285 μg/l. 3BC was accumulated in fish with an average bioconcentration factor of 313 ± 151. Dose-dependent demasculinization in secondary sex characteristics of male fish and dose-dependent induction of plasma vitellogenin occurred, which was significant at 74 μg/l and higher. 3BC had a profound and dose-dependent effect on the histology of gonads of male and female fish at 3 μg/l and higher. At 74 and 285 μg/l, oocyte and spermatocyte development was inhibited in male and female gonads. Testes of exposed males had much fewer spermatogenic cysts, and ovaries of exposed females had much fewer mature but more atretic, follicles. This study shows significant effects of the UV filter 3BC on fertility, gonadal development, and reproduction of fish after short-term exposure that may have negative consequences on the population level.

Key Words: **UV filters; 3-benzylidene camphor; *Pimephales promelas*; reproduction; vitellogenin induction; histological effects.**

Ultraviolet (UV) filters are increasingly used in cosmetics, skin- and hair-care products like sunscreens, shampoos, creams, and fragrances. They are also applied in textiles, optical materials and optics components, household products, fabrics, transdermal drug delivery systems, and for UV stabilization of a variety of materials. By absorbing, scattering, and reflecting UV light they protect the human skin and materials from the negative effects of UV radiation. The amount of UV filters added are increasing, because higher sunlight protection factors are used, and thus generally higher percentages of different UV filters are required in the products.

Consequently, it is not surprising that residues of UV filters have been detected in the environment. They enter the aquatic system either directly into surface water via recreational activities (bathing) or indirectly via wastewater. In lakes and raw wastewater they were detected at concentrations up to 125 ng/l (Poiger *et al.*, 2004) and 19 μg/l (Balmer *et al.*, 2005), respectively. Because they are photostable, often lipophilic (log Kow 3–7), UV filters are relatively stable in the aquatic environment (Balmer *et al.*, 2005; Poiger *et al.*, 2004) and thus critical for bioaccumulation. Indeed, residues of several UV filters have been detected at concentrations of 21–3100 ng/g lipid in fish (Balmer *et al.*, 2005; Buser *et al.*, 2006; Nagtegaal *et al.*, 1997) and even in human milk (Hany and Nagel, 1995).

Besides being present in the environment, UV filters recently gained increasing environmental relevance, because of their reported estrogenic activity *in vitro* (Mueller *et al.*, 2003; Routledge and Sumpter, 1997; Schlumpf *et al.*, 2001; Schreurs *et al.*, 2002; Schultz *et al.*, 2000; Kunz and Fent, in press; Kunz *et al.*, 2006) and *in vivo* in rats (Schlumpf *et al.*, 2004). In particular, the fact that estrogenic activity has been observed in fish *in vitro* (Kunz and Fent, in press) and *in vivo* (Kunz *et al.*, 2006) makes UV filters of potential environmental concern for aquatic organisms. 3-Benzylidene camphor (3BC), benzophenone-1, and benzophenone-2 led to vitellogenin (VTG) induction in juvenile fathead minnows (Kunz *et al.*, 2006), and this was also found for 3BC in rainbow trout (Holbech *et al.*, 2002), 4-methyl benzylidene camphor (4MBC), and octyl-methoxycinnamate (OMC) in male medaka (Inui *et al.*, 2003). Among the UV filters, 3BC showed highest estrogenic potency *in vivo* in fish (Kunz *et al.*, 2006) but also in rats (Schlumpf *et al.*, 2004).

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Currently, it is not known whether UV filters affect the fecundity of fish. This question is of ecotoxicological and ecological importance, because UV filters are present in the aquatic environment and may possess enough potency to adversely affect reproduction and development in fish. Estrogenic effects in several fish species were shown for many chemicals, and for sewage effluents (Rodgers-Gray *et al.*, 2001; Sumpter and Johnson, 2005; Tyler *et al.*, 1998). In the United Kingdom widespread sexual disruption was observed in wild roach (Jobling *et al.*, 2002), likely due to chemicals in sewage. Several different groups of chemicals were found to be estrogenic in short-term exposures by inducing vitellogenin (VTG) in juvenile and male fish. Subsequently, they were demonstrated to negatively affect reproduction, sometimes at environmentally relevant concentrations such as in case of ethinylestradiol (Nash *et al.*, 2004) and 4-*tert*-octylphenol (Gronen *et al.*, 1999), to name a few.

In this work, we evaluate whether a common UV filter, 3BC, adversely affects reproduction in fathead minnows. This UV filter has practical importance as it is added to skin- and hair-care products (allowed up to 2% per weight in Switzerland and European Union), but also to household products, fabrics, and other products. We investigated important parameters for endocrine disruption, such as fecundity, VTG induction, secondary sex characteristics, and gonad histology. The findings of this study show for the first time that a commonly used UV filter adversely affects reproduction in fish in a dose-dependent manner after short-term exposure.

EXPERIMENTAL SECTION

Chemicals. 3BC (MG 240.34, CAS 15087-24-8) was purchased from Induchem (Volketswil, Switzerland) and 17β-estradiol (E2) was purchased from Fluka AG (Buchs, Switzerland). All compounds were > 99% pure. Stock solutions were made in N,N-dimethylformamide (DMF) and stored in the dark at 4°C. Analytical grade DMF was purchased from Arcos Organics (Geel, Belgium).

Fish. Fathead minnows are found as wildfish throughout much of North America and have been chosen because of short reproductive cycles, frequent use in endocrine disrupter research, and established techniques including VTG antibodies (Parks *et al.*, 1999). We used reproductively, mature fathead minnows (*Pimephales promelas*) between 8 and 9 months of age. Fish have not been held in a culture situation conductive to routine spawning before the onset of the experiment.

Newly mature fathead minnows were received from the cultivator (Aquatic Research Organisms, Hampton, NH) and adapted for a minimum of 14 days in our laboratory in a tank prior to the experiment. Fish were fed with Tetramin pellets (Tetra GmbH, Melle, Germany) twice a day with a quantity of 1% of body weight prior to the onset of experiments. During the experiments, fish were fed with brine shrimp (*Artemia salina*, Argent Chemical Labs, Redmond, WA) at a feeding rate of 1% of body weight twice a day.

Exposure. The experimental procedure was similar to that described recently (Ankley *et al.*, 2001; Harries *et al.*, 2000; Panter *et al.*, 2002). During the preexposure and exposure period, fish were held in a 10 l stainless steel aquaria in well-aerated reconstituted tab water medium (total hardness 160 mg/l as $CaCO_3$, total alkalinity 30 mg/l as $CaCO_3$, conductivity 500 µs/cm) and a 16-h/8-h light/dark cycle at 25 ± 1°C. A 48 h static-renewal procedure was used, renewing the total of aquaria water (10 l). After 24 h, food remnants and feces were removed by siphoning a third of the water volume and replacing it by new water containing the appropriate concentrations of 3BC. After 48 h, total aquaria water was replaced by new water containing the appropriate concentrations of 3BC. This administration regime was chosen because of the high lipophilicity of 3BC (log Kow = 5.37), which made it impossible to deliver it by a flow-through system. In order to ensure normal reproductive performance of the fish, unnecessary disturbances were minimized by a static-renewal regime of 48 h instead of 24 h. Thus, water quality was monitored continuously in order to enable conformance with performance-based criteria. Aliquots of 250 ml of water were taken for chemical analysis.

At the beginning of the experiment, four females and two males were randomly assigned to the replicate stainless steel tanks (10 l). Prior to the exposure period, a preexposure phase was initiated in order to establish the reproductive capacity of unexposed fish and to provide tank-specific baseline data for potential statistical comparison after initiation of chemical exposure to 3BC. The concentration of 3BC was zero during the preexposure period. After the preexposure period, treatment groups for the exposure period were randomly assigned to the tanks. During the preexposure and exposure period survival, appearance and behavior of fish, reproductive behavior, secondary sex characteristics, and fecundity (cumulative number of spawned eggs) were determined. Once successful spawning has been established, generally after 14–21 days, 3BC exposure was started. Fish were exposed for 21 days to nominal concentrations of 1, 10, 100, 250, and 500 µg/l 3BC. A control and a solvent control (SC, 1 ml DMF in 10 l of water) were also included. Stock solutions of 3BC were prepared freshly in DMF prior to the start of the experiment and added to the experimental water by mixing. The 3BC concentrations were selected according to concentrations which were found to induce VTG in juvenile fathead minnows (Kunz *et al.*, 2006). For the selected concentrations of 3BC, no toxic side effects (i.e., lethargy, uncoordinated swimming, loss of equilibrium, hyperventilation) were observed.

Physico-chemical measurements and biological observations. Physico-chemical parameters were determined daily. pH and oxygen saturation ranged between 7.2–7.9 and 6.5–8.3 mg/l, respectively, throughout the exposure period. Mortalities and abnormal behavior were recorded daily and dead fish were removed from the tanks. At the end of the experiment (day 21 of the exposure period), all fish were anesthetized with buffered tricaine methanesulfonate (MS-222, 100 mg/l with 200 mg $NaHCO_3$/l). Subsequently, the length of individual fish was measured, they were weighted, and measures of a number of endpoints were made. In order to determine plasma concentrations of VTG, blood was collected from the caudal vein using a heparinized capillary tube (KABE Labortechnik GmbH, Nümbrecht-Elsenroth, Germany) and transferred into a labeled Eppendorf tube. A protease inhibitor (Aprotinin, Fluka AG) was added and plasma was then separated from the blood by centrifugation (10 min at 3000 rpm at 4°C) and stored until analysis at − 80°C. After sampling of the blood, gonads were removed and weighted for determination of the gonado somatic index (GSI = 100 × gonad weight per body weight). Gonads were then placed in Bouin's solution (Fluka AG) for subsequent histological analysis. Photographs of the head (number of nuptial tubercles) of male fathead minnows were taken for further analysis of the secondary sexual characteristics. The remainder of the carcass of the fish was frozen at − 80°C for chemical residue analysis.

VTG analysis. Plasma samples were thawed on ice, and ice-cold assay buffer (Biosense, Bergen, Norway) was added to the individual samples in order to achieve a 1:2 ratio of plasma:buffer. The samples were vortexed and immediately used for VTG analysis. For quantification of VTG a commercially available, homologous enzyme-linked immunosorbent assay for fathead minnow VTG was used as described by Biosense Laboratories AG. Purified fathead minnow VTG from blood plasma (Biosense AG) was used as a standard for quantitation according to the provider's description.

Histology. Routine histological procedures were used to analyze testes and ovaries of the fish. The gonads from the Bouin's solution were embedded in

paraffin. Sections were taken along the long axis of the gonad at 6-µm intervals, in a serial step fashion. Two serial sections were collected from three steps equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of six tissue sections per sample. The sections were stained with haemalaun (Mayer's Haemalaun; Merck, Darmstadt, Germany) and eosin (Fluka AG). Evaluation of the histological effect of 3BC on gonads was based on staging of ovarian and testicular development (similar to Leino *et al.*, 2005; Pawlowski *et al.*, 2004). In order to minimize bias, histological sections were first evaluated in a blinded fashion by two histologists and two members of our group. Subsequently, histological sections were reevaluated for each treatment group independently by these two group members and findings of the two evaluations were compared and controlled for bias.

The ovary was evaluated based upon relative frequencies of oogonia, early vitellogenic, late vitellogenic, and atretic follicles. Degenerative follicles with shrunken, irregular borders were defined as atretic (Miles-Richardson *et al.*, 1999b). Frequencies were evaluated by counting the different stages in three randomly selected fields of vision per female (×90 magnification). Testis staging was based on the relative frequency of spermatogonia, spermatocytes, and spermatides present. Thereby, for each male, pictures of four randomly selected fields of vision (19,276 μm^2) were taken and the areas occupied by the different stages were measured.

Analytical chemistry. During the experiment, 250 ml of exposed water was taken three times from all treatment groups at the beginning (0 h), after siphoning and renewal of the siphoned water (24 h), and prior to water renewal (48 h). The water samples were stored in brown glass flasks at 4°C until analysis by high performance liquid chromatography–diode array detector (HPLC-DAD). For the extraction and analysis of water samples we used the following method, which was developed and validated in our laboratory. Briefly, 25 or 250 ml of water samples, depending on sample concentration, were extracted and concentrated by solid phase extraction, using Sep-Pak Vac 3cc C18 cartridges (Waters AG, Rupperswil, Switzerland). Cartridges were conditioned with dichloromethane, methanol, and H_2O bidistilled prior to the extraction of the water samples. The cartridges were then air dried and eluted with dichloromethane. Dichloromethane was allowed to evaporate to dryness and the dry eluents were resuspended in 100 µl of ethanol and analyzed by HPLC-DAD (Agilent 1100 Series, Agilent Technologies [Schweiz], Basel, Switzerland). Thereby, a concentration of 250–2500 times of the water sample was achieved.

The remainders of the fish carcasses were analyzed for 3BC residues after the 21-day exposure period by means of a liquid-liquid extraction developed and validated in our laboratory. Briefly, a single fish was homogenized together with bidistilled water and ethylacetate in a ratio of 1:2:2 (fish:water:solvent). The homogenate was vortexed until it was mixed thoroughly and then centrifuged (15 min, 4°C, 4500 rpm). The supernatant (3BC solved in ethylacetate) was transferred to another vial and then the solvent was evaporated to dryness. The dry residue was resuspended in 1 ml of ethanol followed by another centrifugation step (30 min, 16,000 rpm). The supernatant was then transferred to a brown vial and analyzed by HPLC-DAD. As a quality control for the used analytical techniques, 4MBC was added to all samples as internal standard with a concentration corresponding to the nominal concentration of the sample. Furthermore, for analysis by HPLC-DAD an external standard with 3BC and 4MBC was included in all sample sets.

Data processing and statistical analysis. The data distribution was tested for normality by using the Kolmogorov-Smirnov test. Differences between treatments were assessed using either analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test to compare the treatment means with respective controls, or a nonparametric test, when a normality distribution of the data was not given. In this case, differences between treatments were assessed by a Kruskal-Wallis test followed by a Dunn's multiple comparison test to compare the treatment means with respective controls. Statistical comparisons were made with the SC as the overall control, given that the results for comparisons with the water control lead to identical findings. The results are given as mean ± standard error of mean (SEM). Differences were considered significant at $p \leq 0.05$. All computations were performed with PRISM 4.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Actual 3BC Concentrations in Aquaria Waters and Concentration in Exposed Fish

Water concentrations of 3BC were determined by HPLC-DAD measured at three time points (0, 24, and 48 h) at all exposure concentrations in order to determine actual effect concentrations. Table 1 shows that actual concentrations at the beginning of the exposure (0 h) were close to nominal values (mean = 112.0 ± 6.9%, $n = 5$). During exposure, 3BC concentrations decreased to a similar extent for all concentrations. After 24 h at partial water renewal, 3BC concentrations decreased to 30–57%, and after 48 h before water renewal, to 20–32% of nominal concentrations. The decrease was due to several factors, including absorption on aquaria walls, spawning substrates, food, fish and eggs, as well as bioaccumulation of 3BC by fish and spawned eggs. To determine the bioaccumulation potential of 3BC, whole-body

TABLE 1
Nominal and Measured Concentrations of 3BC in Exposure Waters and Fish after 21 Days of Exposure

	Exposure waters							Fish	
	Measured concentration (µg/l)								
Nominal concentration (µg/l)	0 h	(%)	24 h	(%)	48 h	(%)	Median	Measured whole-body concentration (µg/g)	BCF
1	1.1 ± 0.1	101	0.5 ± 0.1	45	0.3 ± 0.1	27	0.5	0.16 ± 0.18	399
10	9.9 ± 0.3	99	3.1 ± 1.1	31	2.0 ± 0.4	20	3	0.36 ± 0.27	102
100	118 ± 5	118	44 ± 3	37	22 ± 0.2	18	33	7.22 ± 3.17	230
250	293 ± 34	117	74 ± 3	25	56 ± 1	19	74	36.49 ± 21.91	493
500	547 ± 19	109	285 ± 14	52	160 ± 7	29	285	100.73 ± 47.87	344

Note. BCF, bioconcentration factor calculated by dividing whole-body concentration of each fish by the median water concentration of 3BC. Number of analyzed samples: water ($n = 3$ at each time point and concentration), fish ($n = 8$ fish at each concentration).

residues of 3BC in fish were measured at the end of the experiment after 21 days. Table 1 shows that whole-body residues of 3BC in adult fathead minnows were between 0.16 and 100.73 μg/g depending on the exposure concentration. Based on mean actual water concentrations, this gives an average bioconcentration factor of 313 ± 151.

Fish Survival, Weight, and Length

We observed identical survival rates (94.4 ± 9.6%, $n = 3$ replicates) for the control fish and all exposed fish, except for the fish exposed to 33 μg/l 3BC (100%, $n = 3$ replicates) and 285 μg/l 3BC (88.9 ± 9.6%, $n = 3$ replicates). However, survival did not differ significantly from the control tanks ($p = 0.9306$). No differences were observed in length, weight, and condition factor (CF) of male and female fish at the end of the experiment (Table 2).

Effects on Reproduction

The cumulative fecundity, i.e., the cumulative number of eggs per treatment, differed slightly for all treatments and replicates before exposure (Fig. 1). At the start of 3BC exposure, none of the fish of the water and SC replicates stopped spawning (Fig. 2A). At the lowest exposure concentration of 0.5 μg/l 3BC, fish in one of the three aquaria stopped spawning after 8 days of exposure, whereas fish in the other two replicates continued with slightly reduced spawning activity (Fig. 2B). At 3 μg/l 3BC, fish of one aquarium stopped spawning after 8 days, whereas fish of the other aquaria continued with reduced spawning activity compared to pre-exposure and controls (Fig. 2C). At 33 μg/l 3BC, the fish in two of the three aquaria stopped spawning after 4 and 7 days, respectively, whereas the third aquarium showed reduced spawning with less spawns and eggs per spawn (Fig. 2D). Fish in one aquarium exposed to 74 μg/l 3BC stopped spawning activity immediately, whereas the fish of the second and third aquarium spawned only once after the onset of exposure (Fig. 2E). All fish exposed to 285 μg/l 3BC ceased spawning activity immediately (Fig. 2F). Overall, 3BC led to a dose-dependent

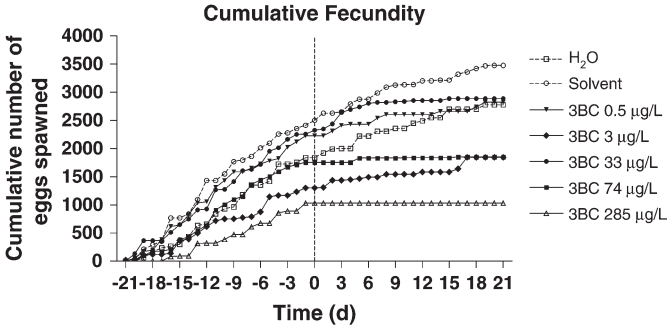

FIG. 1. Cumulative number of eggs spawned per treatment group during preexposure (days −21 to 0) and exposure period (days 0 to 21). Treatments are given in measured median concentrations.

inhibitory effect on fertility and reproduction in fathead minnows. This was already observable at 0.5 μg/l 3BC, but was not significant. Figure 3A-C shows that the number of spawns, number of eggs per spawn, and the number of eggs per female per day were significantly decreased at 74 and 285 μg/l (ANOVA, $p < 0.05$).

In male fathead minnows, exposure to 3BC led to a dose-dependent VTG induction, which was significant at 74 and 285 μg/l 3BC (Fig. 4). In these fish, mean plasma VTG content was between 5272 and 18,020 μg/ml compared to the water and SC having a mean residual level of 15 μg/ml VTG. In female fish, no significant VTG induction was observed. Mean levels ranged between 1300 and 5400 μg/ml for all treatment groups (Fig. 4).

Moreover, exposure to 3BC caused alterations in male secondary sexual characteristics (Fig. 5). At 33 μg/l 3BC and higher, tubercles were less in numbers and seemed less pronounced. The number of tubercles significantly decreased at 74 and 285 μg/l, and the remaining tubercles appeared to be less pronounced. The decrease in the number of tubercles was dose dependent (Fig. 5).

Neither in males nor in females was the GSI altered (Table 2). However, gonadal histology was profoundly affected by 3BC. This UV filter induced a dose-dependent inhibition of spermatogenesis in the testis of male fish. At concentrations

TABLE 2
Body Weight and Length, GSI, and CF of Exposed Fish after 21 Days of Exposure ($n = 18$, 12 Females and 6 Males per Treatment)

				GSI		
	Exposure (μg/l)	Body weight (mg)	Body length (mm)	Males	Females	CF
Controls	Water	211.3 ± 91.1	29.4 ± 2.8	1.5 ± 0.7	16.9 ± 4.4	1.26 ± 0.04
	Solvent	238.0 ± 88.8	30.8 ± 3.5	1.3 ± 0.4	13.6 ± 1.6	1.31 ± 0.22
3BC	0.5	335.9 ± 128.9	32.8 ± 3.1	1.5 ± 0.4	19.4 ± 3.5	1.30 ± 0.07
	3	258.9 ± 95.9	30.9 ± 3.4	1.8 ± 0.4	18.2 ± 2.9	1.34 ± 0.01
	33	115.1 ± 37.4	24.7 ± 2.4	1.5 ± 0.4	14.2 ± 1.9	1.27 ± 0.16
	74	95.8 ± 25.8	23.7 ± 1.8	1.6 ± 0.8	14.9 ± 3.1	1.14 ± 0.08
	285	171.4 ± 35.0	25.9 ± 1.8	1.4 ± 0.4	12.8 ± 2.7	1.23 ± 0.01

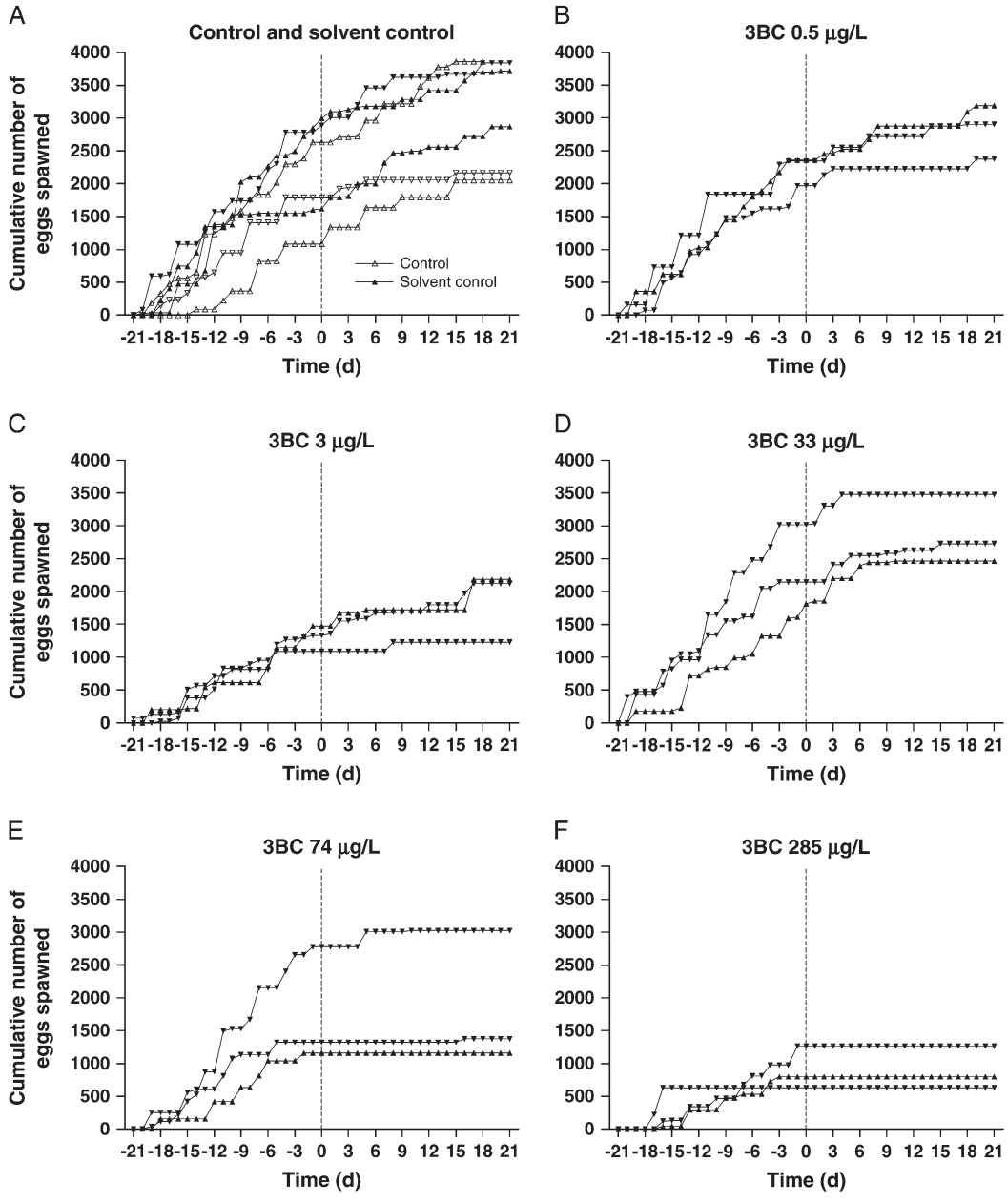

FIG. 2. Cumulative number of eggs spawned in each of the three replicates during preexposure (days −21 to 0) and exposure period (days 0 to 21), shown for controls and each treatment group (A-F). Measured median 3BC concentrations are given.

of 3 µg/l 3BC and higher, testes of exposed males significantly differed from control testes; they possessed reduced tubules with fewer spermatocytic stages and increasing numbers of spermatides. This effect increased with increasing concentrations, and at 74 and 285 µg/l spermatocytic stages decreased by 30–40% compared to the control. Spermatogenesis appeared to be inhibited and testes were characterized by enlarged seminiferous tubules filled with sperms. Moreover, there was a significant lack of primary and secondary spermatocytes accompanied by a relative lack of germinal epithelium. Spermatogonia apparently did not undergo a further differentiation to spermatogenic cysts at 3 µg/l 3BC and higher concentrations ($p < 0.001$). Spermatocytes were less present, whereas the area occupied by spermatides increased in a dose-dependent manner (Figs. 6 and 8A).

In ovaries of treated females exposed to the lowest concentration of 0.5 µg/l 3BC, no discernible differences between treated and control females could be seen. Ovaries of exposed females to 3 µg/l 3BC and higher concentrations were characterized by a significant increase ($p < 0.01$ for 3 and 33 µg/l, $p < 0.05$ for 285 µg/l) in the number of atretic follicles up to 24%, accompanied by a slight decrease of early vitellogenic

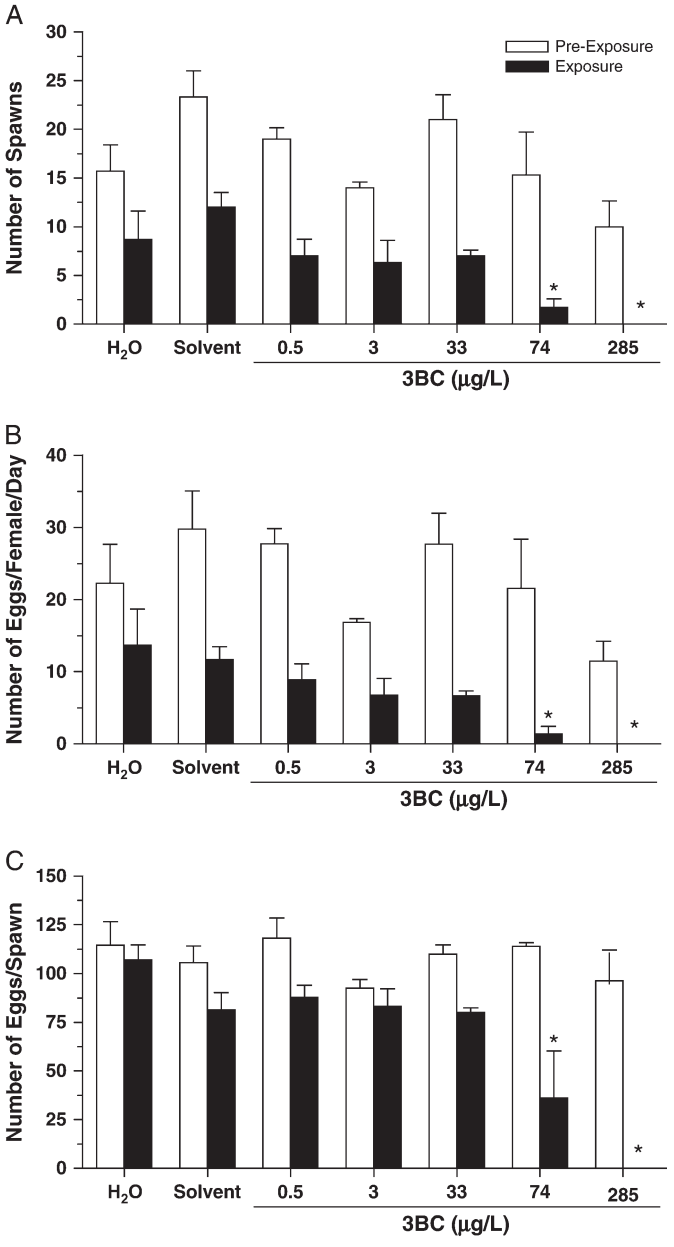

FIG. 3. Fecundity shown as percentage of eggs and spawns during exposure (black bars) relative to preexposure (100%) (white bars). Number of spawns (A). Number of eggs per female per day (B). Number of eggs per spawn (C). Data shown are means and SEM (three replicates per treatment). Asterisks denote significant difference from control at $p \leq 0.05$. Measured median 3BC concentrations are given.

stages and a significant decrease of late vitellogenic stages (Figs. 7 and 8B). Late vitellogenic follicles decreased by 8% at 3 and 33 µg/l 3BC, but then remained stable in number at 74 and 285 µg/l. At 33 µg/l and higher, the relative number of oogonia increased (Fig. 8B). These histological alterations in gonads of male and female fish demonstrate a dose-dependent profound estrogenic effect of 3BC.

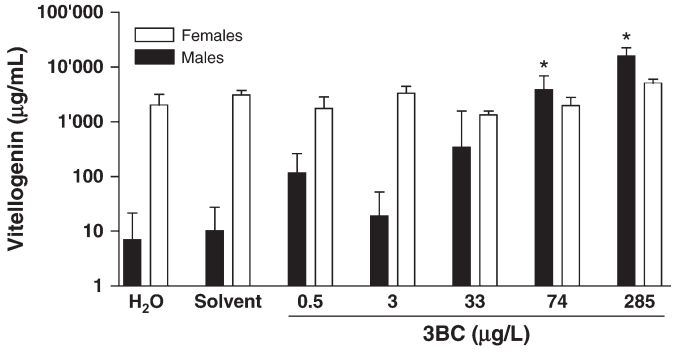

FIG. 4. VTG concentration in female and male fathead minnows exposed to 3BC. Values are means ± SEM (females, $n = 12$; males, $n = 6$). Asterisks denote significant difference from control at $p \leq 0.05$. Measured median 3BC concentrations are given.

DISCUSSION

The UV filter 3BC is used in many products such as sunscreens, skin and hair-care products, household products, textiles, fabrics, optical materials and components, and transdermal drug delivery systems. Although 3BC enters the aquatic system either directly into surface or indirectly via wastewater, environmental concentrations are not yet known. However, its lipophilic nature makes 3BC prone for bioaccumulation in aquatic organisms. 3BC was shown to have estrogenic activity *in vitro* (Holbech *et al.*, 2002; Kunz *et al.*, 2006) and *in vivo* leading to significant VTG induction in fish (Holbech *et al.*, 2002; Kunz *et al.*, 2006). So far, it was unknown whether 3BC affects reproduction in fish in addition.

In the present study, we show for the first time that a commonly used UV filter adversely affects reproduction in fish in a dose-dependent manner. Immediately after the onset of exposure to 285 µg/l 3BC spawning ceased completely, and it was significantly reduced at 74 µg/l. Fish that were successful

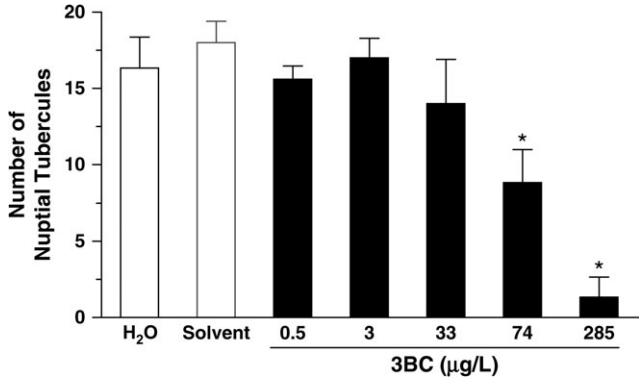

FIG. 5. Number of nuptial tubercles of male fathead minnows. Control males (white bars) and males exposed to 3BC (black bars). Values are means ± SEM ($n = 6$). Asterisks denote significant difference from control at $p \leq 0.05$. Measured median 3BC concentrations are given.

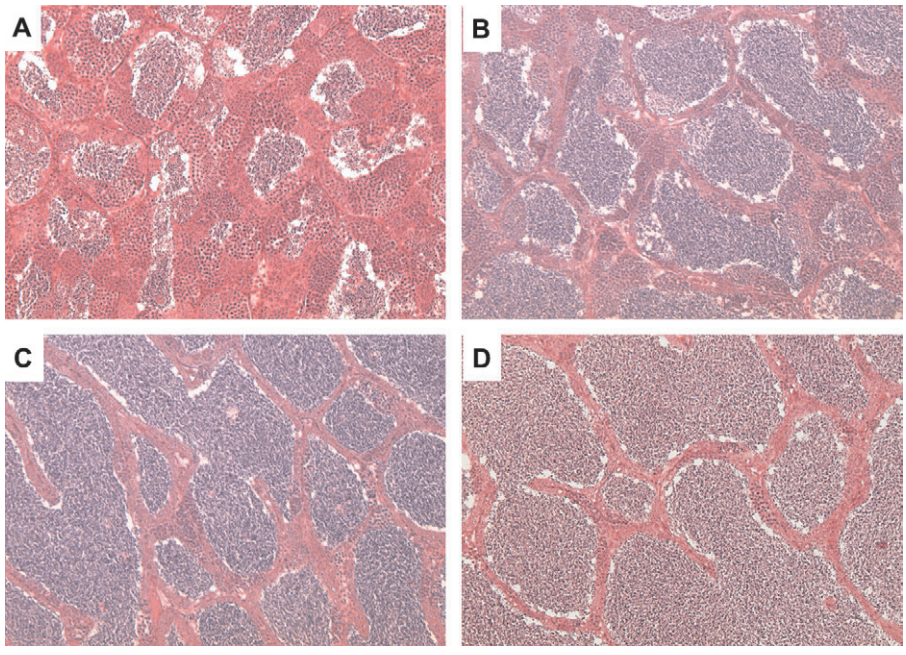

FIG. 6. (A) Section of testis from a control male, typical of active spawning. (B) Section of testis from a males exposed to 3, (C) 33, and (D) 285 µg/l 3BC. Note the enlarged seminiferous tubules filled with sperms and relative lack of spermatocytes and germinal epithelium in the tubules.

spawners during the preexposure period had no or significantly less spawns with significantly fewer eggs per spawn when exposed to 3BC. The immediate and strong impact of 3BC on spawning activity at the highest exposure concentration was surprising. This also holds true for the reduced spawning activity at 3 and 33 µg/l, although it was not significant at these concentrations. Even at the lowest exposure concentration of 0.5 µg/l, fish showed slightly reduced spawning activity and one replicate even stopped spawning after 8 days of exposure, which possibly indicates very weak adverse effects of 3BC on reproduction, but this decrease in spawning activity was not significant. The random assignment of treatment groups to the tanks led to lower preexposure spawning in two groups (3 and 285 µg/l 3BC) compared to the other treatment groups. It is unlikely that this has influenced the strong inhibitory effect of 285 µg/l 3BC, as two of the three replicates spawned with similar frequency but females laid fewer eggs per spawn. Furthermore, spawning activity observed during preexposure completely ceased immediately after onset of exposure in one replicate of the 74 µg/l 3BC exposure group.

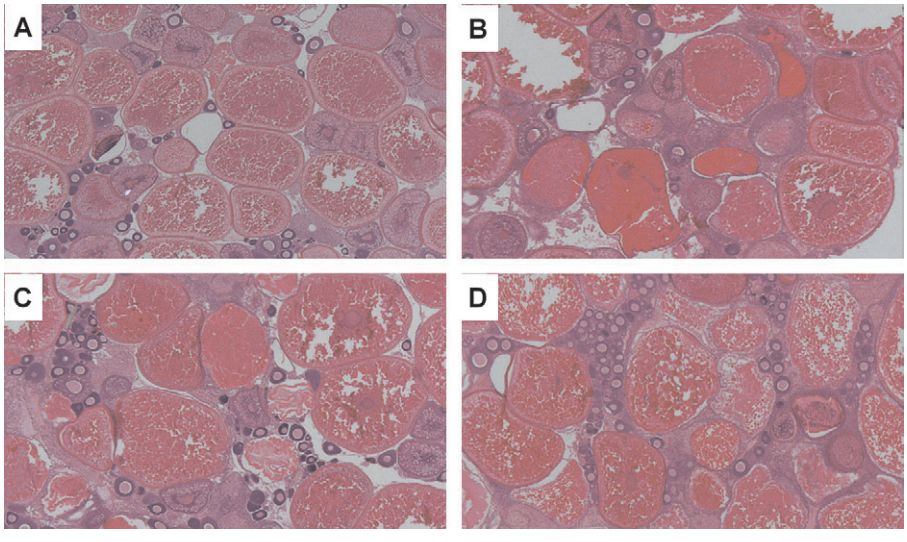

FIG. 7. (A) Section of control ovary, showing mature follicles typical of an actively spawning female. Section of ovaries from females exposed to 3 (B), 33 (C), and 285 µg/l (D) 3BC, showing increased number of atretic follicles and degenerated ovary with only early stages of oogonia.

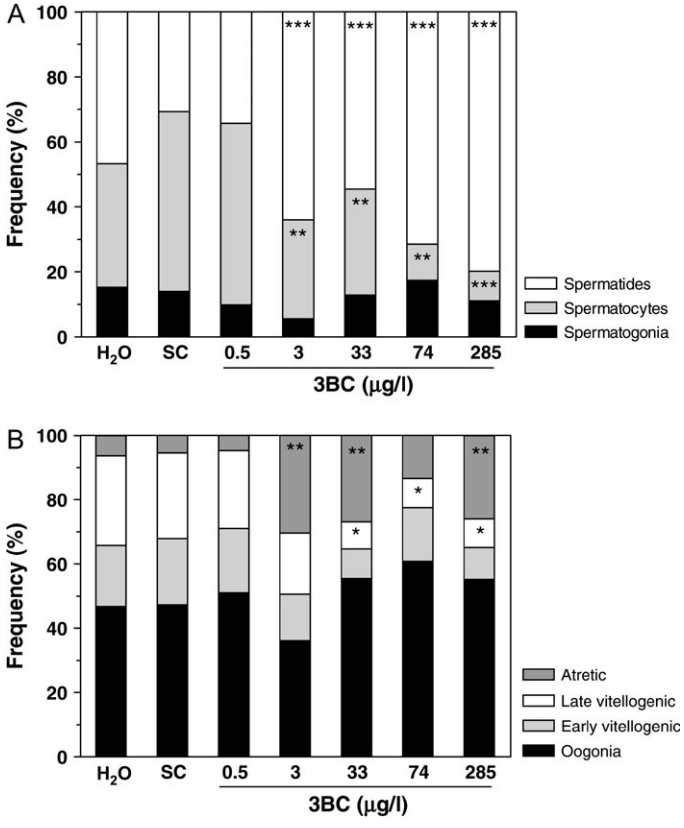

FIG. 8. Relative percentage of the different stages of spermatids in testis of male (A) and oocytes in ovaries of female (B) fathead minnows after 21 days of exposure to 3BC. For males, $n = 4–5$, for females, $n = 4–6$. Asterisks denote significant difference from control, $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

These dose-dependent effects were also observed in gonadal histology. Already at the second lowest exposure concentration of 3 µg/l and at higher concentrations testes of exposed males were clearly distinguishable from control testes, and possessed reduced tubules with fewer spermatocytic stages. This effect increased with increasing concentrations, and at 74 and 285 µg/l, spermatocytic stages decreased by 30–40% compared to the control. Spermatogenesis appeared to be inhibited and testes were characterized by enlarged semiferous tubules filled with spermatids and enlarged spermatocysts with a much greater number of mature sperm contained within and the relative lack of germinal epithelium and primary and secondary spermatocytes. Spermatogonia apparently did not undergo a further differentiation to spermatogenic cysts and spermatids. Possibly, this indicates a standstill of milt production revealed by the accumulation of large spermatocysts and sperms in the lumen of enlarged semiferous tubules and the lack of intermediate stages (spermatogenic cysts). However, the possibility has to be considered that the standstill of milt production is not pathological, but rather the response of males to the stop of spawning activity in females. It is interesting to note that the histological response of testes to 3BC is much like that observed in previous studies with E2 and EE2. These estrogens lead to a full inhibition of testicular development, depending on the dose from a degeneration of spermatozoa to total atrophy (Gimeno *et al.*, 1998; Miles-Richardson *et al.*, 1999a; Pawlowski *et al.*, 2004). The 21 days of exposure to 3BC also inhibited testicular development, but showed less degeneration. Similar inhibition of testicular development was recently reported for fish exposed to the weak estrogen receptor agonist 4-nonylphenol, which led to a significant reduction of fecundity (Harries *et al.*, 2000; Miles-Richardson *et al.*, 1999b) and a significant necrosis of sperm cells and spermatozoa (Miles-Richardson *et al.*, 1999b). Although we did not investigate the state of Sertoli cells, the presence of phagocytic cells in the semiferous tubules and of macrophages in their lumina indicates an extension of the phagocytic role of these cells in males (Miles-Richardson *et al.*, 1999a) exposed to high concentrations of 3BC.

In ovaries, the histological effects of 3BC were just as pronounced. Whereas at 0.5 µg/l 3BC no discernible differences between treated and control females could be seen, 3 µg/l 3BC and higher concentrations led to a significant increase in the number of atretic follicles up to 24%, accompanied by a decrease in early and late vitellogenic stages. Interestingly, from 3 to 33 µg/l 3BC late vitellogenic follicles decreased significantly by 8% but then remained stable in number at 74 and 285 µg/l.

Strong estrogen receptor agonists such as E2 and EE2 have similar effects on ovaries (Kramer *et al.*, 1997; Miles-Richardson *et al.*, 1999a). Similar to these estrogens, 3BC seems to interfere with egg production and this leads to fewer mature, and more atretic follicles in treated ovaries of female fathead minnows. Possibly, sustained abnormally high VTG levels induced by estrogen receptor agonists interfere with final maturation and release of oocytes from the ovary, hypothetically by inhibiting gonadotropin II release by the pituitary (Kramer *et al.*, 1997; Miles-Richardson *et al.*, 1999a). High concentrations of weak estrogen receptor agonists such as methoxychlor also resulted in increased follicular atresia in some females (Ankley *et al.*, 2001), whereas nonylphenol did not induce any histological changes in ovaries (Miles-Richardson *et al.*, 1999b).

The estrogenicity of 3BC was manifested not only in a dose-dependent reduction of spawning activity caused by gonadal degeneration, but also by VTG induction and feminization of male secondary sex characteristics. Significant VTG induction in male fathead minnows was observed at 74 µg/l 3BC and higher. This was observed after 14 days of exposure to 435 µg/l 3BC (Kunz *et al.*, 2006), but also for other estrogenic chemicals acting via the estrogen receptor, such as the pesticide methoxychlor (Ankley *et al.*, 2001). VTG induction in 3BC exposed fish goes in line with a significant decrease in fecundity. The cumulative number of eggs spawned and gonadal histology are considerably more sensitive to the effect of 3BC, contrary to methoxychlor, where VTG induction turned out as the most sensitive endpoint. No significant

VTG induction occurred in females, similar to methoxychlor (Ankley *et al.*, 2001). However, when female fathead minnows are exposed to strong synthetic estrogens, a sustained increase in plasma VTG occurred (Kramer *et al.*, 1997; Miles-Richardson *et al.*, 1999a). The lack of significant VTG increase above the already high VTG level in reproductive females is an indication of the weaker estrogenicity of 3BC compared to EE2.

Besides the reduced fecundity and the clear VTG induction, in male fish the significant decrease of the number of tubercles was another clear indication for the estrogenic activity of 3BC. Males at the highest exposure concentration were visually not discernible from females and all but one have lost all tubercles. Nuptial tubercles in the male fathead minnow can decrease when exposed to both the weak estrogen receptor (ER) agonists 4-nonylphenol (Harries *et al.*, 2000; Miles-Richardson *et al.*, 1999a,b) and the strong ER agonists E2 and EE2 (Miles-Richardson *et al.*, 1999a; Pawlowski *et al.*, 2004). The development of nuptial tubercles and the fatpad in male fathead minnows is stimulated by testosterone produced by the Leydig cells of the testes (Smith, 1978). Testosterone production is under the control of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both of which are regulated by E2 in a feedback mechanism (Junqueira *et al.*, 1986). Thus, atrophy of the nuptial tubercles may have resulted from an inhibition of LH because xenostrogens like 3BC may suppress androgen levels by altering neuroendocrine feedback loops (Ankley *et al.*, 1998; Jobling *et al.*, 1996). The number of tubercles as well as the production of sperms are androgen-dependent processes, they are negatively correlated with exposure to estrogenic chemicals (Jensen *et al.*, 2001; Miles-Richardson *et al.*, 1999a). Thus, demasculinization reflected by these endpoints is an indication for the estrogenic activity of 3BC.

Different effects on nuptial tubercles are observed with other compounds. The antiandrogen flutamide, which has a limited binding capacity to fish androgen receptors (Sperry and Thomas, 2000), causes a significant decrease in number of tubercles in male fathead minnows at high concentrations, but does not induce VTG production in males. On the other hand, the aromatase inhibitor fadrozole leads to a significant VTG induction in males and females, and to a significant induction of tubercles in female fish (Ankley *et al.*, 2002).

Reduced reproduction and even its cessation caused by 3BC may be related to alterations in both males and females. In males we observed a dose-dependent demasculinization, indicated by the loss of secondary sexual characteristics (Fig. 5), and the inhibition of spermatogenesis (Figs. 6 and 8A). This already started at 3 μg/l, and possibly resulted in a loss of gender-specific mating behavior and in a stop of milt reproduction at 74 and 285 μg/l. In females, the inhibition of oogenesis started at 3 μg/l 3BC and was indicated histologically by an increase of atretic and a decrease of early and late vitellogenic follicles in ovaries. At 74 and 285 μg/l, females stopped egg production and the release of mature oocytes. Possibly, this may be a reaction on the missing mating behavior of demasculinized males present in the aquaria.

So far UV filters have been evaluated for estrogenic activity in fish by investigating VTG induction only. This was observed in juvenile fathead minnows for 3BC, BP1, and BP2 (Kunz *et al.*, 2006), and also found after injection of 3BC (68 mg/kg) in rainbow trout (Holbech *et al.*, 2002), and after aqueous exposure to 100 mg/l 4MBC and 10 mg/l OMC in male medaka (Inui *et al.*, 2003). The benefit of measuring VTG induction as a biomarker for estrogenicity is obvious. It is easier to measure and provides information of the estrogenicity of a certain compound with possible adverse effects on reproduction (Sumpter and Johnson, 2005). The lowest observed effect concentrations after exposure to 3BC varies according to the endpoint. For VTG induction and secondary sex characteristics it was 74 μg/l, for effects on fecundity it was 33 μg/l, and histological alterations occurred already at 3 μg/l. VTG determinations in short-term experiments are rather fast, and less manpower and time consuming than a short-term reproduction assay, but our results with 3BC suggest that it possesses lower sensitivity than other parameters. Nevertheless, VTG induction is clearly associated with other direct markers of reproduction effects and thus well suited for screening estrogenic compounds (Sumpter and Johnson, 2005). However, for hazard and risk assessments, short-term reproduction assays provide much more important toxicological and ecological information. They give a more detailed picture of the endocrine activities of a compound and give sensitive information on fecundity, histology, and secondary sex characteristics, and thereby facilitate and refine risk assessment.

Conclusions and Environmental Consequences

Our study demonstrates significant effects of 3BC on the reproduction of fathead minnows. The lowest observed effect concentrations for the most sensitive parameter, gonadal histology, was 3 μg/l. Environmental concentrations of 3BC in water and fish have not yet been reported, but may reach the same level than other UV filters such as 4MBC. In Swiss lakes, UV filters were detected at concentrations up to 125 ng/l (Poiger *et al.*, 2004). In raw and reclaimed drinking water, OMC was found in the range of 0.45–5.61 μg/l in southern California (Loraine and Pettigrove, 2006). In raw wastewater, this and the UV filter oxybenzone (benzophenone-3, BP3) were found in the range of 0.11–10.4 μg/l. In Switzerland, raw wastewater contained UV filters (BP3, 4MBC, OMC, octocrylene [OC]) with a maximum concentration of 19 μg/l for OMC. The concentrations in treated wastewater were lower; 4MBC was usually the most prevalent compound (maximum concentration, 2.7 μg/l), followed by BP3, OMC, and OC (Balmer *et al.*, 2005). Residues of several UV filters have also been detected at concentrations of 21–3100 ng/g lipid in fish (Balmer *et al.*, 2005; Buser *et al.*, 2006; Nagtegaal *et al.*, 1997). If environmental concentrations of 3BC are also in

the low µg range, effects of 3BC on fish reproduction can not be ruled out. Moreover, 3BC is lipophilic and bioaccumulated during 21 days of exposure with an average bioconcentration factor of 313 ± 151. Furthermore, the presence of other UV filters and hormonally active compounds, which may interact additively may adversely affect fish reproduction in contaminated aquatic systems. The pronounced effect of 3BC on reproduction, fecundity, and fertility in fish after a 21 days exposure sheds new light on the potential hazard and risks of widely used UV filters.

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