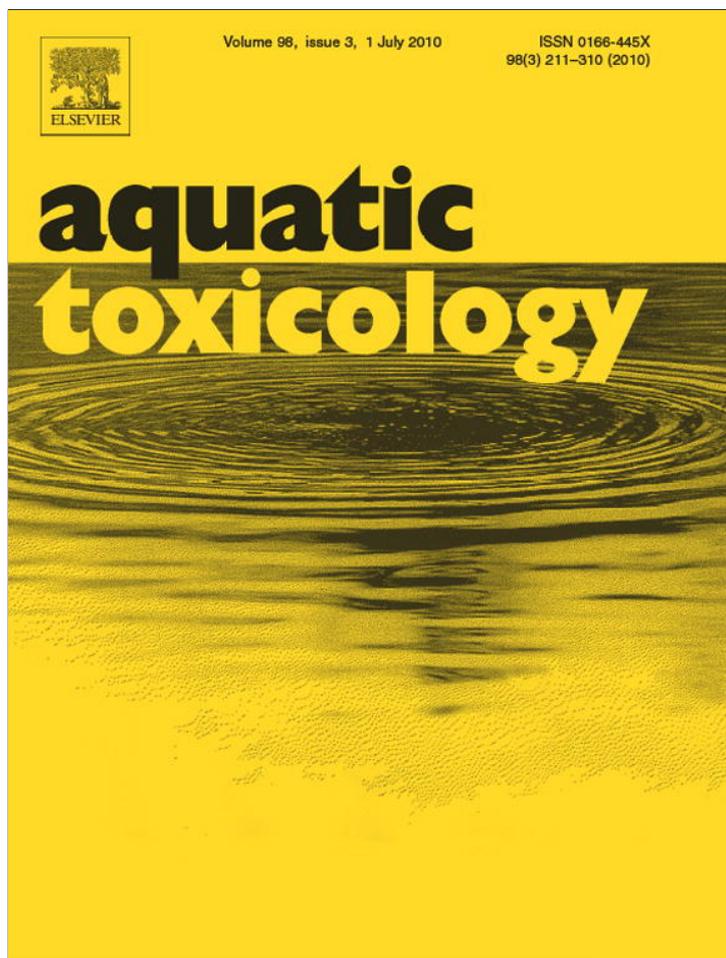


Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*

Sunyoung Han^a, Kyungho Choi^{a,*}, Jungkon Kim^a, Kyunghee Ji^a, Sunmi Kim^a, Byeongwoo Ahn^b, Junheon Yun^c, Kyunghee Choi^c, Jong Seong Khim^d, Xiaowei Zhang^{e,f}, John P. Giesy^{e,f,g,h,i}

^a School of Public Health, Seoul National University, 28 Yunkeon, Chongro, Seoul 110-799, Republic of Korea

^b College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Republic of Korea

^c National Institute of Environmental Research, Incheon 404-708, Republic of Korea

^d Division of Environmental Science and Ecological Engineering, Korea University, Seoul 136-713, Republic of Korea

^e Toxicology Centre and Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Saskatchewan S7J 5B3, Canada

^f State Key Laboratory of Pollution Control and Resource Reuse & School of the Environment, Nanjing University, Nanjing 210093, China

^g Zoology of Department, Center for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, USA

^h Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong SAR, China

ⁱ State Key Laboratory of Marine Environmental Science, College of Oceanography and Environmental Science, Xiamen University, Xiamen, China

ARTICLE INFO

Article history:

Received 9 November 2009

Received in revised form 8 February 2010

Accepted 16 February 2010

Keywords:

Non-steroidal anti-inflammatory drug

Fish

Steroidogenesis

H295R cell

Aromatase

Hormones

ABSTRACT

Despite frequent detection of ibuprofen in aquatic environments, the hazards associated with long-term exposure to ibuprofen have seldom been investigated. Ibuprofen is suspected of influencing sex steroid hormones through steroidogenic pathways in both vertebrates and invertebrates. In this study, the effect of ibuprofen on sex hormone balance and the associated mechanisms was investigated *in vitro* by use of H295R cells. We also conducted chronic toxicity tests using freshwater fish, *Oryzias latipes*, and two freshwater cladocerans, *Daphnia magna* and *Moina macrocopa*, for up to 144 and 21 d of exposure, respectively. Ibuprofen exposure increased 17 β -estradiol (E2) production and aromatase activity in H295R cells. Testosterone (T) production decreased in a dose-dependent manner. For *D. magna*, the 48 h immobilization EC50 was 51.4 mg/L and the 21 d reproduction NOEC was <1.23 mg/L; for *M. macrocopa*, the 48 h immobilization EC50 was 72.6 mg/L and the 7 d reproduction NOEC was 25 mg/L. For *O. latipes*, 120 d survival NOEC was 0.0001 mg/L. In addition, ibuprofen affected several endpoints related to reproduction of the fish, including induction of vitellogenin in male fish, fewer broods per pair, and more eggs per brood. Parental exposure to as low as 0.0001 mg/L ibuprofen delayed hatching of eggs even when they were transferred to and cultured in clean water. Delayed hatching is environmentally relevant because this may increase the risk of being predated. For *O. latipes*, the acute-to-chronic ratio of ibuprofen was estimated to be greater than 1000. Overall, relatively high acute-to-chronic ratio and observation of reproduction damage in medaka fish at environmentally relevant ranges of ibuprofen warrant the need for further studies to elucidate potential ecological consequences of ibuprofen contamination in the aquatic environment.

© 2010 Elsevier B.V. All rights reserved.

Abbreviations: cAMP, cyclic adenosine monophosphate; CF, condition factor; COX, cyclooxygenase; CI, confidence interval; CYP, cytochrome P450; dph, day post-hatch; EC50, median effective concentration; ELISA, enzyme-linked immunosorbent assay; GSI, gonadosomatic index; HSI, hepatosomatic index; LOEC, lowest observed effect concentration; MCIG, minimum concentration to inhibit growth; NOEC, no observed effect concentration; NSAID, non-steroidal anti-inflammatory drug; PGE2, prostaglandin E2; PGR, population growth rate; STP, sewage treatment plant.

* Corresponding author.

E-mail address: kyungho@snu.ac.kr (K. Choi).

1. Introduction

Pharmaceuticals are developed and used for intended biological effects in human and veterinary medicine. The physiologically active nature of pharmaceuticals, however, raised concerns about their potential impacts to non-target species when they were inadvertently discharged into ecosystem (Ankley et al., 2007; Fent et al., 2006). Ibuprofen ((*RS*)-2-(4-isobutylphenyl)propanoic acid, CAS number 15687-27-1) is one of non-steroidal anti-inflammatory drugs (NSAIDs), and is widely used as analgesic, antipyretic and anti-inflammatory purposes to relieve symptoms of arthritis, rheumatic disorders and fever (Hayashi et al., 2008). It is one of the

core medicines included in “Essential Drugs List” of World Health Organization, and therefore produced in large amounts worldwide (Heckmann et al., 2007).

Ibuprofen has frequently been detected in surface water, with as much as 0.1 µg/L detected in surface water of South Wales, UK (Kasprzyk-Hordern et al., 2008). The average concentration detected in major rivers of Korea was 0.03 µg/L (Kim et al., 2007). Relatively greater concentrations of ibuprofen have been reported for effluents (up to 22 µg/L) and influents (up to 84 µg/L) of sewage treatment plants (STPs) (Brun et al., 2006; Gómez et al., 2007). Hence municipal wastewater effluents are an important source of ibuprofen in aquatic environments, especially streams and rivers.

Due to the widespread occurrence of ibuprofen in aqueous environments, its potential for ecological impact has been of growing concern (Christensen et al., 2009). Ibuprofen is known to influence the cyclooxygenase (COX) pathway (Flippin et al., 2007; Heckmann et al., 2008). The COX enzyme could influence the synthesis of eicosanoids, which are important regulators of reproduction in both vertebrates and invertebrates (Hayashi et al., 2008). Therefore, ibuprofen contamination in water environment could affect reproduction of aquatic animals. However, most investigations have been limited to lethal effects during acute exposures. Studies involving long-term exposure have been limited to invertebrates such as *Hydra* (Quinn et al., 2008), mollusks (Pounds et al., 2008) and the freshwater cladoceran, *D. magna* (Halling-Sørensen et al., 1998; Han et al., 2006; Heckmann et al., 2007). In *D. magna*, reproduction and population growth were measured after 14 d exposure to environmentally realistic concentrations of ibuprofen (Heckmann et al., 2007). Only one study reported effects of longer term ibuprofen exposure in freshwater fish: In Japanese medaka (*Oryzias latipes*), reproduction-related endpoints such as blood size and number of broods were evaluated during a 6-week exposure (Flippin et al., 2007).

However, short-term chronic tests covering only partial life stages may not be sufficient for chemicals like ibuprofen that are constantly released from municipal STPs. A combination of a development test with a complementary fish reproduction test, for example, has been suggested in order to understand potential ecological risks of such contaminants, while full life cycle test could be used to refine such assessment (Hutchinson et al., 2000). Therefore, most assessments of the ecotoxicity of ibuprofen conducted to date were not sufficient to understand the potential for chronic effects of ibuprofen exposure on endpoints other than lethality. Significant knowledge gaps in the mechanism of toxicity also exist.

This study was conducted to identify the effects of ibuprofen on steroidogenesis. In addition, we evaluated the effects of chronic exposure of freshwater crustaceans and fish to environmentally relevant concentrations of ibuprofen. For this purpose, the H295R cells, human adrenocortical carcinoma cell line and three model freshwater species, including freshwater macroinvertebrates *D. magna* and *Moina macrocopa*, and fish *O. latipes*, were employed.

2. Material and methods

2.1. Chemicals

Test solutions were freshly prepared by diluting stock solutions with appropriate culture media, immediately before the test or before the renewal of test solutions. Ibuprofen (purity 98%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Solvent-free stock solution of ibuprofen was prepared by dissolving in MilliQ water (Millipore Asia, Yonezawa, Japan) with sonication. Measured concentrations of ibuprofen were 1.13 and 71.9 mg/L for the nominal concentrations of 1 and 100 mg/L, respectively. Concentrations of ibuprofen did not change more than 6% after the 48 h of exposure

of *D. magna* or *O. latipes* (Supplement Table S1). Nominal concentrations are reported throughout the paper.

2.2. Cell culture and maintenance of test organisms

The H295R human adrenocortical carcinoma cell line was obtained from the American Type Culture Collection (ATCC # CRL-2128, ATCC, Manassas, VA, USA) and cultured at 37 °C in a 5% CO₂ atmosphere as previously described (Gracia et al., 2007). Briefly, the cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient mixture (DMEM/F12) (Sigma D-2906; Sigma–Aldrich) supplemented with 1.2 g/L Na₂CO₃, 10 mL/L of ITS+ Premix (BD Bioscience; 354352), and 25 mL/L of Nu-Serum (BD Bioscience; 355100). Medium was changed every 4 d and cells were subcultured every week.

The two freshwater cladocerans (*D. magna* and *M. macrocopa*) and the fish (Japanese medaka fish; *O. latipes*) were cultured and maintained in the Environmental Toxicology Laboratory, Seoul National University (Seoul, Korea) following US Environmental Protection Agency (US EPA, 2002), Oh (2007), and Organization for Economic Cooperation and Development TG 203 (OECD, 1992a,b) protocols, respectively. Both crustaceans were fed daily with a 1:1:1 mixture of yeast (ACH Food Companies, Memphis, TN, USA), cerophyll (Nutraceutical Corporation, Park City, UT, USA), and Tetramin® (Tetra, Melle, Germany). In addition, algae (*Pseudokirchneriella subcapitata*) were also provided. Japanese medaka fish were cultured in filtered tap water after dechlorination by aeration for more than 24 h. Medaka were maintained at 25 ± 1 °C, under a 16:8-h light:dark photoperiod, and were fed twice a day with freshly hatched *Artemia nauplii* (Brine Shrimp Direct, Ogden, UT, USA).

Water quality parameters, including dissolved oxygen, pH, conductivity and temperature, were monitored and logged whenever new batches of media were prepared, following American Public Health Association, American Water Works Association, and Water Pollution Control Federation (1992) protocols. To confirm comparable sensitivity of the test organisms over time, acute lethality tests were conducted with the reference toxicant (zinc chloride) to determine the relative sensitivity of *D. magna*, *M. macrocopa*, and *O. latipes* on a monthly basis (data not shown).

2.3. Steroidogenesis assay using H295R cells

H295R cells were seeded into 24-well plates at a concentration of 3 × 10⁵ cells/mL in 1 mL of medium per well. After 24 h, cells were exposed to ibuprofen dissolved in dimethyl sulfoxide (DMSO; Sigma–Aldrich, St. Louis, MO, USA). The final DMSO concentration in the exposure medium was less than 0.1% (v/v). H295R cells were exposed to various concentrations of ibuprofen for 48 h, and were inspected microscopically for viability. When the exposure resulted in cell viability less than 85% the data were not used to determine the effect on hormone production (Gracia et al., 2007). In addition, to identify the range of ibuprofen concentrations that are non-cytotoxic, a Live/Dead cell viability assay kit (Molecular Probes, Eugene, OR, USA) was used. The culture medium was collected after the exposure and stored at –80 °C for further measurement of hormones produced.

Hormone extraction and quantification were conducted as previously described (Hecker et al., 2006). Briefly, hormones were extracted from the culture medium twice with diethyl ether (5 mL) and the solvent was evaporated under a gentle stream of nitrogen. The residue was reconstituted in ELISA assay buffer and was measured by competitive ELISA following the manufacturer's recommendation (Cayman Chemical, Ann Arbor, MI, USA; Testosterone [T, Cat # 582701], 17β-Estradiol [E2, Cat # 582251]).

Activity of aromatase enzyme was measured using two different methods, direct or indirect assays as previously described

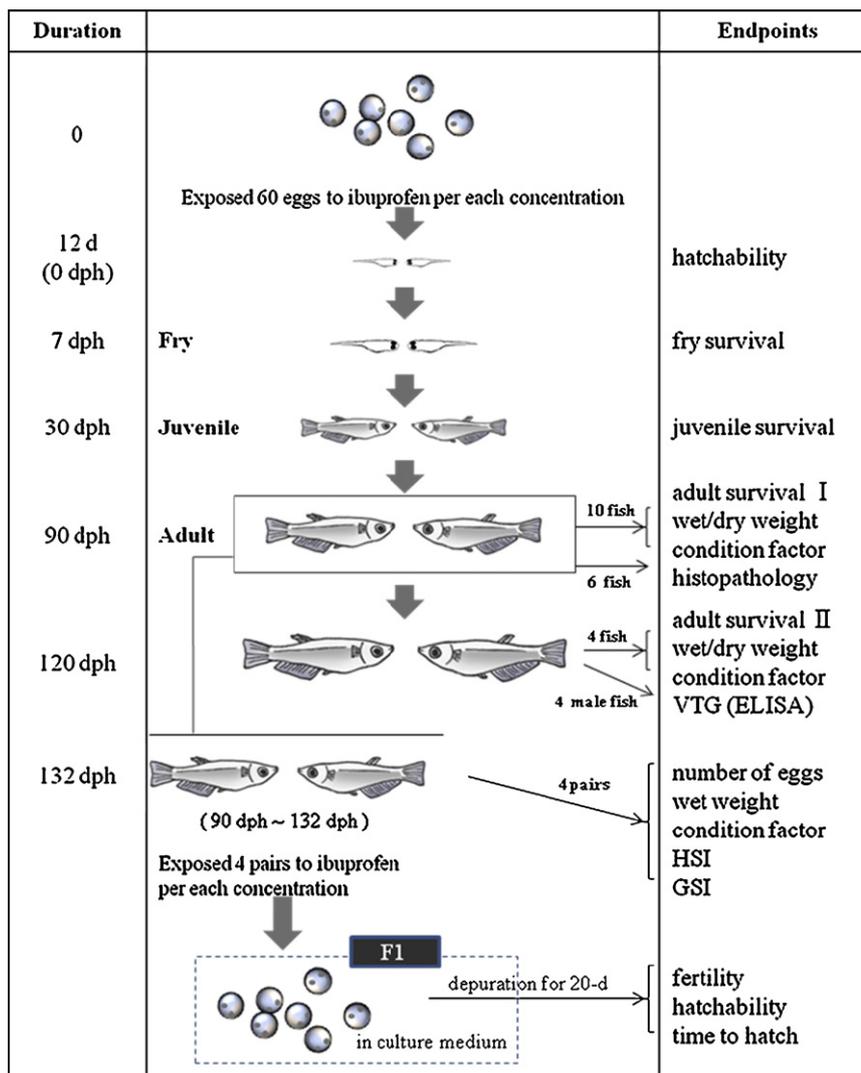


Fig. 1. Experimental procedures for chronic *O. latipes* toxicity test. The 12th day after egg exposure is assigned as the first day post-hatch (dph). Bold letter expresses the development stage. Number of fish that were observed for each endpoint is indicated above the arrows. At 90 dph, four mating pairs from the control or each treatment were selected for the examination of effects on reproduction. To investigate effects of ibuprofen on progeny generation (F1), eggs from four mating pairs were collected and transferred into clean culture water, and fertility, hatchability and time to hatch were determined.

(Sanderson et al., 2001; Higley et al., 2010). For the experiments measuring direct effects of chemicals on aromatase activity, cells were exposed to various concentrations of ibuprofen in a medium containing 54 nM 1 β -³[H]-androstenedione (Perkin Elmer, Boston, MA, USA), with no pre-exposure to ibuprofen. In order to measure indirect effects of ibuprofen on aromatase activity, H295R cells were pre-exposed to ibuprofen for 48 h, and then cells were washed twice and incubated with 0.25 mL of supplemented medium containing 54 nM 1 β -³[H]-androstenedione. DMSO was used as carrier solvent and did not exceed 0.1% (v/v). After 1.5 h incubation at 37 °C with 5% CO₂, cells were placed on ice to stop the reaction. A 200 μ L aliquot of the cell suspension was removed, and chloroform and dextran-coated charcoal were added to remove all remaining 1 β -³[H]-androstenedione. Aromatase activity was determined by the rate of conversion of 1 β -³[H]-androstenedione to estrone. The quantity of ³[H] in extracts of medium was determined by LS 6500 multipurpose scintillation counter (Beckman Coulter, Fullerton, CA, USA). Aromatase activity was expressed as pmoles of androstenedione converted per h per 100,000 cells. Forskolin (10, 1, or 0.1 μ M) was used as a positive control for aromatase induction, while prochloraz (10, 1, or 0.1 μ M) was used as a negative control for aromatase catalytic inhibition.

2.4. *D. magna* and *M. macrocopa* toxicity tests

The 48 h immobility tests were conducted with *D. magna* and *M. macrocopa* to determine the range of ibuprofen concentrations for chronic survival and reproduction tests. Procedures used for 48 h immobility test were in accordance with US EPA (2002). The endpoints for the chronic tests were survival of original neonates, number of young per female, the number of young per brood, time to first reproduction, and population growth rate (PGR). PGR was calculated following the method used in Park and Choi (2008). Basic water chemistries such as dissolved oxygen, pH, temperature, and conductivity were measured and recorded before and after the medium renewal. Chronic *D. magna* toxicity tests (21 d) were conducted as outlined in OECD TG 211 (OECD, 2008). Ten replicates with one neonate each (<24 h old) were exposed to various concentrations of ibuprofen (0, 1.23, 3.70, 11.1, 33.3, or 100 mg/L). Test solutions were renewed three times per week. *D. magna* were fed daily with 300 μ L YCT and 300 μ L algae per each organism. Chronic *M. macrocopa* toxicity tests (7–8 d, three brood) were performed following the protocol by Oh (2007). The test method was similar to the chronic *D. magna* test method except for the test duration (21 d versus 7–8 d) and temperature (20 °C versus 25 \pm 1 °C). Ten

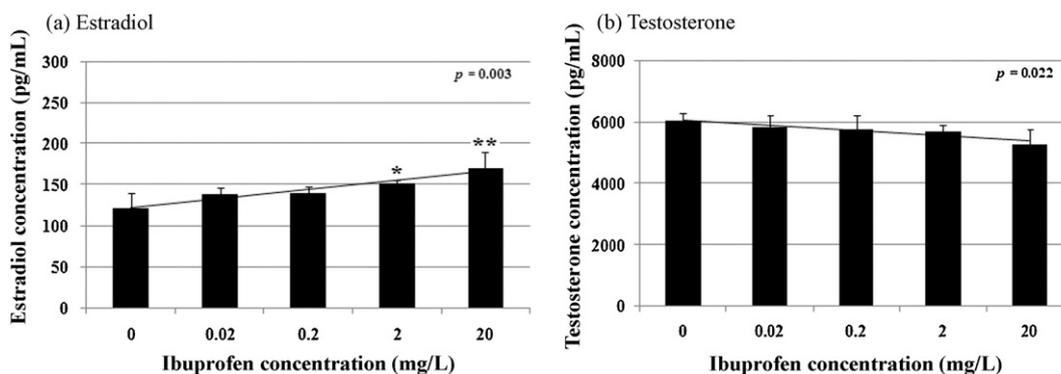


Fig. 2. Mean concentrations of (a) estradiol (E2) and (b) testosterone (T) in H295R cell medium after 48 h exposure to ibuprofen. Error bar represents 1 standard deviation. Asterisk denotes a significant difference from the control (* $p < 0.05$; ** $p < 0.01$). The p value shown on the figure indicates significance of trend line: Significant positive trend was observed for estradiol ($p = 0.003$), while a negative trend was observed for testosterone ($p = 0.022$).

replicate test chambers each containing a single neonate (<24 h old) were exposed to various concentrations of ibuprofen (0, 3.13, 6.25, 12.5, 25.0, or 50.0 mg/L).

2.5. *O. latipes* life cycle toxicity test

A full life cycle chronic toxicity test with a supplementary reproduction test was conducted with fertilized eggs (<24 h of spawning) for 144 d (Fig. 1). This test was an enhancement of the OECD embryo-larval test (TG 210), and was designed to measure gross development, vitellogenin induction, histological manifestations, and reproduction success following the chronic exposure to ibuprofen. During the exposure, fishes were fed freshly hatched *A. nauplii* twice a day.

Initially, 60 fertilized eggs were randomly separated into four test beakers (i.e., 15 eggs per beaker) in each treatment. Fertilized eggs were exposed to 0.01, 0.1, 1, 10, 100, or 1000 $\mu\text{g/L}$ ibuprofen for 12 d. When the eggs hatched, hatchlings were moved into 250 mL beakers. Survival of the juvenile fish was monitored daily until 30 d post-hatch (dph). After 90 and 120 dph, effects on survival, condition factor, histopathology, or vitellogenin induction were determined. To evaluate reproduction effects, four pairs of fish were chosen from each treatment or control after 90 dph, and reproduction endpoints of each mating pair were determined daily for 30 d during the period of 90–132 dph. After this observation, all surviving pairs were euthanized and measured for the condition factor (CF), gonadosomatic index (GSI), and hepatosomatic index (HSI). In addition, the eggs that had been collected from the mating pairs were transferred to clean culture water and were observed for fertility, hatchability and time-to-hatch for additional 20 d.

Vitellogenin in blood plasma was measured using a kit (EnBio Medaka Vitellogenin ELISA system, COSMO BIO, Tokyo, Japan) according to the manufacturer's instructions. A microtitre plate spectrophotometer (Tecan SPECTRAFluor, Tecan, Männedorf, Switzerland) was employed for the measurement. For histological observation, fish were euthanized and fixed in Bouin's solution for 24 h, dehydrated in a series of ethyl alcohol and xylene baths, and embedded in paraffin. H&E stained tissue sections of gonads, livers, and kidneys were examined for histopathology under a light microscope.

2.6. Statistical analysis

The median effective concentrations (EC50) and associated 95% confidence intervals (CI) were calculated by Probit analysis or Spearman–Kärber analysis depending on the normality of distribution and homogeneity of variance of the data. No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were calculated using Fisher's Exact test or Dunnett's one-way analysis of variance (ANOVA) test. For analysis of reproduction data, outliers were identified using Dixon's Q -test (Dean and Dixon, 1951). SPSS 12.0K for Windows (SPSS, Chicago, IL, USA) and ToxStat (ver 3.5. West, Cheyenne, WY, USA) were used.

3. Results

3.1. Hormone production and aromatase activity in H295R cells

Ibuprofen significantly increased the production of E2 in H295R cells in a dose-dependent manner ($p = 0.003$, Fig. 2a). Compared to

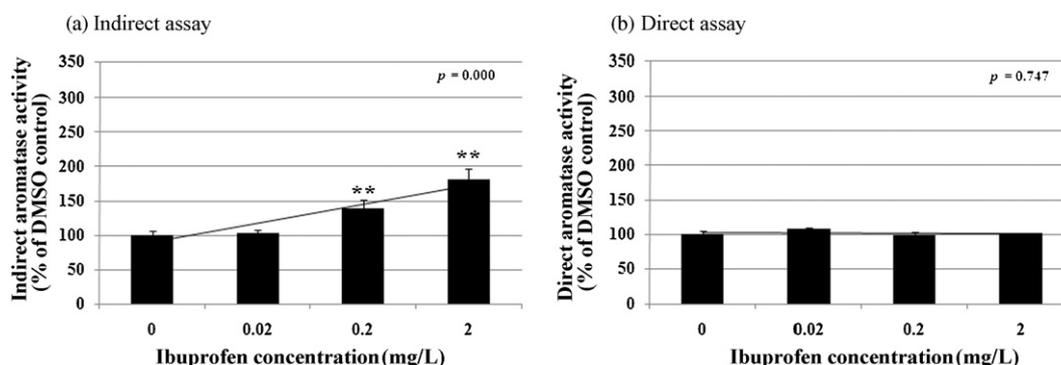


Fig. 3. Induction of aromatase activity by ibuprofen in (a) indirect and (b) direct assay. The induction level was shown as the aromatase activity compared to the 0.1% DMSO solvent control. The mean aromatase activity of triplicate measurements is shown. Error bar represents 1 standard deviation. Asterisks indicate significant difference from the DMSO solvent control (** $p < 0.01$). The p value shown on the figure indicates significance of trend line: Significant positive trend of aromatase activity was observed in indirect assay ($p = 0.000$), while no trend was observed in direct assay ($p = 0.747$).

Table 1
Effects of ibuprofen exposure on survival and reproduction for *D. magna* and *M. macrocopa*^{a, b}.

Species	Concentration (mg/L)	Adult survival (%)	First day of reproduction (d) mean \pm s.d.	No. of young per female mean \pm s.d.	No. of young per brood mean \pm s.d.	PGR ^c
<i>D. magna</i>	Control	100	10.0 \pm 0.0	157.1 \pm 10.7	39.3 \pm 2.7	0.39
	1.23	100	11.0 \pm 1.3	130.8 \pm 17.2*	34.8 \pm 5.5	0.36
	3.70	100	10.1 \pm 0.3	120.2 \pm 24.0*	30.8 \pm 4.7*	0.35
	11.1	100	11.3 \pm 1.5*	111.6 \pm 18.7*	31.2 \pm 5.1*	0.34
	33.3	100	11.2 \pm 1.1*	75.5 \pm 27.6*	21.7 \pm 3.8*	0.32
	100	0*	NA ^d	NA	NA	NA
<i>M. macrocopa</i>	Control	90	3.0 \pm 0.0	69.2 \pm 11.7	16.9 \pm 2.6	0.90
	3.13	100	3.0 \pm 0.0	66.4 \pm 12.4	18.1 \pm 1.7	0.92
	6.25	100	3.0 \pm 0.0	69.5 \pm 12.7	17.9 \pm 2.5	0.92
	12.5	100	3.0 \pm 0.0	60.2 \pm 16.1	16.4 \pm 1.8	0.91
	25.0	70	3.0 \pm 0.0	52.7 \pm 21.0	16.0 \pm 3.2	0.90
	50.0	80	3.0 \pm 0.0	50.7 \pm 12.0*	18.1 \pm 3.0	0.91

^a Asterisk denotes a significant difference compared to the control. * $p < 0.05$.

^b Average measured concentrations were 1.13 and 71.9 mg/L for nominal concentrations of 1 and 100 mg/L, respectively. During 48 h of exposure concentrations of ibuprofen changed <6%.

^c Population growth rate. In *D. magna*, PGR notably decreased in a dose-dependent manner ($p = 0.067$).

^d NA: not applicable due to significant mortality.

control, significantly greater E2 production was observed at concentrations of ibuprofen as little as 2 mg/L and E2 production as proportional to ibuprofen concentration up to 20 mg/L. T concentrations were inversely proportional to ibuprofen concentration ($p = 0.022$, Fig. 2b).

Exposure to 0.2 or 2 mg/L ibuprofen for 48 h resulted in significantly greater aromatase activity compared to the control ($p < 0.01$, Fig. 3a). However, in the direct aromatase assay, aromatase activity did not change ($p > 0.05$, Fig. 3b). This result indicates that ibuprofen affected the catalytic activity of the enzyme indirectly, e.g., through transcriptional level modulation.

3.2. Effects in *Daphnia* and fish

Ibuprofen cause statistically significant effects on survival and reproduction of both medaka and two freshwater cladocerans. The 48 h EC50s were estimated to be 51.4 mg/L (95% confidence interval 40.2–62.7) and 72.6 mg/L (55.0–90.4) for *D. magna* and *M. macrocopa*, respectively. Chronic effects of ibuprofen on survival and reproduction of *D. magna* and *M. macrocopa* exposure are summarized in Table 1. After 21 d exposure with *D. magna*, the NOEC based on survival of the initial neonate was 33.3 mg/L, while the LOEC based on reproduction was 1.23 mg/L which was the least concentration tested. The PGR decreased but the magnitude of the effect was not expected to result in negative population growth. The NOEC based on survival of *M. macrocopa* was >50.0 mg/L, and the NOEC based on reproduction was 25 mg/L. There was no statistically significant effect on PGR.

The effect of ibuprofen on *O. latipes* increased with duration of exposure (Table 2). Survival of adult fish (120 dph) exposed to as little as 1 μ g ibuprofen/L was significantly less than of the controls, while hatchability and survival of fry and juveniles were not affected even at the maximum test concentration of 1000 μ g/L. The length, weight, and condition factor (CF) of surviving adult fish

were not affected by ibuprofen at any of the concentrations tested (Supplement Table S2).

In adult male fish, vitellogenin in blood plasma was greater when exposed to 1000 μ g/L ($p < 0.1$, Fig. 4) than in controls. HSI and GSI were not affected by ibuprofen exposure, except for females exposed to 0.1 μ g/L, but there was no dose-dependent relationship (Fig. 5). Also, because such a change was not observed among male fish, the response was most likely spurious. Histopathological observations of liver, gonad, and kidney found no lesions in exposed fish (Supplement Fig. S1).

Reproduction of eggs was not affected by either of the ibuprofen concentrations. However, the number of eggs per brood was significantly greater at 10 and 100 μ g ibuprofen/L (Table 3). The number of broods per pair exhibited a dose-dependent negative relation-

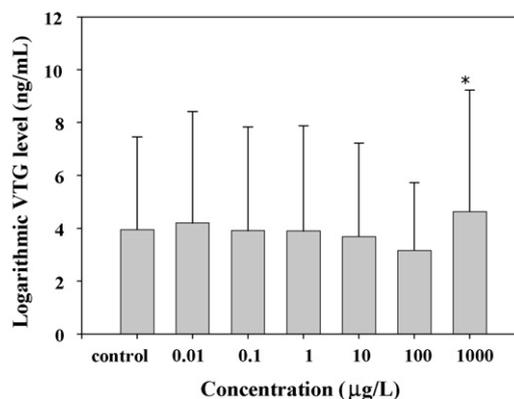


Fig. 4. Blood VTG concentrations in blood plasma of male *Oryzias latipes* among ibuprofen treatments for 120 dph. Error bars represent standard deviation of level of vitellogenin. Vitellogenin level was expressed by logarithmic scale. Asterisk indicates a notable difference from the control ($P < 0.1$) based on Dunnett's ANOVA test. Outliers were controlled by Q test (Dean and Dixon, 1951).

Table 2
Effects of ibuprofen exposure on hatchability and survival of fry, juvenile and adult medaka^{a,b,c}.

Concentration (µg/L)	Hatchability		Fry survival 7 dph		Juvenile survival 30 dph		Adult survival			
	N ^d	Mean ± s.d. (%)	N ^d	Mean ± s.d. (%)	N ^d	Mean ± s.d. (%)	90 dph ^e		120 dph ^e	
							N ^d	Mean (%)	N ^d	Mean (%)
Control	56/60	93.3 ± 9.4	55/56	98.3 ± 3.5	55/56	98.3 ± 3.5	53/56	94.6	37/40	92.50
0.01	46/60	76.8 ± 29.8	44/46	96.3 ± 4.4	44/46	96.3 ± 4.4	41/46	89.1	23/30	76.7
0.1	45/60	75.3 ± 10.0	45/45	100.0 ± 0.0	45/45	100.0 ± 0.0	43/45	95.6	27/29	93.1
1	50/60	83.3 ± 12.5	46/50	93.0 ± 9.9	45/50	91.3 ± 10.5	43/50	86.0	25/34	73.5*
10	53/60	88.3 ± 10.0	50/53	94.8 ± 6.7	48/53	91.5 ± 10.3	42/53	79.3*	26/37	70.3*
100	57/60	95.0 ± 6.3	52/57	91.3 ± 6.4	52/57	89.5 ± 3.5	45/57	79.0*	25/41	61.0*
1000	54/60	89.8 ± 11.6	48/54	88.5 ± 9.8	43/54	79.0 ± 9.9*	39/54	72.2*	12/38	31.6*

^a Hatchability, fry survival and juvenile survival were analyzed by Dunnett's ANOVA test, while adult survival was compared using Fisher's Exact test. Hatchability is estimated as: number of hatched eggs/total eggs × 100, while fry, juvenile, and adult survival is indicated by (number of surviving fries × juveniles × adults)/number of hatched eggs × 100. Refer to Fig. 1.

^b Asterisk denotes a significant difference from the control ($p < 0.05$).

^c Average measured concentrations was 1.13 mg/L for nominal concentrations of 1 mg/L. During 48 h of exposure concentrations of ibuprofen changed <1%.

^d Number of hatched or surviving fish/total number of fish.

^e Standard deviations are not available, because surviving juvenile fishes were combined in one exposure vehicle for adult exposure.

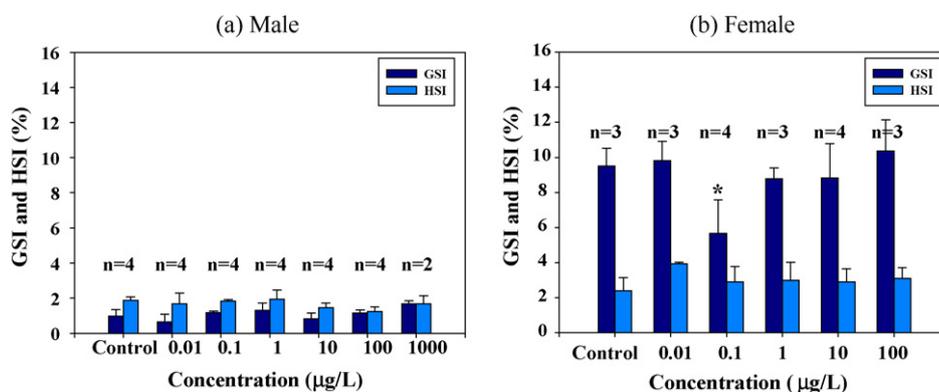


Fig. 5. Gonadosomatic Index (GSI) and hepatosomatic Index (HSI) of male (a) and female (b) Japanese medaka exposed to various concentrations of ibuprofen for 132 dph. The n value represents number of fish used to observe HSI and GSI. Asterisk indicates a significant difference from the control ($p < 0.05$) based on Dunnett's ANOVA test. HSI and GSI are calculated as: gonad weight/total body weight × 100 and liver weight/total body weight × 100. Outliers were controlled by using Q test (Dean and Dixon, 1951).

Table 3
Effect of ibuprofen exposure on reproduction profile of medaka^{a,b,c}.

Concentration (µg/L)	Surviving pairs	No. of total eggs ^d		No. of broods per pair ^d		No. of eggs per brood	
		N ^e	Mean ± s.d.	N ^e	Mean ± s.d.	N ^e	Mean ± s.d.
Control	4	4	243.8 ± 64.9	4	28.5 ± 1.0	4	8.5 ± 2.1
0.01	4	4	155.5 ± 48.1	4	24.8 ± 6.0	4	6.3 ± 1.3
0.1	4	4	212.0 ± 51.1	4	26.5 ± 1.9	4	7.9 ± 1.4
1	3	3	283.3 ± 79.0	3	25.0 ± 6.9	4	11.1 ± 1.1
10	4	4	365.5 ± 90.1	4	27.8 ± 2.5	4	13.0 ± 2.3*
100	3	3	285.7 ± 114.0	3	23.7 ± 6.8	4	11.8 ± 1.6*
1000	2	2	256.5 ± 248.2	2	21.0 ± 9.9	3	5.9 ± 0.2

^a Mean ± standard deviation are derived from the observations from four pairs of adult fish between 90 and 132 dph. Outliers were identified using Q-test and removed from data analyses.

^b Asterisk denotes a significant difference from the control ($p < 0.05$) based on Dunnett's ANOVA.

^c Average measured concentrations was 1.13 mg/L for nominal concentrations of 1 mg/L. During 48 h of exposure concentrations of ibuprofen changed <1%.

^d Number of eggs or broods included those obtained from both surviving and dead fish pairs.

^e Number of replicates.

Table 4
Fertility, hatchability, and time-to-hatch of progeny generation (F1) observed for 20 d in control water^{a,b,c}.

Concentration (µg/L)	N	Fertility (%) mean ± s.d.	Hatchability (%) mean ± s.d.	Time-to-hatch (d) mean ± s.d.
Control	33	100.0 ± 0.0	88.3 ± 20.2	10.9 ± 1.5
0.01	31	100.0 ± 0.0	100.0 ± 0.0	11.5 ± 2.3
0.1	30	100.0 ± 0.0	81.3 ± 23.9	14.7 ± 0.8*
1	29	97.8 ± 3.9	95.2 ± 8.3	12.6 ± 1.3*
10	47	100.0 ± 0.0	79.2 ± 21.5	14.7 ± 0.2*
100	48	100.0 ± 0.0	93.0 ± 7.8	12.2 ± 2.4*
1000	–	–	–	–

^a Eggs were obtained from four mating pairs that were exposed to ibuprofen during 90–132 dph period. Then the eggs were transferred to clean control water and were observed for fertility, hatchability and time-to-hatch for additional 20 d.

^b Asterisk denotes a significant difference from the control ($p < 0.05$).

^c Average measured concentrations was 1.13 mg/L for nominal concentrations of 1 mg/L. During 48 h of exposure concentrations of ibuprofen changed <1%.

Table 5
Acute and chronic toxicity of ibuprofen to aquatic organisms.

Test type	Species	Duration/endpoint	Conc. (CI) (mg/L)	Reference
Acute	<i>Skeletonema costatum</i>	96 h/Growth EC50	7.1	Knoll/BASF (1995) cited in Webb (2004)
	<i>Scenedesmus subspicatus</i>	72 h/Growth EC50	342.2 (242.4–471.5)	Cleuvers (2004)
	<i>Vibrio fischeri</i>	15 min/Bioluminescence EC50	19.1	Farré et al. (2001)
	<i>Lemna gibba</i>	7 d/Wet weight Frond number EC50	>1	Brain et al. (2004)
	<i>Lemna minor</i>	7 d/Growth EC50	4	Pomati et al. (2004)
	<i>Hydra vulgaris</i>	7 d/Survival NOEC	>10	Pascoe et al. (2003)
	<i>Hydra attenuate</i>	96 h/LC50	22.36	Quinn et al. (2008)
	<i>Planorbis carinatus</i>	72 h/LC50	17.08 (5.9–72.3)	Pounds et al. (2008)
	<i>Daphnia magna</i>	48 h/Immobilization EC50	55.6	Knoll/BASF (2009)
	<i>D. magna</i>	48 h/Immobilization EC50	101.2 (89.2–114.9)	Cleuvers (2004)
	<i>D. magna</i>	48 h/LC50	132.6	Han et al. (2006)
	<i>D. magna</i>	48 h/Immobilization EC50	51.44 (40.17–62.71)	This study
	<i>Moina macrocopa</i>	48 h/Immobilization EC50	72.60 (55.0–90.4)	This study
	<i>Xenopus laevis</i>	96 h/Growth Deformity NOEC	20	Richards and Cole (2006)
	<i>X. laevis</i>	96 h/Growth LOEC (MCIG)	30	Richards and Cole (2006)
	<i>X. laevis</i>	96 h/Deformity EC10	30.7	Richards and Cole (2006)
	<i>X. laevis</i>	96 h/LC10	50.8	Richards and Cole (2006)
	<i>X. laevis</i>	96 h/Malformation (Thoracic Edema) EC50	39.9	Richards and Cole (2006)
	<i>Lepomis macrochirus</i>	96 h/LC50	173	Knoll/BASF (1995) cited in Webb (2004)
	Chronic	<i>P. carinatus</i>	21 d/Growth (wet weight) NOEC	1.02
<i>P. carinatus</i>		21 d/Reproduction (hatching success) NOEC	2.43	Pounds et al. (2008)
<i>P. carinatus</i>		21 d/Survival NOEC	5.36	Pounds et al. (2008)
<i>D. magna</i>		21 d/Reproduction NOEC	20	Han et al. (2006)
<i>D. magna</i>		21 d/Reproduction NOEC	<1.23	This study
<i>D. magna</i>		21 d/Survival NOEC	33.3	This study
<i>D. magna</i>		14 d/Reproduction EC50	13.4 (10.7–16.2)	Heckmann et al. (2007)
<i>D. magna</i>		14 d/Survival NOEC	20	Heckmann et al. (2007)
<i>D. magna</i>		14 d/Population growth rate NOEC	<20	Heckmann et al. (2007)
<i>M. macrocopa</i>		7 d/Reproduction NOEC	25	This study
<i>M. macrocopa</i>		7 d/Survival NOEC	>50	This study
<i>H. vulgaris</i>		7 d/Feeding LOEC	<0.01	Pascoe et al. (2003)
<i>H. vulgaris</i>		10 d/Bud formation NOEC	>10	Pascoe et al. (2003)
<i>H. attenuata</i>		96 h/Morphology EC50	1.65 (2.82–0.96)	Quinn et al. (2008)
<i>H. attenuata</i>		96 h/Morphology NOEC	0.1	Quinn et al. (2008)
<i>H. attenuata</i>		96 h/Feeding EC50	3.85	Quinn et al. (2008)
<i>Oryzias latipes</i>		6 weeks/Reproduction LOEC	0.1	Flippin et al. (2007)
<i>O. latipes</i>		30 dph/Survival NOEC	0.1	This study
<i>O. latipes</i>		90 dph/Survival NOEC	0.001	This study
<i>O. latipes</i>		120 dph/Survival NOEC	0.0001	This study
<i>O. latipes</i>		120 dph/Reproduction NOEC	0.001	This study

ship with exposure concentration (p for trend <0.05). Hatching was delayed among the eggs that were spawned from pairs which had been exposed to 0.1 $\mu\text{g/L}$ or greater. However, fertility and hatchability of the eggs were not affected by exposure to ibuprofen (Table 4).

4. Discussion

4.1. Hormone production and aromatase activity in H295R cells

Exposure of H295R cells to ibuprofen leads to not only greater production of E2 but also an increase in aromatase activity in a concentration dependent manner (Figs. 2 and 3). Increased aromatase activity could facilitate the conversion of T to E2 and therefore could lead to a reduction of T. In fact, concentrations of T were inversely proportional to concentration of ibuprofen to which cells were exposed (Fig. 2b). Ibuprofen did not directly affect the activity of the aromatase enzyme (Fig. 3b), but rather, increased aromatase activity by up-regulating transcription of mRNA (Fig. 3a). Changes in E2 concentration and aromatase activity are important because these changes could lead to the alterations in reproduction that have been reported in Flippin et al. (2007) and Heckmann et al. (2007).

It should be noted that the observed increase of E2 in plasma after the exposure to ibuprofen is contrary to what several other reports suggest. Ibuprofen and other NSAIDs can influence estrogen biosynthesis in different way (Brueggemeier et al., 2005; Terry

et al., 2004). Ibuprofen is thought to inhibit the COX pathway via competition with its substrate, arachidonic acid for the active sites of COX. Inhibition of the COX pathway reduces the synthesis of important eicosanoids such as prostaglandin E2 (PGE2). PGE2 increases intracellular cyclic adenosine monophosphate (cAMP) levels which up-regulates aromatase expression, which in turn, increases conversion of T into E2, and reduced PGE2 synthesis results in inhibition of estrogen production through modulation of aromatase (Brueggemeier et al., 2005). Aromatase (CYP19) is a cytochrome P450 enzyme that converts androstenedione into estrone (E1), or T into E2. Because E1 and E2 are two major estrogens in humans, inhibition of aromatase activity could reduce the concentration of estrogens and influence hormone balance and the physiological processes and behaviors that they control.

The mechanisms of E2 increase by ibuprofen exposure are not clear. One possibility is that pathways other than the COX enzyme might be associated with the ibuprofen-caused increase in aromatase activity and subsequent production of E2 by H295R cells. Another possibility is an interspecies variation of COX enzyme activity in response to ibuprofen exposure. Although the COX enzyme is known to influence synthesis of eicosanoids in both vertebrates and invertebrates (Hayashi et al., 2008), the effect of ibuprofen on COX activity does not appear to be the same among organisms: Ibuprofen lead to reduction of COX activity in female medaka that were exposed at 100 $\mu\text{g/L}$ for 6 weeks (Flippin et al., 2007). In *D. magna*, however, microarray experiments showed increased transcription of the COX gene after the exposure to

ibuprofen (Heckmann et al., 2008). This suggests that ibuprofen might influence the COX pathway differently in medaka (fish) than in *Daphnia* (crustacean). Further investigations are needed for verifying sex hormone induction in medaka, and elucidating the underlying mechanisms of steroidogenic alteration of ibuprofen exposure.

4.2. Effects on *Daphnia* and fish

Longer durations of exposure (21 d) of *D. magna* to ibuprofen resulted in greater effects. The LOEC for effects on reproduction was 1.23 mg/L, which was the least concentration studied, while the 48 h immobility EC50 was 51.4 mg/L. However for *M. macrocopa*, reproduction NOEC was observed at 25 mg/L, and it was not much different from 48 h immobility EC50 of 72.6 mg/L. Acute and chronic effects of ibuprofen or reproduction of other aquatic invertebrates have been reported to be in the mg/L range (Table 5). Considering reproduction-related effects due to chronic exposure, the sensitivity of *D. magna* observed in this study was close to that of the freshwater gastropod *Planorbis carinatus*. However, *M. macrocopa* appeared to be more tolerant of chronic ibuprofen exposure compared to other invertebrates. Lethality of *Daphnia* would not be expected at concentrations of ibuprofen observed in the environment.

Mortality of medaka due to ibuprofen exposure increased in a time-dependent manner as fish matured (Table 2). At 120 dph, exposure to as low as 1 µg/L ibuprofen resulted in significantly less survival of medaka than controls, while survival of 7 dph fry was not affected even at the maximum exposure concentration of 1000 µg/L. Considering the pseudo-persistent nature of pharmaceutical exposure and a relatively high acute-to-chronic ratio of ibuprofen as great as 1000, potential hazard of long-term exposure to ibuprofen needs to be further investigated.

In addition, reproduction-related endpoints like vitellogenin were affected by ibuprofen exposure in medaka. Vitellogenin is one of the most well documented biomarkers of exposure to endocrine disruptors, and is widely accepted as a valid biomarker for estrogenic chemicals in oviparous species that has been validated to be an indicator of population-level effects on reproduction (Sumpter and Jobling, 1995).

To date, the longest term study of the effects of ibuprofen on fish has been a 6-week exposure with medaka (Flippin et al., 2007). In that study exposure to 100 µg/L ibuprofen resulted in fewer spawning events, but increased number of eggs per brood. This is consistent with the results of the present study, in which the number of broods per pair of fish was inversely proportional to ibuprofen concentration, while the number of eggs per brood was directly proportional to concentration of ibuprofen with a threshold of 10 µg/L ibuprofen (p for trend <0.01, Table 3). The larger brood size in fish might be a compensatory response to reproductive damage caused by ibuprofen exposure. In *D. magna*, a similar compensatory increase in fecundity has been reported during a 10 d depuration period after 10 d exposure to up to 80 mg/L ibuprofen (Hayashi et al., 2008). In addition, the inhibition of COX pathway by ibuprofen is thought to be reversible.

It is noteworthy that parental exposure to 0.1 µg/L ibuprofen appeared to influence the time required for hatching of eggs even when the eggs were relocated to clean water. The concentration of 0.1 µg/L is within the ecologically relevant range observed in surface waters (Kasprzyk-Hordern et al., 2008). In the ecosystem such delays in time-to-hatch may increase the risk of being predated because of lack of mobility, thus PGR might be influenced.

Overall, our results suggest that reproduction-related effects of chronic exposure of medaka to ibuprofen may be in part explained by its effects on estrogen homeostasis. The relatively great acute-to-chronic ratio and observation of reproduction damage in fish

at environmentally relevant concentrations of ibuprofen warrant further studies to elucidate potential ecological consequences of ibuprofen contamination in water environment.

Acknowledgement

This study was supported by National Institute of Environmental Research and National Research Foundation of Korea (Project # 2009-0080808). The research was also supported by a Discovery Grant from the National Science and Engineering Research Council of Canada (Project # 326415-07) and a grant from the Western Economic Diversification Canada (Project # 6578 and 6807) to the University of Saskatchewan. The authors wish to acknowledge the support of an instrumentation grant from the Canada Foundation for Infrastructure. Prof. Giesy was supported by the Canada Research Chair program and an large Chair Professorship at the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2010.02.013.

References

- American Public Health Association, American Water Works Association, Water Pollution Control Federation, 1992. Standard Methods for the Examination of Water and Wastewater, 18th ed., American Public Health Association, Washington, DC, USA.
- Ankley, G.T., Brooks, B.W., Huggett, D.B., Sumpter, J.P., 2007. Repeating history: pharmaceuticals in the environment. *Environ. Sci. Technol.* 41, 8211–8217.
- Brain, R.A., Johnson, D.J., Richards, S.M., Sanderson, H., Sibley, P.K., Solomon, K.R., 2004. Effects of 25 pharmaceutical compounds to *Lemma gibba* using a seven-day static-renewal test. *Environ. Toxicol. Chem.* 23, 371–382.
- Brueggemeier, R.W., Hackett, J.C., Diaz-Cruz, E.S., 2005. Aromatase inhibitors in the treatment of breast cancer. *Endocr. Rev.* 26, 331–345.
- Brun, G.L., Bernier, M., Losier, R., Doe, K., Jackman, P., Lee, H.B., 2006. Pharmaceutically active compounds in Atlantic Canadian sewage treatment plant effluents and receiving waters, and potential for environmental effects as measured by acute and chronic aquatic toxicity. *Environ. Toxicol. Chem.* 25, 2163–2176.
- Christensen, A.M., Markussen, B., Baun, A., Halling-Sørensen, B., 2009. Probabilistic environmental risk characterization of pharmaceuticals in sewage treatment plant discharges. *Chemosphere* 77, 351–358.
- Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. *Ecotox. Environ. Safe.* 59, 309–315.
- Dean, R.B., Dixon, W.J., 1951. Simplified statistics for small numbers of observations. *Anal. Chem.* 23 (4), 636–638.
- Farré, M., Ferrer, I., Ginebreda, A., Figueras, M., Olivella, L., Tirapu, L., Vilanova, M., Barceló, D., 2001. Determination of drugs in surface water and wastewater samples by liquid chromatography–mass spectrometry: methods and preliminary results including toxicity studies with *Vibrio fischeri*. *J. Chromatogr. A* 938, 187–197.
- Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. *Aquat. Toxicol.* 76, 122–159.
- Flippin, J.L., Huggett, D., Foran, C.M., 2007. Changes in the timing of reproduction following chronic exposure to ibuprofen in Japanese medaka, *Oryzias latipes*. *Aquat. Toxicol.* 81, 73–78.
- Gómez, M., Bueno, M.M., Lacorte, S., Fernández-Alba, A., Agüera, A., 2007. Pilot survey monitoring pharmaceuticals and related compounds in a sewage treatment plant located on the Mediterranean coast. *Chemosphere* 66, 993–1002.
- Gracia, T., Hilscherova, K., Jones, P.D., Newsted, J.L., Higley, E.B., Zhang, X., Hecker, M., Murphy, M.B., Yu, R.M.K., Lam, P.K.S., Wu, R.S.S., Giesy, J.P., 2007. Modulation of steroidogenic gene expression and hormone production of H295R cells by pharmaceuticals and other environmentally active compounds. *Toxicol. Appl. Pharm.* 225, 142–153.
- Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lützhøft, H.C., Jørgensen, S.E., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment—a review. *Chemosphere* 36, 357–393.
- Han, G.H., Hur, H.G., Kim, S.D., 2006. Ecotoxicological risk of pharmaceuticals from wastewater treatment plants in Korea: occurrence and toxicity to *Daphnia magna*. *Environ. Toxicol. Chem.* 25, 265–271.
- Hayashi, Y., Heckmann, L.H., Callaghan, A., Sibly, R.M., 2008. Reproduction recovery of the crustacean *Daphnia magna* after chronic exposure to ibuprofen. *Ecotoxicology* 17, 246–251.
- Hecker, M., Newsted, J.L., Murphy, M.B., Higley, E.B., Jones, P.D., Wu, R., Giesy, J.P., 2006. Human adrenocarcinoma (H295R) cells for rapid in vitro determination

- of effects on steroidogenesis: Hormone production. *Toxicol. Appl. Pharm.* 217, 114–124.
- Heckmann, L.H., Callaghan, A., Hooper, H.L., Connon, R., Hutchinson, T.H., Maund, S.J., Sibly, R.M., 2007. Chronic toxicity of ibuprofen to *Daphnia magna*: effects on life history traits and population dynamics. *Toxicol. Lett.* 172, 137–145.
- Heckmann, L.H., Sibly, R.M., Connon, R., Hooper, H.L., Hutchinson, T.H., Maund, S.J., Hill, C.J., Bouetard, A., Callaghan, A., 2008. Systems biology meets stress ecology: linking molecular and organismal stress responses in *Daphnia magna*. *Genome Biol.* 9, R40.
- Higley, E.B., Newsted, J.L., Zhang, X., Giesy, J.P., Hecker, M., 2010. Assessment of chemical effects on aromatase activity and E2 and T production using the H295R cell line. *Environ. Sci. Pollut. Res.* doi:10.1007/s11356-009-0285-3.
- Hutchinson, T.H., Brown, R., Brugger, K.E., Campbell, P.M., Holt, M., Länge, R., McCahon, P., Tattersfield, L.J., Egmond, R.V., 2000. Ecological risk assessment of endocrine disruptors. *Environ. Health Perspect.* 108, 1007–1014.
- Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2008. The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. *Water Res.* 42, 3498–3518.
- Kim, S.D., Cho, J., Kim, I.S., Vanderford, B.J., Snyder, S.A., 2007. Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. *Water Res.* 41, 1013–1021.
- Knoll/BASF, 2009. Safety data sheet: Ibuprofen 25 US Quality. Knoll pharmaceuticals, Nottingham. Issue/revision date 10 August 2009.
- OECD, 1992a. OECD Guideline for testing of chemicals, Test No. 210: Fish, Early-Life Stage Toxicity Test. Section 2: Effects on Biotic Systems. Organization of Economic Cooperation and Development, Paris.
- OECD, 1992b. OECD Guidelines for testing of chemicals, Test No. 203: Fish, Acute Toxicity test. Section 2: Effects on Biotic systems. Organization of Economic Cooperation and Development, Paris.
- OECD, 2008. OECD Guideline for testing of chemicals, Test No. 211: *Daphnia magna* Reproduction Test. Section 2: Effects on Biotic Systems. Organization of Economic Cooperation and Development, Paris.
- Oh, S., 2007. Development of a Standard 7-day Chronic Toxicity Test Method using indigenous aquatic Macroinvertebrate *Moina macrocopa*. Master of Public Health thesis. Seoul National University, Seoul.
- Park, S., Choi, K., 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. *Ecotoxicology* 17, 526–538.
- Pascoe, D., Karntanut, W., Müller, C.T., 2003. Do pharmaceuticals affect freshwater invertebrates? A study with the cnidarians *Hydra vulgaris*. *Chemosphere* 51, 521–528.
- Pomati, F., Netting, A.G., Calamari, D., Neilan, B.A., 2004. Effects of erythromycin, tetracycline and ibuprofen on the growth of *Synechocystis* sp. and *Lemna minor*. *Aquat. Toxicol.* 67, 387–396.
- Pounds, N., Maclean, S., Webley, M., Pascoe, D., Hutchinson, T., 2008. Acute and chronic effects of ibuprofen in the mollusc *Planorbis carinatus* (Gastropoda: Planorbidae). *Ecotox. Environ. Saf.* 70, 47–52.
- Quinn, B., Gagné, F., Blaise, C., 2008. An investigation into the acute and chronic toxicity of eleven pharmaceuticals (and their solvents) found in wastewater effluent on the cnidarian, *Hydra attenuata*. *Sci. Total. Environ.* 389, 306–314.
- Richards, S.M., Cole, S.E., 2006. A toxicity and hazard assessment of fourteen pharmaceuticals to *Xenopus laevis* larvae. *Ecotoxicology* 15, 647–656.
- Sanderson, J.T., Letcher, R.J., Heneweer, M., Giesy, J.P., van den Berg, M., 2001. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. *Environ. Health Perspect.* 109, 1027–1031.
- Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect.* 103, 173–178.
- Terry, M.B., Gammon, M.D., Zhang, F.F., Tawfik, H., Teitelbaum, S.L., Britton, J.A., Subbaramaiah, K., Bannenberg, A.J., Neugut, A.I., 2004. Association of frequency and duration of aspirin use and hormone receptor status with breast cancer risk. *JAMA* 291, 2433–2440.
- US Environmental Protection Agency, 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. US EPA, Washington DC, USA.
- Webb, S.F., 2004. A data-based perspective on the environmental risk assessment of human pharmaceuticals 1—collation of available ecotoxicity data. In: Kümmerer, K. (Ed.), *Pharmaceuticals in the Environment*, 2nd ed. Springer-Verlag, Berlin, Heidelberg, New York, pp. 317–343.