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Ibuprofen reduces zebrafish PGE₂ levels but steroid hormone levels and reproductive parameters are not affected

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1. Introduction

Human pharmaceuticals are excreted and eliminated into wastewaters either as the parent compound or as metabolites. Removal rates of pharmaceuticals in sewage treatment plants (STPs) are generally high although not 100%. Hence, persistent or highly consumed pharmaceuticals like analgesics have been detected in freshwater systems at ng/L–μg/L levels. We investigated whether ibuprofen would affect prostaglandin and sex steroid hormone levels in adult zebrafish (Danio rerio) and if expression levels of genes involved in steroidogenesis and prostaglandin synthesis were affected. Zebrafish were exposed to moderate concentrations of ibuprofen (21, 201 or 506 μg/L) for 7 days in a semi-static test system. Ibuprofen concentrations were close to nominal levels and decreased by a maximum of 12–13% over 24 h. Prostaglandin E₂ (PGE₂) levels in whole body homogenates of males and ovaries of females decreased in a monotonic dose–response relationship whereas male 11-ketotestosterone levels and ovarian 17β-estradiol levels remained unchanged. Ibuprofen did not have an influence on vitellogenin levels, female gonadosomatic index or cumulative egg production and no dose–response relationship in ovarian and testicular expression levels of the investigated genes was observed. This study shows that ibuprofen reduces PGE₂ levels in male and female zebrafish but has no consistent effects on other investigated reproductive parameters.

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2. Materials and methods

2.1. Animals and experimental design

Zebrafish (D. rerio) were purchased from DAP International (Etobicoke, ON, Canada) and held at the Hagen Aquaslab at the University of Guelph in an A-HAB fish containment unit (Aquatic Habitats, Apopka, FL, USA) with re-circulated well water at 26 °C. The photoperiod was 12 h light:12 h dark. Fish were acclimatized for one week in the test aquaria and fed three times daily: twice with commercial salmon fry pellets (Martin Mills, Elmira, ON, Canada) and once with bloodworms (Oregon Desert Brine Shrimp Co., Lakeview, OR, USA).

The exposure was performed as a 7-day semi-static exposure in 20 L glass aquaria each containing 15 L of water. The water was changed every day and water temperature and oxygen saturation were measured every second day. Each aquarium was aerated and the oxygen level was 91% ± 1.04 (mean ± SEM) of the air saturation and the temperature was 25.5 ± 0.8 °C. Egg trays with artificial plants were placed in the tanks and eggs were collected and counted every day after spawning. Egg collection started four days before the exposure. Each exposure was performed in triplicate with 26 fish (12 females and 14 males) in each aquarium.

Ibuprofen (CAS no. 15687-27-1, Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved in methanol and added to the test aquaria every day when water was renewed. The nominal ibuprofen concentrations were 0, 20, 200 and 500 μg/L and the final solvent concentration was <0.01%.

2.2. Actual water concentrations of ibuprofen

Water samples (2 mL) were collected from each aquarium three times during 24 h (0.5–1, 3–4 and 24 h after water renewal). Water samples were passed through PVDF filters (pore size 0.45 μm, Frisenette, Denmark) before the actual concentrations were determined on a Triple Quad LC/MS (Agilent, Wilmington, DE, USA). Isocratic elution was performed using an Agilent Zorbax Eclipse XDB C-18 Rapid Resolution HT column (50 × 4.6 mm i.d., 1.8 μm particle size) and column temperature was set at 70 °C. Injection volume was 30 μL. The mobile phase consisted of a 40:60 (v/v) mixture of 0.1% formic acid and acetonitrile (pH = 3.0) and a flow rate of 1.0 mL/min (retention time: 1.39 min). Analysis was done using electrospray ionization (ESI) in positive ion mode. Drying gas flow was 11.0 L/min and drying gas temperature was set at 350 °C. The nebulizer pressure was set at 35 psi and capillary voltage at 4000 V. Fragmentor voltage was 90 V and collision energy 15 V. The mass-to-charge-ratio (m/z) of precursor ion and quantifier ion was respectively 224.3 and 161.2. The detection limit (signal-to-noise-ratio > 10) for ibuprofen in sample matrix was 11.7 pg on column (0.39 pg/μL).

2.3. Sampling

After seven days of exposure the fish were anesthetized in MS-222 (0.1 g/L), blot dried on KIM™ wipes, and weighed. Individuals used for gene expression had their head and tail separated from the trunk with cuts made right behind the pectoral fins and right behind the dorsal fin, respectively. The head and tail fraction was weighed and immediately put on dry ice and stored at −80 °C until vitellogenin analysis was performed. The ovaries were removed from the trunk and put in RNase-free microcentrifuge tubes, weighed, and frozen in dry ice. The trunks of the males were placed on dry ice with the body cavity opened and intestines removed in order to make the testes easier to locate. The testes were dissected out and placed in RNase-free microcentrifuge tubes. All female fish for gene expression studies were sampled between 10:00 and 11:30 h and all males between 12:00 h and 14:00 h. Male fish for whole body steroid determination were snap frozen in microcentrifuge tubes after the presence of testis had been verified. Ovaries for steroid determination were dissected as described above and GSI was calculated for all female fish by the following equation: GSI = (gonad mass (mg)/[(body mass (mg) − gonad mass (mg))] × 100.

2.4. RNA extractions

Ovarian tissue from 6 females and testis tissue from 7 to 8 males per aquarium were used for gene expression studies. Gonad tissue was homogenized with a handheld homogenizer and RNA was extracted from the tissue with Trizol® (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. Chloroform and isopropanol were purchased from Sigma-Aldrich Co. During extraction of testes 3.75 μL of a 10 times diluted glycogen solution (Roche, 20 mg/mL) was added to the aqueous phase to aid in RNA precipitation. The RNA pellets from ovary extractions were re-suspended in 30–50 μL Gibco™ water (Invitrogen) whereas the volume used for tests was 10 μL.

RNA concentrations were quantified on a NanoDrop-8000 spectrophotometer (Agilent Technologies, Mississauga, ON, Canada) and diluted to 1000 ng/μL (females) or 800 ng/μL (males). RNA quality of each sample was determined by the ratio of absorbance at 260 nm and 280 nm. Further, RNA integrity of a subset of RNA samples was assessed using an Agilent 2100 Bioanalyzer and a RNA 6000 Nano LabChip kit (Agilent Technologies). Before reverse transcription (RT) the samples were quantified again with the NanoDrop-8000 spectrophotometer and DNase treated by mixing each sample with 2 μL DNase I (AMP-D1: Sigma-Aldrich) according to manufacturer’s instructions. Primers were designed according to Ings and Van Der Kraak (2006) and the sequences are shown in Table 2. In order to prevent genomic DNA amplification primers were designed to span an exon–exon boundary in the mRNA sequence.

2.5. Reverse transcription and real-time PCR (RT-PCR)

For the reverse transcription (RT) reaction a total of 2 and 1.6 μg RNA for each sample was used for females and males, respectively. RT water controls were prepared by adding 2 μL of Gibco™ water instead of sample. The samples were incubated for 5 min at 70 °C with 2 μL (0.1 μg) random primer (Promega, Madison, WI, USA) per tube and immediately transferred to ice. The total reaction volume was
brought to 25 μL and contained the following final concentrations: 5 × RT first strand buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM MgCl₂; Invitrogen), DTT (10 mM; Invitrogen), dNTPs (0.5 mM; Roche Molecular Biochemicals, Laval, PQ, Canada), M-MLV reverse transcriptase (200 U; Invitrogen) and Gibco™™ water. The reaction was performed at 37 °C for 1 h followed by 5 min at 90 °C and subsequently cooling down to 4 °C.

qPCR was performed using ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) and each sample was run in duplicate. The following was added to each reaction: 5 μL cDNA template (diluted 5, 10 or 20 × depending on gene of interest), 2.5 μL of both forward and reverse primers (0.4 μM) and 10 μL SYBR® Green PCR Master Mix. The qPCR cycling conditions were: 2 min at 95 °C; 40 cycles of 15 s at 95 °C and 1 min at 60 °C (Lister and Van Der Kraak, 2009). To demonstrate primer specificity and amplification of a single product, a melting curve analysis was applied to all reactions.

2.6. Determination of steroid hormones and PGE₂

Six females and six males from each aquarium were used for steroid and PGE₂ measurements. Extraction of steroids was performed on dissected ovaries and whole body homogenate of males. The ovary tissue was sonicated after addition of 100 μL of a 0.1 M PBS buffer (Na₂HPO₄, NaH₂PO₄ and NaCl; pH 7.4) with 1 mM EDTA and 10 μM indomethacin. Whole body male fish were pulverized in a chilled ceramic mortar filled with liquid nitrogen and the homogenate was mixed with 4 × homogenate weight (v/v) of PBS buffer and then sonicated.

Ovaries and whole body homogenates were extracted with 400 μL of a 0.1 M PBS buffer (NaCl, 75 mM KCl, 3 mM MgCl₂; pH 7.4). Ethyl acetate (1% methanol) was used to collect the final elute from ovaries and 50:50 methanol/acetonitrile (1% acetic acid) was used to elute the male samples. This fraction was dried down under a stream of nitrogen or in a TurboVap®. Ovary samples were reconstituted in 300 μL EIA buffer whereas males were reconstituted in 1000 μL E₂, 11-KT and PGE₂ levels were analyzed by enzyme immuno assays (EIA; Cayman Chemical, Ann Arbor, MI, USA) as per manufacturer’s instructions. Plate development times for E₂, 11-KT and PGE₂ were 120, 30 and 90–120 min respectively.

2.7. Vitellogenin by ELISA

Homogenization and preparation of the samples for vitellogenin analysis was performed according to Kinberg et al. (2007). Direct non-competitive sandwich ELISA was used to quantify vitellogenin concentrations in the supernatant of the sample homogenate according to Holbech et al. (2001) with the modifications described in Morthorst et al. (2010).

2.8. Data analysis and statistics

Prior to analyses all data sets were screened for homogeneity of variance and normality and if necessary data were log transformed. To make sure the replicates in each exposure group did not differ from each other a one-way ANOVA was performed before values from the replicates were pooled. A Bonferroni–Holm corrected one-way ANOVA was performed to compare the exposure groups with the control. The software package SigmaStat® Statistical Software version 2.0 was used in all statistical analyses and statistical differences were considered significant if p < 0.05.

3. Results

3.1. Chemical analysis of ibuprofen concentrations in water

The measured concentrations of ibuprofen during the 24-hour period between water renewals are shown in Table 3. The variation between the replicates within a treatment was minimal. Ibuprofen concentrations were constant throughout the experiment and the
Similar to the control, whole body male and ovarian PGE2 levels were significantly different in any of the treatment groups. No effect was observed on the expression levels of the remaining investigated genes in ovaries (\(cpla2\), \(cyp11\), \(17\beta\)-\(hsd2\), \(17\beta\)-\(hsd3\), \(star\) and \(ef1\)) (Figs. S2 and S3). No effect was observed in the expression levels of the remaining investigated genes in ovaries (\(ptgs1\), \(ptgs2\), \(cyp19a\), \(\alpha\)-\(11\)-\(cyp\), \(\beta\)-\(11\)-\(cyp\), \(\alpha\)-\(17\)-\(cyp\), \(\beta\)-\(17\)-\(cyp\), \(\alpha\)-\(fl\)-\(cyp\), \(\beta\)-\(fl\)-\(cyp\), \(\alpha\)-\(sh\)-\(cyp\), \(\beta\)-\(sh\)-\(cyp\)).

3.5. Egg production and GSI

Egg production was not affected at any of the exposure concentrations (Fig. 4). GSI of the females was not affected in a significant way but a slight increase in GSI was observed in the high exposure group (Fig. 5).

4. Discussion

Due to high consumption rates and insufficient removal in STPs human and veterinary pharmaceuticals have been detected in aquatic ecosystems and aquatic organisms might therefore be continuously exposed to highly consumed pharmaceuticals like analgesics (Ankley et al., 2007). However, only few experiments have investigated the possible effects of analgesics on aquatic vertebrates. Mild analgesics inhibit the enzymes responsible for prostaglandin synthesis, cyclooxygenase-1 and -2 (COX-1 and COX-2). Prostaglandins and their precursor arachidonic acid play key roles in vertebrate reproduction for example oocyte maturation, ovulation and regulation of gonadal steroidogenesis (Van Der Kraak and Chang, 1990; Wade and Van Der Kraak, 1993; Sorbera et al., 2001; Patino et al., 2003). Both steroid- and prostaglandin-derived sex pheromones have been identified in fish (Stacey, 2003), and exposure to prostaglandin F\(_{2\alpha}\) (PGF\(_{2\alpha}\)) increases goldfish testoste-

### Table 3

<table>
<thead>
<tr>
<th>Nominal concentration (µg/L)</th>
<th>Actual concentration (µg/L)</th>
<th>Actual concentrations over time between water renewals (µg/L)</th>
<th>n=17 n=18 n=18 n=17</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>20</td>
<td>21.7 ± 0.5</td>
<td>22.7 ± 0.4</td>
<td>21.3 ± 0.7</td>
</tr>
<tr>
<td>200</td>
<td>201.5 ± 7.8</td>
<td>215.5 ± 21.1</td>
<td>200.4 ± 8.8</td>
</tr>
<tr>
<td>500</td>
<td>506 ± 10.9</td>
<td>547.8 ± 6.1</td>
<td>487.9 ± 4.6</td>
</tr>
</tbody>
</table>

Fig. 1. Male and female PGE2 concentrations. PGE2 concentrations in (A) male whole body homogenate and (B) ovaries of adult zebrafish. Data represent mean ± SEM. The number of male and female fish in each exposure group is indicated in the bottom of the figure. * indicates significantly different from the control group (p<0.05).
long-term study (132 days) ibuprofen did also not affect GSI in medaka (Han et al., 2010) but reduced spawning events and increased number of eggs was observed which is in accordance with another study (Flippin et al., 2007). The effect of ibuprofen on spawning in the medaka could be due to a longer exposure period but also zebrafish egg production can be difficult to assess, as the daily egg production is variable and affected by temperature, age, spawning strategy etc. (Paull et al., 2008). Indomethacin caused reduced egg production and reduced PGE2 levels in female zebrafish after 16 days of exposure (Lister and Van Der Kraak, 2008), but it remains unclear whether reduced egg production was caused by changes in male reproductive behavior or oocyte maturation and ovulation.

Analgesics impair testosterone synthesis in male rats (Didolkar et al., 1980; Kristensen et al., 2011, 2012) and Fernandes et al. (2011) recently showed, that ibuprofen inhibits enzymes involved in androgen synthesis in gonads of male carp in vitro. On the other hand ibuprofen did not influence testosterone levels in male mice (Martini et al., 2008) and generally mammalian studies have provided conflicting results regarding effects of analgesics on steroid production, which could be explained by the fact that analgesics have different mechanisms of action even though acting on the same enzyme and in addition test species, timing and duration of exposure may also play a role (Didolkar et al., 1981; Gupta and Goldman, 1986; Wise et al., 1991). Altered steroid production due to short-term exposure to endocrine disrupters has been demonstrated (Andersen et al., 2006) but in our experiment E2 and 11-KT levels in gonads of male carp were not affected. (Andersen et al., 2006) but in our experiment E2 and 11-KT levels in gonads of male carp were not affected.

Fig. 2. Male and female 11-KT and E2 concentrations. (A) 11-KT concentrations in male whole body homogenate and (B) E2 concentrations in ovaries. Data represent mean±SEM. The number of male and female fish in each exposure group is indicated in the bottom of the figure.

Alteration in levels of the estrogen-dependent yolk precursor vitellogenin is a well-known biomarker for exposure to endocrine disrupters in fish (Tyler et al., 1999; Holbech et al., 2012). Ibuprofen did not affect male or female vitellogenin levels (Fig. 3), which suggests that ibuprofen does not affect steroidogenesis through the estrogen receptor pathway. This is in accordance with results from the yeast estrogen screen assay (YES assay) in our lab where ibuprofen and other analgesics failed to induce a response by binding to the human estrogen receptor (data not published).

Fig. 3. Male and female vitellogenin concentrations. Vitellogenin concentrations in (A) males and (B) females. Four outlying male values were removed. Data represent mean±SEM. The number of male or female fish in each exposure group is indicated in the bottom of the figure.

Steroids are synthesized through multiple steps and numerous enzymes are involved and changes in expression levels of involved enzymes and substrate levels can influence hormone levels. C17, 20-lyase (also called P450c17 and CYP17) and 11β-hydroxylase (CYP11β) are involved in androgen synthesis. In an in vitro system with male carp gonads analgesics impaired the activity of CYP11β and CYP17 (Fernandes et al., 2011) but ibuprofen did not influence adult male 11-KT levels in our study. Altered steroid production due to short-term exposure to endocrine disrupters has been demonstrated (Andersen et al., 2006) but in our experiment E2 and 11-KT levels of adult fish were not affected by ibuprofen (Fig. 2). If long-term depression could influence steroid metabolism and subsequent reproductive processes related to hormone levels was beyond the scope of this experiment.

In vitro
Both cpla2 and ptgs2 are involved in prostaglandin synthesis and ovarian expression levels of those genes fluctuate during the ovulatory cycle of spawning zebrafish whereas expression of ptgs1 remains fairly stable (Lister and Van Der Kraak, 2009). In this experiment ovarian expression of cpla2 in the intermediate exposure group (201 µg/L) was significantly different from the control (Fig. S2). Zebrafish ovarian development is asynchronous with follicles in different stages. The proportions of follicles in different stages vary between individuals and could explain the significant difference of cpla2 expression even though sampling occurred at the same time every day. Ibuprofen inhibits the COX enzymes by substrate competition and a response to overcome sampling occurred at the same time every day. Ibuprofen inhibits the COX enzyme catalyzing the conversion of androgens to estrogens. Arachidonic acid and its metabolites are involved in regulation of STAR, the enzyme responsible for transport of cholesterol from the outer to the inner mitochondrial membrane (Wang et al., 2003) and hence changes in prostaglandin levels could also influence cholesterol transport and thereby steroid synthesis. However, ibuprofen did not influence female star and cyp19a1 or male star gene expression (Fig. S2) and also not 11-KT and E2 levels. Ibuprofen significantly increased both E2 production and aromatase activity by up-regulation of mRNA transcription in H295R cells (Han et al., 2010) and a tendency to decreased testosterone production was also observed, which could be due to increased aromatase activity. Those abovementioned equivocal results suggest that NSAIDs may influence vertebrate species differently and illustrate that further investigations are needed to understand the impact of NSAIDs on aquatic wildlife.

Prostaglandins are involved in many pathways and processes and their synthesis is regulated through different pathways. In the present experiment we observed a significant effect of ibuprofen on PGE2 levels at concentrations also detected in effluent from STPs (Metcalfe et al., 2003; Camacho-Munoz et al., 2010) but no consistent effects on other reproductive endpoints were found. Prostaglandins have been shown to affect several sperm parameters in mammals and a prolonged reduction of prostaglandin levels could lead to more severe effects due to changes in fertilization success. More research is needed in the field of secondary effects of prostaglandin disruption in fish, as wild fish populations are exposed to numerous pharmaceuticals including several NSAIDs in concentrations able to affect endogenous prostaglandin levels.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.

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