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Two common mild analgesics have no effect on general endocrine mediated endpoints in zebrafish (*Danio rerio*)

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Abstract

Mild analgesics such as acetylsalicylic acid (ASA) and acetaminophen (APAP) exert their pain-relieving effect in humans by inhibition of prostaglandin synthesis. Prostaglandins play key roles in developmental and reproductive processes in vertebrates, and in recent years, it has been suggested that weak analgesics might also act as endocrine disruptors. In a set of experiments we investigated if ASA and APAP affect well-established endocrine endpoints in zebrafish (*Danio rerio*), which is a commonly used model organism in the investigation of endocrine disrupting chemicals. Zebrafish were exposed to APAP (0.22, 2.3, and 30 mg/L) or ASA (0.2, 0.5, 1.7, and 8.2 mg/L) from hatch to sexual maturity in a test design resembling the OECD Fish Sexual Development Test. No effects on sex ratio and vitellogenin levels were observed. Adult zebrafish were exposed to high concentrations (mg L^{-1}) of ASA or APAP for eight or 14 days. ASA reduced the levels of prostaglandin E_2 , but had no effect on the concentration of 11-ketotestosterone and vitellogenin. Overall, ASA decrease prostaglandin E_2 concentrations, but well-established endpoints for endocrine disruption in zebrafish are generally not affected by aquatic exposure neither during development nor adulthood. According to the WHO/IPCS definition of an endocrine disrupter, the present results do not define APAP and ASA as endocrine disrupters.

Introduction

Mild analgesics are sold over-the-counter under various trade names, and the most common active ingredients in non-prescription mild analgesics are acetylsalicylic acid (ASA), ibuprofen, and paracetamol also called acetaminophen (APAP). The sales of mild analgesics have been increasing in many countries during the latest decades (Hudec *et al.*, 2012; Kristensen *et al.*, 2016). For example, the annual sale in Denmark in 2015, including both over-the-counter and by prescription sales, was 155,000,000 DDD (defined daily dose) corresponding to >27 DDD per person in 2015 (The Danish Health Data Authority).

Mild analgesics exert their analgesic and antipyretic effects by inhibiting the synthesis of prostaglandins, which are modified fatty acids. Arachidonic acid is the primary precursor of prostaglandins, and it is converted into various prostaglandins by action of the bi-functional cyclooxygenase enzymes. There are two isoforms of the cyclooxygenase enzymes, Cox-1 and Cox-2, in humans and also in zebrafish (*Danio rerio*) (Grosser *et al.*, 2002; Ishikawa *et al.*, 2007). Prostaglandins play an important role in several reproductive processes such as ovulation and sexual behaviour in both mammals and fish (reviewed by Cha *et al.* (2006) and Corcoran *et al.* (2010)). Because prostaglandins play a role in the masculinisation of the male foetus during mammalian development concern has also been raised over human prenatal exposure to analgesics (reviewed by Kristensen *et al.* (2016)). Recently, mild analgesics were shown to have an endocrine disrupting potential during foetal life: intake of analgesics during pregnancy was associated with cryptorchidism in humans (Jensen *et al.*, 2010; Kristensen *et al.*, 2011), exposure to APAP during foetal development de-masculinised male rats (Kristensen *et al.*, 2011; Hay-Schmidt *et al.*, 2017), and anti-estrogenic effects were observed in testes of humans and rats exposed to mild analgesics (Jensen *et al.*, 2010; Kristensen *et al.*, 2011; Albert *et al.*, 2013). Prostaglandins are involved in both

ovulation and gonadal differentiation in zebrafish, and they have also been demonstrated to influence steroidogenesis in cultured goldfish testis (Wade & Van Der Kraak, 1993; Lister & Van Der Kraak, 2008; Pradhan & Olsson, 2014). Sexual development of fish is under strong influence of sex steroids, and several endocrine disrupters affect the sex ratio and yolk protein (vitellogenin) synthesis in zebrafish (Kinnberg *et al.*, 2007; Morthorst *et al.*, 2010; Holbech *et al.*, 2012; Holbech *et al.*, 2013), which are both well-established and recognised endocrine mediated endpoints included in the OECD Test Guideline 234 Fish Sexual Development Test (FSDT) (OECD, 2011). Because prostaglandins are involved in reproductive processes, gonadal differentiation, and steroid synthesis the prostaglandin inhibitors, such as APAP and ASA, might also affect sexual development of zebrafish, and therefore the aims of the experiments were to investigate 1) if exposure to APAP and ASA affected prostaglandin synthesis, steroid production and vitellogenin levels in adult zebrafish, and 2) if exposure to APAP and ASA during sex determination and differentiation affected sex ratio and vitellogenin levels.

Here we attempt to summarise the data obtained from of a series of experiments carried out over a period of several years during the mid 2000s, when the OECD test guideline 234 was still undergoing validation and information about e.g. the aquatic toxicity of analgesics was scarce.

Materials and methods

An overview of the experiments, including experimental details and endpoints, is provided in Table 1.

2.1. Chemicals

Acetaminophen (APAP) (99%, Cas No 103-90-2) and acetyl salicylic acid (ASA) (99%, Cas No 50-78-2) were purchased from Sigma-Aldrich (Vallensbæk Strand, Denmark). Stock solutions were

made in ASTM type-1a water (Elga, PURELAB), and fresh stock solutions were prepared once or twice per week.

2.2. *Animals and housing*

Adult zebrafish were obtained from a local supplier (credofish.dk) and the fish were acclimatized for several weeks in the zebrafish unit at University of Southern Denmark in tap water (ground water) mixed with deionized water. The test systems in all experiments consisted of 8 L glass aquaria with 6 L of water and a water exchange of 18 L per 24 hours (flow-through), and the test systems had been running for two days before the embryos or adult fish were added. The water temperature and oxygen saturation was measured once or twice per week. The temperature was $27\pm 1^\circ\text{C}$ and the average oxygen saturation level in the tanks was between 66 and 73% of the air saturation value (on the day of sampling single oxygen saturation levels of 55% were measured in the APAP FSDT because the pumps were paused for a few hours). The photoperiod was 14:10 h (light:dark), except in the ASA FSDT and the 8 days adult exposure where it was 12:12 h. The pH and conductivity of the water were kept within the recommended range for zebrafish husbandry (Brand *et al.*, 2002). The supply of both water and test compounds was controlled by peristaltic pumps (Ole Dich Instrument Makers, Denmark).

2.3. *Fish Sexual Development Test (FSDT) exposures with APAP and ASA*

Overall, the FSDT experiments followed the OECD test guideline 234 but with smaller differences regarding the experimental design because the experiments were performed before the guideline was validated. These differences are mentioned in the text below. To obtain zebrafish eggs, breeding boxes with artificial plants were put in a breeding tank with parent fish late in the afternoon. The following morning (0 days post fertilization (dpf)) eggs were collected and unfertilized eggs were removed. The fertilised eggs were kept in groups of 100 in 400 mL beakers at $27.5\pm 1^\circ\text{C}$ for two to three days to minimize embryo and larvae mortality in the tanks during the

exposure period, and every morning the undeveloped embryos were removed and substituted with embryos from a reservoir. A total of 40 (APAP FSDT) or 80 (ASA FSDT) embryos were added to each tank (Table 1). At the age of 4 dph (=7 dpf) larvae were fed once or twice daily with Sera Micron powdered food for fry (Heisenberg, Germany) and at 7 dph this was supplemented with freeze dried decapsulated *Artemia* eggs. At 9 dph the dry food was supplemented with newly hatched *Artemia* sp. nauplii (Inter Yyba GmbH, Germany) once a day. From 20 dph (APAP FSDT) or 15 dph (ASA FSDT) the juvenile fish were fed twice daily with a combination of crushed TetraMin® flakes (Tetra GmbH, Melle, Germany) and decapsulated *Artemia* eggs and once a day with *Artemia* sp. nauplii. The exposure periods were from 2 dpf to 74-75 dph (APAP FSDT) and 3 dpf to 60 dph (ASA FSDT), respectively (Table 2). Triplicate tanks were used for each of the FSDT exposure concentrations.

2.4. Adult exposure with APAP and ASA

Groups of eight adult males and eight adult females were exposed to ASA or APAP for 14 days, and groups of ten males were exposed to ASA for eight days. The exposure concentrations are shown in Table 3.

2.5. Quantification of APAP and ASA in water samples

A one-week pilot study with zebrafish larvae exposed to ASA (1, 10 and 100 mg/L) and APAP (1, 10 and 100 mg/L) showed that only 100 mg ASA/L had an effect on mortality (data not shown). Based on the results from the pilot study and a literature survey the nominal exposure concentrations in the experiments shown in Table 1 were selected. These concentrations were chosen to investigate potential effects on endocrine endpoints, and not to reflect environmentally relevant concentrations. The nominal concentrations selected for the two weeks' and 8 days' exposures of adult fish are shown in Table 2; duplicate tanks for each concentration were used in the two weeks' exposure while single tanks were used in the 8 days' exposure.

Water samples from the exposure tanks were collected with a 3 mL syringe and passed through a 45µm filter. During the FSDT experiments water samples were collected 12 or 13 times from each tank and in the 14 and 8 days exposure experiments with adult fish samples were collected 6 to 10 times (Table 2 and 3). At the beginning of the FSDT experiments the pump flow was adjusted in order to have exposure concentrations close to the nominal concentrations.

Actual concentrations of APAP and ASA were measured on a Triple Quad LC/MS (Agilent, Wilmington, DE), and the protocol described for quantification of IBU in Morthorst *et al.* (2013) was followed with the following modifications for quantification of APAP and ASA. Mobile phases consisted of a 80:20 (v/v) mixture of 1% formic acid and methanol. The injection volume was 6 µL. Fragmentor voltage was 110 V and collision energy was 20 V (ASA), 15 V (APAP). The mass-to-charge-ratios (m/z) of precursor and quantifier ions were respectively 121.1 and 65.3 (ASA) and 152.1 and 110.1 (APAP). The detection limit of all three compounds in the sample matrix was 11.7 pg on the column, corresponding to 1.95 µg/L (Signal-to-noise-ratio > 10).

2.6. Sampling and histology

Sampling and preparation of tissue for histology was performed as described in Kinnberg *et al.* (2007) and Morthorst *et al.* (2010). Briefly, head and tail were separated from the trunk after the fish were euthanized with bicarbonate buffered MS-222 (0.1g L⁻¹). The trunk was fixated in Bouin's fixative, dehydrated in graded ethanol solutions, sectioned longitudinally and stained with hematoxylin and eosin. The gonads were examined by light microscopy and the sex was determined by visual examination.

2.7. Vitellogenin analysis

The use of head and tail for vitellogenin analysis is validated and recommended in the OECD Test Guideline 234 (OECD, 2011). Briefly, the head and tail of each fish was snap frozen in liquid nitrogen and stored at -80°C. After homogenization of the head and tail the vitellogenin

concentrations were measured by means of a direct non-competitive sandwich ELISA with biotin conjugated antibodies and streptavidin-HRP conjugated antibodies as outlined in Holbech *et al.* (2001) and with the modifications described in Morthorst *et al.* (2010). Because of the high number of male fish in the ASA FSDT experiment, vitellogenin was analysed in randomly selected subsamples of approximately one quarter of the male fish.

2.8. Enzyme Immuno Assay (EIA) in the 14 days exposure with ASA

The concentrations of 11-ketotestosterone (11-KT) and prostaglandin E₂ (PGE₂) were measured on the trunk part of male fish, and the samples were treated as described in Morthorst *et al.* (2013). Briefly, the trunks were homogenized in liquid nitrogen, extracted three times with methanol and re-dissolved in acetate buffer (pH 4.0) after evaporation of methanol. A fraction (250 µL) of each sample was loaded on StrataTM-X columns with 30 mg mL⁻¹ sorbent mass (Phenomenex, Denmark) as per manufacturer's instructions for reversed phase solid phase extraction (SPE) of neutral compounds. The samples were re-dissolved in EIA buffer (Cayman Chemical, MI, USA) after evaporation of the elution solvent and stored at -80°C for later analysis. Male concentrations of PGE₂ and 11-KT were determined with Cayman Chemical EIA kits as per manufacturer's prescription. Plate development times for 11-KT and PGE₂ were 30 and 90-120 min, respectively.

2.9. Statistics

Prior to the statistical analyses the data was screened for homogeneity of variance and normality and if necessary the data was log transformed. Differences in vitellogenin concentrations, PGE₂, 11-KT levels and body weights between the groups were evaluated by a Bonferroni-Holm adjusted one-way ANOVA or, when variances were unequal, a non-parametric Kruskal-Wallis one-way ANOVA. The Bonferroni-Holm adjusted χ^2 test was used to examine differences in sex ratio and mortality between the control group and the exposure groups. Analyses were performed with

SigmaStat® Statistical Software version 2.0 and statistical difference was considered at $p < 0.05$.

Outliers were not removed from the datasets and they are shown in the figures as filled circles.

Results

3.1. Actual concentrations of APAP and ASA

The nominal and actual water concentrations of APAP and ASA are shown in Table 2 and 3. The actual concentrations of APAP and ASA determined in the replicate tanks for each exposure concentration were not significantly different in any of the experiments (Table 2 and 3). The mean actual concentrations are used throughout the manuscript, and the results are evaluated based on mean measured concentrations. The actual concentrations were very close to nominal at the beginning of the FSDT exposures, but towards the end of the experiments the exposure concentrations dropped most likely because the fish have grown adult and thereby increased the uptake and metabolism of the exposure compound.

3.2. Survival and growth

The survival and body weight of fish in the FSDT experiments are shown in Table 2. At 13 dph all fish in one of the 30 mg L⁻¹ tanks in the APAP FSDT experiment were dead, and the following two days the fish in the remaining replicates died too. No abnormal behaviour was observed in the preceding days. The survival in the remaining APAP FSDT exposure groups was 32-41% and with no difference between the control group and exposure groups (Table 2). The survival in the ASA FSDT was 46-60% and there was also no difference between the control group and exposure groups (Table 2). At the time of sampling the average bodyweight of fish in the APAP and ASA groups in the FSDT experiments did not differ from their respective control groups (Table 2).

The survival was 97% in the 14 days experiment with 3.8 mg ASA L⁻¹, while the mortality among the fish exposed to 1.9 mg APAP L⁻¹ was high; only 28% survived (Table 3). Due to the low survival in the APAP group, no further analyses were performed on fish from this group.

3.3. Sex ratio in the FSDT

The FSDT control groups had 78.9% (APAP) and 79.3% (ASA) males, respectively (Figure 1). Between 3% and 9.7% of the fish in the ASA FSDT groups were undifferentiated at 60 dph when the exposure ended (Fig. 1A), whereas maximum 2% of the fish were undifferentiated in the APAP FSDT exposure (Figure 1B). Two intersex fish were found in the group exposed to 1.7 mg ASA L⁻¹ (Figure 1A). The sex ratio in the exposed groups did not differ from the sex ratios in the control groups in any of the experiments (Figure 1A and B).

3.4. Vitellogenin levels

Neither male nor female vitellogenin concentrations of exposed fish differed from the control groups after the APAP or ASA FSDT exposures (Figure 2A-D). The same result was seen after the 8 days of ASA exposure in adult males (Figure 3).

3.5. Concentrations of 11-KT and PGE₂

The PGE₂ levels in males exposed to ASA for 14 days were significantly reduced (Figure 4B), but 11-KT levels were not affected (Figure 4A).

Discussion

Besides the analgesic and antipyretic effects of mild analgesics it has been suggested that these pharmaceuticals might also have endocrine disrupting properties in humans and animals (reviewed by Kristensen *et al.* (2016)). Based on the present four experiments (Table 1) we did not observe

any effects of acetaminophen (APAP) and acetylsalicylic acid (ASA) on endocrine mediated endpoints in the zebrafish.

Before discussing the results it is important to address some issues that could influence the interpretation of the results. The experiments were carried out over a period of several years in the mid 2000s while the OECD Fish Sexual Development Test (FSDT) test guideline (TG) 234 was still undergoing validation, and therefore the experimental protocols deviate slightly from the recommendations in the guideline (OECD, 2011). The APAP and ASA FSDT experiments had three replicates with 40 and 80 larvae respectively, compared to the four replicates with 30 larvae recommended in the TG 234, and thereby the initial number of fish per test concentration was equal to or higher than recommended. The statistical power to detect a change in sex ratio in the APAP experiment was lower than required because of a high mortality in the control group. In the TG 234, a total of 64 fish provides sufficient power to detect a 31% change in sex-ratio for example from 50% (fe)males to 81% or 19% (OECD, 2012). Only 38 control fish survived in the APAP FSDT, but the difference in sex ratio between the control group and the group exposed to 2.3 mg L⁻¹ APAP (Figure 1) is very small, and even an extremely powerful test design would not detect a significant effect in this experiment. The survival in the ASA FSDT was markedly higher, and the power of the test was higher than required; the survival of control fish in the ASA FSDT met the TG 234 survival criteria of 56% (80% hatching success and 70% post hatch survival), whereas the survival of the control group in the APAP FSDT was only 32%. A low survival can affect the sex ratio if the mortality is gender specific. However, this seems not to be the case because the sex ratio was similar in the ASA FSDT, which had lower and acceptable control group mortality (Figure 1).

Mild analgesics have been detected in effluents from wastewater treatment plants, surface water, groundwater, and even drinking water (reviewed by der Beek *et al.* (2016)), and they are fairly water soluble (APAP: 14000 mg L⁻¹ and ASA: 4600 mg L⁻¹) and easily biodegradable, but because of the widespread consumption these compounds are continuously discharged into the aquatic environment. Pharmaceuticals are designed to affect specific targets and they have the potential to seriously affect wildlife species because many mechanisms of action are well conserved across animal phyla; e.g. zebrafish have predicted orthologs to 86% of the 1318 investigated human drug targets (Gunnarsson *et al.*, 2008).

Mild analgesics act by inhibiting the synthesis of prostaglandins, which are involved in several reproductive processes including oocyte maturation, sexual behaviour and gonadal differentiation in fish (Kobayashi *et al.*, 2002; Lister & Van Der Kraak, 2008; Pradhan & Olsson, 2014).

Prostaglandin inhibitors have been demonstrated to influence enzymes involved in steroidogenesis, steroid levels and reproduction in mammals but the results have been contradictory (Didolkar *et al.*, 1980; Kumar & Chinoy, 1988; Adams & McLaren, 2002; Frungieri *et al.*, 2006; Kristensen *et al.*, 2011). The zebrafish is a well-known model organism (OECD, 2011) and several endocrine disrupting compounds have been shown to alter sex ratio and vitellogenin levels of zebrafish (Kinnberg *et al.*, 2007; Morthorst *et al.*, 2010; Holbech *et al.*, 2012; Holbech *et al.*, 2013). The present experiments aimed to investigate possible endocrine disrupting effects of mild analgesics in this model species.

Analgesics are designed to reduce prostaglandin levels in humans and they also reduce prostaglandin levels in zebrafish; ASA significantly reduced male PGE₂ levels in the present experiment (Figure 4A) and ibuprofen concentrations in the µg L⁻¹ range reduced PGE₂ levels of both males and females (Morthorst *et al.*, 2013). Prostaglandins have been shown to play a role in

the steroid synthesis of fish (Wade & Van Der Kraak, 1993), but 11-KT levels of males remained unchanged after ASA exposure (Figure 4B) and likewise, the before mentioned ibuprofen-induced reduction in PGE₂ levels did also not lead to concurrent changes in 17 β -oestradiol (E2) and 11-KT levels (Morthorst *et al.*, 2013). However, ibuprofen has been reported to affect 17 β -oestradiol (E2), 11-KT and vitellogenin levels in adult zebrafish (Ji *et al.*, 2013).

Because the vitellogenin synthesis is regulated by E2, an alteration of vitellogenin levels is closely related to endogenous E2 levels or exposure to endocrine active substances. In the present experiments adult exposure to ASA and lifelong exposure to APAP or ASA did not affect the vitellogenin concentration (Figure 2 and 3). The agonistic and antagonistic receptor-binding activities of chemicals can be tested in human cell lines and yeast reporter gene assays with human steroid receptors. APAP and ASA had no or a very weak estrogenic response in the Yeast Estrogenic Screen Assay (YES-assay) both when tested in our own lab (unpublished data) and by Fent *et al.* (2006). However, an anti-estrogenic and anti-androgenic response of ibuprofen, but not APAP, was demonstrated recently; ibuprofen acted as an antagonist to the human oestrogen and androgen receptors in two *in vitro* assays (Ezechias *et al.*, 2016).

The different effects of the prostaglandin inhibitors could be explained by differences in their mechanisms of action and how they bind to the COX enzymes. Concomitant reductions in prostaglandin levels, COX activity and COX gene expression have not been found in fish (Flippin *et al.*, 2007; Lister & Van Der Kraak, 2008; Morthorst *et al.*, 2013) and this could indicate that prostaglandin inhibitors influence the prostaglandin synthesis without affecting expression of the COX genes. That mild analgesics display a wide diversity in their responses is demonstrated in an *ex vivo* human foetal testis system (Ben Maamar *et al.*, 2017). The effects on e.g. testosterone production varied, and the only consistent effect was reduced PGE₂ levels, which is also what we observed in the zebrafish exposed to ASA (Figure 4A) and ibuprofen (Morthorst *et al.*, 2013). An

anti-androgenic effect of mild analgesics has been demonstrated in *ex vivo* foetal testes of rats and humans (Kristensen *et al.*, 2012; Ben Maamar *et al.*, 2017), but the mechanism of action is likely to be uncoupled from the inhibition of the prostaglandin synthesis. How the inhibition of prostaglandin synthesis is related to steroids levels, steroidogenesis and reproduction remains unknown.

Sexual differentiation in zebrafish is very sensitive to exposure to exogenous hormones and zebrafish populations can be made all female by exposure to natural and synthetic steroid estrogens (Nash *et al.*, 2004; Holbech *et al.*, 2006). During the period of sexual differentiation all male or male biased populations can be produced by exposure to androgens (Orn *et al.*, 2003; Morthorst *et al.*, 2010) or aromatase inhibitors (Kinnberg *et al.*, 2007; Thorpe *et al.*, 2011; Holbech *et al.*, 2012). Chemicals (e.g. 4-nonylphenol, 4-*tert* octylphenol and 4-*tert* pentylphenol) with weaker estrogenic effects than the steroid hormones have previously been shown to alter the sex ratio in zebrafish (OECD, 2011). The sex ratio in the control and exposure groups in the current experiments did not vary significantly (Figure 1), and the sex ratio was generally skewed towards males, which has previously been seen in untreated zebrafish populations (Orn *et al.*, 2006; Larsen *et al.*, 2008; Hensley & Leung, 2010). A sex ratio significantly skewed towards males (72%) was observed in an inbred line of zebrafish (Brown *et al.*, 2012). The breeding history of the parental fish in the present experiments is unknown because the local supplier does not register the breeding history, which means that we unfortunately cannot relate the sex ratio to the inbreeding status of the parental fish. It has been proposed that the growth rate could influence the sex ratio in fish populations (Lawrence, 2008; Gomez-Requeni *et al.*, 2010). Because large females are able to produce more gametes than small females, individuals with slow growth probably have a higher reproductive success as small males than as small females, and a slow growth could lead to a male biased sex

ratio as observed in the present experiment. The average bodyweight of fish exposed to APAP is higher because the experiment was ended on 70 dph compared to 60 dph in the ASA exposure and also the number of fish in each tank was lower in the APAP FSDT (Table 2). In a similar experiment in the same time period we observed a skewed sex ratio towards males in the control groups but exposure to the phytoestrogen biochanin A (209 µg/L) pushed the sex ratio significantly towards females (Holbech *et al.*, 2013). Therefore, if APAP and ASA were able to influence the sexual development in zebrafish, we believe the sex ratio would have changed. According to the WHO/IPCS definition of an endocrine disrupter (WHO, 2002), both an endocrine mechanism and an adverse effect should be observed. According to this definition the present results do not define APAP and ASA as endocrine disrupters because the population relevant adverse endpoint sex ratio was not affected. Changes in vitellogenin levels are generally not regarded as a population relevant adverse endpoint in hazard assessment of endocrine disrupters all though a decline in female vitellogenin could affect reproduction (Miller *et al.*, 2007). Further, neither male nor female vitellogenin levels were affected by exposure to APAP or ASA (Figure 2A-D). However, an induction in liver vitellogenin has been observed in the Japanese medaka (*Oryzias latipes*) after exposure to a high APAP concentration (Kim *et al.*, 2012).

Fish exposed to 30 mg APAP L⁻¹ died between 13 and 15 dph even though a 7-day range finding test (highest test concentration: 100 mg L⁻¹) with larvae had been performed before the experiment started (Data not shown). Survival of adult fish exposed for APAP was significantly reduced at 1.9 mg L⁻¹, whereas larvae exposed to 2.26 mg L⁻¹ until 74-75 dph survived. Thus, APAP toxicity seems to be different in juvenile and adult fish, which could be due to differences in the metabolism or a result of adaptation. An age specific difference in sensitivity has previously been demonstrated in zebrafish exposed to biochanin A (Holbech *et al.*, 2013) and Kim *et al.* (2012) did not observe

increased mortality in medaka larvae at 95 mg APAP L⁻¹ but the juvenile survival (33 dpf) was reduced at this concentration.

Conclusion

Based on the present experiments it is clear, that ASA decrease PGE₂ levels significantly without affecting 11-KT and vitellogenin levels of male zebrafish, and APAP and ASA exposure of zebrafish from fertilization to adulthood do not influence sex ratio or vitellogenin levels. Mild analgesics have showed endocrine disrupting potential in vertebrates, but the mechanisms of action and the effects seem to be different for each compound, and the effects on the prostaglandin and steroid systems may not be directly coupled. The equivocal results obtained from animal studies illustrate the complex interaction between the arachidonic acid and steroidogenic pathways.

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Table 1 Overview of the FSDT and adult exposure experiments with APAP and ASA. dpf: days post fertilisation; dph: days post hatch (3 dpf = 0 dph). Only 28% of the fish survived in the APAP adult exposure and further analyses were therefore not performed.

§ one of the replicates (3.8 mg L⁻¹) had 7 females and 9 males, because one male was mistaken as a female when sexing the fish before the experiment started.

Adult	Duration	Replicates	Fish per	Endpoints	
ASA	8 days	1 replicate	10	Males only	Vtg
Control					
0.4 mg L ⁻¹					
0.8					
3.0					
9.5					
ASA	14 days	2 replicates	16	8 females	11-KT and PGE ₂
Control					
3.8 mg L ⁻¹					
APAP	14 days	2 replicates	16	8 females 8 males	
Control					
1.9 mg L ⁻¹	Most died				
FSDT	Duration	Replicates	Fish per	Sex ratio	Endpoints
ASA	3 dpf to	3 replicates	80		Sex ratio and
Control				18:82	
0.2 mg L ⁻¹				29:71	
0.5				29:71	
1.7				29:71	
8.2				30:70	
APAP	2 dpf to	3 replicates	40		Sex ratio and
Control				21:79	
0.22 mg L ⁻¹				34:64	
2.26				24:73	
30	(Died)				

Table 2 Nominal and actual concentrations of ASA and APAP in the FSDT experiments; the number of water samples is written in brackets. The number of fertilized eggs and the average weight of the surviving fish are shown. The error in percent survival is based on the results from three replicates in each treatment. Mean \pm SEM.

Nominal concentration	Actual concentration	Initial number of fertilized	Fish weight (mg)	Surviving fish (n)	Surviving fish (%)
ASA FSDT					
Control	-	240	107 \pm 5	135	56.3 \pm 4.1
0.32	0.17 \pm 0.020 (39)	240	108 \pm 5	132	55.0 \pm 2.1
1.0	0.48 \pm 0.064 (39)	240	108 \pm 4	144	60.0 \pm 4.7
3.2	1.67 \pm 0.21 (39)	240	111 \pm 4	130	54.2 \pm 0.7
10	8.2 \pm 0.64 (39)	240	118 \pm 5	111	46.3 \pm 5.3
APAP FSDT					
Control		120	270 \pm 16	38	31.7 \pm 3.8
0.25	0.22 \pm 0.03 (36)	120	225 \pm 13	50	40.8 \pm 3.8
2.5	2.26 \pm 0.18 (36)	120	240 \pm 20	45	37.5 \pm 5.4
25	30 \pm 2 (9)	120	-	0	0

Table 3 Nominal and actual concentrations of ASA and APAP in the adult exposure experiments.

The number of water samples is written in brackets. Mean \pm SEM.

Nominal exposure concentrations	Actual exposure concentrations	Surviving fish (%)
14 days exposure		
Control	-	100
ASA 5.0	3.8 \pm 0.36 (20)	97
APAP 2.5	1.9	28
8 days ASA exposure		
Control	-	100
0.32	0.4 \pm 0.04 (6)	80
1.0	0.8 \pm 0.12 (6)	100
3.2	3.0 \pm 0.29 (6)	90
10	9.5 \pm 0.81 (6)	100

Figure 1

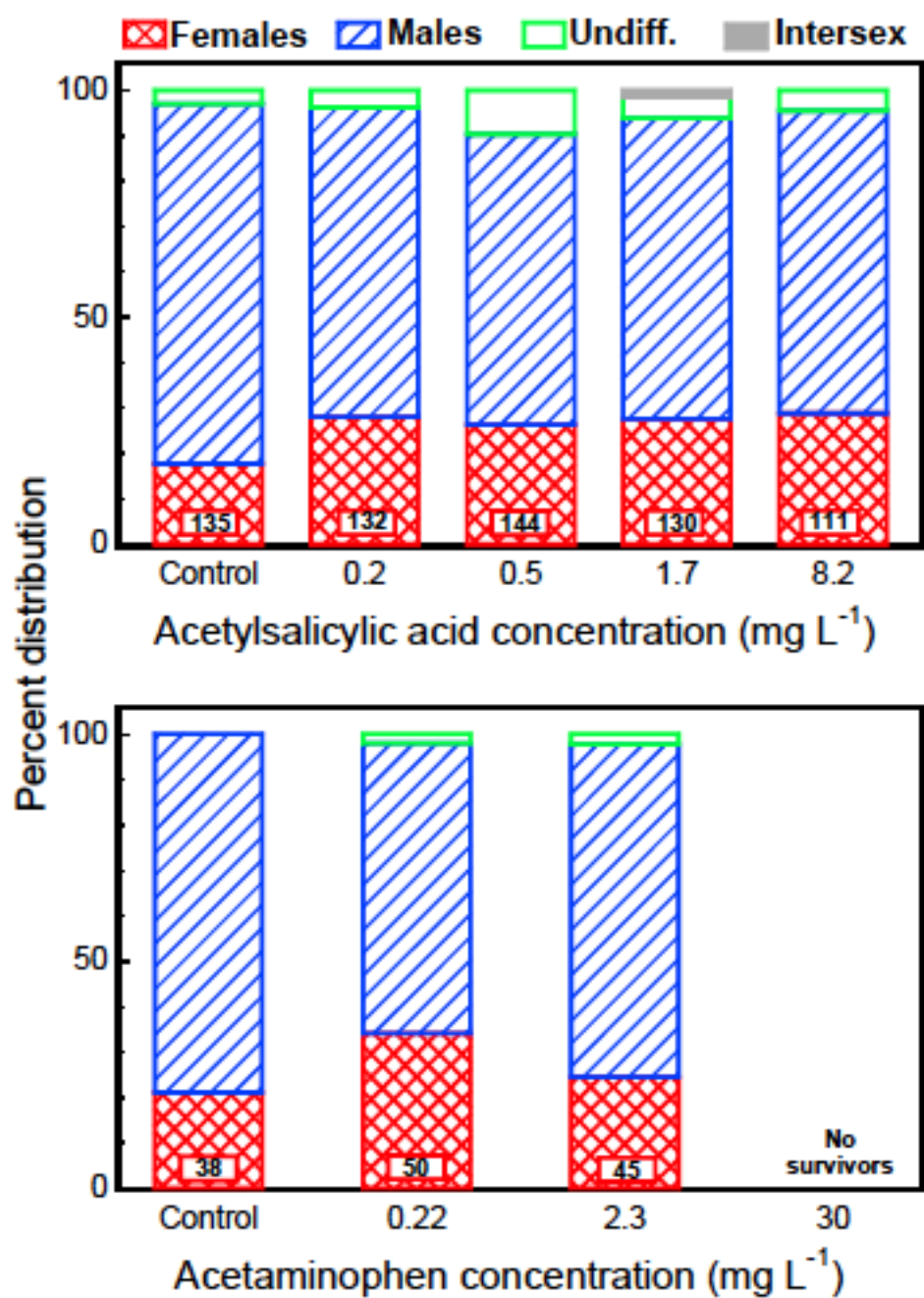


Figure 2

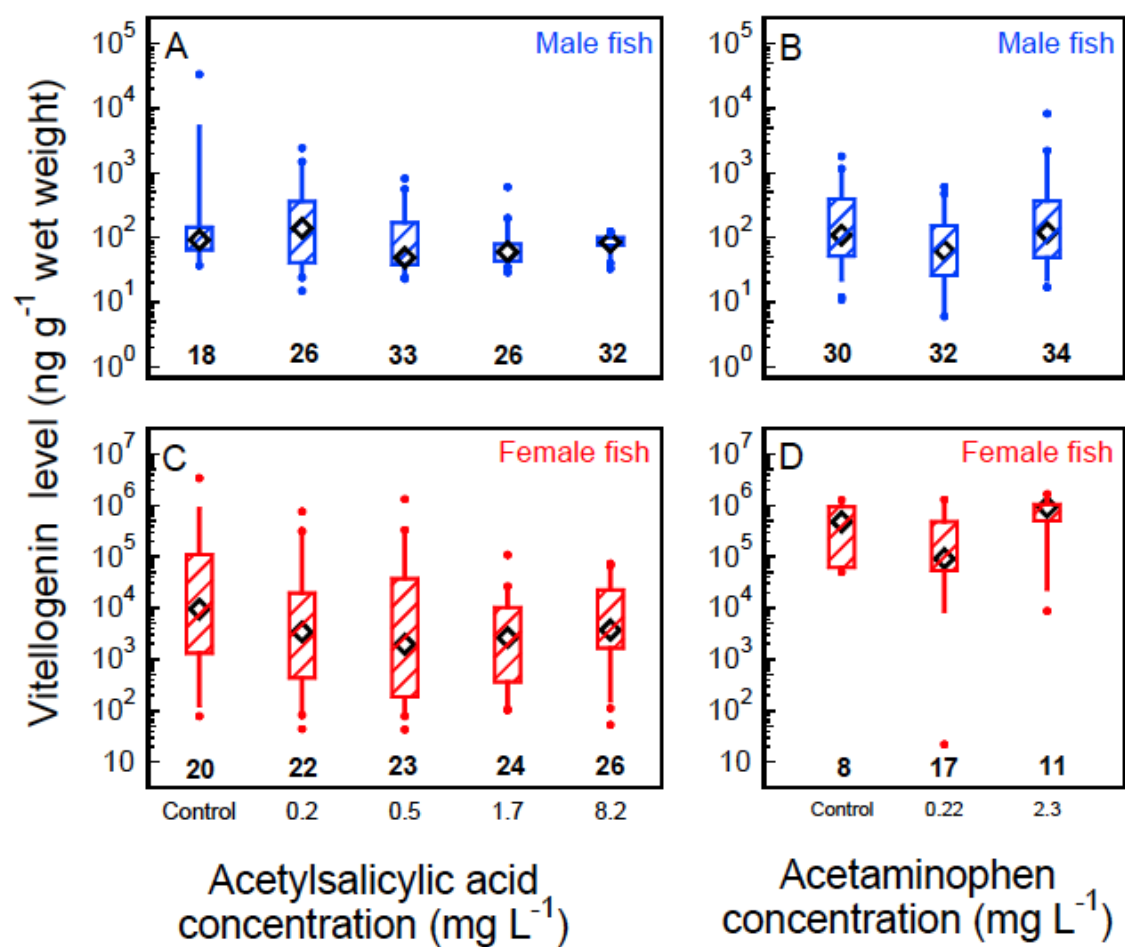
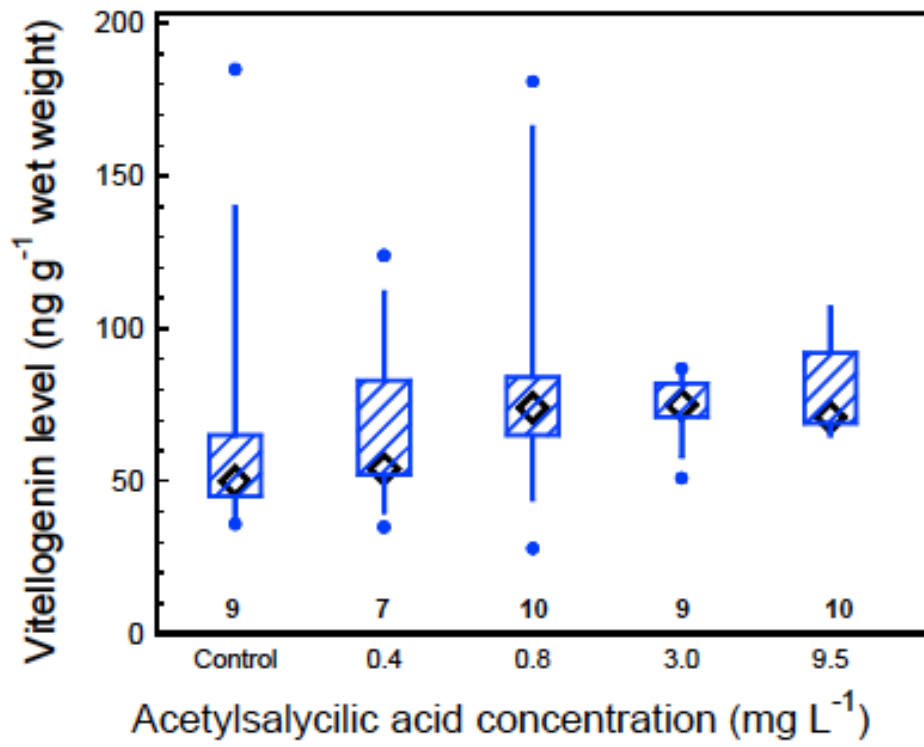


Figure 3



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Figure 4

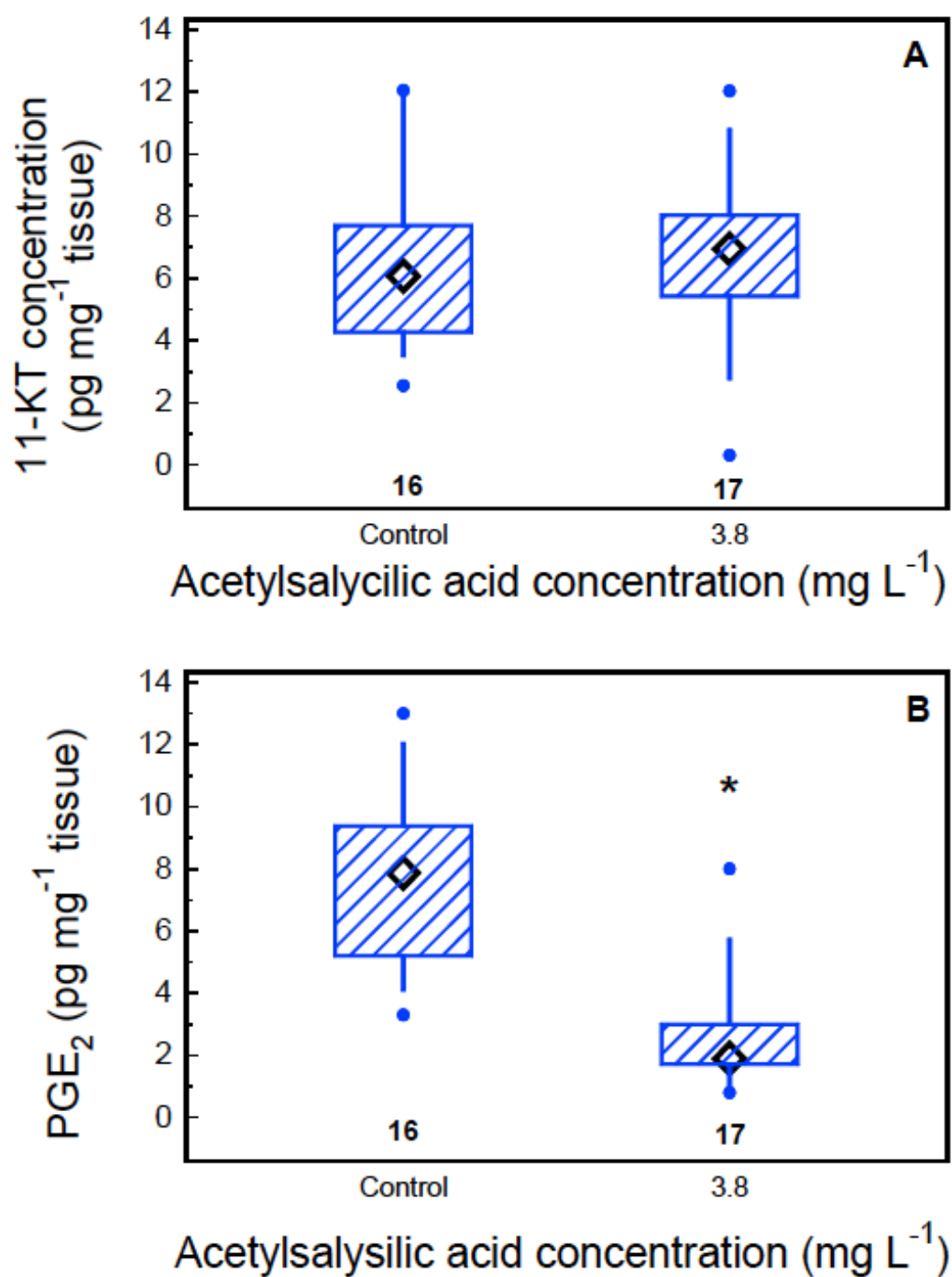


Figure legends

Figure 1. Sex ratios in the FSDT experiments with ASA and APAP. The percentages of females, males, undifferentiated, and intersex fish in the FSDT exposure experiments with ASA (A) and APAP (B). The total number of fish in each exposure group is shown at the bottom of the bars.

Figure 2. Vitellogenin in male and female zebrafish. The vitellogenin concentrations in male (A and B) and female (C and D) zebrafish in the FSDT exposure experiments with ASA (A and C) and APAP (B and D). The diamonds within the boxes represents the median, the lower and upper boundaries of boxes show the 25th and 75th percentiles, and the lower and upper whiskers show the 10th and 90th percentiles. The outliers are marked as filled circles. The number of male or female fish in each exposure group is shown at the bottom of the boxes.

Figure 3. Vitellogenin in male zebrafish. The vitellogenin concentrations in adult male zebrafish after exposure to ASA for eight days. The diamonds within the boxes represents the median, the lower and upper boundaries of boxes show the 25th and 75th percentiles, and the lower and upper whiskers show the 10th and 90th percentiles and the outliers are marked as filled circles. The number of fish in each exposure group is indicated in the bottom of the boxes.

Figure 4. 11-KT and PGE₂ concentrations in males. The concentrations of 11-KT (A) and PGE₂ (B) in adult male zebrafish exposed to 3.8 mg L⁻¹ ASA for 14 days. The diamonds within the boxes represents the median, the lower and upper boundaries of boxes show the 25th and 75th percentiles, and the lower and upper whiskers show the 10th and 90th percentiles. * (p<0.05) means significantly different from the control, and the outliers are marked as filled circles. The number of fish in each exposure group is indicated in the bottom of the boxes.