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Investigation of the potential endocrine effect of nitrate in zebrafish *Danio rerio* and brown trout *Salmo trutta*

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ABSTRACT

Nitrate has the potential to affect steroid production. Nitrate concentrations in streams in agricultural areas may exceed concentrations showing effects in laboratory studies. The effects of nitrate and/or nitrite on endocrine relevant endpoints were tested in zebrafish and brown trout. Zebrafish were exposed in two experiments to nitrate (8.8 to 89 mg NO$_3$/L) and nitrite (3.6 to 19 mg NO$_2$/L) during the period of sexual differentiation and sex ratios were determined. Vitellogenin concentrations were determined in the second experiment. The sex ratio was unaffected by the exposure to nitrate and nitrite. Vitellogenin concentrations were slightly elevated in males (but not females) in all of the groups exposed to nitrate. Juvenile brown trout were exposed to 5.7, 14, and 31 mg NO$_3$/L for 8 days and vitellogenin levels in liver were determined. Vitellogenin concentrations in the females were not affected by exposure, but in the males, there was an overall statistically significant effect of exposure to nitrate with the group exposed to 5.7 mg NO$_3$/L showing a trend of higher vitellogenin concentrations than the control group; levels in the males of the groups exposed to 14 and 31 mg NO$_3$/L were not statistically different from those of the control group. In conclusion, some marginal effect of nitrate in male fish on endocrine activity was observed but the present results for zebrafish, using environmentally relevant concentrations, do not define nitrate and nitrite as endocrine disrupting chemicals according to the generally accepted WHO/IPCS definition because no adverse effects (altered sex ratios) were demonstrated.

Keywords:

Zebrafish
Brown trout
Nitrate
Endocrine
1. Introduction

Feminization of male fish by natural or synthetic estrogenic chemicals released from waste water treatment plants (WWTPs) has become a worldwide issue of concern (Desforges et al., 2010; Jobling et al., 1998; Sumpter, 2005). Due to the efficiency of Danish WWTPs with tertiary treatment, the estrogenic activity in the treated wastewater is generally too low to feminize male fish (Stuer-Lauridsen et al., 2005) but, nevertheless, surveys carried out between 2000 and 2004 showed that the majority of juvenile brown trout in Danish streams had elevated levels of vitellogenin (Bjerregaard et al., 2006b; Bjerregaard et al., 2008), a well-recognized biomarker of estrogenic exposure (Sumpter, 1995). Also, intersex was recorded in roach (*Rutilus rutilus*) from Danish streams during the same time interval (Bjerregaard et al., 2006a), albeit with a lower severity than detected in British rivers (Jobling et al., 1998; Jobling and Tyler, 2003; Jobling et al., 2006).

Exposure to nitrate and nitrite is known to have the potential to interfere with steroid concentrations proposedly via the effect of NO (to which NO$_2^-$ and NO$_3^-$ can be reduced) as a controlling factor in the regulation of enzymes in the steroidogenic pathways in the gonads as well as hepatic CYP-enzymes degrading the steroids (reviewed by Guillette and Edwards, 2005). Laboratory studies show clear effect of nitrate or nitrite exposure on endocrine related endpoints (e.g. Kellock et al., 2018) - especially testosterone (T) production and levels (i.e. Freitag et al., 2016; Freitag et al., 2015; Hamlin et al., 2016; Hamlin et al., 2008; Panesar, 1999; Panesar and Chan, 2000; Zraly et al., 1997) have been investigated. Field investigations show various correlations between nitrate concentrations in nitrate contaminated springs and lakes in Florida and various sexual traits in fish and alligators (Edwards and Guillette, 2007; Edwards et al., 2006; Guillette and Edwards, 2005).

Approximately 61% of the area of Denmark is used for agriculture and the intense agricultural practices lead to leakage of nutrients from the agricultural fields via drains to the streams. Nitrate concentrations in Danish streams not affected by agriculture are typically in the range 3 to 6 mg NO$_3^-$/L, whereas concentrations in streams affected by agriculture are in the range 20 to 40 mg NO$_3^-$/L (Wiberg-Larsen, 2015); this is within or above the range for which associations with
reproductive endpoints were found in mosquitofish (*Gambusia holbrooki*) in Florida (Edwards and Guillette, 2007; Edwards et al., 2006).

Sexual development in zebrafish *Danio rerio* is very sensitive to perturbations in androgen-estrogen sex hormone balance and changes in the sex ratio have been established as an endpoint showing endocrine disruption in the Fish Sexual Development Test – OECD TG 234 (OECD, 2011a).

The primary aim of the present study was to investigate if exposure to nitrate or nitrite could affect endocrine relevant endpoints such as sex ratios and vitellogenin concentrations in zebrafish and the secondary aim was to investigate if exposure of juvenile brown trout to environmentally relevant concentrations of nitrate might help explain the elevated levels of vitellogenin found in previous surveys in Danish streams.

2. Materials and methods.

2.1. Sexual Development Test in zebrafish

The experiments generally followed the procedure outlined in OECD Test Guideline 234 (OECD, 2011a) but since the zebrafish experiments were carried out before the final adoption of the guideline there are some modifications.

Adult zebrafish were obtained from local suppliers and kept at 26±1 °C and a light:dark regime of 12:12 h as a stock for breeding. Late in the afternoon spawning chambers with artificial plants were placed in the aquarium containing approximately 50 adult zebrafish of mixed sex and removed again the following morning where fertilized eggs were collected for the experiments. Newly hatched larvae were fed with TetraMin® Baby (Tetra GmbH) until 2 weeks post hatch. After 1 week, TetraMin® Baby was supplemented with newly hatched *Artemia salina* nauplii (San Francisco Bay brand). Two weeks post hatch and throughout the test, the larvae were fed with *Artemia salina* nauplii.

The exposure systems were flow-through test systems with 10-L glass aquaria containing 8 L water. Administration of water and test compound was controlled by peristaltic pumps (Ole Dich Instrument Makers); flow rate was 40 L per 24 h. Water was a 3:1 mixture of deionized (ASTM
type 4) water and tap water (groundwater). Temperature was 28.2±0.5°C in Exp. 1 and 26±1 °C in Exp. 2. The light:dark regime was 12:12 h. The aquaria were aerated. pH and conductivity were kept within the recommended range for zebrafish husbandry (Brand, 2002). Water samples were taken regularly for determination of actual exposure concentrations (Table 1).

At 60 dpf all fish were euthanized in an overdose of bicarbonate buffered 3-aminobenzoic acid ethyl ester, methane sulfonate salt (MS-222) and length and weight were recorded.

Two experiments were carried out and the exposure concentrations are given in Table 1.

In zebrafish experiment 1, exposure took place from 20 dph to 60 dph; one tank was used for each exposure concentration and 2 replicate tanks were used for the control. A total of 15 water samples was taken from each aquarium at 2-3 d intervals. Ninety fish larvae were randomly added to each tank on day 20 post hatch.

In zebrafish experiment 2, exposure took place from 0 dpf till 60 dpf. At 0 dpf, 95 fertilized eggs were placed in each tank. Two replicate tanks were used for all groups. A total of 6 water samples were taken from each aquarium at approximately 10 d intervals.

2.2. Brown trout experiments

Juvenile brown trout originating from a population of parental wild brown trout sampled around Funen, Denmark, and hatched during the winter 2014/2015 were collected at Fyns Laksefisk where they had been hatched and raised on commercial food (Aller Aqua, Futura Granulate 2) in recirculated groundwater. The fish were collected at Fyns Laksefisk, November 2015 and brought to the laboratory where they were acclimated for five days before the experiment was initiated. On the third day of the acclimation and first day of the experiment the fish were fed 1% of the body weight with the food mentioned above.

Approximately 20 brown trout were placed in each of nine 80 L steel tanks with Plexiglas lids. The tanks were supplied with flowing tap water (groundwater) at 80 L/24h. The water was aerated. Temperature was 14±1 °C and the light:dark regime was 12:12. The addition of water and tested compounds were controlled by peristaltic pumps (Ole Dich Instrument Makers). The brown trout were exposed to nominal concentrations of 0, 10, 25 and 50 mg NO₃/L. Replicate tanks were used
for each treatment. A reference group was exposed to 100 ng/L 17β-estradiol (E2) to ensure that the fish responded to estrogens (one tank, only). Water samples were taken from each of the nitrate exposure tanks at day 1, 6 and 8 (and also after removal of the fish at day 9 and 10) for determination of actual exposure concentrations (Table 1). Exposure to nitrate was carried out for eight days. After the feeding on the first day of the experiment, the fish were not fed further during the exposure period.

At the end of each experiment, the fish were anesthetized in MS-222 (3-aminobenzoic acid ethyl ester, methane sulfonate salt) and length and weight were recorded. The liver was dissected out, weighed and frozen at -80 °C until further processing. The liver was homogenised in two volumes of buffer (50 mM Tris-HCl pH 7.4, 1% protease inhibitor Cocktail) by means of a pistil and centrifuged (50,000 g * 30 min) and the supernatant was taken out and stored at -80 °C until the vitellogenin concentration was measured. The sex of each fish was determined by visual examination of the gonads under a stereo microscope.

2.1. Vitellogenin analysis and histology

Zebrafish were cut into three sections, head, trunk (containing the gonads) and tail; vitellogenin levels were determined in the homogenates of the tail and head sections in zebrafish experiment 2 as described by Holbech et al. (2001; 2006) and Morthorst et al. (2010) with a quantification limit of 40 ng/g. In brown trout, vitellogenin levels were determined in liver homogenate according to Bjerregaard et al. (2006b; Bjerregaard et al., 2008) with a quantification limit of 20-40 ng/g. Intra- and inter assay coefficients of variation have been determined to 8.1% and 16.7% for the trout ELISA (Bjerregaard et al., 2006b) and 5.8% and 10.4% for the zebrafish ELISA (Holbech et al., 2001).

The sex of the zebrafish was determined by histological examination of gonads as described by Kinnberg et al. (2007). In zebrafish experiment 1, the maturation stages of the ovaries were characterized according to Selman et al. (1993). Ovaries were divided into five maturation stages: 0: oogonia exclusively, I: primary growth, II: cortical alveolus, III: vitellogenic, or IV: mature. Testes
were divided into three stages according to the extent of spermatozoa: I: no spermatozoa, II: moderate spermatozoa, or III: abundant spermatozoa.

**Determination of nitrate and nitrite concentrations**

Exposure concentrations were determined by means of Lachat QuikChem® Series 8500 (method no. 10-107-04-1-C: Nitrate is reduced to nitrite by passage of the sample through a copperized cadmium column. The nitrite is then determined by diazotizing with sulfanilamide followed by coupling with N-(1-naphthyl)ethylenediamine dihydrochloride. Absorption is read at 520 nm.). The limit of detection was 10 µg N/L – corresponding to 44.2 µg NO$_3$\text{-}L$^{-1}$ and 32.9 µg NO$_2$\text{-}L$^{-1}$.

**2.3. Chemicals**

NaNO$_3$ (CAS 7631-99-4) was supplied by Sigma and NaNO$_2$ (CAS 7632-00-0), MS-222 (3-aminobenzoic acid ethyl ester, methane sulfonate salt – CAS 886-86-2), protease inhibitor Cocktail [Sigma-Aldrich, product P 8340)] and 17β-estradiol (CAS 50-88-2) were supplied by Sigma-Aldrich.

**2.4. Data handling and statistical treatment**

Vitellogenin data: Normality and variance homogeneity were checked and data were logarithmically transformed if necessary. If normality and variance homogeneity could be obtained, a one-way ANOVA followed by a parametric Dunnett test was used. If not, a non-parametric Kruskal-Wallis ANOVA followed by Dunns test was used. Sex ratio data and gonadal maturation stages: $\chi^2$ tests were used. Significance level: $\alpha$=0.05. Unless otherwise stated, mean±standard deviation is given in the text.

**3. Results**

**3.1. Nitrate and nitrite water concentrations**

The actual exposure concentrations of nitrate and nitrite were typically somewhat lower than the nominal values and they ranged between 57 and 95 percent of the nominal values (Table 1). Approximately 1 mg NO$_3$\text{-}L$^{-1}$ was found in the tap water of the brown trout control tanks and –
consistent with the dilution with deionized water – nitrate concentrations were lower in the water of the zebrafish control groups (Table 1).

3.2. Zebrafish experiment 1

Sixty-one percent control fish (109 of 180) and 60 percent (429/720) of the total fish number survived between 20 and 60 dph and the average weight was 104±58 mg with no statistically significant difference between control and exposed groups for either weight, length (Table 2) or survival. There were no statistically significant differences in the weight and length of males and females. No statistically significant difference was found between the sex ratios of the two replicate control groups. The control groups had 55 females, 53 males and 1 undifferentiated fish and sex ratios in the groups exposed to nitrate or nitrite did not show any statistically significant deviations from this ratio (Fig. 1). A few undifferentiated fish were found in the exposed groups, apparently independent of exposure concentrations; undifferentiated fish were smaller than average (65±35mg; n=7). One intersex fish was registered in the group exposed to the highest (14 mg/L) concentration of nitrite (Fig. 1).

All stages of ovaries were found in the female fish but stage 2 was the predominant (Fig. 2A); no statistically significant differences were found between control and exposed groups. Testes of the male fish were predominantly stage 2 and 3 (Fig. 2B) and there were no statistically significant differences between control and exposed groups.

3.3. Zebrafish experiment 2

Twenty-seven percent control fish (52 of 190) and thirty percent (286/950) of the total fish number survived between fertilization and 60 dpf and the average weight of the fish was 142±78 mg (n=286) with no statistically significant difference between control and exposed groups for neither weight, length (Table 2) nor survival. There was no difference between male and female fish; undifferentiated fish were generally small (36±25 mg; n=15).

The control groups had 56 percent males, 36 percent females, 6 percent undifferentiated fish and 2 percent intersex fish (Fig. 3) and similar skewed sex ratios were also found in the exposed groups with no statistically significant differences from the control groups. No statistically significant
differences were found between the sex ratios of the replicates of any exposure group. Median vitellogenin levels in the female fish ranged between $7 \times 10^4$ and $27 \times 10^4$ ng/g and there were no statistically significant differences between exposure groups or within replicate groups (Fig. 4A). Median vitellogenin levels in the male fish ranged from 88 to 281 ng/g and a statistically significant difference was found between the control group and the groups exposed to 19, 46 and 89 mg NO$_3^-$ /L (Fig. 4B), the latter 3 groups showing elevated concentrations. There was no effect of nitrite exposure (Fig. 4B).

3.4. Brown trout experiment

No mortality was registered during the experiment. The average length and weight of the brown trout were 7.4±0.6 cm and 4.2±1.0 g with no statistically significant differences between the groups (Table 2). Thirty-two percent of the 196 fish were males; the weight of males and females was not different.

Concentrations of vitellogenin in the liver of the control group ranged from 5 to 880 ng/g for the females and 30 to 560 ng/g for the males (Fig. 5) with no statistically significant differences between the two sexes (p=0.19). Liver vitellogenin levels in the E2-reference group ranged from 204 to 9040 ng/g in the males and 142 to 28320 ng/g in the females with no statistically significant differences between the two sexes (p=0.2). For both sexes, the increases in the vitellogenin concentrations in the liver of the E2 exposed group relative to the control were statistically significant (p < 0.0001; not shown).

Liver vitellogenin levels in the female fish in the groups exposed to nitrate were not affected (p=0.37) relative to the controls (Fig. 5A). In the males, exposure to nitrate showed an overall effect (p=0.027) with the group exposed to 10 mg NO$_3^-$ /L showing a trend (p=0.062) of higher vitellogenin concentrations in the liver than the control group (Fig. 5b).

4. Discussion

Exposure to nitrate and nitrite at environmentally realistic concentrations did not affect the sex ratio in zebrafish but exposure to nitrate caused a slight, but statistically significant increase in vitellogenin levels in male – but not in female - zebrafish and juvenile brown trout.
The test acceptability criteria for control fish in OECD TG 234 is an average weight at 60 dph of at least 75 mg blotted wet weight and a length of at least 14 mm; with average weights > 92 mg and lengths of > 16mm in all groups, this criterion was met in the present experiments. However, the growth of zebrafish larvae also depends on the population density (Tsai et al., 2007) such that zebrafish in heavily populated aquaria grow to a smaller size than fish in less densely populated ones. Zebrafish experiment 1 and 2 had 54±6 and 29±7 fish, respectively, in each tank at 60 dpf and in concordance with (Tsai et al., 2007), the fish in experiment 2 grew larger than the fish in experiment 1.

Exposure to nitrate and nitrite did not affect the survival of the zebrafish and this is consistent with the No Observable Effect Concentration (NOEC) for nitrate exposure of 4761 mg NO₃⁻/L for swim-up larvae which were the most sensitive life stage (Learmonth and Carvalho, 2015) and no effect of exposure to 300 mg NO₂⁻/L on 5 dph survival (Simmons et al., 2012). The overall control fish survival in zebrafish experiment 2 was lower than the OECD TG 234 test acceptability criteria (survival should be at least 56% - 80% hatching success and 70% post hatch survival). The reason for the low control survival was a high mortality occurring during the very sensitive period of transition between internal- and external food intake around 5-10 dpf. We do not consider this to affect the overall conclusion from the experiments. Excess mortality would only affect the overall conclusion concerning the effects on the sex ratio if gender specific mortality occurred. Mortality predominantly took place during the very early larval stages (before initiation of sexual differentiation at 20 dpf) so mortality took place - not among males or females but - among undifferentiated larvae. The toxicological data on nitrate and nitrite as well as the results of experiment 1 (where survival did meet the TG234 criteria) with zebrafish also do not support gender specific mortality. The survival in the experiment 2 (27%) is only slightly lower than it was (31.7 %) in an investigation of the effects of biochanin A in the Fish Sexual Development Test (Holbech et al., 2013).

Nitrate and nitrite are proposed to be able to affect steroid production and concentration subsequent to reduction to NO which plays a role in the regulation of enzymes in the steroidogenic pathways in the gonads as well as in the regulation of hepatic CYP-enzymes degrading the steroids.
(reviewed by Guillette and Edwards, 2005). The majority of studies in which the effects of nitrate or nitrite on steroidogenesis have been investigated in male mammals have shown inhibitory effects in Leydig cells and/or decreases in plasma concentrations of T - if any effects have been found (Panesar, 1999; Panesar and Chan, 2000; Zraly et al., 1997). Four weeks’ exposure of male rats to 33 mg NO$_3$-/L or 37 mg NO$_2$-/L in the drinking water reduced the plasma T concentrations to approximately 55 and 71%, respectively, of that of the controls (Panesar and Chan, 2000). In field investigations with mosquito fish (Gambusia holbroooki), some male reproductive characteristics and ambient nitrate concentrations appear correlated (Edwards and Guillette, 2007) but somewhat confusingly, gonopodium length and testis weight appear positively correlated to ambient nitrate concentrations whereas sperm counts show a negative correlation (Edwards and Guillette, 2007). For female mosquito fish, the proportion of non-pregnant females was positively correlated to ambient nitrate concentrations (Edwards et al., 2006). For juvenile alligators (Alligator mississippiensis) in seven Florida lakes, concentrations of nitrate or total-N were negatively correlated with female plasma T concentrations and positively correlated with male E2 concentrations and there was a trend that male T correlated negatively with the ambient concentration of total–N (Guillette and Edwards, 2005). Conversely, in an experimental study, increased plasma T concentrations were observed in young female alligators (Alligator mississippiensis) exposed to 443 mg NO$_3$-/L for five weeks or five months (Hamlin et al., 2016). In experimental studies on female sturgeon (Acipenser baeri), 30 days exposure to 252 mg NO$_3$-/L increased plasma concentrations of E2, T and 11-ketotestosterone (the latter only in one experiment of two) (Hamlin et al., 2008). Exposure of juvenile Atlantic salmon, Salmo salar, to 45.6 mg NO$_3$- /L for 27 days resulted in increased plasma T concentrations whereas exposure to 451 mg NO$_3$-/L did not (Freitag et al., 2015). Exposure of juvenile Labeo rohita to 6.6 mg NO$_2$-/L for 45 d caused dramatic reductions (male T: 97% and female E2: 93%) in plasma steroid concentrations (Ciji et al., 2013). Exposure to nitrate may also affect steroid production and development of male sexual traits in amphibians: ex vivo steroid production was inhibited in female Xenopus laevis exposed to 25 and 50 mg NO$_3$-/L for 7 days (Barbeau and Guillette, 2007) and expression of male sexual traits were altered by exposure of newts Triturus helveticus to 75 mg NO$_3$-/L for three weeks (Secondi et al.,
Exposure to 130 mg NO$_3$-L reduced reproductive output in medaka _Oryzias latipes_ (Shimura et al., 2002) and exposure to 50 and 250 mg NO$_3$-L for 209 d after hatch increased vitellogenin levels in both male and female fathead minnow, _Pimephales promelas_ (Kellock et al., 2018) and increased the concentration of 11-ketotestosterone in the males.

It is apparent from the above that the effects of nitrate or nitrite exposure on steroid concentrations and/or sexual traits vary widely among the various investigations and it is currently challenging to generate a general pattern for the responses. Induction of male vitellogenin synthesis (as seen in some of the groups in the present experiments) is known to be a response elicited by an E2-signal or an exposure to chemicals with oestrogenic activities and it may indicate that the exposure causes minor increases in the intrinsic E2 concentrations in the males. It is noteworthy that Kellock et al. (2018) observed increased levels of vitellogenin in both male and female fathead minnow exposed to nitrate (50 and 250 mg NO$_3$-L) for 209 d after hatch whereas this was only registered in the males of the present investigation. The biological relevance of the statistically significant effect of nitrate on vitellogenin concentrations in the male brown trout and zebrafish exposed to nitrate in the present investigation is questionable. The two higher nitrate exposures did not result in vitellogenin levels in the male brown trout that were different from the control. Non-monotonic dose response relationships have been documented in the area of endocrine disrupting effects (Vandenberg et al., 2012), but such datasets should also be interpreted cautiously (Beausoleil et al., 2013) - especially if only one dose or concentration deviates from the general pattern such as the vitellogenin results from the male brown trout in the present experiments and the increased T concentration for one of the exposure concentrations in Atlantic salmon (Freitag et al., 2015).

Concerning the effects of potentially altered steroid concentrations on the sex ratio in zebrafish, it is a well-established fact that sexual differentiation in zebrafish is very sensitive to exposure to chemicals that one way or another interfere with sex hormones. Sexual differentiation in zebrafish takes place between 20 and 40 dpf and although the sensitivity to exposure to exogenous hormones or endocrine disrupting chemicals (EDCs) is well-established, the exact biochemical mechanisms by which this takes place are still unknown. Zebrafish populations can be made all female or female biased by exposure to natural and synthetic steroid estrogens (Holbech et al., 2006; Nash et al.,
2004) and anti-androgenic chemicals (Kinnberg et al., 2015) during the period of sexual differentiation and all male or male biased populations can be produced by exposure to androgens (Morthorst et al., 2010; Orn et al., 2003) or aromatase inhibitors (Holbech et al., 2012; Kinnberg et al., 2007; Thorpe et al., 2011). 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 have the ability to alter the sex ratio in zebrafish (OECD, 2011b, c) which is the major apical endpoint in OECD TG 234. During the initial phase of the development and validation of OECD TG 234, exposure took place between 20 and 60 dpf (e.g. Holbech et al., 2006) whereas in the final guideline exposure takes place 0 to 60 dpf (OECD, 2011a). The background for the final TG 234 exposure regime from 0-60 dpf was that Japanese medaka (Oryzias latipes) and three spined stickleback (Gasterosteus aculeatus) were included as test species and medaka gonadal sex differentiation is initiated before hatch at around 8-9 dpf (Kobayashi et al., 2004) whereas the most sensitive window for sex differentiation in stickleback is within the first two weeks (Hahlbeck et al., 2004). For zebrafish, both exposure regimes are equally suited to reveal effects on sexual differentiation by EDCs (Andersen et al., 2004) and the choice of the two different exposure regimes in the two zebrafish experiments in the present investigation is not considered to have any influence on the results. The sex ratios in the control groups in the present experiments were close to unity in zebrafish experiment 1 whereas there was a bias towards males in zebrafish experiment 2. The test acceptability criteria for control fish in OECD TG 234 is a sex ratio within 30/70 for males and females (by histological evaluation) which is met in both experiments. Skewed control sex ratios have been noticed in other studies (Hensley and Leung, 2010; Holbech et al., 2013; Larsen et al., 2008; Orn et al., 2006), and Brown et al. (Brown et al., 2012) observed a significant male-biased (72%) sex ratio in the most inbred line of zebrafish lines studied. In the present study, the parental fish were purchased from a local supplier who does not register the breeding history of the fish so we cannot connect the inbreeding status to the sex ratio. We did, however, observe in an investigation where the controls were even more male-biased than in zebrafish experiment 2 of the present experiment that exposure to the phytoestrogen biochanin A could shift the sex ratio towards
more females (Holbech et al., 2013). Therefore, there is no reason to assume that the lack of effect of nitrate and nitrite on the sex ratio in experiment 2 is caused by the somewhat skewed sex ratio in the control group. Even if steroid concentrations have been slightly altered in the zebrafish males of the nitrate exposed groups (as indicated by the slight increase in male vitellogenin concentrations) it can be concluded that this potential alteration has not been high enough to affect the sexual differentiation.

Surveys carried out between 2000 and 2004 showed that the majority of juvenile brown trout in Danish streams had elevated levels of vitellogenin (Bjerregaard et al., 2006b; Bjerregaard et al., 2008) although Danish WWTPs were shown only to discharge negligible amounts of estrogenic activity (Stuer-Lauridsen et al., 2005). Concentrations of nitrate in streams affected by agriculture (which is the majority of the Danish area) are typically in the range 20 to 40 mg NO$_3$-L (Wiberg-Larsen, 2015) and the fairly high nitrate concentrations might be hypothesized to play a role for the elevated brown trout vitellogenin levels. The field studies (Bjerregaard et al., 2006b; Bjerregaard et al., 2008) showed elevated concentrations of vitellogenin in both male and female juvenile brown trout whereas the slightly increased levels in the lowest exposure group were only found in the males in the present investigation. Also, average concentrations of total nitrogen in Danish streams affected by agriculture were 6.5 mg total-N/L (corresponding to 29 mg NO$_3$-L) in the period between 2000 and 2004 – or somewhat higher than the low concentration with a marginal effect in the present investigation. Therefore, the results of the present investigation do not indicate that the nitrate present in the Danish streams is responsible for the elevated vitellogenin levels demonstrated in the 2000-2004 surveys (Bjerregaard et al., 2006b; Bjerregaard et al., 2008). Corroborating this conclusion, Morthorst et al. (2018) recently showed that vitellogenin concentrations have decreased between 2004 and 2010 and it is indicated that this change is caused by a reduction in the discharges of wastewater with little purification from houses in the open land. The present results do, however, also indicate that the potential role of nitrate as an endocrine active substance in male fish is not unambiguously elucidated.

In conclusion, some marginal endocrine activity of nitrate in male fish was observed at environmentally relevant concentrations but the present results for zebrafish do not define nitrate
and nitrite as endocrine disrupting chemicals according to the generally accepted WHO/IPCS definition (OECD, 2012; WHO/IPCS, 2002; WHO/UNEP, 2013) because no adverse effects (altered sex ratios) were demonstrated.

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Figure legends

**Fig. 1.** *Danio rerio*. Percentages of females, males, undifferentiated and intersex zebrafish after exposure to nitrate or nitrite in the water from 20 to 60 days post hatch (zebrafish experiment 1). The number of fish in each group is given in histogram inserts. Actual mean concentrations of nitrate or nitrite are shown on the x-axis.

**Fig. 2.** *Danio rerio*. Percentages of gonads in the various maturity stages in female (A) and male (B) zebrafish in zebrafish experiment 1. The number of fish in each group is given in histogram inserts. Actual mean concentrations of nitrate or nitrite are shown on the x-axis.

**Fig. 3.** *Danio rerio*. Percentages of females, males, undifferentiated and intersex zebrafish after exposure to nitrate or nitrite in the water from 0 to 60 days post hatch (zebrafish experiment 2). The number of fish in each group is given in histogram inserts. Actual mean concentrations of nitrate or nitrite are shown on the x-axis.

**Fig. 4.** *Danio rerio*. Vitellogenin concentrations in female (A), male (B) and undifferentiated and intersex (C) zebrafish exposed to nitrate or nitrite in the water from 0 to 60 days post hatch (zebrafish experiment 2). Fifty and 90 percentile box plot with median (◊) and outliers (●). The number of fish in each group is shown above the x-axis. Groups without common letters are significantly different (P< 0.05). Actual mean concentrations of nitrate or nitrite are shown on the x-axis.

**Fig. 5.** *Salmo trutta*. Vitellogenin concentrations in the liver of female (A) and male (B) juvenile brown trout exposed to nitrate in the water for 8 days. Fifty and 90 percentile box plot with median (◊) and outliers (●). The number of fish in each group is shown above the x-axis. Actual mean concentrations of nitrate are shown on the x-axis.
Table 1. Nominal and actual concentrations in the 3 experiments. Number of determinations are given in parentheses. Concentrations are given as mg NO$_3$/$L$ or mg NO$_2$/$L$.

<table>
<thead>
<tr>
<th></th>
<th>NO$_3^-$</th>
<th>NO$_2^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nominal</strong></td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Zebrafish1</td>
<td>0.4±0.04 (30)</td>
<td>3.6±0.4 (10)</td>
</tr>
<tr>
<td></td>
<td>10.0±1.0 (15)</td>
<td>18.5±0.7 (12)</td>
</tr>
<tr>
<td></td>
<td>14.7±1.3 (15)</td>
<td>46±3.3 (12)</td>
</tr>
<tr>
<td>Trout</td>
<td>1.1±0.07 (12)</td>
<td>13.7±0.6 (12)</td>
</tr>
<tr>
<td></td>
<td>5.7±0.3 (12)</td>
<td>31±1.3 (12)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Zebrafish2</td>
<td>0.7±0.1 (6)</td>
<td>18.9±2.2 (12)</td>
</tr>
<tr>
<td></td>
<td>18.5±0.7 (12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>46±3.3 (12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>89±3.5 (12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.9±1.1 (12)</td>
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</table>
Table 2. Weight and length of the fish in the 3 experiments (mean±SEM).

<table>
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<tr>
<th>Nominal</th>
<th>mg NO$_3$/L</th>
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<th></th>
<th></th>
<th>mg NO$_2$/L</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>25</td>
<td>50</td>
<td>100</td>
<td>5</td>
<td>20</td>
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<tr>
<td>Zebrafish1</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>112±6</td>
<td>119±9</td>
<td>101±8</td>
<td>95±7</td>
<td>109±8</td>
<td>93±7</td>
<td>94±8</td>
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<tr>
<td>Length (mm)</td>
<td>17.3±0.3</td>
<td>18.0±0.4</td>
<td>16.6±0.4</td>
<td>16.7±0.5</td>
<td>17.1±0.4</td>
<td>16.7±0.4</td>
<td>16.4±0.4</td>
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<tr>
<td>Zebrafish2</td>
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</tr>
<tr>
<td>Weight (mg)</td>
<td>144±12</td>
<td>141±11</td>
<td>145±9</td>
<td>137±12</td>
<td>140±8</td>
<td>18.8±0.4</td>
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<tr>
<td>Length (mm)</td>
<td>18.4±0.5</td>
<td>18.2±0.6</td>
<td>18.7±0.5</td>
<td>18.2±0.5</td>
<td>18.8±0.4</td>
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<tr>
<td>Brown trout</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Weight (g)</td>
<td>3.9±0.16</td>
<td>4.4±0.16</td>
<td>4.3±0.14</td>
<td>4.0±0.19</td>
<td></td>
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<tr>
<td>Length (cm)</td>
<td>7.2±0.1</td>
<td>7.6±0.1</td>
<td>7.4±0.1</td>
<td>7.5±0.1</td>
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</tr>
</tbody>
</table>
Figure 1

![Bar chart showing the percent distribution of females, males, intersex, and undifferentiated individuals across different nitrate/nitrite concentration levels. Each bar is divided into segments representing these categories, with labels indicating the concentration levels (e.g., Control, 8.8 NO₃⁻, 15 NO₃⁻, etc.) and the corresponding percent distribution values.]
Figure 2

(A) Ovaries

(B) Testes

Nitrate/nitrite concentration (mg/L)
Figure 3: Percent distribution of Nitrate/nitrite concentration (mg/L) for different categories: Females, Males, Intersex, and Undiff. The concentrations are as follows:

- Control: 52
- 19 NO$_3^-$: 56
- 46 NO$_3^-$: 55
- 89 NO$_3^-$: 59
- 19 NO$_2^-$: 64
Figure 4

(A) Female fish

(B) Male fish

(C) Undifferentiated & intersex fish

Vitelligenin level (ng g$^{-1}$ wet weight)

Nitrate/nitrite concentration (mg/L)

Control 19 NO$_3^-$ 46 NO$_3^-$ 89 NO$_3^-$ 19 NO$_2^-$