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Disruption of deiodinase type 2 in zebrafish disturbs male and female reproduction

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Abstract

Thyroid hormones are crucial mediators of many aspects of vertebrate life, including reproduction. The key player is the biologically active 3,5,3'-triiodothyronine (T₃), whose local bio-availability is strictly regulated by deiodinase enzymes. Deiodinase type 2 (Dio2) is present in many tissues and is the main enzyme for local T₃ production. To unravel its role in different physiological processes, we generated a mutant zebrafish line, completely lacking Dio2 activity. Here we focus on the reproductive phenotype studied at the level of offspring production, gametogenesis, functioning of the hypothalamic–pituitary–gonadal axis and sex steroid production. Homozygous Dio2-deficient zebrafish were hypothyroid, displayed a delay in sexual maturity and the duration of their reproductive period was substantially shortened. Fecundity and fertilization were also severely reduced. Gamete counts pointed to a delay in oogenesis at onset of sexual maturity and later on to an accumulation of oocytes in mutant ovaries due to inhibition of ovulation. Analysis of spermatogenesis showed a strongly decreased number of spermatogonia A at onset of sexual maturity. Investigation of the hypothalamic–pituitary–gonadal axis revealed that dysregulation was largely confined to the gonads with significant upregulation of *igf3*, and a strong decrease in sex steroid production concomitant with alterations in gene expression in steroidogenesis/steroid signaling pathways. Rescue of the phenotype by T₃ supplementation starting at 4 weeks resulted in normalization of reproductive activity in both sexes. The combined results show that reproductive function in mutants is severely hampered in both sexes, thereby linking the loss of Dio2 activity and the resulting hypothyroidism to reproductive dysfunction.

Key Words

- ▶ thyroid hormone
- ▶ deiodinase
- ▶ deficiency
- ▶ reproduction
- ▶ zebrafish

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Introduction

Vertebrate reproduction is controlled by several endocrine factors, among which thyroid hormones (THs) are indispensable. They play an essential role in gametogenesis, steroidogenesis, embryogenesis, fetal development and growth, always with 3,5,3'-triiodothyronine (T₃) as a key factor (Jana & Bhattacharya 1994, Kirby *et al.* 1996, Tovo-Neto *et al.* 2018). Balanced T₃ levels are therefore of crucial importance, since both too high or too low levels can have detrimental consequences for reproduction/fertility,

as shown from fish to humans (Beamer *et al.* 1981, Carr & Patino 2011, Mintziori *et al.* 2016).

The link between THs and reproduction is being investigated in different animal models, including zebrafish. Gametogenesis in teleosts, being oviparous and anamniote vertebrates, differs somehow from mammals. Oogonia continue to proliferate throughout adulthood, oocytes store a large amount of yolk during vitellogenesis and postvitellogenic follicles contain no antrum

(Jalabert 2005). Spermatogenesis occurs in cysts, enveloping a single germ cell clone, and cyst-forming Sertoli cells retain their capacity to proliferate throughout adulthood (Schulz *et al.* 2010). Zebrafish are sexually mature from approximately 3 months onward, they can spawn all year round and evidence for TH action in the gonads is evident: mRNA for both TH receptors (*thraa* and *thrb*) has been detected in zebrafish ovaries and testes (Morais *et al.* 2013, Marelli *et al.* 2016) and specifically in males it was shown that T₃ stimulates the formation of new cysts via proliferation of Sertoli cells and undifferentiated spermatogonia A, mediated by insulin-like growth factor 3 (Igf3) (Morais *et al.* 2013).

An often used approach to study the role of THs in fish reproduction is goitrogen treatment. These chemicals lower systemic TH levels by blocking hormone production in the thyroid gland. This adversely affects reproductive success, as shown by decreased fecundity and gonadosomatic index, altered sex steroid levels and decreased sperm count (Mukhi & Patino 2007, Naderi *et al.* 2014, Forsatkar *et al.* 2018). Next to systemic TH levels, deiodinases are important players in regulating tissue TH content. By intracellular activation and inactivation of THs, these enzymes function to adapt the amount of active TH to the local needs. Deiodinases are structurally and functionally conserved among vertebrates and exist in three different types (Darras & Van Herck 2012). Deiodinase type 2 (Dio2) is the most important one for catalyzing the conversion of the prohormone 3,5,3',5'-tetraiodothyronine (T₄) into T₃, whereas deiodinase type 3 (Dio3) is the main inactivator of T₃. Deiodinase type 1 (Dio1) can both activate and inactivate THs but has a lower substrate affinity (Darras & Van Herck 2012).

We recently generated Dio2-knockout (KO) zebrafish (*dio2*^{-/-}) and initial characterization of the phenotype revealed a severe disruption of reproduction (Houbrechts *et al.* 2016). This is in strong contrast to DIO2KO mice that do not show any obvious reproductive defects (Schneider *et al.* 2001). On the other hand, reproduction is severely hampered in both male and female DIO3KO mice (Hernandez *et al.* 2006), showing that in mammals too, deiodinase deficiency can have detrimental effects on reproduction. One factor that could play a role in the divergence between the three models are the differences observed in TH status. *dio2*^{-/-} mutant zebrafish are severely hypothyroid (Houbrechts *et al.* 2016) while DIO2KO mice are euthyroid (Schneider *et al.* 2001). DIO3KO mice experience thyrotoxicosis in early life, but this shifts to hypothyroidism from postnatal day 15 onward throughout adulthood (Hernandez *et al.* 2006).

These data raise the question about the relative importance of deiodinases versus inappropriate systemic TH levels in the regulation of reproduction.

In the present study we investigated the contribution of Dio2 in reproduction, using our *dio2*^{-/-} mutant zebrafish. These animals are permanently Dio2 deficient, suffer from severe hypothyroidism and reproduce poorly (Houbrechts *et al.* 2016). We first studied their reproductive phenotype at different levels and subsequently investigated whether reversing their overall hypothyroidism by T₃ supplementation was able to rescue this phenotype.

Materials and methods

Zebrafish husbandry

Our research group recently generated two *dio2*^{-/-} zebrafish lines (Houbrechts *et al.* 2016). For the present study we used the deletion mutant (lv1) which is missing 3 conserved amino acids upstream of selenocystein, leading to a complete abolishment of Dio2 activity. Wild type (WT), heterozygous and homozygous mutant zebrafish were maintained in circulating freshwater aquaria at 27.5 ± 1°C under a 14L/10D regimen. Fish were fed *ad libitum* with formulated feed in the morning and *Artemia salina* larvae in the evening. Embryos and larvae (until 7 days post fertilization) were kept in 0.3 × Danieau's medium after spawning and incubated at 28 ± 0.5°C. Animal experiments were approved by the Institutional Ethical Committee of the KU Leuven (P239/2013 and P091/2017) and executed in strict accordance with the European Council Directive (2010/63/EC).

Fecundity and fertilization rate assessment

Sexually mature fish were allowed to spawn either via natural group matings or via individual mating set-ups. After 3 h the eggs were collected and counted. Fertilized/unfertilized eggs were discerned under a stereomicroscope and fertilization percentages were determined. Group spawning experiments were conducted as previously described (Houbrechts *et al.* 2016). For individual mating set-ups five male and five female *dio2*^{+/-} and *dio2*^{-/-} siblings were paired alternately with the same and the opposite genotype.

Thyroid hormone measurements

For TH extractions, 3 pools of 3–4 ovaries per genotype and 4–7 pools of 10–14 testes per genotype were collected

from 1-year-old fish and snap-frozen on dry ice. THs were extracted and measured by radioimmunoassay as previously described (Reyns *et al.* 2002).

RNA isolation and quantitative PCR

Ovaries, testes, liver and brain were dissected from 7-month-old and 1-year-old WT and *dio2*^{-/-} fish and snap-frozen on dry ice. For pooled samples, 3 replicates (pools of 3 fish) were used per genotype. When analyzing individual samples, quantitative PCR (qPCR) was performed on 5–7 replicates per genotype. *vtg1* (liver), *igf3*, *ar*, *esr2a*, *esr2b*, *hsd11b2*, *hsd17b3*, *cyp19a1b* (gonads), *gnrh2*, *gnrh3*, *kiss1* and *kiss2* (brain) mRNA was quantified using SYBR Green-based qPCR as explained in detail in Supplementary data and Supplementary Table 1 (see section on [supplementary data](#) given at the end of this article).

Histology

Adult WT and *dio2*^{-/-} fish were killed using 0.1% tricaine. Gonads were sampled, fixed in 4% phosphate-buffered paraformaldehyde at 4°C, dehydrated, embedded in paraffin and sectioned at 7 µm. Three different stages were investigated for females: 3/4 months (i.e. onset of sexual maturity of WT and *dio2*^{-/-} respectively), 1 year (i.e. age that *dio2*^{-/-} no longer reproduce but WT still do), and for WT also 2 years (after reproductive arrest). Testis histology was performed at 3/4 months and 1 year. All sections were stained using hematoxylin (1 min) and 1% acidified eosin (5 s) and surface measurements and gamete counts were performed. Details on histological analysis can be found in [Supplementary data](#). For each genotype and sex, gonads from 3 to 4 fish were analyzed.

Steroid hormone measurements

We measured sex steroid levels (estradiol (E2), 11-ketotestosterone (11-KT) and testosterone) in gonads from 1-year-old *dio2*^{+/+} and *dio2*^{-/-} fish. Ovaries were collected individually ($n=12$ for *dio2*^{+/+}; $n=9$ for *dio2*^{-/-}). For testes we pooled tissues from 3 fish per replica ($n=7$ for *dio2*^{+/+}; $n=10$ for *dio2*^{-/-}). Gonad tissue was extracted twice with 4 volumes of diethyl ether. The combined supernatant was dried and dissolved in 200 µL buffer after which enzyme-linked immunosorbent assay (ELISA; kits from Cayman Chemical Company) was performed. These kits have previously been used for sex steroid measurement in zebrafish (Liu *et al.* 2009, Felix *et al.* 2013). Additionally, sex steroid secretion in the holding water was determined.

Seven-month-old sexually active *dio2*^{+/+} and *dio2*^{-/-} fish (7 males, 7 females) were placed individually in a glass container with 110 mL system water for 4 h without visual contact. Hormones were extracted from the holding water and ELISA was performed according to the methods described by Felix *et al.* (2013) and Morthorst *et al.* (2013) for 11-KT and E2 respectively.

T₃ rescue treatment

Two separate batches of *dio2*^{+/+} ($n=41$ –48) and *dio2*^{-/-} ($n=13$ –44) 4-week-old juveniles (F2 generation from siblings) were divided in half and placed in 8 L tanks. One group received T₃ supplementation (10 nM) while the other group served as control (10⁻⁵ N NaOH). Solutions were refreshed 3 times a week and treatment continued until 6 months of age. Fecundity and fertilization rates were assessed starting from 3 months of age. Fish were set up to reproduce once weekly.

Statistical analysis

Statistical analysis for qPCR, gamete counts and surface measurements was performed using GraphPad Prism for Windows version 5.00 (GraphPad Software). If data passed the Kolmogorov–Smirnov normality test, they were analyzed by unpaired Student's *t* test (2 groups) or one-way ANOVA with Tukey *post hoc* test (>2 groups). When data did not follow Gaussian distribution, the non-parametric Mann–Whitney *U* test (1 variable) or Kruskal–Wallis test (2 variables) was performed. Data from mating set-ups were analyzed by repeated-measures two-way ANOVA in GraphPad. A *P* value ≤0.05 was considered statistically significant for all analyses.

Results

General reproductive phenotype

WT zebrafish typically start reproducing around the age of 3 months. The onset of sexual maturity in *dio2*^{-/-} fish was delayed by approximately 2 months. Furthermore, WT zebrafish can reproduce for 18 months or more, while the reproductive period of *dio2*^{-/-} fish was severely shortened to 2–3 months. This implies that by 8 months of age, mutants normally no longer show sexual activity. Moreover, more than half of the mutants systematically failed to spawn during this 2- to 3-month period, which renders them infertile. Survival rates in embryos/larvae of fertilized mutant eggs were similar to

those for WT. We did however observe a clear shift toward a male population. Sex determination of 179 fish (combination of 2 batches) after heterozygous intercross showed the following male-to-female ratios: *dio2*^{+/+}: 51:49; *dio2*^{+/-}: 68:32; *dio2*^{-/-}: 76:24.

Thyroid status in the gonads

T₃ is the most active TH and is mainly produced peripherally by the Dio2 enzyme. Therefore, tissue T₃ levels are the most representative for local thyroid status. T₃ levels measured in 1-year-old ovaries and testes were significantly reduced by 94 and 79% in *dio2*^{-/-} zebrafish respectively (Fig. 1A and B). For ovaries we also measured T₄ levels, which were reduced by 48% (0.96 ± 0.23 pmol/g for *dio2*^{-/-} vs 1.83 ± 0.18 pmol/g for *dio2*^{+/+}; $P=0.04$). T₄ data for testes are not available due to lack of material. These data show that the gonads of *dio2*^{-/-} zebrafish are in a hypothyroid status, but that the effect is most pronounced in females where T₃ levels are extremely reduced.

Effects on the expression of other genes regulating TH levels and action were investigated by qPCR in 1-year-old fish. In the ovaries we found a significant upregulation of *dio1* and *thraa* expression in *dio2*^{-/-} mutants compared to WT, while *dio3a*, *dio3b* and *thrb* expression were unaffected (Fig. 1C). For testes a significant downregulation was observed in *dio1* and *thrb* expression (Fig. 1D).

Effects on spawning

Our previous study briefly mentioned that reproduction is hampered in *dio2*^{-/-} mutants (Houbrechts *et al.* 2016). We now followed spawning in three batches, containing

different male-to-female ratios (i.e. 22:9, 5:12 and 15:15). Regardless of the sex ratio, matings of *dio2*^{-/-} fish yielded significantly less eggs compared to WT (17 ± 5 for *dio2*^{-/-} vs 152 ± 17 for *dio2*^{+/+}) and fertilization percentages were significantly reduced ($21 \pm 5\%$ for *dio2*^{-/-} vs $76 \pm 4\%$ for *dio2*^{+/+}).

Since these results suggested that fertility might be hampered in both sexes, we subsequently crossed individual *dio2*^{+/+} and *dio2*^{-/-} siblings (Fig. 2). As expected, the best reproductive performance was obtained when pairing WT males and females. For the mating between mutant males and females both egg production and fertilization were significantly reduced. Combination of WT fish with the opposite mutant sex also adversely affected fecundity and fertilization with values between those of pure WT or *dio2*^{-/-} combinations.

Effects on gonad size and gametogenesis

Since weight but not length of female *dio2*^{-/-} mutants increases drastically from sexual maturity onward (Houbrechts *et al.* 2016), we hypothesized this was caused by the accumulation of eggs in the abdomen. We determined the gonad section surface area of mutant and WT fish at 3/4 months and 1 year as a proxy for ovary/testis size. Ovary size was significantly increased in *dio2*^{-/-} fish at both ages (Fig. 3A) while testis size was not affected (Fig. 3E). Representative pictures of ovaries and testes are shown in Fig. 4. To corroborate our surface measurements, we also weighed gonads of a different 1-year-old batch. These data substantiate the surface measurements, showing significantly increased ovarian weight in *dio2*^{-/-} fish (30.1 ± 3.2 mg for WT vs 85.1 ± 13.1 mg for mutant; $n=5$ per genotype; unpaired Student's *t* test; $P=0.008$),

Figure 1

TH status in gonads of 1-year-old *dio2*^{+/+} and *dio2*^{-/-} zebrafish. (A and B) T₃ levels (pmol per gram wet weight) in ovary (3 replicates of 3–4 ovaries) and testis (4–7 replicates of 10–14 testes) extracts of WT and *dio2*^{-/-} fish. (C and D) qPCR analysis in WT and *dio2*^{-/-} fish, showing relative mRNA expression of *dio1*, *dio3a*, *dio3b*, *thraa* and *thrb* in ovary and testis. Data were normalized based on the mean expression of three housekeeping genes (*eef1a11l*, *rpl13a* and *eif1b* for ovary and *eef1a11l*, *rpl13a* and *hprt1* for testis). Average WT (*dio2*^{+/+}) values were set to 1 and *dio2*^{-/-} values were rescaled accordingly. All data are expressed as mean $\pm$ s.e.m. (three replicates with three animals pooled per replicate). Per tissue and per gene, differences between both genotypes were analyzed by an unpaired Student's *t* test. * $P < 0.05$; ** $P < 0.01$.

Figure 2

Spawning experiment of *dio2*^{+/+} and *dio2*^{-/-} siblings. Five males and five females of each genotype were individually crossed in two combinations: male and female of the same genotype or male and female of different genotypes. (A) Fecundity: total number of eggs per spawning; (B) fertilization percentages. Values represent mean ± S.E.M. Data were analyzed by repeated-measures ANOVA with Tukey *post hoc* test. Groups with no common letter are significantly different ($P < 0.05$).

while testis weight did not differ (2.7 ± 0.3 mg/animal for WT ($n=5$, pools of 6) vs 2.9 ± 0.3 mg/animal for mutant ($n=4$, pools of ≥ 5); Mann–Whitney U test; $P=0.206$).

Counting different oocyte stages, we only observed significant differences at 3/4 months showing a higher percentage of primary oocytes and a lower percentage of vitellogenic oocytes in *dio2*^{-/-} compared to WT (Fig. 3B and C). To make an accurate comparison at the stage of reproductive arrest, we also investigated 2-year-old WT females. This showed a strong decrease in oocyte number in WT fish when they stopped reproducing: 49, 59 and 43% reduction from 1 towards 2 years for primary, vitellogenic and mature oocytes, respectively. This is in strong contrast to the also non-active 1-year-old mutants, where oocyte numbers remained high, confirming they have problems with ovulation/egg deposition.

For testes, comparison between genotypes revealed a significant reduction in the percentage of area covered by spermatogonia A at 3/4 months in *dio2*^{-/-} fish, while the

opposite was observed at 1 year (Fig. 3F). Spermatozoa area on the other hand was significantly increased in *dio2*^{-/-} fish at reproductive onset, but significantly decreased at 1 year compared to WT (Fig. 3G). The remaining area (containing spermatogonia B, spermatocytes, spermatids, Leydig and Sertoli cells) was significantly lower at 3/4 months in *dio2*^{-/-} fish (Fig. 3H).

Effects on the hypothalamic–pituitary–gonadal axis

To identify potential disturbances in the functioning of the hypothalamic–pituitary–gonadal (HPG) axis, we measured the expression levels of relevant genes in tissues of 7-month-old sexually active siblings (Fig. 5). Two primary components were measured: kisspeptin (*kiss1* and *kiss2*) and gonadotropin-releasing hormone (*gnrh2*, *gnrh3*). No differences were observed for both sexes, except for a significant increase in *kiss2* in male *dio2*^{-/-} brain (Fig. 5B). Due to lack of biological material, we were not able to obtain data on gonadotropins in the pituitary gland. Therefore, gonadal expression of *igf3*, a gonadotropin-regulated gene, was measured, revealing a significant upregulation in *dio2*^{-/-} mutants of both sexes (Fig. 5A and B).

Next, we measured intra-gonadal sex steroid levels in 1-year-old WT and *dio2*^{-/-} fish (F1 generation from siblings). E2 levels in ovarian extracts were significantly reduced in *dio2*^{-/-} fish (Fig. 6A). Similarly, 11-KT and testosterone levels were significantly decreased in mutant testis extracts (Fig. 6A). Since these sex steroids also act as pheromones that could also be involved in spawning failure, we determined the amount of E2 (females) and 11-KT (males) secreted into the water by WT fish and infertile mutants at 7 months of age (F1 generation from siblings). E2 as well as 11-KT secretion was significantly reduced in *dio2*^{-/-} fish (Fig. 6B).

To find out the reason and consequences of the decreased sex steroid levels, we tested a set of genes belonging to the steroid biosynthesis and signaling pathway: 2 estrogen receptors (*esr2a*, *esr2b*), the androgen receptor (*ar*), the aromatase enzyme-converting testosterone into E2 (*cyp19a1b*), and 2 enzymes involved in 11-KT production (*hsd11b2* and *hsd17b3*), the most abundant and most potent androgen in fish. Steroidogenesis and steroid signaling seemed to be similarly affected in *dio2*^{-/-} ovaries and testes compared to WT siblings (Fig. 6C and D). A general trend toward upregulation was observed, with a significant increase for *ar* (both sexes), *esr2b* (ovary) and *hsd17b3* (testis). One remarkable exception was the significant reduction in

Figure 3

Gametogenesis stage counting in *dio2*^{+/+} and *dio2*^{-/-} fish. (A) Ovary size, presented as average ovarian section surface (mm²); Percentage of primary (B), vitellogenic (C) and mature (D) oocytes determined on five sections per female at 3/4 months (3/4 M) and 1 year (1 Y); (E) Testis size, presented as average testicular section surface (mm²); Percentage of spermatogonia A (F), spermatozoa (G) and remaining area (H) determined on four sections per male at 3/4 months and 1 year. Data are expressed as mean $\pm$ s.e.m. ($n = 3-4$) and were analyzed by Mann-Whitney *U* test. * $P < 0.05$.

hsd11b2 levels in both sexes. Hepatic expression of *vtg1*, a major component of egg yolk, showed a significant upregulation in *dio2*^{-/-} fish (Fig. 5A). We also analyzed heterozygous individuals and found that these fish showed expression profiles very similar to their WT siblings (data not shown).

The effect of T₃ supplementation on reproductive status

dio2^{-/-} zebrafish suffer from severe peripheral hypothyroidism. To find out if the defects caused by insufficient Dio2-dependent T₃ production in the gonads and/or hypothalamus/pituitary could be overcome by systemic hormone treatment, T₃ was added to the holding water of developing WT and mutant zebrafish (F2 generation from siblings) from 4 weeks post fertilization. This treatment did not only normalize the onset of sexual maturity in *dio2*^{-/-} fish to 3 months instead of 5 months, but both egg production and fertilization rates also increased up to levels of WT fish (Fig. 7). As expected, mutants receiving a vehicle treatment only started to reproduce after 5 months of age for a duration of 2 months or less.

Discussion

Deiodinases are important to ensure the correct TH balance throughout the body and activity-reducing deiodinase polymorphisms have a negative impact on different

aspects of human health (Verloop *et al.* 2014). This has however not yet been thoroughly studied in relation to reproduction. Data from mouse studies show that reproduction is severely hampered in DIO3KO mice while fertility is unimpeded in DIO2KO mice (Schneider *et al.* 2001, Hernandez *et al.* 2007). We studied reproduction in a Dio2-deficient zebrafish line where adults, in contrast to euthyroid DIO2-deficient mice, display severe hypothyroidism throughout the body. Our results clearly demonstrated adverse effects of Dio2 deficiency and the resulting hypothyroid status on reproduction but also showed that reversing systemic hypothyroidism prior to gonad maturation rescues reproductive activity.

dio2^{-/-} zebrafish show a delay in onset of reproduction

The onset of sexual maturity is delayed in *dio2*^{-/-} zebrafish. Female mutants only start laying eggs around 5 months of age. At 3/4 months, corresponding to the onset of sexual maturity, we found a shift in oocyte development, with more primary oocytes at the expense of vitellogenic oocytes, indeed pointing to a delay. This shift may be due to a delay in vitellogenin incorporation in hypothyroid *dio2*^{-/-} females. It was indeed found in Japanese eel that T₃ accelerates vitellogenin incorporation and increases the percentage of vitellogenic oocytes (Kayaba *et al.* 2008). Therefore we would expect hepatic *vtg1* expression to be lower in mutants but unfortunately, we do not have expression data at this early age. Analysis at 7 months

Figure 4

Representative pictures of gonads of *dio2*^{+/+} and *dio2*^{-/-} fish at 1 year of age. (A and B) Ovaries showing primary oocytes, vitellogenic oocytes (cortical alveolus stage + vitellogenesis) and mature oocytes; (C and D) Testes showing spermatogonia A and spermatozoa. For counts, all four types of spermatogonia A (1 undifferentiated and 3 differentiated) were included.

showed a significant increase in *vtg1* expression, which points in a different direction and should be further investigated.

Pronounced hypothyroidism is known to disturb spermatogenesis, as observed for instance in young goitrogen-treated tilapia showing complete absence of spermatids and spermatozoa (Matta *et al.* 2002). The effects of Dio2 deficiency in young zebrafish were less severe, showing a reduced percentage of spermatogonia A but an increased percentage of spermatozoa in mutants right before the onset of apparent sexual activity. One possibility is that the available pool of spermatogonia in hypothyroid *dio2*^{-/-} fish is reduced. Since stimulation of spermatogonial proliferation by T₃ occurs via Igf3 (Morais *et al.* 2017) and *igf3* expression is increased in the mutants (although only measured at a later stage), this is doubtful. Alternatively, high Igf3 levels might stimulate differentiation of spermatogonia toward mature sperm cells (Nobrega *et al.* 2015), which would fit with the observed shift in relative proportion of spermatogonia/spermatozoa.

Following the definition of teleostean puberty, which starts with the onset of gametogenesis and ends with first reproduction (Schulz & Goos 1999), we can hypothesize that *dio2*^{-/-} fish are delayed in puberty since they did not reproduce at 3 months of age. This corresponds to the observed delay in sexual activity in hypothyroid mammals (Bakke *et al.* 1976, Weber *et al.* 2003). Another zebrafish study similarly showed that goitrogens delay male pubertal development (Sharma & Patino 2013). In contrast to hypothyroid DIO3KO mice with delayed puberty, we did not find indications for a lack of testis growth spurt (Martinez *et al.* 2016). Rather, diminished production of sex steroids (specifically 11-KT in males) could be the underlying cause (Schulz & Goos 1999).

Reproduction remains impaired at later age

The apparent delay in oogenesis was caught up at later age, since relative oocyte counts at 1 year were very similar for WT and mutant fish. The maturation process of the oocytes is thus fully implemented in *dio2*^{-/-} females,

Figure 5

Components of HPG-axis in 7-month-old *dio2*^{+/+} and *dio2*^{-/-} siblings. Expression of *gnrh2*, *gnrh3*, *kiss1*, *kiss2* (brain), *igf3* (gonad) and *vtg1* (liver) in female (A) and male (B) fish. Data were normalized based on the mean expression of different housekeeping genes (*ef1a111*, *rpl13a* and *eif1b* for brain, liver and ovary; *ef1a111*, *rpl13a* and *hprt1* for testis). Average WT (*dio2*^{+/+}) values were set to 1 and *dio2*^{-/-} values were rescaled accordingly. Data are expressed as mean ± S.E.M. (5–7 individual animals per condition). Per tissue and per gene, differences between both genotypes were analyzed by unpaired Student's *t* test (Gaussian distribution) or Mann-Whitney *U* test (non-parametric). **P* < 0.05; ***P* < 0.01.

which corresponds to the significant upregulation at 7 months of ovarian *igf3*, a growth factor with a potent stimulatory action on oocyte maturation (Li *et al.* 2015), as well as *ar*, which positively regulates oocyte maturation and fecundity in females (Crowder *et al.* 2018). In contrast, loss of *Ar* function results in fewer mature oocytes in zebrafish as well as mice (Shiina *et al.* 2006, Walters *et al.* 2010).

While mutant females possess high numbers of (mature) oocytes, they spawn poorly and completely fail to spawn from the age of 8 months. When comparing fish in reproductive arrest (i.e. 2 year for WT; 1 year for mutants), we found that oocyte numbers were drastically decreased in WT fish while numbers remained high in *dio2*^{-/-} fish, indicating that females cannot deposit their

eggs and have ovulatory problems. It has been shown in many non-mammalian vertebrates that egg laying is a parameter susceptible to hypothyroidism, proportional to the degree of severity (Mukhi & Patino 2007, Chen *et al.* 2008, Dumont 2008). This may be linked to the decrease in E2 in *dio2*^{-/-} mutants, since sex steroids play an essential role in several aspects of the reproductive process and are modulated by THs (Cyr & Eales 1988). E2 is known to peak before the ovulatory phase, as demonstrated in zebrafish via intra-ovarian measurements (Lister & Van Der Kraak 2009). The significant decrease in intra-ovarian as well as secreted E2 in our *dio2*^{-/-} females observed at different stages may thus in part explain the ovulatory problems and account for the significant upregulation of *esr2b*. Furthermore, egg deposition requires the contractile action of muscles and we already showed in our previous work that muscle development/contraction and locomotor behavior is adversely affected by *Dio2* deficiency (Bagci *et al.* 2015, Houbrechts *et al.* 2016). Lastly, the combination of male sexual behavior and pheromone secretion normally triggers female ovulation (Hisoka & Firlit 1962). Sex steroids are known to control courtship behavior in zebrafish (Pradhan & Olsson 2015) and their production/secretion is in general stimulated by THs, either directly or indirectly via the HPG axis (Swapna & Senthilkumaran 2007). Sex steroid production and release is disturbed in *dio2*^{-/-} mutants and this indeed seemed to diminish male-typical courtship behavior (personal observations), potentially leading to an inadequate stimulation of female ovulation.

Unlike oogenesis, mutant spermatogenesis still shows dysregulation at 1 year of age when they are in reproductive arrest. In contrast to the situation at 3/4 months, the percentage of spermatogonia A was now increased in *dio2*^{-/-} males compared to WT. This could be related to their androgen insufficiency (low 11-KT levels) which was previously shown in zebrafish to induce accumulation of spermatogonia A (de Waal *et al.* 2009). The premature reproductive arrest in males was also accompanied by a significantly decreased percentage of spermatozoa. Similar findings occurred in zebrafish and another teleost, the fathead minnow, exposed to goitrogens. They displayed reduced *T*₃ and *T*₄ levels (Noyes *et al.* 2013, Naderi *et al.* 2014), accompanied by a decreased number of mature spermatozoa, leading to male infertility (Muirhead *et al.* 2006, Naderi *et al.* 2014). Another known thyroid-blocking chemical, 2,3,7,8-tetrachlorodibenzo-p-dioxin, also impaired zebrafish reproductive capacity by decreasing spermatozoa number with a concurrent increase in spermatogonial cells (Baker *et al.* 2016).

Figure 6

Sex steroid profiles from *dio2*^{+/+} and *dio2*^{-/-} fish (F1 generation from siblings). (A) Intra-gonadal levels (pg/mg tissue) of E2 in ovarian tissue (*n* = 9–12) and 11-KT and testosterone in testicular tissue (*n* = 7–12); (B) Concentration (pg/mL) of E2 and 11-KT in the holding water of female and male fish respectively (*n* = 7 individual fish per genotype). (C and D) *esr2a*, *esr2b*, *ar*, *hsd11b2*, *hsd17b3* and *cyp19a1b* expression in ovary and testis. Data were normalized based on the mean expression of different housekeeping genes (*eef1a11*, *rpl13a*, and *eif1b* for ovary; *eef1a11*, *rpl13a* and *hprt1* for testis). Average WT (*dio2*^{+/+}) values were set to 1 and *dio2*^{-/-} values were rescaled accordingly (*n* = 5–7 individual animals per condition). Data are expressed as mean + s.e.m. Differences between both genotypes were analyzed either by unpaired Student's *t* test (Gaussian distribution) or Mann-Whitney *U* test (no Gaussian distribution). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Despite some changes in spermatogenesis, *dio2*^{-/-} mutants still possess a substantial amount of spermatozoa. This is in strong contrast to the recurring fertilization problems. Fertilization percentages are low in sexually active fish and from 8 months onward reproduction is halted. This could reflect problems and (at later stages) even defects in sperm ejaculation. Moreover, reduced sperm quality and/or motility can cause additional complications. The latter hypothesis is substantiated by the fact that, even when paired with WT females, mutant male fertilization percentages on average did not increase drastically (from 40 to 53%). It has been shown in different mammalian species that hypothyroidism lowered the quality and motility of spermatozoa (while spermatogenesis was only sporadically affected), often in combination with reduced testosterone levels (Chandrasekhar *et al.* 1985, Mallem *et al.* 2006). The stronger impact of Dio2 deficiency on spermatogenesis compared to oogenesis may relate to differences in local TH signaling. 1-year-old ovaries show a possible compensatory response to the reduction in T₃ availability by a significant increase in *thraa* expression. The opposite effect is found in testis where the decrease in *thrb* expression may further reduce TH action.

It thus seems that the spermatogenic process in the *dio2*^{-/-} mutants continues at later stages while reproductive behavior is absent. This is similar to the

phenotypic characteristics observed in mutant zebrafish and medaka with deficiencies in steroidogenic enzymes (Sato *et al.* 2008, Zhai *et al.* 2018). Fertile males (normal spermatogenesis) were compromised in their male-typical reproductive behavior, caused by insufficient sex steroid production. Our sex steroid measurements were performed at 7 months and 1 year of age, corresponding to sexually active and non-active fish respectively. Although the values represent either secretion (water) or production (intra-gonadal) levels, the difference seems much larger in older fish. This could mean that sex steroid production is progressively decreasing with age and once it falls below a minimum (maybe around 8 months) it can no longer sustain reproductive behavior and sexual activity ceases. In this context it would be interesting to find out if sex steroid supplementation could rescue sexual behavior in the *dio2*^{-/-} mutants at later age.

The upregulation of *ar* and *hsd17b3* found in *dio2*^{-/-} males might be a compensatory reaction to the low intra-testicular 11-KT levels in order to stimulate the reproductive axis. This is comparable to findings in *ar*^{-/-} mutant zebrafish and ARKO mice, displaying upregulated expression levels of steroidogenic genes such as *hsd17b3* (De Gendt *et al.* 2005, Tang *et al.* 2018). Moreover, mRNA levels of hypothalamic genes were not markedly changed in the *ar*^{-/-} zebrafish model, while these fish, similar to our 3/4-month-old mutants, displayed significantly

Figure 7

Rescue by T₃ supplementation. (A) fecundity and (B) fertilization percentages of *dio2*^{+/+} and *dio2*^{-/-} fish (F2 generation from siblings) treated or not with 10 nM T₃. Statistical analysis was performed by one-way ANOVA with Tukey *post hoc* test. Groups with no common letter are significantly different ($P < 0.05$).

reduced 11-KT levels together with reduced numbers of spermatogonia (Tang *et al.* 2018).

Elucidating the reproductive phenotype at different levels of the HPG axis

The clear decrease in gonadal steroids is likely to result in a reduced negative feedback on different regulators in the HPG axis. Except for an increase in *kiss2* in male mutants, we found no apparent dysregulation at the hypothalamic level. This is not necessarily surprising, since the *kiss/gnrh* system is considered less crucial in zebrafish. Null mutants for *gnrh3*, *kiss1+kiss2* and *gnrh3+kiss1+kiss2* display normal gonad maturation and reproductive capacity (Tang *et al.* 2015, Liu *et al.* 2017). We think alterations rather occur at the pituitary level for the gonadotropins. As Igf3, a growth factor controlled by Fsh in males (Nobrega *et al.* 2015), is clearly upregulated, we expect this gonadotropin to be elevated. This could reflect a situation of primary hypogonadism, like in male DIO3KO mice characterized by an increase in *Fsh*/FSH and *Lh*/LH, but a decrease in testosterone (Martinez *et al.* 2016).

Sex steroid production in the gonads and subsequent secretion in the water is diminished by Dio2 deficiency, especially in males. The low secretion levels of 11-KT in sexually active *dio2*^{-/-} males implies impaired pheromone production from the Leydig cells. It was shown in zebrafish that this specific cell type is TH responsive through the presence of Thrb (Morais *et al.* 2013). In zebrafish ovaries, both *thraa* and *thrb* were detected, but no cell types were specified (Marelli *et al.* 2016). Zebrafish steroidogenesis is thus positively correlated to T₃ levels as demonstrated in different fishes and mammals, where hypothyroidism leads to a reduction in 11-KT and testosterone tissue levels and/or circulating testosterone and E2 levels (Swapna *et al.* 2006, Sahoo *et al.* 2008, Tomza-Marciniak *et al.* 2014).

Recovery of reproductive capacity after T₃ supplementation

Reproductive activity in Dio2-deficient fish was remarkably well recovered by systemic T₃ supplementation starting at 4 weeks. This suggests that although Dio2 is expressed in zebrafish ovary and testis (unpublished data), and therefore, normally contributes to T₃ availability in these organs, the lack of local T₃ production can be overcome by providing enough external T₃, implying the local presence of one or more transporters with good affinity for T₃. Mct8 is a likely candidate, but its presence in adult zebrafish gonads remains to be confirmed. In mouse, several TH transporters have been identified in testis and ovary (Kinne *et al.* 2011) and also in another teleost, the fathead minnow, *mct8*, *mct10* and especially *oatp1c1* were found to be expressed in the gonads (Muzzio *et al.* 2014).

Although the present rescue experiment suggests that the negative impact of Dio2 deficiency on reproduction can be overcome by correcting systemic hypothyroidism, a few questions remain. We started T₃ supplementation prior to sexual maturity when maturation of the gonads was still ongoing. It would be interesting to test whether treating fully mature zebrafish is also capable of normalizing reproductive activity. If not, this would point to persistent defects originating during gonad development and maturation. We also found a skewed sex ratio with male predominance in the offspring of *dio2*^{-/-} fish. Interestingly, this is exactly the opposite of what has been observed due to hypothyroidism in other non-mammalian studies. In fish and *Xenopus*, lower T₄ and/or T₃ levels resulted in a female-skewed sex ratio, while male predominance was found when T₄ levels were increased (Goleman *et al.* 2002, Zhong *et al.* 2005, Sharma & Patino 2013, Naderi *et al.* 2014). Another dissimilarity compared

to hypothyroidism is found in the ovarian size. Ovaries of *dio2*^{-/-} fish more or less doubled in size, in contrast to available data from hypothyroid animals (fish and mammals) showing reduced ovarian weight (Dijkstra *et al.* 1996, Noyes *et al.* 2013). These differences suggest that local Dio2 activity may still have a role of its own. Last but not least, we need to identify changes at the level of the hypothalamus and pituitary in more detail to find out whether they are the cause or rather the consequence of impaired gonadal function. This is essential in unraveling the molecular mechanisms underlying the negative impact of impaired T₃ signaling on reproduction.

Conclusion

Permanent disruption of Dio2 activity hampers both male and female fertility at different levels of the reproductive axis. Counteracting the hypothyroid status by T₃ supplementation from early stages remarkably well recovered reproductive function. This implies that gonadal TH activation by Dio2 is not indispensable when T₃ can be supplied via an alternative way. It also explains the striking difference between the *dio2*^{-/-} zebrafish reproductive phenotype and normal reproduction in the euthyroid DIO2KO mice. Some subtle differences with the effects of goitrogen-induced hypothyroidism remain, suggesting that local Dio2 action might be needed at some stages of gonadal development and function. This needs to be further investigated, for instance by starting T₃ rescue treatment at different stages, such as immediately after fertilization or only during the reproductive period. Generating a conditional KO line would also be extremely helpful in this regard, something that should be possible in the near future thanks to the rapid evolution in techniques for genome editing.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-18-0549>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A M H performed all experiments, acquired and analyzed the data and was responsible for writing the manuscript. J V H assisted in the histological studies. V M D (supervisor) designed the experiments together with A M H and participated in writing the manuscript.

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References

- Bagci E, Heijlen M, Vergauwen L, Hagenaars A, Houbrechts AM, Esguerra CV, Blust R, Darras VM & Knapen D 2015 Deiodinase knockdown during early zebrafish development affects growth, development, energy metabolism, motility and phototransduction. *PLoS ONE* **10** e0123285. (<https://doi.org/10.1371/journal.pone.0123285>)
- Baker BB, Yee JS, Meyer DN, Yang D & Baker TR 2016 Histological and transcriptomic changes in male zebrafish testes due to early life exposure to low level 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Zebrafish* **13** 413–423. (<https://doi.org/10.1089/zeb.2016.1275>)
- Bakke JL, Lawrence NL, Robinson S & Bennett J 1976 Lifelong alterations in endocrine function resulting from brief perinatal hypothyroidism in the rat. *Journal of Laboratory and Clinical Medicine* **88** 3–13.
- Beamer WJ, Eicher EM, Maltais LJ & Southard JL 1981 Inherited primary hypothyroidism in mice. *Science* **212** 61–63. (<https://doi.org/10.1126/science.7209519>)
- Carr JA & Patino R 2011 The hypothalamus-pituitary-thyroid axis in teleosts and amphibians: endocrine disruption and its consequences to natural populations. *General and Comparative Endocrinology* **170** 299–312. (<https://doi.org/10.1016/j.ygcen.2010.06.001>)
- Chandrasekhar Y, Holland MK, D'Occhio MJ & Setchell BP 1985 Spermatogenesis, seminal characteristics and reproductive hormone levels in mature rams with induced hypothyroidism and hyperthyroidism. *Journal of Endocrinology* **105** 39–46. (<https://doi.org/10.1677/joe.0.1050039>)
- Chen Y, Sible JC & McNabb FM 2008 Effects of maternal exposure to ammonium perchlorate on thyroid function and the expression of thyroid-responsive genes in Japanese quail embryos. *General and Comparative Endocrinology* **159** 196–207. (<https://doi.org/10.1016/j.ygcen.2008.08.014>)
- Crowder CM, Lassiter CS & Gorelick DA 2018 Nuclear androgen receptor regulates testes organization and oocyte maturation in zebrafish. *Endocrinology* **159** 980–993. (<https://doi.org/10.1210/en.2017-00617>)
- Cyr DG & Eales JG 1988 In vitro effects of thyroid hormones on gonadotropin-induced estradiol-17 beta secretion by ovarian follicles of rainbow trout, *Salmo gairdneri*. *General and Comparative Endocrinology* **69** 80–87. ([https://doi.org/10.1016/0016-6480\(88\)90055-X](https://doi.org/10.1016/0016-6480(88)90055-X))
- Darras VM & Van Herck SL 2012 Iodothyronine deiodinase structure and function: from ascidians to humans. *Journal of Endocrinology* **215** 189–206. (<https://doi.org/10.1530/JOE-12-0204>)
- De Gendt K, Atanassova N, Tan KA, de Franca LR, Parreira GG, McKinnell C, Sharpe RM, Saunders PT, Mason JI, Hartung S, *et al.* 2005 Development and function of the adult generation of Leydig cells in mice with Sertoli cell-selective or total ablation of the androgen receptor. *Endocrinology* **146** 4117–4126. (<https://doi.org/10.1210/en.2005-0300>)

- de Waal PP, Leal MC, Garcia-Lopez A, Liarte S, de Jonge H, Hinfray N, Brion F, Schulz RW & Bogerd J 2009 Oestrogen-induced androgen insufficiency results in a reduction of proliferation and differentiation of spermatogonia in the zebrafish testis. *Journal of Endocrinology* **202** 287–297. (<https://doi.org/10.1677/JOE-09-0050>)
- Dijkstra G, de Rooij DG, de Jong FH & van den Hurk R 1996 Effect of hypothyroidism on ovarian follicular development, granulosa cell proliferation and peripheral hormone levels in the prepubertal rat. *European Journal of Endocrinology* **134** 649–654. (<https://doi.org/10.1530/eje.0.1340649>)
- Dumont J 2008 *The Effects of Ammonium Perchlorate on Reproduction and Development of Amphibians*, p 44. Stillwater, OK, USA: Oklahoma State University. (available at: <https://apps.dtic.mil/dtic/tr/fulltext/u2/a495519.pdf>)
- Felix AS, Faustino AI, Cabral EM & Oliveira RF 2013 Noninvasive measurement of steroid hormones in zebrafish holding-water. *Zebrafish* **10** 110–115. (<https://doi.org/10.1089/zeb.2012.0792>)
- Forsatkar MN, HedayatiRad M & Luchiaro AC 2018 “Not tonight zebrafish”: the effects of *Ruta graveolens* on reproduction. *Pharmaceutical Biology* **56** 60–66. (<https://doi.org/10.1080/13880209.2017.1421234>)
- Goleman WL, Carr JA & Anderson TA 2002 Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. *Environmental Toxicology and Chemistry* **21** 590–597. (<https://doi.org/10.1002/etc.5620210318>)
- Hernandez A, Martinez ME, Fiering S, Galton VA & St Germain D 2006 Type 3 deiodinase is critical for the maturation and function of the thyroid axis. *Journal of Clinical Investigation* **116** 476–484. (<https://doi.org/10.1172/JCI26240>)
- Hernandez A, Martinez ME, Liao XH, Van Sande J, Refetoff S, Galton VA & St Germain DL 2007 Type 3 deiodinase deficiency results in functional abnormalities at multiple levels of the thyroid axis. *Endocrinology* **148** 5680–5687. (<https://doi.org/10.1210/en.2007-0652>)
- Hisoaka KK & Firlit CF 1962 Ovarian cycle and egg production in the zebrafish, *Brachydanio rerio*. *Copeia* **1962** 788–792. (<https://doi.org/10.2307/1440680>)
- Houbrechts AM, Delarue J, Gabriels IJ, Sourbron J & Darras VM 2016 Permanent deiodinase type 2 deficiency strongly perturbs zebrafish development, growth, and fertility. *Endocrinology* **157** 3668–3681. (<https://doi.org/10.1210/en.2016-1077>)
- Jalabert B 2005 Particularities of reproduction and oogenesis in teleost fish compared to mammals. *Reproduction, Nutrition, Development* **45** 261–279. (<https://doi.org/10.1051/rnd:2005019>)
- Jana NR & Bhattacharya S 1994 Binding of thyroid hormone to the goat testicular Leydig cell induces the generation of a proteinaceous factor which stimulates androgen release. *Journal of Endocrinology* **143** 549–556. (<https://doi.org/10.1677/joe.0.1430549>)
- Kayaba T, Sasaki N, Adachi S & Yamauchi K 2008 Effects of pituitary glycoprotein hormones and thyroid hormones on in-vitro vitellogenin incorporation into organ-cultured oocytes in the Japanese eel, *Anguilla japonica*. *Zoological Science* **25** 334–343. (<https://doi.org/10.2108/zsj.25.334>)
- Kinne A, Schulein R & Krause G 2011 Primary and secondary thyroid hormone transporters. *Thyroid Research* **4** (Supplement 1) S7. (<https://doi.org/10.1186/1756-6614-4-S1-S7>)
- Kirby JD, Mankar MV, Hardesty D & Kreider DL 1996 Effects of transient prepubertal 6-N-propyl-2-thiouracil treatment on testis development and function in the domestic fowl. *Biology of Reproduction* **55** 910–916. (<https://doi.org/10.1095/biolreprod55.4.910>)
- Li J, Chu L, Sun X, Liu Y & Cheng CH 2015 IGFs mediate the action of LH on oocyte maturation in zebrafish. *Molecular Endocrinology* **29** 373–383. (<https://doi.org/10.1210/me.2014-1218>)
- Lister AL & Van Der Kraak GJ 2009 Regulation of prostaglandin synthesis in ovaries of sexually-mature zebrafish (*Danio rerio*). *Molecular Reproduction and Development* **76** 1064–1075. (<https://doi.org/10.1002/mrd.21072>)
- Liu C, Yu L, Deng J, Lam PK, Wu RS & Zhou B 2009 Waterborne exposure to fluorotelomer alcohol 6:2 FTOH alters plasma sex hormone and gene transcription in the hypothalamic-pituitary-gonadal (HPG) axis of zebrafish. *Aquatic Toxicology* **93** 131–137. (<https://doi.org/10.1016/j.aquatox.2009.04.005>)
- Liu Y, Tang H, Xie R, Li S, Liu X, Lin H, Zhang Y & Cheng CH 2017 Genetic evidence for multifactorial control of the reproductive axis in zebrafish. *Endocrinology* **158** 604–611. (<https://doi.org/10.1210/en.2016-1540>)
- Mallem L, Boulakoud MS & Franck M 2006 Hypothyroidism after medium exposure to the fungicide maneb in the rabbit *Cuniculus lepus*. *Communications in Agricultural and Applied Biological Sciences* **71** 91–99.
- Marelli F, Carra S, Agostini M, Cotelli F, Peeters R, Chatterjee K & Persani L 2016 Patterns of thyroid hormone receptor expression in zebrafish and generation of a novel model of resistance to thyroid hormone action. *Molecular and Cellular Endocrinology* **424** 102–117. (<https://doi.org/10.1016/j.mce.2016.01.020>)
- Martinez ME, Karaczyn A, Stohn JP, Donnelly WT, Croteau W, Peeters RP, Galton VA, Forrest D, St Germain D & Hernandez A 2016 The Type 3 deiodinase is a critical determinant of appropriate thyroid hormone action in the developing testis. *Endocrinology* **157** 1276–1288. (<https://doi.org/10.1210/en.2015-1910>)
- Matta SL, Vilela DA, Godinho HP & Franca LR 2002 The goitrogen 6-n-propyl-2-thiouracil (PTU) given during testis development increases Sertoli and germ cell numbers per cyst in fish: the tilapia (*Oreochromis niloticus*) model. *Endocrinology* **143** 970–978. (<https://doi.org/10.1210/endo.143.3.8666>)
- Mintziori G, Kita M, Duntas L & Goulis DG 2016 Consequences of hyperthyroidism in male and female fertility: pathophysiology and current management. *Journal of Endocrinological Investigation* **39** 849–853. (<https://doi.org/10.1007/s40618-016-0452-6>)
- Morais RD, Nobrega RH, Gomez-Gonzalez NE, Schmidt R, Bogerd J, Franca LR & Schulz RW 2013 Thyroid hormone stimulates the proliferation of Sertoli cells and single type A spermatogonia in adult zebrafish (*Danio rerio*) testis. *Endocrinology* **154** 4365–4376. (<https://doi.org/10.1210/en.2013-1308>)
- Morais RDVS, Crespo D, Nobrega RH, Lemos MS, van de Kant HJG, de Franca LR, Male R, Bogerd J & Schulz RW 2017 Antagonistic regulation of spermatogonial differentiation in zebrafish (*Danio rerio*) by Igf3 and Amh. *Molecular and Cellular Endocrinology* **454** 112–124. (<https://doi.org/10.1016/j.mce.2017.06.017>)
- Morthorst JE, Lister A, Bjerregaard P & Van Der Kraak G 2013 Ibuprofen reduces zebrafish PGE(2) levels but steroid hormone levels and reproductive parameters are not affected. *Comparative Biochemistry and Physiology. Toxicology and Pharmacology* **157** 251–257. (<https://doi.org/10.1016/j.cbpc.2012.12.001>)
- Muirhead EK, Skillman AD, Hook SE & Schultz IR 2006 Oral exposure of PBDE-47 in fish: toxicokinetics and reproductive effects in Japanese Medaka (*Oryzias latipes*) and fathead minnows (*Pimephales promelas*). *Environmental Science and Technology* **40** 523–528. (<https://doi.org/10.1021/es0513178>)
- Mukhi S & Patino R 2007 Effects of prolonged exposure to perchlorate on thyroid and reproductive function in zebrafish. *Toxicological Sciences* **96** 246–254. (<https://doi.org/10.1093/toxsci/kfm001>)
- Muzzio AM, Noyes PD, Stapleton HM & Lema SC 2014 Tissue distribution and thyroid hormone effects on mRNA abundance for membrane transporters Mct8, Mct10, and organic anion-transporting polypeptides (Oatps) in a teleost fish. *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology* **167** 77–89. (<https://doi.org/10.1016/j.cbpa.2013.09.019>)
- Naderi M, Wong MY & Gholami F 2014 Developmental exposure of zebrafish (*Danio rerio*) to bisphenol-S impairs subsequent reproduction potential and hormonal balance in adults. *Aquatic Toxicology* **148** 195–203. (<https://doi.org/10.1016/j.aquatox.2014.01.009>)

- Nobrega RH, Morais RD, Crespo D, de Waal PP, de Franca LR, Schulz RW & Bogerd J 2015 Fsh stimulates spermatogonial proliferation and differentiation in zebrafish via Igf3. *Endocrinology* **156** 3804–3817. (<https://doi.org/10.1210/en.2015-1157>)
- Noyes PD, Lema SC, Macaulay LJ, Douglas NK & Stapleton HM 2013 Low level exposure to the flame retardant BDE-209 reduces thyroid hormone levels and disrupts thyroid signaling in fathead minnows. *Environmental Science and Technology* **47** 10012–10021. (<https://doi.org/10.1021/es402650x>)
- Pradhan A & Olsson PE 2015 Zebrafish sexual behavior: role of sex steroid hormones and prostaglandins. *Behavioral and Brain Functions* **11** 23. (<https://doi.org/10.1186/s12993-015-0068-6>)
- Reyns GE, Janssens KA, Buyse J, Kuhn ER & Darras VM 2002 Changes in thyroid hormone levels in chicken liver during fasting and refeeding. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* **132** 239–245. ([https://doi.org/10.1016/S1096-4959\(01\)00528-0](https://doi.org/10.1016/S1096-4959(01)00528-0))
- Sahoo DK, Roy A, Bhanja S & Chainy GB 2008 Hypothyroidism impairs antioxidant defence system and testicular physiology during development and maturation. *General and Comparative Endocrinology* **156** 63–70. (<https://doi.org/10.1016/j.ygcen.2007.11.007>)
- Sato T, Suzuki A, Shibata N, Sakaizumi M & Hamaguchi S 2008 The novel mutant scl of the medaka fish, *Oryzias latipes*, shows no secondary sex characters. *Zoological Science* **25** 299–306. (<https://doi.org/10.2108/zsj.25.299>)
- Schneider MJ, Fiering SN, Pallud SE, Parlow AF, St Germain DL & Galton VA 2001 Targeted disruption of the type 2 selenodeiodinase gene (*DIO₂*) results in a phenotype of pituitary resistance to T4. *Molecular Endocrinology* **15** 2137–2148. (<https://doi.org/10.1210/mend.15.12.0740>)
- Schulz RW & Goos HJT 1999 Puberty in male fish: concepts and recent developments with special reference to the African catfish (*Clarias gariepinus*). *Aquaculture* **177** 5–12. ([https://doi.org/10.1016/S0044-8486\(99\)00064-2](https://doi.org/10.1016/S0044-8486(99)00064-2))
- Schulz RW, de Franca LR, Lareyre JJ, Le Gac F, Chiarini-Garcia H, Nobrega RH & Miura T 2010 Spermatogenesis in fish. *General and Comparative Endocrinology* **165** 390–411. (<https://doi.org/10.1016/j.ygcen.2009.02.013>)
- Sharma P & Patino R 2013 Regulation of gonadal sex ratios and pubertal development by the thyroid endocrine system in zebrafish (*Danio rerio*). *General and Comparative Endocrinology* **184** 111–119. (<https://doi.org/10.1016/j.ygcen.2012.12.018>)
- Shiina H, Matsumoto T, Sato T, Igarashi K, Miyamoto J, Takemasa S, Sakari M, Takada I, Nakamura T, Metzger D, *et al.* 2006 Premature ovarian failure in androgen receptor-deficient mice. *PNAS* **103** 224–229. (<https://doi.org/10.1073/pnas.0506736102>)
- Swapna I & Senthilkumaran B 2007 Thyroid hormones modulate the hypothalamo-hypophyseal-gonadal axis in teleosts: molecular insights. *Fish Physiology and Biochemistry* **33** 335–345. (<https://doi.org/10.1007/s10695-007-9165-2>)
- Swapna I, Rajasekhar M, Supriya A, Raghuveer K, Sreenivasulu G, Rasheeda MK, Majumdar KC, Kagawa H, Tanaka H, Dutta-Gupta A, *et al.* 2006 Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish, *Clarias gariepinus*. *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology* **144** 1–10. (<https://doi.org/10.1016/j.cbpa.2006.01.017>)
- Tang H, Liu Y, Luo D, Ogawa S, Yin Y, Li S, Zhang Y, Hu W, Parhar IS, Lin H, *et al.* 2015 The kiss/kissr systems are dispensable for zebrafish reproduction: evidence from gene knockout studies. *Endocrinology* **156** 589–599. (<https://doi.org/10.1210/en.2014-1204>)
- Tang H, Chen Y, Wang L, Yin Y, Li G, Guo Y, Liu Y, Lin H, Cheng CHK & Liu X 2018 Fertility impairment with defective spermatogenesis and steroidogenesis in male zebrafish lacking androgen receptor. *Biology of Reproduction* **98** 227–238. (<https://doi.org/10.1093/biolre/iox165>)
- Tomza-Marciniak A, Pilarczyk B, Stankiewicz T, Blaszczyk B & Kuba J 2014 The effect of chitosan on the concentration of 17beta-estradiol and free triiodothyronine in mice exposed to polychlorinated biphenyls (PCBs). *Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances and Environmental Engineering* **49** 376–382. (<https://doi.org/10.1080/10934529.2014.854568>)
- Tovo-Neto A, da Silva Rodrigues M, Habibi HR & Nobrega RH 2018 Thyroid hormone actions on male reproductive system of teleost fish. *General and Comparative Endocrinology* **265** 230–236. (<https://doi.org/10.1016/j.ygcen.2018.04.023>)
- Verloop H, Dekkers OM, Peeters RP, Schoones JW & Smit JW 2014 Genetics in endocrinology: genetic variation in deiodinases: a systematic review of potential clinical effects in humans. *European Journal of Endocrinology* **171** R123–R135. (<https://doi.org/10.1530/EJE-14-0302>)
- Walters KA, Simanainen U & Handelsman DJ 2010 Molecular insights into androgen actions in male and female reproductive function from androgen receptor knockout models. *Human Reproduction Update* **16** 543–558. (<https://doi.org/10.1093/humupd/dmq003>)
- Weber G, Vigone MC, Stroppa L & Chiumello G 2003 Thyroid function and puberty. *Journal of Pediatric Endocrinology and Metabolism* **16** (Supplement 2) 253–257.
- Zhai G, Shu T, Xia Y, Lu Y, Shang G, Jin X, He J, Nie P & Yin Z 2018 Characterization of sexual trait development in *cyp17a1*-deficient zebrafish. *Endocrinology* **159** 3549–3562. (<https://doi.org/10.1210/en.2018-00551>)
- Zhong X, Xu Y, Liang Y, Liao T & Wang J 2005 The Chinese rare minnow (*Gobiocypris rarus*) as an in vivo model for endocrine disruption in freshwater teleosts: a full life-cycle test with diethylstilbestrol (*Gobiocypris rarus*). *Aquatic Toxicology* **71** 85–95. (<https://doi.org/10.1016/j.aquatox.2004.10.014>)

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