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Peripheral T3 signaling is the target of pesticides in zebrafish larvae and adult liver

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Abstract

The intra-tissue levels of thyroid hormones (THs) regulate organ functions. Environmental factors can impair these levels by damaging the thyroid gland and/or peripheral TH metabolism. We investigated the effects of embryonic and/or long-life exposure to low-dose pesticides, ethylene thiourea (ETU), chlorpyrifos (CPF) and both combined on intra-tissue T_4/T_3 metabolism/signaling in zebrafish at different life stages. Hypothyroidism was evident in exposed larvae that showed reduced number of follicles and induced *tshb* mRNAs. Despite that, we found an increase in free T_4 (fT4) and free T_3 (fT3) levels/signaling that was confirmed by transcriptional regulation of TH metabolic enzymes (deiodinases) and T3-regulated mRNAs (*cpt1*, *igfbp1a*). Second-generation larvae showed that thyroid and TH signaling was affected even when not directly exposed, suggesting the role of parental exposure. In adult zebrafish, we found that sex-dependent damage of hepatic T_3 level/signaling was associated with liver steatosis, which was more pronounced in females, with sex-dependent alteration of transcripts codifying the key enzymes involved in '*de novo* lipogenesis' and β-oxidation. We found impaired activation of liver T_3 and PPARα/Foxo3a pathways whose deregulation was already involved in mammalian liver steatosis. The data emphasizes that the intra-tissue imbalance of the T_3 level is due to thyroid endocrine disruptors (THDC) and suggests that the effect of a slight modification in T_3 signaling might be amplified by its direct regulation or crosstalk with PPARα/Foxo3a pathways. Because T_3 levels define the hypothyroid/hyperthyroid status of each organ, our findings might explain the pleiotropic and site-dependent effects of pesticides.

Key Words

- HPT axis-disrupting chemicals
- lipid metabolism
- peripheral T3 metabolism/signaling
- liver steatosis, deiodinases

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Introduction

Thyroid diseases and associated pathologies, such as obesity, non-alcoholic fatty liver disease (NAFLD), and so on, are widespread (Mehran *et al.* 2017). Genetic and environmental factors contribute to their onset by damaging the synthesis of thyroid hormones (THs) or their metabolism/signaling in peripheral organs (Vanderpump 2011).

The thyroid secretes the prohormone thyroxine (T_4), and to a lesser extent triiodothyronine (T_3), which is the most active form. T_3 is produced mainly in peripheral organs (e.g. liver) in which TH metabolizing enzymes (deiodinases) can either activate or inactivate them, without necessarily affecting the circulating T_3 level. TH signaling relies on both the circulating and the tissue/cell-specific levels of T_3 and, finally, on TH receptors (THRs). Local T_3 levels depend on an ensemble of tissue/cell-specific factors, such as deiodinases and TH transporters, which is relatively independent of serum TH levels (Colella *et al.* 2020). The peripheral modification of T_4 and T_3 defines the hypothyroidism or hyperthyroidism of different organs and the resulting dysfunction of target cells (i.e. hepatocytes). Hepatic T_3 signaling (hT_3 signaling) is modulated by the binding of T_3 to THRB, which regulates the expression of several hepatic genes, including transcripts involved in lipid metabolism (Oppenheimer *et al.* 1987).

Epidemiological and experimental data provide evidence that different environmental pesticides, including carbamates, such as mancozeb and ethylene thiourea (ETU; its metabolite), and organophosphorus pesticides, such as chlorpyrifos (CPF), might impair TH levels (Chhabra *et al.* 1992, IARC 2001, Belpoggi *et al.* 2002, Jeong *et al.* 2006, De Angelis *et al.* 2009, Axelstad *et al.* 2011). Although CPF has been banned in some countries, both CPF and ETU are widely used, and it is practically impossible not to be exposed from an early age. Studies conducted in developmentally exposed mice pointed to potential effects on thyroid function at otherwise non-toxic levels (Porreca *et al.* 2016). However, rodent models did not indicate if the thyroid damage was due to the direct activity of pesticides on fetuses or on maternal TH level/signaling.

Because of the external development and the transparency of developing embryos, zebrafish are a useful model for examining the effects of exposure to environmental factors during embryonic development in promoting disease in adult life. This permits easy incubation of the embryos in a chemical solution for the examination of stage-specific exposure effects (Eddins *et al.* 2010, Levin *et al.* 2011, Richendrfer *et al.* 2012, Richendrfer & Creton 2013, Clift *et al.* 2014). Thyroid development in zebrafish closely resembles that of higher vertebrates. Despite some anatomical differences, the feedback loop active in the HPT axis and the intra-tissue/cell TH metabolism/signaling pathways are both evolutionarily conserved between fish and mammals. Some studies have reported differences (Darras *et al.* 2015, Marelli & Persani 2017, Zada *et al.* 2017); tshβ transcription is stimulated by *crh* (De Groef *et al.* 2006), and zebrafish deiodinase 1 (*dio1*) has little affinity for T_4 and is mainly involved in T_3 deactivation (Guo *et al.* 2014).

The data reported here demonstrate the impairment of thyroid development and of whole body T_3 level/signaling in zebrafish larvae exposed to pesticides, and emphasizes the role of parental exposure. The data provides evidence that the T_3 and PPARalpha/Foxo3a signaling pathways are targets of pesticides in adult livers.

Materials and methods

Zebrafish stocks and husbandry

Adult fish (AB line) were maintained according to standard procedures on a 14 h light:10 h darkness cycle at 28°C as previously described (Westerfield 1995). Embryos were obtained by natural spawning and staged based on hours post-fertilization (hpf) or days post-fertilization (dpf) according to morphological criteria (Kimmel *et al.* 1995). Animal experiments were performed in accordance with the European Council Directive 2010/63/EU. Procedures were approved by the Italian Ministry of Health (IMH, ID number 78-17).

Chemical treatment

Chlorpyrifos (CasN. 2921-88-2) and ethylene thiourea (CasN. 96-45-7), purchased from Greyhound Chromatography and Allied Chemicals and Sigma Aldrich, respectively, were stored as recommended. F1 larvae were obtained by placing adult males and females in the breeding box overnight. Spawning was triggered under light and was completed within 30 min. The collected embryos were examined under a stereomicroscope at 2 hpf and blastulae were selected. At 6 hpf embryos were randomly assigned to experimental groups by placing them in separate glass Petri dishes

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(three plates/experimental group), with each dish containing 85 embryos in 100 mL of fish water containing ETU (100 µM), CPF (30 or 300 nM), or ETU and CPF combined (100 µM + 30 nM or 100 µM + 300 nM). We kept them in an incubator at 28°C, and the exposure solutions were changed daily. Larvae at 7 dpf were anesthetized with tricaine and processed for subsequent analysis. This procedure was performed three times. In parallel, a set of 7 dpf larvae (n = 45/group per 100 mL) were kept and treated until adulthood, and the exposure solutions were changed daily (180 dpf). At 10 dpf, larvae were transferred to 3.5 L tanks of the ZebTec toxicology stand-alone unit (Tecniplast, Italy).

F2 larvae were produced by mating with similarly exposed F1 animals (n = 20 males and n = 20 females), as reported above (Supplementary Fig. 1, see section on supplementary materials given at the end of this article). At 6 hpf, embryos (n = 85/group) were exposed as above (F2-larvae) or left untreated (F2-ND larvae, n = 85/group). At 7 dpf, F2 and F2-ND larvae were processed as described above. The detailed experimental plan is presented in Supplementary Fig. 1.

RNA extraction and RT-qPCR analysis

RNA from whole larvae (85 pooled larvae/group for three biological replicates) and adult livers (n = 5/group), was prepared with TRIzol reagent (Invitrogen). RNA (1 µg) was reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen) and real-time qPCR (RT-qPCR) was performed using PowerUP SYBR Green Master Mix (Applied Biosystems with Applied Biosystem QuantStudio 7 Flex System). Primer sequences are listed inSupplementary Table 1. Data were normalized through the level of internal control *tubaI* (for larval samples) and *actb1*, for liver samples, as suggested by McCurley & Callard 2008. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression changes.

Measurement of fT3 and fT4 hormones

TH levels were measured in homogenates of pooled larvae (85 larvae/group, for three biological replicates) and adult livers (n = 3/group) using ELISA, according to the published protocol with minor changes (Chang *et al.* 2012). Briefly, larvae and adult livers were homogenized in PBS (10 mM K_2HPO_4, 100 mM KCL, 1 mM DTT, pH 7.4) (1 mL/85 larvae, 300 µL/liver). After centrifugation (10 min, 5000 ***g*** at 4°C), the supernatants were collected and stored at −80°C until TH levels were measured using an ELISA kit (Diametra). In total, 100 µL was used for ELISA.

Oil red O (ORO) staining

Frozen livers from adult males and females (n = 3/group) were subjected to ORO staining as detailed in the Supplementary materials. Briefly, liver cryosections (10 µm) were incubated in 60% isopropanol (5 min) and stained with ORO solution (0.5% isopropanol for 30 min, Sigma Aldrich) as indicated by the manufacturer. After mounting in glycerol, samples were imaged on a Zeiss AxioImager M1 microscope (20× and 63× objectives).

Western blot analysis

Proteins were prepared by lysing frozen livers (n = 3/group) in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% SDS, 0.5% Na-deoxycholate, Nonidet P-40, protease and phosphatase inhibitor mixture (Sigma)) and analyzed by Western blotting as detailed in the Supplementary materials. The primary antibodies used were P-Foxo3a (S253), Foxo3a (75D8) and SREBP1c (28481) (Abcam). B-actin (Cell Signalling) was used to normalize data. The secondary antibodies used were anti-rabbit (G21234) and anti-mouse (G21D40) (Life Technologies).

Immunohistochemical staining of thyroid follicles

Immunostaining for T_4 and thyroglobulin (TG) was performed on fixed and bleached larvae as detailed in the Supplementary materials. Staining was performed with the following antibodies: rabbit anti-T4 (1:4000, MP Biochemicals) and anti-human thyroglobulin (1:6000, Dako), respectively, as previously described (Elsalini *et al.* 2003, Porreca *et al.* 2012). Zebrafish samples were imaged on a Zeiss AxioImager M1 microscope, with 20× and 40× objectives.

Statistical analysis

The number of enrolled adults was determined by the parameters indicated by the IMH for G*Power analysis (Faul *et al.* 2007), as required for *in vivo* experiments in Italy.

Statistical analyses of the data were performed using GraphPad Prism 5 (GraphPad Software Inc). Student's *t*-test or ANOVA with Dunnett's *post hoc* correction test for multi-group comparison was performed to evaluate the

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significance of differences. RTqPCR data were expressed as the fold change of the mean ± S.D. Probability values <0.05 were considered significant.

Results

Early embryonic exposure to pesticides impairs TH peripheral thyroid metabolism/signaling and thyroid development

To investigate T_3 and T_4 levels/signaling and thyroid differentiation in zebrafish larvae exposed to CPF, ETU or both ETU and CPF combined, we designed an experimental plan which included embryos generated from unexposed parents (F1-larvae) or exposed ones (F2-larvae) obtained by inter-group mating, as better specified in Supplementary Fig. 1 and the Materials and methods section. Briefly, 6 hpf embryos were exposed to ETU 100 µM, CPF 30 nM, CPF 300 nM or both ETU and CPF combined until killing at 7 dpf (F1-larvae) or 180 dpf (F1-adults). F2 embryos were exposed as described for F1-larvae or left untreated (ND-F2 larvae). Doses of both compounds were chosen according to published data (Wang *et al.* 2017, Cao *et al.* 2018, Jarque *et al.* 2018, Yang *et al.* 2011), considering the need to investigate synergic/competitive activities in co-exposure conditions. No major effect was found in survival and morphology of larvae even in co-exposure conditions in either generation (data not shown).

Peripheral TH signaling is the result of the circulating level of T_4, relying on thyroid function, and T_3 that is mainly dependent on peripheral TH metabolism. We tested the l-fT4 (larval free T_4) and l-fT3 levels using ELISA on samples prepared by whole body lysing. FT4 and fT3 levels were increased in (ETU)-, (CPF 300 nM)- and (ETU + CPF 30 nM)-F1 larvae (Fig. 1A and B). FT3 was also induced in larvae exposed to CPF 30 nM.

The expression of T3-targets, such as insulin growth factor binding protein 1a gene (*igfbp1a*) (Houbrechts *et al.* 2016) and carnitine palmitoyltransferase I (*cpt1*) (Jansen *et al.* 2000), was tested to verify the effects of fT3 increase. Both were induced in (CPF 30 nM)- and (ETU + CPF 300 nM)-F1 larvae (Fig. 1C and D, respectively). The increase was statistically significant only for *igfbp1a* in larvae

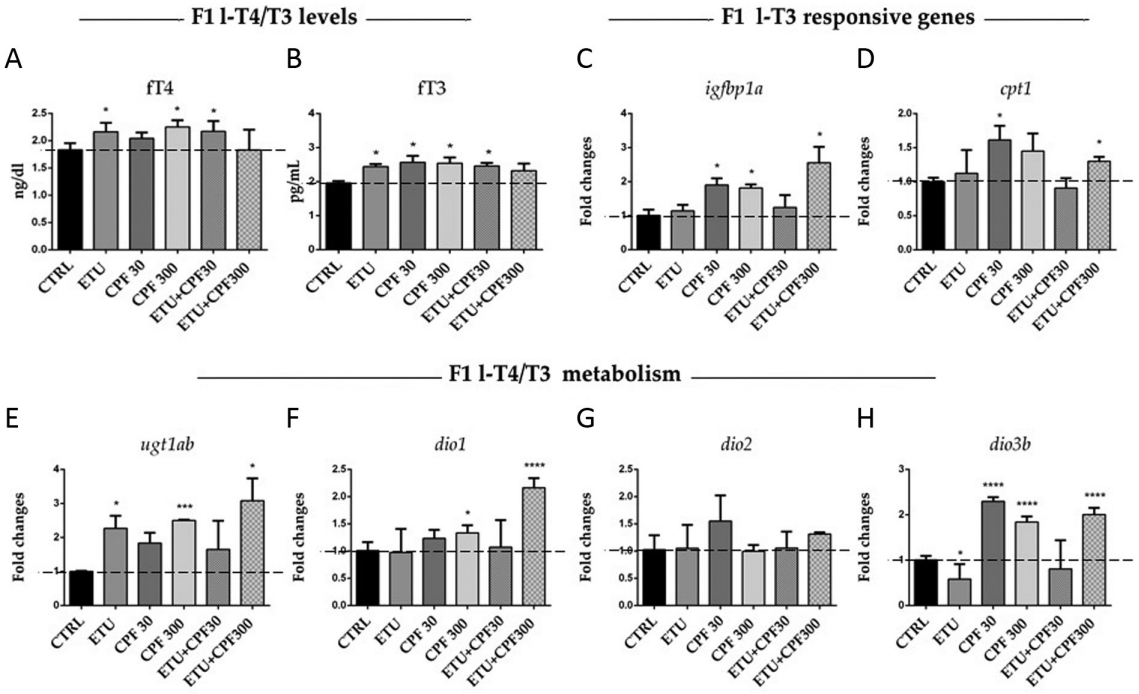

Figure 1
Larval fT4- and fT3-level/metabolism and signaling is altered in F1 larvae exposed to CPF, ETU or both ETU and CPF combined. (A and B) L-fT4 and l-fT3 levels were determined by ELISA in F1 zebrafish larvae. (C and D) Levels of *igfbp1a* and *cpt1* mRNAs (l-T3 responsive genes) and (E–H) *ugt1ab*, *dio1*, *dio2*, *dio3b* transcripts (l-T4/T3 metabolism) were assayed by RTqPCR, as reported in the Materials and methods section. Experiments were conducted on larvae exposed to pesticides from 6 hpf to the time of killing (7 dfp) or left untreated (CTRL). RTqPCR tests were performed on pools of 85 larvae from three independent experiments. Data analysis was conducted as described in the Materials and methods section. Data are reported as fold change values calculated as a ratio between average relative gene expression in treated and control larvae, after normalization on *tubal* mRNA. Mean and S.D. are reported. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

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exposed to CPF 300 nM. Subsequently, we analyzed the expression of the genes involved in TH metabolism, such as iodothyronine deiodinase 2 (*dio2* – converting T_4 in T_3), iodothyronine deiodinase 1 and 3b (*dio1*, *dio3b*, inactivating T_3) and UDP glucuronosyltransferase 1 family a, b (*ugt1ab*, involved in T_3 inactivation via *dio1*). As expected, these last three transcripts were induced with T_3 increment. Concordantly, we found the induction of their mRNAs in (CPF-300 nM) and (ETU+CPF 300 nM)-F1 larvae. *dio3b* mRNA was increased in larvae exposed to CPF 30 nM, whereas it was reduced in ETU-F1 larvae. Its regulation was the opposite of *ugt1ab* only in (ETU)-F1 larvae. No major change in *dio2* transcript was found (Fig. 1E, F, G and H). The levels of transthyretin (*ttr*, T_4 transporter) and thyroid receptor alpha a/beta (*thraa/b*) transcripts were evaluated. *thraa* and *thrb* mRNAs were both induced in (CPF 30 nM)- and (ETU+CPF 300 nM)-F1 samples, whereas the first was also induced in (CPF 300 nM)-larvae. *ttr* RNAs showed a pathway of regulation similar to *dio3b* (Supplementary Fig. 2D–F). Overall, the data suggested that early life exposure to CPF, ETU or both ETU and CPF combined altered thyroid status in zebrafish larvae.

We then investigated thyroid development/function. We determined the number of thyroid follicles by immunohistochemistry (IHC) using an antibody against T_4 (Fig. 2A, B, C, D, E, F and G) or against thyroglobulin (TG, Supplementary Fig. 2A). T_4 staining revealed the reduction of the follicle number in all the conditions compared to control, except for

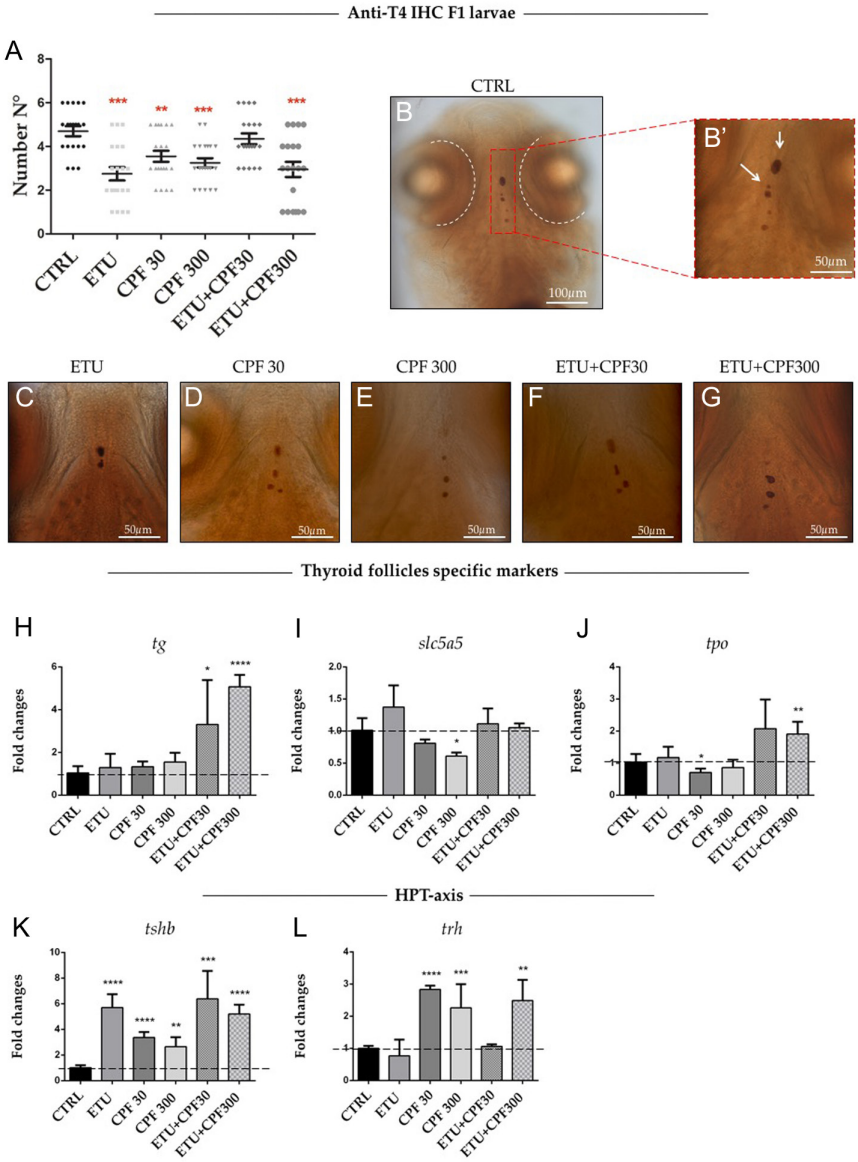

Figure 2
Thyroid development and HPT-axis gene expression is damaged in F1 larvae exposed to pesticides. (A) Number of thyroid follicles in control (CTRL) and exposed (from 6 hpf to 7 dpf) F1 larvae, stained by immunohistochemistry (IHC) using an antibody against T_4, as reported in the Materials and methods section. 20 larvae/group collected from three independent experiments were used. Each data point is a single larva. Mean and S.D. are reported, $^*P < 0.05$. (B and B′) Representative image of thyroid follicles in control F1 zebrafish larvae in a ventral view (B, 20× magnification, B′, 40× magnification). (C–G) Representative images of thyroid follicles in F1 larvae exposed to pesticides (40× magnification). (H–J) Transcripts of *tg*, *slc5a5*, *tpo* (thyroid follicles-specific markers); (K and L) *tshb* and *trh* (HPT-axis) were assayed by RTqPCR on pools of 85 larvae from three independent experiments. Data analysis was conducted as described in the Materials and methods section. Data are reported as fold change values calculated as the ratio between average relative gene expression in treated and control larvae, after normalization on *tubal* mRNA. Mean and S.D. are reported. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0134.

(ETU+CPF 30 nM)-F1 samples (Fig. 2A). TG staining confirmed the result (Supplementary Fig. 2A). Thyroglobulin (*tg*) and thyroid peroxidase (*tpo*) transcripts were induced in larvae exposed to ETU+CPF 300 nM. *tg* mRNA was also positively regulated in (ETU+CPF 30 nM)-larvae (Fig. 2H and J), whereas (Na)-iodide symporter (*slc5a5*) mRNA was reduced in larvae exposed to CPF 300 nM (Fig. 2I). The expression of *pax8* and *nkx2.4b*, transcriptional regulators of these thyroid-specific genes, was impaired. *pax8* mRNA increased in (CPF 30 nM)- and (CPF 300 nM)-F1 larvae while *nkx2.4b* mRNA was decreased in all exposure conditions, except for (ETU)-F1 larvae (Supplementary Fig. 2B and C). Finally, we evaluated the levels of *tshb* and *trh* transcripts, enhanced in the pituitary and hypothalamus, of hypothyroid animals. We observed an increase of *tshb* mRNA in all treatments (Fig. 2K), whereas *trh* mRNA was increased only in (CPF 30 nM)-, (CPF 300 nM)- and (ETU+CPF 300 nM)-F1 samples (Fig. 2L).

The group of regulated mRNAs specific to the HPT-axis in F1 larvae emphasized the impact of pesticide exposure, in that thyroid development and HPT function was damaged.

Parental exposure results in impairment of body larval T3-level/signaling and thyroid development/function in the offspring even without further exposure

We investigated the effects of parental exposure on the offspring obtained via F1 adult inter-group mating and further exposed (F2)- or untreated (ND F2)-larvae, as detailed in Supplementary Fig. 1.

L-fT4 levels were increased in (CPF 30 nM)-, (CPF 300 nM)- and (ETU+CPF 300 nM) F2-larvae (Fig. 3A). Although not statistically significant, the increase was also evident in (CPF 300 nM)- and (ETU+CPF 300 nM) ND-F2 larvae. L-fT4 and l-fT3 levels were decreased in (ETU+CPF 30 nM)-F2 larvae and only the first was even strongly reduced in the ND-groups (Fig. 3B). L-fT3 was reduced in (ETU+CPF 30 nM)-F2 larvae and showed an increasing trend in all the other groups of exposure. This result was corroborated by

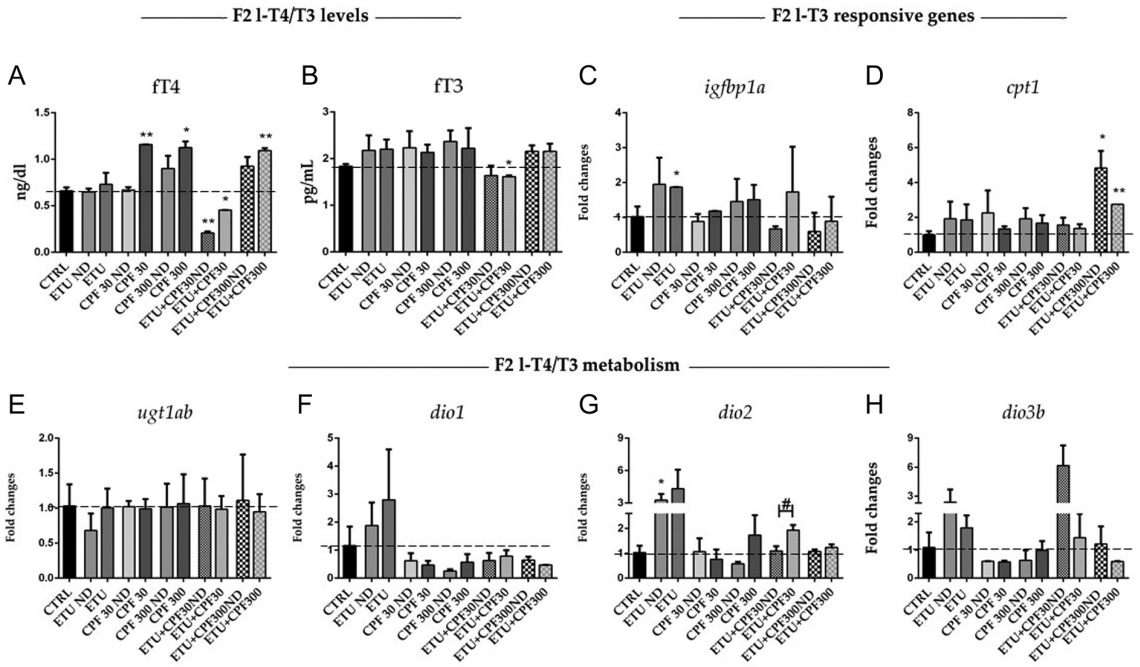

Figure 3
Larval T4/T3-levels and expression of genes involved in lT3-levels/responsive gene/thyroid metabolism in F2 zebrafish larvae exposed or not exposed to pesticides. (A and B) L-fT4 and l-fT3 levels were detected by ELISA in zebrafish larvae from exposed parents and further exposed from 6 hpf to 7 dpf (F2-larvae) or not exposed (ND F2-larvae). (C and D) mRNAs of l-T3-regulated transcripts (*igfbp1a* and *cpt1*) and (E–H) enzymes involved in TH metabolism (*ugt1ab*, *dio1*, *dio2*, *dio3b*) were assayed using RTqPCR in F2- and ND F2-larvae as reported in the Materials and methods section. RTqPCR tests were performed on pools of 85 larvae from three independent experiments. Data analysis was conducted as described in the Materials and methods section. Data are reported as fold change values calculated as a ratio between average relative gene expression in treated and control larvae, after normalization on *tubal* mRNA. Mean and S.D. are reported as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

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the expression profile of T3-responsive genes, *igfbp1a* and *cpt1*. *igfbp1a* mRNA level was found to be increased in a statistically significant manner in (ETU)-F2 larvae, although only a trend towards induction was observed in (ETU ND), (CPF300 ND and CPF300) and (ETU+CPF30)-F2 samples (Fig. 3C). *cpt1* mRNA was augmented in (ETU + CPF 300 nM)-F2 larvae, and this increase was even stronger in ND-larvae. An increasing trend in *cpt1* mRNA was also observed in the other exposure conditions (Fig. 3D).

Expression of *dio2* was increased in (ETU ND)-larvae (Fig. 3G). A trend toward an increase was evident in (ETU)-, (CPF300 nM)- and (ETU + CPF 30n M)-F2 groups, but was only statistically significant in (ETU ND)-larvae (Fig. 3G). No modification was observed for *ugt1ab* gene (Fig. 3E), whereas a trend toward *dio1* mRNA reduction was found in all samples, except for (ETU)- and (ETU ND)-F2 larvae, in which it was regulated in the opposite manner (Fig. 3F). Although not statistically significant, *dio3b* mRNA was enhanced in both (ETU)- and (ETU + CPF 30 nM)-exposure groups (Fig. 3H). *thrb* mRNA was induced in (ETU) and (CPF-30 nM) F2 larvae and in the respective ND-F2 samples (Supplementary Fig. 3E). *thraa* transcript was increased only in (CPF 300 nm)-F2 larvae and in (ETU ND)- and (CPF 300 nM ND)-F2 samples.

The thyroid follicles were counted upon staining with an antibody against T_4 (Fig. 4A) and TG (Supplementary Fig. 3A). We noted a reduction in the number of follicles in (ETU)-, (ETU + CPF 30 nM)- and (ETU + CPF 300 nM)-F2 larvae (Fig. 4A and H, I, J, L) vs control (Fig. 4B). Interestingly, the number of follicles was restored in ND-F2 larvae in the last two conditions (Fig. 4C, D, E, F and G). *tg*, *scl5a5* and *tpo* mRNAs were induced in (ETU)-samples and only *scl5a5* in (ETU ND)-F2 larvae (Fig. 4M, N and O). *scl5a5* mRNA was increased in (ETU + CPF 30 nM ND)-F2 larvae and also showed a trend toward induction in the co-exposed samples as *tpo* mRNA (Fig. 4N and O). Unlike the F1 larvae, we found a different expression of *pax8* and *nkx2.4b* genes. Indeed, both were inhibited in (CPF 30 nM) and (ETU + CPF 300 nM)-F2 samples. *pax8* increased in (ETU ND), while *nkx2.4b* was upregulated in (ETU)-, (ETU + CPF30)-, (CPF 300 ND)-, and (ETU+CPF 300ND)-F2 larvae (Supplementary Fig. 3B and C). *pax8* mRNA was positively regulated in (ETU ND)- and (ETU)-F2 larvae, though this was not statistically significant in the second group (Supplementary Fig. 3B). *nkx2.4b* mRNA increased in the (ETU)- and (ETU + CPF 30 nM)-F2 group (Supplementary Fig. 3C). The level of *tshb* mRNA showed a trend toward upregulation (Fig. 4P), whereas *trh* mRNA increased in (ETU)-, (ETU ND)-, (ETU + CPF 30 nM)- and (ETU + CPF 300 nM ND)-F2.

The data in F2 larvae confirmed the effects of pesticide exposure on TH metabolism/signaling, demonstrating that parental exposure was sufficient to promote them in different conditions.

Hepatic T_3-level/signaling in F1-adult livers is damaged by early embryonic exposure to pesticides

T_3 regulates metabolic processes and its altered level/signaling during early life damages metabolic health during adulthood (Mullur *et al.* 2014). Therefore, we evaluated the effects of exposure on liver metabolic health in adult (180 dpf) F1-males ($n = 15$) and females ($n = 15$). No striking phenotypic abnormality, including in reproductive ability, was found in three different matings, nor was any difference found in mortality rate. An increase in body weight and length at the time of killing (180 dpf) was observed in (ETU + CPF 300 nM)-F1 females. In the same exposure group, body weight was increased in the F1-males and in (ETU + CPF 30 nM) animals (Fig. 5A and B).

Hepatic lipid accumulation was confirmed by ORO of liver sections prepared from cryopreserved samples. No major effect of ETU was found in females ($n = 3$, Fig. 5C and D) or males ($n = 3$, Fig. 5I and J), whereas a dose-dependent activity of CPF was found in both sexes (Fig. 5E and F females; Fig. 5K and L males), although less severe in males. Representative images are shown in Fig. 5. Lipid accumulation was restricted to some areas of the liver in (CPF 300 nM)- and (ETU + CPF 30 nM)-males (Fig. 5L and M). Milder sex-dependent differences were detected in co-exposed fish: ETU alleviated CPF 300 nM activity in both sexes (Fig. 5H and N) and CPF 30 nM only in females (Fig. 5E).

We determined the hepatic (h)-fT4 and h-fT3 levels using ELISA ($n = 3$ males and females/groups). Their profiles were partially overlapping in males: fT4 increased by exposure to CPF 300 nM and ETU + CPF 30 nM, and fT3 increased by exposure to ETU and ETU + CPF 30 nM (Fig. 6I and J). The overlap was not found in females because fT4 was increased in animals exposed to CPF 30 nM and ETU + CPF 300 nM, whereas fT3 was not regulated in either condition. Its reduction was evident in (ETU + CPF 30 nM)-females (Fig. 6A and B). Although slight and often not statistically significant, the increase in h-fT3 (Fig. 6J) was concordant with the upregulation of *igfbp1a* and *cpt1* mRNAs in male livers ($n = 5$, Fig. 6K and L). In females ($n = 5$), the expression of *igfbp1a* increased only in (ETU)- and (ETU + CPF 300 nM)-livers (Fig. 6C), with a concordant increase in the fT3 level only in the

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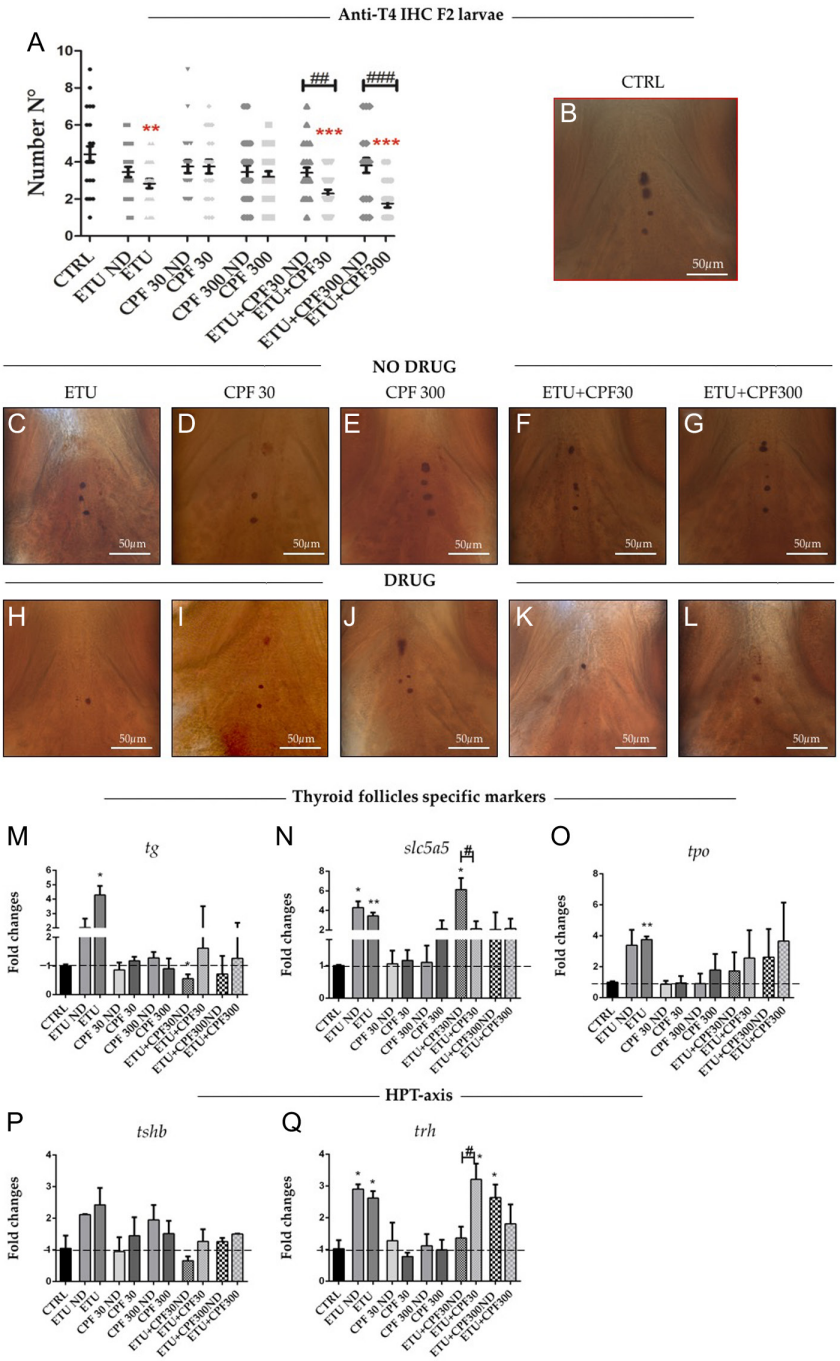

Figure 4
Thyroid development and HPT-axis gene expression are damaged in F2 zebrafish larvae. (A) The graph represents the number of thyroid follicles in control (CTRL) and exposed larvae left untreated (ND F2) or exposed (F2) from 6 hpf to 7 dpf (Supplementary Fig. 1), as reported in Fig. 2. Staining was performed with an antibody against T_4 on 20 larvae/group from three independent experiments. Each data point is a single larva. Mean and S.D. are reported. (B) Representative images of thyroid follicles in control F2 larvae (B, 40× magnification). (C–L) Representative images (40× magnification) of thyroid follicles in F2 larvae left unexposed (ND, C–G) or exposed (H–L). A significant difference in the control group is indicated by *, $^{*}P < 0.01$, $^{***}P < 0.001$, whereas a significant difference in the ND-group is reported by #, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. (M–O) Thyroid-specific mRNAs (*tg*, *slc5a5* and *tpo*), (P and Q) pituitary- and hypothalamus-specific (*tshb* and *trh*, respectively) were assayed by RTqPCR. Pools of 85 larvae/group were left untreated (ND) or exposed to pesticides, as described in the Materials and methods section. RTqPCR tests were performed separately on pools of 85 larvae from three independent experiments. Data analysis was conducted as described in the Materials and methods section. Data are reported as fold change values calculated as a ratio between average relative gene expression in treated and control adults, after normalization on *tubal* mRNA. Mean and S.D. are reported. A significant difference in the control group is indicated by *, $^{*}P < 0.05$, $^{**}P < 0.01$, whereas a significant difference in the ND-group is indicated by #, $^{\#}P < 0.05$. A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0134.

former group (Fig. 6B). Furthermore, it was inhibited in (CPF 300 nM)- and (ETU+CPF 30 nM)-female livers, whereas *cpt1* mRNA was always found to be reduced (Fig. 6D). We verified the expression of genes involved in '*de novo* lipogenesis'. *acc* mRNA was increased in all conditions in females, although this was not statistically significant in (CPF 300 nM) and (ETU+CPF 300 nM) females. *fasn* transcription was inhibited at both doses in CPF and in (ETU+CPF 30 nM)-livers (Supplementary Fig. 4C). Both genes were induced only in (ETU)-male livers and reduced in (CPF 30 nM)-samples (Supplementary Fig. 4K). Furthermore, *fasn* was reduced in co-exposed males of both groups.

As hepatic β-oxidation is directly targeted by T_3 (Sinha *et al.* 2018), we assessed the expression of genes involved in T_3 metabolism and transport. *dio3b* transcript was induced in (ETU)- and (CPF-300 nM)-female livers and decreased in (ETU+CPF 300 nM)-samples (Fig. 6H).

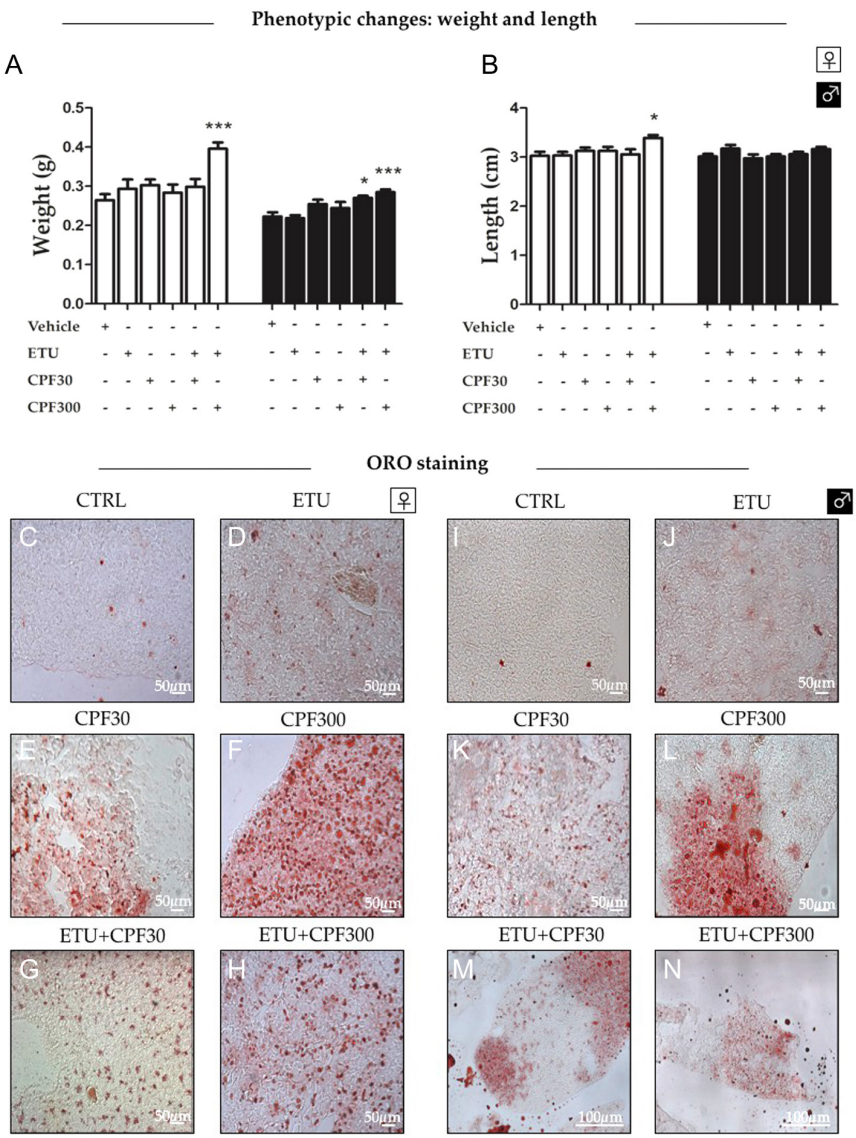

Figure 5
Embryonic exposure to pesticides induces liver steatosis in a sex- and exposure-dependent manner. (A and B) Weight gain and length were measured in control (CTRL) and exposed zebrafish, from 6 hpf to the 180 dpf, males ($n = 15$) and females ($n = 15$). Mean and S.D. are reported. A significant difference from the control group is indicated by $^{*}P < 0.05$, $^{***}P < 0.001$. Lipid accumulation in the liver was confirmed by ORO staining of sections obtained from samples stored in the OCT of adult females (C–H) and males (I–N), as reported in the M&M section. Staining was performed on at least three animals/groups. 20× and 63× Magnification images are shown. A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0134.

Concordantly, *dio2* mRNA was inhibited in the first two conditions (Fig. 6G). *ugt1ab* mRNA was upregulated in (CPF-30 nM)- and (ETU + CPF 30 nM)-females (Fig. 6E), whereas *dio1* transcript was inhibited in exposed females (Fig. 6F). In males, hepatic *dio2* and *dio3b* mRNAs were always increased (Fig. 6O and P, respectively) and *dio1* expression was augmented in all exposure groups, except for (CPF 30 nM)-male livers (Fig. 6N). *ugt1ab* was inhibited in (CPF 30 nM)-male liver in a statistically significant manner, while a decrease was observed in the other groups with the exception of (CPF 300 nM) livers (Fig. 6M). *ttr* and *thrb* transcripts were similarly reduced in all the exposed female livers (Supplementary Fig. 4G and H), whereas this was significant only for (CPF 30 nM)- and (ETU + CPF 300 nM) livers in males (Supplementary Fig. 4O and P).

This data suggests that T_3 signaling in male livers increases, probably in order to protect against the effects of CPF exposure.

Hepatic PPARa/Foxo3a pathway is damaged by early embryonic exposure to pesticides

The discordance between T_3 levels and *cpt1* mRNA, especially in female livers, suggested damage to other signaling pathways. To get new insights, we analyzed with the JASPAR tool (Fornes *et al.* 2020) a sequence of 5000 bp upstream of the zebrafish *cpt1a* gene to retrieve the binding sites for its putative transcriptional regulators. We found binding sites for THRB/PPAR/NR1I3, SREBFs and FOXO3 (Fig. 7A). As PPARα is the major regulator of *cpt1* gene expression,

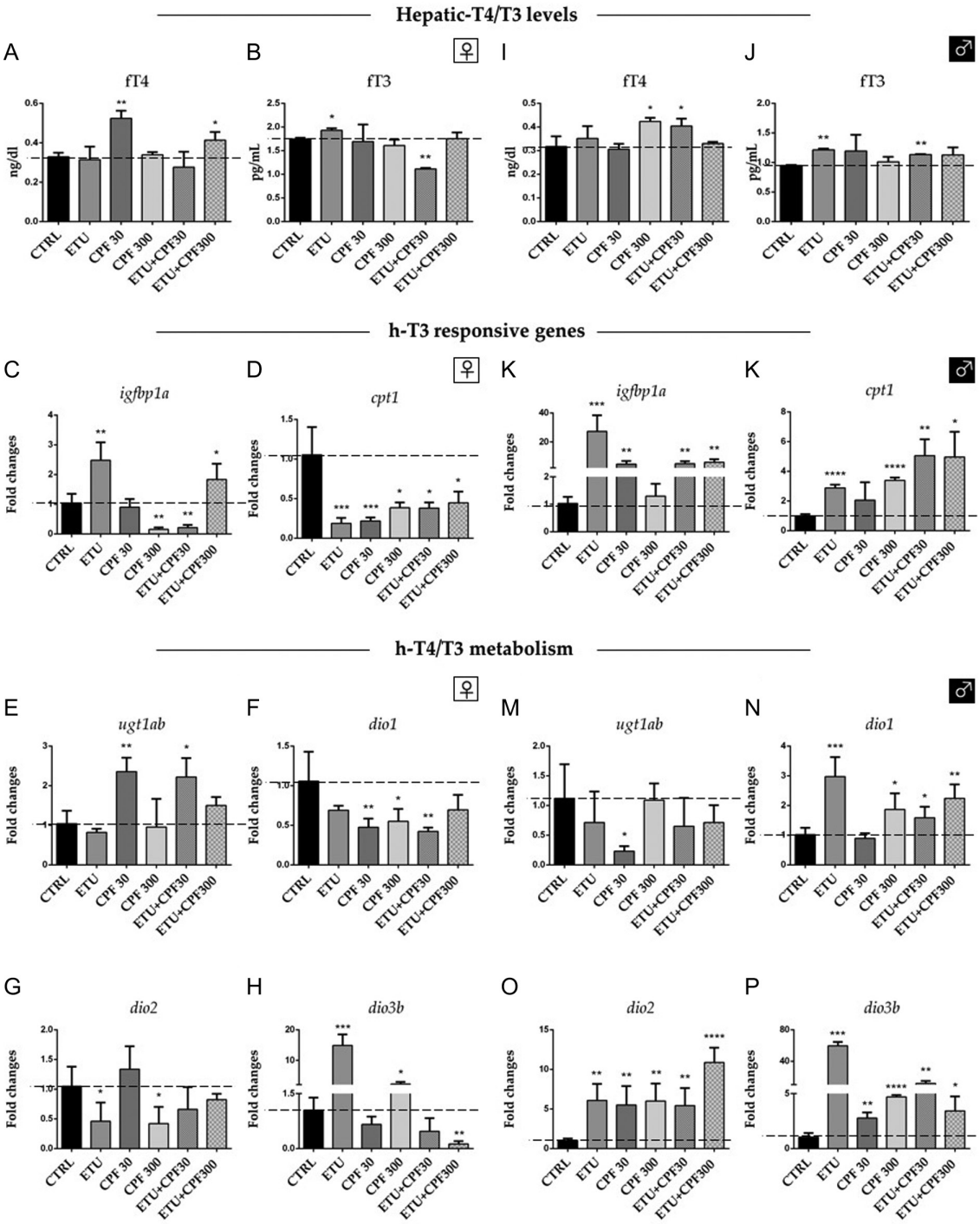

Figure 6

Sex-dependent modulation of hT4/T3-level and regulation of TH metabolic enzymes/T3-regulated transcripts in livers of adult zebrafish exposed to pesticides. H-fT4 and h-fT3 levels were determined by ELISA in females ($n = 3$; A, B) and males ($n = 3$; I, J), exposed from 6 hpf to 180 dpf, and prepared as described in the Materials and methods section. (C, D) Female and (K, L) male mRNA level of h-T3 responsive genes *igfbp1a* and *cpt1* was assayed by RTqPCR, as reported in the Materials and methods section. (E–H) Female and (M–P) male mRNAs of TH metabolizing enzymes, *ugt1ab*, *dio1*, *dio2*, *dio3b*, in livers of adult fishes. RTqPCR tests were performed on livers of five males and five females/groups. Data analysis was conducted as described in the Materials and methods section. Data are reported as fold change values calculated as a ratio between average relative gene expression in treated and control adults, after normalization on *actb1* mRNA. Mean and S.D. are reported. Significant difference from the control group is indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

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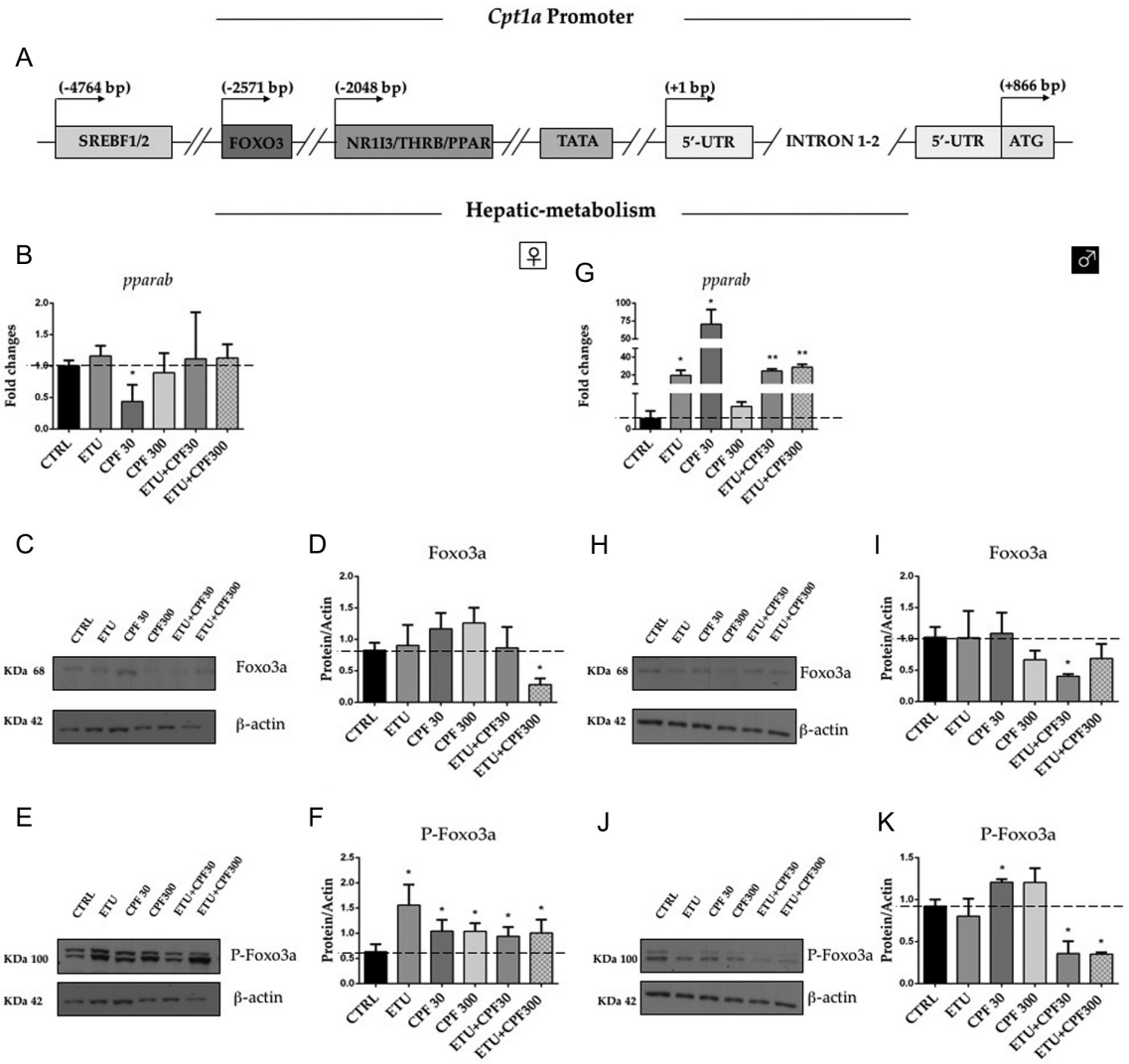

Figure 7

Pesticides deregulate the PPARα/Foxo3a axis in zebrafish liver. (A) Schematic representation of the zebrafish *cpt1a* promoter (5000 bp upstream of the *cpt1* gene, ENSEMBL, transcript ID ENSDART00000145375.5) as found with bioinformatics analysis conducted with JASPAR. Levels of *pparab* transcripts in female ($n = 5$; B) and male ($n = 5$; G) livers of fish exposed to pesticides from 6 hpf to 180 dpf. Data analysis was conducted, as described in the Materials and methods section. Data are reported as fold change values calculated as a ratio between average relative gene expression in treated and control adults, after normalization on *actb1* mRNA. Mean and S.D. are reported. A significant difference from the control group is indicated by $*P < 0.05$, $**P < 0.01$. Representative images of Western blotting analyses of Foxo3a and P-Foxo3a (Ser253) conducted in females ($n = 3$; C, E) and males ($n = 3$; H, J). Samples were processed as described in the Materials and methods section. Each line represents a single fish. Semiquantitative analysis of the Western blotting results was conducted upon normalization on β-actin level and densitometric analyses. Statistical analysis is shown for females (D, F) and males (I, K). Data analysis was conducted as described in the Materials and methods section. Mean and S.D. are reported. A significant difference from the control group is indicated by $*P < 0.05$.

we determined its level by RTqPCR. Indeed, the PPARα antibody did not work in zebrafish (Schlaepfer & Joshi 2020). *ppar alpha* (*pparab*) mRNA was inhibited in (CPF 30 nM)-female livers (Fig. 7B), whereas its expression was strongly induced in all the exposed males (Fig. 7G), except for (CPF 300 nM)-male livers. We also assessed the level of *fasn* and *acc* mRNAs, because it was regulated by *thrb* and PPARγ. Interestingly, the increase of *acc* mRNA in females (Supplementary Fig. 4C) could not be explained by *ppar gamma* mRNA (*pparg*) and SRBF1 protein (Supplementary Fig. 4A, D and E, respectively) regulation: the first was always inhibited and the second was induced only in (ETU + CPF 300 nM)-female livers. In males, *fasn* and *acc* mRNAs increased only in (ETU)-livers (Supplementary Fig. 4J and K) even though the *pparg* mRNA increase was not statistically significant in

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these samples (Supplementary Fig. 4I). No major effect was detected for SRBF1 protein in male livers.

The Foxo3 pathway has a role in *cpt1* mRNA regulation (Shao *et al.* 2016). Since the phosphorylation by Akt of Foxo3 at Ser S253 (P-Foxo3a) has been shown to inactivate the pathway by promoting protein export from the nucleus, we analyzed the levels of P-Foxo3a and Foxo3a by Western blotting. Although the cellular content of Foxo3a was diminished only in (ETU + CPF 300 nM) female livers (Fig. 7C and D), P-Foxo3a levels were increased in all the conditions in this sex (Fig. 7E and F). P-Foxo3a in males was induced in (CPF 30 nM)-livers (Fig. 7J and K). Concordant with the increase in lipid accumulation detected by ORO staining, a trend toward its increase was also evident in (CPF 300 nM)-male livers.

Finally, we evaluated the hepatic expression of TNF alpha, which is considered a negative factor in hepatic steatosis (Copaci *et al.* 2006). We observed a major increase in TNF alpha mRNA in all conditions in females (Supplementary Fig. 5A) but this was not statistically significant in (CPF 300 nM) and (ETU) and (ETU + CPF 300 nM)-male livers (Supplementary Fig. 5B).

Overall, the data suggested that males were protected from hepatic steatosis because of the hyperthyroid status of the liver concomitant with the activation of the PPAR/Foxo3a pathway.

Discussion

The mechanisms which regulate thyroid development/function and the processes controlled locally by THs are conserved in vertebrates. Several rodent studies provide evidence that both are targets of thyroid-hormone-disrupting chemicals (THDCs) such as CPF and ETU (Leemans *et al.* 2019). The imbalance of TH signaling is a factor contributing to several diseases, including hepatic lipid dysmetabolism, regardless of whether it is the result of the alteration of thyroid function and/or of peripheral TH metabolism. By simultaneous analysis of thyroid gland morphology/development, TH levels/signaling and HPT-axis activity, we report evidence that adaptive processes are established to ensure the correct level of T_3 is maintained following exposure to low doses of CPF, ETU or both ETU and CPF combined, from early developmental stages. The slight increase in l-fT4 and l-fT3 might be the result of such a process, as suggested by the upregulation of several thyroid-specific transcripts and *tshb* mRNAs. The detected increase in l-fT3 and the levels of deiodinase transcripts in whole larvae can depend on the need to stabilize the T_3 levels up to the tissue-specific demand (Table 1). This does not imply that hypothyroidism can be promoted in specific organs, as shown by *tshb* mRNA induction in the pituitary (Table 1).

Table 1 Summary of results of F1-larvae and F2-larvae.

Sample name	Anterior pituitary *tshb*	Thyroid Number of follicles *tg*, *nis*, *tpo*	Whole body L-fT4, L-fT3, *dio2*, *dio3b*, *igfbp1a*, *cpt1*	Thyroid function/whole body thyroid status
F1-Larvae				
ETU	*tshb* ↑[d]	↓,[c] *tg* (–), *nis* ↑, *tpo* (–)	L-fT4 ↑,[a] L-fT3 ↑,[a] *dio2* (–), *dio3b* ↓,[a] *igfbp1a* (–), *cpt1* (–)	↓/↑
CPF 30	*tshb* ↑[d]	↓,[b] *tg* (–), *nis* ↓, *tpo* ↓[a]	L-fT4 ↑, L-fT3 ↑,[a] *dio2* ↑, *dio3b* ↑,[d] *igfbp1a* ↑,[a] *cpt1* ↑[a]	↓/↑
CPF 300	*tshb* ↑[b]	↓,[c] *tg* (–), *nis* ↓,[a] *tpo* (–)	L-fT4 ↑,[a] L-fT3 ↑,[a] *dio2* (–), *dio3b* ↑,[d] *igfbp1a* ↑,[a] *cpt1* ↑	↓/↑
ETU + CPF 30	*tshb* ↑[c]	(–), *tg* ↑,[a] *nis* (–), *tpo* ↑	L-fT4 ↑,[a] L-fT3 ↑,[a] *dio2* (–), *dio3b* (–), *igfbp1a* (–), *cpt1* (–)	↓/↑
ETU + CPF 300	*tshb* ↑[d]	↓,[c] *tg* ↑,[d] *nis* (–), *tpo* ↑[b]	L-fT4 (–), L-fT3 ↑, *dio2* (–), *dio3b* ↑,[d] *igfbp1a* ↑,[a] *cpt1* ↑[a]	↓/↑
F2-Larvae DRUG				
ETU	*tshb* ↑	↓,[b] *tg* ↑,[a] *nis* ↑,[b] *tpo* ↑[b]	L-fT4 (–), L-fT3 ↑, *dio2* ↑, *dio3b* ↑, *igfbp1a* ↑,[a] *cpt1* ↑	↓/↑
CPF 30	*tshb* ↑	↓, *tg* (–), *nis* (–), *tpo* (–)	L-fT4 ↑,[b] L-fT3 ↑, *dio2* (–), *dio3b* ↓, *igfbp1a* (–), *cpt1* (–)	↓/↑
CPF 300	*tshb* ↑	↓, *tg* (–), *nis* ↑, *tpo* ↑	L-fT4 ↑,[a] L-fT3 ↑, *dio2* ↑, *dio3b* (–), *igfbp1a* ↑ *cpt1* ↑	↓/↑
ETU + CPF 30	*tshb* ↑	↓,[c] *tg* (–), *nis* ↑, *tpo* ↑	L-fT4 ↓,[a] L-fT3 ↓,[a] *dio2* ↑, *dio3b* (–), *igfbp1a* ↑, *cpt1* (–)	↓/↑
ETU + CPF 300	*tshb* ↑	↓,[c] *tg* (–), *nis* ↑, *tpo* ↑	L-fT4 ↑,[b] L-fT3 ↑, *dio2* (–), *dio3b* ↓, *igfbp1a* (–), *cpt1* ↑[b]	↓/↑

Schematic representation of the results for different treatments. Symbol: ↑, upregulation; ↓, downregulation; (–), no change. Significant difference from the control group is indicated by [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$; [d]$P < 0.0001$.

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The timing of thyroid developmental steps in zebrafish has been established using temporal tracking of the stages impaired by pesticides. Our analyses at 7 dpf showed no major effect on thyroid specification and migration, until the complete formation of the first and single follicle, which normally concluded in 72 hpf (Alt *et al.* 2006). THs are supplied by the yolk until that time. The detection of a reduced number of follicles suggests that major effects could involve growth and proliferation. Although we cannot distinguish between a lack of follicle development and their death, their numeric reduction implies limited T_4 production that is not sufficient to counteract the decrement of the TH maternal level due to the depletion of the yolk. The observed maintenance relies on the activation of the feedback loop that stimulates T_4 production by the remaining follicles. We observed a reduction in the number of follicles in F1 and F2 larvae. *tshb* mRNA induction may intervene in compensating for the alteration of follicle formation at late stages. Indeed, TSH regulates thyroid growth and differentiation, but it is not involved in organogenesis or cell migration (Marians *et al.* 2002, Postiglione *et al.* 2002). Interestingly, *tshr* morphants showed a reduction in the size and number of follicles, downregulation of differentiation markers (such as *slc5a5* and to a lesser extent *tg*), and reduced expression of thyroid transcription factors in zebrafish (Opitz *et al.* 2011). Thus, *tshb* transcript induction may participate in the establishment of an an efficient thyroid late differentiation, independently from the raising of TH levels (Table 1). Although we can suggest that early exposure to pesticides might damage late thyroid differentiation and peripheral TH metabolism/signaling, future studies are needed to analyze this feature in depth.

Because of its external development, zebrafish permits larval damage due to direct embryonic exposure to be investigated. The data reported here demonstrate that parental exposure worsens the embryonic effects of pesticides in terms of reduction of follicle number and the activation of the feedback loop, which is less effective in F2 vs F1 (Table 1). Indeed, the increase in l-fT4 and l-fT3 is less evident in F2 larvae and is restricted to a few conditions, such as in the (ETU+CPF 30 nM)-group. Differences could also be detected in the regulation of *dio3b* induced in ETU-F2 larvae and inhibited in F1-samples (Table 1). The small increase in *tshb*, which regulates the expression of the thyroid-specific genes such as *scl5a5*, suggests that other pathways might be involved, such as NF-kB. We have proposed it as a regulator of *scl5a5* expression in mice exposed to p-dioxin and have shown that CPF reduces p65 nuclear level in rat follicular cells (Reale *et al.* 2018, 2019). The results found in F2-larvae provide evidence that parental exposure might itself cause the reduction of the number of follicles and hypothyroidism, although we cannot distinguish between the impairment of the TH reserve in the yolk or germline epigenetic changes. This point should be specifically addressed.

Different human and rodent studies pointed out that the embryonic impairment of TH levels might modulate organ development and activity in adulthood (Colborn *et al.* 1993, Zoeller 2010, Gutleb *et al.* 2016, Porreca *et al.* 2017). Here, we focus on liver metabolic activity in adults, because it is a major target of T3-signaling. The data suggest a sex- and exposure-dependent promotion of liver steatosis, which is summarized in Table 2. CPF and ETU differ in such activity, with CPF being more effective than ETU. Both compounds exert a milder effect in male livers, which are protected by the increase in hepatic T3 levels/signaling, resulting in the accumulation of *cpt1* mRNA. In steatotic female livers, the balance between the transcripts of the T3-activating enzymes (*dio2*) and inactivating ones (*dio1* and *dio3b*) is indicative of its reduction (Fig. 6). We found a significant discrepancy between the sex-dependent differences in T_3 level/signaling and an opposite trend in regulation of *cpt1* mRNA (Table 2). This emphasizes the possible involvement of other pathways, such as PPARα and Foxo3a, both shown as regulators of *cpt1* expression in mammals but not in zebrafish (Asaoka *et al.* 2013). The increase of P-Foxo3a in female liver agrees with the *cpt1* mRNA downregulation reported in rodents. Indeed, P-Foxo3a cytoplasmic retention resembles the mouse loss-of-function mutation (Tzivion *et al.* 2011) because its transcriptional activity is inhibited. Concordantly P-Foxo3a is less phosphorylated in exposed males and a strong induction of PPARα was also described (Radenne *et al.* 2008). This is relevant because *thrb*, whose expression is reduced in females and is weakly expressed in males, acts in synergy with PPARα in regulating gene expression (Table 2). The T_3 increase in males might enhance PPARα/Thrb complex formation and activity (Videla *et al.* 2016). PPARα regulation might play a double role because it could inhibit AKT/Foxo3a phosphorylation, as reported in kidney (Chung *et al.* 2012). Indeed, we report an increase in PPARα and reduced Foxo3a phosphorylation in males. Furthermore, Foxos can interact with several nuclear receptors including TRs and PPARs and, within the complex, the activity of both proteins can be differently modulated (van der Vos & Coffer 2008). Finally, T_3 and

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Table 2 Summary of the results of F1-adult female and male.

Sample name	Lipid accumulation	Hepatic T4/T3 levels and TH responsive transcripts	Pathways involved in hepatic steatosis	Hepatic thyroid status/hepatic lipid accumulation
	ORO staining	H-fT4, H-fT3, *igfbp1a*, *cpt1*	*thrb*, *pparab*, P-Foxo3a, *tnfa*	↑ or ↓/slight, severe or moderate steatosis
Fl-Adult Female				
ETU	Slight effect	H-fT4 (–), H-fT3 ↑,[a] *igfbp1a* ↑,[b] *cpt1* ↓[c]	*thrb* ↓,[c] *pparab* (–), P-Foxo3a ↑,[a] *tnfa* ↑[a]	↑/slight steatosis
CPF 30	Severe effect	H-fT4 ↑,[b] H-fT3 (–), *igfbp1a* (–), *cpt1* ↓[c]	*thrb* ↓,[b] *pparab* ↓,[a] P-Foxo3a ↑,[a] *tnfa* ↑[a]	↓/severe steatosis
CPF 300	Severe effect	H-fT4 (–), H-fT3 (–), *igfbp1a* ↓,[b] *cpt1* ↓[a]	*thrb* ↓,[b] *pparab* (–), P-Foxo3a ↑,[a] *tnfa* ↑	↓/severe steatosis
ETU + CPF 30	Moderate effect	H-fT4 (–), H-fT3 ↓,[b] *igfbp1a* ↓,[b] *cpt1* ↓[a]	*thrb* ↓,[b] *pparab* (–), P-Foxo3a ↑,[a] *tnfa* ↑[b]	↓/moderate steatosis
ETU + CPF 300	Moderate effect	H-fT4 ↑,[a] H-fT3 (–), *igfbp1a* ↑,[a] *cpt1* ↓[a]	*thrb* ↓,[c] *pparab* (–), P-Foxo3a ↑,[a] *tnfa* ↑[a]	↑/moderate steatosis
Fl-Adult Male				
ETU	Slight effect	H-fT4 (–), H-fT3 ↑,[b] *igfbp1a* ↑,[c] *cpt1* ↑[d]	*thrb* (–), *pparab* ↑,[a] P-Foxo3a (–), *tnfa* ↓	↑/slight steatosis
CPF 30	Moderate effect	H-fT4 (–), H-fT3 ↑, *igfbp1a* ↑,[b] *cpt1* ↑	*thrb* ↓,[c] *pparab* ↑,[a] P-Foxo3a↑,[a] *tnfa* ↑b	↑/moderate steatosis
CPF 300	Moderate effect	H-fT4 ↑,[a] H-fT3 (–), *igfbp1a* (–), *cpt1* ↑[d]	*thrb* (–), *pparab* ↑, P-Foxo3a ↑, *tnfa* ↓[b]	↑/moderate steatosis
ETU + CPF 30	Moderate effect	H-fT4 ↑,[a] H-fT3 ↑,[b] *igfbp1a* ↑,[b] *cpt1* ↑[b]	*thrb* ↓,[b] *pparab* ↑,[b] P-Foxo3a ↓,[a] *tnfa* ↓	↑/moderate steatosis
ETU + CPF 300	Moderate effect	H-fT4 (–), H-fT3 ↑, *igfbp1a* ↑,[b] *cpt1* ↑[a]	*thrb* ↓,[b] *pparab* ↑,[b] P-Foxo3a ↓,[a] *tnfa* ↑[a]	↑/moderate steatosis

Schematic representation of the results for different treatments. Symbol: ↑, upregulation; ↓, downregulation; (–), no change. A significant difference from the control group is indicated by [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$; [d]$P < 0$.

Foxo3a signaling might be involved in the sex-dependent regulation of TNFα mRNA (Table 2) because T_3 could reduce the production of pro-inflammatory cytokines in the liver (Taki-Eldin *et al.* 2012) and the retention of Foxo3a in the nuclei of male hepatocytes could contribute (Xia & Zhu 2013). The role of other pathways, such as estrogen signaling, could be explored (Marlatt *et al.* 2012).

In conclusion, hyperthyroidism presents at larval stages, as a result of alteration in thyroid development/function and peripheral TH metabolism/signaling and results in the impairment of β-oxidation and '*de novo* lipogenesis at larval stages (Supplementary Figs 2G, H and 3G, H) and in adult livers (Supplementary Fig. 4B, C, J and K). Mechanistically, sex-dependent regulation of lipid metabolism is the result of the organ/tissue-specific crosstalk between T_3 signaling and other pathways, such as Foxo3a and PPARs, and is involved in the preservation of hepatic health in mammals. Here, we report evidence of their activity in zebrafish adult livers, which emphasizes the vast potential of zebrafish in investigating pathways involved in complex metabolic diseases such as hepatic steatosis.

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 C, P I and N V involved in conceptualization. C M, N V and P A performed methodology. C M and N V involved in software technique. C M and N V helped in validation. M M, C M and N V involved in formal analysis. P I, R C, R F, R N A, R L and A F performed investigation. C M, N V and M M involved in data curation. C M, N V and C A involved in writing and drafted the original manuscript. de F M, M M, and A C involved in writing, review and editing. M M and A C involved in supervision. A C performed project administration. M M and A C involved in funding acquisition. All authors have read and agreed to the published version of the manuscript. M M and C A contributed equally as last authors to this work.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-20-0134.

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