Concomitant exposure to benzo[a]pyrene and triclosan at environmentally relevant concentrations induces metabolic syndrome with multigenerational consequences in Silurana (Xenopus) tropicalis

Marie Usal, Christophe Regnault, Sylvie Veyrenc, Karine Couturier, Cécile Batandier, Anne-Laure Bulteau, David Lejon, Bruno Combourieu, Thomas Lafond, Muriel Raveton, et al.

Concomitant exposure to benzo[a]pyrene and triclosan at environmentally relevant concentrations induces metabolic syndrome with multigenerational consequences in *Silurana (Xenopus) tropicalis*

Marie Usal[a1], Christophe Regnault[a1], Sylvie Veyrenc[a], Karine Couturier[b], Cécile Batandier[b], Anne-Laure Bulteau[c], David Lejon[d], Bruno Combourieu[d], Thomas Lafond[e], Muriel Raveton[a] and Stéphane Reynaud[a2].

[a] Univ. Grenoble-Alpes, Univ. Savoie Mont Blanc, CNRS, LECA, 38000 Grenoble, France.

[b] Univ. Grenoble-Alpes, INSERM, LBFA, 38000 Grenoble, France.

[c] Institut de Génomique Fonctionnelle de Lyon, Univ. Lyon 1, CNRS UMR5242, Ecole Normale Supérieure de Lyon, 69000 Lyon, France.

[d] Rovaltain Research Company, F26300 Alixan, France

[e] Centre de Ressources Biologiques Xénopes, Univ. Rennes 1, CNRS, UMS 3387, Rennes France.

[1] Contributed equally to the work

[2] Corresponding Author:
stephane.reynaud@univ-grenoble-alpes.fr
+33 (0)4 76 51 46 80

Email addresses: MU: marie.usal@hotmail.fr, CR: christophe.regnault@gmail.com, SV: sylvie.veyrenc@univ-grenoble-alpes.fr, KC: karine.couturier@univ-grenoble-alpes.fr, CB: cecile.batandier@univ-grenoble-alpes.fr, ALB: anne-laure.bulteau@ens-lyon.fr, DL: dlejon@rovaltainresearch.com, BC: Bruno.combourieu@rovaltainresearch.com, TL, thomas.lafond@univ-rennes1.fr, MR: muriel.raveton@univ-grenoble-alpes.fr, SR: stephane.reynaud@univ-grenoble-alpes.fr

ABSTRACT:

Numerous studies suggest that amphibians are highly sensitive to endocrine disruptors (ED) but their precise role in population decline remains unknown. This study shows that frogs exposed to a mixture of ED throughout their life cycle, at environmentally relevant concentrations, developed an unexpected metabolic syndrome. Female *Silurana* (*Xenopus) tropicalis* exposed to a mixture of benzo[a]pyrene and triclosan (50 $ng.L^{-1}$ each) from the tadpole stage developed liver steatosis and transcriptomic signature associated with glucose intolerance syndrome, and pancreatic insulin hyper secretion typical of pre-diabetes. These metabolic disorders were associated with delayed metamorphosis and developmental mortality in their progeny, both of which have been linked to reduced adult recruitment and reproductive success. Indeed, F1 females were smaller and lighter and presented reduced reproductive capacities, demonstrating a reduced fitness of ED-exposed *Xenopus*. Our results confirm that amphibians are highly sensitive to ED even at concentrations considered to be safe for other animals. This study demonstrates that ED might be considered as direct contributing factors to amphibian population decline, due to their disruption of energetic metabolism.

Key words: endocrine disruptors, metabolic syndrome, amphibian population decline, transgenerational, *Silurana* (*Xenopus) tropicalis*

1. Introduction

In recent decades, amphibian wetland populations have declined dramatically worldwide, with an extinction rate 211 times faster than for other species (Roelants et al., 2007). Fragmentation and loss of habitat has been described as one of the main causes, along with other threats such as the introduction of exotic species, emerging infectious diseases, and global climate change (Blaustein and Bancroft, 2007; Hayes et al., 2010). In addition to these well-described factors, several studies suggest that wetland pollution by endocrine disruptors (ED) is also a key contributing factor to this decline by altering amphibian fitness (Fedorenkova et al., 2012). Although several authors claim that the vulnerability of amphibians to ED is relatively similar to that of other species (Kaplan, 2009), there is growing evidence that once the symptoms of ED toxicity are detected in amphibian populations, population decline and community destructuring are unavoidable (Hayes et al., 2010). ED may act on crosstalk between the thyroid and reproductive axes (Duarte-Guterman et al., 2014) thus disrupting organogenesis and gonadal differentiation in larvae (Crump et al., 2002; Veldhoen et al., 2006; Kashiwagi et al., 2008; Kloas et al., 2009; Langlois et al., 2010; Lorenz et al., 2011; Shi et al., 2014). ED may also interfere with the gonadotrop axis in adults during the breeding season, causing disruptions of egg and sperm maturation (Hayes et al., 2002, 2011; Mosconi et al., 2002; Urbatzka et al., 2006, 2007). In addition, there are growing concerns that ED might also be involved in metabolic disorders (Casals-Casas and Desvergne, 2011). However, despite experimental evidence from mammals and epidemiological correlations in humans suggesting that ED could induce metabolic diseases (Alonso-Magdalena et al., 2010; Jiang et al., 2014; Chevalier and Fénichel, 2015a; Chevalier and Fénichel, 2015b; Susiarjo et al., 2015; Chevalier and Fénichel, 2016, Medic-Stojanoska, 2019), their potential involvement in metabolic disorders associated with amphibian population decline has been largely neglected. The first evidence that ED could alter frog metabolism was shown in *S. tropicalis* after acute exposure to the suspected ED triclosan

(TCS) (Regnault et al., 2016) and the well-known benzo[a]pyrene (BaP) (Regnault et al., 2014, 2016). More recently, two independent controlled exposures of *S. tropicalis* throughout their life cycle to these two ED at environmentally relevant concentrations induced a metabolic syndrome featuring a pre-diabetes state. The exposed animals produced progeny that metamorphosed later, were smaller and lighter at metamorphosis, and had reduced reproductive success, thus linking metabolic disorders to putative population decline (Regnault et al., 2018).

In situ, TCS is found in wastewater effluent due to its use as an antimicrobial agent in household and personal-care products such as cosmetics, soap, toothpaste, shampoo and textiles (Levy et al., 1999; Piccoli et al., 2002; McAvoy et al., 2009; Møretrø et al., 2011; McArthur et al., 2012). It is released into natural aquatic environments and, in 2000, it was among the top seven organic wastewater pollutants found in streams by the United States Geological Survey (McAvoy et al., 2009; Barber et al., 2015). In wetlands, this emerging contaminant is therefore mixed with other ED, including the well-known BaP, classified as a reproductive ED by several countries, including the United States and all the European Union member states (Petersen and Gleeson, 2007; United States Government, 2010). BaP enters surface water mainly *via* atmospheric fallout, urban run-off, municipal and industrial effluent, and oil spillages or leakages (Srogi, 2007). TCS and BaP are both found in effluent from wastewater treatment plants (Weir et al., 2010; Gardner et al., 2012), in water streams (Galloway et al., 2005) and in lakes (Whitacre, 2010). Although it is common practice to assess the possible environmental risk of chemicals individually, this approach may over or underestimate the real environmental impact of mixtures of xenobiotics (Relyea, 2009; Godoy and Kummrow, 2017). BaP and TCS concentrations in surface water vary from 0.1 to 237 $ng.L^{-1}$ (Trapido and Veldre, 1996; Olivares-Rubio et al., 2015) and 0.8 to 74 $ng.L^{-1}$ (Lindström et al., 2002; Blair et al., 2013) respectively. Thus the impact of these chemicals in mixture should be studied within this range of concentrations.

In this context, this study aims at understanding the precise impact of a mixture of BaP and TCS on *S. tropicalis* at environmental concentrations. The effects of BaP and TCS were studied over two generations of *S. tropicalis*. The parental F0 generation of *Xenopus* was exposed to the mixture (50 ng.L^{-1} of each ED) for 12 months from the tadpole stage to the mature adult stage. The impacts of these contaminants on the *Xenopus'* metabolism were studied by examining both the transcriptome and overall liver physiology. Multigenerational impacts were studied by monitoring the development and reproductive capacity of their progeny not exposed to the ED (F1 generation).

2. Methods

2.1. Animals

Seven-day old *Silurana* (*Xenopus) tropicalis* were acclimatized for one week in Marc's Modified Ringer's (MMR) 0.1X medium under controlled conditions (25°C, photoperiod 12h:12h). The frogs were fed daily *ad libitum* with trout food (pellets Aquatic 3, Special Diets Services). The density of *Xenopus* was 50 tadpoles per 2 L of 0.1X MMR (two tanks per exposure condition). Between 30 (prior to metamorphosis) to 150 days (complete metamorphosis), the density of frogs was adjusted to 30 animals per 4 L. After 150 days, the density of animals was adjusted to 10 animals per 4 L (four tanks per conditions). All experiments were performed in an accredited animal house (Rovaltain research company, n° A 26 004 1401) and approved by the ethics committee ComEth Grenoble - C2EA – 12 (animal welfare agreement n° 02545.03).

2.2. Exposure

The *Xenopus* were collectively exposed to the ED-mixture under the controlled conditions, in identical tanks, with identical animal densities as described above. One-quarter of the medium was renewed daily using an MMR solution containing BaP (Sigma-Aldrich) and TCS

(Sigma-Aldrich) at an equimolar concentration of 200 ng.L^{-1} and the medium in its entirety was changed weekly. The control frogs were exposed to dimethyl sulfoxide (DMSO, vehicle) at a concentration of 4/1000 (v:v). As described in Regnault et al. (2018), pollutant concentrations in the medium were continually monitored using GC-MS/MS, and confirmed that the average weekly BaP/TCS concentrations were both equal to 50 ng.L^{-1}. These concentrations match the BaP and TCS concentrations found in polluted ponds (Trapido and Veldre, 1996; Lindström et al., 2002; Blair et al., 2013; Olivares-Rubio et al., 2015). *Xenopus* mortality and development were monitored weekly and the body mass and snout-vent length of each animal was measured at the metamorphic stage. After 12 months' exposure, four females from each exposure condition were used for glucose tolerance test. Eight others females were rapidly killed by concussion and immediately dissected to sample the organs. Blood was collected through an intra-cardiac puncture.

2.3. Mating

At the adult stage, five F0 females and ten F0 males were selected from the ED-mixture batch and from control batch to mate naturally. Two injections of human chorionic gonadotropin (hCG) were performed in the dorsal lymph sac of animals to induce the mating behavior (Showell and Conlon, 2009). After hatching, the tadpoles were bred in the same control conditions as described above and the F1 development was monitored weekly. The following criteria were used to characterize the development of F1 *Xenopus*: time from hatching to metamorphosis, snout-vent length and weight at the metamorphic and adult stages. Afterwards, the mature adults of the F1 generation were mated, as described above, to assess their reproductive capacity.

2.4. Glucose tolerance test

Glycaemia was measured by digital sampling at 0, 1, 2, and 4 hours after intraperitoneal glucose injection (1 $mg.g^{-1}$ fresh mass) using a glucometer (Accu-Chek performa, Roche Diagnostics, Meylan, France). The statistical comparison was performed on the area under the curve obtained after glucose injection.

2.5. Hepatosomatic index and hepatotoxicity

The hepatosomatic index (HSI) was calculated by dividing the weight of the liver by the body weight and hepatoxicity was evaluated by measuring alanine aminotransferase activity in serum as previously described in Regnault et al. (2018).

2.6. Histological observations

The liver (same lobe/frog) and the pancreas were collected, embedded in Tissue OCT (Labonord - VWR, Templemars, France), rapidly frozen in liquid nitrogen, and conserved at -80°C. Using a cryostat (CM3050 S, Leica, Nussloch, Germany), histological transverse sections 7 µm thick were produced and mounted on Super-Frost plus microscope slides (Labonord). The frozen sections of liver were stained with Hematoxylin and Erythrosin (HE) and Gomori's trichrome to study tissue organization, with Oil Red O (ORO) to assess total lipid hepatic content and with Periodic Acid Schiff (PAS) staining to assess glycogen content. All staining and procedures have been previously described in Regnault et al. (2014). PAS scoring was performed by two people independently. The PAS staining score (0–4) was assigned according to the percentage of glycogen in the cells, with a score of 0 corresponding to no glycogen, and a score of 4 corresponding to a cell full of glycogen. For ORO, lipid quantifications were performed as previously described (Regnault et al., 2014). In short, an image segmentation was processed using R, G and B layer similarity, with the default sharpness and compactness parameters. The object size parameter was set to 10. Then, using a 2-class typology (lipid or not), several training

parcels were selected manually for each class. An image classification was then run using the nearest neighboring algorithm. The results of the classifications were rasterized and the proportion of each class estimated.

Insulin production was quantified by immunohistochemistry on frozen pancreas sections as previously described (Regnault et al., 2014).

2.7. Hepatic mitochondrial respiration and mitochondrial oxidative stress evaluation

The mitochondria were prepared as described in Garait et al. (2005). Complex I and complex II–mediated mitochondrial respiration (State 2) was evaluated with 5 mM glutamate, 2.5 mM malate, and 5 mM succinate. State 3 respiration was measured after the addition of adenosine diphosphate (ADP, 500 mM). The respiratory control index (RCI) was defined as the ratio: state 3/state 2.

To evaluate the mitochondrial oxidative stress, aconitase activity was measured spectrophotometrically at 340 nm as previously described (Bulteau et al., 2003). Citrate synthase activity was measured as control of mitochondria density by following the rate of appearance of thionitrobenzoic acid at 412 nm in the presence of 10 µg protein, 100 mM Tris–HCl pH 8.0, 0.1%Triton X100, 300 mM acetylCoA, 100µM DTNB, and 500 µM oxaloacetate (Srere, 1969).

2.8. Liver proteasome activity

As previously described in Bulteau et al. (2002), the peptidase activity of the proteasome was assayed using a fluorogenic peptide, succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin (LLVYAMC) (Sigma-Aldrich, Saint Quentin Falavier, France).

2.9. Liver RNA extraction and sequencing

The complete method is largely detailed in Regnault et al. (2018). Briefly, for each biological replicate, the RNAqueous®-4PCR Kit (Ambion, USA) was used to extract the total RNA from 15 mg of liver and according to the manufacturer's instructions. RNA-seq libraries were prepared using the TruSeq Stranded mRNA sample Prep kit (Illumina) and sequenced on an Illumina GAIIx sequencer as 75 bp reads by Hybrigenics-Helixio (Clermont-Ferrand, France). Read mapping and gene expression quantification and differential analysis were performed using Trimmomatic (v0.32.1) (Bolger et al., 2014), Bowtie-0.12.7 / TopHat 2 (v0.6) software (Trapnell et al., 2009), Cufflinks (v0.0.7), Cuffmerge (v0.0.6) and Cuffdiff (v0.0.7) (Trapnell et al., 2012) tools implemented in a Galaxy pipeline(http://galaxyproject.org).Differentially expressed genes were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and General Ontology terms (GO-term) enrichment analysis, using the online functional annotation tool DAVID (Database for Annotation, Visualisation and Integrated Discovery, http://david.abcc.ncifcrf.gov) (Huang et al., 2009) as previously described (Regnault et al., 2014). The significance was calculated using a modified Fisher's Exact test ($p < 0.05$). Heat maps reflecting gene expression profiles sharing enriched annotation pathways or other gene expression associated with these pathways, were generated using TM4 Multi experiment Viewer (MeV) software (Saeed et al., 2003).

2.10. Muscle glycogen content

Forty mg of thigh muscle were lysed in 40% KOH (100°C, 30 minutes) and cooled, before glycogen precipitating with 100% ethanol (-20°C, overnight). After centrifugation at 10,000 x g for 15 minutes at 4°C, the glycogen pellet was hydrolyzed into glucose by 2M HCl at 100°C for 3 hours. After 2M NaOH addition, the concentration of glucose was measured using the GAGO-20 kit (Sigma Aldrich, France), in accordance with the manufacturer's instructions.

2.11. Transcription profile of muscle glucose transporter

Total RNA was isolated from 40 mg muscle using TRIzol RNA isolation reagent (Invitrogen, Life Technologies, Carlsbad, USA) according to the manufacturer's instructions. RNA was purified with an RNA Clean & Concentrator kit to remove sugars present in large quantities (R1015, Ozyme zymo Research). After DNAse I treatment of 1.5 µg total RNA, cDNA synthesis was performed with SuperScript III and oligo-dT_{20} primer (Invitrogen) according to the manufacturer's instructions. Real-time quantitative PCR reactions were performed on the iQ5 system (Bio-Rad, Hercules, USA) with 0.45 µM of each primer, 7.5 µL of iQ SYBR Green supermix (Bio-Rad) and 3 µL of cDNA diluted (1:100). The primers used (Table S1) were designed and validated *in silico* with NCBI primer blast. For each gene analyzed, a melt curve analysis was performed to check for the unique presence of the targeted PCR product and the absence of significant primer dimers. The selected housekeeping genes (*RPS7* and *RPL27*) were validated with several RNAseq experiments (Regnault et al., 2014, 2018). The ΔΔCT method (Pfaffl, 2001) taking Into account PCR efficiency was used for data analysis was used for data analysis.

2.12. Oocyte lipid content

Five to ten mg of oocytes were crushed in 500 µl of 1:1 chloroform/methanol then kept at 4°C for one hour. The samples were centrifuged at 18 000g for 30 minutes then 100 µl of supernatant was placed in a glass tube. The samples were placed in a dry bath at 95°C for 10 minutes to obtain lipid deposition. Once the solvent evaporated, 200 µl of 95° sulfuric acid was added and the samples are kept at 95°C for 10 minutes. Once the samples had cooled, 4.8 mL of phospho-vanillin reagent (Sigma, V1104) was added. After 10 minutes, lipids were measured spectrophotometrically at 535 nm.

2.13. Statistics

For F0 females, phenotypic data are expressed as the mean ± SEM and derived from 4 individual experiments. Since the statistical distribution of the data was unknown (Gaussian or not) for four replicates, Wilcoxon's test was used to compare the ED-mixture exposure population to the control population.

The distributions of animal development times (mortality, age to metamorphosis) and female maturity time for F0 and F1 generations were compared between the control populations and the ED-exposed population using a Kaplan-Meyer log-rank test.

3. Results and discussion

3.1. Effect of the ED-mixture on the development of F0 animals and their progeny (F1)

The ED-mixture at environmental concentrations did not significantly impact the mortality of F0 exposed animals compared to the F0 control (Fig. 1A). *Xenopus* exposed to the ED-mixture showed no delay in metamorphosis (Fig. 1B) nor any significant modification in size or weight at the time of metamorphosis (Fig. 1C and D). Furthermore, the ED-mixture did not impact the time to maturity of F0 females (Fig. 1E). Taken together, these results confirmed those obtained for exposure to a single ED, with the exception of the time to metamorphosis, which has been shown to be significantly delayed for *Xenopus* exposed to BaP and TCS individually (Regnault et al., 2018).

In order to test the effect of parental exposure on their progeny, five pairs of F0 *Xenopus* were mated. Despite their sexual maturity, one of the five exposed females rejected the male leading to 80% successful amplexus at 3 hours post-stimulation compared to 100% for the control (Fig. 1F). In addition, a lower number of hatched eggs per amplexus was observed in ED-mixture- exposed animals (Fig. 1G and S1A).

The F1 progeny, only exposed to the ED-mixture at the gamete stage, showed significant mortality during the first 150 days of development (Fig. 1H) and significantly delayed metamorphosis (the 100% population metamorphosis level was reached 20 days later) (Fig. 1I). Moreover, F1 metamorphic *Xenopus* were lighter but there was no significant decrease in size (Fig. 1J and 1K). In comparison to single exposure to BaP or TCS, the effect of exposure to the ED-mixture on progeny age at metamorphosis appeared to be equivalent to BaP exposure, whereas the effect on progeny weight was equivalent to TCS exposure (Regnault et al., 2018). These disturbances could play a major role in the ongoing decline of the amphibian population. Delayed metamorphosis could affect progeny survival in cases of early desiccation of temporary ponds (Rumrill et al., 2018). Along with lower body weight at metamorphosis, it could also reduce adult recruitment and the chances of successful reproduction (Smith, 1987, Todd et al., 2011). At the adult stage, F1 females ancestrally exposed to the ED-mixture were smaller and lighter (Fig. S2A and B), factors which have been shown to impact on overwintering survival rates in several amphibian species in temperate environments (Hayes et al., 2006). Moreover, reduced post-metamorphic growth has previously been showed to impact reproductive success since female weight correlates with clutch weight in several amphibian species (Kuramoto, 1978; Browne, 2007). F1 females from ED-mixture-exposed progenitors also showed delayed sexual maturity, taking more than 80 days to reach 80% of mature *Xenopus* in the population compared to the controls (Fig. 1L). These effects were not found in genitors suggesting a multigenerational worsening of the phenotype. In addition, a reduction in F1 reproductive success and consequent decrease in the fitness of F0 progenitors was observed. Indeed, whereas amplexus was successful for 100% of controls at 3 hours post-stimulation, this was the case for only 40% of F1 progeny from exposed progenitors (Fig. 1M). F1 mating produced eggs with highly variable hatching rates, although the overall trend showed a dramatic decrease in the number of hatched eggs from the F1 offspring of ED-mixture-exposed parents (Fig. 1N and S1B). Variable hatching rates have been described in natural mating in *S. tropicalis* (Showell and Conlon, 2009) and

these results should be interpreted with caution. However, since the same transgenerational effects have previously been observed in *S. tropicalis* exposed to each ED separately (Regnault et al., 2018) reaching a total of 15 mating pairs for exposed animals, these results still suggest a decrease in reproduction success that will need to be confirmed in future studies.

Previous studies have established a correlation between a decrease in maternal egg provisioning and disturbances in offspring development such as decreased size and weight at metamorphosis, a longer larval period and decreased tadpole survival rates (Dziminski and Roberts, 2006). Indeed, oocytes need amino acids, carbohydrates and lipids from the maternal liver to ensure normal development (Brooks et al., 1997). In this study, a downward trend in the lipid content in the oocytes of ED-mixture-exposed F0 females was detected (Fig. S3). Therefore, the observed impacts on the F1 progeny could be explained by changes in lipid and carbohydrate metabolism in exposed F0 females, leading to a decrease in maternal provisioning.

3.2. Effect of ED-mixture on F0 female metabolism

Endocrine disruptor-mixture exposure induced an increase in the hepatosomatic index (HSI) of F0 *Xenopus* (Fig. 2A) correlated with marked liver steatosis (Fig. 2B) (Teubner et al., 2015). Moreover, lipid metabolism disruption has been recently described in aquatic animals (e.g. *Gobiocypris rarus*, *Danio rerio*, *Xenopus laevis*, *S. tropicalis*) exposed to ED (Grün et al., 2006; Guan et al., 2016; Santangeli et al., 2018; Regnault et al., 2018). Liver steatosis is a hallmark of non-alcoholic fatty liver disease (NAFLD) and one of the major disturbances associated with dyslipidemia (Neuschäfer-Rube et al., 2015; Liu et al., 2016; Hua et al., 2017) demonstrating the link between a hypertrophic liver and the accumulation of lipid droplets. Hepatic lipids can be hydrolyzed by mitochondria which perform fatty acid β-oxidation to produce ATP (Ferré and Foufelle, 2010) but excess lipid accumulation during chronic liver steatosis has been associated with mitochondrial dysfunction (Jiang et al., 2014). In this study, exposure to

the ED-mixture led to a 1.64-fold elevated state 2 respiratory rate (Fig. 2C) along with a 1.5-fold decreased respiratory control index (Fig. 2D) and aconitase inactivation (Fig. 2E) showing mitochondrial uncoupling and oxidative stress (Koliaki et al., 2015). These results are confirmed by the stability of citrate synthase activity (Fig. 2F) suggesting that the observed decrease in aconitase activity was not related to variations in mitochondrial density in the liver. Lipid accumulation in the liver has been previously associated with hepatotoxicity (Yamaguchi et al., 2007). The 2.5-fold increase in serum alanine amino transferase activity (Fig. 2G) confirmed this phenomenon in the ED-mixture-exposed *Xenopus*. Liver disruption (Fig. 2H-K) is characterized by ballooning hepatocytes (Fig. 2I), increasing melanomacrophage centers (Fig. 2I, arrow), swollen blood vessels (Fig. 2K, full arrow), necrosis and leukocyte infiltrates (Fig. 2K, dotted arrows). NAFLD encompasses steatosis and the accumulation of glycogen (Bedossa, 2016) whereas ballooned hepatocytes, inflammation, and necrosis are indicative of progression from NAFLD to non-alcoholic steatohepatisis (NASH) (Day and James, 1998; Day, 2006). Therefore, the hepatic damage in the ED-mixture-exposed animals is typical of the progression between NAFLD to NASH and corresponds to a combination of the disturbances previously described for *Xenopus* under single exposure to BaP and to TCS (Regnault et al., 2018). The liver damage observed in the animals exposed to the ED-mixture was accompanied by a decrease in liver proteasome activity (Fig. 2L) suggesting endoplasmic reticulum stress and the possible development of insulin resistance (IR) (Otoda et al., 2013). This metabolic syndrome is confirmed by the accumulation of glycogen in the liver which has been associated with NAFLD-induced IR (Fig. 2M) (Han et al., 2016). Moreover, despite no difference in basal serum glucose levels, ED-mixture exposure led to glucose intolerance with a 1.8-fold increase in blood glucose (Fig. 2N). This glucose intolerance is confirmed by a threefold increase in insulin production by the pancreatic β-cells (Fig. 2O). In type 2 pre-diabetes the onset of hyperglycaemia is preceded by an increased metabolic demand for insulin due to IR. This leads to a period of normal blood glucose levels, during which the insulin hypersecretion by pancreatic β-cells compensates for IR

(Kasuga, 2006). In addition, the decrease in muscular glycogen content (Fig. 2P) and the reduced transcription of *slc2a4* (Fig. 2Q) (coding glut4 protein; the main transporter of glucose under insulin stimulation) in ED-mixture-exposed animals confirmed the metabolic syndrome and the development of peripheral IR (Petersen et al., 2007).

3.3. Effect of ED-mixture on F0 female liver transcriptome

The liver transcriptome of ED-mixture-exposed *Xenopus* showed 120 genes differentially transcribed. This corresponds to 1.3% of the total number of genes detected with about 78% of genes up-regulated (Table S2). KEGG pathway enrichment indicated a link between ED-mixture exposure and “transcriptional misregulation in cancer” (5-fold) and “ABC transporters” (8-fold) (Table 1). GOterm enrichment analysis of the differentially transcribed genes indicates a link between ED mixture exposure and “nucleotide binding” (4-fold), “steroid hydroxylase activity” (18-fold), “ATPase activity coupled to transmembrane movement of substances” (10-fold), “chloride channel activity” (8-fold), “carbohydrate binding” (4-fold), “regulation of hormone secretion” (64-fold), “negative regulation of T-helper 2 cell differentiation” (80-fold), “transport” (3.2-fold), “amino acid transport” (13-fold), “cell proliferation” (3-fold), “negative regulation of apoptotic process” (3-fold), “liver regeneration” (16-fold), and “multicellular organism development” (2-fold) (Table 1). Several of the enriched KEGG pathways and GOterms have to be taken as a whole and suggest that a metabolic syndrome associated with liver steatosis and histopathological consequences occurred in the ED-mixture-exposed animals. Moreover, more than 32% of the differentially transcribed genes not necessarily highlighted by the enrichments are associated with NAFLD/NASH and their consequences including inflammation, cell death/proliferation and cancer (Fig. 3) and confirmed the global IR phenotype. This IR phenotype is characterized by a global dysregulation of genes involved in hepatic carbohydrate and lipid metabolism (Bechmann et al., 2012). One of the first symptoms of IR in the liver is the activation of neoglucogenic genes due to the absence of insulin signaling (Bechmann et al.,

2012), and ED-mixture-exposed *Xenopus* showed a marked over-transcription of the three genes encoding glucose-6-phosphatase (*G6PC*, *g6pc and g6pc2*) (3.59-fold, 2.94-fold and 3.04-fold respectively) (Fig. 3). IR syndrome has also been associated with a dysregulation of genes regulating lipid metabolism, leading to the accumulation of triglycerides (main form), free fatty acids or cholesterol in the liver (Naik et al., 2013). Several genes involved in *de novo* fatty acid synthesis appeared over-transcribed in ED-mixture-exposed animals including ATP-citrate lyase (*acly*, 2.86-fold) and lipase hormone-sensitive (*lipe*, 3.59-fold) (Fig. 3). It has been demonstrated that *acly* is a key lipogenic enzyme for fatty acid biosynthesis (Wang et al., 2009) and that the increased activity of hepatic lipases positively correlates with steatosis in humans (Miksztowicz et al., 2012). Moreover, the scaffold attachment factor B (*safb*) and the SREBF chaperone (*scap*) genes associated with type 2 diabetes and lipid accumulation appear to be stimulated (2.61-fold and 3.60-fold respectively) (Fig. 3) (Felder et al., 2007; Yuan et al., 2011; Mastrocola et al., 2015) . This dysregulation of lipid metabolism can be explained by BaP's capacity to activate the arylhydrocarbon receptor (AhR) (Neuschäfer-Rube et al., 2015; Moyer et al., 2016). In addition to the over-transcription of genes involved in lipid synthesis, fatty acid binding protein 1 (*fapb1*) (3.76-fold) and low-density lipoprotein receptor (*ldlr,* 2.41-fold) genes coding proteins involved in fatty acid and cholesterol uptake appeared over-transcribed (Fig. 3). This phenomenon is a key aggravating factor in NAFLD (Zhang et al., 2016; Mukai et al., 2017) and has been already described in the development of type 2 diabetes (Mansego et al., 2012; Ramakrishnan et al., 2012).

Several studies have shown that steatosis is associated with reactive oxygen species (ROS) production by mitochondria which causes cell damage (Vial et al., 2011; Lockman et al., 2012). Nevertheless, mitochondria control ROS levels by inducing their scavenging system as the uncoupling protein 2 (*ucp2*) which plays a protective role by ameliorating oxidative stress (Rousset et al., 2004). Here, we found an under-transcription of *ucp2* (0.19-fold) in ED-mixture-exposed animals suggesting a lack of protective effect (Fig. 3). Moreover, this phenomenon was

accompanied by the under-transcription of glutathione peroxidase 4 (g*px4)*, which has also associated with ROS protection in fatty livers (0.40-fold) (Ucar et al., 2013). A breakdown in antioxidant defenses has been shown to play a significant role in oxidative stress associated with NASH (Ucar et al., 2013) and confirmed the observed inactivation of aconitase in ED-mixture-exposed animals (Fig. 3).

In type 2 diabetes, lipotoxicity and oxidative stress are key pathogenic processes in NAFLD which, by inducing inflammatory processes, induce progression to NASH (Takahashi and Fukusato, 2014). Leukocyte infiltrates and the dysregulation of key genes involved in inflammation such as *bcl6* (2.64-fold), *nfkbiz*, (2.12-fold), *cd40* (4.02-fold) and *fcn3* (12.24-fold) were both observed in ED-mixture-exposed *Xenopus* (Fig. 3). In NASH, the inflammatory processes correlate with hepatocellular proliferation (VanSaun et al., 2013). Hepatic transcriptomic data indicated the over-transcription of genes involved in the inhibition of apoptosis (*araf, birc7, fabp1, fmn2*), the under-transcription of genes activating apoptosis (*anxa1, anxa5, ucp2, cyr61, myc*), along with the over-transcription of genes involved in cell proliferation and cancer (*cuzd1, eps8, glul, srrt, tacc2, cd40, etv6, bcl6*) in ED-mixture-exposed animals (Fig. 3). Taken together, these results suggest a global dysregulation of genes involved in the balance between apoptosis and proliferation in ED-mixture-exposed *Xenopus*, with a trend towards the upregulation of proliferation processes which confirms the progression from NAFLD to NASH.

4. Conclusion

In this study, we focused on finding links between exposure to a mixture of ED and amphibian population decline. We found that exposure to an environmentally realistic mixture of ED, can lead to severe metabolic impairments in amphibians. Understanding the impact of the mixture is very complex since exposure to each individual compound can induce very different effects. According to Relyea et al. (2009), in some taxa such as amphibians, the impact of

pollutant mixtures cannot be predicted from the effects observed from exposure to an individual compound. Here, we found that exposure to the ED-mixture induced a similar phenotype to exposure to the same ED individually (Regnault et al., 2018). Our results also provide insight into the transgenerational effects of ED-mixtures, which might negatively affect the fecundity of the progeny only exposed at the gamete stage. Therefore, the results of this study highlighting metabolic disorders induced by exposure to the ED-mixture might confim the role of ED as a cause contributing to amphibian population decline.

Acknowledgements

This work was funded by the ComUE Université Grenoble-Alpes IRS IDEX Grant, by the CNRS-INSU EC2CO program and by the Federative Structure Environmental and Systems Biology (BEeSy) of the Grenoble-Alpes University. Marie Usal was funded by the Région Auvergne-Rhône-Alpes. Christophe Regnault received funding from the French Ministry of Higher Education and Research. We thank Kim Barrett (Version Originale) for the English review of the manuscript.

Data access

RNA-seq sequence data have been deposited at EMBL-EBI (http://www.ebi.ac.uk/ena) under the accession number PRJEB18463.

References

Alonso-Magdalena, P., Vieira, E., Soriano, S., Menes, L., Burks, D., Quesada, I., Nadal, A., 2010. Bisphenol A exposure during pregnancy disrupts glucose homeostasis in mothers and adult male offspring. Environ. Health Perspect. 118, 1243–1250.

Barber, L.B., Loyo-Rosales, J.E., Rice, C.P., Minarik, T.A., Oskouie, A.K., 2015. Endocrine disrupting alkylphenolic chemicals and other contaminants in wastewater treatment plant effluents, urban streams, and fish in the Great Lakes and Upper Mississippi River Regions. Sci. Total Environ. 517, 195–206.

Bechmann, L.P., Hannivoort, R.A., Gerken, G., Hotamisligil, G.S., Trauner, M., Canbay, A., 2012. The interaction of hepatic lipid and glucose metabolism in liver diseases. J. Hepatol. 56, 952–964.

Bedossa, P., 2016. Histological Assessment of NAFLD. Dig. Dis. Sci. 61, 1348–1355.

Blair, B.D., Crago, J.P., Hedman, C.J., Klaper, R.D., 2013. Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. Chemosphere 93, 2116–2123.

Blaustein, A.R., Bancroft, B.A., 2007. Amphibian Population Declines: Evolutionary Considerations. BioScience 57, 437–444.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinforma. Oxf. Engl. 30, 2114–2120.

Brooks, S., Tyler, C.R., Sumpter, J.P., 1997. Egg quality in fish: what makes a good egg? Rev. Fish Biol. Fish. 7, 387–416.

Browne, R.K., 2007. Reproduction and larval rearing of amphibians. ILAR Journal/National Research Council, Institute of Laboratory Animal Resources. pp. 214–234.

Bulteau, A.-L., Ikeda-Saito, M., Szweda, L.I., 2003. Redox-Dependent Modulation of Aconitase Activity in Intact Mitochondria. Biochemistry 42, 14846–14855.

Bulteau, A.-L., Szweda, L.I., Friguet, B., 2002. Age-dependent declines in proteasome activity in the heart. Arch. Biochem. Biophys. 397, 298–304.

Casals-Casas, C., Desvergne, B., 2011. Endocrine disruptors: from endocrine to metabolic disruption. Annu. Rev. Physiol. 73, 135–162.

Chevalier, N., Fénichel, P., 2016. Obésité, diabète de type 2 et perturbateurs endocriniens. Presse Médicale, Médecine et environnement 45, 88–97.

Chevalier, N., Fénichel, P., 2015a. Endocrine disruptors: new players in the pathophysiology of type 2 diabetes? Diabetes Metab. 41, 107–115.

Chevalier, N., Fénichel, P., 2015b. Bisphenol A: Targeting metabolic tissues. Rev. Endocr. Metab. Disord. 16, 299–309.

Crump, D., Werry, K., Veldhoen, N., Van Aggelen, G., Helbing, C.C., 2002. Exposure to the herbicide acetochlor alters thyroid hormone-dependent gene expression and metamorphosis in *Xenopus Laevis*. Environ. Health Perspect. 110, 1199–1205.

Day, C.P., 2006. From fat to inflammation. Gastroenterology 130, 207–210.

Day, C.P., James, O.F.W., 1998. Steatohepatitis: A tale of two “hits”? Gastroenterology 114, 842–845.

Duarte-Guterman, P., Navarro-Martín, L., Trudeau, V.L., 2014. Mechanisms of crosstalk between endocrine systems: Regulation of sex steroid hormone synthesis and action by thyroid hormones. Gen. Comp. Endocrinol., 2nd Meeting of The North American Society for Comparative Endocrinology 203, 69–85.

Dziminski, M.A., Roberts, J.D., 2006. Fitness consequences of variable maternal provisioning in quacking frogs (Crinia georgiana). J. Evol. Biol. 19, 144–155.

Fedorenkova, A., Vonk, J.A., Lenders, H.J.R., Creemers, R.C.M., Breure, A.M., Hendriks, A.J., 2012. Ranking ecological risks of multiple chemical stressors on amphibians. Environ. Toxicol. Chem. 31, 1416–1421.

Felder, T.K., Oberkofler, H., Weitgasser, R., Mackevics, V., Krempler, F., Paulweber, B., Patsch, W., 2007. The SREBF-1 locus is associated with type 2 diabetes and plasma adiponectin levels in a middle-aged Austrian population. Int. J. Obes. 31, 1099–1103.

Ferré, P., Foufelle, F., 2010. Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c. Diabetes Obes. Metab. 12, 83–92.

Galloway, J.M., Haggard, B.E., Meyers, M.T., Green, W.R., 2005. Occurrence of pharmaceuticals and other organic wastewater constituents in selected streams in northern Arkansas, 2004. (USGS Numbered Series No. 2005–5140), Scientific Investigations Report. www.pubs.usgs.gov/sir/2005/5140/SIR2005-5140.pdf.

Garait, B., Couturier, K., Servais, S., Letexier, D., Perrin, D., Batandier, C., Rouanet, J.-L., Sibille, B., Rey, B., Leverve, X., Favier, R., 2005. Fat intake reverses the beneficial effects of low caloric intake on skeletal muscle mitochondrial H(2)O(2) production. Free Radic. Biol. Med. 39, 1249–1261.

Gardner, M., Comber, S., Scrimshaw, M.D., Cartmell, E., Lester, J., Ellor, B., 2012. The significance of hazardous chemicals in wastewater treatment works effluents. Sci. Total Environ. 437, 363–372.

Godoy, A.A., Kummrow, F., 2017. What do we know about the ecotoxicology of pharmaceutical and personal care product mixtures? A critical review. Crit. Rev. Environ. Sci. Technol. 47, 1453–1496.

Grün , F., Watanabe, H., Zamanian, Z., Maeda, L., Arima, K., Cubacha, R., Gardiner, D.M., Kanno, J., Iguchi, T., Blumberg, B., 2006. Endocrine-disrupting organotin compounds are potent inducers of adipogenesis in vertebrates. Mol. Endocrinol. 20, 2141-2155.

Guan, Y., Gao, J., Zhang, Y., Chen, S., Yuan, C., Wang, Z., 2016. Effects of bisphenol A on lipid metabolism in rare minnow *Gobiocypris rarus*. Comp. Biochem. Physiol. C 179: 144-149.

Han, X., Liu, C., Xue, Y., Wang, J., Xue, C., Yanagita, T., Gao, X., Wang, Y., 2016. Long-term fatty liver-induced insulin resistance in orotic acid-induced nonalcoholic fatty liver rats. Biosci. Biotechnol. Biochem. 80, 735–743.

Hayes, T., Haston, K., Tsui, M., Hoang, A., Haeffele, C., Vonk, A., 2002. Herbicides: Feminization of male frogs in the wild. Nature 419, 895–896.

Hayes, T.B., Anderson, L.L., Beasley, V.R., de Solla, S.R., Iguchi, T., Ingraham, H., Kestemont, P., Kniewald, J., Kniewald, Z., Langlois, V.S., Luque, E.H., McCoy, K.A., Muñoz-de-Toro, M., Oka, T., Oliveira, C.A., Orton, F., Ruby, S., Suzawa, M., Tavera-Mendoza, L.E., Trudeau, V.L., Victor-Costa, A.B., Willingham, E., 2011. Demasculinization and feminization of male gonads by atrazine: Consistent effects across vertebrate classes. J. Steroid Biochem. Mol. Biol. 127, 64–73.

Hayes, T.B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., Lee, M., Mai, V.P., Marjuoa, Y., Parker, J., Tsui, M., 2006. Pesticide Mixtures, Endocrine Disruption, and Amphibian Declines: Are We Underestimating the Impact? Environ. Health Perspect. 114, 40–50.

Hayes, T.B., Falso, P., Gallipeau, S., Stice, M., 2010. The cause of global amphibian declines: a developmental endocrinologist's perspective. J. Exp. Biol. 213, 921–933.

Hua, M.-C., Su, H.-M., Yao, T.-C., Kuo, M.-L., Lai, M.-W., Tsai, M.-H., Huang, J.-L., 2017. Alternation of plasma fatty acids composition and desaturase activities in children with liver steatosis. PloS One 12, e0182277.

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 37, 1–13.

Jiang, Y., Xia, W., Zhu, Y., Li, X., Wang, D., Liu, J., Chang, H., Li, G., Xu, B., Chen, X., Li, Y., Xu, S., 2014. Mitochondrial dysfunction in early life resulted from perinatal bisphenol A exposure contributes to hepatic steatosis in rat offspring. Toxicol. Lett. 228, 85–92.

Kaplan, M., 2009. Amphibians rarely give earliest warning of pollution. Nat. News. http://www.nature.com/news/2009/091029/full/news.2009.1048.html (accessed 11.6.18).

Kashiwagi, A., Utsumi, K., Kashiwagi, K., Ohta, S., Sugihara, K., Hanada, H., Kitamura, S., 2008. Effects of Endocrine Disrupting Chemicals on Amphibian Metamorphosis and Mitochondrial Membrane Permeability Transition. J. Health Sci. 54, 273–280.

Kasuga, M., 2006. Insulin resistance and pancreatic β cell failure. J. Clin. Invest. 116, 1756–1760.

Kloas, W., Urbatzka, R., Opitz, R., Würtz, S., Behrends, T., Hermelink, B., Hofmann, F., Jagnytsch, O., Kroupova, H., Lorenz, C., Neumann, N., Pietsch, C., Trubiroha, A., Van Ballegooy, C., Wiedemann, C., Lutz, I., 2009. Endocrine Disruption in Aquatic Vertebrates. Ann. N. Y. Acad. Sci. 1163, 187–200.

Koliaki, C., Szendroedi, J., Kaul, K., Jelenik, T., Nowotny, P., Jankowiak, F., Herder, C., Carstensen, M., Krausch, M., Knoefel, W.T., Schlensak, M., Roden, M., 2015. Adaptation of Hepatic Mitochondrial Function in Humans with Non-Alcoholic Fatty Liver Is Lost in Steatohepatitis. Cell Metab. 21, 739–746.

Kuramoto, M., 1978. Correlations of quantitative parameters of fecundity in amphibians. Evolution 32, 287–296.

Langlois, V.S., Duarte-Guterman, P., Ing, S., Pauli, B.D., Cooke, G.M., Trudeau, V.L., 2010. Fadrozole and finasteride exposures modulate sex steroid- and thyroid hormone-related gene expression in *Silurana (Xenopus) tropicalis* early larval development. Gen. Comp. Endocrinol. 166, 417–427.

Levy, C.W., Roujeinikova, A., Sedelnikova, S., Baker, P.J., Stuitje, A.R., Slabas, A.R., Rice, D.W., Rafferty, J.B., 1999. Molecular basis of triclosan activity. Nature 398, 383–384.

Lindström, A., Buerge, I.J., Poiger, T., Bergqvist, P.-A., Müller, M.D., Buser, H.-R., 2002. Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater. Environ. Sci. Technol. 36, 2322–2329.

Liu, X.L., Cao, H.X., Fan, J.G., 2016. MicroRNAs as biomarkers and regulators of nonalcoholic fatty liver disease. J. Dig. Dis. 17, 708–715.

Lockman, K.A., Baren, J.P., Pemberton, C.J., Baghdadi, H., Burgess, K.E., Plevris-Papaioannou, N., Lee, P., Howie, F., Beckett, G., Pryde, A., Jaap, A.J., Hayes, P.C., Filippi, C., Plevris, J.N., 2012. Oxidative stress rather than triglyceride accumulation is a determinant of mitochondrial dysfunction in in vitro models of hepatic cellular steatosis. Liver Int. Off. J. Int. Assoc. Study Liver 32, 1079–1092.

Lorenz, C., Contardo-Jara, V., Pflugmacher, S., Wiegand, C., Nützmann, G., Lutz, I., Kloas, W., 2011. The Synthetic Gestagen Levonorgestrel Impairs Metamorphosis in *Xenopus laevis* by Disruption of the Thyroid System. Toxicol. Sci. 123, 94–102.

Mansego, M.L., Martínez, F., Martínez-Larrad, M.T., Zabena, C., Rojo, G., Morcillo, S., Soriguer, F., Martín-Escudero, J.C., Serrano-Ríos, M., Redon, J., Chaves, F.J., 2012. Common Variants of the Liver Fatty Acid Binding Protein Gene Influence the Risk of Type 2 Diabetes and Insulin Resistance in Spanish Population. PLOS ONE 7, e31853.

Mastrocola, R., Collino, M., Nigro, D., Chiazza, F., D'Antona, G., Aragno, M., Minetto, M.A., 2015. Accumulation of Advanced Glycation End-Products and Activation of the SCAP/SREBP Lipogenetic Pathway Occur in Diet-Induced Obese Mouse Skeletal Muscle. PLOS ONE 10, e0119587.

Medic-Stojanoska, M., Milosevic, N., Milanovic, N., Stojanoski, S., Vukovic1, B., Icin, T., Bajkin, I., Stepanovic, K., Milic, N. 2019. Can phthalates impair liver function? Endocrine Abstracts (2019) 63 GP232

McArthur, J.V., Tuckfield, R.C., Baker-Austin, C., 2012. Antimicrobial Textiles, in: Antibiotic Resistance, Handbook of Experimental Pharmacology. Springer, Berlin, Heidelberg, pp. 135–152.

McAvoy, D.C., Schatowitz, B., Jacob, M., Hauk, A., Eckhoff, W.S., 2009. Measurement of triclosan in wastewater treatment systems. Environ. Toxicol. Chem. 21, 1323–1329.

Miksztowicz, V., Lucero, D., Zago, V., Cacciagiú, L., Lopez, G., Gonzalez Ballerga, E., Sordá, J., Fassio, E., Schreier, L., Berg, G., 2012. Hepatic lipase activity is increased in non-alcoholic fatty liver disease beyond insulin resistance. Diabetes Metab. Res. Rev. 28, 535–541.

Møretrø, T., Høiby-Pettersen, G.S., Habimana, O., Heir, E., Langsrud, S., 2011. Assessment of the antibacterial activity of a triclosan-containing cutting board. Int. J. Food Microbiol. 146, 157–162.

Mosconi, G., Carnevali, O., Franzoni, M.F., Cottone, E., Lutz, I., Kloas, W., Yamamoto, K., Kikuyama, S., Polzonetti-Magni, A.M., 2002. Environmental Estrogens and Reproductive Biology in Amphibians. Gen. Comp. Endocrinol. 126, 125–129.

Moyer, B.J., Rojas, I.Y., Kerley-Hamilton, J.S., Hazlett, H.F., Nemani, K.V., Trask, H.W., West, R.J., Lupien, L.E., Collins, A.J., Ringelberg, C.S., Gimi, B., Kinlaw, W.B., Tomlinson, C.R., 2016. Inhibition of the aryl hydrocarbon receptor prevents Western diet-induced obesity. Model for AHR activation by kynurenine via oxidized-LDL, TLR2/4, TGFβ, and IDO1. Toxicol. Appl. Pharmacol. 300, 13–24.

Mukai, T., Egawa, M., Takeuchi, T., Yamashita, H., Kusudo, T., 2017. Silencing of FABP1 ameliorates hepatic steatosis, inflammation, and oxidative stress in mice with nonalcoholic fatty liver disease. FEBS Open Bio 7, 1009–1016.

Naik, A., Košir, R., Rozman, D., 2013. Genomic aspects of NAFLD pathogenesis. Genomics, SI:Clinical and Translational Genomics 102, 84–95.

Neuschäfer-Rube, F., Schraplau, A., Schewe, B., Lieske, S., Krützfeldt, J.-M., Ringel, S., Henkel, J., Birkenfeld, A.L., Püschel, G.P., 2015. Arylhydrocarbon receptor-dependent mIndy (Slc13a5) induction as possible contributor to benzo[a]pyrene-induced lipid accumulation in hepatocytes. Toxicology 337, 1–9.

Olivares-Rubio, H.F., Dzul-Caamal, R., Gallegos-Rangel, M.E., Madera-Sandoval, R.L., Domínguez-López, M.L., García-Latorre, E., Vega-López, A., 2015. Relationship between biomarkers and endocrine-disrupting compounds in wild Girardnichthys viviparus from two lakes with different degrees of pollution. Ecotoxicol. Lond. Engl. 24, 664–685.

Otoda, T., Takamura, T., Misu, H., Ota, T., Murata, S., Hayashi, H., Takayama, H., Kikuchi, A., Kanamori, T., Shima, K.R., Lan, F., Takeda, T., Kurita, S., Ishikura, K., Kita, Y., Iwayama, K., Kato, K., Uno, M., Takeshita, Y., Yamamoto, M., Tokuyama, K., Iseki, S., Tanaka, K., Kaneko, S., 2013. Proteasome dysfunction mediates obesity-induced endoplasmic reticulum stress and insulin resistance in the liver. Diabetes 62, 811–824.

Petersen, A.M., Gleeson, T.T., 2007. Characterization of circannual patterns of metabolic recovery from activity in Rana catesbeiana at 15°C. J. Exp. Biol. 210, 1786–1797.

Petersen, K.F., Dufour, S., Savage, D.B., Bilz, S., Solomon, G., Yonemitsu, S., Cline, G.W., Befroy, D., Zemany, L., Kahn, B.B., Papademetris, X., Rothman, D.L., Shulman, G.I., 2007. The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. Proc. Natl. Acad. Sci. U. S. A. 104, 12587–12594.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29, e45.

Piccoli, A., Fiori, J., Andrisano, V., Orioli, M., 2002. Determination of triclosan in personal health care products by liquid chromatography (HPLC). Il Farm. 57, 369–372.

Ramakrishnan, G., Arjuman, A., Suneja, S., Das, C., Chandra, N.C., 2012. The association between insulin and low-density lipoprotein receptors. Diab. Vasc. Dis. Res. 9, 196–204.

Regnault, C., Usal, M., Veyrenc, S., Couturier, K., Batandier, C., Bulteau, A.-L., Lejon, D., Sapin, A., Combourieu, B., Chetiveaux, M., Le May, C., Lafond, T., Raveton, M., Reynaud, S., 2018. Unexpected metabolic disorders induced by endocrine disruptors in *Xenopus*

tropicalis provide new lead for understanding amphibian decline. Proc. Natl. Acad. Sci. U. S. A. 115, E4416–E4425.

Regnault, C., Willison, J., Veyrenc, S., Airieau, A., Méresse, P., Fortier, M., Fournier, M., Brousseau, P., Raveton, M., Reynaud, S., 2016. Metabolic and immune impairments induced by the endocrine disruptors benzo[a]pyrene and triclosan in *Xenopus tropicalis.* Chemosphere 155, 519–527.

Regnault, C., Worms, I.A., Oger-Desfeux, C., MelodeLima, C., Veyrenc, S., Bayle, M.-L., Combourieu, B., Bonin, A., Renaud, J., Raveton, M., Reynaud, S., 2014. Impaired liver function in *Xenopus tropicalis* exposed to benzo[a]pyrene: transcriptomic and metabolic evidence. BMC Genomics 15.

Relyea, R.A., 2009. A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. Oecologia 159, 363–376.

Roelants, K., Gower, D.J., Wilkinson, M., Loader, S.P., Biju, S.D., Guillaume, K., Moriau, L., Bossuyt, F., 2007. Global patterns of diversification in the history of modern amphibians. Proc. Natl. Acad. Sci. 104, 887–892.

Rousset, S., Alves-Guerra, M.-C., Mozo, J., Miroux, B., Cassard-Doulcier, A.-M., Bouillaud, F., Ricquier, D., 2004. The biology of mitochondrial uncoupling proteins. Diabetes 53 Suppl 1, S130-135.

Rumrill, C.T., Scott, D.E., Lance, S.L., 2018. Delayed effects and complex life cycles: How the larval aquatic environment influences terrestrial performance and survival. Environ. Toxicol. Chem. 37, 2660–2669.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., Quackenbush, J., 2003. TM4: a free, open-source system for microarray data management and analysis. BioTechniques 34, 374–378.

Santangeli, S., Notarstefano, V., Maradonna, F., Giorgini, E., Gioacchini, G., Forner-Piquer, I., Habibi, H.R., Carnevali, O., 2018. Effects od diethylene glycol dibenzoate and Bisphenol A on the lipid metabolism of Danio rerio. Sci. Total Environ. 636, 641-655.

Shi, H., Zhu, P., Guo, S., 2014. Effects of tributyltin on metamorphosis and gonadal differentiation of *Xenopus laevis* at environmentally relevant concentrations. Toxicol. Ind. Health 30, 297–303.

Showell, C., Conlon, F.L., 2009. Natural Mating and Tadpole Husbandry in the Western Clawed Frog *Xenopus tropicalis.* Cold Spring Harb. Protoc. 2009, pdb.prot5292.

Smith, D.C., 1987. Adult Recruitment in Chorus Frogs: Effects of Size and Date at Metamorphosis. Ecology 68, 344–350.

Srogi, K., 2007. Monitoring of environmental exposure to polycyclic aromatic hydrocarbons: a review. Environ. Chem. Lett. 5, 169–195.

Susiarjo, M., Xin, F., Bansal, A., Stefaniak, M., Li, C., Simmons, R.A., Bartolomei, M.S., 2015. Bisphenol a exposure disrupts metabolic health across multiple generations in the mouse. Endocrinology 156, 2049–2058.

Srere, P.A., 1969 Citrate synthase. Methods Enzymol 13: 3-5

Takahashi, Y., Fukusato, T., 2014. Histopathology of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World J. Gastroenterol. 20, 15539–15548.

Teubner, D., Paulus, M., Veith, M., Klein, R., 2015. Biometric parameters of the bream (Abramis brama) as indicators for long-term changes in fish health and environmental quality--data from the German ESB. Environ. Sci. Pollut. Res. Int. 22, 1620–1627.

Todd B.D., Bergeron C.M., Hepner M.J., Hopkins, W.A., 2011. Aquatic and terrestrial stressors in amphibians: a test of the double jeopardy hypothesis based on maternally and trophically derived contaminants. Environ. Toxicol. Chem. 30, 2277-2284.

Trapido, M., Veldre, I., 1996. On polynuclear aromatic hydrocarbons contamination levels in the ecosystem of Lake Peipsi in the 1970s-1980s. Hydrobiologia 338, 185–190.

Trapnell, C., Pachter, L., Salzberg, S.L., 2009. TopHat: discovering splice junctions with RNA-Seq. Bioinforma. Oxf. Engl. 25, 1105–1111.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., Pachter, L., 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7, 562–578.

Ucar, F., Sezer, S., Erdogan, S., Akyol, S., Armutcu, F., Akyol, O., 2013. The relationship between oxidative stress and nonalcoholic fatty liver disease: Its effects on the development of nonalcoholic steatohepatitis. Redox Rep. 18, 127–133.

United States Government, 2010. Endocrine Disruptor Screening Program: Second List of Chemicals for Tier 1 Screening. https://www.federalregister.gov/documents/2010/11/17/2010-28818/endocrine-disruptor-screening-program-second-list-of-chemicals-for-tier-1-screening (accessed 11.7.18).

Urbatzka, R., Bottero, S., Mandich, A., Lutz, I., Kloas, W., 2007. Endocrine disrupters with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of *Xenopus laevis*: I. Effects on sex steroid levels and biomarker expression. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 144, 310–318.

Urbatzka, R., Lutz, I., Opitz, R., Kloas, W., 2006. Luteinizing hormone, follicle stimulating hormone, and gonadotropin releasing hormone mRNA expression of *Xenopus laevis* in response to endocrine disrupting compounds affecting reproductive biology. Gen. Comp. Endocrinol. 146, 119–125.

VanSaun, M.N., Mendonsa, A.M., Gorden, D.L., 2013. Hepatocellular Proliferation Correlates with Inflammatory Cell and Cytokine Changes in a Murine Model of Nonalchoholic Fatty Liver Disease. PLOS ONE 8, e73054.

Veldhoen, N., Skirrow, R.C., Osachoff, H., Wigmore, H., Clapson, D.J., Gunderson, M.P., Van Aggelen, G., Helbing, C.C., 2006. The bactericidal agent triclosan modulates thyroid

hormone-associated gene expression and disrupts postembryonic anuran development. Aquat. Toxicol. 80, 217–227.

Vial, G., Dubouchaud, H., Couturier, K., Cottet-Rousselle, C., Taleux, N., Athias, A., Galinier, A., Casteilla, L., Leverve, X.M., 2011. Effects of a high-fat diet on energy metabolism and ROS production in rat liver. J. Hepatol. 54, 348–356.

Wang, Q., Jiang, L., Wang, J., Li, S., Yu, Y., You, J., Zeng, R., Gao, X., Rui, L., Li, W., Liu, Y., 2009. Abrogation of hepatic ATP-citrate lyase protects against fatty liver and ameliorates hyperglycemia in leptin receptor-deficient mice. Hepatol. Baltim. Md 49, 1166–1175.

Weir, A., Moiles, W.E., Brockman, B., Mattick, C.S., McClellan, K., Gerwe, L., Deo, R.P., Halden, R.U., 2010. Concentrations of Hydrophobic Organic Pollutants in U.S. Wastewater Treatment Plants and in Receiving Surface Waters Modeled from EPA Biosolids Monitoring Data, in: Halden, R.U. (Ed.), Contaminants of Emerging Concern in the Environment: Ecological and Human Health Considerations. American Chemical Society, Washington, DC, pp. 421–436.

Whitacre, D.M. (Ed.), 2010. Reviews of Environmental Contamination and Toxicology Volume 206, Springer-Verlag, New York.

Yamaguchi, K., Yang, L., McCall, S., Huang, J., Yu, X.X., Pandey, S.K., Bhanot, S., Monia, B.P., Li, Y.-X., Diehl, A.M., 2007. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. Hepatol. Baltim. Md 45, 1366–1374.

Yuan, Y., Zhao, L., Chen, Y., Moorhead, J.F., Varghese, Z., Powis, S.H., Minogue, S., Sun, Z., Ruan, X.Z., 2011. Advanced glycation end products (AGEs) increase human mesangial foam cell formation by increasing Golgi SCAP glycosylation in vitro. Am. J. Physiol.-Ren. Physiol. 301, F236–F243.

Zhang, W., Guo, R., Yang, Y., Ding, J., Zhang, Y., 2016. Long-term effect of heavy-metal pollution on diversity of gastrointestinal microbial community of Bufo raddei. Toxicol. Lett. 258, 192–197.

FIGURE CAPTIONS

Fig.1. Impact of the ED-mixture on the development of exposed *Xenopus* (F0) and their progeny (F1). (A) Proportion of surviving F0 individuals from hatching to metamorphosis. (n = 82 and 62 for the control and ED-mixture exposure groups, respectively). (B) Kinetic of F0 juvenile females' development. (n = 34 per exposure group). (C) Snout-vent length of F0 metamorphic individuals (n = 34). (D) Body mass of F0 metamorphic animals (n = 34 per exposure group). (E) Kinetic of F0 juvenile females' development from hatching to sexual maturity. (n = 34 per exposure group). (F) Proportion of successful amplexus at 3 hours poststimulation of F0 animals (n = 5 for each exposure). (G) Relative number of hatched eggs per spawn. (n = 4 and 3 for control and ED-mixture, respectively). Control values were used for normalizing data. (H) Proportion of surviving F1 individuals from hatching to metamorphosis. Asterisks indicate statistical difference from the control evaluated through Kaplan-Meyer log-rank test: *P < 0.05 (n = 300 for progeny from control and ED-mixture parental exposure groups). (I) Kinetic of of F1 individuals' development from hatching to metamorphosis. Asterisks indicate statistical difference from the control evaluated through Kaplan-Meyer log-rank test: *P < 0.05 (n = 46 and 47 for progeny from the control and ED-mixture parental exposure populations, respectively). (J) Snout-vent length of F1 metamorphic individuals (n = 46 and 32 for progeny from the control and ED-mixture parental exposure groups, respectively). (K) Body mass of F1 metamorphic individuals. Asterisks indicate statistical difference from the control evaluated through a Wilcoxon test: **P < 0.01 (n = 46 and 32 for progeny from the control and ED-mixture parental exposure groups, respectively). (L) Kinetic of F1 juvenile females' development from hatching to sexual maturity. Asterisks indicate statistical difference from the control evaluated through a Kaplan-Meyer log-rank test: *P < 0.05 (n = 10 and 16 for progeny from the control and ED-mixture parental exposure groups, respectively). (M) Proportion of successful amplexus at 3 hours post-stimulation of F1 animals (n = 5 for each parental exposure). (N) Relative number of hatched eggs per spawn (n = 4 and 3

for progeny from the control and ED-mixture parental exposure groups, respectively). Control values were used for normalizing data. MIX: ED-mixture.

Fig. 2. Impact of the ED-mixture on F0 female metabolism. (A) Hepatosomatic index of control and ED-mixture-exposed animals. (B) Hepatic lipid content of control and ED-mixture-exposed animals. The images reveal the lipid droplets (red staining, Oil Red O; Scale bars, 25 µm.). The histogram represents the proportion of stained area in the livers. (C) Hepatic mitochondrial state 2 respiration rate of the control and ED-mixture-exposed animals. (D) Hepatic mitochondrial respiratory control indices (RCI) of the control and ED-mixture-exposed animals. (E) Hepatic aconitase activity in the control and ED-mixture-exposed animals. (F) Hepatic citrate synthase activity in the control and ED-mixture-exposed animals. (G) Serum ALAT activity in the control and ED-mixture-exposed animals. (N) Hepatic glycogen content of control and ED-mixture-exposed animals. (H) Liver sections of control animals stained with HE. (Scale bars, 70 µm.). (I) Liver sections of ED-mixture-exposed animals stained with HE. The arrow indicates an area of melanocytes. (Scale bars, 70 µm.). (J) Liver sections of control animals stained with Gomori's Trichrom. (Scale bars, 70 µm.). (K) Liver sections of ED-mixture-exposed animals stained with Gomori's Trichrom. Dotted arrows indicate leukocyte infiltrates in a necrotic area and the arrow shows a swollen blood vessel. (Scale bars, 70 µm). (L) Hepatic proteasome activity of control and ED-mixture-exposed animals. (M) Hepatic glycogen content of control and ED-mixture-exposed animals. Asterisks indicate statistical difference from the control evaluated through a Wilcoxon test: *$P < 0.05$; $n = 4$ per exposure group. (N) Glucose tolerance test. (O) Insulin immunostaining of the pancreas of the control and ED-mixture-exposed animals. The images reveal insulin content by red staining. (Scale bars, 25 µm.) The histograms show the insulin quantity estimated as area × intensity of islet stained for insulin. (P) Muscle glycogen content of the control and ED-mixture-exposed animals. (Q) Transcription of slc2a4 involved in glucose assimilation in skeletal muscle. Asterisks indicate statistical difference from the control evaluated

through a Wilcoxon test: *P < 0.05; (n = 3 for control animals and n = 4 for ED-mixture- exposed animals). Control values were used for normalizing data. MIX: ED-mixture.

Fig. 3. Circos plot representing the overlap between liver transcriptome of F0 females exposed to the ED-mixture and metabolic pathways. The genes considered are those involved in metabolic disorders, NAFLD/NASH pathologies and immune system pathways and histopathological consequences. Outside the circle dysregulated genes and pathways are indicated. The color scale indicates transcription ratios (log_2 fold change) relative to the controls. (n = 4 per exposure group).

Table 1 - Functional pathway enrichment analysis based on hepatic genes differentially transcribed between ED-mixture-exposed and control F0 animals.

The 120 genes showing a significant differential transcription in ED-mixture-exposed animals were used for annotation enrichment using the Database for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation tool (modified Fisher's exact test with $P < 0.05$). The table indicates the number of genes involved in each enriched KEGG pathway or GOTerm, and the fold enrichment along with its associated P value. MF: Molecular Function and BP: Biological Process.

	Term	Count	Fold enrichment	P-Value
KEGG	ABC transporters	3	8.9	4.3E-02
	Spliceosome	5	4.9	1.7E-02
	Transcriptional misregulation in cancer	7	5.4	1.5E-03
GOterm MF	ATPase activity, coupled to transmembrane movement of substances	3	10.8	3.1E-02
	Chloride channel activity	3	8.8	4.5E-02
	Steroid hydroxylase activity	3	18.2	1.2E-02
	Carbohydrate binding	5	4	3.5E-02
	Nucleotide binding	10	4.5	3.4E-04
GOterm BP	Negative regulation of T-helper 2 cell differentiation	2	80	2.5E-02
	Regulation of hormone secretion	2	64	3.1E-02
	Amino acid transport	3	13.7	2.0E-02
	Liver regeneration	3	16.5	1.4E-02
	Cell proliferation	7	3.1	2.6E-02
	Transport	7	3.2	2.1E-02
	Multicellular organism development	8	2.5	4.3E-02
	Negative regulation of apoptotic process	9	3.2	7.3E-03

Highlights

Frogs were exposed to a mixture of ED throughout their life cycle

Females exposed to ED displayed a marked metabolic syndrome typical of a prediabetes

This was associated with delayed metamorphosis and mortality in their progeny

F1 animals had reduced reproductive success showing reduced fitness in F0 frogs

Thus, direct and transgenerational effects of ED may contribute to amphibian declines

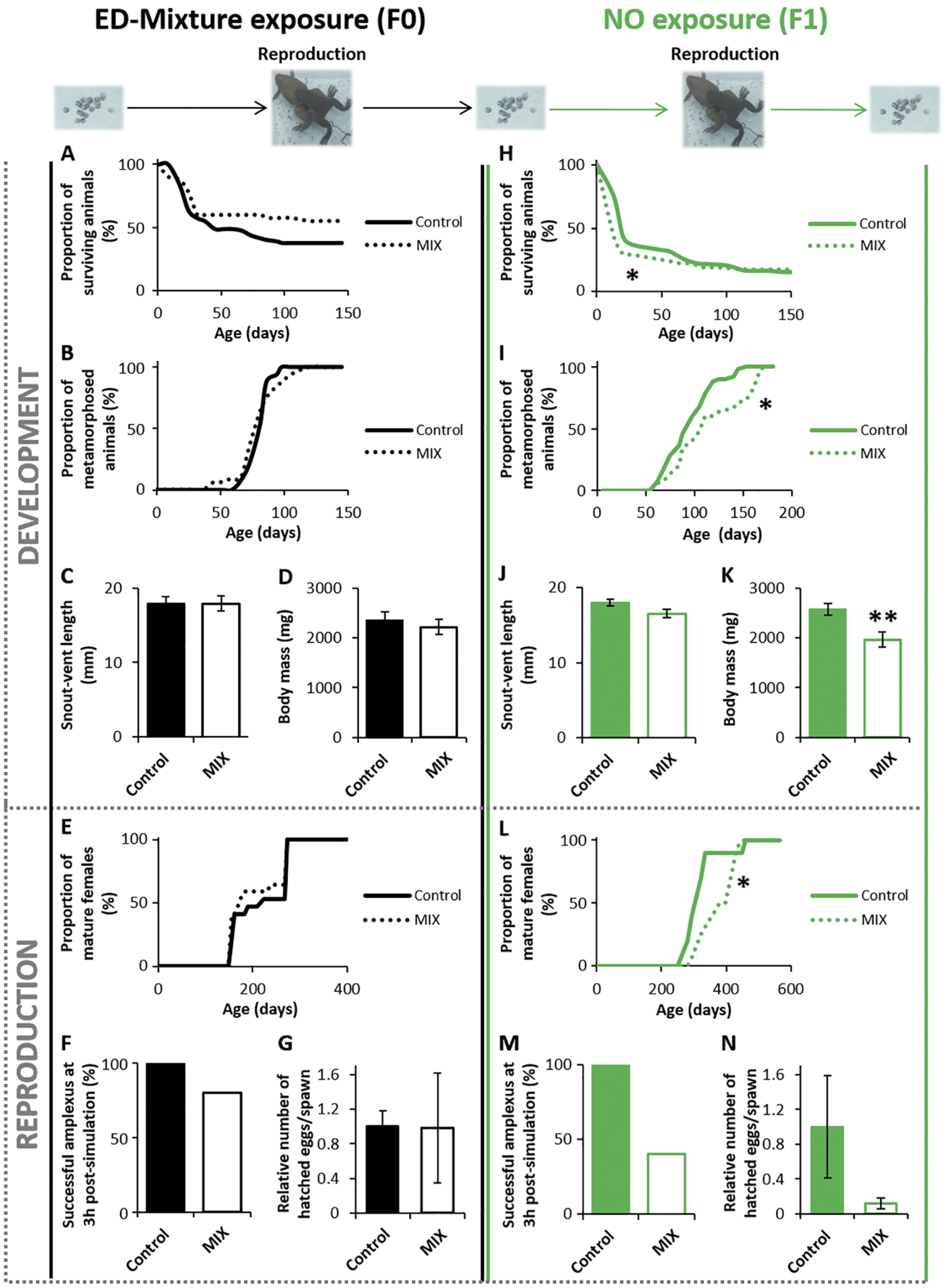
ED-Mixture exposure (F0)
NO exposure (F1)
Reproduction
Reproduction
DEVELOPMENT
REPRODUCTION
A
B
C
D
E
F
G
H
I
J
K
L
M
N
Proportion of surviving animals (%)
Proportion of metamorphosed animals (%)
Snout-vent length (mm)
Body mass (mg)
Proportion of mature females (%)
Successful amplexus at 3h post-simulation (%)
Relative number of hatched eggs/spawn
Age (days)
Control
MIX
*
**

Figure 1

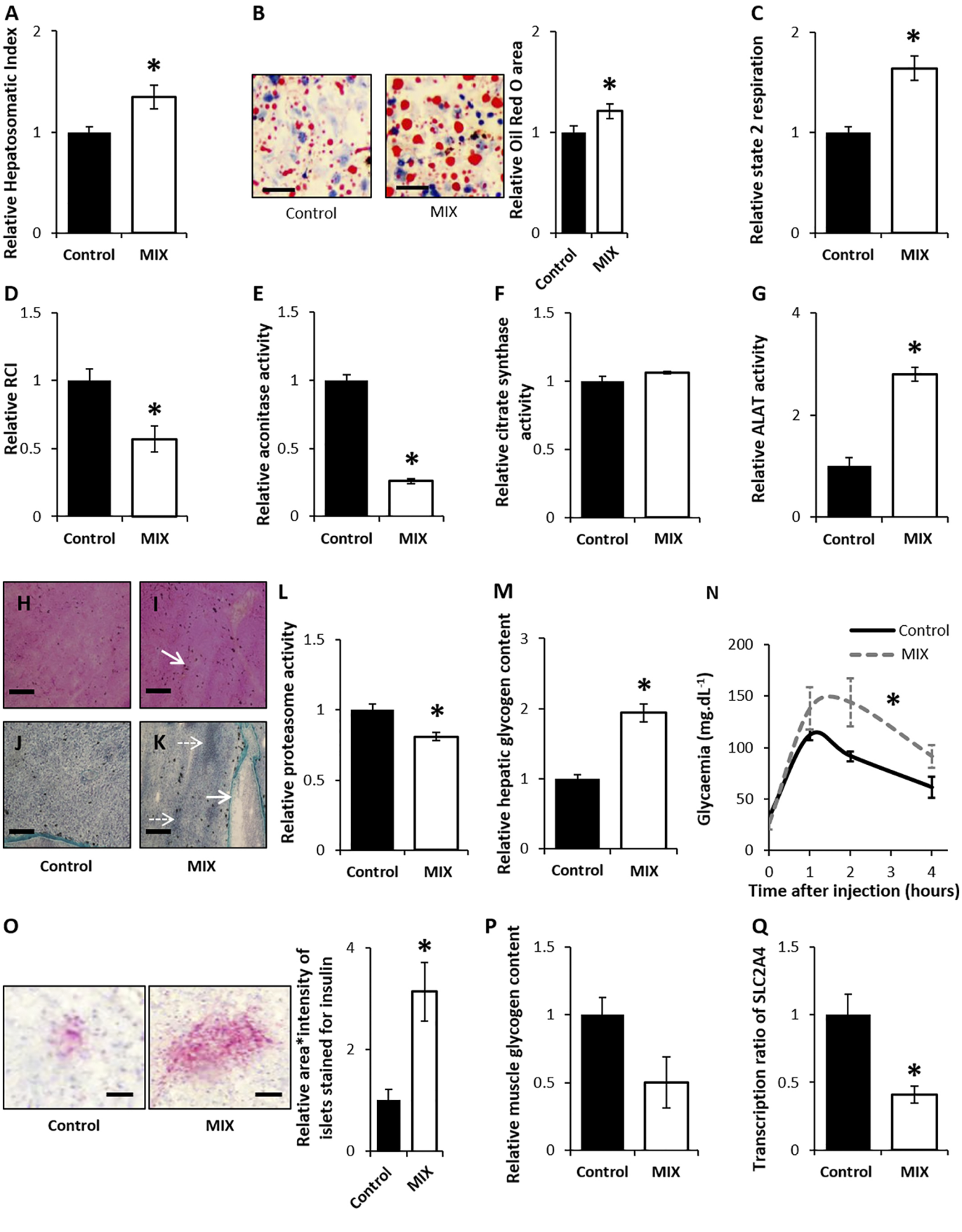
A
Relative Hepatosomatic Index
2
1
0
*
Control
MIX
B
Control
MIX
Relative Oil Red O area
2
1
0
*
Control
MIX
C
Relative state 2 respiration
2
1
0
*
Control
MIX
D
Relative RCI
1.5
1
0.5
0
*
Control
MIX
E
Relative aconitase activity
1.5
1
0.5
0
*
Control
MIX
F
Relative citrate synthase activity
1.5
1
0.5
0
Control
MIX
G
Relative ALAT activity
4
2
0
*
Control
MIX
H
I
J
K
Control
MIX
L
Relative proteasome activity
1.5
1
0.5
0
*
Control
MIX
M
Relative hepatic glycogen content
3
2
1
0
*
Control
MIX
N
Control
MIX
Glycaemia (mg.dL-1)
200
150
100
50
0
*
0
1
2
3
4
Time after injection (hours)
O
Control
MIX
Relative area*intensity of islets stained for insulin
4
2
0
*
Control
MIX
P
Relative muscle glycogen content
1.5
1
0.5
0
Control
MIX
Q
Transcription ratio of SLC2A4
1.5
1
0.5
0
*
Control
MIX

Figure 2

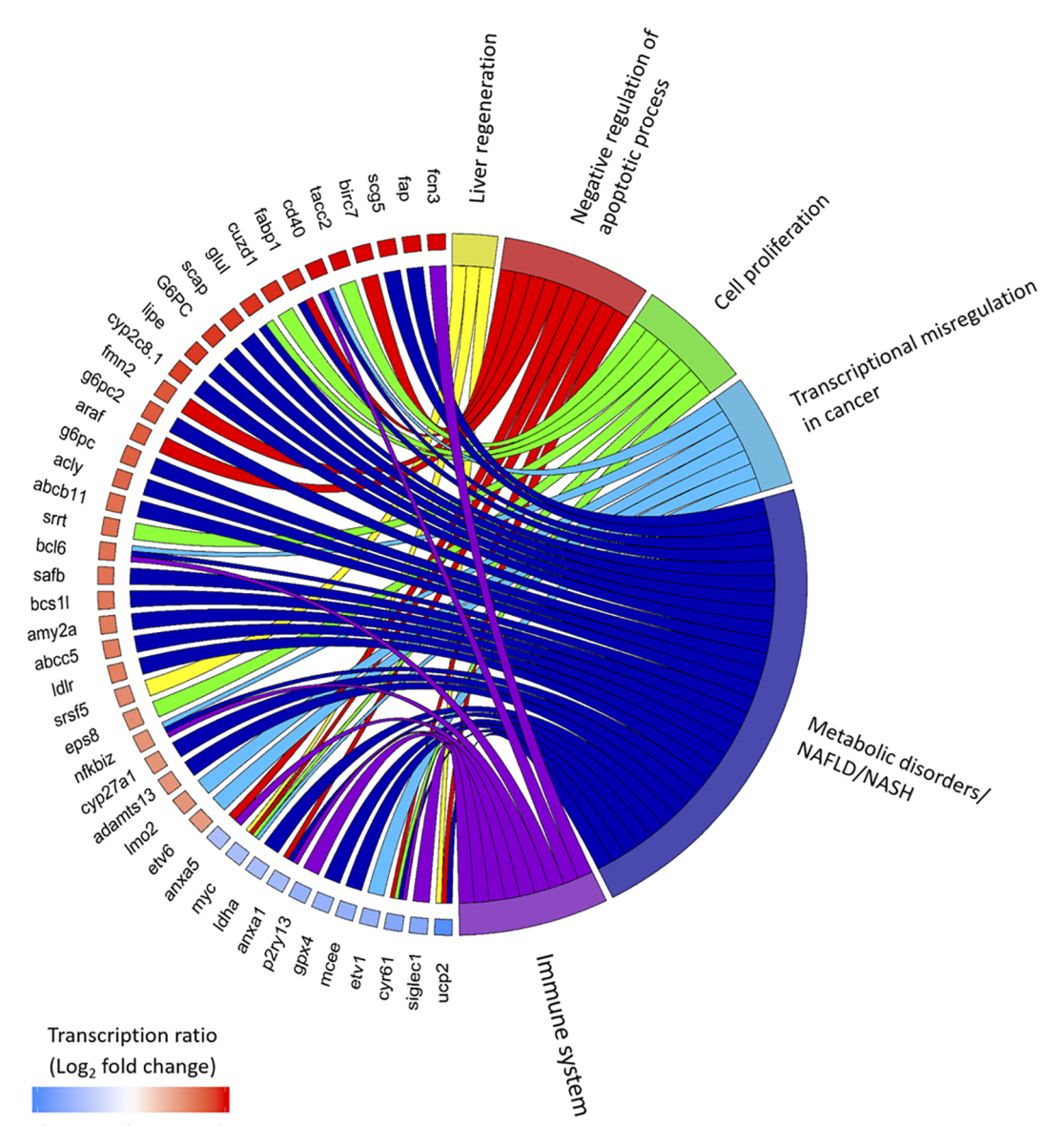
Liver regeneration
Negative regulation of apoptotic process
Cell proliferation
Transcriptional misregulation in cancer
Metabolic disorders/ NAFLD/NASH
Immune system
fcn3
fap
scg5
birc7
tacc2
cd40
fabp1
cuzd1
glul
scap
G6PC
lipe
cyp2c8.1
fmn2
g6pc2
araf
g6pc
acly
abcb11
srrt
bcl6
safb
bcs1l
amy2a
abcc5
ldlr
srsf5
eps8
nfkbiz
cyp27a1
adamts13
lmo2
etv6
anxa5
myc
ldha
anxa1
p2ry13
gpx4
mcee
etv1
cyr61
siglec1
ucp2
Transcription ratio
(Log2 fold change)
-2
0
2

Figure 3