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ABSTRACT

Salmonids are ecologically, economically and culturally important fish species in North America, but whether contaminants in the environment may play a role in their population decline is unclear. We tested the hypothesis that BPA deposition in eggs, mimicking a maternal transfer scenario, compromises the stress axis functioning and target tissues stress response in two generations of a model salmonid species, the rainbow trout (*Oncorhynchus mykiss*). Eggs were enriched with 0, 4 or 40 ng BPA, fertilized, and reared in clean water for two generations. The fish were subjected to an acute stressor after a year in both generations to test their stress performances. Trout raised from BPA-enriched eggs showed impaired stressor-mediated plasma cortisol and lactate response in the F1 and F2 generation, respectively. Key genes involved in cortisol biosynthesis in the head kidney, as well as stress- and growth-related transcripts in the liver and muscle were impacted either in the F1 and/or F2 generations. Our results underscore the long-term impact associated with BPA in eggs, mimicking a maternal transfer scenario, on the stress performance of trout in two generations. The results highlight the need for developing novel biomarkers to predict long-term and generational toxicities in salmonids.

KEYWORDS: Salmonids, BPA, Cortisol, Stress response, Gene expression, Transcriptomics

INTRODUCTION

Bisphenol A (BPA), an organic compound used in the production of plastics and epoxy resins, is ubiquitously distributed in the aquatic environment with mounting evidence of its impact on the endocrine system of animals [1,2]. Global production of BPA has increased substantially over the years, and over 500 tons of BPA are released into the environment annually [2]. A large body of work has provided insight into the toxicities of BPA in both aquatic and terrestrial animals [1,3]. In addition, maternal transfer of BPA has been reported in humans, rodents and fish [3–6], but the long-term developmental effects are far from clear. Recent studies have also described that exposure to BPA during critical early developmental periods may lead to stable epigenetic modifications that are passed on to the next generation [7,8].

As in mammals, BPA is an estrogen mimic in fishes and impacts reproduction [9–11]. Recently studies also highlight developmental toxicities related to growth and stress response activation in fish [12–14]. Furthermore, multigenerational impact of BPA on reproduction was shown in a model small-bodied fish with short life spans and generation times [9]; however, no information currently exists on multigenerational impacts of BPA in ecologically relevant fish species with longer life spans. Indeed, the potential for chemicals to cause adverse effects that persists in multiple generations are of concern, as it highlights the profound and sustained environmental health dysfunction [15,16], especially when observed in ecologically-relevant species.

The physiological response to stressors is highly conserved among vertebrates [17,18], as an evolutionary consequence of its crucial role in animals fitness. Any perturbations in the cortisol stress response, as seen with contaminant exposure [19], may negatively impact growth and development [20,21] and survival of the animal [22,23]. In anadromous salmonids, stress axis function and its ability to respond to an acute stressor are also considered a good determinant of reproductive outcome and progenies fitness [23,24]. In addition, studies have shown that cortisol plays a significant role in the upstream migration of salmonids to their spawning grounds [25,26]. Therefore, ability of fish to display a normal stress axis activity provides a good marker of global health of the species in a given environment [19].

Rainbow trout (*Oncorhynchus mykiss*) is considered an excellent model for toxicological studies [27,28], and a model salmonid given its genotypic resemblance with other migratory salmonids [29,30]. Our companion studies in trout recently showed that BPA in eggs, mimicking maternal transfer of this contaminant, affects the ontogeny of growth and stress response in the F1 generation [13,14]. Also, we showed changes in growth and metabolism during development in the two generation of trout raised from BPA-enriched eggs [21]. However, we have not shown before whether the stress performance, a key aspect of animal fitness, is impacted in multiple generations by BPA exposure in this species. Against this backdrop, we tested the hypothesis that BPA deposition in trout eggs, mimicking a maternal transfer scenario, compromises the long-term stress performance of the progeny in two generations. Plasma cortisol response to an acute stressor, the head kidney capacity to produce cortisol in response to adrenocorticotropic hormone (ACTH) stimulation *in vitro*, and the transcript abundance of corticosteroidogenic genes in the head kidney were used as markers of stress axis activity, while plasma glucose, lactate levels and tissue glycogen content were measured as indicators of metabolic stress response [17,31]. In addition, changes in transcript abundances of stress- and growth-related genes, and epigenetic markers in the liver and muscle in response to acute stressor exposure were used as biomarkers of target tissue responses.

MATERIALS AND METHODS

Experimental Animals and Treatments

The experimental details, including BPA exposure, fish maintenance and breeding of fish to obtain F1 and F2 generations have been published already [14,21]. Briefly, pooled oocytes from four females were fertilized with pooled milt from four male rainbow trout (3+ year class brood stock). Ovarian fluid was also collected from these four females for BPA treatment. Pooled oocytes were immersed in 50 ml of ovarian fluid containing vehicle alone (<0.01% ethanol; control group) or vehicle containing BPA at 3 or 30 μg ml^{-1} for 3 h at 6-8 °C with gentle shaking every 30 min. After the exposure, the oocytes were mixed with 1-2 ml of milt for fertilization, after which the embryos were rinsed several times with clean water. This treatment resulted in an egg BPA content of 4 and 40 ng egg^{-1} in the 3 and 30 μg ml^{-1} exposure groups, respectively [14]. The embryos were maintained in a Heath chamber incubator receiving clean groundwater at a rate of 10 l min^{-1} (6-8 °C). Larvae were maintained in the incubator for a week after hatch, after which they were moved to holding tanks (3 × 200 l tanks per treatment; n=277-299 larvae per replicate) receiving flow-through water at a rate of 10 l min^{-1}, under a 12h L: 12h D photoperiod. At 1 year, fish were sampled before and after an acute stress challenge (see below).

To study the BPA effects in the second generation, oocytes from the F1 generation adult female rainbow trout, kept separate based on the F0 egg BPA concentration, were fertilized with pooled milt from a stock of unexposed male rainbow trout. There was no detectable BPA in the eggs of any treatment groups. The experimental condition and fish rearing was similar to the F1 generation trout, except only one tank per treatment was maintained for the F2 generation. At 1 year, trout in the F2 generation were also sampled before and after an acute stress challenge (see below). Experiments were conducted at the Alma Research Station (ARS) (Alma, ON, Canada), and the experimental procedures were approved by the Animal Care and Use Committees at the University of Guelph and the University of Waterloo, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Stress Sampling

Trout (82.2 ± 5.0 g) from F1 and F2 generations were sampled 365 days post fertilization (dpf) to investigate the effects of BPA in eggs on long-term stress performance in trout. We examined primary and secondary stress response in control and trout raised from BPA accumulated eggs after an acute stress challenge. The stressor consisted of a 3 min handling disturbance, which elicited a transient rise in plasma cortisol levels, as described previously [12]. Food was withheld 48 h prior to the commencement of the stress experiment. Fish were sampled either prior to the stressor protocol (0 h time-point) or at 1, 4 and 24 h post-stressor exposure. Fish were euthanized with buffered Tricaine methanesulfonate (MS-222) and blood was collected by caudal severance in tubes containing EDTA as the anticoagulant. Blood samples were centrifuged at 5000 x g for 5 min to separate plasma and stored at -80 °C for later analysis of cortisol, glucose and lactate levels. Tissues (head kidney, liver and muscle) were quickly excised, flash frozen in dry ice, and stored at -80 ºC until transcript analysis. We measured the physiological markers of stress response (plasma cortisol, glucose and lactate levels), along with the molecular markers of stress response in the liver and muscle (glucocorticoid receptor 1 [*gr1*], glucocorticoid receptor 2 [*gr2*], and mineralocorticoid receptor [*mr*]) and head kidney (genes related to corticosteroid biosynthesis: melanocortin 2 receptor (*mc2r*), cytochrome P450 side-chain cleavage (*p450scc*) and steroidogenic acute regulatory protein (*star*) in the two generations of trout. Also, molecular markers of growth (insulin-like growth factor-1 [*igf1*], insulin-like growth factor-2 [*igf2*], insulin-like growth factor 1a receptor [*igf1ra*], insulin-like growth factor 1b receptor [*igf1rb*], growth hormone receptor 1[*gh1r*], growth hormone receptor 2 [*gh2r*]) and epigenetics (DNA methyltransferase 1 [*dnmt1*], DNA methyltransferase 2 [*dnmt2*] and liver specific methionine adenosyltransferase 1 alpha [*mat1a*]) were measured in the liver and muscle in the two generations of trout before and after an acute handling stress challenge.

In Vitro Cortisol Production

Cortisol production was measured as previously described [32], with minor modifications. Briefly, head kidney tissue, containing interrenal steroidogenic cells, was removed from unstressed trout from control and BPA treated groups in both F1 and F2 generations (n= 5-6) and placed in a petri dish containing Hank's buffer. The tissue was finely minced, washed in Hank's buffer three times to remove any blood clots, and equally distributed into 24 well plates (3 wells per fish). Tissues slices were pre-incubated for 2 h at 13 °C with gentle shaking to equilibrate. The tissue from each fish was then exposed to either fresh buffer only (no stimulus group) or fresh buffer containing 0.5IU ml^{-1} ACTH for 4 h at 13 °C, with gentle shaking. The concentration of ACTH chosen was based on a previous study [32]. At the end of the exposure, samples were collected, quickly centrifuged at 13,000 × g for 1 min, and supernatant stored frozen at -80 °C for later cortisol determination. Lactate dehydrogenase (LDH) leakage was used to confirm tissue viability [32], and there was no effect on tissue viability due to the incubation protocol.

Plasma, Medium and Tissue Analyses

Cortisol analysis in the plasma and medium (*in vitro* assay) were carried out by radioimmunoassay (RIA) as described previously [33]. Plasma glucose and lactate levels were measured enzymatically as described previously [34,35]. Liver glycogen content was determined by measuring glucose levels before and after amyloglucosidase hydrolysis as described before [35], while protein was measured using the bicinchoninic acid method using bovine serum albumin as the standards [35].

Tissue Transcript Analysis

Tissue RNA extraction, cDNA synthesis and the quantitative real-time PCR (qPCR) protocol have been described in detail previously [12]. Briefly, the transcript levels were analyzed using the iQ™ SYBR® green supermix fluorescent dye with the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each sample was assayed in duplicate and the following thermal cycling protocol was followed: 2 min at 94 °C; 40 cycles of: 30 s at 95 °C, followed by 30 s at the melting temperature for each gene (Supporting Information [SI] Table 1); 1 min at 95 °C; 1 min at 55 °C, followed by melt curve analysis starting from 55- 95 °C in increments of 0.5 °C every 10 s. Copy number for each gene was determined using plasmid standard curves previously established in our laboratory following the protocol described previously [12]. All samples were assayed for the genes of interest and for the housekeeping gene, elongation factor 1α (*ef1α*), which did not change between treatments.

Transcriptome Analysis of Stress Related Genes in the Liver

Liver stress transcriptome of 4 fish prior to the stressor exposure per treatment in both generations were described using expression results from a previous study [21], deposited into the Gene Expression Omnibus (GEO) database (Accession #: GSE94281; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94281). Protocol for identification, annotation, and enrichment of differentially expressed genes between treatments in 365 dpf F1 and F2 generations was described previously [21]. For the present study, only genes differentially expressed in at least one of BPA treatment and enriched for the GO term "response to stress" (GO:0006950) were used.

Statistical Analysis

Statistical analyses were performed by use of SigmaPlot 13.0 software (Systat Software Inc., San Jose, CA, USA). All transcript abundance results were presented as fold change compared to the treatment control (BPA0) at time 0 h. Heatmaps represent mean values per treatment for each time point pre and post-stressor exposures within each generation. All heatmaps were plotted using the function heatmap.2 from the gplots package in R [36]. A two way analysis of variance (ANOVA) followed by Holm–Sidak post hoc test was used to determine significant effect of BPA exposure and handling stressor on plasma cortisol, glucose, and lactate concentrations, liver glycogen content, and transcript abundance of genes in the liver, muscle and head kidney tissues. When there was a significant interaction between BPA exposure and handling stressor, a one-way ANOVA followed by Holm–Sidak post hoc test was used to separately test the effect of BPA exposure or time on those parameters. Data were log-transformed wherever necessary to meet the assumptions of normality and equal variance. Only non-transformed data are shown in the figures. Figures were plotted either using SigmaPlot 13.0 or R 3.3.1 (http://cran.r-project.org/). A probability level of $p < 0.05$ was considered significant. All data (except transcript abundance and liver transcriptome) are shown as mean ± standard error of the mean (S.E.M.). Transcript abundance results with mean ± S.E.M can be found in the SI Tables 2, 3 and 4.

RESULTS

Plasma Cortisol Response

There was a significant interaction between BPA exposure and acute handling stressor on plasma cortisol concentrations in the F1 generation of trout. BPA treatment did not alter resting plasma cortisol concentration at 365 dpf in the F1 generation (Fig. 1A). However, BPA significantly impacted the ability of these fish to respond to an acute handling stressor (Fig. 1A). Trout from the control treatment group showed a significantly greater cortisol response 1 h after an acute stressor exposure, which returned to unstressed levels at 4 h post-stressor exposure (Fig. 1A). Although F1 generation trout from the 4 ng BPA group showed a plasma cortisol response to stress similar to that of the control group, the cortisol levels at 1h after the acute handling stressor was significantly lower in that group when compared to the controls. In the F1 40 ng BPA group, the cortisol levels 1 h post-stress were not significantly different than those of unstressed fish (Fig. 1A). In the F2 generation, acute handling stressor significantly increased plasma cortisol levels at 1 h post-stressor and this steroid level dropped to basal level at 4 and 24 h post-stressor exposure in all treatment groups (Fig. 1B). BPA had no significant impact on either the unstressed or stressed levels of plasma cortisol concentrations in the F2 generation (Fig. 1B).

Interrenal Cortisol Production and Transcript Abundance

To determine if BPA accumulation in eggs impacts cortisol production capacity, this steroid production was monitored in the head kidney of F1 and F2 rainbow trout following *in vitro* stimulation with ACTH (Figs. 1C & D). When compared to the un-stimulated head kidney tissues, ACTH stimulation for 4 h significantly increased cortisol levels in all treatment groups, and this response was not modified by BPA accumulation in eggs (Fig. 1C). A similar response was also seen in the F2 generation head kidney tissues and the basal or ACTH-stimulated cortisol production was not altered by BPA in eggs (Fig. 1D).

Significantly greater transcript abundance of *mc2r*, *p450scc* and *star* was observed in the head kidney tissues of BPA40 group compared to the control group in the F1 generation trout (Fig. 1E). Acute handling stressor had no effects on *mc2r* and p*450scc* transcript abundance, but it significantly upregulated transcript abundance of *star* in all treatment groups at 24 h post-stress when compared to expression of *star* in the pre-stressed trout (Fig. 1E).

In the F2 generation, BPA had no significant effect on *mc2r* and *p450scc* transcript abundance in the head kidney of trout (Fig. 1E). Acute handling stressor significantly upregulated transcript abundance of *p450scc* at 1 and 4 h post-stressor when compared to unstressed fish. However, BPA treatment impacted the stressor-mediated transcript abundance of *star* in the BPA40, but not the BPA4 group (Fig. 1E). In the 40 ng BPA group, *star* mRNA abundance was upregulated by approximately 2-fold at 1 h post-stress when compared to controls at the same time point and to unstressed individuals from the same treatment group (Fig. 1E & SI Table 2).

Plasma Secondary Stress Response

There were no significant effects of BPA on plasma glucose and lactate concentrations in the F1 generation fish at 365 dpf (Fig. 2A & B). The acute stressor significantly increased plasma glucose concentration at 4 h in all groups. Similarly, plasma lactate concentrations were increased by 2-3 fold at 1h post-stress in all treatment groups (Fig. 2B). Liver glycogen content was significantly lower in 24 h post-stress trout from all the treatment groups when compared to the unstressed and 1h post-stress trout, but this was not impacted by BPA treatment (Fig. 2C).

In the F2 generation, BPA had no effect on plasma glucose levels in trout from unstressed or post-stressor groups (Fig. 2D). Acute stressor significantly decreased the plasma glucose concentrations 24 h after post stress in all treatment groups when compared to the 0, 1 and 4h trout (Fig. 2D). There was a significant main effect of BPA on stressor-mediated plasma lactate concentrations in the F2 generation trout (Fig. 2E). Similar to F1 generation trout, plasma lactate was significantly greater 1h after the acute handling stressor in all treatment groups. Ancestral exposure to 40 ng BPA increased plasma lactate concentrations by approximately 2-fold in F2 trout when compared to the controls and the 4 ng BPA group (Fig. 2E). Liver glycogen concentrations were significantly lower 24 h after post-stress in all treatment groups when compared to the unstressed and 1h post-stress trout, but this was not impacted by BPA treatment (Fig. 2F).

Liver Stress Transcriptomics and Targeted Genes Expression

The effect of BPA accumulation on stress related genes was determined in the liver of unstressed F1 and F2 generation trout using a transcriptomics approach [21]. Only differentially expressed genes related to the GO term 'response to stress' were selected for this study (Fig. 3A - D & SI Table 5). There were a total of 35 and 66 stress-related genes that were differentially expressed between at least one BPA treatment compared to the control in F1 and F2 generations, respectively (Fig. 3A, C & D). 17 of those differentially expressed genes were identical in both F1 and F2 generations (Fig. 3A). Based on gene ontology terms, the six most represented biological functions in the F1 generation trout were defense response, innate immune response, response to organic substance, cellular nitrogen compound metabolic process, macromolecule metabolic process and regulation of cellular process. In the F2 generation, the six most represented biological functions were defense response, regulation of cellular process, macromolecule metabolic process, innate immune response, signal transduction, and cellular macromolecule metabolic process. The majority of differentially expressed stress-related genes in the F1 and F2 generations trout participated in the defense response (Fig. 3B).

The stressor-mediated growth and stress related transcript changes were also assessed using qPCR. In the F1 generation trout, 40 ng BPA egg accumulation upregulated transcript abundance of *igf1* and *igf2* (Fig. 4A). Acute handling stressor significantly increased (~1.5 to 3 fold) transcript abundance of *igf1*, *igf2* and *gh2r* in all treatment groups at all time-points post-stressor exposure (Fig. 4A & SI Table 3). Transcript abundance of *gh1r* was significantly increased (~2 to 3 fold) in all groups at 24h post-stress when compared to the unstressed trout (Fig. 4A & SI Table 3). There were no interactive effects of BPA accumulation in eggs and acute handling stressor on transcript abundance of *igf1ra* and *igf1rb* in trout (Fig. 4A). Also, BPA had no significant effect on the expression of stress related genes (*gr1*, *gr2* and *mr*) in the trout liver (Fig. 4A). However, an acute handling stressor significantly increased *gr1* and *gr2* (~1.5 to 4 fold), but not *mr*, transcript levels in all the post-stress treatment groups when compared to the unstressed trout (Fig. 4A & SI Table 3).

In the F2 40 ng BPA group, transcript abundance of genes involved in growth (*igf2* and *igf1ra*), and stress response (*gr1* and *mr*) were modified (Fig. 4B). Transcript levels of *igf1ra* were significantly increased, while those for *igf2* were significantly decreased in pre and post stress time periods when compared to the control trout (Fig. 4B). A 50 % reduction in transcript abundance of *igf1ra* was observed at 1, 4 and 24 h post stress trout from all treatment groups when compared to unstressed fish. Acute handling stressor, but not maternal ancestral exposure to BPA, significantly increased *igf1* transcript abundance at 24 h post stress in trout from all treatment groups. In the liver of F2 40 ng BPA group, there was a significant upregulation of *mr* and downregulation of *gr1* transcript abundance in both pre and post stress time points when compared to the control trout (Fig. 4B). An acute stressor significantly increased *gr1* transcript abundance in the liver of F2 generation trout from all treatment groups when compared to the unstressed individuals, but *gr2* transcript abundance significantly increased only at 1h post-stress when compared to the unstressed fish (Fig. 4B).

Muscle Transcript Abundance

BPA accumulation in eggs had no effect on transcript abundance of genes related to growth (*igf1rb*, *gh1r*, *gh2r* and *igf1ra*) and stress response (*mr*, *gr2* and *gr1*) in the muscle of F1 rainbow trout (Fig. 5A). *Igf1* and *2* are not expressed in muscle tissues and hence we did not measure transcript abundance of those genes. An acute handling stressor significantly upregulated transcript abundance of all above mentioned genes in the muscle at all post-stressor time-points when compared to the unstressed trout (Fig. 5A).

In the F2 generation, elevated transcript abundance of muscle *igf1rb* was noticed in BPA4 and 40 groups compared to the controls (Fig. 5B). Also, BPA40 group had significantly higher *igf1ra* and *mr* compared to the control group. An acute handling stressor significantly increased transcript abundance of *igf1rb*, *gh1r*, *gh2r*, *igf1ra*, *mr* and *gr2* in trout muscle at all post-stressor time-points when compared to the unstressed trout (Fig. 5B).

Epigenetic Markers in the Liver and Muscle

BPA accumulation in eggs did not significantly affect the transcript abundance of liver epigenetic markers, including *dnmt1*, *dnmt2* and liver specific *mat1a*, in the F1 generation (Fig. 4A). An acute handling stressor challenge significantly increased *dnmt2* transcript levels at 4 and 24 h post-stressor in the liver of trout from all treatment groups when compared to the unstressed trout (Fig. 4A). No significant changes were observed in *mat1a* and *dnmt1* transcript abundance

(Fig. 4A). In the F2 generation, BPA treatment significantly increased liver *dnmt2*, but not *mat1a* and *dnmt1* transcript levels only in the BPA40 group compared to the controls (Fig. 4B). The acute handling stressor, regardless of BPA treatment, reduced the transcript abundance of *dnmt1* at all post-stressor time-points compared to the unstressed trout (Fig. 4B). The transcript abundance of *dnmt2* was significantly higher only at 4 h post-stressor time-point when compared to the unstressed trout, while *mat1a* was not affected by the stress challenge.

BPA accumulation in eggs had no significant effect on the transcript abundance of muscle *dnmt1* and *dnmt2* in the F1 generation (Fig. 5A). An acute handling stressor significantly upregulated the transcript abundance of muscle *dnmt1*, but not *dnmt2*, at all time-points post-stressor compared to the unstressed trout (Fig. 5A). In the F2 generation, trout from the BPA40 group demonstrated significantly greater increase in transcript abundance of muscle *dnmt1* compared to the control group, but not such treatment effect was noticed for *dnmt2* (Fig. 5B). An acute handling stressor significantly up regulated transcript abundance of muscle *dnmt1*, but not *dnmt2* in post stress trout from all treatment groups when compared to the unstressed group (Fig. 5B).

DISCUSSION

The most significant finding of this study was that BPA accumulation in eggs, mimicking maternal transfer of this contaminant, altered the acute stress performances in two generations of rainbow trout. The longer-term and generations effects in plasma stress parameters and the target tissue molecular effects were more evident in the 40 ng BPA per egg compared to the 4 ng BPA per egg groups. The teleost stress axis functioning is highly conserved [37], and we have shown previously that the developmental programming of the cortisol stress axis was disrupted by BPA accumulation in eggs in the F1 generation [12,14]. To our knowledge this is the first study to demonstrate BPA impact not only on the plasma stress response, but also acute stress-related transcript changes in the liver, muscle and head kidney of trout in successive generations. The concentrations of BPA reported in trout embryos in the present study are environmentally realistic, as similar BPA concentrations were found in wild fish and zooplankton collected from the BPA-impacted sites [38,39]. In addition, recent studies have provided evidence of maternal transfer of BPA from the exposed adult female fish to eggs [3,6], and hence understanding the generational toxicities of maternally deposited BPA is highly relevant from a risk assessment stand-point.

In our study, the accumulated BPA in eggs was rapidly cleared during embryogenesis with levels below detection at hatch (42 dpf) [13]. The low level exposure of BPA during early embryogenesis was shown previously to impact the developmental programming of the growth and stress axes [12–14]. This disruption of stress axis development in early life stages may have played a role in the altered stressor-mediated plasma cortisol and/or metabolite levels seen in the 1 yr old fish in the F1 and F2 generations. The suppression of stressor-induced plasma cortisol response in the BPA40 group is consistent with an earlier study showing a similar response in the F1 generation [12]. The lower steroid response corresponded with an upregulation of genes encoding proteins critical for steroid biosynthesis in the interrenal tissue in BPA40 group in the F1 generations. A similar mismatch in steroidogenic gene expression and cortisol output was also observed in progenies (baseline group only) of sockeye salmon (*Oncorhynchus nerka*) exposed to maternal stress [40], and 65 dpf trout exposed to BPA during embryogenesis [14], suggesting contaminant impact on the transcript stability or turnover. However, our results reveal that the attenuation of the cortisol response in the F1 generation may not be due to disruption in steroid biosynthesis as the BPA fish were able to evoke an ACTH-stimulated cortisol response similar to that of the control group (Fig. 1C). This suggests that BPA impacts the hypothalamus-pituitary axis development, leading to disruption in either the CRF and/or ACTH production. This notion was supported by a recent study demonstrating that BPA modifies both CRH and ACTH transcripts in rats [41], and such changes may affect the developmental programming of the cortisol stress axis. The developmental impact of BPA on cortisol stress functioning seen in F1 generation was not transferred to the F2 generation of trout in the present study. This was also the case with the secondary stress response indicators, including plasma glucose and liver glycogen content, as BPA accumulation in eggs did not modify the stressor-mediated changes in these parameters in the F1 and F2 generation trout. However, this was not the case with stressor-mediated plasma lactate level, which was higher in the BPA40 group in the F2 generation. As elevated lactate level is an indicator of anaerobic metabolism and altered secondary stress response in fish [31,42], our results suggest adverse effects on muscle energy metabolism in the F2 generation in response to ancestral exposure to BPA (see below).

In the present study liver transcriptomic analysis revealed that a total of 35 and 66 stress related genes were differentially expressed between at least one BPA treatment and the control (0 BPA) in F1 and F2 generations, respectively. In the F2 generation liver, approximately a two-fold increase in stress-related genes, including genes related to host defense, regulation of cellular process and macromolecule metabolic process, suggest that BPA impacts on molecular programming events are more evident in the F2 generation trout [21]. The majority of differentially expressed stress-related genes in both F1 and F2 generations participate in the host defense response. Examples of differentially expressed stress genes with host defense response functions include, among others, mx proteins, retinoic acid inducible protein-i, interferon inducible mx proteins, toll-like receptors genes. All these genes participate in immune response and are essential for limiting viral and bacterial infection or diseases [43]. A bidirectional communication between stress axis and immune system has been reported in fish [44] and modification of this communication by contaminants may lead to organismal level impact. A number of previous studies have demonstrated that exposure to pollutants or stress can modify the host immune response, which increases susceptibility of salmonids to both viral and bacterial infections [45–47]. Future studies should test the hypothesis that whether developmental exposure to BPA in trout increases the risk of infections in exposed generations and their progenies, and the underlying mechanism needs to be elucidated.

While the hormonal regulation of growth has been extensively reviewed [48], the alterations in the response of somatotropic axis genes to acute stressor exposure is far from clear. Our results reveal for the first time acute changes in the stress- and growth-related transcripts in the liver and muscle of fish in response to an acute handling stressor. The majority of transcripts were upregulated at 1 h and they stayed elevated over the 24 h period after an acute stressor, the only exception was liver *igf1ra* in the F2 generation that was significantly downregulated post-stressor exposure. Acute handling stress challenge in Coho salmon (*Oncorhynchus kisutch*) increased hepatic *igf1* expression without an increase in *ghr* at 1.5 h post-stressor, but transcript levels of the two genes were dropped to the control levels at 16 h post-stressor challenge [49]. As in Coho salmon, we observed similar trend in *igf1* and *ghr1* gene expression in the liver of F1 generation trout at 1 h, but we did not observe a drop in transcript abundance of those genes at 24 h, suggesting that the temporal profile of *ghr1* and *igf1* genes respond differentially to an acute stressor challenge. We saw greater transcript abundance of growth-related genes in trout muscle after an acute handling stress challenge. Greater muscle GHR protein expression was reported in fish after heat shock [50]. On the other hand, no change in *ghr1* and down regulation of *ghr2* were observed in muscle of *Pampus argenteus* underwent a handling stress challenge [51]. Collectively, the results suggest that the transcript abundance of growth-related genes in the liver and muscle are modified by acute stressor in fish, and these changes may be related to acute stress hormone stimulation of muscle metabolism [20,31].

Our results reveal that BPA in eggs disrupt the acute stressor-mediated changes in molecular growth targets in the liver and muscle of the trout progeny in two generations. Given these tissues are the major metabolic targets for stress hormones action during acute stress recovery and adaptation in fish [31,52], the results suggest a compromised stress performance. The lower transcript abundance of *igf2* and a trend for reduced transcript abundance of *igf1* in the liver, as well as a significant increase in transcript abundance of *igf1ra* and *igf1rb* in the muscle of F2 generation trout in the BPA40 group supports disruption in molecular programming of the growth axis by BPA, as studies have shown that stressors impair growth axis development and function [20,21]. Similarly, both g*r1* and *mr* were differentially expressed in the liver of F2 generation trout from the BPA40 group underscoring possible changes in target tissue stress steroid responsiveness that are evident in the F2 generation. The overall increase in transcript abundance in the muscle and liver in the BPA group reflects a higher tissue metabolic demand, as transcription and/or translation are energy demanding [20,52], and contributes to the increased energy demand during stress in fish [31,52]. Our results suggest that BPA accumulation in eggs disrupts the metabolic adjustments that are essential during acute stress adaptation [52], leading to the proposal that the overall stress performance will be compromised by BPA even in the F2 generation of trout.

Recent studies have suggested BPA-induced epigenetic modifications for generational toxicities in mammals [8,53], and we propose a similar mode of action for the observed modification of stress response in F2 generation trout. To this end, we investigated the impact of BPA in eggs on transcript abundance of genes involved in epigenetic modification in both liver and muscle of the F1 and F2 generations trout before and after an acute handling stress challenge. We observed a significant upregulation of *dnmt2* in the liver and *dnmt1* in the muscle of F2 generations trout from the BPA40 group. Role of *dnmt1* in DNA methylation is well established whereas *dnmt2* has a weak DNA methylation activity but is involved in the transfer RNA (tRNA) methylation [8,53–55]. Methylation of tRNA by *dnmt2* has been demonstrated to promote tRNA stability as it protects tRNA against ribonuclease cleavage during thermal or chemical stress [54]. tRNA-derived small RNAs has been suggested to trigger gene silencing [55], suggesting that altered *dnmt2*-induced tRNA methylation during stress may indirectly affect gene expression. In addition, a role for *dnmt2* in transgenerational epigenetic modification was recently demonstrated in mice [56]. Hyper DNA methylation of the promotor region of a gene causes transcriptional repression, and this may be involved in the down-regulation of *igf2* and *gr1* in the liver of F2 generation trout from the BPA40 group, but this needs to be further tested and validated. However, we also saw a significant number of growth and stress response genes upregulated in both liver and muscle of F2 generation trout from the BPA40 group, leading to the proposal that epigenetics mechanisms other than DNA methylation, including histone modification [57], may also be involved in the BPA-induced generational toxicities in trout. Taken together, epigenetic modification, including DNA methylation may potentially be involved in the BPA-induced generational toxicities in fish.

Overall, the study provided evidence that BPA (~ 40 ng) accumulation in eggs, mimicking a maternal transfer scenario, leads to impairment of the primary and secondary stress response over two generations of rainbow trout. Given the importance of trout as a model species to investigate stressor effects in salmonids [27,58], observed BPA-induced generational impairment in the stress response may be reflective of the potential impact this environmental contaminant may exert on salmonid fitness, including reducing their ability to respond to additional stressors such as climate change, pollution, disease or predation. To this end, studies have shown that exposure to endocrine disruptors affect fish performances, including development, stress reactivity, behaviour, disease susceptibility, reproduction and fitness [12,16,40,47,59]. However, toxicities associated with parental and ancestral exposures are not currently included in the ecological risk assessment framework. Our finding that BPA accumulation in eggs can have long-term and multigenerational adverse effects, in spite of complete lack of tissue contaminant burden [12,13], suggests that the current risk assessment framework may not protect aquatic animals against chemicals such as BPA in contaminated sites. Our study underscores the need for developing biomarkers to predict generational toxicities in aquatic animals, and include that information in ecological risk assessment for management of such chemicals.

ASSOCIATED CONTENT

Supporting Information

Information on primer sequences (Table 1), mean values of stress-related genes in head kidney (Table 2), and mean values of growth, stress and epigenetic-related genes in liver (Table 3) and muscle (Table 4) of the F1 and F2 generations trout, and the gene ontology (GO) term description of differentially expressed stress-related genes in the liver of two generations of trout (Table 5).

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Figure Legends

Fig. 1. <u>Primary stress response</u>. Plasma cortisol levels (A and B), head kidney cortisol production (C and D), and transcript abundance of key cortisol biosynthesis genes in head kidney (E) were determined in F1 and F2 trout raised from either the control (0) or BPA-treated (4 and 40 ng) eggs in the F0 generation. For A, B and E, time 0 represents changes in variables in the unstressed fish, whereas rest of the time-points (1, 4 and 24 h) represent post-stressor responses. The heatmap represents mean fold changes of key cortisol biosynthesis genes in each treatment groups at each time periods (0, 1, 4, and 24 h) when compared to the unstressed (0 h) control trout. For figure A, different lower case letters denote significant difference between treatment groups across the time periods, while an asterisk represents a significant difference between the control and BPA groups within that time period; for figure B, different lower case letters denote significant difference between the time periods; for figure C and D, different lower case letters denote significant differences between ACTH treated and non-treated groups within each treatment group; and for figure E, different lower case letters denote significant difference between the time periods at the given BPA concentration, an asterisk represents a significant treatment effect, while different uppercase letters denote significant differences within control and BPA treatment groups across the time periods, and a hashtag denotes significant difference between control and given BPA exposed trout at that time-point. All data are shown as mean ± standard error of the mean (S.E.M.; n = 4-6 samples in each treatment and time points).

Fig. 2. <u>Secondary stress response.</u> Plasma glucose (A&D), lactate (B&E) and liver glycogen (C&F) levels were determined in F1 and F2 generation trout raised from eggs containing 0, 4 or 40 ng BPA. Time '0' represents changes in plasma or liver variables in the unstressed fish from all the treatment groups, whereas the other time points (1, 4 and 24 h) represent post-stressor

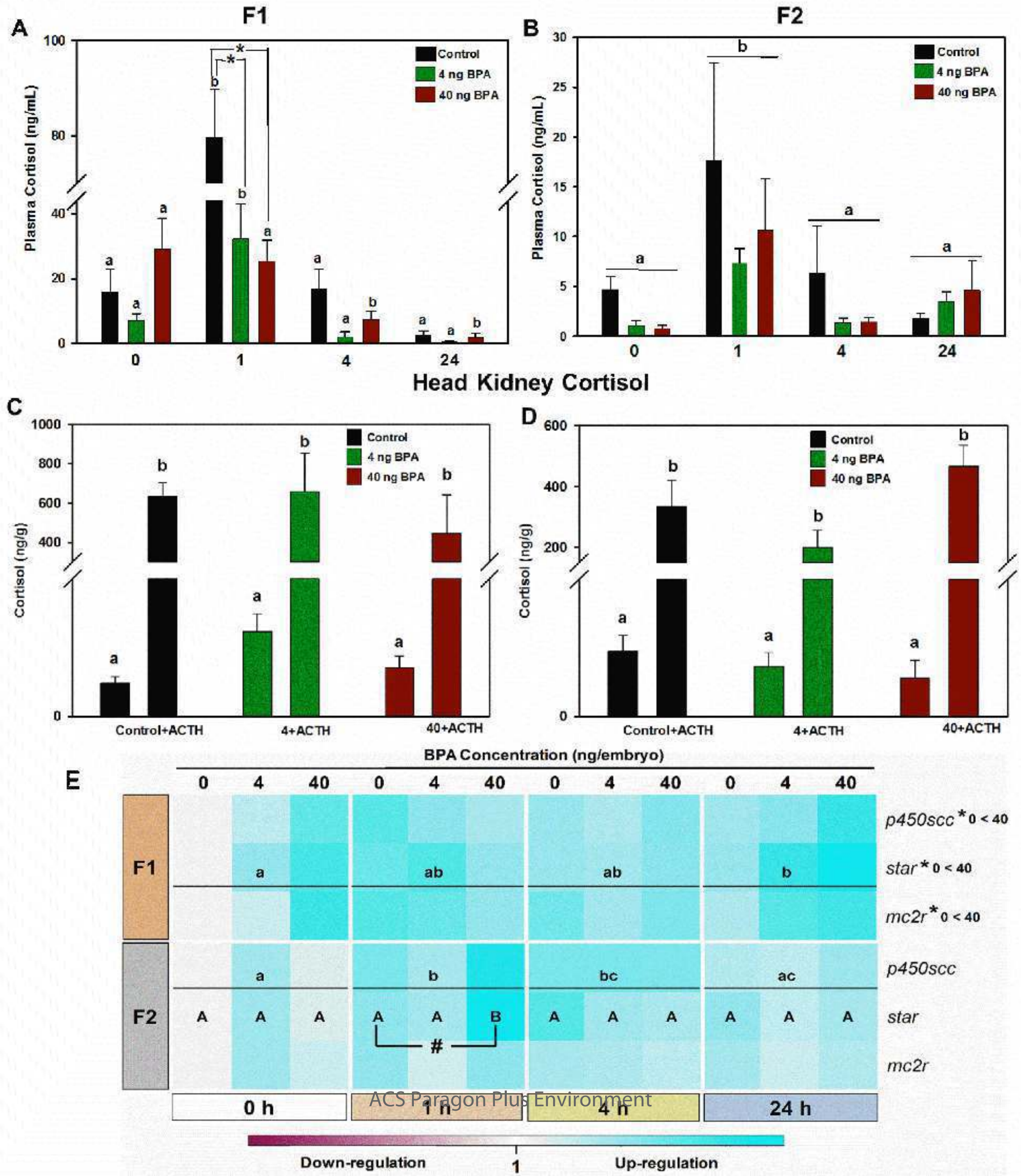
responses. A two way analysis of variance (ANOVA) followed by Holm–Sidak post hoc test was used to determine significant effect of BPA exposure and time on secondary stress response biomarkers in plasma and liver of two generations of trout (n= 5-6). Different lower case letters denote significant differences with the time periods and an asterisk represents a significant difference between control and given BPA exposed trout.

Fig. 3. **Transcriptome analysis of stress related genes in the liver.** Venn diagram of differentially expressed stress-related genes in the liver of F1 and F2 generations trout (A). Only genes differentially expressed in at least one of BPA treatment and enriched for the GO term "response to stress" were selected in our study. Bar graph illustrates the six most represented biological functions of differentially expressed stress response genes in the liver of both generations of trout (B). Differentially expressed stress related genes in F1 (C) and F2 (D) generations trout were shown in the Heatmaps. Each box represents average expression of stress related genes (n=4).

Fig. 4. Liver transcripts of growth- and stress-related genes. Heatmaps illustrate multigenerational effects of egg BPA accumulation on key growth-, stress- and epigenetics-related genes in the liver of two generations of rainbow trout. Transcript abundance of growth (insulin-like growth factor-1 [*igf1*], insulin-like growth factor-2 [*igf2*], insulin-like growth factor 1a receptor [*igf1ra*], insulin-like growth factor 1b receptor [*igf1rb*], growth hormone receptor 1 [*gh1r*], growth hormone receptor 2 [*gh2r*]), stress (glucocorticoid receptor 1 [*gr1*], glucocorticoid receptor 2 [*gr2*], and mineralocorticoid receptor [*mr*]) and epigenetics (DNA methyltransferase 1 [*dnmt1*], DNA methyltransferase 2 [*dnmt2*] and liver specific methionine adenosyltransferase 1 alpha [*mat1a*]) related transcripts were measured in trout livers raised from eggs containing 0, 4 and 40 ng BPA before and after an acute handling stress challenge in the F1

(A) and F2 (B) generations. Each small box in the heatmaps represents mean fold changes (n=4-6) of key growth, stress and epigenetic related genes in each treatment groups at each time periods (0, 1, 4, and 24h) when compared to the unstressed (0 h) control trout. In the analysis of transcript abundance of growth, stress and epigenetics related genes, different lower case letters denote significant differences with the time periods (two-way ANOVA with Holm–Sidak post hoc test, $p < 0.05$). An asterisk represents a significant difference between control and given BPA exposed trout (two-way ANOVA with Holm–Sidak post hoc test, $p < 0.05$).

Fig. 5. Muscle transcripts of growth- and stress-related genes. Heatmaps illustrate multigenerational effects of egg BPA accumulation on key growth-, stress- and epigenetics-related genes in the muscle of two generations of rainbow trout. Transcript abundance of growth (insulin-like growth factor 1a receptor [*igf1ra*], insulin-like growth factor 1b receptor [*igf1rb*], growth hormone receptor 1 [*gh1r*], growth hormone receptor 2 [*gh2r*]), stress (glucocorticoid receptor 1 [*gr1*], glucocorticoid receptor 2 [*gr2*], and mineralocorticoid receptor [*mr*]) and epigenetics (DNA methyltransferase 1 [*dnmt1*], DNA methyltransferase 2 [*dnmt2*] related transcripts were measured in trout muscles raised from eggs containing 0, 4 and 40 ng BPA before and after an acute handling stress challenge in the F1 (A) and F2 (B) generations. Each small box in the heatmaps represents mean fold changes (n=4-6) of key growth, stress and epigenetics related genes in each treatment groups at each time periods (0, 1, 4, and 24h) when compared to the unstressed (0 h) control trout. In the analysis of transcript abundance of growth and stress related genes, different lower case letters denote significant differences with the time periods (two-way ANOVA with Holm–Sidak post hoc test, $p < 0.05$). An asterisk represents a significant difference between control and given BPA exposed trout (two-way ANOVA with Holm–Sidak post hoc test, $p < 0.05$).



A
F1
Plasma Cortisol (ng/mL)
Control
4 ng BPA
40 ng BPA
0
1
4
24
B
F2
Head Kidney Cortisol
C
D
Cortisol (ng/g)
Control+ACTH
4+ACTH
40+ACTH
E
BPA Concentration (ng/embryo)
F1
F2
p450scc *0 < 40
star *0 < 40
mc2r *0 < 40
p450scc
star
mc2r
0 h
1 h
4 h
24 h
Down-regulation
1
Up-regulation

Figure 1

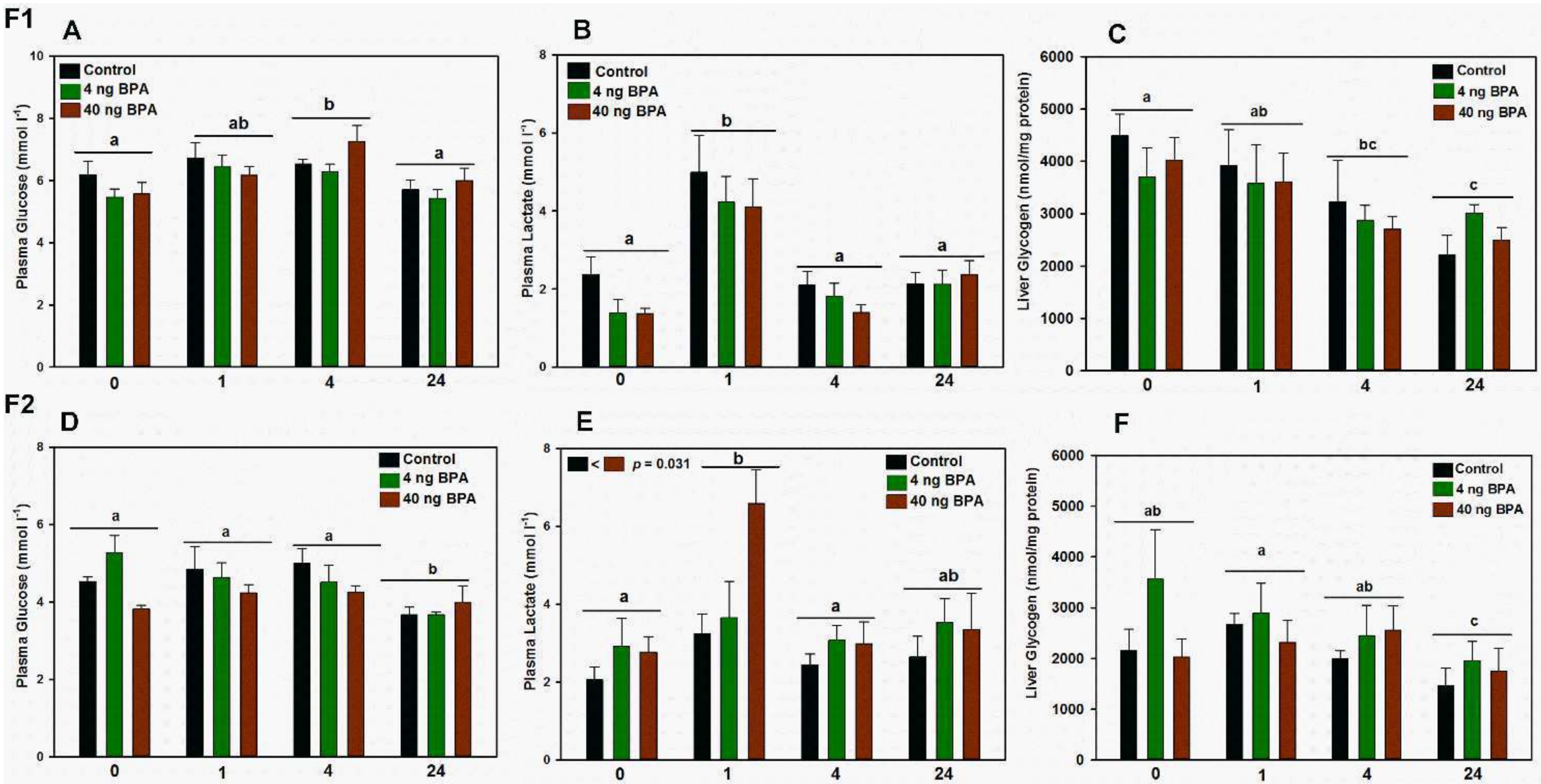

F1
A
Control
4 ng BPA
40 ng BPA
Plasma Glucose (mmol l-1)
a
ab
b
a
0
1
4
24
B
Plasma Lactate (mmol l-1)
a
b
a
a
C
Liver Glycogen (nmol/mg protein)
a
ab
bc
c
F2
D
Plasma Glucose (mmol l-1)
a
a
a
b
E
< p = 0.031
Plasma Lactate (mmol l-1)
a
b
a
ab
F
Liver Glycogen (nmol/mg protein)
ab
a
ab
c

Figure 2

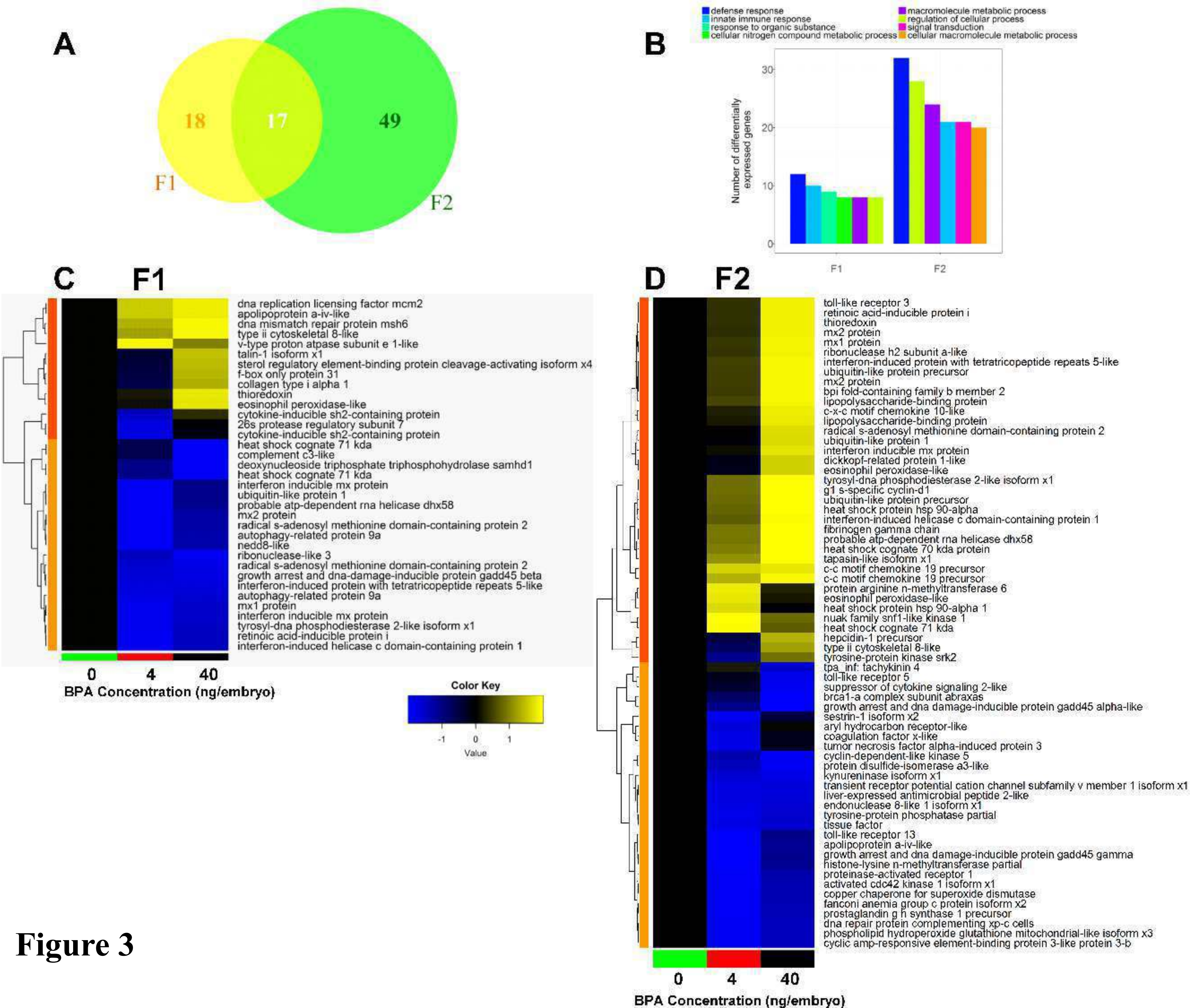
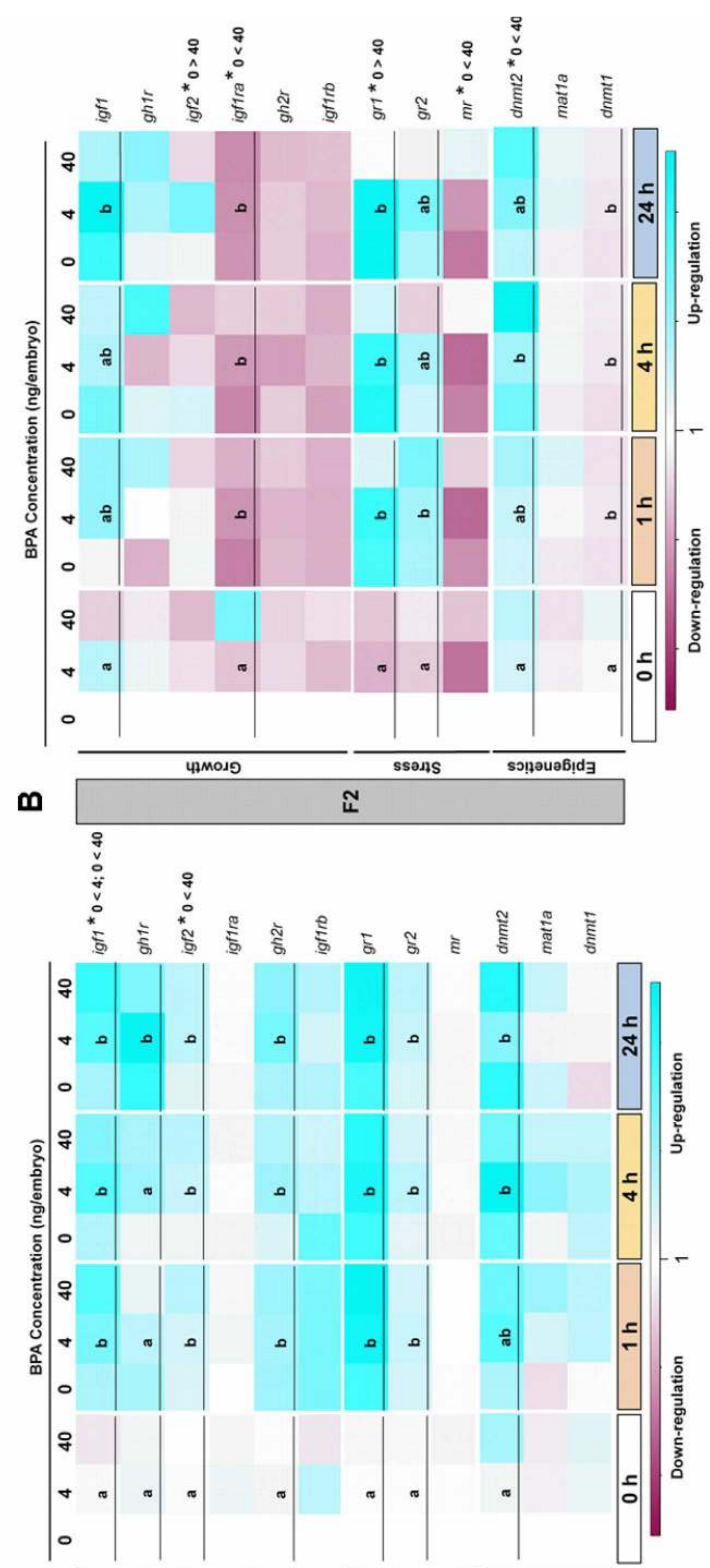

A
18
17
49
F1
F2
B
defense response
innate immune response
response to organic substance
cellular nitrogen compound metabolic process
macromolecule metabolic process
regulation of cellular process
signal transduction
cellular macromolecule metabolic process
Number of differentially expressed genes
30
20
10
0
F1
F2
C
F1
dna replication licensing factor mcm2
apolipoprotein a-iv-like
dna mismatch repair protein msh6
type ii cytoskeletal 8-like
v-type proton atpase subunit e 1-like
talin-1 isoform x1
sterol regulatory element-binding protein cleavage-activating isoform x4
f-box only protein 31
collagen type i alpha 1
thioredoxin
eosinophil peroxidase-like
cytokine-inducible sh2-containing protein
26s protease regulatory subunit 7
cytokine-inducible sh2-containing protein
heat shock cognate 71 kda
complement c3-like
deoxynucleoside triphosphate triphosphohydrolase samhd1
heat shock cognate 71 kda
interferon inducible mx protein
ubiquitin-like protein 1
probable atp-dependent rna helicase dhx58
mx2 protein
radical s-adenosyl methionine domain-containing protein 2
autophagy-related protein 9a
nedd8-like
ribonuclease-like 3
radical s-adenosyl methionine domain-containing protein 2
growth arrest and dna-damage-inducible protein gadd45 beta
interferon-induced protein with tetratricopeptide repeats 5-like
autophagy-related protein 9a
mx1 protein
interferon inducible mx protein
tyrosyl-dna phosphodiesterase 2-like isoform x1
retinoic acid-inducible protein i
interferon-induced helicase c domain-containing protein 1
0
4
40
BPA Concentration (ng/embryo)
Color Key
-1
0
1
Value
D
F2
toll-like receptor 3
retinoic acid-inducible protein i
thioredoxin
mx2 protein
mx1 protein
ribonuclease h2 subunit a-like
interferon-induced protein with tetratricopeptide repeats 5-like
ubiquitin-like protein precursor
mx2 protein
bpi fold-containing family b member 2
lipopolysaccharide-binding protein
c-x-c motif chemokine 10-like
lipopolysaccharide-binding protein
radical s-adenosyl methionine domain-containing protein 2
ubiquitin-like protein 1
interferon inducible mx protein
dickkopf-related protein 1-like
eosinophil peroxidase-like
tyrosyl-dna phosphodiesterase 2-like isoform x1
g1 s-specific cyclin-d1
ubiquitin-like protein precursor
heat shock protein hsp 90-alpha
interferon-induced helicase c domain-containing protein 1
fibrinogen gamma chain
probable atp-dependent rna helicase dhx58
heat shock cognate 70 kda protein
tapasin-like isoform x1
c-c motif chemokine 19 precursor
c-c motif chemokine 19 precursor
protein arginine n-methyltransferase 6
eosinophil peroxidase-like
heat shock protein hsp 90-alpha 1
nuak family snf1-like kinase 1
heat shock cognate 71 kda
hepcidin-1 precursor
type ii cytoskeletal 8-like
tyrosine-protein kinase srk2
tpa_inf: tachykinin 4
toll-like receptor 5
suppressor of cytokine signaling 2-like
brca1-a complex subunit abraxas
growth arrest and dna damage-inducible protein gadd45 alpha-like
sestrin-1 isoform x2
aryl hydrocarbon receptor-like
coagulation factor x-like
tumor necrosis factor alpha-induced protein 3
cyclin-dependent-like kinase 5
protein disulfide-isomerase a3-like
kynureninase isoform x1
transient receptor potential cation channel subfamily v member 1 isoform x1
liver-expressed antimicrobial peptide 2-like
endonuclease 8-like 1 isoform x1
tyrosine-protein phosphatase partial
tissue factor
toll-like receptor 13
apolipoprotein a-iv-like
growth arrest and dna damage-inducible protein gadd45 gamma
histone-lysine n-methyltransferase partial
proteinase-activated receptor 1
activated cdc42 kinase 1 isoform x1
copper chaperone for superoxide dismutase
fanconi anemia group c protein isoform x2
prostaglandin g h synthase 1 precursor
dna repair protein complementing xp-c cells
phospholipid hydroperoxide glutathione mitochondrial-like isoform x3
cyclic amp-responsive element-binding protein 3-like protein 3-b
0
4
40
BPA Concentration (ng/embryo)

Figure 3



A
F1
B
F2
BPA Concentration (ng/embryo)
0 4 40 0 4 40 0 4 40 0 4 40
Growth
Stress
Epigenetics
igf1 * 0 < 4; 0 < 40
gh1r
igf2 * 0 < 40
igf1ra
gh2r
igf1rb
gr1
gr2
mr
dnmt2
mat1a
dnmt1
igf1
gh1r
igf2 * 0 > 40
igf1ra * 0 < 40
gh2r
igf1rb
gr1 * 0 > 40
gr2
mr * 0 < 40
dnmt2 * 0 < 40
mat1a
dnmt1
0 h
1 h
4 h
24 h
Down-regulation
1
Up-regulation

Figure 4

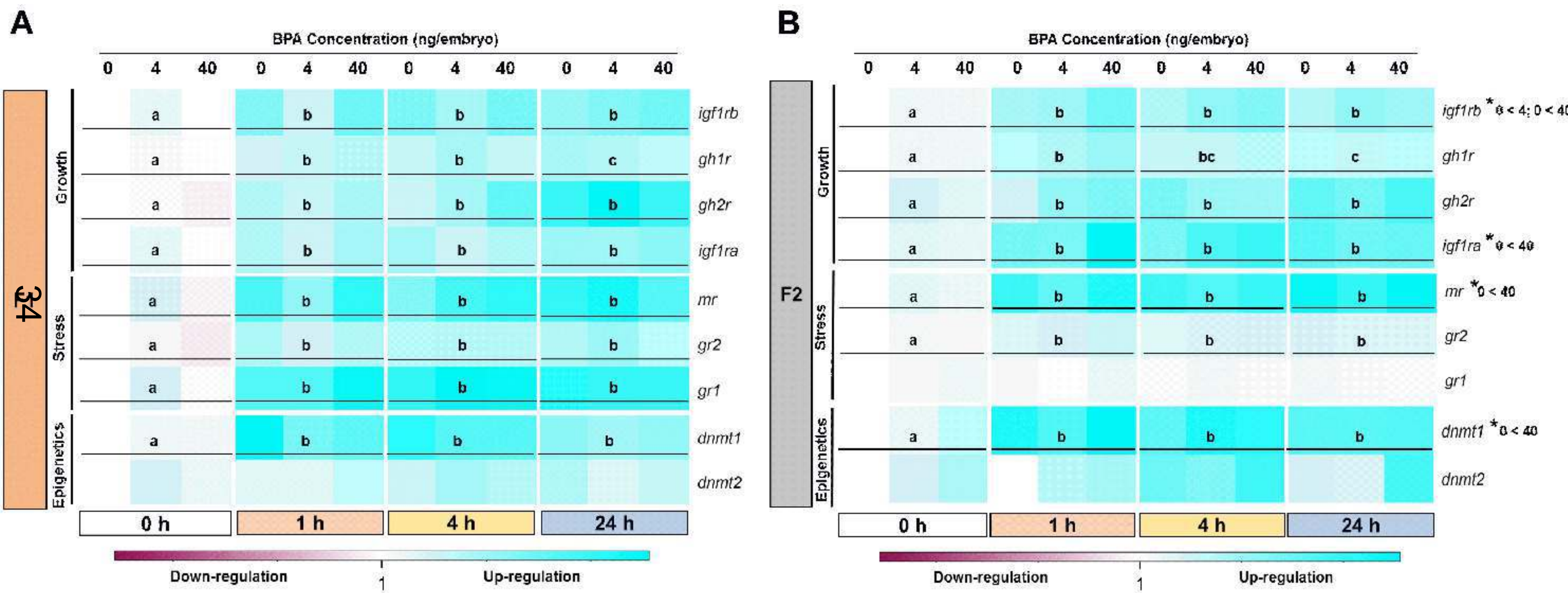
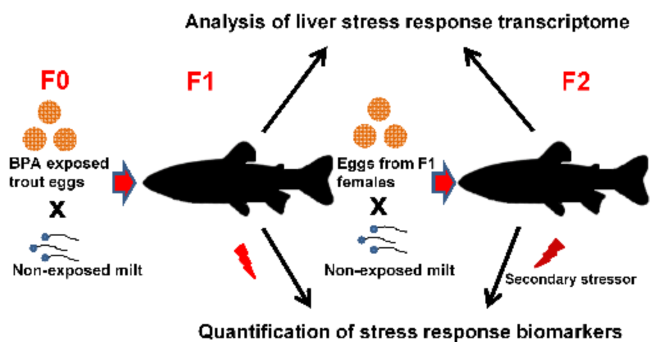

A
BPA Concentration (ng/embryo)
0 4 40 0 4 40 0 4 40 0 4 40
Growth
Stress
Epigenetics
igf1rb
gh1r
gh2r
igf1ra
mr
gr2
gr1
dnmt1
dnmt2
0 h
1 h
4 h
24 h
Down-regulation
1
Up-regulation
B
F2
igf1rb *0 < 4; 0 < 40
igf1ra *0 < 40
mr *0 < 40
dnmt1 *0 < 40

Figure 5


Analysis of liver stress response transcriptome
F0
F1
F2
BPA exposed
trout eggs
X
Non-exposed milt
Eggs from F1
females
X
Non-exposed milt
Secondary stressor
Quantification of stress response biomarkers