Generational Effects of Bisphenol A on Growth and Stress Performance in Rainbow Trout

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The aquatic environment is severely impacted by xenobiotics that are released due to anthropogenic activities, threatening ecosystem health. Some of these contaminants accumulate in lipophilic fish tissues and are maternally transferred to developing offspring, affecting their growth and performance. However, knowledge about the long-term and generational impacts associated with maternal transfer of contaminants is limited in fish. In this thesis, the hypothesis tested was that maternal transfer of bisphenol A (BPA) leads to disruption in the developmental programing of growth and stress axes functioning in rainbow trout (*Oncorhynchus mykiss*), and that these changes are passed on to the next generation. This was tested by exposing oocytes to either control (vehicle; <0.01% ethanol) 0.3, 3.0, and 30.0 mg l^{-1} BPA in ovarian fluid for 3 h, prior to fertilization, to mimic maternal transfer. This led to the accumulation of 0, 0.8, 4.4 and 41.3 ng BPA $embryo^{-1}$. Oocytes were fertilized with milt from clean males, and offspring growth, development and stress performances were assessed in a clean environment for a year (F1 generation). For F2 generation, oocytes collected from F1 females, raised from the different BPA accumulated eggs, were fertilized with milt from clean males and raised in a clean environment for one year as described for F1 generation.

The accumulated BPA in eggs was quickly cleared and it was no longer detected in the F1 embryos at hatch. BPA exposure reduced specific growth rate and increased food conversion ratio in larvae reared from BPA-laden oocytes. Moreover, BPA-exposed fish had an altered cortisol developmental profile and a delay in stress axis maturation. In addition, the mRNA abundance of genes involved in somatotropic [insulin-like growth factor (IGF) -1; IGF-2; IGF receptor b (IGF-1rb)] and stress axes functioning [steroidogenic acute regulatory protein (StAR); cytochrome P450 side chain cleavage (P450scc)] were altered. Also, changes in thyroid signaling [thyroid receptor (TR) mRNA levels] and cortisol signaling [glucocorticoid receptor (GR) protein expression] were disrupted temporally during development. These results demonstrate that BPA accumulation in eggs, mimicking maternal transfer, impacts growth and development, and delays stress axis maturation via non-reproductive endocrine disrupting routes in trout.

Some of the BPA changes seen in F1 generation also persisted in the F2 generation. For instance, ancestral exposure to BPA led to reduced growth and whole body glycogen content prior to feeding in the F2 fish. The developmental transcript profile of growth hormone-1and -2, IGF-1 and -2 and IGF-1rb, along with whole body cortisol levels were impacted by ancestral exposure to BPA. Moreover, a delay in cortisol dynamics post-stress was noted in the F2 fish of BPA exposure lineage. Our results show that ancestral exposure to BPA leads to effects on growth and stress performance in rainbow trout, but the mechanism is not known.

To further investigate the long-term effect of BPA accumulation in eggs on stress performances, F1 and F2 juvenile fish were subjected to an acute stressor. Also, head kidney tissues from these juvenile fish were subjected to adrenocorticotrophic hormone (ACTH) stimulation in vitro to assess cortisol production capacity. BPA accumulation in eggs led to a reduced acute handling stressor-induced plasma cortisol response in trout from the F1 and F2 (only high BPA group) generations. Also, BPA exposure had a pronounced impact on acute handling stressor-mediated plasma glucose (only F2 generation) and lactate levels, indicative of a metabolic disturbance. BPA exposure (only the 4.4 ng group) did affect unstimulated but not stimulated [ACTH or 8-bromo-cyclic AMP (8-B-cAMP)] cortisol production from head kidney slices of juvenile fish from F1 generation. In the F2 generation, there was an increase in ACTH-stimulated cortisol production only from the high BPA-exposed group. Overall, BPA in eggs disrupts long-term cortisol and metabolic stress performances in rainbow trout. While the impaired plasma cortisol stress performance was dose-related in the F1, the effect was apparent only for high BPA group in the F2 generation, suggesting that the generational effects on cortisol stress axis functioning may be concentration-dependent.

A metabolomics approach further confirmed multigenerational effects associated with BPA accumulation in eggs. Analysis of the metabolome profile at hatch and prior to first feed, using gas chromatography-time of flight-mass spectrometry (GC-TOF-MS), revealed a BPA-mediated metabolic disruption, including changes in pathways involved in carbohydrate, lipid and amino sugar metabolism, and amino acid metabolism and synthesis. Pathways involved in citric acid cycle and alanine, aspartate and glutamate metabolism were

altered in both generations, suggesting that these pathways have the potential to be markers with predictive value for multigenerational effects of BPA in fish. Altogether, the study provides novel insights on the impact of BPA on rainbow trout metabolome at hatch and first feed. The results suggest that pathways involved in energy metabolism are targets for BPA impact and should be investigated as potential markers for BPA toxicity.

Overall, BPA accumulation in oocytes induces long-term delays in growth and stress axis maturation in F1 generations fish, and these effects persist in the F2 generation. The developmental profiles of key genes of the somatotropic and HPI axes were altered by BPA, along with whole body composition, suggesting that BPA exposure leads to a metabolic disturbance in fish, resulting in reduced growth. Additionally, the altered plasma cortisol response to acute stress in F1 and F2 juveniles provides evidence for multigenerational effects of BPA on stress axis functioning. The current study proposes that BPA-induced epigenetic modifications during early development may be playing a key role in the generational effects on growth and stress axes disruption in trout. The finding that the growth and developmental changes to BPA exposure also corresponds with endocrine and metabolome changes in multiple generations in trout is novel, and underscores the necessity to develop new risk assessments tools for chemicals that are maternally transferred in fish.

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I am especially grateful to my supervisor, Dr. Matt Vijayan, without whom this project never would have come to life. His constant guidance and support over the past several years have made me believe in this project and take it to completion. I am sincerely thankful to my committee members for their constant guidance and wonderful discussions over the years. In particular, I am grateful to Dr. Mark Servos for his support and assistance with analysis; to Dr. John Leatherland for the entertaining hallway discussions on IGF signaling, and to Dr. Heidi Engelhardt for her challenging questions on epigenetics. I would also like to extend my gratitude and appreciation to past and current members of the Vijayan lab, who have graciously taken time away from their work to assist me with sampling. In particular, I would like to thank Dr. Laura Dindia for providing insightful comments on my thesis writing. Lastly, a big thank you to all of my friends and family for their encouragement and support. In particular, I am grateful to my wonderful family, for always putting a smile on my face.

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8-B-cAMP – 8-bromo-cyclic adenosine monophosphate

3β-HSD – 3β-hydroxysteroid dehydrogenase

AARS – Alma Aquaculture Research Station

ACTH – adrenocorticotropic hormone

ANOVA – analysis of variance

BPA – bisphenol A

BPA-G – bisphenol A-glucuronide

BPAGA – bisphenol A glucuronic acid

CF – condition factor

CRF – corticotropin releasing factor

CV-ANOVA – cross-validated analysis of variance

dpf – days post-fertilization

DWTP – drinking water treatment plant

dw – dry weight

E2 - 17β estradiol

EF1α – elongation factor 1α

EDC/S – endocrine disrupting compound/substance

ER – estrogen receptor

FCR – food conversion ratio

FSH-β – follicular stimulating hormone β

GC-TOF-MS – gas chromatography-time of flight-mass spectrometry

GH – growth hormone

GHr – growth hormone receptor

GH-RH – growth hormone releasing hormone

GnRH – gonadotropin releasing hormone

GR – glucocorticoid receptor

HPI axis – hypothalamus-pituitary-interrenal axis

HPLC – high-pressure liquid chromatography

HSP90 – heat shock protein 90

IGF – insulin-like growth factor

IGFr – insulin-like growth factor receptor

LDH – lactate dehydrogenase

LH – luteinizing hormone

MC2R – melanocortin-2 receptor

MFC – median full range

MR – mineralocorticoid receptor

MSEA – metabolite set enrichment analysis

MS222 – tricaine methanesulfonate 222

OPLS-DA – orthogonal partial least squares discriminant analysis

P450scc – cytochrome P450 side chain cleavage

P450c17 – 17α-hydroxylase

PCA – principal component analysis

PCR – polymerase chain reaction

PKA – protein kinase A

PKC – protein kinase C

qRT-PCR – quantitative real-time polymerase chain reaction

QEA – quantitative enrichment analysis

RIA – radio-immuno assay

SEM – standard error of the mean

SGR – specific growth rate

ss – somatostatinergic system

SOP – standard operating procedure

StAR – steroidogenic acute regulatory protein

TCA – citric acid cycle

TDI – tolerable daily intake

TH – thyroid hormone

TR – thyroid hormone receptor

US EPA – United States Environmental Protection Agency

VTG – vitellogenin

WHO – World Health Organization

ww – wet weight

WWTP – wastewater treatment plant

Chapter 1: General Introduction

1.1 Introduction

Growth and stress response in fish are mainly mediated by the activation of somatotropic and hypothalamus-pituitary-interrenal (HPI) axes, respectively, leading to physiological responses that are highly conserved in vertebrates (Björnsson et al., 2004; Nesan & Vijayan, 2013; Reinecke, 2010; Vijayan et al., 2010). Recent studies have identified the growth and stress axes as targets for xenobiotics impact in fish, and exposure during early developmental stages appears to elicit long-term and possibly permanent alterations in their functioning, bringing about defects in growth and stress performances in fish (McCormick et al., 2010; Aluru et al., 2010; Corrales et al., 2014). Exposures during early developmental stages in fish are of great concern, as contaminants have been detected in oocytes of wild fish (Ostrach et al., 2008) and can, thus, impact the developmental programing of the somatotropic and HPI axes, but the long-term implications are unknown.

Bisphenol A (BPA), an organic contaminant with endocrine disrupting properties, is a bi-product of plastic degradation and it enters the aquatic environment via both point (specific location of chemical input) and non-point (run-off) sources (Cao et al., 2008; Cao et al., 2009). Studies have shown that BPA can mimic the action of the female hormone 17β-estradiol (E2; Kang et al., 2002), but its effects are not limited to its interaction with the estrogen receptor (Vandenberg et al., 2009). BPA is known to have a plethora of effects in aquatic vertebrates, including impaired sperm motility in male fish (Hatef et al., 2010), alterations in thyroid hormone signaling in amphibians (Heimeier and Shi, 2010), developmental defects in zebrafish (*Danio rerio*; Duan et al., 2008; McCormick et al., 2010), and reduced growth and gonad size in fathead minnows (*Pimephales promelas*; Sohoni et al., 2001). While BPA in the water can accumulate in embryos of salmonids (Honkanen et al. 2001), recent studies have shown that this chemical can also be transferred from the mother to the offspring, and persist in the embryo during key developmental stages (Takao et al., 2008; Aluru et al., 2010).

A recent study in our laboratory showed that BPA accumulation in trout oocytes, mimicking maternal transfer, disrupted growth and stress response in juvenile rainbow trout (*Oncorhynchus mykiss*), thereby identifying the somatotropic and HPI axes as potential targets of

BPA toxicity during development (Aluru et al., 2010). The results from this study suggests that embryonic BPA exposure has long-term implications for growth and development, possibly through modifications of somatotropic and HPI axes programming during embryogenesis. Research to date has analyzed the long-term effects of BPA exposure during embryonic development in fish (Sohoni et al., 2001; Duan et al., 2008; McCormick et al. 2010), but few have looked at how maternal deposition of this chemical impacts subsequent generations. Therefore, the purpose of this thesis was to investigate whether the long-term effects associated with BPA accumulation in oocytes noted by Aluru et al. (2010) were due to re-programming of the growth and HPI axes during development, and whether these alterations persist in subsequent generations of rainbow trout.

1.2 A brief review of the life cycle of the rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) has been widely used as a model organism for toxicological research. However, few studies have looked at the multigenerational effects of contaminants in this species (Aluru et al., 2010). One of the main reasons for such scarce research in this area has to do with the life cycle of the trout as it takes ~3 years to reach reproductive maturity. This section gives a brief overview of the rainbow trout life cycle, in an attempt to familiarize the reader with the various life stages that are mentioned throughout this thesis.

Mature rainbow trout spawn in early to late spring, in shallow, well oxygenated rivers, at a water temperature of 6-8 ˚C (Behnke, 2002). Prior to spawning, a female uses its tail to dig a depression in the gravel, called a red, where the eggs are deposited and quickly fertilized by an attending male (Staley and Mueller, 2000). Within 10-14 days, most of the embryos develop sufficiently for the eyes to be seen, and by 28 days all the embryos have reached the eyed stage. Hatching in rainbow trout occurs anywhere between 1-3 months from fertilization and it is dependent on the water temperature; higher temperatures increasing the developmental rate (Raine et al., 2004; Zubair et al., 2011). Once hatched, the larvae (alevin or yolk-sac fry) remain in the gravel until first feed, at which time point the yolk sac is reabsorbed and exogenous feeding begins. First feed usually occurs within 10-20 days after hatching, when the larvae emerge from the gravel as fry (often referred to as swim-up fry; Michael Burke, AARS, personal

communication). When the fish are about one year old they are termed the fingerlings, and then they are called juveniles from >1.5 years. The juveniles have all the markings of an adult rainbow trout, but are reproductively immature, and maturity is reached by 3 - 4 year after fertilization. The timing of the various developmental stages in rainbow trout is highly dependent on both endogenous and exogenous cues, including maternal deposition of hormones (e.g. growth hormone, cortisol and thyroid hormone), water temperature and light cycle (Barry et al., 1995; Reincke et al., 2005; Reinecke, 2010). Integration of these signals is critical for proper development of the embryos, and this thesis will focus on two key endocrine axes, the somatotropic and the hypothalamus-pituitary-interrenal (HPI) axes.

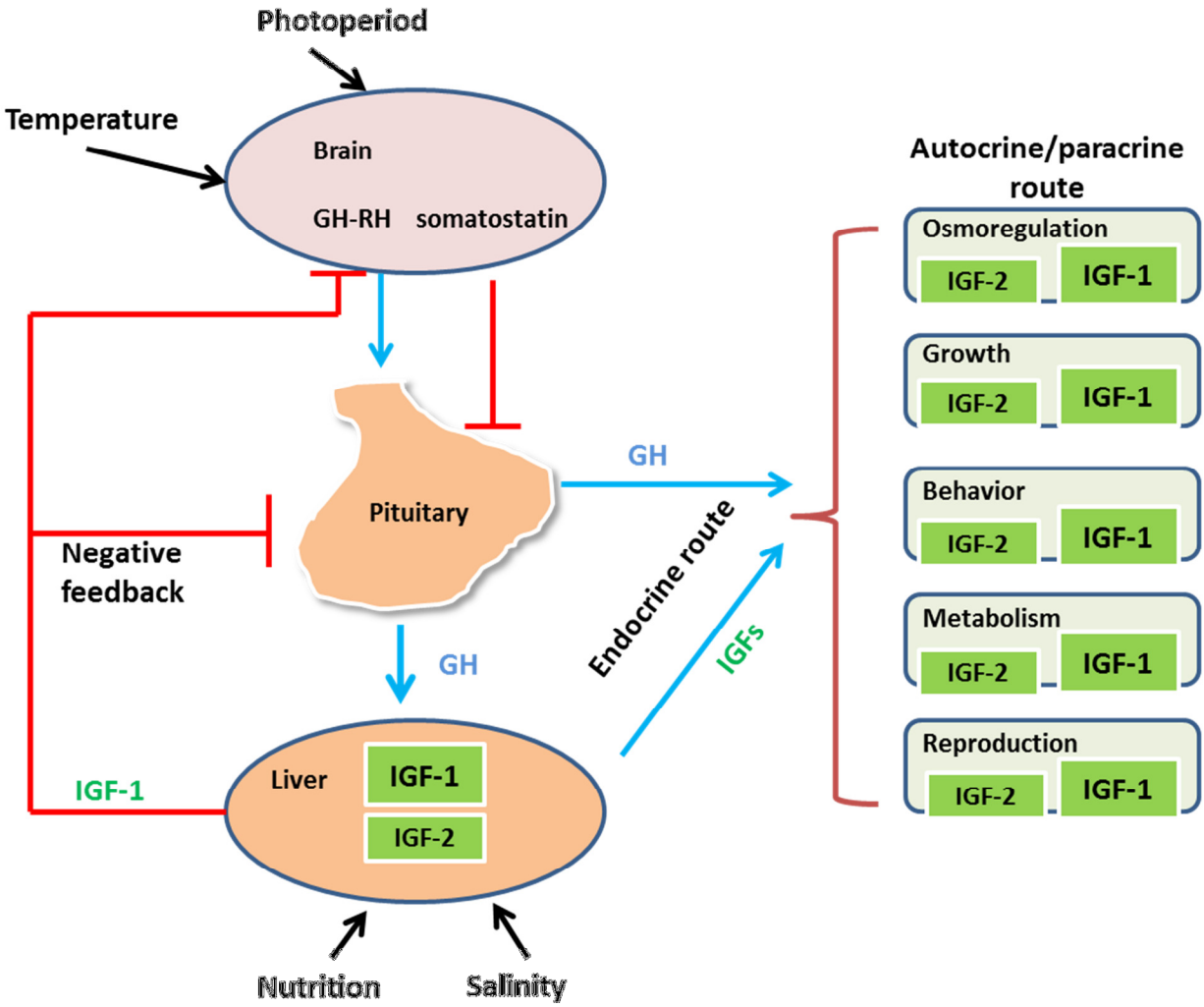
1.3 The somatotropic axis in teleosts

The somatotropic (growth) axis in teleosts is highly dependent on endogenous and exogenous cues perceived by the central nervous system, that bring about effects on growth, development, metabolism and ion regulation (Fig. 1-1). The major hormone involved in this particular endocrine response is growth hormone (GH), and its release is under the control of the hypothalamus. In teleosts, the GH-producing somatotrophs lie in the vicinity of these hypothalamic fibers in the anterior pituitary (Björnsson et al., 2002). Once a cue is received by the brain, the primary secretagogues, GH releasing hormone (GH-RH) and dopamine (DA), signal the pituitary to produce and release GH into the circulation (Luo et al. 1989; Agústsson and Björnsson 2000). GH targets a multitude of organs and it exerts its actions via the GH receptors (GH-r), leading to activation of intracellular pathways that mediate cellular metabolism, enzyme activity and production of insulin-like growth factor-1 (IGF-1) and IGF-2 (Björnsson et al., 2002).

In fish, the liver and muscle have been identified as major targets of GH and IGF-1, although these hormones have been shown to target other tissues, particularly the ones involved in osmoregulation (Yao et al., 1991; Pérez-Sánchez et al., 2002; Reinecke et al., 2005). While the liver and muscle have a greater abundance of receptors for GH (GH-r) and IGF-1 (IGF-1r), they are also widely distributed in a variety of other tissues in fish (Reinecke et al., 2005, 2010). The interaction of GH and IGF-1 leads to growth and developmental effects, changes in metabolism, osmoregulatory and ionoregulatory capacity and behavior, as well as sexual maturation in fish

Figure 1-1. The somatotropic (growth hormone/insulin-like growth factor 1; GH/IGF-1) axis in teleosts.

Following the perception of an environmental cue (e.g. light cycle, temperature, etc.), or signals from endogenous rhythms, or negative feed-back from biological processes (e.g. energy metabolism), neural, neuroendocrine and endocrine signals are integrated in the central nervous system and in the pituitary to regulate growth hormone (GH) secretion (Björnsson et al., 2002). Once the levels of GH in the circulation have increased due to release from the pituitary, ligand-receptor interaction occurs at the level of target tissues, which, depending on the cell type, leads to stimulation of intracellular signaling pathways that mediate enzyme activity, cellular metabolism and activity, and secretion of insulin-like growth factor 1 (IGF-1). In teleosts, the liver appears to be a major target organ for GH (Björnsson et al., 2002). Both GH and IGF-1 stimulate growth, osmoregulation, energy metabolism, behaviour and reproductive maturity in fish. Similarly, both hormones are involved in the negative feed-back that regulates GH secretion from the pituitary, by acting directly on their respective receptors in the brain and pituitary. Figure adapted from Reinecke (2010). Blue arrows indicate stimulation of hormone release via and endocrine route. Red lines represent suppression of GH release from the pituitary, either via negative feedback from circulating IGF levels and the subsequent suppression of GH-RH, or through release of somatostatin from the brain.


Photoperiod
Temperature
Brain
GH-RH somatostatin
Pituitary
GH
Negative feedback
GH
Endocrine route
IGFs
IGF-1
Liver
IGF-1
IGF-2
Nutrition
Salinity
Autocrine/paracrine route
Osmoregulation
IGF-2
IGF-1
Growth
IGF-2
IGF-1
Behavior
IGF-2
IGF-1
Metabolism
IGF-2
IGF-1
Reproduction
IGF-2
IGF-1

(Reinecke et al., 2005; Reinecke, 2010). In addition, IGF-1 regulates GH secretion by negative feed-back (Fig. 1-1; Blaise et al., 1995; Ágústsson and Björnsson, 2000).

1.4 Growth hormone

Growth hormone plays a central role in somatotropic axis signaling. GH is secreted by the pituitary in response to stimuli received from the hypothalamus, which integrates exogenous (i.e. as salinity, photoperiod, temperature) and endogenous cues (i.e. immune response, negative feedback from other processes), to bring about a somatotropic axis response (Reinecke et al., 2005). GH secretion is regulated by hypothalamic peptides and neurotransmitters. Somatostatin is a strong inhibitor of GH release, as studies have reported lower GH secretion in its presence *in vitro* (Agustsson et al., 2000). Growth hormone releasing hormone (GH-RH) and dopamine, are GH-secretagogues in salmonids. In trout, treatment with GH-RH induced GH release (Luo and McKeown, 1991; Blaise et al., 1995), while dopamine increased GH levels in the pituitary and attenuated the effects of somatostatin in salmonids (Agustsson et al., 2000). Other peptides, such as ghrelin and gonadotropin-releasing hormone (GnRH), are known to induce GH release in fish tissues. GH secretion by the pituitary increases in the presence of GnRH (Holloway and Leatherland, 1997), underscoring the important role that GH plays in fish maturation. Indeed, GH levels have been shown to increase in maturing fish, along with an increase in GnRH (Bjornsson et al., 2002). The link between ghrelin and GH release establishes a role for the somatotropic axis in modulating growth during long-term food deprivation and following periods of starvation (Bjornsson et al., 2002).

1.5 Insulin-like growth factor (IGF)

The action of GH at the tissue level is mediated by the GH receptor, GH-r. In fish, GH targets a multitude of organs, leading to activation of intracellular pathways by binding to the two GH receptors, GH-1r and GH-2r (Reinecke et al., 2005). While the principal action of GH in fish is activation of the synthesis and release of insulin-like growth factor 1 (IGF-1) and 2 (IGF-2), mainly from the liver (Reinecke et al., 2005; Reinecke, 2010), the presence of GH receptors in fish tissues suggest that this hormone can also act directly on the tissues, and not solely through induction of IGF (Reinecke, 2010). Together with GH, IGFs are involved in metabolism, osmoregulation, growth and development (Reinecke, 2010). The action of IGFs occurs via the

two IGF receptors (IGF-1ra and IGF-1rb) in the liver and other tissues. While GH is the main secretagogue for IGF-1, studies have suggested that IGF-1 can be regulated independently of GH, as IGF receptors have been identified in several tissues in fish (for a thorough review, see Bjornsson et al., 2002). At the level of the tissues, IGF can mediate process locally, thorough autocrine/paracrine pathways. A clear role for IGF-1 has been established in growth and metabolic processes, as fish treated with IGF-1 implants showed growth and metabolic effects (McCormick et al., 1992; Castillo et al., 2004). Fish muscle has been shown to have a great abundance of IGF-1r transcripts, underscoring the role of IGF-1 in regulating muscle development (Castillo et al., 2002). In addition to IGF-1, IGF-2 also mediated growth and development, but its role is more apparent during early developmental stages (Li et al., 2010) and it is not entirely understood.

1.6 Stress response and the hypothalamus-pituitary-interrenal (HPI) axis in fish

In fish, a series of generalized physiological and biochemical changes occur in response to endogenous (i.e. infection) or exogenous (i.e. handling, crowding, predation, changes in salinity, nutrition, chemical exposure) stressors (Mommsen et al., 1999; Iwama et al., 2006; Flik et al., 2006). These changes attempt to restore homeostasis and/or compensate for the challenge that the organisms are faced with. In fish, the stress response has been categorized into primary, secondary and tertiary responses.

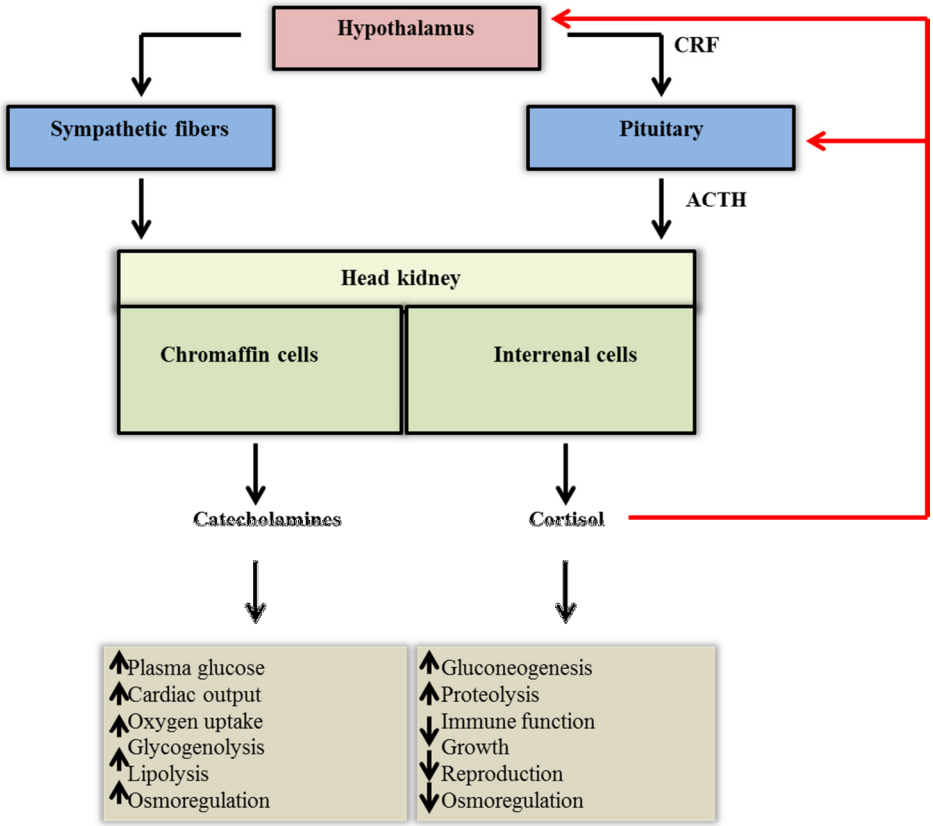
The primary stress response involves the initial perception of a stressor by the brain and the activation of the neuroendocrine response associated with the general stress response pathway (Fig. 1-2). Upon perception of an endogenous or exogenous cue, the hypothalamic nuclei involved in stress response, process and evaluate the information, directing the production of pituitary adrenocorticotropic hormone (ACTH) via the release of corticotropin-releasing factor (CRF; Flik et al., 2006; Fuzzen et al., 2011). In addition to CRF signaling, the brain also sends signals via the sympathetic fibers (Fig. 1-2), to induce a rapid release of catecholamines from the chromaffin cells of the head kidney (the equivalent of the mammalian adrenal gland; Mommsen et al., 1999) and from the adrenergic nerves (Randall and Perry, 1992). This stress response is more rapid (within seconds to minutes) than the response mediated by the activation of the hypothalamus-pituitary-interrenal (HPI) axis (within minutes to hours; Iwama et al., 2006).

Within the HPI axis, CRF acts on the anterior pituitary to release ACTH in the circulation (Flik et al., 2006; Fuzzen et al., 2011), which then induces *de novo* synthesis and secretion of cortisol by binding to the melanocortin-2-receptor (MC2R) in the interrenal cells of the head kidney (Iwama et al., 2006; Aluru and Vijayan, 2009; Alsop and Vijayan, 2009). Upon their release into the circulation, catecholamines and cortisol mediate a variety of physiological and biochemical changes that are part of the secondary stress response, assisting organisms to cope with stress. These changes include enhanced metabolic capacity, effects on osmoregulation and ionoregulation, increased cardiac output and altered energy metabolism (Vijayan and Moon, 1994; Vijayan et al., 1996; Mommsen et al., 1999; Aluru et al., 2010). If the stressor persists, it will compromise the health performance of the organism leading to tertiary responses, including altered growth and development, increased disease susceptibility and morality. This will be reflected not only at the individual levels, but also will lead to population declines (Barton, 2002).

HPI axis development has been well studied in teleosts. Cortisol in oocytes has been established to be of maternal origin in various fish species, including rainbow trout (Barry et al., 1995; Auperin and Geslin, 2008) and zebrafish (Nesan and Vijayan, 2012; Nesan and Vijayan, 2013), but the levels decrease during embryo development. In trout, endogenous cortisol production begins around hatching (de Jesus and Hirano, 1992; Auperin and Geslin, 2008), when interrenal cells differentiate in the head kidney (Barry et al., 1995). However, trout are not able to elicit a cortisol stress response until several days to weeks after hatch, due to a delay in HPI axis maturation (Barry et al., 1995; Auperin and Geslin, 2008). This delay has also been reported in other fish species, including zebrafish (Alsop and Vijayan, 2008) and perch (*Perca fluviatili*; Jentoft et al., 2003), and it appears to be well conserved in higher vertebrates (Nesan and Vijayan, 2013). The activity of the HPI axis increases around first-feed, suggesting that HPI axis maturation may be associated with the transition from endogenous to exogenous feeding, as

Figure 1-2. The stress axis in fish.

Upon the perception of a stressor, the hypothalamus signals the chromaffin cells in the head kidney via the sympathetic fibers, to induce a rapid (within seconds to minutes) response. Consequently, the chromaffin cells produce catecholamines, which rapidly induce physiological and biochemical changes in response to the challenge. In addition, the hypothalamus releases corticotropin-releasing factor (CRF) to induce adrenocorticotropic hormone (ACTH) release from the pituitary. ACTH then targets the interrenal (steroidogenic) cells in the head kidney and induces cortisol production by binding to the membrane receptor melanocortin-2- receptor (MC2R). Once in the general circulation, cortisol induces a variety of biochemical changes in response to stress. In addition, cortisol controls its own production and secretion by negative feed-back to the brain and pituitary (red arrows), to induce a decrease in CRF and ACTH production and secretion, respectively.


Hypothalamus
CRF
Sympathetic fibers
Pituitary
ACTH
Head kidney
Chromaffin cells
Interrenal cells
Catecholamines
Cortisol
Plasma glucose
Cardiac output
Oxygen uptake
Glycogenolysis
Lipolysis
Osmoregulation
Gluconeogenesis
Proteolysis
Immune function
Growth
Reproduction
Osmoregulation

proper regulation of metabolic pathways may be needed during periods of fasting (Barry et al., 1995).

1.7 Cortisol biosynthesis in fish

As previously mentioned, cortisol production in fish occurs at the level of the head kidney, in the interrenal cells (Mommsen et al., 1999; Iwama et al., 2006; Aluru and Vijayan, 2009; Alsop and Vijayan, 2009). Once ACTH binds to MC2R on the steroidogenic cell membrane, the G-protein response pathway is activated, leading to production of cyclic adenosine monophosphate (cAMP) from ATP by the adenylyl cyclase (Fig. 1-3). Protein kinase A (PKA) is then activated, leading to the phosphorylation of the cytoplasmic steroidogenic acute regulatory protein (StAR), which shuttles cholesterol across the mitochondrial membrane and into the matrix. Once in the mitochondrial matrix, cholesterol is converted to pregnenolone by cholesterol side chain cleavage enzyme (P450scc), the first rate-limiting step in corticosteroid synthesis (Mommsen et al., 1999; Aluru and Vijayan, 2008). Pregnenolone is then transported outside of the mitochondrion and into the endoplasmic reticulum, where 17α-hydroxylase (P450c17) converts it to 17-OH-pregnenolone. 17-OH-pregnenolone is then transported back into the mitochondrion, where 3β-hydroxy steroid dehydrogenase (3β-HSD) converts it to 17-OH-progesterone. The last steps of corticosteroid biosynthesis are mediated by P450c21 and P450c11, after which cortisol is produced and released into the circulation (Fig. 1-3; Mommsen et al., 1999).

1.8 Genomic signaling of cortisol

The action of cortisol is wide ranging in fish (Mommsen et al., 1999), but in response to stress the primary role is to bring about physiological and biochemical changes aimed at adapting to the stressor challenge. These include changes in osmoregulation, immune response, metabolism, growth and reproduction (Vijayan et al., 1996; Mommsen et al., 1999; Vijayan et al., 2005; Aluru et al 2010). The signaling pathway for cortisol involves the activation of the glucocorticoid receptor, a ligand-activated transcription factor that binds to the promoter region of target genes and regulates their transcription. Because this pathway involves gene regulation, it is called the genomic pathway of steroid action (Bury and Sturm, 2007). The genomic pathway has been well characterized and it involves ligand-activated transcription factors that belong to

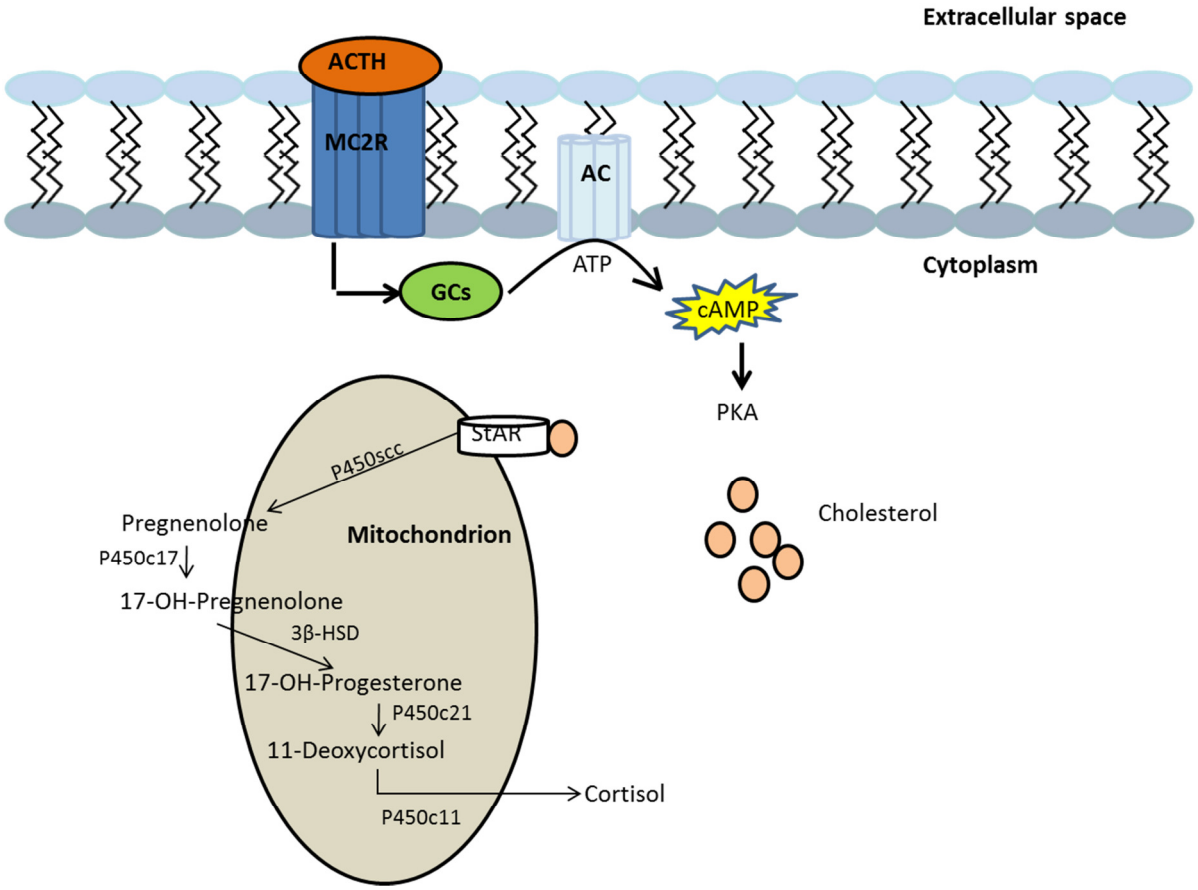


Figure 1-3. Cortisol biosynthesis in fish.

ACTH binding to the membrane receptor MC2R leads to an increase in cAMP, which stimulates StAR to shuttle cholesterol from the cytoplasm into the mitochondria. Through a series of hydroxylations and cleavages, cholesterol is converted to cortisol, which is then released in the cytoplasm. Reactions occurring inside the mitochondria are depicted in the shaded area delimited by the organelle, while those occurring in the cytosol are shown outside of the mitochondrion. Abbreviations: GCs: G-proteins; ACTH: adrenocorticotropic hormone; MC2R: melanocortin 2 receptor; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; StAR: steroidogenic acute regulatory protein; P450scc: cytochrome P450 side chain cleavage; 3β-HSD: 3β-hydroxysteroid dehydrogenase. Figure adapted from Mommsen et al. (1999).

the steroid nuclear receptor family (Mommsen et al., 1999; Aluru and Vijayan, 2009). Cortisol binds to both nuclear receptors: glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), although the role of the latter in mediating cortisol effects is not well understood (Vijayan et al., 2005; Prunet et al., 2006; Iwama et al., 2006; Alsop and Vijayan, 2008; Aluru and Vijayan, 2009).

At the target tissue, cortisol diffuses through the plasma membrane of target cells into the cytoplasm, where it binds to GR (Fig. 1-4). Several studies have shown that GR transcript levels are increased by stress and cortisol administration, further emphasizing the importance of this receptor in the cortisol mediated stress response (Sathiyaa and Vijayan, 2003; Vijayan et al., 2005). Upon binding to GR, the cortisol-GR heterocomplex is translocated into the nucleus, where it binds to the glucocorticoid response element (GRE) in the promoter region of genes, leading to increased or decreased expression of those particular genes involved in various physiological processes, which have been previously summarized (Vijayan et al., 2005; Prunet et al., 2006; Aluru and Vijayan, 2009).

Of great importance to cortisol signaling are the cofactors/coactivators that are involved in steroid signaling, such as the heat shock proteins (HSPs). These are small-molecular weight proteins that act as chaperones for various enzymes and ligands. With respect to GR signaling, HSP70 and HSP90 have been shown to play crucial roles. Sathiyaa and Vijayan (2003) have shown that GR degradation was increased when HSP90 was blocked from binding to the receptor. HSP90 is believed to aid in folding and maturation of GR, prior to ligand binding, and lead to a decrease in proteosome degradation of the receptor (Sathiyaa and Vijayan, 2003). While the role of HSP90 in GR signaling is clear, the role of other HSPs in fish has not been thoroughly investigated. Basu et al. (2001) have shown that there is an association between HSP70 and GR in rainbow trout liver, while Boone & Vijayan (2002) have shown that the glucocorticoid-induced glucose release *in vitro* was reduced in the presence of high levels of HSP70, suggesting that the roles that HSPs play in GR signaling might be dependent upon the stress levels of the cell (Iwama et al., 2006).

While few studies have shown the interaction of cortisol with MR *in vivo*, it is clear that the mode of action of cortisol is mediated through the various isoforms of the GR receptor in trout (Vijayan et al., 2005; Aluru and Vijayan, 2009). In teleosts, there are two genes encoding

for GR (GR1 and GR2), with the exception of zebrafish, that have only one GR-encoding gene (Bury et al., 2003; Alsop and Vijayan, 2009; Nesan and Vijayan, 2013). Although a distinct role for each of the GR-encoding genes has not yet been elucidated, GR1 and GR2 activation is dependent on the circulating cortisol levels in the body: GR2 is more sensitive, being activated at lower cortisol levels, while GR1 is activated at higher cortisol levels brought upon by a stressor challenge (Bury et al., 2003; Prunet et al., 2006; Alderman et al., 2012). While trout GR mRNA and proteins are detected in all major tissues, recent studies have shown that central and peripheral GR is essential in the negative regulation of plasma cortisol levels (Alderman et al., 2012).

In addition to the activation of genomic pathways leading to physiological effects, cortisol also acts on the central nervous system by binding to GR to regulate its own production via a negative feed-back pathway (Fig. 1-2). In the brain, cortisol reduces CRF mRNA abundance in the pre-optic area in rainbow trout (Doyon et al., 2006) and in goldfish (Bernier et al., 1999), and it appears to reduce (Doyon et al., 2006) and maintain basal (Alderman et al., 2012) neuropeptide Y levels in rainbow trout. In addition, cortisol also modulates StAR transcript levels in the interrenal tissue, thus regulating its own biosynthesis (Alderman et al., 2012).

In peripheral tissues, the effects of glucocorticoids on metabolism have been well documented (Aluru and Vijayan, 2009). Briefly, in teleosts, GR signaling in the liver has been shown to be involved in energy metabolism, cell stress response, reproduction and metabolism of xenobiotics (Wiseman et al., 2007; Aluru and Vijayan, 2007; 2009). Moreover, cortisol has recently been shown to play a critical role in teleost development (Nesan and Vijayan, 2013). Knock down of GR using morpholinos in zebrafish led to defects in mesoderm formation and muscle development (Nesan et al., 2012). Also, abnormal cortisol levels during embryogenesis led to developmental defects, including cardiac malformation and impaired stress axis function (Nesan and Vijayan, 2012, 2013). Altogether, cortisol is very important for early developmental programming in zebrafish, and any disruption in cortisol levels during embryogenesis may have long-term implications (Nesan et al., 2012).

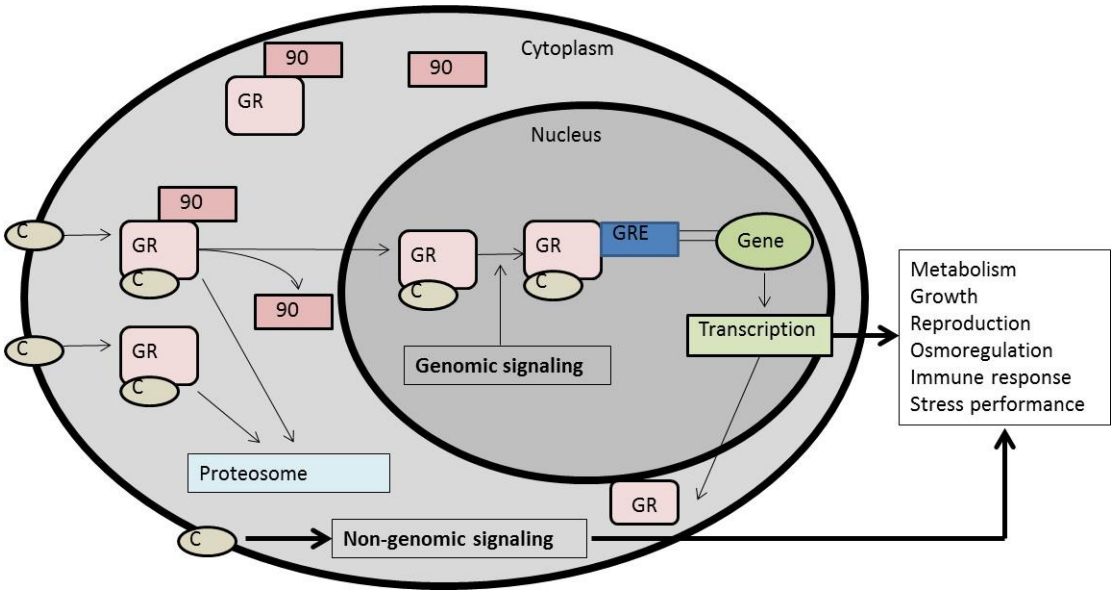


Figure 1-4. Genomic pathway of cortisol action.

Cortisol (C) diffuses through the plasma membrane into the target cell. Cortisol binds to GR/HSP90 complex in the cytosol. The cortisol-GR complex is then translocated into the nucleus, where it binds to GRE in the promoter region of target genes, leading to an increase or a decrease in gene expression. HSP90 (90) is released from the cortisol-GR complex prior to nuclear translocation. Cortisol production also induces GR transcription in the nucleus. Once GR is released from the nucleus into the cytosol, HSP90 acts as a chaperone by preventing GR degradation by the proteasome and aiding in maturation of the receptor. In addition to genomic signaling, cortisol is believed to also induce non-genomic signaling pathways that are involved in a variety of physiological processes. Figure adapted from Vijayan et al. (2005), Iwama et al. (2006), Aluru and Vijayan (2009).

1.9 Non-genomic signaling of cortisol

In addition to genomic effects, cortisol has been shown to elicit additional, more rapid non-genomic actions, that may play a role in stress adaptation (Borski, 2000; Dindia et al., 2012). Studies have shown that the non-genomic action of glucocorticoids in general, and cortisol in particular, involves plasma membrane receptors (although none are currently known), and changes in membrane fluidity that rapidly activate various metabolic signaling pathways (Lösel et al., 2003; Lösel and Wehling, 2003; Dindia et al., 2012). Lösel and Wehling (2003) have proposed that steroid action, in addition to the genomic signaling, can occur via two non-genomic pathways: one that is through a classical steroid membrane receptor and it is second-messenger mediated, and another that occurs through non-classical receptors. Indeed, Dindia et al. (2012) have clearly shown, for the first time, that stress levels of cortisol caused rapid changes in plasma membrane fluidity in rainbow trout liver, triggering acute stress-related signaling pathways. The authors have shown that, similar to the action of the known membrane fluidizer benzyl alcohol, cortisol induced rapid phosphorylation of putative protein kinases A and C (PKA and PKC) and Akt, which are involved in the stress-response pathway (Lösel et al., 2003; Lösel and Wehling, 2003). Although the overall scope of the current thesis will not be focusing on the non-genomic signaling of cortisol, it is worthwhile noting the importance of this pathway, as exposure to xenobiotics, such as endocrine disrupting compounds, that alter cortisol production will, undoubtedly, interfere with non-genomic signaling and with the overall stress response of the organism.

1.10 Endocrine disrupting chemicals

There are thousands of chemicals being released into the aquatic environment yearly. The majority of these anthropogenic chemicals are endocrine disrupting substances/chemicals (EDSs or EDCs; Canadian Environmental Protection Act (CEPA), 1999; Vandenberg et al., 2009, 2012; Vandenberg, 2013). According to the World Health Organization (WHO), an EDC is defined as an exogenous substance or mixture that interferes with the functioning of the endocrine systems of organisms, leading to short- and/or long-term disturbances at in the organism, its progeny and, eventually, at the level of the entire population (WHO, 2013). The endocrine system is responsible for regulating a multitude of functions in living organisms, including growth and

development, digestion, sexual differentiation and reproduction. An endocrine disrupting substance binds to or blocks the endocrine receptors, thus impairing the synthesis, secretion, transport, action and metabolism of hormones that are involved in these processes (Daston et al., 2003; Hotchkiss et al., 2008; Vandenberg et al., 2012). According to the 2013 assessment on EDCs conducted by the WHO and the United Nations Environment Program, there are approximately 800 chemicals that are currently either known or suspected to affect the endocrine system, with a very small number of them having been investigated and demonstrated to affect whole organisms (WHO, 2013).

Endocrine disrupting chemicals are released in the environment through both diffuse and point sources. Examples of diffuse sources include urban runoff, degradation of plastics and the conversion of more complex EDCs into less complex ones though natural degradation, while examples of point sources include wastewater treatment plant (WWTP) discharges and emissions into the atmosphere from chemical plants. Examples of endocrine disrupting substances include surfactants, pesticides, preservatives, additive and plasticizers, such as bisphenol A (BPA), to name a few (Daston et al., 2003; Vandenberg et al., 2012).

Over the past ten years, however, there has been a shift in the way scientists analyze the effects of EDCs. While in the past chemical doses higher than or equal to the lowest observable adverse effect level (LOAEL) to humans and wildlife were used in toxicity testing, it has recently become apparent that different approaches need to be taken when analyzing the effects of EDCs: the analysis of low dose effects (WHO, 2013). A low dose effect is defined as any change induced by a compound in the range of human and wildlife exposure (Vandenberg et al., 2012; Vandenberg, 2013; WHO, 2013). Since EDCs behave like hormones, they are active at low doses and are capable of inducing a variety of changes at such low concentrations. Moreover, a variety of EDCs are capable of interacting with each other to produce additive and synergistic effects on a system, while others can affect more than one hormonal pathway by interacting with more than one hormone receptor (Mathews et al., 2001; Kitamura et al., 2005; Zoeller, 2005; vom Saal and Hughes, 2005; Nishizawa et al., 2005; Alonso-Magdalena et al., 2006; Wetherill et al., 2007; Vandenberg et al., 2009, 2012; Li et al., 2012; WHO, 2013).

Along with focusing on the low dose effects of EDCs, a second shift on how scientists analyze chemical exposure has occurred with respect to EDC toxicity. It has become apparent

that adult sensitivity to the exposure does not correlate with the sensitivity of the individual to the chemical during development (Vandenberg et al., 2009). Therefore, timing of exposure has become another factor in how the study of a chemical with potential EDC activity is approached. While the effects on adults appear to dissipate over time, as the exposure is either reduced or eliminated entirely, effects resulting from exposure during critical time points of development are long-lasting and often permanent (WHO, 2013). Indeed, evidence emerging from human and animal studies has identified fetal development and puberty as being highly sensitive periods of development with respect to EDC exposure (WHO, 2013). Correlations have been established between EDC exposure during early development and the increase in incidence of various health conditions in humans, such as cancer, obesity, diabetes, asthma, neurological disorders and decreased fertility rates (WHO, 2013). With respect to wildlife, EDCs have been identified in various tissues, such as in the liver of marine mammals (Houde et al., 2011), in the egg sac of striped bass females (Ostrach et al., 2008), as well as in the muscle, brain and liver of various fish species across the world (Belfroid et al., 2002; Barber et al., 2006; Wang et al., 2011; Flint et al., 2012; Renz et al., 2013). Conditions such as imposex, superimposition of male sexual characters in females, leading to reproduction failure, has been attributed to EDC exposure in gastropods (Lemos and Esteves, 2009). and intersex in fish has been attributed to WWTP effluents, a major source of EDCs (Tetreault et al., 2011).

1.11 A general overview of bisphenol A

Bisphenol A (2,2-bis[4-hydroxyphenyl]propane; BPA; Fig. 1-5a; Table 1-1) is one of the most well–studied EDCs. It was first discovered by A.P. Dianin in 1891, as part of an effort to develop synthetic estrogens to be used for pharmaceutical purposes. In the 1930s, however, it was abandoned due to the higher estrogenic activity of diethylstilbestrol (DES; Dodds and Lawson, 1936; Vandenberg et al., 2009; Vandenberg, 2013). It has since been used in the production of plastics and epoxy resins, which made it ubiquitously present in the environment (Table 1-2, 1-3; Staples et al., 1998; Sajiki and Yonekubo, 2004; Cao et al., 2008; Cao et al., 2009). While BPA is rapidly metabolized by the human body, recent studies have found it in the urine of over 90% of Americans, suggesting that the population is continuously exposed to the chemical (Calafat et al., 2005). In addition, BPA has been found in various water bodies across

the globe (Table 1-3) and in fish tissues (Table 1-4), and it has been correlated with a variety of effects in fish (Table 1-5). It is apparent that the effects of low dose and early life stage BPA exposures, in both humans and wildlife, are of great concern. Therefore, the overall objective of this thesis is to determine whether BPA exposure during critical time points of development in rainbow trout affects growth, development and performance in fish, and whether any changes observed affect a second, naïve generation of trout.

Bisphenol A (Fig 1-5a; Table 1-1) is a white, solid compound that is a common monomer used in the industry to manufacture plastics and epoxy resins, which are then further used as coatings on food containers, as sealants in dentistry, and as antioxidants and stabilizing materials for plastics (Staples et al., 1998; Sajiki and Yonekubo, 2004; Cao et al., 2008, 2009). Residues of BPA can leak out of plastic food containers, particularly at high temperatures and with frequent use (Krishnan et al., 1993; Sajiki and Yonekubo, 2004; Cooper et al., 2011), and accumulate in lipid-rich animal tissues at concentration that have the potential to elicit long-term impacts on development and performance, particularly if exposure occurs during early development (Lindholst et al., 2000; Ostrach et al., 2008; Cao et al., 2008, 2009; Aluru et al., 2010; Wei et al., 2011). In the United States and Europe, the tolerable daily intake (TDI) dose for humans has been established at 50 µg kg^{-1} body weight day^{-1} by the U.S. Environmental Protection Agency (US EPA; US EPA, 1993) and the European Commission Scientific Committee on Food (SCF; EU RAR, 2003), respectively. In Canada, the TDI dose established by Health Canada is at 25 µg kg^{-1} body weight day^{-1} (Health Canada, 2009).

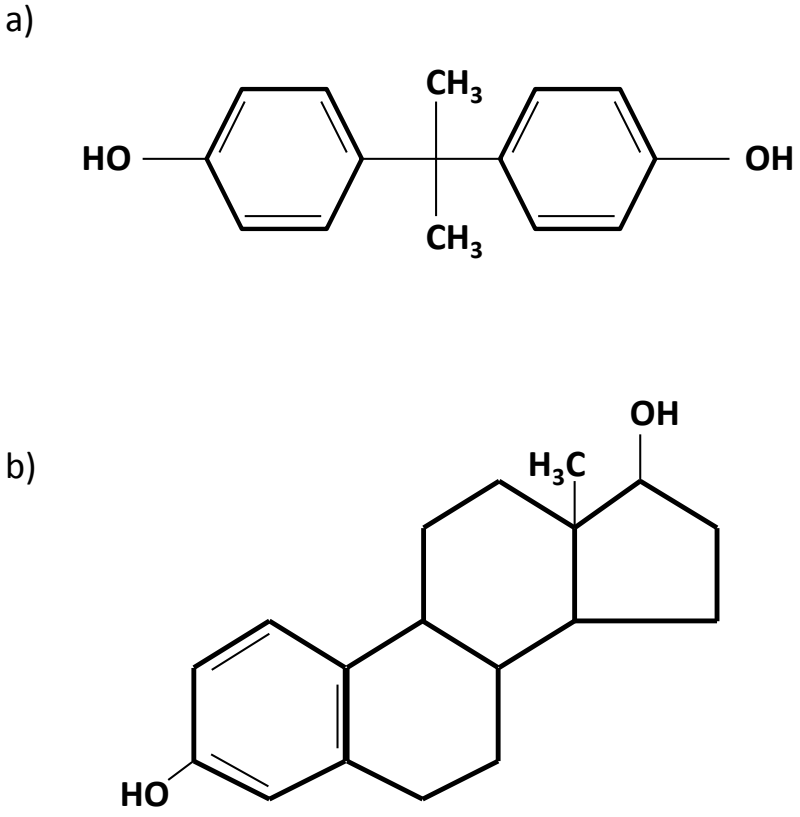


Figure 1-5. The molecular structures of (a) bisphenol A (BPA) and (b) 17β-estradiol (E2).

Bisphenol A has two phenol functional groups, composed of two benzene rings and two –OH substituents, which enable it to fit in the binding pocket of the estrogen receptor (ER) and mimic the effects of the endogenous female hormone, E2.

Table 1-1. Chemical and physical properties of bisphenol A.

Property	Value/description	Reference
CAS #	80-05-07	Sigma Aldrich MSDS
Molecular formula	$C_{15}H_{16}O_2$	
Molecular weight	228.90 g mol^{-1}	
Color	White, solid	
Melting point	158-159 ºC	
Boiling point	220 ºC	
Freezing point	-156.5 ºC	Dorn et al., 1987
Specific gravity	1.195 at 25 ºC	
Vapor pressure	0.2 mm Hg at 170 ºC	
Solubility	120 mg l^{-1} (25 ºC)	
	257 mg l^{-1} (22-24 ºC)	Lee and Peart, 2000
Log K_{ow}	3.3	
	2.2 (exp.), 3.84 (calc.)	Dorn et al., 1987
Half-life	1-4 days in ambient waters	Staples et al., 1998

1.12 On the mode of action of BPA as an endocrine disrupting compound

BPA has been of particular interest to the scientific community for more than a decade due to its xenoestrogenic properties, which enable it to mimic the effects of the female hormone 17β-estradiol (E2; Fig. 1-5b; Krishnan et al., 1993; Crain et al., 2007; Wetherill et al., 2007; Huang et al., 2010; Flint et al., 2012). Bisphenol A has two phenol functional groups, composed of two benzene rings and two –OH substituents (Fig.1-5a), which enable it to fit in the binding pocket of the estrogen receptor (ER) and, hence, mimic the effects of the endogenous female hormone, E2. Several studies have determined that, although BPA binds to both types of ER receptors (ERα and ERβ), it has a higher affinity for ERβ (Gould et al., 1998; Kuiper et al., 1998; Sohoni et al., 2001; Matthews et al., 2001). Recent *in vitro* studies have shown that at low concentrations ($\leq$ 10 nmol l^{-1}), BPA acts as an antagonist of both estrogen receptors, while at higher doses ($\geq$ 10 nmol l^{-1}), it appears to have agonistic properties (Li et al., 2012). Moreover, the type of action that BPA exerts on the estrogen receptors is not only dose-dependent, but also tissue- and life stage-dependent (Wetherill et al., 2007). In addition, BPA toxicity appears to be strongly affected by the route of exposure, as Mathews et al. (2001) found that rats metabolize BPA to the low-estrogenic activity metabolite BPA-glucuronide (BPA-G) faster when BPA is administered by gavage than by subcutaneous injection.

Overall, the interaction of BPA with the estrogen receptors appears to be one of the most well, although not completely, understood modes of action of this chemical. BPA has been shown to affect the availability of endogenous estrogens in rainbow trout, impact estrogen biotransformation and excretion (Jurgella et al., 2006), and ultimately interfere with the mode of action of endogenous estrogens by either mimicking, enhancing or inhibiting their activity (Gould et al., 1998; Kuiper et al., 1998; Lindholst et al., 2000; Matthews et al., 2001; Sohoni et al., 2001; Van den Belt et al., 2003; Olsen et al., 2005; Crain et al., 2007; Kang et al., 2007; Wetherill et al., 2007; Li et al., 2012). In addition to this mode of action, BPA has been shown to affect other endocrine pathways. Studies have shown that BPA exhibits anti-androgenic effects in fish (Sohoni and Sumpter, 1998; Wetherill et al., 2002; Lee et al., 2003; Sun et al., 2006; Sohoni et al., 2001). BPA effects have also been associated with disruptions in thyroid hormone function (Moriyama et al., 2002; Zoeller, 2005; Zoeller et al., 2005; Wetherill et al., 2007), as

well as with impaired development and function of the nervous system (MacLusky et al. 2005; Leranth et al. 2008; Kim et al. 2011).

While BPA is clearly an endocrine disrupting compound, defining it exclusively as a xenoestrogen would be inaccurate. Recent studies have identified additional mechanisms of action of BPA that appear to be independent of the nuclear estrogen receptors, and which tend to differ in magnitude of effect, depending on the dose of BPA (low vs. high), duration of the exposure, and on the life stage of the organism being exposed (Whetherill et al., 2001; Vanderberg et al., 2012). Moreover, BPA has been shown to be transferred from the mother to the offspring *in utero*, suggesting the possibility that BPA toxicity is magnified by exposure during critical periods of development, which can then lead to long-term and possibly multi-generational effects (vom Saal et al., 1998; Yokota et al., 1999; Schönfelder et al., 2002; Demoradzki et al., 2003; Balakrishnan et al., 2010; Nishikawa et al., 2010). Indeed, based on their review of *in vitro* studies conducted on the mode of action of BPA, Wetherill and colleagues (2001) identified cell, tissue and life stage sensitivity as being key aspects to be pursued in future research focusing on the mode of action of BPA. In addition, the authors also suggested low, environmentally relevant doses as being the focus when analyzing the mode of action of this chemical, as BPA exerts pleiotropic, tissue-specific effects that appear to be life stage dependent.

1.13 Bisphenol A in freshwaters

Most of the studies carried out on the low-dose exposure to BPA and on its long-term impacts, as well as on the maternally transferred BPA and its effects in the offspring, have used mammalian models, while few studies have utilized fish. Testing BPA effects in fish is important, especially since a significant quantity of BPA (12-17,200 µg BPA l^{-1}) is released into the aquatic environment from various point sources (Table 1-2; Yamamoto et al., 2001; Crain et al., 2007; Xi et al., 2011; Wang et al., 2011). Moreover, recent studies have raised the issue of long-term effects of low-doses of BPA in aquatic organisms (Aluru et al., 2010; Zhou et al., 2011).

Bisphenol A enters the aquatic environment from point sources, such as waste landfill leachates (Yamamoto et al., 2001), waste water effluents, and sewage (Crain et al., 2007; Wei et

al., 2011). The BPA in landfill leachates is derived from degradation of plastics via hydrolysis prior to release in the watershed, while the BPA in the sewage effluent is derived mostly from human-ingested BPA (Meesters and Wa, 2002; Wintgens et al., 2003). Plastic debris enters the watershed from urban runoff and other non-point sources (Crain et al., 2007; Cooper et al., 2011). Studies have shown that BPA can leach from the plastic debris when exposed to heat (Krishnan et al., 1993; Cooper et al., 2011), therefore contaminating the watershed. BPA derived from the degradation of plastic debris found in fresh and marine waters contributes significantly to the total concentration of BPA present in the aquatic environment, as plastics constitute approximately 60-80% of the total anthropogenic debris (Gregory and Bryan, 1997). Furthermore, a high percentage of these plastics are found on the ocean floors and on the bottom of lakes and rivers, further contributing to the release of the chemical in the aquatic environment. BPA has been shown to be degraded by gram-negative bacteria under aerobic conditions (Lobos et al., 1992; Kang and Ha, 2002), with BPA having a half-life of ~4.5 days under these conditions (Cousins et al., 2002). Aerobic BPA degradation occurs mainly in oxygen rich waters, such as rivers, but it is limited in anaerobic environments, such as sediments and estuaries (Ike et al., 2006). Indeed, studies have shown that sediments in BPA-contaminated waters hold a much higher BPA concentration than the water itself (Heemken et al., 2001; Peng et al., 2007; Flint et al., 2012). Reports on the environmental concentration of BPA in rivers and marine environments appear to vary in the literature. Concentrations ranging from 18 ng BPA l^{-1} to >1000 ng BPA l^{-1} have been reported in freshwaters (Table 1-3).

Table 1-2. Reported concentrations (in µg l^{-1}) of bisphenol A in various representative point and non-point sources.

Country	BPA	Source	Reference
Canada	0.193-3.81	Municipal sewage treatment plant influent	Lee and Peart, 2000
	0.031-59.1	Municipal sewage treatment plant effluent	
	n.d.*-0.590	WWTP** influent	Fernandez et al., 2007
	n.d.-1.054	WWTP effluent	
	0.011-0.040	Kraft mill effluent	
	0.094-0.172	Sewage effluent	Wang et al., 2011
USA	0.11-1.7	Untreated septage	Rudel et al., 1998
	0.094-0.15	Untreated wastewater	
	0.02-0.055	Treated septage/wastewater	
	n.d.-1.41	Plume from landfill/septage lagoon	
	<1.0	Receiving waters of BPA manufacturing and processing facilities	Staples et al., 2000
	0.088-0.74	Urban runoff	Kolpin et al., 2004
	n.d.-3.6	Primary effluent in WWTPs	Drewes et al., 2004
	n.d.-0.05	Secondary effluent in WWTPs	
Japan	n.d.-17.2	Waste landfill leachates	Yamamoto et al., 2001
	8.0-370	Final effluent from paper recycling plants	Fukazawa et al., 2001
Germany	max: 0.7	Sewage treatment effluent	Fromme et al., 2002
UK	0.891-1.105	WWTP influent	Hernando et al., 2004
	0.013-0.019	WWTP effluent	
	0.378-0.89	WWTP effluent	Jiang et al., 2005
	0.046	Treated WWTP effluent	
Spain	0.07-10.3	Sewage treatment influent	Céspedes et al., 2006
	0.07-3.45	Sewage treatment effluent	
Sweden	0.49	Sewage treatment effluent	Larsson et al., 1999

*n.d. = not detectable; **WWTP = waste water treatment plant

1.14 Bisphenol A accumulation in fish tissues

In fish, BPA exposure occurs either directly from the water or via bioaccumulation through the food chain. Freshwater and marine fish tend to accumulate BPA in their tissues, with BPA concentrations varying from one species of fish to another, and from one tissue to another (Table 1-4). It appears that BPA accumulates mostly in the liver (Pedersen and Lindholst, 1999), but significant BPA levels were also reported in the fish muscle, plasma and brain (Lindholst et al., 2001; Table 1-4). In 2001, Lindholst and colleagues determined that exposure of trout to BPA, either in the water or through intraperitoneal injection, led to accumulation of the chemical in fish tissues (Lindholst et al., 2001). In trout exposed to 100 $\mu g\ l^{-1}$ waterborne BPA, the plasma concentration at 48 h post-exposure was 0.35 μg BPA ml^{-1}, while the liver and the muscle concentrations reached 540 and 380 μg BPA ml^{-1}, respectively. Similar to the waterborne exposure, the authors observed that when BPA was administered intraperitoneally to the trout, the liver and the muscle accumulated the largest amount of this chemical (Lindholst et al., 2001). However, it appears that these tissues are not the only ones where BPA accumulates in fish. In Atlantic salmon (*Salmo salar*), BPA has been shown to accumulate in all parts of the developing egg, and its uptake rate is directly proportional to the ambient water temperature (Honkanen et al., 2001). Moreover, Renz et al. (2013) have recently shown that BPA accumulates the brain tissue of three wild freshwater fish species in Pittsburgh (Table 1-4), suggesting that this chemical accumulated preferentially in lipid-rich tissues of fishes.

Although fish do accumulate BPA in their bodies, a large part of the chemical is also eliminated over time. In fish, degradation of BPA occurs via phase II degradation enzymes, such as UDP-glucuronosyltransferases and aryl sulfotransferases. The polar moieties of BPA are conjugated by the glucuronyl and sulfonyl transferases, a group of enzymes that add glucuronyl and sulfonyl groups to the parent molecule, thereby increasing its solubility in water and facilitating its subsequent excretion (Lindholst et al., 2001). Lindholst et al. (2001) have reported that the concentration of the less toxic bisphenol A glucuronic acid (BPAGA; glucuronidated form of BPA) in rainbow trout exposed to BPA increases above the levels of the parent compound within 2-6 h of a waterborne exposure to the chemical. The authors also determined that approximately 92% of the BPA is converted to BPAGA in the trout liver after 12 h from the

Table 1-3 Reported concentrations of bisphenol A (in ng l^{-1}) in freshwaters.

Country	BPA	Water body	Reference
Canada	230-149,200	Wastewater from industrial facilities	Lee and Peart, 2000
	0.45-18.8	Bow and Elbow Rivers, Calgary, Alberta	Chen et al., 2006
	<90-800	Great Lakes Basin	Klecka et al., 2009
USA	<1000-8000	Rivers near BPA manufacturing and processing facilities	Staples et al., 2000
	9.0-44.0	Bayou River	Boyd et al., 2004
	6.0-113	Mississippi River	
	<1.5-57	Lake Pontchartrain	
	88-740	Water from various rivers in Iowa	Kolpin et al., 2004
	25-130	Tres Rios Wetlands, Arizona	Barber et al., 2006
	1.900	Drinking water sources across the USA	Focazio et al., 2008
	5-25	Water from various DWTPs* in USA	Benotti et al., 2009
	37-183	Little Calumet River	Schultz et al., 2013
	15-181	Chicago Sanitary & Ship Canal	
	n.d-15.4	Allegheny and Monongehela Rivers, Pittsburgh	Renz et al., 2013
China	1040	Shijing River	Zhao et al., 2009
	43.5-639.1	Pearl River Delta	Gong et al., 2009
Taiwan	200-1970	Kao Pin River	Chen et al., 2010
	70-3940	Wulo Creek	
Japan	20.2-30.1	Tamagawa River	Hashimoto et al., 2005
	<500-900	Kaeda River	Kang & Kondo, 2006
Germany	<50-272	Streams in South West Germany	Bolz et al., 2001
	0.5-14	Streams in Southern Germany	Kuch & Ballschmiter, 2001
Spain	10-20	River Ebre in Catalonia	Brossa et al., 2005
Netherlands	130	Dommel River	Belfroid et al., 2002
	<18	Bergemeer Lake	
	<18-170	Eysden	

*DWTP = drinking water treatment plant

Table 1-4. Measured BPA levels in tissues of freshwater and marine wild fish.

Water	Country	Fish common name	Species name	BPA* (ng g^{-1} ww)	Tissue	Reference
Freshwater	Netherlands	Bream	*Abramis brama*	1-5 ng g^{-1} dw	Muscle	Belfroid et al., 2002
	Norway	Lake trout	*Salvelinus namaycush*	2.67-10.4	Muscle	Schlabach et al., 2004
		Perch	*Perca fluviatilis*	1.2-7.7	Muscle	
		Pike	*Esox lucius*	1.0-13.7	Muscle	
	China	High: Mud fish	*Cirrhina molitorella*	0.20	Muscle	Wei et al., 2011**
		Low: Rice field eel	*Monopterus albus*	0.05	Muscle	
	USA	Alewife	*Alosa pseudoharengus*	n.d.-120 pg g^{-1}	Brain	Renz et al., 2013 ***
		Smallmouth bass	*Micropterus dolomieu*		Brain	
		Gizzard shad	*Dorosoma cepedianum*		Brain	
Marine	Netherlands	Flounder	*Platichthys flesus*	2-75 ng g^{-1} dw	Liver	Belfroid et al., 2002
				5-11 ng g^{-1} dw	Muscle	
	China	Various market fish		n.d.-1.01	Muscle	Shao et al., 2007
		High: Tongue sole	*Cynoglossus robustus*	0.11	Muscle	Wei et al., 2011**
		Low: Yellow seafin	*Acanthopagrus latus*	0.05	Muscle	
	Italy	Salpa	*Sarpa salpa*	6.0	Liver	Mita et al., 2011**
				5.0	Muscle	
		Bass	*Dicentrarchus labrax*	1.5	Liver	

*Unless specified otherwise, all tissue BPA concentrations are in ng g^{-1} wet weight (ww) or dry weight (dw).

**These studies analyzed BPA levels in multiple marine and freshwater species. In the interest of space, only species with the highest and lowest tissue BPA levels are reproduced in this table.

***The authors in this study did not differentiate between the three fish species when they presented their data.

initial BPA exposure. BPA levels also decreased in the muscle and in the plasma of the fish over time, but the changes were not as rapid as in the liver, suggesting that the liver is the main detoxification organ for BPA in the fish body. Although detoxification of BPA occurs in fish, when exposure to this chemical occurs during early developmental stages, the BPA-induced changes may disrupt developmental programming events, leading to long-term impact on fish performances (Aluru et al., 2010).

1.15 Non-reproductive effects of BPA in fish

Due to its xenoestrogenic properties, BPA is known to impact reproduction in fish. While Flint et al. (2012) gave a through summary of the effects of BPA in fish, Table 1-5 summarizes the studies published in the last ten years. Overall, BPA is considered a weak estrogen in fish, which leads to the feminization of males (Sumpter and Jobling, 1995; Jobling et al., 1996; Sohoni et al., 2001; Kang et al., 2002). However, recent studies have shown that BPA has non-reproductive effects in fish that are quite diverse, including formation of blood vessel edema, disturbances in cell morphology and disruption in metabolism (McCormick et al., 2010; Lam et al., 2011; Jordan et al., 2012).

Briefly, exposure of newly hatched salmon fry to 1000 $\mu g\ l^{-1}$BPA led to edema and damaged blood vessels in the yolk sac, haemorrhages around the gill arches, phlegmatic behaviour and morphological changes in liver cells (Honkanen et al., 2004). Furthermore, BPA has been shown to affect the fish endocrine system by increasing the mRNA abundance of gonadotropin-α, FSH-β and LH-β in a hermaphroditic fish, the mangrove killifish (*Kryptolebias marmoratus*; Rhee et al., 2009). These genes play an important role in sex differeniation, and the presence of BPA could affect sexual maturation and embryonic development by altering the expression of these genes.

In chronic studies, BPA has been shown to produce biochemical alterations in fish. In the Japanese medaka (*Oryzias latipes*), a 60 day exposure to environmentally relevant concentration of BPA (1, 10 and 100 $\mu g\ l^{-1}$) induced an increase in catalase activity in the liver, while the activity of liver superoxide dismutase was increased only at the 100 $\mu g\ l^{-1}$ concentration (Minghong et al., 2011). Surprisingly, a higher concentration of BPA of 1000 $\mu g\ l^{-1}$ did not induce any changes in enzyme activity, suggesting that the effects of BPA on the hepatic

antioxidases are more pronounced at the lower doses of this chemical. Alo' et al. (2005) analyzed the effects of BPA on the stomatostatinergic (ss) system of the hermaphroditic fish, *Coris julis*, over a 14-day exposure to the chemical. The authors determined that BPA is capable of modifying the binding affinities of two somatostatin receptor subtypes (sst_2 and sst_5) in the brain of *C. julis*. Since the ss system has a potent inhibitory effect on the basal and stimulated growth hormone (GH) release in the brain of teleosts (Lin et al., 1999), it is quite possible that one of the mechanisms by which BPA impairs growth and development in fish may be by impacting the ss system, but such an effect remains to be determined.

In zebrafish (*Danio rerio*), Duan et al. (2010) used a metabonomics approach to demonstrate that BPA disturbs metabolism. The authors analyzed the production of various metabolites, such as saturated fatty acids and amino acids, by the zebrafish 8-day old larvae using a gas-chromatography mass spectroscopy (GC-MS) approach. Embryos were exposed to 0.5, 1.5 and 4.5 mg l^{-1} BPA in water from fertilization to 8 days post-fertilization and metabolite content was extracted and measured in the larvae. The authors noted an increase in 3 saturated fatty acids, which are associated with cardiac defects, and a decrease in semi-volatile and volatile metabolites associated with muscular movements. Indeed, Duan et al (2010) noted pericardiac edema in the BPA-exposed fish, while Lam et al. (2011) noted pericardiac edema in the larvae exposed to BPA, along with a decrease in independent swimming. Recent studies have also suggested that exposure to BPA during critical periods of development can impact fish growth, development and performance, implying that BPA may target the fish somatotropic axis (Aluru et al., 2010).

The BPA-induced reproductive and non-reproductive effects previously mentioned in this review could lead into long-term or permanent changes in the fish. Several studies have focused on how this compound affects the development of aquatic organisms by impacting other endocrine systems, following both chronic and acute exposures. Jiao and Cheng (2010) found that exposure of cultured primary black seabream (*Acanthopagrus schelgeli*) hepatocytes to BPA caused marked decreases in receptors of genes involved in growth and development. In another study examining the effects of BPA on fish development, McCormick et al. (2010) exposed zebrafish (*Danio rerio*) embryos (3-h post-fertilization) to BPA for 5 days and monitored fish development and survival. The study found that BPA caused hemorrhage and edema in 48-h old

Table 1-5. Reported effects of various exposure concentrations of BPA on fish species, in the last 10 years.

Fish species	BPA exposure; time	Reported effects of BPA	Reference
Atlantic cod	50 µg l^{-1}; 3 wks	VTG induction	Larsen et al., 2006
Atlantic salmon	1000 µg l^{-1}; 6 days	Yolk sac edema and hemorrhage	Honkanen et al., 2004
Brown trout	1.75-5 µg g^{-1}; 3.5 months	Reduced sperm quality, delayed and inhibited ovulation	Lahnsteiner et al., 2005
Carp	1,10,100,1000 µg l^{-1}; 2 wks	VTG induction, gonadal changes, increased oocyte atresia, intersex, exposure-dependent changes in estrogen to androgen ratios	Mandich et al., 2007
European seabass	10 µg l^{-1}; 2 wks	VTG induction	Correia et al., 2007
Fathead minnow	160 µg l^{-1}; 2 wks	VTG induction	Brian et al., 2005
Japanese medaka	100 µg l^{-1}; 60 days	Induction of oxidative stress enzymes	Minghong et al., 2011
Japanese seabream	1-10 µM; 24 h	*In vitro* reduction of mRNA levels of GHr and IGF-1 in hepatocytes	Jiao & Cheng, 2010
Longchin goby	0.44 nM; 48 h	Stimulated germinal vesicle breakdown	Baek et al., 2007
Mediterranean rainbow wrasse	80 µg l^{-1}; 2 wks	Modifications of binding affinity of two somatostatin receptors in the brain	Alo' et al., 2005
Mangrove killifish	0.6 µg l^{-1}; 24 h	Disruption of endocrine system	Rhee et al., 2009
Perch	0.12-2 mM	*In vitro* sperm motility was reduced	Hatef et al., 2010
Rainbow trout	30 and 100 µg ml^{-1}; 3 h oocyte loading with BPA	Increased mortality, VTG induction, altered growth, GH/IGF axis genes, and stress response	Aluru et al., 2010
Turbot	59 µg l^{-1}; 2 wks	Changes in sex steroid concentrations	Labadie and Budzinski, 2006
Zebrafish	228 µg l^{-1}; 48 h	Feminized embryo brains, increased female to male ratio	Crain et al., 2007
	50-4500 µg l^{-1}; 7 days	Disruption of genes involved in growth, development and reproduction	Lam et al., 2011
	5-25 µM; 5 days	Edema, hemorrhage	McCormick et al., 2010

embryos, leading to decreased survival. Additionally, Baek et al. (2007) determined that BPA induces germinal vesicle breakdown, thus promoting oocyte maturation in the longchin goby (*Chasmichthys dolichognathus*).

In a recent study, Aluru et al. (2010) addressed the issue of maternally transferred chemicals (Ostrach et al., 2008) and demonstrated that such an exposure to BPA affects the GH/IGF (somatotropic) axis in trout (*Oncorhynchus mykiss*), similar to what was observed in mammalian *in vitro* studies (Wetherill et al., 2007). Furthermore, fish body mass was reduced in the BPA groups starting at 124 days post-fertilization (dpf), and it was maintained until the end of the experiment, at 400 dpf. Growth hormone levels were induced by the BPA exposure, and these changes were also maintained from 13 to 400 dpf. Key genes involved in development and their respective receptors were also impacted by the exposure to BPA compared to controls, similar to what Jiao and Cheng (2010) found. Lastly, the stress performance of the fish exposed to BPA was also altered, as cortisol production and uptake appeared to be impaired in the BPA-exposed groups. The Aluru et al. (2010) study suggests that exposure to BPA during critical periods of development could disrupt the somatotropic and stress axes during early development, leading to defects in growth and stress performance. This particular study suggests that BPA-induced re-programing of the two axes during embryogenesis is due to epigenetic modifications. However, there has been no study yet that has examined the impact of ancestral exposure to contaminant, including BPA, on fish health performances.

1.16 Thesis objectives

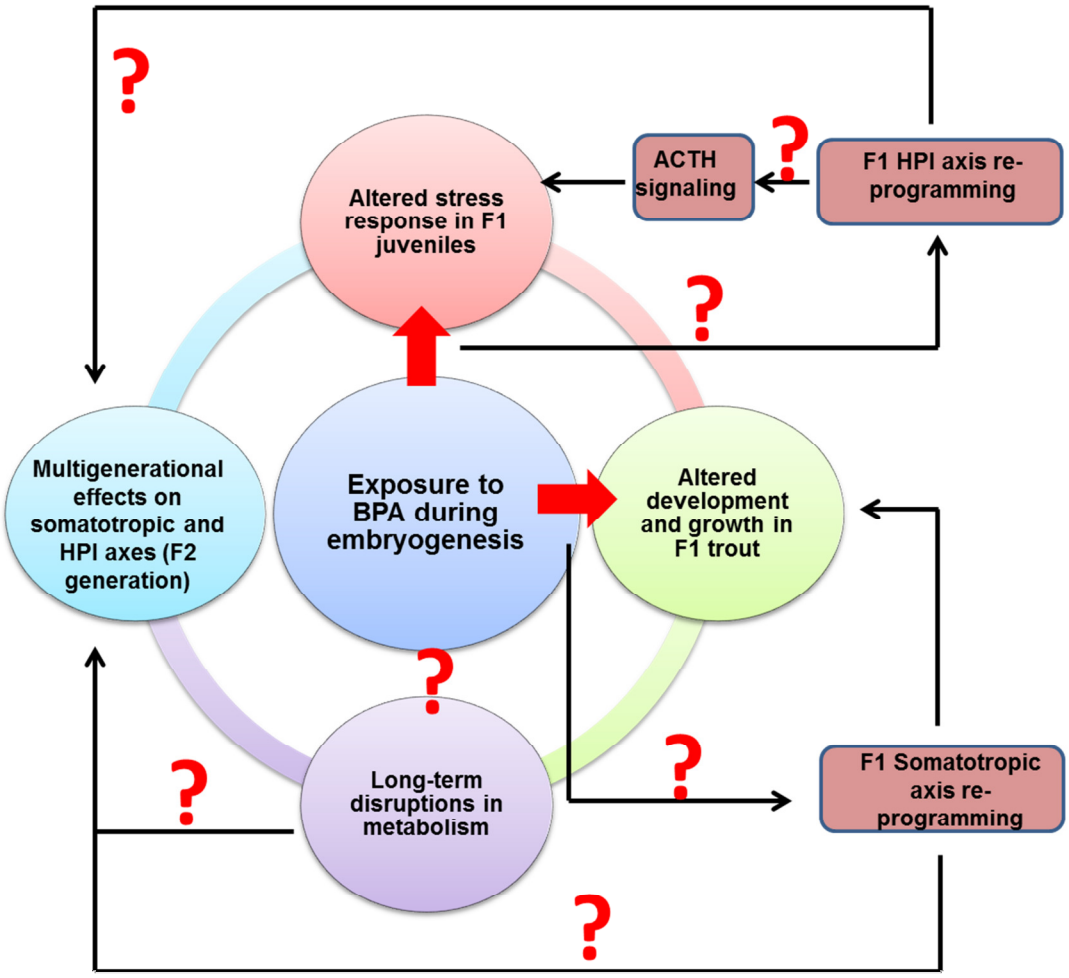
Given that contaminants are maternally transferred to offspring in wild fish (Ostrach et al., 2008), and that maternal transfer of contaminants has recently been shown to have multigenerational effects (Corrales et al., 2014), the objective of this thesis was to characterize the long term impact associated with BPA exposures in fish (Fig. 1-6). Identifying whether somatotropic and HPI axes genes are targets for BPA impact during early development, and whether the BPA-mediated changes translate to longer-term and generational effects on growth and stress performances, will lead to identification of candidate genes as early warning biomarkers of BPA effects in fish. Insights on how metabolic pathways may be impacted in the developing fish due to the deposition of BPA into oocytes will allow the determination of possible mechanisms of action that lead to long term effects of this contaminant in fish.

Therefore, the overall hypothesis of my thesis is that BPA accumulation in oocytes, mimicking maternal transfer, and its subsequent presence in the growing embryos, disrupts the development and functioning of the somatotropic and stress axes in two generations of rainbow trout. The specific objectives are to determine whether BPA accumulation in oocyte disrupts:

- somatotropic axis development in the F1 generation (Chapter 2);
- stress axis development in the F1 generation (Chapter 3);
- somatotropic and HPI axes development in the F2 generation (Chapter 4);
- HPI axis functioning in F1 and F2 juveniles (Chapter 5);
- the whole body metabolome profile at hatch and just prior to first feed in the F1 and F2 trout (Chapter 6).

Figure 1-6. Schematic diagram depicting the known long-term effects of embryonic BPA exposure in trout and identifying where knowledge gaps are.

Oocyte loading with BPA, mimicking maternal transfer, induced delays in development in the offspring, affecting growth and development in trout (Aluru et al., 2010). In addition, juveniles that were exposed to BPA as embryos presented alterations in the highly conserved acute stress response cortisol and glucose profiles. Since BPA was no longer detected in the embryos after hatch, these findings suggest that BPA interferes with somatotropic and HPI axes signaling during development. Whether this is due to BPA-induced re-programming of the two axes during embryogenesis, and whether these alterations are multigenerational in trout, was investigated in the current thesis. Studies in other fish species have found that BPA in embryos induced significant alterations in the metabonome (Duan et al., 2010), while the effects of maternally transferred contaminants have recently been related to multigenerational effects in fish (Corrales et al., 2014). The current thesis aims to determine whether similar situations are present in trout, by examining the metabolome profile and determining whether the changes noted in F1 generation fish persist in F2 (multigenerational effects).


Altered stress response in F1 juveniles
ACTH signaling
F1 HPI axis re-programming
Multigenerational effects on somatotropic and HPI axes (F2 generation)
Exposure to BPA during embryogenesis
Altered development and growth in F1 trout
Long-term disruptions in metabolism
F1 Somatotropic axis re-programming

Chapter 2: Bisphenol A in Oocytes Impacts Growth and Energy Reserves in Rainbow Trout

2.1 Overview

Bisphenol A (BPA), a monomer used in the production of plastics and epoxy resins, is ubiquitously present in the aquatic environment. BPA is considered a weak estrogen in fish, but the effects of this chemical on early developmental events are far from clear. We tested the hypothesis that BPA accumulation in eggs, mimicking maternal transfer, leads to long-term defects in growth and energy reserves, due to impact on the development of the somatotropic axis. Oocytes were exposed to 0, 0.3, 3, and 30 mg l^{-1} BPA for 3 h prior to fertilization. The BPA-exposed oocytes accumulated levels of 0.8, 4.4 and 41.3 ng BPA $embryo^{-1}$, respectively, which declined and were completely eliminated by hatch [42 days post-fertilization (dpf)]. Oocyte BPA accumulation led to delayed yolk absorption, increased larval water and reduced total energy contents. This corresponded with reduced specific growth rate and higher food conversion ratio in larvae reared from BPA-laden oocytes. In addition to growth defects, BPA accumulation in oocytes impacted the mRNA abundance of genes involved in the somatotropic axis function in a life-stage dependent manner. Growth hormone isoforms (GH-1 and -2) and their receptors were impacted in a life-stage dependent manner, and insulin-like growth factor (IGF) -1 and IGF-2 transcript levels in the higher BPA group increased 20 days post-feed. The transcript levels of the IGF receptor b were impacted by BPA, decreasing by ~10-30% across all life stages, in all the treatments. In addition, the developmental profile of the thyroid receptors (TR) mRNA abundance in the BPA-exposed fish was also altered. Our results demonstrate that oocyte enrichment of BPA, mimicking maternal transfer, has the potential to impact growth and development through disruption of the somatotropic and thyroid hormone axes in trout.

2.2 Introduction

Bisphenol A (2,2-bis[4-hydroxyphenyl]propane; BPA), a monomer used in the production of common plastics and epoxy resins, is ubiquitously present in the environment (Staples et al., 1998; Sajiki & Yonekubo, 2004; Cao et al., 2008, 2009). BPA levels ranging from 0.45 to 8000 ng l^{-1} have been reported in freshwater bodies across North America (Staples et al. 2000; ; Chen et al., 2006), while tissue levels of BPA as high as 10.4 ng g^{-1} ww in wild fish have been detected in the muscle, brain and liver (Wei et al., 2011; Renz et al., 2013). BPA has been labeled as an endocrine disrupting compound, mainly based on its ability to weakly mimic the effects of the female hormone 17β-estradiol (E2), including production of the egg yolk protein vitellogenin (VTG) in fish (Brian et al., 2005; Larsen et al., 2006; Correia et al., 2007; Alonso-Magdalena et al., 2012; Hanson et al., 2014). However, recent research has shown that the effects of this chemical in fish extend beyond its xenoestrogenic role, with BPA toxicity being pleiotropic and life stage dependent (Vandenberg et al., 2009; Vandenberg, 2013). Chronic exposures to waterborne BPA during early developmental stages of fishes have been shown to induce abnormal development, embryonic deformities and mortality (Honkanen et al., 2004; McCormick et al., 2010), while exposure of adults has led to marked changes in reproduction and growth (Crain et al., 2007; Mandich et al., 2007; Lam et al., 2011; Hanson et al. 2012, 2014). A recent study showed that egg accumulation of BPA, mimicking maternal transfer, leads to long-term effect on somatotropic axis function in rainbow trout (Aluru et al., 2010).

The major hormone involved in somatotropic axis function is growth hormone (GH; Björnsson et al., 2002). In fish, GH targets a multitude of organs, leading to activation of intracellular pathways by binding to the two GH receptors, GH-1r and GH-2r (Reinecke et al., 2005). The growth promoting effect of GH is mediated by its binding to GH-Rs and activating the synthesis and release of insulin-like growth factor 1 (IGF-1) and 2 (IGF-2) from the liver (Reinecke et al., 2005; Reinecke, 2010). Together with GH, IGFs are involved in metabolism, osmoregulation, growth and development (Reinecke, 2010). In addition to the GH-IGF axis, thyroid hormones (THs) also play an important role in growth differentiation, metabolism and organismal maturation. Thyroid hormone action is mediated via the activation of the thyroid hormone receptors (TRs), encoded by two genes, TRα and TRβ (Raine & Cameron, 2004; Terrien & Prunet, 2013). A role for GH, IGFs and THs in growth regulation during

embryogenesis and early development is well established (for detailed reviews, see Power et al., 2001; Reinecke, 2010; Terrien and Prunet, 2013).

Recent studies have identified the somatotropic and TH axes as targets of xenobiotic toxicity. For instance, environmental estrogens impact growth, salinity adaptation and GH and IGF sensitivity in trout post-hatch (Hanson et al., 2012; Hanson et al., 2014) Also, BPA acts as a TR antagonist in *Xenopus laevis* (Moriyama et al., 2002; Zoeller, 2005) and disrupts thyroid function in zebrafish (*Danio rerio*; Terrien et al., 2011). While exposure of contaminants through the water column is of main concern for fish, maternal accumulation of contaminants in lipid depots and the subsequent transfer of these chemicals to the offspring through eggs have recently gained attention (Takao et al., 2008; Ostrach et al., 2008; Aluru et al., 2010). Indeed, exposure of early developing embryos to contaminants led to long-term abnormalities in growth and development (Westerlund & Billsson, 2000; N ye et al., 2007; McCormick et al., 2010; Aluru et al., 2010; Corrales et al., 2014), but the mechanism are far from clear.

We tested the hypothesis that BPA accumulation in oocytes, mimicking maternal contaminant transfer, leads to reduced growth and energy reserves and this involves disruption of genes essential for somatotropic and thyroid axes functioning in fish. To test this, rainbow trout oocytes were exposed to different concentrations of BPA for 3 h, prior to fertilization, to mimic maternal transfer, as described previously (Aluru et al., 2010). The offspring growth, development, body composition and energy reserves were monitored for 112 days. Temporal changes in mRNA abundance of GH, IGF and their receptors and TRs were measured to evaluate if somatotropic and thyroid axes development and signaling are impacted by oocyte accumulation of BPA.

2.3 Materials and Methods

2.3.1 Materials and Chemicals

Unless otherwise specified, bisphenol A and all other chemicals and enzymes were purchased from Sigma–Aldrich (St. Louis, MO, USA). D-Glucose was purchased from Bioshop (Burlington, ON, CA), while monobasic and dibasic sodium phosphate, sodium bicarbonate and borosilicate tubes were purchased from Fisher Scientific (Ottawa, ON, CA). The 96-Well plates for quantitative real-time PCR (qRT-PCR), along with the iQ SYBR® green fluorescent dye mix

were purchased from Bio-Rad (Mississauga, ON, CA). The TRIol used for RNA extraction was purchased from Invitrogen (Burlington, ON, CA), while the first strand cDNA synthesis kit and the DNase I for treating the RNA prior to making cDNA were purchased from Fermentas (Burlington, ON, CA). All the solutions used for BPA extraction were HPLC grade and were purchased from Sigma-Aldrich.

2.3.2 Experimental Fish

Experiments were conducted at Alma Aquaculture Research Station (AARS), Alma, Ontario, Canada, and the protocol was approved by the Animal Care and Use Committees at the University of Guelph and the University of Waterloo. Gametes were pooled from reproductive age rainbow trout (3+ year class brood stock). Oocytes from four female and milt from four male rainbow trout were pooled and used for all treatments. Upon collection, oocytes were visually assessed for quality (color, size, buoyancy) by well-trained AARS personnel and assigned a number from 1 to 3 (1 = lowest quality; 3 = highest quality). Only those meeting the highest quality were used for experiments. Ovarian fluid was collected from all four females (a total of ~ 200 ml), and it was stored at 6-8˚C prior to the beginning of the experiment.

2.3.3 Bisphenol A Treatments and Sampling Protocol

The pooled oocytes were distributed into four treatment groups (12 ml of oocytes per treatment for a total of 875 oocytes/treatment). The oocytes were immersed in 50 ml of ovarian fluid containing vehicle alone (<0.01% ethanol; 0 BPA group) or BPA concentrations of 0.3, 3 or 30 µg ml^{-1} for 3 h at 6-8 ˚C. During the exposure, the containers were gently shaken every 30 min to ensure equal distribution of the vehicle- or BPA-enriched ovarian fluid throughout. At the end of the treatment period, the oocytes were fertilized by the addition of 2 ml of milt and water (to activate the sperm), they were thoroughly mixed, and then were allowed to sit for one min to ensure maximal fertilization. After fertilization, the embryos were rinsed with clean fresh water several times before incubation. The embryos were then incubated in a Heath chamber incubator receiving AARS water at a rate of 10 l min^{-1}, at a temperature of 8 ˚C. Samples of embryos, larvae and juveniles were collected at time 0 (just prior to fertilization), 7, 14 (organogenesis), 28 (eyed egg stage), 42 (hatching), 65 (just prior to first feed), 85 and 112 days post-fertilization (dpf).

Larvae were maintained in the incubator for a week after first hatch (until 49 dpf), after which they were moved to holding tanks (3 × 200 l tanks per treatment) receiving flow-through water at a rate of 10 l min^{-1}, under a 12hL: 12hD photoperiod, and their health was monitored by AARS personnel. Tank assignment for each treatment was done using a random number generator to minimize tank bias (M. Burke, AARS personnel, personal communication). Fish were weighed weekly during the experiment. After 65 dpf, larvae were fed to satiation daily, using mechanical feeders, at a rate proportional to their predicted growth rate that was calculated by AARS personnel on a weekly basis (Aluru et al., 2010). Food was only withheld for 48 h prior to sampling at the post-feeding life stages (85 and 112 dpf).

Sampling protocol involved anesthetizing the embryos and juveniles in a lethal dose of methane-tricaine sulphonate (MS222; 1.0 g l^{-1}) buffered with sodium bicarbonate (2.0 g l^{-1}). A total of n=6 (pool of 5-10 embryos each) were collected at each sampling time point prior to hatch, dried on a paper towel, weighed, frozen on dry ice and stored at -80 ºC. Individual hatch sac fry and swim-up fry were collected at and post-hatch, respectively (n=24, after 42 dpf), from each treatment. All analysis in this study was conducted on whole bodies, without yolk. A sub-group of n=6 swim-up fry were collected for imaging at 65 dpf from each treatment, and they were anesthetized as previously described. The embryos were then imaged using the Leica MZ16 microscope, with a Leica DFC 320 camera, and the images were captured at a magnification of 7.5X. After 65 dpf, the fry (n=24) were collected at each time point, dried, their weights and lengths were recorded, and stored at -80 ˚C until further analysis.

2.3.4 Analytical Techniques

2.3.4.1 BPA extraction and quantification

BPA extraction from the embryos and larvae was carried out as previously described by Aluru et al. (2010) with minor modifications. Briefly, fertilized eggs (pool of 5-10 frozen eggs and embryos for 0-28 dpf) and just-hatched embryos (n=3 pooled 4-6 larvae without yolk at 42 dpf; n=3 pooled yolks from 4-6 individuals at 42 dpf) were pulverized on dry ice using a mortar and a pestle, after which they were dissolved in 20 ml of a dichloromethane:methanol (2:1) solution, to which an internal standard of deuterated BPA (BPA-d16) was added as an internal control. The samples were then vortexed for 30 s. A KCl solution (0.9%) was added to the mixture (20% of total volume) as a polar solvent, to aid in the separation of the organic and

aqueous phases. The samples were vortexed again for 30 s. The homogenate was then centrifuged for 10 min at 1000 × *g*, at room temperature, and two distinct phases were obtained. The dichloromethane (organic) phase was then removed with a glass pipette and evaporated to dryness under nitrogen gas, on a shaker, at room temperature. The samples were then re-dissolved in 1.0 ml methanol: hexane (1:20) by vortexing them for 30 s and then sonicating for 5 min in a water bath, at room temperature, to ensure that all the BPA from the sides of the glass tube had dissolved in the mixture. To separate the BPA from the other organic compounds in the solution, a Sep-Pak Vac 1cc (100 mg) NH2 cartridge (Waters, Canada) was used. Prior to the addition of the sample, the cartridge was pre-conditioned with 5.0 ml methanol:hexane (1:20), under a water vacuum, at 1.0 ml min^{-1}. The sample was then applied to the cartridge and the filtrate was discarded. The cartridge was then washed with 7.5 ml hexane and then allowed to dry for 3 min under water vacuum. The sample was then desorbed with 4.0 ml of 100% methanol and the filtrate was collected. The solution was then dried under nitrogen gas to concentrate the BPA, and then it was re-suspended in 300 µl of 100% methanol. The solution was stored in the -20 ˚C, in amber vials, to prevent light degradation of the BPA until analysis was performed.

Quantification of BPA was carried out as previously described by Aluru et al. (2010), without any modifications. Briefly, BPA quantification was conducted using the LC-MS/MS Method, with Agilent 1200 used for LC, and Applied Biosystems MDS Sciex API 3200 QTrap used for MS analysis. The Agilent Eclipse XDB C18 column (5 µm particles, 4.5 mm diameter × 150 mm length) was used for chromatography, following established protocols, and the BPA calibration curve was established using BPA-d16 as a standard. The ratios of the peak areas and the standards were calculated using Agilent Chemstation software. The BPA quantification limit was 75-80 ng g^{-1} and it was determined at a signal to noise ratio >3. Figure 2-1a only represents the BPA treatments, while all the other figures that have BPA on the x-axis show the actual BPA accumulation in the oocytes immediately after the 3 h treatment, with the average life stage (0-28 dpf) control value subtracted.

2.3.4.2 Whole body water and gross energy determination

Whole body water and energy contents were determined at 42, 65 and 112 dpf. Fish were weighed (initial weight), placed in individually labelled tubes and dried at 100 ºC for 48 h. Their final weight was then recorded, and the difference was used to determine the whole body water

content, which was then expressed as %wet weight (refer to equation 5). The whole body energy content was determined using a bomb calorimeter (6725 bomb semimicro calorimeter; Parr Instrument Company, Moline, IL, USA). The calorimeter calibration and sample analysis protocols were followed exactly as outlined by the manufacturer (SOP#1109). Briefly, the bomb was calibrated with a series of ten benzoic acid standards for caloric purposes and provided by the manufacturer. Calibration was performed in the thermal jacket containing 450 ml water. Prior to weighing and analysis, the samples were pressed into a pellet using the Parr pellet press (Parr Instrument Company, Moline, IL, USA). Standards and samples were weighed prior to analysis by using an XS205 dual range balance (Mettler-Toledo International Inc., Mississauga, ON, CA), and the weight was used for the final calculation of the gross heat. All analysis of gross heat was conducted in the presence of 35 atm of oxygen. The instrument was calibrated every ten samples to ensure that the deviation of the measurements from the initial calibration was less than 0.67%, as stated in the SOP. All gross heat values are represented in kilo calories g^{-1} dry weight (kcal g^{-1} dw).

2.3.4.3 Tissue and whole body processing and analysis for glycogen, glucose and lactate

Whole body (42, 65, 112 dpf) analysis of glucose, glycogen and lactate were carried out as described by Birceanu et al. (2014), with minor modifications. Briefly, fish were weighed and homogenized on dry ice prior to processing, using a mortar and a pestle. This was followed by sonication in five volumes of Tris buffer (50 mmol l^{-1}, pH 7.4), containing 1.0 mg ml^{-1} protease inhibitor, and the samples were then centrifuged at 500 × *g* for 30 sec, to remove any debris. Samples were deproteinated with perchloric acid (35% w/v), to a final PCA concentration of 8% in the sample and neutralized with 3.0 mol l^{-1} K_2CO_3 for glucose and lactate determination. For glycogen determination, samples were mixed with three parts sodium acetate buffer (2.0 mol l^{-1}, pH 4.8) and then incubated at 37 ºC for 2 h with amyloglucosidase (20 U per sample). The digestion was terminated by adding 70% perchloric acid and the samples neutralized with 3.0 mol l^{-1} K_2CO_3. All samples were stored at -80 ºC until analysis.

Endogenous and total glucose, along with whole body lactate levels were determined enzymatically as previously described (Birceanu et al., 2014) using a 96-well microplate spectrophotometer (VersaMax ROM v3.13, Molecular Devices, CA, USA). Whole body protein

levels were determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA) with bovine serum albumin as the standard.

2.3.4.4 RNA extraction and first strand cDNA synthesis

Total RNA from whole body was extracted using the TRIzol Reagent following the manufacturers' protocols. The RNA was dissolved in 50 µl RNase free water (Qiagen, Mississauga, ON, CA) and quantified spectrophotometrically at 260 nm using the NanoDrop™ spectrophotometer (ThermoScientific, Nepean, ON, CA). Prior to first strand cDNA synthesis, 1.0 µg RNA was treated with DNase I (1.0 µl) in $MgCl_2$ buffer (1.0 µl). RNase-free water was then added to bring the volume to 10.0 µl. The reaction was incubated at 37 ºC for 30 min, after which it was terminated by the addition of 1.0 µl 25 mmol l^{-1} EDTA and incubated at 65 ºC for 10 min. First strand cDNA was synthesized following the manufacturer's instructions (Fermentas, Burlington, ON, CA). Briefly, a master mix was prepared containing the following ingredients per sample: 2 µl 10X RT buffer, 0.8 µl 25X dNTP Mix (100 mM), 2 µl 10X RT Random Primers, 1 µl Mulstiscribe Reverse Transcriptase and 4.2 µl RNase-free water. Next, 10 µl of the master mix were added to each sample and gently mixed by pipetting. The tubes were then placed in the thermocyler (Mastercycler, Eppendorf, CA) and the following temperature cycle was used: 25 ˚C for 10 min, 37 ˚C for 120 min and 85 ˚C for 5 min, after which the tubes were stored at -20 ˚C for later analysis.

2.3.4.5 Primers and Quantitative Real-Time Polymerase Chain Reaction

The primers were designed based on rainbow trout growth hormone (GH-1 and GH-2), insulin-like growth factor (IGF-1 and IGF-2) and their respective receptors (GH-1r, GH-2r and IGF-1Ra, IGF-1Rb), thyroid hormone receptor (TRα and TRβ), and EF1α sequences from rainbow trout (Table 2-1; Raine et al., 2004; Aluru et al., 2010). The transcript levels were analyzed using the iQ SYBR® green fluorescent dye master mix with the iCycler real-time PCR detection system (Bio-Rad, Hercules, CA). Each sample was assayed in duplicate and the following PCR conditions were used for amplification: 3 min at 95 ˚C; 40 repeats of 2 steps: 20 s at 95 ˚C, followed by 20 s at the melting temperature for each gene (refer to Table 2-1); 1 min at 95 ˚C; 1 min at 55 ˚C, followed by melt curve analysis starting at 55 ˚C and increasing to 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 by

Aluru et al. (2010). All the samples were assayed for the genes of interest and for the housekeeping gene, elongation factor 1α (EF1α). They were all then normalized to EF1α, as expression of this gene did not change across life stages, and the data was reported as % change from the 42 dpf control samples for all the life stages.

2.3.5 Calculations and Statistical Analysis

Condition factor (CF), specific growth rate (SGR), along with food conversion ratio (FCR) and whole body water content were determined using the following formulae:

(1) $CF = (10^5 \times W_i) / (L_i^3)$

(2) $SGR = Ln[W_2 / W_1] / (t_2 - t_1) \times 100\%$

(3) $FCR = W_f / (W_2 - W_1)$

(4) Body water content $= (W_i - W_{i\text{-}dry}) / W_i \times 100\%$

where W_i is the wet weight of individual fish in the tank in g, L_i is the fish's respective length in mm; W_1 and W_2 represent the total dry weight in g of the fish in the tank at the beginning (t_1) and at the end (t_2) of the interval, respectively;; W_f is the total weight of food consumed over the experimental period; $W_{i\text{-}dry}$ is the dry weight for each individual fish of wet weight W_i.

Statistical analysis was performed using SigmaPot 11.0 software (Systat Software Inc., San Jose, CA, USA). All data are shown as mean ± standard error of the mean (S.E.M.). A two-way analysis of variance (ANOVA) was used to determine significance of BPA burden, of BPA treatment on gene transcripts, whole body water content, dry weight and whole body gross energy at various developmental stages. A one-way ANOVA was used for analyzing the effects of BPA exposure on various parameters, such as CF, SGR, and FCR. With both types of ANOVAs, a Tukey's post-hoc test was used whenever interactions were detected, and a probability level of $p < 0.05$ was considered significant. The data was log-transformed wherever necessary to meet the assumptions of normality and equal variance. Only non-transformed data are shown in the figures. An outlier test was performed on the non-transformed that using the ©2014 GraphPad Software. Only one outlier was identified and then eliminated from the GH-1 mRNA levels data set. For figures showing % change, the S.E.M. is to be used only as reference and it is not a true reflection of the data. For simplicity purposes, whenever there was no significant difference noted between treatments within one time point, no letters were added to the bars on the figures.

Table 2-1. Forward (F) and reverse (R) sequences, amplicon size, accession number and annealing temperature for the primers used in real-time quantitative PCR.

Gene of interest	Sequence	Amplicon size (bp)	Accession #	Annealing temperature (˚C)
EF1α	F: 5′-CATTGACAAGAGAACCATTGA-3′ R: 5′-CCTTCAGCTTGTCCAGCAC-3′	95	AF498320.1	56
IGF-1	F: 5′-TGGACACGCTGCAGTTTGTGTGT-3′ R: 5′-CACTCGTCCACAATACCACGGTT-3′	120	EF450071	68
IGF-2	F: 5′-CGGCAGAAACGCTATGTGGA-3′ R: 5′-TGCTGGTTGGCCTACTGAAA-3′	79	EF450072	58
IGF-1Ra	F: 5′-AGAGATAGACGACGCCTCCTA-3′ R: 5′-CACCAAATAGATCCCTACGT-3′	104	AF062499	58
IGF-1Rb	F: 5′-CCTAAATCTGTAGGAGACCTGGAG-3′ R: 5′-GTTAGCCACGCCAAATAGATCC-3′	139	AF062500	58
GH-1	F: 5′-TTCAAGAAGGACATGCACAAGGTC-3′ R: 5′-CTCCAGCCCACGTCTACAGA-3′	97	AF005923	66
GH-2	F: 5′-CCCACGTTTACAGAGTGCAGTTG-3′ R: 5′-GCTTCAAGAAGGACATGCATAAGGTT-3′	93	DQ294400	66
GH-1r	F: 5′-TGAACGTTTTTGGTTGTGGTCTA-3′ R: 5′-CGCTCGTCTCGGCTGAAG-3′	61	AY861675.1	60
GH-2r	F: 5′-CATGGCAACTTCCCACATTCT-3′ R: 5′-GCTCCTGCGACACAACTGTTAG-3′	65	AY751531.1	60
TRα	F: 5′-GCACAACATTCCCCACTTCT-3′ R: 5′-AGTTCGTTGGGACACTCCAC-3′	117	AF146777	60
TRβ	F: 5′-TCACCTGTGAAGGATGCAAG-3′ R: 5′-GACAGCGATGCACTTCTTGA -3′	152	AF146775	60

2.4 Results

2.4.1 Bisphenol A (BPA) accumulation in oocytes

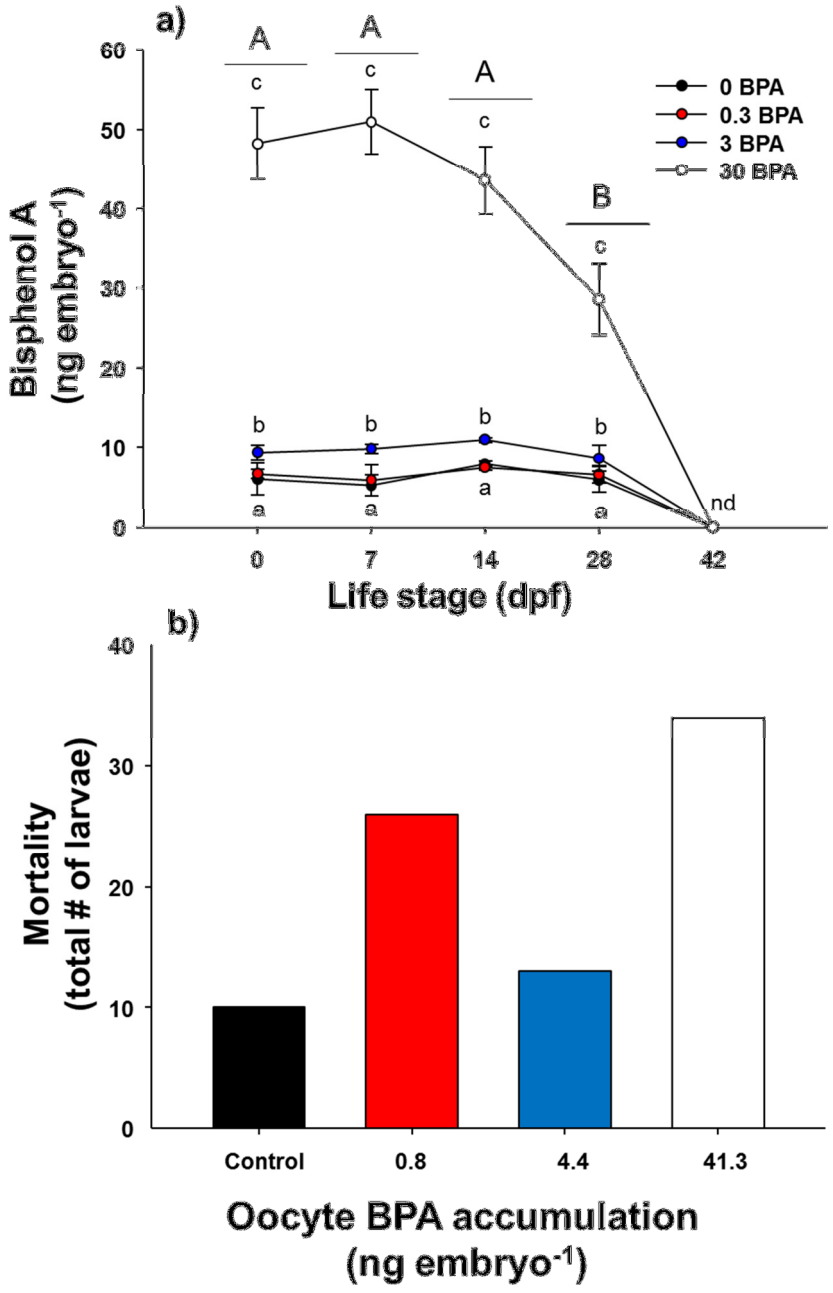
Bisphenol A concentrations in control embryos were at a background level of approximately 6.04 ± 2.0 ng embryo^{-1} at all the sampling time points. After 3 h of exposure, BPA concentrations reached 6.7± 1.8, 10.3 ± 2.1, 47.2 ± 5.7 ng embryo^{-1} in the 0.3, 3.0 and 30.0 mg l^{-1} BPA groups, respectively (Fig. 2-1a). After subtracting the control values, which were considered background, the net BPA accumulation in the oocytes exposed to the three concentrations were 0.8 ± 0.6, 4.4 ± 0.9 and 41.3 ± 4.5 ng embryo^{-1}, respectively. Only the 3 and 30.0 BPA groups had significantly higher BPA levels compared to the control group, while the 0.3 BPA group was not significantly different from controls (Fig. 2-1a). The BPA levels in the embryos did not change up to 14 dpf, after which they dropped by approximately 40% in the 30 BPA group, measuring 22.7 ± 3.4 ng embryo^{-1} (background subtracted) at the 28 dpf time point. Bisphenol A was not detected in the embryos or in the yolks at hatch (42 dpf; Fig. 2-1a). However, only embryo (without yolk) BPA levels are shown in Fig 2-1a.

2.4.2 Phenotypic changes, survival and growth

Bisphenol A treatments did not affect fertilization rate (as assessed by AARS personnel) or the overall development of the embryos prior to hatch (data not shown). There were no observed delays in hatching or first feeding in the exposed individuals when compared to controls (data not shown). However, within the first 14 days after hatch, the recorded mortality rate increased in the oocytes that accumulated 0.8 and 41.3 ng embryo^{-1} BPA by 2.6 and 3.4 fold, respectively, when compared to controls (Fig. 2-1b). There was no mortality recorded after that in any of the groups. In addition, the higher BPA group appeared to retain more yolk at 65 dpf (first feed) when compared to controls, which lost their entire yolk almost entirely by that time (Fig. 2-2).

Figure 2-1. Bisphenol A (BPA) concentration in trout embryos and mortality 14 days after hatch.

(a) BPA depuration and (b) # of dead larvae in the first 14 days after hatch (42-56 dpf) following a 3 h oocyte loading with vehicle (<0.01% ethanol) or 0.3, 3 and 30 mg l^{-1} BPA in ovarian fluid. For (a), data are shown as mean ± S.E.M. (n = 3 pools of 5-10 embryos from 0-28 dpf; n = 5 individual larvae at 42 dpf). Lower case letters represent significant difference within each life stage, while upper case letters denote the effect of time on BPA levels. Data points or groups of data points sharing the same letter are not significantly different (p<0.05, one-way ANOVA, Tukey's post-hoc test).


a)
A
A
A
B
c
c
c
c
0 BPA
0.3 BPA
3 BPA
30 BPA
b
b
b
b
a
a
a
a
nd
Bisphenol A
(ng embryo-1)
60
50
40
30
20
10
0
0
7
14
28
42
Life stage (dpf)
b)
Mortality
(total # of larvae)
40
30
20
10
0
Control
0.8
4.4
41.3
Oocyte BPA accumulation
(ng embryo-1)

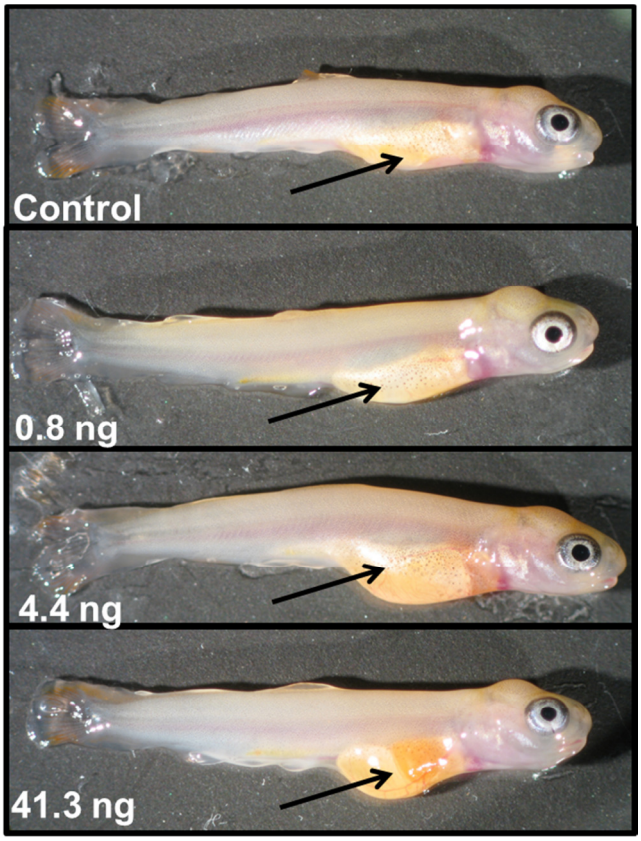


Figure 2-2. Bisphenol A (BPA) impact on embryo development.

Phenotypic changes in 65 dpf (first feed) larvae, following the initial BPA accumulation in oocytes (control; 0.8, 4.4 and 41.3 ng embryo^{-1}). Following the exposure, the oocytes were fertilized, water hardened and allowed to develop in a flow-through system, at 8.0 ˚C. This is a representative image of the presence of yolk (arrows) volume in the control and the BPA groups.

Whole body water content in control individuals increased by 15% from 42 dpf (hatch) to 65 dpf (first feed), reaching approximately 86% of the total wet weight, while at 112 dpf (post-feed) it decreased to the levels seen in larvae at 42 dpf (~74% ww; Fig. 2-3a). In the BPA-exposed fish, whole body water burden increased by 14-16% at 42 dpf (hatch) when compared to controls. Moreover, the profile of % whole body water during development was quite different in the treatment groups, remaining unchanged at 42 and 65 dpf, and then significantly decreasing at 112 dpf compared to the other time points (Fig. 2-3a).

The dry weight of the larvae at 42, 65 and 112 dpf was not significantly affected by the BPA treatments, with the dry weight steadily increasing from 42 to 112 dpf in all the groups (Fig. 2-3b). The whole body gross energy profile also increased during development, from 5.34 ± 0.19 kcal g^{-1} dw at 65 dpf, to 6.00 ± 0.04 at 112 dpf in control fish (Fig. 2-3c). At 65 dpf, the groups that accumulated 4.4 and 41.3 ng BPA $embryo^{-1}$ were significantly impacted by the exposure, with their whole body gross energy being ~15% and 18% lower, respectively, when compared to controls. By 112 dpf, however, the whole body gross energy of the BPA-treated fish was not significantly different from that of controls.

Fish weight and length at 85 and 112 dpf were not significantly affected by the oocyte BPA treatment, although condition factor (CF) increased in the higher BPA group at 85 dpf, but not 112 dpf (Table 2-2). BPA, however, had a significant impact on specific growth rate (SGR) over a 47 d period from first feed (65 dpf) to 112 dpf (Fig. 2-4a). SGR was significantly reduced by ~15% in all the BPA groups when compared to the controls. In addition, the food conversion ratio (FCR) during this period (from 65 to 112 dpf) was significantly higher in the BPA groups by ~ 22% when compared to controls (Fig. 2-4b).

Figure 2-3. Effects of BPA on whole body water content, dry weight and whole body gross energy content.

Changes in (a) whole body water content, (b) dry weight and (c) whole body gross energy content in trout from 42 to 112 dpf, following the initial BPA accumulation in oocytes (control; 0.8, 4.4 and 41.3 ng BPA embryo^{-1}). Data are shown as mean ± S.E.M. (n = 4-6 fish). Lower case letters represent significant difference within each life stage, while upper case letters denote the effect of time on whole body water and dry weight. Asterisks (*) denote a significant time effect within one treatment. Bars or groups of bars sharing different letters are statistically significantly (p<0.05, one-way ANOVA, Tukey's post-hoc test). For simplicity purposes, whenever there was no significant difference noted between treatments within one time point, no letters were used.

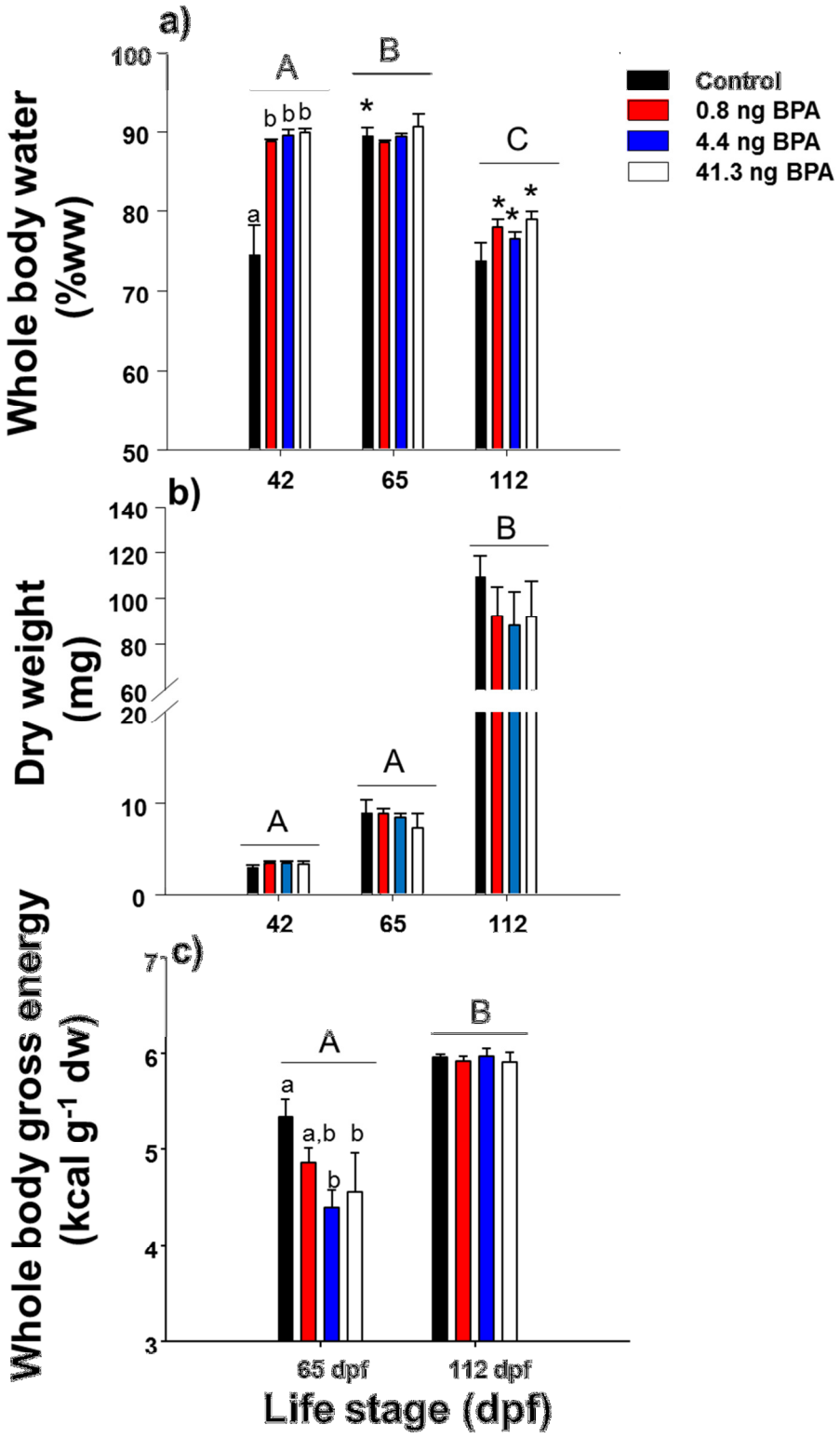
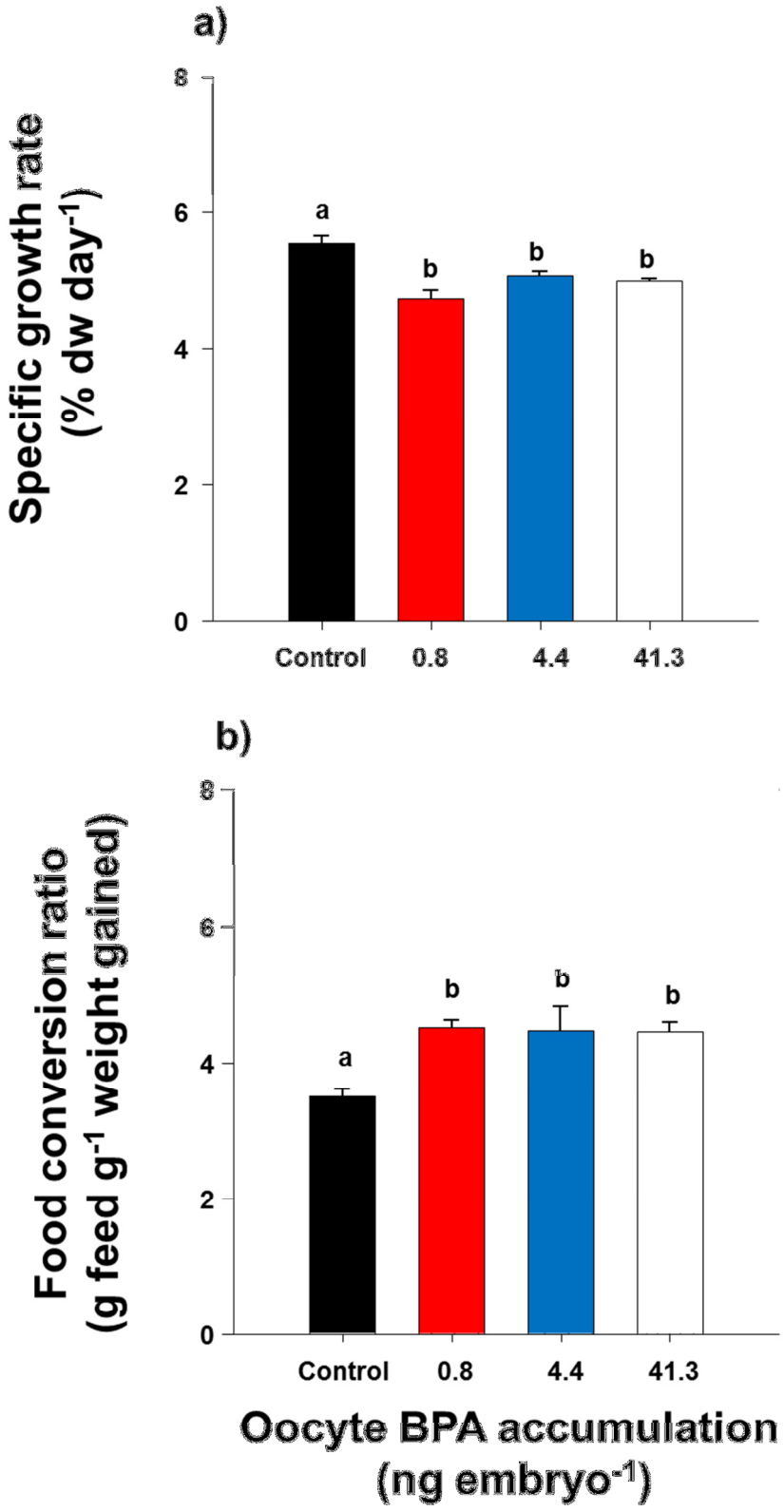

a)
Whole body water (%ww)
100
90
80
70
60
50
A
B
C
b b b
a
*
*
*
*
42
65
112
Control
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
b)
Dry weight (mg)
140
120
100
80
60
20
10
0
A
A
B
42
65
112
c)
Whole body gross energy (kcal g-1 dw)
7
6
5
4
3
A
B
a
a,b
b
b
65 dpf
112 dpf
Life stage (dpf)

Table 2-2. Body characteristics of rainbow trout exposed to BPA as oocytes, prior to fertilization. Data points sharing the same letters are not statistically different (one-way ANOVA, Tukey's post-hoc test, $p<0.05$; n =18 for 85 dpf and n=30 for 112 dpf).

		Oocyte BPA Accumulation after 3 h of incubation with background subtracted (ng embryo^{-1})			
	Days post-fertilization (dpf)	**Control**	**0.8**	**4.4**	**41.3**
Length (mm)	85	28.00 ± 0.50^{a}	28.24 ± 0.54^{a}	27.21 ± 0.62^{a}	27.18 ± 0.68^{a}
	112	41.07 ± 0.75^{a}	40.97 ± 0.75^{a}	40.86 ± 0.75^{a}	40.90 ± 0.74^{a}
Weight (g)	85	0.20 ± 0.01^{a}	0.20 ± 0.01^{a}	0.19 ± 0.01^{a}	0.21 ± 0.01^{a}
	112	0.70 ± 0.03^{a}	0.69 ± 0.03^{a}	0.68 ± 0.03^{a}	0.68 ± 0.03^{a}
CF	85	0.87 ± 0.02^{a}	0.87 ± 0.03^{a}	0.93 ± 0.04^{ab}	1.01 ± 0.04^{b}
	112	1.00 ± 0.03^{a}	0.93 ± 0.03^{a}	0.95 ± 0.02^{a}	0.96 ± 0.01^{a}

Figure 2-4. Bisphenol A effects on growth and food conversion ratio.

(a) Specific growth rate (SGR) and (b) food conversion ratio (FCR) in trout from 65 to 112 dpf, following the initial BPA accumulation in oocytes (control; 0.8, 4.4 and 41.3 ng BPA embryo^{-1}). Data are shown as mean ± S.E.M. (n = 3 tanks per treatment). Bars or groups of bars with different letters are significantly different (p<0.05, one-way ANOVA, Tukey's post-hoc test).


a)
Specific growth rate
(% dw day-1)
8
6
4
2
0
a
b
b
b
Control
0.8
4.4
41.3
b)
Food conversion ratio
(g feed g-1 weight gained)
8
6
4
2
0
a
b
b
b
Control
0.8
4.4
41.3
Oocyte BPA accumulation
(ng embryo-1)

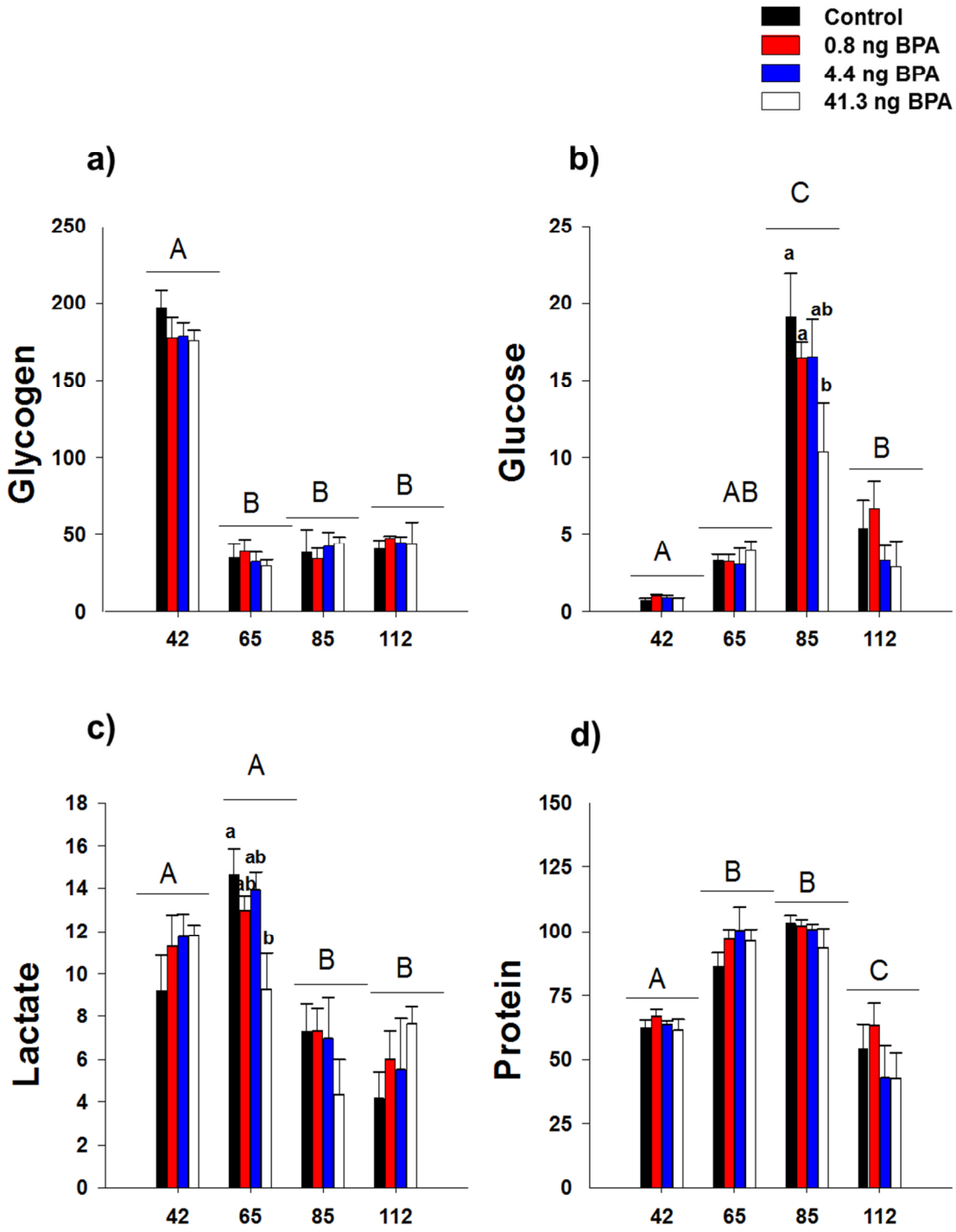
2.4.3 Whole body metabolites and energy reserves

Whole body metabolites and energy reserves were impacted by exposure of the oocytes to BPA in maternal fluid (Fig. 2-5). In control fish, whole body glycogen levels at first feed (65 dpf) decreased by ~5-fold when compared to 42 dpf levels, and remained relatively unchanged post-feed (Fig. 2-5a). The whole body glycogen levels in the BPA-exposed groups were not significantly different from the controls at each of the life stages, and they followed an identical temporal trend. In contrast to the glycogen levels, whole body glucose did not show a similar profile across life stages (Fig. 2-5b). Whole body glucose levels in control fish increased by ~5-fold at 65 dpf when compared to their hatch levels. At 85 dpf, glucose levels increased by ~30-fold while at 112 dpf they were only 8-fold higher than the levels measured at 42 dpf. BPA did not significantly impact glucose levels at 42, 65 and 112 dpf. However, at 85 dpf, glucose levels were 2.5-fold lower in when compared to controls at the same life stage (Fig. 2-5b).

Whole body lactate was not impacted by BPA exposure at 42, 85 and 112 dpf. Control lactate levels remained relatively unchanged at hatch (42 dpf) and first feed (65 dpf), but they decreased post-feed, being ~1.3 and 2.2-fold lower at 85 and 112 dpf, respectively, when compared to 42 dpf levels. At 65 dpf, BPA decreased whole body lactate by ~1.4-fold when compared to controls at the same life stage (Fig. 2-5c). Lastly, whole body protein concentration was significantly increased by ~1.4-fold at and post-hatch, and its levels were not impacted by BPA exposure during embryogenesis (Fig. 2-5d).

Figure 2-5. Bisphenol A effect on whole body glycogen, glucose, lactate and protein levels.

Changes in whole body (a) glycogen, (b) glucose, (c) lactate and (d) protein in trout following BPA accumulation in oocytes (control; 0.8, 4.4 and 41.3 ng BPA embryo^{-1}). Data are expressed as (a-c) µmol g ww^{-1} and (d) mg protein g ww^{-1}, and are shown as mean ± S.E.M (n = 5-6 fish). Lower case letters represent significant difference within one life stage, while upper case letters denote the effect of time on each variable. Asterisks (*) denote a significant time effect within one treatment. Bars or groups of bars with different letters are significantly different (p<0.05, one-way ANOVA, Tukey's post-hoc test).


Control
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
a)
Glycogen
250
200
150
100
50
0
A
B
B
B
42
65
85
112
b)
Glucose
25
20
15
10
5
0
C
AB
A
B
a
ab
a
b
42
65
85
112
c)
Lactate
18
16
14
12
10
8
6
4
2
0
A
A
B
B
a
ab
ab
b
42
65
85
112
d)
Protein
150
125
100
75
50
25
0
A
B
B
C
42
65
85
112

2.4.4 Growth hormone (GH) and insulin-like growth factor (IGF) mRNA abundance

GH-1 mRNA abundance was not impacted by the oocyte exposure to BPA. Although the data shows a high degree of variability, the developmental mRNA profile of GH-1 did show a temporal trend, peaking at 65 and 85 dpf, then decreasing at 112 dpf (Fig. 2-6a). GH-2 mRNA abundance, however, was impacted by the BPA accumulation in oocytes (Fig. 2-6b). At 65 dpf, the oocytes that accumulated 41.3 ng oocyte^{-1} had significantly higher levels of GH-2 transcripts overall than the controls and the 4.4 ng BPA group, irrespective of the life stage. At 65 dpf, the same group had a ~200-fold increase in transcript levels when compared to controls at the same life stage. The GH-receptor transcripts levels appeared to vary with life stage, with the transcript levels increasing at 65 dpf, decreasing at 85 dpf, and then increasing again at 112 dpf (Fig. 2-6c,d). In addition, GH-1r mRNA levels were impacted by the BPA treatment at 65 dpf. The higher BPA exposure induced a significant increase in the GH-1r transcripts at that time point, with the effect dissipating at the later life stages.

IGF-1 and IGF-2 transcript levels showed a temporal trend, increasing across life stages. BPA treatment had a significant impact on IGF-1 mRNA abundance at 85 and 112 dpf (Fig. 2-7a). At 85 dpf, the oocytes that accumulated 41.3 ng oocyte^{-1} had ~50% more IGF-1 transcripts in their whole bodies when compared to controls. At 112 dpf the impact of BPA was different, with transcript levels decreasing by ~30-40% in the oocytes that accumulated 0.8 and 41.3 ng BPA embryo^{-1}. Similar to its effect on IGF-1 transcripts, BPA exposure led to ~50% increase in IGF-2 transcripts at 85 dpf (Fig. 2-7b). IGF-1-receptor a (IGF-1ra) was not affected by BPA, and its transcript levels peaked at 65 dpf, after which they decreased at 85 dpf, and then further at 112 dpf (Fig. 2-7c). IGF-1rb was impacted by BPA across all the life stages. Regardless of the BPA exposure, the transcripts decreased by ~10-30% across all treatments when compared to controls at each of the life stages (Fig. 2-7d).

Figure 2-6. Bisphenol A effects on growth hormone and growth hormone receptor genes.

Changes in (a) growth hormone 1 (GH-1), (b) GH-2, (c) GH-1 receptor (GH-1r) and (d) GH-2r mRNA abundance in trout, following the initial BPA accumulation in oocytes (control; 0.8, 4.4 and 41.3 ng BPA embryo^{-1}). Data are expressed as % of the control value at 42 dpf and are shown as mean ±S.E.M. (n = 5-6 fish). Therefore, the S.E.M. should be used as a guide, and not as a true representation of the data. Lower case letters represent significant difference within one life stage, while upper case letters denote the effect of time on mRNA abundance. Bars or groups of bars with different letters are significantly different (p<0.05, one-way ANOVA, Tukey's post-hoc test).

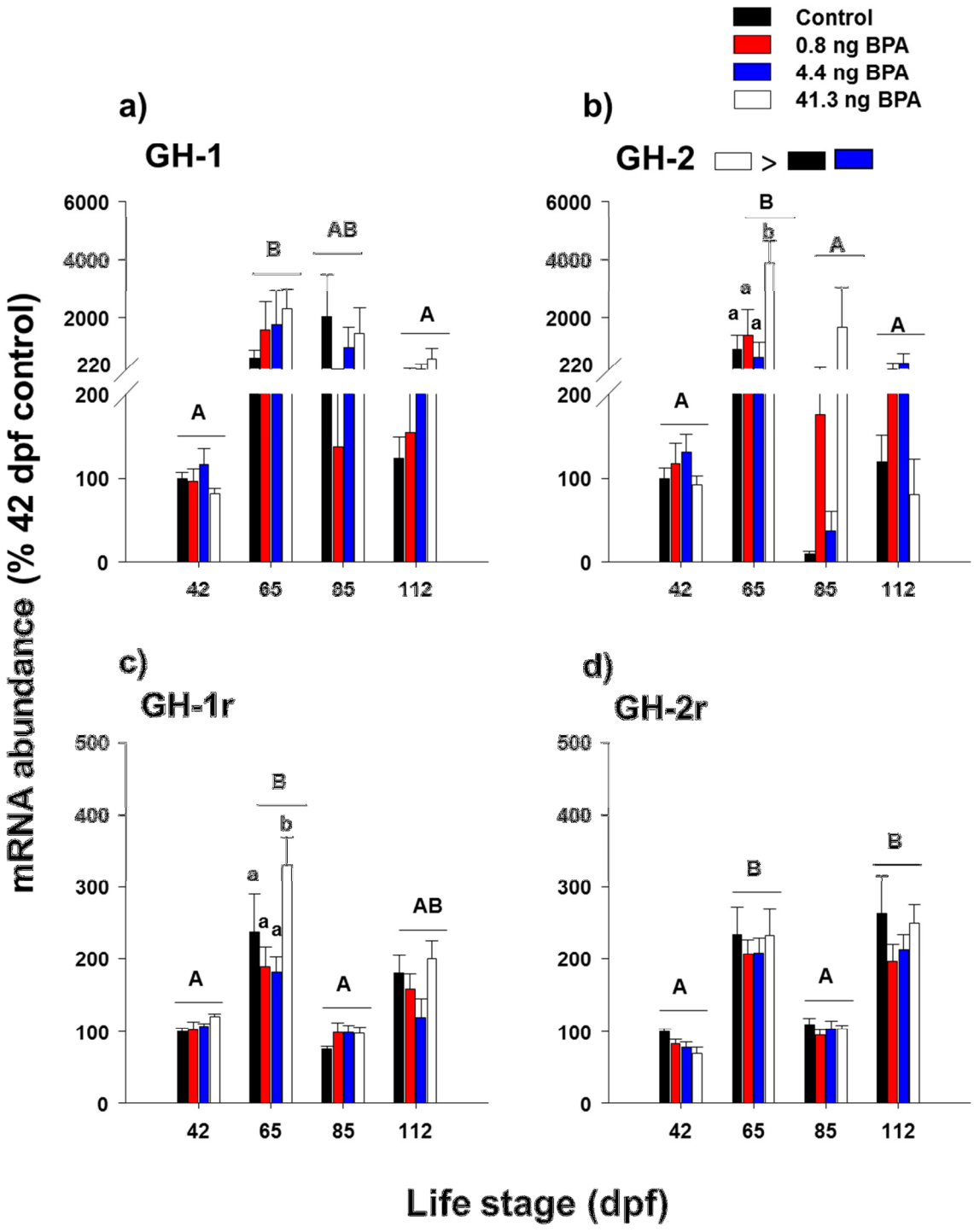
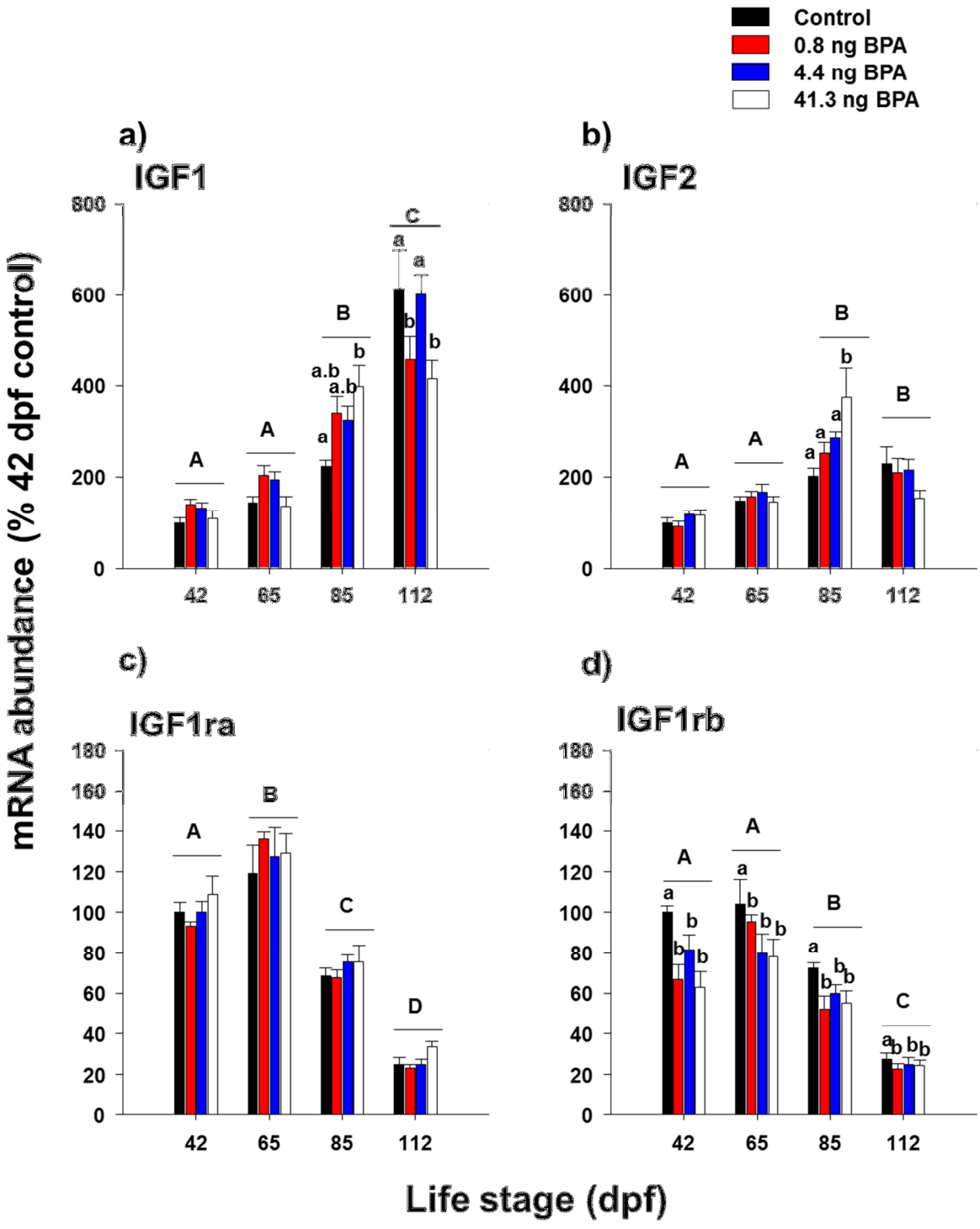

Control
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
a)
GH-1
b)
GH-2
c)
GH-1r
d)
GH-2r
mRNA abundance (% 42 dpf control)
Life stage (dpf)
42
65
85
112

Figure 2-7. Bisphenol A effects on insulin-like growth factor (IGF) and IGF-receptor genes.

Changes in (a) growth hormone 1 (IGF-1), (b) IGF-2, (c) IGF-1 receptor α (IGF-1Ra) and (d) IGF-1Rb mRNA abundance in trout, following BPA accumulation in oocytes (control; 0.8, 4.4 and 41.3 ng BPA embryo^{-1}). Data are expressed as % of the control value at 42 dpf and are shown as mean ±S.E.M. (n = 6 fish). The S.E.M. should be used as a guide, and not as a true representation of the data. Lower case letters represent significant difference within one life stage, while upper case letters denote the effect of time on mRNA abundance. Bars or groups of bars with different letters are significantly different (p<0.05, one-way ANOVA, Tukey's post-hoc test).

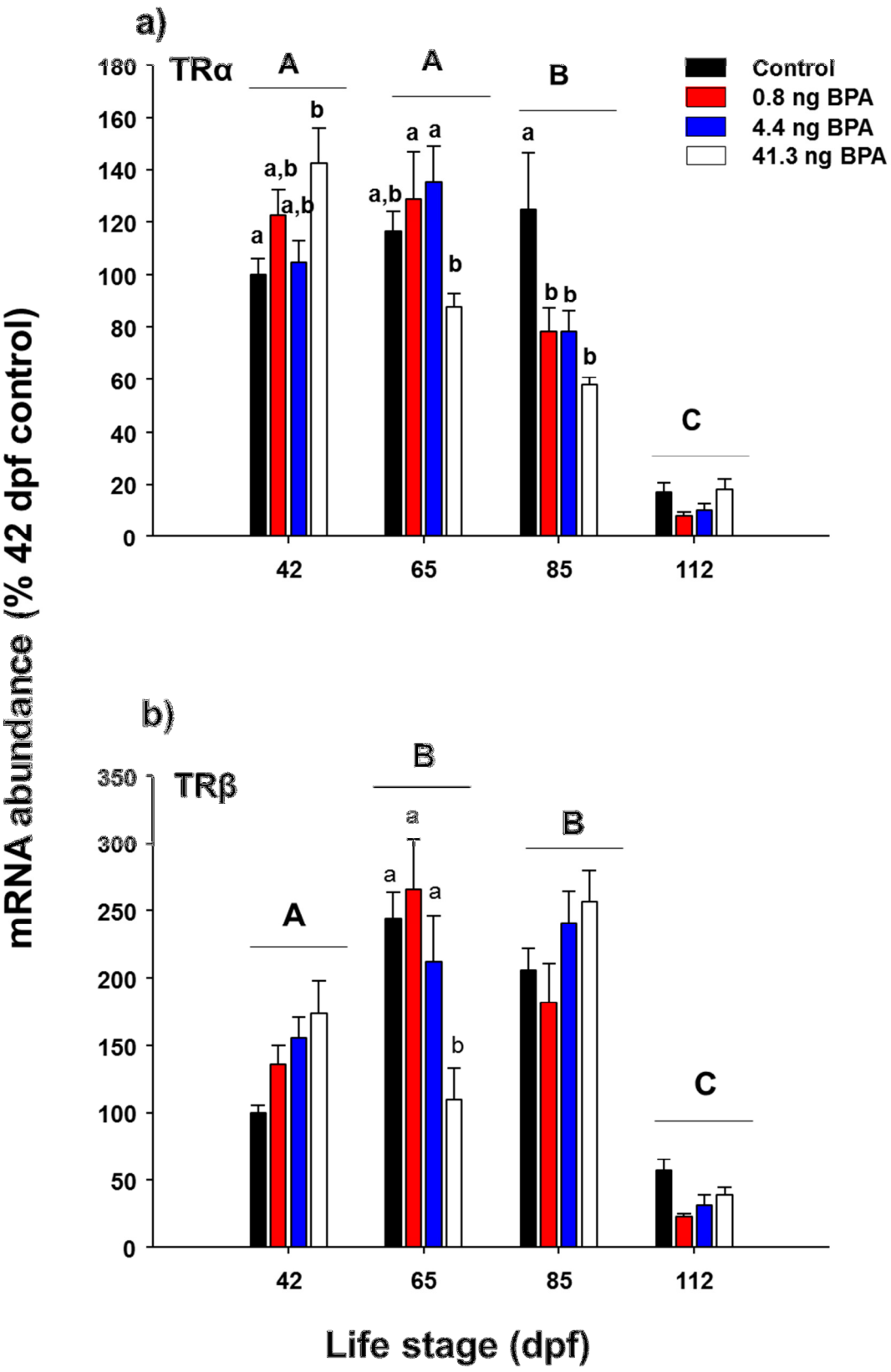

Control
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
a)
IGF1
b)
IGF2
c)
IGF1ra
d)
IGF1rb
mRNA abundance (% 42 dpf control)
Life stage (dpf)
42
65
85
112

2.4.5 Thyroid hormone receptor (TR) mRNA abundance

Thyroid hormone receptors (TRα and TRβ) transcripts were significantly impacted by oocyte BPA exposure (Fig. 2-8). TRα transcript levels in control fish peaked at 42 and 65 dpf, after which they decreased at 85 dpf, and then even more so at 112 dpf (Fig. 2-8a). TRβ transcript levels in controls also peaked at 65 dpf, after which they decreased at 85 and 112 dpf when compared to their 42 dpf levels (Fig. 2-8b). Bisphenol A exposure did have an effect on the thyroid receptor mRNA abundance in a life stage-dependent manner. At 42 dpf, TRα transcripts in the larvae of the highest BPA group were significantly higher than controls. Furthermore, at 65 dpf, the larvae that had accumulated 4.4 ng BPA embryo^{-1} as oocytes had higher levels of receptor transcripts than controls. The treatments significantly decreased TRα mRNA abundance across all treatments at 85 dpf, while at 112 dpf there was no BPA effect (Fig. 2-8a). While BPA treatment had no effect on TRβ at 42, 85 and 112 dpf, the mRNA abundance of the receptor decreased at 65 dpf in the 41.3 ng embryo^{-1} group when compared to controls.

Figure 2-8. Bisphenol A effects on thyroid hormone receptor genes.

Changes in thyroid hormone receptors (a) TRα and (b) TRβ mRNA abundance in trout, following the BPA accumulation in oocytes (control; 0.8, 4.4 and 41.3 ng BPA embryo^{-1}) Data are expressed as % of the control value at 42 dpf and are shown as mean ±S.E.M. (n = 6 fish). The S.E.M. should be used as a guide, and not as a true representation of the data. Lower case letters represent significant difference within one life stage, while upper case letters denote the effect of time on mRNA abundance. AsterisksBars or groups of bars with different letters are significantly different (p<0.05, one-way ANOVA, Tukey's post-hoc test).


a)
TRα
A
A
B
C
Control
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
180
160
140
120
100
80
60
40
20
0
42
65
85
112
b)
TRβ
B
A
B
C
350
300
250
200
150
100
50
0
mRNA abundance (% 42 dpf control)
Life stage (dpf)

2.5 Discussion

Our results demonstrate that BPA accumulation in oocytes impacts offspring growth and development in rainbow trout. While BPA was no longer detected in the larvae post-hatch, disturbances in growth and body composition persisted even after the initiation of endogenous feeding, implicating long-term effects of this chemical exposure on growth and development. The BPA-induced alterations in the developmental profiles of GH, IGFs and their receptor transcripts appear to be life stage- and dose-dependent, underscoring the somatotropic axis as a target for BPA toxicity (Aluru et al., 2010). The alteration in thyroid hormone receptor profiles during development post-hatch supports TH signaling as key target for BPA impact during embryogenesis in fish (Moriyama et al., 2002; Zoeller, 2005; Terrien et al., 2011; Terrien and Prunet, 2013). Together, these results underscore a critical role for BPA accumulation in oocyte on offspring growth dysfunction, and the mode of action involves disruption of the somatotropic and thyroid axes functioning in trout.

The concentrations of BPA reported in the oocytes after the acute 3 h exposure were comparable to those reported in the tissues of fishes. While no study has reported BPA content in wild salmonid eggs, the level seen here are comparable to other contaminants (i.e. polychlorinated biphenyls and pesticides) that were maternally transferred to oocytes in wild fish (Ostrach et al., 2008). The maternal transfer of BPA was confirmed by laboratory studies with medaka (*Oryzias latipes*; Takao et al., 2008). A 4 day waterborne parental exposure to BPA resulted in the transfer of this chemical to the oocytes and persisted in the developing embryos for up to 6 days in a clean environment (Takao et al., 2008). The concentration of BPA reported in fish tissues, including muscle (*Salvelinus namaycush*: 2.67-10.4 ng g^{-1} ww; *Esox Lucius*:1.0-13.7 ng g^{-1} ww; Schlabach et al., 2004) and liver (*Platichthys flesus*: 2-75 ng g^{-1} dw; Belfroid et al., 2002) are comparable to the values recorded in the present study, suggesting possible environmental relevance of our oocyte model for mimicking maternal transfer. The transfer of contaminants from the mother to the eggs and their presence during critical period of organogenesis may lead to long-term defects in growth and development (Takao et al., 2008; Ostrach et al., 2008; Aluru et al., 2010).

The importance of the somatotropic axis during teleost development has been well documented. Studies have analyzed the GH-IGF axis functioning and regulation during development by using morpholino oligonucleotides in zebrafish models (Schlueter et al., 2006;

Zhu et al., 2007), by injecting/exposing trout to GH and GH-antagonists to determine the overall effects on growth (Poppinga et al., 2007; Kling et al., 2012), or by subjecting the fish to various starving conditions and measuring the GH-IGF axis components (Montserrat et al., 2007). The developmental profiles of GH and IGF transcripts in the current study are in agreement with those observed in salmonids (Li et al., 2006; 2007; Aluru et al., 2010). The increase in IGF mRNA abundance with development post-hatch is in agreement with a role for IGFs in regulating somatic growth and post-hatch development (Wood et al., 2005). While the role of IGF-2 in embryos before hatch is well documented (Wood et al., 2005), our results suggest a possible role for IGF-2 signaling after first feed in trout.

While the functioning of somatotropic axis is essential for growth and early development, this axis is also a target for environmental contaminants (Aksakal et al., 2010; Aluru et al., 2010; Vandenberg et al., 2012; Hanson et al., 2012; Hanson et al., 2014). It has been recently shown that BPA accumulation in eggs also disrupts GH/IGF axis leading to growth defects in trout (Aluru et al., 2010). Any defects in GH-IGF signaling during critical periods of development may have permanent effects on the growth and performance in fishes (Reinecke, 2010). This is supported by the results in the current study, where loading of oocytes with environmentally relevant BPA concentrations disrupted IGF signaling and this corresponded with delayed yolk absorption prior to first feed and specific growth rate post feed in trout larvae. Despite the higher IGF levels seen post-feed in trout larvae, the suppression of the IGF-1Rb receptor transcript profile in larvae hatched from BPA laden eggs suggests disruption in IGF signaling. The lack of changes in hatching rate or first feed following BPA exposure leads to the proposal that BPA does not delay development, but may be impacting the energy reallocation and growth associated with exogenous feeding in trout. Indeed IGF-1 signaling is thought to play a key role in energy substrate allocation and growth in fish (Reinecke, 2010; Leatherland et al., 2010). Consequently, the decrease in IGF receptor transcript levels in the BPA group reflects an overall decrease in the SGR from first feed to 112 dpf in trout. BPA accumulation in eggs did not significantly impact GH-1 and GH receptor transcripts, but it did, however, induce an increase in GH-2 mRNA abundance in the highest BPA group, independent of life stages. While Aluru et al (2010) did not report any effect on the GH-2 mRNA developmental profile at their lowest BPA dose, the current findings propose that perhaps GH levels may be impacted by accumulation of BPA in

oocytes. Together, the results suggest that BPA in eggs leads to disruption in IGF signaling and growth performance in trout, while the mechanism of action of BPA remains to be elucidated.

Interestingly, the food conversion ratio (FCR) from 65-112 dpf significantly increased in BPA-exposed individuals when compared to controls. The decreased SGR, coupled with an increased FCR, suggests poor feed utilization and this may be responsible for the reduced growth rate due to BPA exposure. The lower energy status along with higher water content and the corresponding changes in whole body glucose and lactate content, especially in the high BPA groups, post-feeding points to a metabolic disturbance. While the mechanism leading to poor feed utilization due to BPA is unknown, our findings suggest a fasting response in these animals despite higher feed intake. It remains to be seen if the higher IGF levels seen in the BPA groups post-feeding are a compensatory response to feed deprivation. In support, studies have shown that plasma IGF levels are modulated by fasting in rainbow trout (Reinecke et al., 2010).

In addition to the GH-IGF axis, the thyroid also plays a pivotal role in early fish development (Leatherland et al., 2010). Thyroid hormones (THs) have been shown to be involved in the changes associated with metamorphosis and in the transition from larvae to juveniles in fish (Power et al., 2001). In rainbow trout, TH signaling occurs through the thyroid hormone receptors (TRs), TRα and TRβ and the presence of thee receptors during early trout development has been clearly established (Jones et al., 2002; Raine et al., 2004). While several studies have implicated BPA and other endocrine disrupting compounds in disrupting thyroid hormone signaling in fish (Zoeller, 2005; Heimeier & Shi, 2010; Carr & Patiño, 2011); Terrien and Prunet, 2013), the current study is the first to show that maternally transferred BPA impacts TR transcript levels during offspring development. The higher TRα and lower TRβ mRNA abundance observed in the higher BPA groups at hatch and first feed, respectively, along with an overall down regulation of these transcripts in the BPA groups post-hatch suggests that the TH disruption seen with this contaminant is life-stage dependent. While the mechanism leading to TR disruption by BPA is unknown in fish, studies in mammalian and cell models indicate that BPA in the micromolar range inhibits TR-mediated gene activation *in vitro* (Moryiama et al., 2002), and acts as a TRβ antagonist in rats *in vivo* during development (Zoeller et al., 2005). The temporal changes in TR expression led us to hypothesize that BPA targets the thyroid system development in trout, and this may lead to modifications in TR signaling in later life stages. It

remains to be seen if the TR disruption may also be contributing to the changes in SGR seen post-feeding in trout larvae.

In conclusion, maternal transfer of BPA to oocytes and its subsequent presence in the developing embryos has long-term effects on growth in fish. The lower total energy content coupled with a higher feed conversion ratio post-feed in trout developed from BPA-laden eggs suggests a disturbed metabolism and reduced nutrient utilization. This leads us to propose that the fish are exhibiting a fasting response in the BPA group, and this may be playing a role in the reduced SGR. While the mechanism leading to the developmental effects of BPA is unknown, clearly this contaminant is an endocrine disruptor of the somatotropic and thyroid axes in trout. The long term changes observed after the chemical is no longer detected in the fish leads to the hypothesis that BPA in eggs may cause epigenetic changes in genes involved in somatotropic and thyroid axes functioning, inducing long-term and generational defects in growth and development.

2.6 Acknowledgements

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Chapter 3: Bisphenol A in Oocytes Impacts the Development and Function of the Hypothalamus-Pituitary-Interrenal Axis in Rainbow Trout

3.1 Overview

Bisphenol A is a chemical ubiquitously present in the aquatic environment and early developmental stage exposure has led to growth and developmental defects in fish. Recent studies have suggested that maternal deposition of BPA in oocytes and its subsequent presence in the embryos during development alters stress performance in juveniles, but no study has examined the effect of this contaminant on stress axis development and function. The hypothesis tested in this study was that maternal transfer of BPA impacts the development and function of the hypothalamus-pituitary-interrenal (HPI) axis during in trout. Oocytes had either no BPA (controls) or were loaded with 0.8 ± 0.6, 4.4 ± 0.9 and 41.3 ± 4.5 ng BPA embryo^{-1}. The temporal changes in whole body cortisol, steroidogenic gene profiles and corticosteroid receptor responses were monitored during development. In addition, fish were exposed to an acute handling stressor challenge at key developmental stages (hatch, just prior to first feed and post hatch) to assess HPI axis function by measuring whole body cortisol levels. The developmental profile of cortisol was altered in the 0.8 and 41.3 ng groups. StAR transcript levels were significantly reduced in the 0.8 and 4.4 ng groups, while P450scc mRNA abundance was elevated in the 41.3 ng group prior to the first feed. Whole body mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) protein expression were significantly higher in the 41.3 ng group prior to the first feed, while in the 4.4 ng group GR protein expression was higher at hatch. The onset of the cortisol stress response in controls and 0.8 ng group was seen at 65 dpf, but this was delayed in the 4.4 and 41.3 ng fish. However, all groups showed a cortisol response 10 weeks post-feed. Overall, the findings suggest that deposition of BPA in oocytes delays HPI axis development and functioning and also disrupts corticosteroid receptors expression in rainbow trout.

3.2 Introduction

The hypothalamus-pituitary-interrenal (HPI) axis in fish plays a dynamic role in development of the larvae and juveniles. Cortisol, the key glucocorticoid hormone released upon perception of a stressor, is a determining factor in redirecting energy flow within the organism, in an attempt to cope with stress (Flick et al., 2006; Vijayan et al., 2010). Regulation of cortisol production in teleosts during development and in response to a stressor is under the control of the HPI axis functioning (Barton et al., 2002; Vijayan et al., 2010).

The synthesis of cortisol by the interrenal tissue of fishes is modulated by ACTH, a proopiomelanocortin (POMC)-derived peptide from the pituitary (Sewer and Waterman, 2003; Flick et al., 2006). Upon release in the body, ACTH binds to the melanocortin 2 receptor (MC2R), a transmembrane G-protein coupled receptor, leading to cholesterol mobilization and the subsequent triggering of the cortisol synthesis pathway. First, steroidogenic acute regulatory protein (StAR) mediates the translocation of cholesterol in the inner mitochondrial membrane. Next, cholesterol is converted to pregnenolone, a reaction that is mediated by cytochrome P450 side chain cleavage (P450scc), the first rate-limiting enzymatic reaction of cortisol biosynthesis (Vijayan et al., 2010). This first enzymatic reaction is followed by several reactions catalyzed by a series of cytochrome P450 enzymes and hydroxysteroid dehydrogenases that ultimately lead to cortisol production (Sewer and Waterman, 2003). Cortisol signaling in target tissues occurs via the ligand-activated transcription factors, the corticosteroid receptors known as the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR; Bury et al., 2003; Alsop ad Vijayan, 2007; Aluru and Vijayan, 2007, 2008).

In most teleosts, there are two genes encoding for GR (GR1 and GR2), but only one gene for MR. In zebrafish, there is only one GR-encoding gene (Bury et al., 2003; Prunet et al., 2006; Alsop and Vijayan, 2009), Although a distinct role for each of the GR-encoding genes has not yet been elucidated, GR1 and GR2 activation is dependent on the circulating cortisol levels in the body: GR2 is more sensitive, being activated at lower cortisol levels, while the expression of GR1 appears to be induced at higher cortisol levels brought upon by a stressor challenge (Bury et al., 2003; Prunet et al., 2006; Alderman et al., 2012). While trout GR mRNA and proteins are detected in all major tissues, recent studies have shown that central and peripheral GR is essential in negative regulation of plasma cortisol levels (Alderman et al., 2012). In contrast to

GR, a clear role for MR in mediating the cortisol stress effect is not well understood (Vijayan et al., 2005).

HPI axis development has been well studied in teleosts. Cortisol in oocytes has been established to be of maternal origin in various fish species, including rainbow trout (*Oncorhynchus mykiss*; Barry et al., 1995; Auperin and Geslin, 2008) and zebrafish (*Danio rerio*; Nesan and Vijayan 2012, 2013), but the levels decrease during embryo development. In trout, endogenous cortisol production begins around hatching, (de Jesus and Hirano, 1992; Auperin and Geslin, 2008), when interrenal cells differentiate in the head kidney (Barry et al., 1995). However, trout are not able to elicit a cortisol stress response until several days to weeks after hatch, due to a delay in HPI axis maturation (Barry et al., 1995; Auperin and Geslin, 2008). This delay has also been reported in other fish species, such as zebrafish (Alsop and Vijayan, 2008) and perch (*Perca fluviatili*; Jentoft et al., 2002), and it appears to be conserved in higher vertebrates as well (Nesan and Vijayan, 2013). The activity of the HPI axis increases around first-feed, suggesting that HPI axis maturation may be associated with transition from endogenous to exogenous feeding, as proper regulation of gluconeogenic pathways may be needed during periods of fasting (Barry et al., 1995).

Prolonged exposure to stressors, including xenobiotics, and the associated disturbances in HPI axis activity can have deleterious effects on survival, growth, performance, immune function and reproduction (Vijayan et al., 2005; Iwama et al., 2006; Ings et al., 2011; Nesan and Vijayan, 2012). Recent studies in fish have shown that xenobiotics, including bisphenol A (BPA) and polychlorinated biphenyls (PCBs), can be transferred from the mother to the offspring (Ostrach et al., 2008; Takao et al., 2010). This finding raises concerns about the potential impact that these chemicals may have on the development of the fish HPI axis and the associated target tissue cortisol signaling, which play an important role in directing proper development (Vijayan et al., 2010; Nesan and Vijayan, 2013).

BPA is ubiquitously present in the environment (Cao et al., 2008, 2009), particularly in fresh waters (Chen et al., 2006) and in fish tissues (Wei et al., 2007; Renz et al., 2013). Exposure of fish to BPA leads to abnormal development and growth (McCormick et al., 2010), reproductive impairment and increased mortality (Lam et al., 2011; Hanson et al., 2012, 2014). While most *in vivo* and *in vitro* studies in fish have focused on the estrogenic properties of this chemical, comparatively little research has been conducted on how BPA deposition in the eggs,

mimicking maternal transfer, impacts the development of the HPI axis in fish. A recent study showed that oocyte loading with BPA leads to long-term effect on HPI axis functioning in response to a stressor in juvenile rainbow trout, *Oncorhynchus mykiss* (Aluru et al., 2010). However, whether the observed BPA-induced changes in plasma cortisol and glucose levels in this study are due to reprograming of the HPI axis during development is unknown.

This study tests the hypothesis that BPA deposition in fish eggs, mimicking maternal transfer, disrupts HPI axis programming during embryogenesis in rainbow trout. To this end, our two objectives were: 1) to assess the effects of early developmental BPA exposure on HPI axis development; and 2) to determine if there are any long term effects on HPI axis functioning. Trout oocytes were loaded with varying concentrations of BPA (0.8, 4.4 and 41.3 ng embryo^{-1}), followed by fertilization and development in clean water. The developmental profile of HPI axis activity at key developmental stages, including at hatch, first feed and post-feed in trout was monitored. Temporal changes in larval whole body cortisol levels, mRNA abundances of genes involved in corticosteroidogenesis (MC2R, StAR and P450scc) and expression patterns of key cortisol signaling proteins (GR and MR) to assess disruption in HPI axis development (objective 1) were examined. The effect of BPA on HPI axis functioning during development (objective 2) was ascertained by subjecting the embryos and larvae to a stressor and monitoring the whole body cortisol and glucose response over a 24 h period. Evoking a corticosteroid response post-stress is a well-established marker of stress performance and HPI axis activity, as this response is evolutionarily conserved and is an indication of the animal's adaptive ability to cope with stressors (Iwama et al., 2006; Vijayan et al., 2010).

3.3 Materials and methods

3.3.1 Materials and chemicals

The scintillation cocktail and the cortisol antibody were purchased from MP Biomedicals (Solon, OH, USA), while [1,2,6,7-^{3}H] cortisol tracer was purchased from GE Healthcare (Upsala, Sweden). Reagents for the bicinchoninic protein assay were purchased from Thermo Scientific (Rockford, IL, USA), while the 96-well plates for quantitative real-time PCR (qRT-PCR), along with the iQ SYBR® green fluorescent dye mix were purchased from Bio-Rad (Mississauga, ON, CA). The TriZol used for RNA extraction was purchased from Invitrogen (Burlington, ON, CA), and the first strand cDNA synthesis kit and the DNase I for treating the

RNA prior to making cDNA were purchased from Fermentas (Burlington, ON, CA). Unless otherwise specified, all other chemicals were purchased from Sigma-Aldrich.

3.3.2 Experimental fish

Experiments were conducted at Alma Aquaculture Research Station (AARS), Alma, Ontario, Canada, in accordance with the Animal Care and Use Committee of the University of Guelph, Guelph, Ontario, Canada and the Animal Care Committee of the University of Waterloo, Waterloo, Ontario, Canada. Egg pooling for BPA loading was conducted exactly as described previously (see Chapter 2). Larvae and fry used for Objective 1, measuring whole body developmental profiles of cortisol, steroidogenic genes and corticosteroid receptors, were obtained from fertilization of oocytes collected in September, while those used for Objective 2, acute stress responses, were obtained from fertilization of oocytes in February. AARS has bred over the years two populations of rainbow trout to sustain their stock: one that spawns in the fall and one in early spring. Both populations are healthy, with no reported difference in fecundity, gonad size and egg quality (M. Burke, personal communication). The sampling that was conducted on the September group did not leave enough fish to carry out the stress studies. Therefore, a second set of oocytes were BPA-treated and fertilized in February, as described above, and this group was used for the stressor exposure studies.

3.3.3 Experimental protocols

3.3.3.1 Objective 1- BPA effect on HPI axis development

The fish used for developmental profile monitoring of the HPI axis and cortisol signaling were from the same group of fish that were exposed to BPA as oocytes in Chapter 2, while those used for stress response measurements were from a different batch. Regardless, bisphenol A loading of oocyte, to mimic maternal transfer, along with the fish sampling protocol, was conducted exactly as previously described (see Chapter 2). Embryos and fish were as previously described (see Chapter 2). Oocytes accumulated 0.8, 4.4 and 41.3 ng BPA embryo^{-1} (background subtracted) following the 3 h incubation with 0.3, 3.0 and 30.0 µg ml^{-1} BPA in maternal fluid (see Chapter 2).

3.3.3.2 Objective 2 – BPA effect on HPI axis functioning

Stress performance experiments were conducted at three distinct life stages: 42 dpf (hatching), 65 dpf (first feed) and 140 dpf (post feed). Prior to the application of the stress, fish (n=6 individual fish) were collected (0 h time) from each of the treatments (controls, 0.8, 4.4 and 41.3 ng groups). The stress protocol used was that of Auprin and Geslin (2008) for 42 and 65 dpf fish, and Aluru et al. (2010) for 140 dpf fish, with some modifications. Briefly, just hatched embryos and larvae prior to first feed (65 dpf) were removed from their tray with a net and exposed to air for 1 min, after which they were placed in a glass beaker and manually swirled for another minute. At the end of the stress period, they were placed back in their respective trays and allowed to recover. Fish (n=6 per treatment at each time point) were then collected at 15 min (0.25 h), 30 min (0.5 h), 60 min (1 h), 120 min (2 h) and 24 h post-stress. Fish total weight (body + yolk) and length was recorded, after which the yolk was separated from the body. The samples were then placed on dry ice and stored in the -80 ˚C for later analysis of whole body cortisol and glucose levels.

Fish were also stressed at 140 dpf to assess if the cortisol production in response to an acute stressor had been impacted by BPA treatment. The stress protocol involved a handling and netting disturbance that has been previously described by Aluru et al. (2010). Briefly, a total of 6 fish were sampled from each of the replicate tanks prior to the application of the stress (0 h group). Fish were anesthetized with a dose of MS222, as previously described. The remaining fish were chased with a net and scooped out of the water 40 times for 3 min, after which they were allowed to recover. Sampling occurred at 1, 4 and 24 h post-stress and consisted in quickly netting the fish and placing them in a lethal dose of MS222 (1.0 g l^{-1} buffered with 2.0 g l^{-1} sodium bicarbonate). Whole fish were then flash frozen on dry ice and stored in the -80 ˚C for later analysis.

3.3.4 Analytical techniques

3.3.4.1 Whole body cortisol extraction and measurement

Cortisol measurements were carried out by radioimmunoassay (RIA) as described by Ings et al. (2012). The cortisol antibody was diluted 1:250 prior to use, while a dilution of 60.0 µl to 11.0 ml RIA buffer (40.0 mmol l^{-1} Na_2HPO_4, 10.0 mmol l^{-1} $NaH_2PO_4 \cdot H_2O$, 1.0 g l^{-1} gelatin

and 0.1 g l^{-1} thimerasol) was used for the [1,2,6,7-^{3}H] cortisol tracer. All dilutions were done in RIA buffer and both the antibody and tracer were tittered to ensure 35-50% binding prior to use.

Cortisol was extracted from the whole body of the fish following a protocol adapted from Alsop and Vijayan (2007) and Ings et al. (2011). Briefly, whole bodies were weighed and then crushed on dry ice using a mortar and a pestle. To this, 50 mmol l^{-1} Tris buffer (pH 7.4) with protease inhibitor (Roche Diagnostics GmBH, Mannheim, Germany) was added at 1:5 ratio according to Vijayan et al. (2006). The mixture was sonicated for 30 s on ice and 25-100 µl homogenate was then added to 900-975µl water and the mixture was vortexed for 30 s. Cortisol was extracted from the diluted homogenate using a 5 ml laboratory grade ethyl ether and the samples were vortexed for 45 s, incubated at room temperature for 10 min, after which the tubes were placed in dry ice for 5 min. After the bottom aqueous layer froze, the top ethyl ether layer was decanted in a pre-labeled glass vial. This was repeated two more times for a total of 3 extractions per sample. The samples were then left overnight to dry at room temperature and the steroid was re-suspended in 500 µl RIA buffer. Three 0 h control samples were spiked with 2 µg l^{-1} cortisol to determine the extraction efficiency of the assay (calculation 3.1). The cortisol extraction efficiency was 64.8 ± 9.2% and only non-corrected data are shown in the current study.

3.3.4.2 Measuring whole body protein and glucose levels

Protein levels in whole embryos or larvae were determined using the bicinchoninic acid (BCA) method (Smith et al., 1985) using bovine serum albumin as the standard. Plasma glucose levels were determined enzymatically as previously described (Bergmeyer, 1985; Birceanu et al., 2014) using a 96-well microplate spectrophotometer (VersaMax ROM v3.13, Molecular Devices, CA, USA). Whole body protein levels were determined by the bicinchoninic protein method with bovine serum albumin as the standard.

3.3.4.3 SDS-PAGE and western blotting

Larvae were homogenized in Tris (50 mmol l^{-1}, pH 7.4) buffer supplemented with protease inhibitor and the protein concentration determined as described above. The SDS-PAGE and western blotting followed the protocols described before (Sathiyaa and Vijayan, 2003). Briefly, homogenate was diluted with 2X Laemmli's sample buffer (0.5 mol l^{-1} Tris-HCl, pH 6.8, 60 mmol l^{-1} 25% glycerol, 2% SDS, 0.1% bromophenol blue, 14 mmol l^{-1} β-mercaptoethanol)

and then 1X Laemmli's sample to obtain a protein concentration of 2 mg ml^{-1}. Proteins in the samples (40 µg total protein loaded) were separated on an 8% polyacrylamide gel along with a pre-stained molecular mass ladder (FroggaBio Scientific Solutions, Toronto, ON, CA). The gel was run at 200 V for 42 min using 1X TGS (250 mM Tris, 1.92 M glycine, 1% SDS). Proteins were then transferred onto a 0.2 µm pore nitrocellulose membrane (BioRad) with a semidry transfer unit (BioRad) using a transfer buffer (25 mmol l^{-1} Tris pH 8.3, 192 mmol l^{-1} glycine, and 10% (v/v) methanol), at 20 V for 26 min. The transfer was confirmed by protein visualization with Ponceau S solution. Blots were washed with TTBS (3 × 10 min; 20 mmol l^{-1} Tris pH 7.5, 300 mmol l^{-1} NaCl and 0.1% (v/v) Tween 20). Membrane blocking was done with 5% skim milk in TTBS containing 0.05% sodium azide, for 60 min on a shaker, at room temperature. The rabbit polyclonal anti-trout GR (1:1000; Sathiyaa and Vijayan, 2003) used was diluted in the 5% skim milk blocking solution containing 0.05% sodium azide, while the MR antibody used was zebrafish affinity-purified (1:1000; 21st Century Biochemicals, Marlboro, MA, USA). The membranes were incubated in primary antibody (60 min at room temperature for GR and overnight at 4 ˚C for MR), then washed in TTBS (3 × 10 min). They were then incubated in secondary antibody (goat-anti-rabbit IgG coupled to horseradish peroxidase; BioRad, Hercules, CA, USA) for 60 min at room temperature, and washed with TTBS (3×10 minutes). Band detection was carried out using an ECL-Plus Western blotting detection system (GE Healthcare Life Sciences, Piscataway, NJ, USA) and scanned by PharosFX^{TM} Molecular Imager (BioRad), equipped with the Quantity One software (version 4.6.9). All bands were quantified with Chemi-imager using AlphaEase software (Alpha Innotech, Santa Clara CA, USA). Equal loading of samples was confirmed by first washing the membranes in TTBS (3 × 10 min), and then probing them with β-actin (Cy3-coupled monoclonal primary antibody produced in mouse, 1:1000). Protein intensity was normalized to β-actin.

3.3.4.4 RNA extraction and first strand cDNA synthesis

Total RNA from whole body was extracted using the TriZol Reagent, by following the manufacturers' protocols. RNA extraction, DNAase treatment and first strand cDNA synthesis were conducted as previously described (see Chapter 2).

3.3.4.5 Primers and quantitative real-time polymerase chain reaction

The primers were designed using MC2R, StAR, P450scc and EF1α sequences from rainbow trout (Table 3-1). The mRNA abundance was analyzed using the iQ SYBR® green fluorescent dye master mix with the Bio-Rad iCycler real-time PCR detection system as described before (Aluru et al., 2010). Copy number for each gene was determined using plasmid standard curves previously established in our laboratory following the protocol described by Aluru et al. (2010). All the samples were assayed for the genes of interest and for the housekeeping gene (EF1α) and the relative transcript levels determined by quantitative real-time PCR, as previously described (Aluru et al., 2010). EF1a was used as the housekeeping gene because the threshold cycle values were similar between the different developmental stages and treatments and hence used for normalization. The values in the figures are shown as % of 42 dpf control values for all the life stages.

3.3.5 Calculations and statistical analysis

Cortisol extraction efficiency was calculated using the following formula:

(3.1) Extraction efficiency = measured $_{[cortisol]}$ / expected $_{[cortisol]}$ × 100%

Statistical analysis was performed using SigmaPot 11.0 software (Systat Software Inc., San Jose, CA, USA). All data are shown as mean ± standard error of the mean (S.E.M.). Two-way analysis of variance (ANOVA) was used to determine the effects of BPA treatment and temporal changes on cortisol profiles, gene transcripts and whole-body protein expression. Whenever interactions were detected, a Tukey's post-test was used, and a probability level of $p < 0.05$ was considered significant. The data were log-transformed and square-root transformed wherever necessary to meet the assumptions of normality and equal variance. Only non-transformed data are shown in the figures. For figures showing percentages, the S.E.M. is to be used only as reference and it is not a true reflection of the data variance. For simplicity purposes, whenever there was no significant difference noted between treatments within one time point, no letters were added to the bars on the figures.

Table 3-1. Forward (F) and reverse (R) sequences, amplicon size, accession number and annealing temperature for the primers used in real-time quantitative PCR.

Gene of interest	Sequence	Amplicon size (bp)	Accession #	Annealing temperature (˚C)
MC2R	F: 5′-GAGAACCTGTTGGTGGTGGT-3′ R: 5′-GAGGGAGGAGATGGTGTTGA-3′	105	EU119870.1	60
StAR	F: 5′-TGGGGAAGGTGTTTAAGCTG-3′ R: 5′-AGGGTTCCAGTCTCCCATCT-3′	101	AB047032.2	60
P450scc	F: 5′-GCTTCATCCAGTTGCAGTCA-3′ R: 5′-CAGGTCTGGGGAACACATC-3′	140	S57305.1	60
EF1α	F: 5′-CATTGACAAGAGAACCATTGA-3′ R: 5′-CCTTCAGCTTGTCCAGCAC-3′	95	AF498320.1	56

3.4 Results

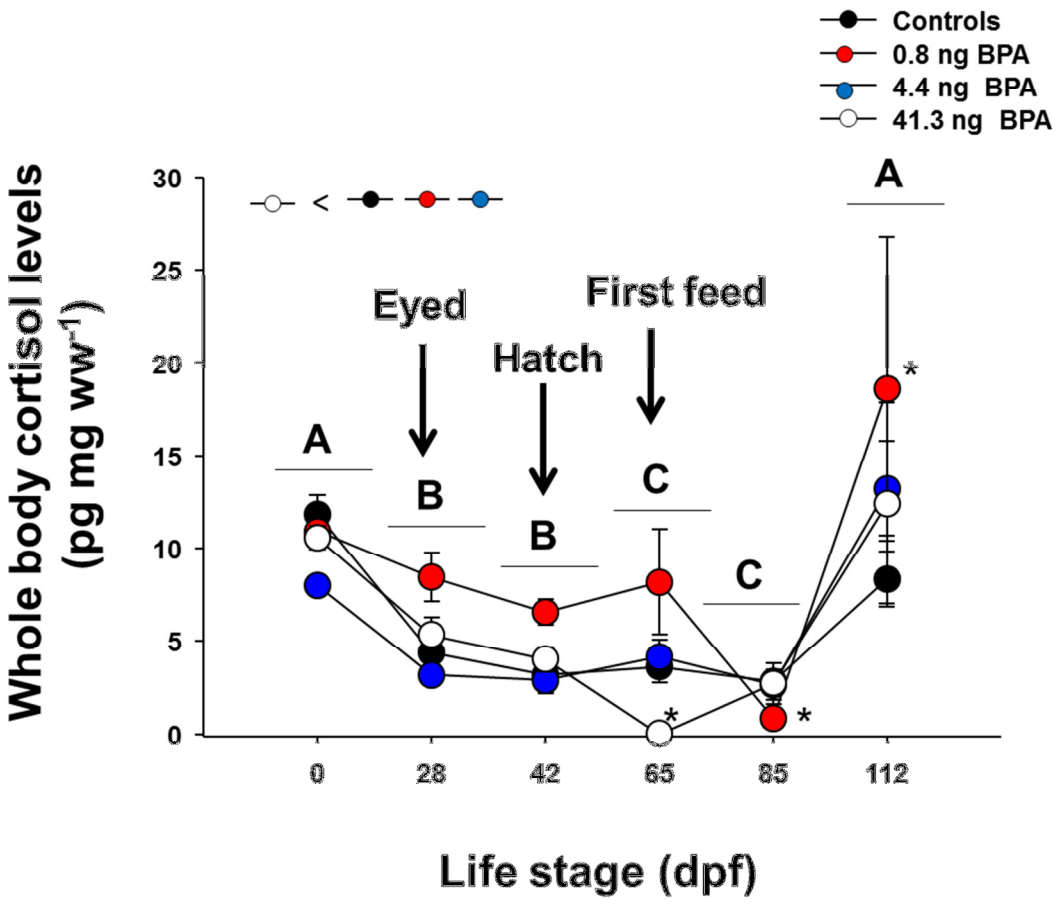
3.4.1.1 Objective 1 – BPA effect on HPI axis development

3.4.1.1.1 Developmental profile of cortisol

The basal concentrations of cortisol from fertilization to 112 dpf are shown in Fig. 3-1. Whole body cortisol levels in control embryos and larvae decreased 3.6-fold from fertilization to 28 dpf (eyed stage), and then remained stable at similar levels at 42 dpf (hatch). At 112 dpf, the control cortisol levels increased approximately 10-fold when compared to the 85 dpf levels. The larvae in the highest BPA treatment had significantly lower cortisol levels at first feed when compared to their respective controls, while the oocytes that accumulated 0.8 ng BPA embryo^{-1} had reduced cortisol at 85 dpf and a 2-fold increase in basal cortisol levels at 112 dpf when compared to controls at the same life stages. Overall, the 41.3 ng group had significantly lower cortisol levels than controls and the other BPA groups.

Figure 3-1. Developmental profile of cortisol.

Whole body cortisol levels (pg mg^{-1} ww) at key developmental stages in control (black circle) and BPA-exposed oocytes. Oocytes exposed to 0.3, 3.0 ad 30.0 mg l^{-1} BPA accumulated 0.8 (red circle), 4.4 (blue circle) and 41.3 (white circle) ng BPA $embryo^{-1}$, respectively, immediately following the 3 h incubation in maternal fluid. Each value represents the mean ± S.E.M. for a sample size of n=5-6 pools of 5-10 eggs until hatching, or individual larvae and fry after hatching. Upper case letters denote a life stage effect on whole body cortisol levels, while Asterisks (*) denote a treatment effect within one life stage that is significantly different from their respective controls (two-way ANOVA, Tukey's post-hoc test, $p<0.05$).

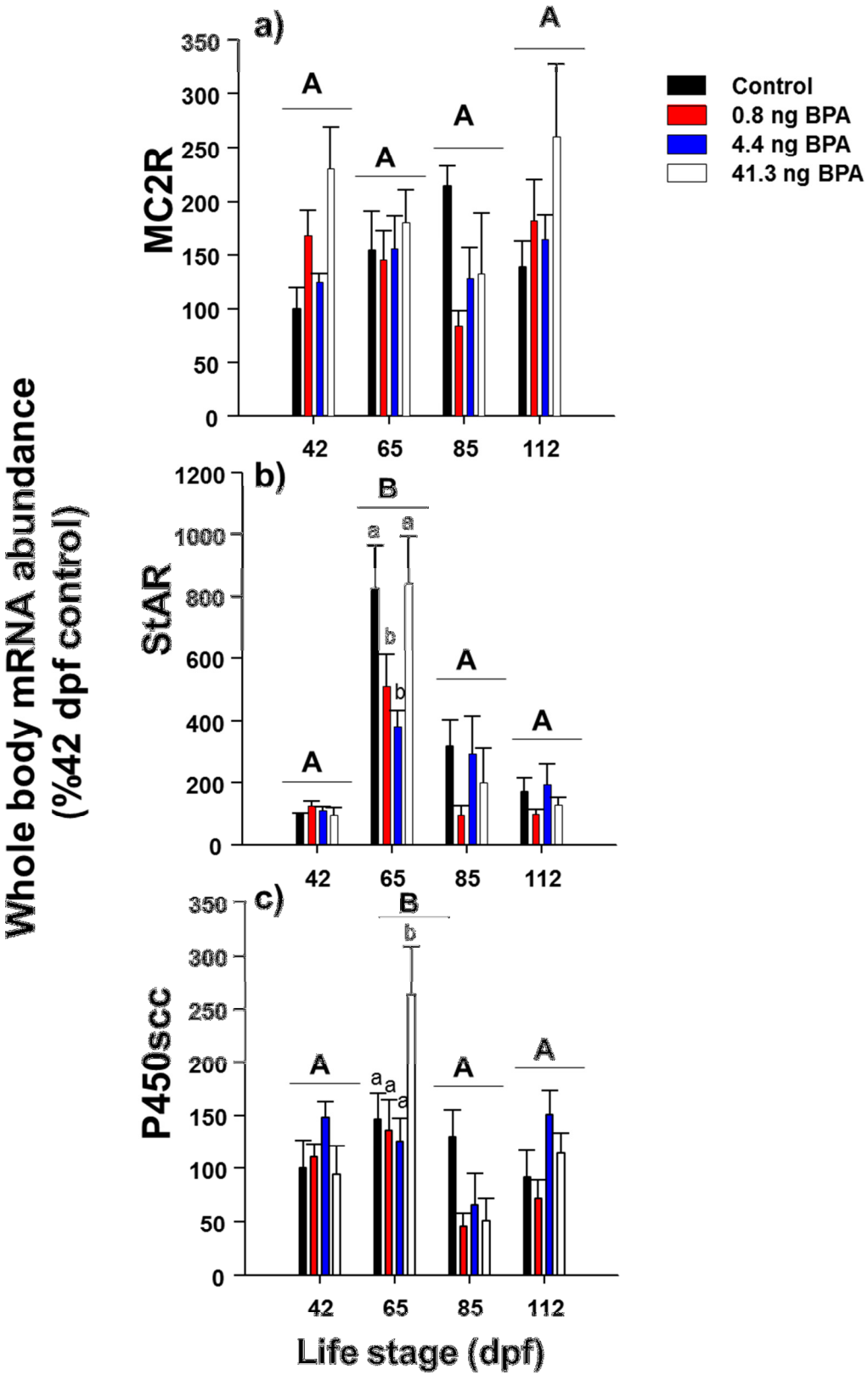

Controls
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
Whole body cortisol levels
(pg mg ww-1)
30
25
20
15
10
5
0
Eyed
Hatch
First feed
A
B
B
C
C
A
*
*
*
0
28
42
65
85
112
Life stage (dpf)

3.4.1.1.2 Developmental profile of MC2R, StAR and P450scc

The developmental profile of key genes involved in cortisol production was examined (Fig. 3-2). The mRNA abundance of MC2R remained unchanged throughout development and it was not affected by oocyte loading with BPA (Fig. 3-2a). The transcript abundance for StAR in control fish, however, increased 8-fold at 65 dpf when compared to the levels at hatch (42 dpf), after which the levels dropped again post-feed (Fig. 3-2b). Oocytes that had initially accumulated 0.8 and 4.4 ng BPA embryo^{-1} had approximately 1.5 and 2.0-fold, respectively, reduction in StAR transcript levels at 65 dpf when compared to the control fish at the same life stage, while the transcript levels in oocytes that accumulated the highest BPA levels were not impacted by the exposure. P450scc mRNA levels did not change across life stages in controls (Fig. 3-2c); however, the oocytes that accumulated 41.3 ng BPA embryo^{-1} had an approximately 2-fold increase in transcript levels at 65 dpf, while the other BPA treatments did not affect P450scc transcripts at any life stage.

Figure 3-2. Developmental profile of mRNA abundance of MC2R, StAR and P450scc.

Whole body transcript levels of (a) MC2R, (b) StAR and (c) P450scc measured at key developmental stages, following the 3 h oocyte loading with BPA in maternal fluid, prior to fertilization. Upper case letters denote a life stage effect on transcript levels, while lower case letters denote a treatment effect within one life stage (n=5-6 individuals, two-way ANOVA, Tukey's post-hoc test, $p<0.05$).

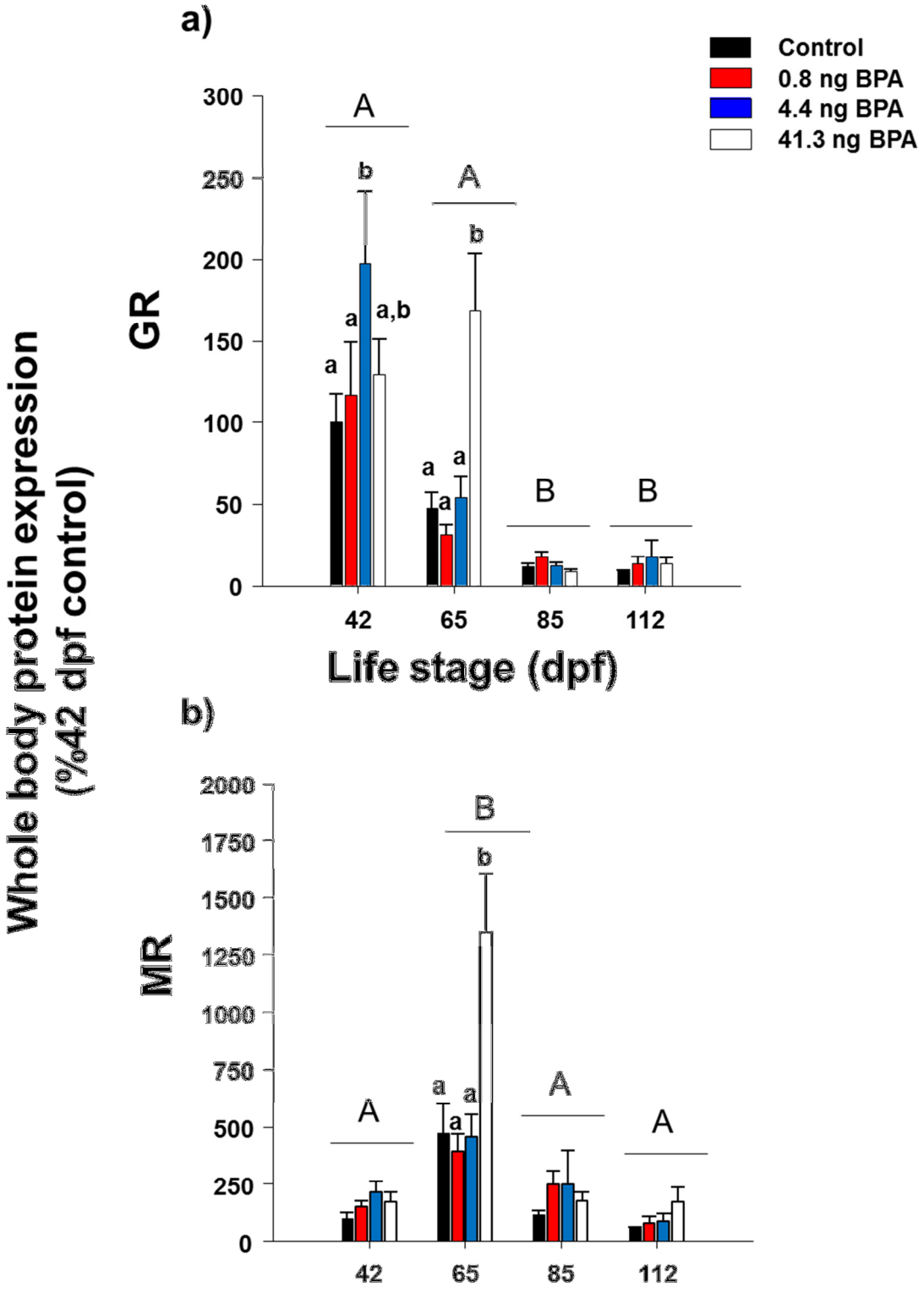

Whole body mRNA abundance
(%42 dpf control)
a)
MC2R
b)
StAR
c)
P450scc
Life stage (dpf)
42
65
85
112
Control
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA

3.4.1.1.3 Developmental profile of MR and GR protein expression

The whole body developmental profile of MR and GR protein levels at key developmental stages is shown in Fig. 3-3. In controls, MR levels peaked at 65 dpf (first feed), after which they returned to hatch levels across all the life stages examined. The highest BPA exposure induced an approximately 3-fold increase in MR protein expression at 65 dpf, when compared to controls (Fig. 3-3a). GR protein levels in control individuals decreased 2-fold from 42 dpf to 65 dpf, and then they decreased approximately 10-fold at 85 and 112 dpf, when compared to the 42 dpf levels (Fig. 3-3b). At hatch (42 dpf), oocytes that accumulated 4.4 ng BPA embryo^{-1} had a 2-fold increase in GR protein levels when compared to the controls at the same life stage. At first feed (65 dpf), however, the highest BPA group had a 3-fold increase in protein expression when compared to its respective control levels.

Figure 3-3. Developmental profile of MR and GR protein expression.

Whole body protein expression for (a) MR and (b) GR at key developmental stages, in controls and BPA-treated oocytes (refer to Fig 3-1 for more detailed methods). Upper case letters denote a life stage effect on whole body protein levels, while lower case letters denote a treatment effect within one life stage (n=4-6 individuals, two-way ANOVA, Tukey's post-hoc test, $p<0.05$).


a)
Control
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
GR
300
250
200
150
100
50
0
A
A
B
B
b
a
a,b
a
a
b
a
a
42
65
85
112
Life stage (dpf)
b)
MR
2000
1750
1500
1250
1000
750
500
250
0
B
A
A
A
b
a
a
a
42
65
85
112
Whole body protein expression
(%42 dpf control)

3.4.2 Objective 2 – BPA effects on HPI axis functioning

Whole body cortisol levels following a stressor challenge were measured at 42 dpf (hatch), 65 dpf (first feed) and 140 dpf (post feed) to determine the effects of oocyte BPA accumulation on HPI axis functioning (Fig. 3-4). There was no stress response elicited at 42 dpf, although control levels of the steroid were approximately 3-fold higher than those of the BPA-treated larvae (Fig. 3-4a). At 65 dpf, however, cortisol levels in control fry increased 10-fold at 0.5 h post-stress when compared to non-stressed fish (0 h) and to the 2 and 24 h samples (Fig. 3-4b). The 0.8 ng group showed a stress response comparable to controls, while no increase in cortisol levels were noted in the 4.4 and 41.3 ng groups. Overall, the cortisol levels post-stress were significantly lower in the 41.3 ng group when compared to controls. At 140 dpf there was a stress response elicited in all the groups, regardless of treatment, and there were no statistically significant differences in cortisol levels among the treatment groups (Fig. 3-4c).

Overall, whole body glucose levels were significantly higher at 1 h and 2 h after stress when compared to earlier time points at 42 dpf (Fig. 3-5). Control glucose levels were lower prior to stress, after which they significantly increased by 2-fold at 24 h. The BPA-induced effects were dependent upon the sampling time. The 41.3 ng group had significantly higher glucose levels pre-stress (0 h) when compared to controls and 4.4 ng groups at the same time point, while at 1 h they were significantly higher than control values only (Fig. 3-5a). In the 0.8 ng BPA group, glucose levels peaked at 2 h, when they were approximately 2-fold higher than the controls. At 65 dpf, control glucose levels increased by 1.5-fold at 0.5 h post-stress, after which the levels decreased. There was an overall treatment effect, with the 41.3 ng group having higher levels than the controls and the 4.4 ng group, irrespective of the sampling time (Fig. 3-5b). At 140 dpf, the cortisol profile following a stressor was different than at 65 dpf. In controls, glucose levels were high prior to stress, , remained constant 1 h post-stress, after which they decreased by 1.5-fold at 4 h and 24 h (Fig. 3-5c). There was an overall BPA-induced effect, with the whole body glucose levels in the 4.4 ng group being significantly lower than control values, irrespective of time.

Figure 3-4. Cortisol profile following acute stress.

Whole body cortisol levels at (a) 42 dpf, (b) 65 dpf and (c) 140 dpf in control and BPA-treated oocytes (refer to Fig. 3-1 for more detailed methods). Upper case letters denote a life stage effect on whole body cortisol levels (n=5-6 individuals, two-way ANOVA, Tukey's post-hoc test, $p<0.05$).

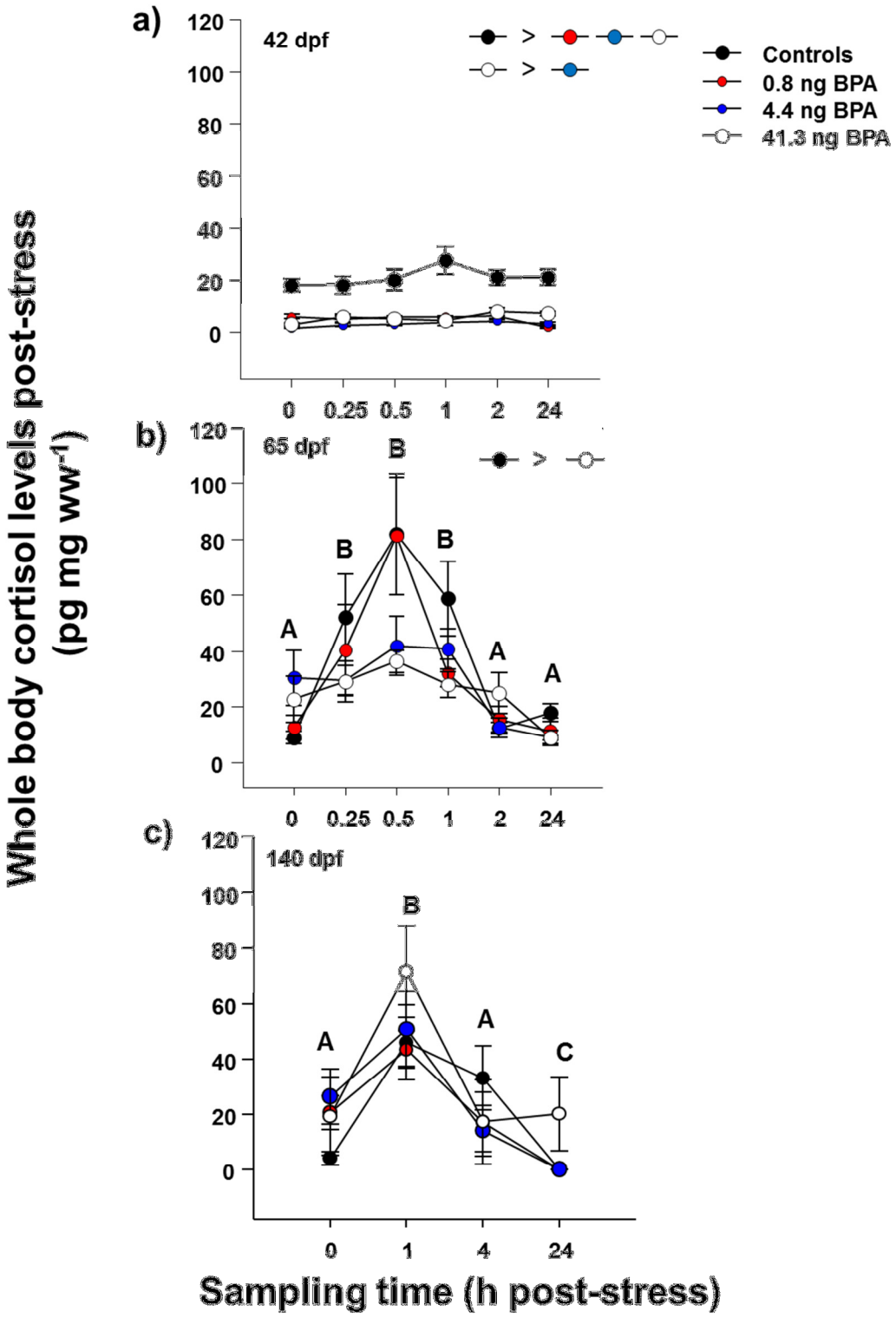
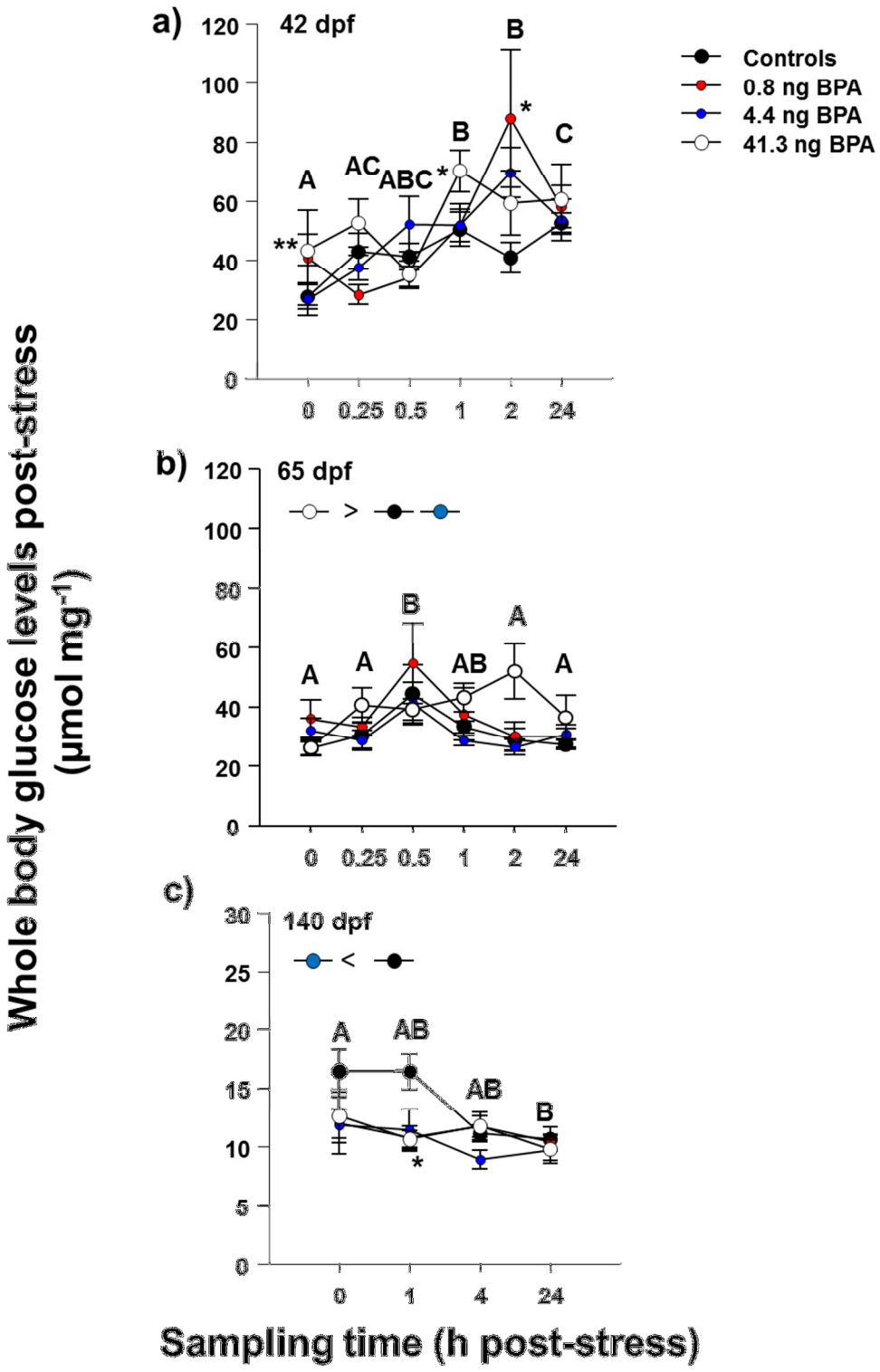

a)
42 dpf
Controls
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
b)
65 dpf
c)
140 dpf
Whole body cortisol levels post-stress
(pg mg ww-1)
Sampling time (h post-stress)

Figure 3-5. Glucose profile following acute stress.

Whole body glucose levels at (a) 42 dpf, (b) 65 dpf and (c) 140 dpf in control and BPA-treat oocytes (refer to Fig. 3-1 for more detailed methods). Inserts denote a time effect. One asterisk (*) denotes a significant difference between the treatment it marks and the control, while two asterisks (**) denote a significant difference in 41.3 ng group from controls and 4.4 ng BPA (n=5-6 individuals, two-way ANOVA, Tukey's post-hoc test, $p<0.05$).


a)
42 dpf
b)
65 dpf
c)
140 dpf
Controls
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
Whole body glucose levels post-stress
(µmol mg-1)
Sampling time (h post-stress)

3.5 Discussion

BPA loading of oocytes, mimicking maternal transfer, significantly altered the developmental profile of cortisol and steroidogenic genes, although the chemical was no longer detected in the embryos at 42 pdf (hatch; see Chapter 2, Fig. 2-1a). To our knowledge, this is the first study to examine the developmental profile of HPI axis in response to contaminant accumulation in eggs. Several studies have demonstrated that contaminants impact stress response in juvenile fish, suggesting HPI axis as a target for endocrine disruption (see Hontela and Vijayan, 2008 for a review; Ings et al., 2011; Sandhu and Vijayan, 2014). These effects can have long-term implications, since cortisol signaling via the GR and MR plays a key role in regulation of growth, development and metabolism (Vijayan et al., 2005; Aluru and Vijayan, 2010).

In the current study, cortisol levels measured at key developmental stages in fish were in agreement with those previously described in trout (Barry et al., 1995; Auperin and Geslin, 2008). In general, cortisol levels in developing fish embryos decline after fertilization until several days post-hatch, after which the cortisol levels rise again due to activation of endogenous cortisol production pathways (Barry et al., 1995; Auperin and Geslin, 2008; Zubair et al., 2012; Nesan and Vijayan, 2013). The cortisol content in the oocytes and freshly fertilized embryos is of maternal origin, and it is thought to drive the early developmental events in fish (Nesan and Vijayan, 2013). While studies suggest that *de novo* synthesis of cortisol commences several days after hatching (Barry et al., 1995; Auperin and Geslin, 2008; Li et al., 2012; Alsop and Vijayan, 2008; Nesan and Vijayan, 2013), the capacity to synthesize cortisol to stress and ACTH challenge prior to hatching have also been reported in fish (Barry et al., 1995; Auperin and Geslin, 2008). Our experiments demonstrated unequivocally that trout larvae do not exhibit a cortisol response to an acute stressor challenge at hatch, but they do so prior to the commencement of feeding (65 dpf). Auperin and Geslin (2008) did not observe an increase in cortisol in embryos and larvae exposed to an acute stressor until 9 days post-hatch, while Barry et al. (1995) suggested that trout are not capable of responding to a stressor until several days post-hatch, proposing that perhaps the HPI axis is not mature enough to elicit a signal to induce cortisol production in the interrenals immediately after hatch. Indeed, research suggests that, although trout interrenal cells were capable of corticosteroid biosynthesis and ACTH was present

in the pituitary, there was a 2-week hyporesponsiveness period to an acute stressor after hatch (Barry et al., 1995), which is in line with the findings of the current study.

The loading of the oocytes with BPA altered the cortisol profile during development, and the effects appear to be dose-specific. The oocytes that accumulated 0.8 and 41.3 ng BPA embryo^{-1} showed a similar trend of decreased cortisol after fertilization (0 dpf) when compared to controls. However, the decrease in cortisol levels was not as steep in the 0.8 ng group as it was in the controls during development, suggesting that these embryos might not have been able to metabolize the maternally derived steroid as efficiently. Indeed, Aluru et al. (2010) noted a delayed reduction in cortisol levels following a stressor applied to BPA-exposed fish reared in identical conditions, possibly due to an inability of the individuals to metabolize the compound, as a result of a BPA-induced reprogramming of the HPI axis; whether this is the case, however, needs further investigating.

Although the 0.8 ng group had higher than control cortisol levels throughout early development, these fish exhibited lower levels of the steroid at 85 dpf when compared to controls at the same life stage. Similarly, cortisol levels in the 41.3 ng group continuously decreased up to 65 dpf, when BPA was no longer detected in the larvae, only to increase at 85 and 112 dpf to control levels. Overall, the 41.3 ng group had reduced cortisol levels when compared to controls, with the levels being lowest at 65 dpf (just prior to first feed). These findings suggest that perhaps there is a BPA-mediated delay in *de novo* cortisol synthesis in these fish (a delay to first feed in the 41.3 ng group and to early post-feed in the 0.8 ng group), as they are unable to maintain constant cortisol levels after the maternally-deposited reserves are depleted. Unlike the BPA-exposed fish, the basal control cortisol levels remain constant at 65 and 85 dpf, suggesting that controls are able to synthesize the steroid at these time points, as previously reported (Barry et al., 1995; Alsop and Vijayan, 2009; Nesan and Vijayan, 2013). Inability of the fish to sustain cortisol levels post-hatch is indicative of a delay in HPI axis functioning. Contaminants, such as metals, have been shown to disrupt cortisol production *in vitro* in trout (Sandhu and Vijayan, 2011; Miller and Hontella, 2011), while BPA has been shown to affect the stress response profile in a concentration-specific manner in juvenile trout at 400 dpf, in an oocyte loading experiment similar to the current study (Aluru et al., 2010).

At the level of the genome, the expression patterns of the steroidogenic genes varied with developmental stage. Although control MC2R mRNA levels followed an upward trend from

hatch to 85 dpf, this change was not significantly different across the time points examined. This result is not surprising, since Alsop and Vijayan (2007) reported a significant increase in the MC2R transcript levels at hatch in the zebrafish (*Danio rerio*), suggesting that hatching is the time when the steroidogenic pathway is activated in teleosts (Barry et al., 1995). Indeed, our sampling time points are all at- or post-hatch, after the onset of the steroidogenic pathway, when a levelling off of the basal transcript levels of MC2R was reported in previous studies (Alsop and Vijayan, 2007). In contrast to MC2R, control StAR and P450scc transcript levels peaked just prior to first feed (65 dpf), an increase that was not mirrored in the cortisol profile of the fish. While the role that the steroidogenic genes play in trout follicle development and maturation has been established (Nakamura et al., 2005), little is known about the steroidogenic gene expression profiles during trout development post-hatch. Nevertheless, the expression patterns of StAR transcripts noted in the current study are in line with those reported by Alsop and Vijayan (2007) in zebrafish, with the transcript abundance peaking prior to first feed. This pattern could be related to the onset of the acute stress response in trout, which occurs approximately 2 weeks after hatch. The transcript profiles of StAR and P450scc in the current study further suggest that the onset of feeding may be a critical time in the activation of the higher brain and the hypothalamic centers responsible for mediating a stress response in teleosts (Nesan and Vijayan, 2013).

The onset of exogenous feeding (at or after 65 dpf) appears to be sensitive to BPA-induced reprogramming of the stress axis, as it appears that there is a delay in HPI axis activation in the 41.3 ng group, as implied by the barely detectable basal cortisol levels in this group at 65 dpf, and the overall reduced cortisol levels during development. This is further supported by the lack of a cortisol production following an acute stress just prior to first feed, which is then followed by a normal cortisol profile when a stressor is applied at 140 dpf. While the transcript levels of MC2R were not affected by BPA loading of oocytes, those of StAR and P450scc were impacted. StAR mRNA abundance decreased in the lower BPA groups, while in the 41.3 ng group there was no change. Taken together with the increase in P450scc transcripts, and with the much higher MR and GR protein expression in these fish when compared to 65 dpf controls, BPA exposure (41.3 ng) impacts HPI axis function and may disrupt GR and MR signaling during development. However, the BPA effect on HPI axis functioning was transient during development, and by 140 dpf there was no effect of BPA on the stress response in trout.

MR and GR are ligand activated transcription factors that translate the cortisol signal to the genome (see Chapter 1, Fig. 1-5), bringing about the activation or suppression of various pathways involved in development (Alsop and Vijayan, 2009; Nesan and Vijayan, 2013). Although similar studies have been done in zebrafish (Alsop and Vijayan, 2009), to our knowledge, this study is first to show the developmental profile of MR and GR protein expression in rainbow trout post-hatch. In the trout larvae, MR levels increased just prior to first feed, mirroring the increases observed in StAR and P450scc transcripts, suggesting that MR signaling may play a role in the onset of stress response in trout. Indeed, a similar profile was noted in MR transcripts of zebrafish embryos from 1.5 to 25 hours post-fertilization (hpf), suggesting a role for MR in teleost stress axis development (Stolte et al., 2008; Alsop and Vijayan, 2009; Takahashi and Sakamoto, 2013). The increased MR protein expression at 65 dpf suggests that this receptor may also have a role in initiation of exogenous feeding, and the associated osmoregulation (Kiilerich et al., 2011), as well as feeding behavior (Takahashi and Sakamoto, 2013). In contrast to MR, control GR protein expression was highest at hatch, while decreasing at the later life stages. This decrease may be related to the elevation in whole body cortisol levels. Down regulation of GR transcripts in the presence of higher levels of circulating cortisol have been previously reported in salmonids (Shrimpton, 1996; Shrimpton and McCormick, 1998), while *in vitro* hepatocyte studies have reported elevated GR mRNA levels, but decreased GR protein content with high cortisol (Sathiyaa and Vijayan, 2003). Taken together, the data suggests that cortisol levels may be playing a role in the post-hatch regulation of GR expression during trout development.

The results suggest that BPA affects MR and GR protein expression at 65 dpf. While the cortisol stress response was completely abolished in the 41.3 ng group, these fish had higher basal MR and GR protein levels when compared to controls and the lower BPA groups. MR and GR modulate glucocorticoid signaling in fish, which impacts osmoregulation, growth development and metabolism (Mommsen et al., 1999). Therefore, the disruption in corticosteroid receptors expression prior to first feed may lead to metabolic and growth disturbances. Indeed, Chapter 2 showed that whole body energy is reduced in the 41.3 ng group, and this correlated with a reduced specific growth rate in these fish (Chapter 2, Figs. 2-3c, 2-4a). Together, these results suggest that BPA-mediated metabolic and growth dysfunctions may involve changes in MR and GR protein expression in trout.

The current study has shown, for the first time, how the developmental profile of steroidogenic genes and corticosteroid receptors in rainbow trout post-hatch is impacted by BPA deposition in oocytes. This study provides evidence that HPI axis development and function is impacted by BPA accumulation in eggs. The longer term effects of BPA exposure on stress axis activation leads to the hypothesis that epigenetic changes in genes involved in proper HPI axis functioning and in cortisol signaling may be playing a role in the impaired stress response (Aluru et al., 2010). Whether these changes are passed onto the offspring remains to be resolved. Knowledge on the developmental profiles of key genes and proteins involved in HPI axis development and function, as well as on how contaminants disrupt those profiles, can offer valuable insights on the mode of action of chemicals. Such an understanding of the mode of action of contaminants like BPA can bring about better guidelines for evaluating risk assessment.

3.6 Acknowledgements

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Chapter 4: Bisphenol A in F0 Oocytes Impacts Growth and Stress Performance in F2 Generation Rainbow Trout

4.1 Overview

Previous research has shown that BPA exposure during embryogenesis, mimicking maternal transfer, has long term impacts on growth and stress performance in F1 fish, but studies analyzing the effects in subsequent generations (F2) are scarce. Therefore, the hypothesis was that BPA accumulation in oocytes, mimicking maternal transfer of contaminants, leads to defects in growth and stress performance in the F2 generation of rainbow trout (*Oncorhynchus mykiss*). Trout F0 oocytes were loaded with either vehicle (control; <0.01% ethanol) or 0.8, 4.4 and 41.3 ng BPA embryo^{-1} and offspring in the F2 generation were assessed for impact on the somatotropic and hypothalamus-pituitary-interrenal (HPI) axes functioning. There was a significant reduction in specific growth rate of offspring in the F2 generation that were from the 4.4 ng BPA-exposed lineage. Also, these same fish showed reduced whole body glycogen content just prior to first feed. Growth hormone isoforms (GH-1 and -2) mRNA abundance profiles were altered during development, while their receptor profiles remained unchanged in the BPA-exposed lineages. Insulin-like growth factor (IGF)-1 and -2 transcript levels were reduced in the lower BPA groups, while IGF-1 receptor-b abundance was lower than controls in the 4.4 ng group throughout development. With respect to the HPI axis, there was a reduction in cortisol levels at hatch in the offspring from BPA lineage. Melanocortin 2 receptor (MC2R) transcript abundance during development was significantly higher in the lower BPA group, while StAR and P450scc levels were unaffected. Exposure of juveniles to an acute handling stressor resulted in delayed cortisol recovery to pre-stress levels in the 4.4 ng BPA lineage over a 24 h period. These results demonstrate for the first time that ancestral exposure to BPA leads to the disruption of growth and stress performance in rainbow trout.

4.2 Introduction

Bisphenol A (BPA) is a chemical that is ubiquitously present in the aquatic environment. Concentrations as high as 14.9 ng l^{-1} (Lee and Peart, 2000) have been reported in fresh waters, while concentrations reaching 11-75 ng g^{-1} dry weight (dw) have been reported in fish tissues (Belfroid et al., 2002). Although BPA is classified as a weak estrogenic compound, recent research has focused more on its non-estrogenic properties, as this chemical has been shown to have pleiotropic effects, independent of the estrogen receptor (Duan et al., 2008; Vandenberg et al., 2009; McCormick et al., 2010). Recently, Aluru et al. (2010) showed that exposure of fish embryos to BPA during critical periods of development leads to long-term effects on growth and stress performance in rainbow trout (*Oncorhynchus mykiss)*, suggesting that BPA induces epigenetic changes that will lead to generational effects. This hypothesis is further supported by evidence from mammals, where BPA administered to female mice, at pre-mating, gestation and lactation stages, has been shown to alter epigenetic patterning during early stem cell development of the fetus, leading to alterations in offspring phenotype (Dolinoy et al., 2008). In fish, few studies have shown that BPA is transferred from the mother to the offspring (Takao et al., 2008), and that the chemical persists in the developing embryo for several days post-fertilization, being detected during critical periods of development, including organogenesis and eyed stage in rainbow trout (Takao et al., 2008; Aluru et al., 2010). However, studies on generational effects of BPA in fish, following maternal exposure to contaminants during their critical developmental stages, are scarce.

Somatotropic and HPI axes functioning are used as markers of long-term fish performances. (Barry et al., 1995; Auperin and Geslin, 2008; Reinecke, 2010; Nesan and Vijayan, 2012; see Chapter 1). With respect to the somatotropic axis, growth hormone (GH) secreted by the pituitary gland in response to endogenous or exogenous cues exerts action by binding to its receptors (GH-R) in target tissues. They play a key role in osmoregulation, metabolism, tissue growth, reproduction and immune function, either directly or indirectly by stimulating the release of insulin-like growth factor 1 (IGF-1) and IGF2 from the liver (Reinecke et al., 2005). The IGFs exert their effect by biding to IGF receptors, IGF-1rb and -1ra, and they have a key role in growth and development (Reinecke et al., 2005; Reinecke, 2010). Recent studies have shown that the somatotropic axis can indeed act as a target of contaminant toxicity (Aluru et al., 2010; Hanson et al., 2014; see Chapter 2). The HPI axis also plays a critical role in

development, with cortisol being involved in major physiological processes, including growth, metabolism, immune function and osmoregulation (Mommsen et al., 1999). In addition, cortisol plays a central role in the organismal acute response to a stressor, bringing about metabolic and physiological changes that offer an adaptive advantage to dealing with the stressor challenge.

The synthesis of cortisol by the interrenal tissue of fishes is modulated by ACTH, a proopiomelanocortin (POMC)-derived peptide from the pituitary (Sewer & Waterman, 2003; Flick et al., 2006). Upon release in the body, ACTH binds to the melanocortin 2 receptor (MC2R), a transmembrane G-protein coupled receptor, leading to cholesterol mobilization and the subsequent triggering of the cortisol synthesis pathway. First, steroidogenic acute regulatory protein (StAR) mediates the translocation of cholesterol in the inner mitochondrial membrane. Next, cholesterol is converted to pregnenolone, a reaction that is mediated by cytochrome P450 side chain cleavage (P450scc), the first rate-limiting enzymatic reaction of cortisol biosynthesis (Vijayan et al., 2010). This first enzymatic reaction is followed by several reactions catalyzed by a series of cytochrome P450 enzymes and hydroxysteroid dehydrogenases that ultimately lead to cortisol production (Sewer and Waterman, 2003). Therefore, any alterations in the HPI axis functioning due to contaminants will have long-term implication and will lead to reduced fitness (Ings et al., 2012).

Against this background, the hypothesis was that BPA accumulation in oocytes (F0 generation), mimicking maternal transfer of the contaminant, leads to modifications in growth, stress response and developmental profiles of the somatotropic (growth) and hypothalamus-pituitary-interrenal (HPI) axes components in the F2 generation of rainbow trout. Offspring hatched from mothers that were grown from eggs exposed to BPA (ancestral exposure) were used to assess impact on F2 generation. Markers of growth, including specific growth rate (SGR), food conversion ratios (FCR), body mass, whole body water content and condition factors were monitored post-feed in F2 fish. The developmental profiles of genes involved in somatotropic (GHs, IGFs and their receptors) and HPI (MC2R, StAR, P450scc) axes functioning were examined, along with whole body metabolite composition (glycogen, glucose, lactate and protein), at key developmental stages, including hatch (42 dpf), prior to first feed (65 dpf) and post-feed (85, 112 and 140 dpf). To determine impact on stress response, fish were exposed to an acute handling stressor at 140 dpf and the whole body cortisol and glucose response was monitored over a 24 h period during recovery.

4.3 Materials and Methods

4.3.1 Materials and Chemicals

Unless otherwise specified, all chemicals and enzymes were purchased from Sigma–Aldrich (St. Louis, MO, USA). The scintillation cocktail and the cortisol antibody were purchased from MP Biomedicals (Solon, OH, USA), while [1,2,6,7-^{3}H] cortisol tracer was purchased from GE Healthcare (Upsala, Sweden). D-Glucose was purchased from Bioshop (Burlington, ON, CA), while monobasic and dibasic sodium phosphate, sodium bicarbonate and borosilicate tubes were purchased from Fisher Scientific (Ottawa, ON, CA). The 96-Well plates for quantitative real-time PCR (qRT-PCR), along with the iQ SYBR® green fluorescent dye mix were purchased from Bio-Rad (Mississauga, ON, CA). The TRiZol used for RNA extraction was purchased from Invitrogen (Burlington, ON, CA), while the first strand cDNA synthesis kit and the DNase I for treating the RNA prior to making cDNA were purchased from Fermentas (Burlington, ON, CA). All the solutions used for BPA extraction were HPLC grade.

4.3.2 Experimental Fish

Experiments were conducted at Alma Aquaculture Research Station (AARS), Alma, Ontario, Canada, in accordance with the Animal Care and Use Committee of the University of Guelph, Guelph, Ontario, Canada and the Animal Care Committee of the University of Waterloo, Waterloo, Ontario, Canada.

Gametes were pooled from 3-year-old rainbow trout females (F1 generation, previously exposed to BPA as oocytes; see Chapter 2). Oocytes from females were collected and labeled based on the maternal BPA exposure concentration. Milt from stock male rainbow trout was pooled and used for all treatments. Upon collection, oocytes were visually assessed for quality by well-trained AARS personnel and assigned a number from 1 to 3 (1 = lowest quality; 3 = highest quality). Only those meeting the highest quality were used for experiments. Ovarian fluid was collected from all females (a total of ~ 200 ml), and it was stored at 6-8˚C prior to the beginning of the experiment. The ovarian fluid was also labeled according to the female's initial BPA accumulation. Therefore, the BPA numbers (in ng embryo^{-1}) reflect the BPA accumulated by the oocytes in F0 oocytes (0.8, 4.4 and 41.3; see Chapter 2) and are not a reflection of the BPA accumulated by the fish in this experiment.

4.3.3 Experimental protocols

4.3.3.1 Oocyte incubation and sampling protocol

The pooled oocytes were distributed among pre-labeled containers and immersed in 50 ml of their respective ovarian fluid, without any BPA, for 3 h at 6-8 ˚C to mimic the conditions followed for F0 generation oocyte loading experiment (Chapter 2). The containers were gently shaken every 30 min, as previously described for the maternal exposure (see Chapter 2). At the end of the 3 h period, the oocytes were fertilized and incubated as previously described, and sampling was done exactly as before, without any deviations. After hatch, fish were moved to holding tanks identical to their parental holding tanks at the same life stage, and kept under identical conditions. Sampling protocol involved anesthetizing the fish in a lethal dose of methane-tricaine sulphonate (MS222; 1.0 g l^{-1}) buffered with sodium bicarbonate (2.0 g l^{-1}). All sampling was conducted as previously described, without any deviations (see Chapter 2).

4.3.3.2 Experiment 1 - Developmental profile of the somatotropic axis in F2 generation

To determine whether ancestral exposure to BPA impacted the somatotropic axis function, fish were collected at identical developmental stages (days post-fertilization, dpf) as the F1 generation fish (see Chapter 2). Briefly, embryos were collected at 7, 14, 28 (eyed stage), 42 (hatch), 65 (first feed), 85, 112 and 140 dpf for whole body analysis of various parameters. Samples were immediately frozen on dry ice and stored at -80 ˚C. Phenotypic changes in F2 generation fish were monitored, along with whole body water, dry weight, specific growth rate (SGR), as well as food conversion ratio (FCR) and whole body developmental profiles of glycogen, glucose and lactate levels. At the level of the genome, the developmental profile mRNA abundance of key genes of the somatotropic axis (GH-1, -2 and GH-1r, GH-2r; IGF-1 and -2, IGF-1ra and -1rb) were measured.

4.3.3.3 Experiment 2 – Developmental profile and functioning of the hypothalamus-pituitary-interrenal (HPI) axis

To assess whether HPI axis programming has been altered by ancestral exposure to BPA, the whole body cortisol profile and the mRNA abundance of key steroidogenic genes (MC2R; StAR; P450scc) were monitored at 42, 65, 85, 112 and 140 dpf. In addition, fish were exposed to an acute stressor at 140 dpf, identical to the protocol previously described (Aluru et al., 2010;

see Chapter 3). Briefly, a total of six fish were sampled from each tank prior to the application of the stress (0 h group). Fish were anesthetized with a dose of MS222, as previously described. The remaining fish were acutely stressed (netting and repeated air exposure (40 times) for 3 min) after which they were allowed to recover. Sampling occurred at 1, 4 and 24 h post-stress and consisted of quickly netting the fish and placing them in a lethal dose of MS222 (1.0 g l^{-1} buffered with 2.0 g l^{-1} sodium bicarbonate). Whole fish were then flash frozen on dry ice and stored at -80 ˚C for later analysis.

4.3.4 Analytical techniques

4.3.4.1 BPA extraction and quantification

BPA extraction from the embryos and larvae was carried out as previously described by Aluru et al. (2010), with the minor modifications described in Chapter 2. BPA was measured only in 0 dpf oocytes, to ensure the chemical was not present in the oocyte at the beginning of the experiment. Quantification of BPA was carried out as previously described by Aluru et al. (2010) and Chapter 2, without any modifications. The extraction efficiency for BPA was 79.3 ± 0.6%. Only BPA numbers not corrected for extraction efficiency are shown in this study.

4.3.4.2 Tissue and whole body processing and analysis for glycogen, glucose, lactate and protein

Whole body analysis of glucose, glycogen and lactate developmental profiles were determined enzymatically as previously described (Bergmeyer, 1985; Birceanu et al., 2014; Chapter 2). Where levels were below detection (lactate assay only), an arbitrary number of 0.001 was assigned to the data points, to allow for statistical analysis. Whole body protein levels were determined at 562 nm by the bicinchoninic protein method (Thermo Scientific, Rockford, IL, USA), with bovine serum albumin as the standard.

4.3.4.3 RNA extraction and first strand cDNA synthesis

Total RNA from whole body was extracted using the TRIzol reagent following the manufacturers' protocols. RNA quantification and DNase treatment, along with cDNA synthesis were conducted exactly as previously described (see Chapter 2).

4.3.4.4 Primers and Quantitative Real-Time Polymerase Chain Reaction

The primers were designed and the transcript levels were analyzed as previously described (see Chapter 2; Table 4-1; Aluru et al., 2010). All data was then normalized to EF1α, as this gene did not change across life stages, and the data was reported as % change from the 42 dpf control samples for all the life stages.

4.3.5 Calculations and statistical analysis

Condition factor (CF), specific growth rate (SGR), along with food conversion ratio (FCR) and whole body water content were determined using the following formulas:

$$(4.1) \quad CF = (10^5 \times W_i) / (L_i^3)$$

$$(4.2) \quad SGR = Ln[W_2 / W_1] / (t_2 - t_1) \times 100\%$$

$$(4.3) \quad FCR = W_f / (W_2 - W_1)$$

$$(4.4) \quad \text{Body water content} = (W_i - W_{i\text{-dry}}) / W_i \times 100\%$$

where W_i is the wet weight of individual fish in the tank in g, L_i is the fish's respective length in mm; W_1 and W_2 represent the total weight in g of the fish in the tank at the beginning (t_1) and at the end (t_2) of the interval, respectively; W_f is the total weight of food consumed over the experimental period; $W_{i\text{-dry}}$ is the dry weight for each individual fish of wet weight W_i.

Statistical analysis was performed using SigmaPot 11.0 software (Systat Software Inc., San Jose, CA, USA). All data are shown as mean ± standard error of the mean (S.E.M.). A two-way analysis of variance (ANOVA) was used to determine significance of BPA treatment on gene transcripts, whole body water content and dry weight. A one-way ANOVA was used for analyzing the effects of BPA exposure on various parameters, such as CF, SGR and FCR. With both types of ANOVAs, a Tukey's post-hoc test was used whenever interactions were detected, and a probability level of $p < 0.05$ was considered significant. The data was log-transformed wherever necessary to meet the assumptions of normality and equal variance. Only non-transformed data are shown in the figures. For figures showing %change, the S.E.M. is to be used only as reference and it is not a true reflection of the data. For simplicity purposes, whenever there was no significant difference noted between treatments within one time point, no letters were added to the bars on the figures.

Table 4-1. Forward (F) and reverse (R) sequences, amplicon size, accession number and annealing temperature for the primers used in real-time quantitative PCR.

Gene of interest	Sequence	Amplicon size (bp)	Accession #	Annealing temperature (˚C)
EF1α	F: 5′-CATTGACAAGAGAACCATTGA-3′ R: 5′-CCTTCAGCTTGTCCAGCAC-3′	95	AF498320.1	56
IGF-1	F: 5′-TGGACACGCTGCAGTTTGTGTGT-3′ R: 5′-CACTCGTCCACAATACCACGGTT-3′	120	EF450071	68
IGF-2	F: 5′-CGGCAGAAACGCTATGTGGA-3′ R: 5′-TGCTGGTTGGCCTACTGAAA-3′	79	EF450072	58
IGF-1Ra	F: 5′-AGAGATAGACGACGCCTCCTA-3′ R: 5′-CACCAAATAGATCCCTACGT-3′	104	AF062499	58
IGF-1Rb	F: 5′-CCTAAATCTGTAGGAGACCTGGAG-3′ R: 5′-GTTAGCCACGCCAAATAGATCC-3′	139	AF062500	58
GH-1	F: 5′-TTCAAGAAGGACATGCACAAGGTC-3′ R: 5′-CTCCAGCCCACGTCTACAGA-3′	97	AF005923	66
GH-2	F: 5′-CCCACGTTTACAGAGTGCAGTTG-3′ R: 5′-GCTTCAAGAAGGACATGCATAAGGTT-3′	93	DQ294400	66
GH-1r	F: 5′-TGAACGTTTTTGGTTGTGGTCTA-3′ R: 5′-CGCTCGTCTCGGCTGAAG-3′	61	AY861675.1	60
GH-2r	F: 5′-CATGGCAACTTCCCACATTCT-3′ R: 5′-GCTCCTGCGACACAACTGTTAG-3′	65	AY751531.1	60
MC2R	F: 5′-GAGAACCTGTTGGTGGTGGT-3′ R: 5′-GAGGGAGGAGATGGTGTTGA-3′	105	EU119870.1	60
StAR	F: 5′-TGGGGAAGGTGTTTAAGCTG-3′ R: 5′-AGGGTTCCAGTCTCCCATCT-3′	101	AB047032.2	60
P450scc	F: 5′-GCTTCATCCAGTTGCAGTCA-3′ R: 5′-CAGGTCTGGGGAACACATC-3′	140	S57305.1	60

4.4 Results

4.4.1 Embryo phenotypic changes and growth

There were no delays in hatch or first feed observed in these fish with any treatment. All fish hatched at 42 dpf and the yolk was fully utilized by 65 dpf. Fish weight at 140 dpf was significantly lower in the 41.3 ng BPA lineage compared to controls (Table 4-2). Similarly, body length was significantly lower in the two higher BPA lineages compared to control and 0.8 ng groups, while condition factor (CF) was significantly higher in the 4.4 ng group when compared to controls at 140 dpf.

Whole body water levels and dry weight developmental profiles were life-stage dependent (Fig. 4-1). Whole body water in controls significantly increased from 42 dpf to 65 dpf, but at 85 dpf the levels were not significantly different than at hatch (Fig. 4-1a). At 112 dpf, control fish whole body water content significantly decreased when compared to 85 and 42 dpf levels. At 85 dpf, the whole body water content in the BPA groups were 6-8% lower than control levels. There was a clear ancestral BPA exposure (0.8 ng BPA embryo^{-1}) effect on whole body water content that was independent of the life stage (Fig. 4-1a).

The dry weight was life-stage dependent and the levels were significantly higher at 85 and 112 dpf compared to the 42 and 65 dpf values (Fig. 4-1b). At 85 dpf, there was a significant treatment effect with ancestral exposure to BPA increasing (3-fold) dry weight content compared to the control group. Overall, ancestral exposure to 0.8 ng BPA embryo^{-1} resulted in a significantly higher dry weight content compared to the controls and this was life stage-independent (Fig. 4-1b).

There was a significant reduction in specific growth rate (SGR) from 65 to 112 dpf due to ancestral exposure to 4.4 ng BPA, but not the other BPA concentrations, compared to the control (Fig. 4-2a). Food conversion ratio (FCR) was not significantly impacted by ancestral BPA exposures (Fig. 4-2b).

Table 4-2. Body characteristics of F2 generation rainbow trout at 140 dpf (n=24). Data points sharing the same letter are not statistically different (one-way ANOVA, Tukey's post-hoc test, $p<0.05$).

BPA accumulation in F1 oocytes after 3 h of incubation (background subtracted) (ng embryo^{-1})				
	Control	**0.8**	**4.4**	**41.3**
Length (mm)	47.6 ± 0.8^{a}	49.2 ± 0.7^{a}	44.2 ± 0.8^{b}	44.7 ± 0.7^{b}
Weight (g)	1.1 ± 0.04^{a}	1.2 ± 0.04^{a}	1.0 ± 0.05^{ab}	1.0 ± 0.04^{b}
CF	1.1 ± 0.03^{a}	1.0 ± 0.02^{a}	1.2 ± 0.03^{b}	1.1 ± 0.02^{ab}

Figure 4-1. Effect of ancestral exposure to BPA on whole body water and dry weight in trout.

Changes in (a) whole body water (% wet weight, ww) and (b) dry weight (mg) in F2 generation, following exposure at the oocyte stage (see Materials and Methods for details). Data are shown as mean + S.E.M. (n=6 individuals). Upper case letters denote a time effect, while lower case letters denote a treatment effect within one time point. Bars or groups of bars with different letters are statistically significant (two-way ANOVA, with Tukey's post-hoc test, $p<0.05$). For simplicity purposes, wherever there was no statistical significance noted between treatments within one time point, no letters were used.

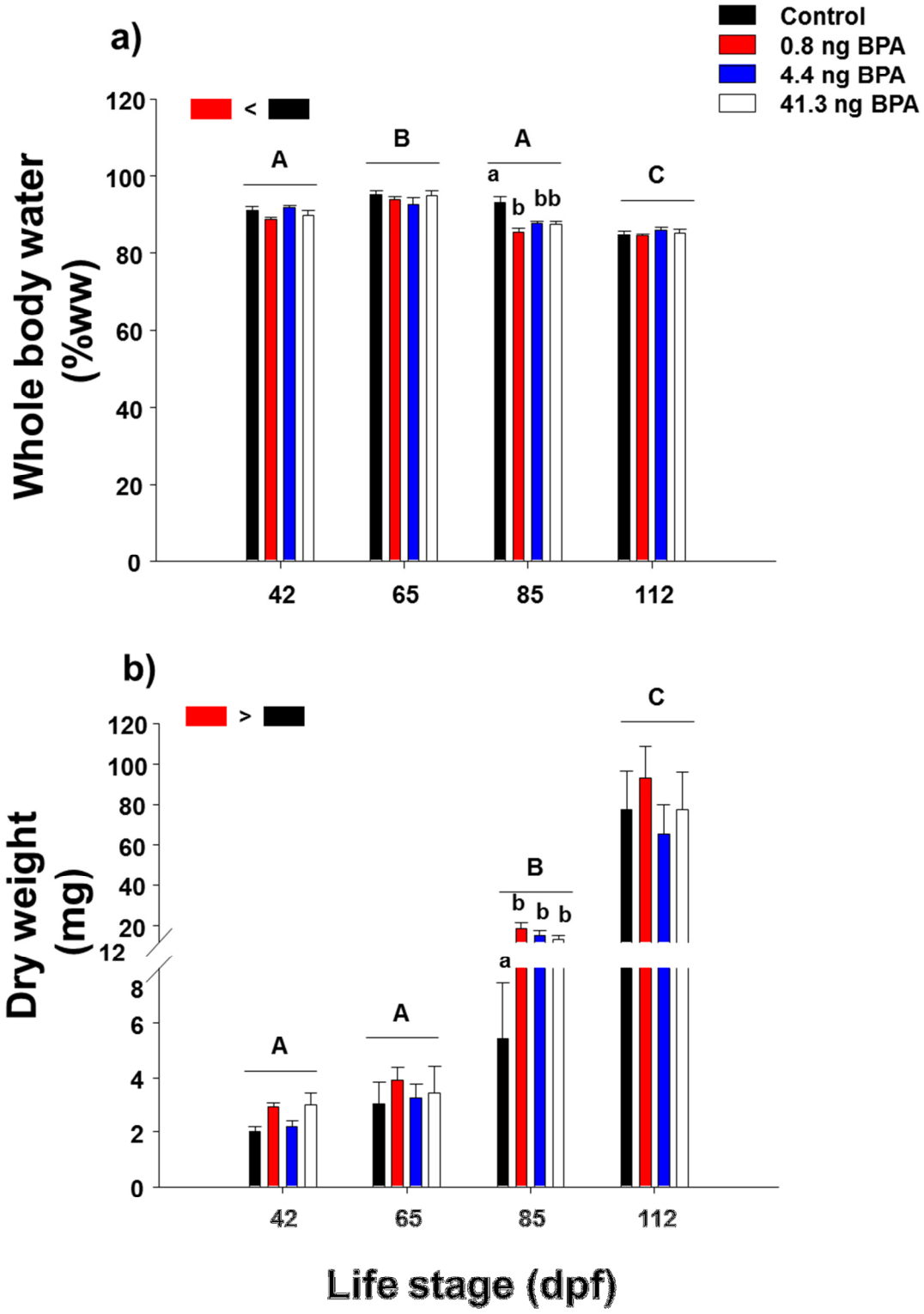
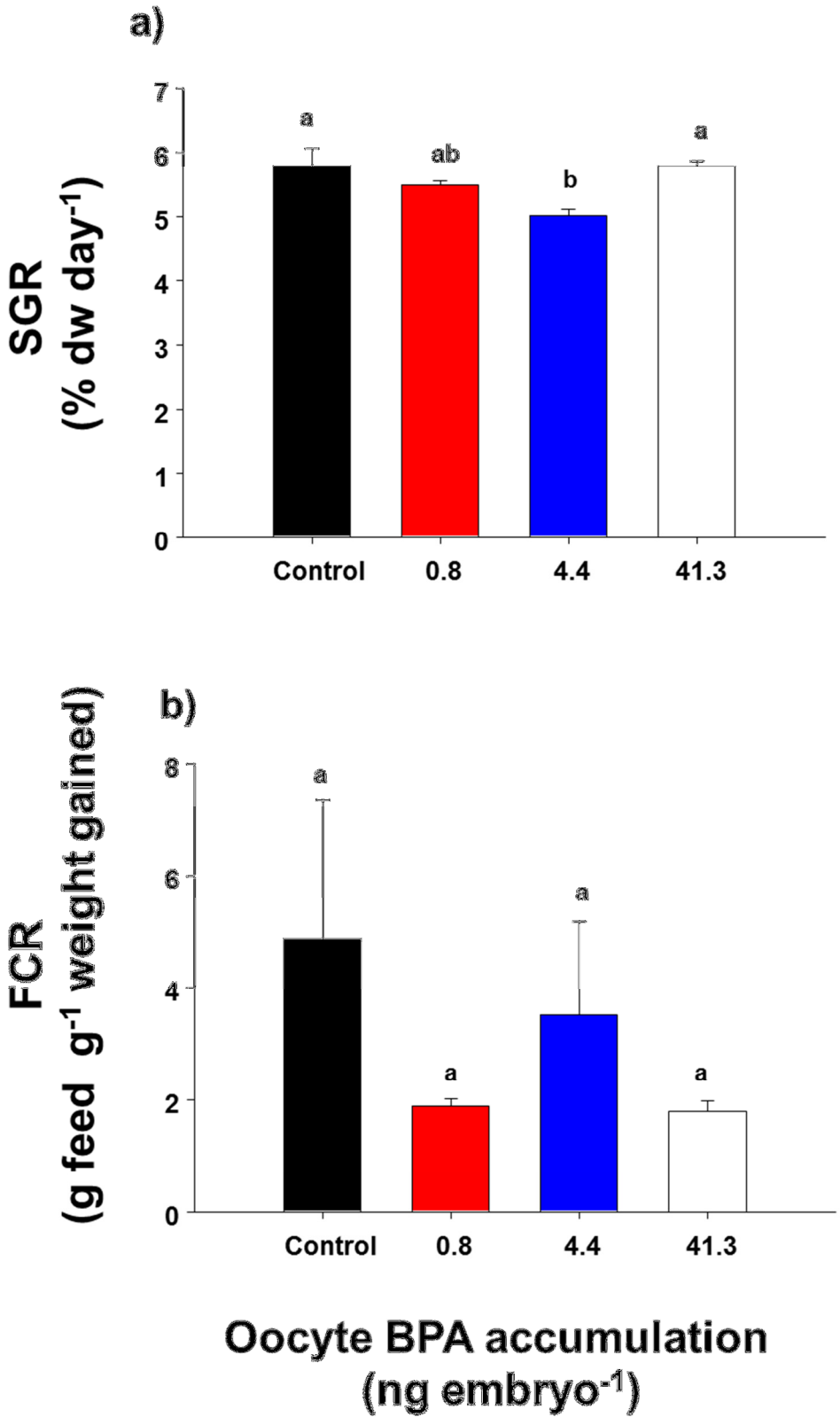

a)
Control
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
Whole body water (%ww)
120
100
80
60
40
20
0
<
A
B
A
C
a
b
bb
42
65
85
112
b)
Dry weight (mg)
>
120
100
80
60
40
20
12
8
6
4
2
0
A
A
B
C
b
b
b
a
42
65
85
112
Life stage (dpf)

Figure 4-2. Effects of ancestral exposure to BPA on specific growth rate and food conversion ratio in F2 individuals.

Changes in (a) specific growth rate (SGR) and (b) food conversion ratio (FCR) from 65 dpf (first feed) to 112 dpf, in F2 generation, following maternal loading with BPA (for an expanded description of the protocol, refer to Fig. 4-1 and Materials and Methods section). Data are shown as mean + S.E.M. (n=3-4 tanks). Letters denote statistically significant differences between treatments. Bars with different letters are statistically significant (one-way ANOVA, Tukey's post-hoc test, $p<0.05$).


a)
SGR
(% dw day-1)
7
6
5
4
3
2
1
0
a
ab
b
a
Control
0.8
4.4
41.3
b)
FCR
(g feed g-1 weight gained)
8
6
4
2
0
a
a
a
a
Control
0.8
4.4
41.3
Oocyte BPA accumulation
(ng embryo-1)

4.4.2 Whole body glycogen, glucose, lactate and protein levels

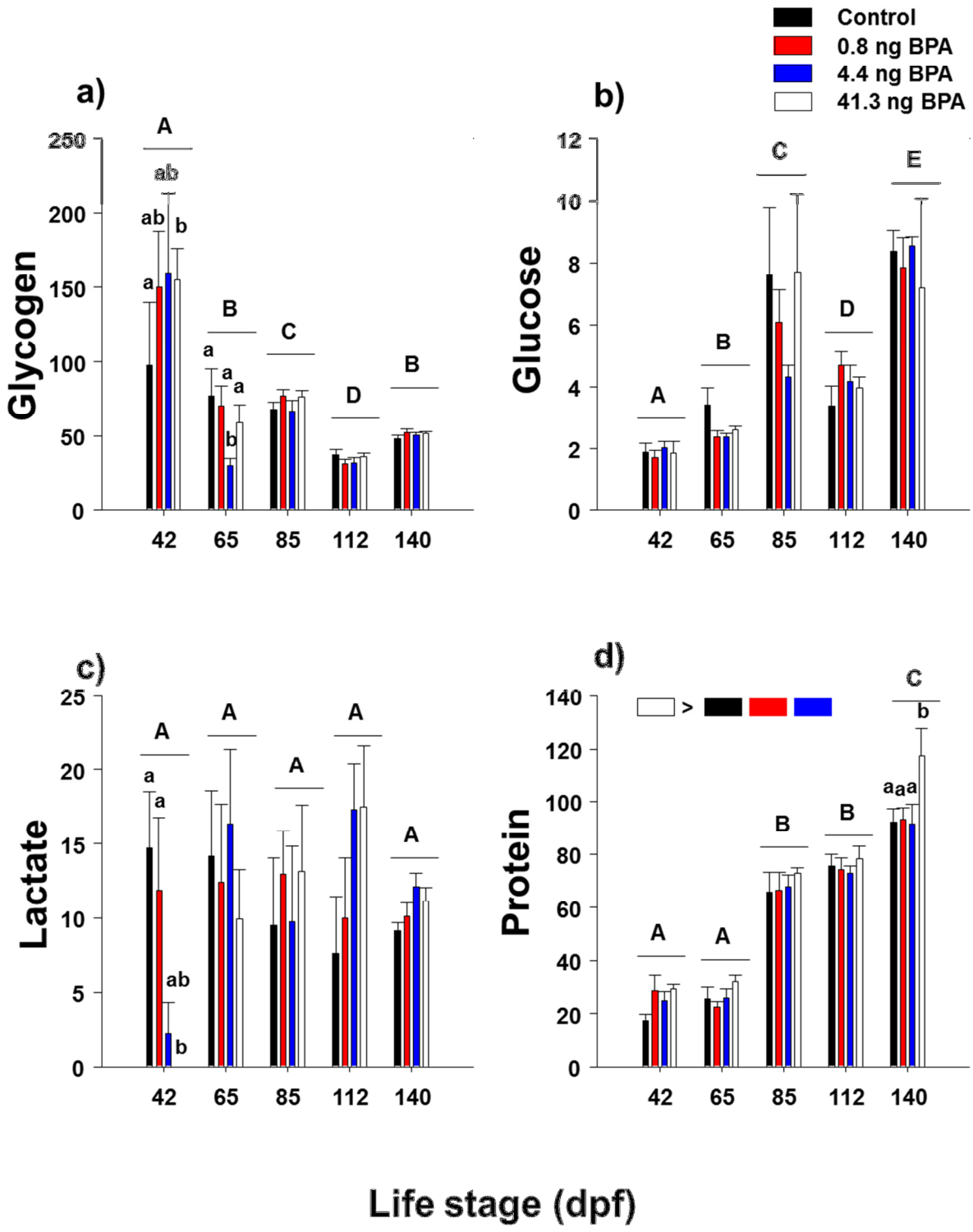
The profiles of whole body energy reserves, lactate and protein levels were life stage-dependent (Fig. 4-3). Whole body glycogen levels in the control group were highest at 42 dpf, and then significantly decreased at each life stage, reaching their lowest levels at 112 dpf, where they were 2.6-fold lower than at 42 dpf (Fig. 4-3a). While there were no effects of ancestral exposure to BPA in F2 generation post-feed, whole body glycogen levels in the 41.3 ng group at hatch (42 dpf) were 1.6-fold higher than the controls at the same life stage. At first feed (65 dpf), the 4.4 ng group had whole body glycogen levels that were 2.6-fold lower than all the other groups (Fig. 4-3a).

Whole body glucose levels significantly increased at each life stage (Fig. 4-3b). Glucose levels increased 2.0-fold at first feed and 4.0-fold at 85 dpf when compared to hatch levels. At 112 dpf, however, whole body glucose significantly decreased, only to increase again at 140 dpf. There was no measurable effect of maternal exposure to BPA during development on whole body glucose in F2 generation. Whole body lactate control levels, although variable, were not dependent on developmental stages (Fig. 4-3c). At 42 and 65 dpf, lactate levels remained relatively stable and although they were variable post-feed, no statistical difference was noted. At hatch (42 dpf), however, the lactate levels were below detection in the 41.3 ng group, and they were significantly lower than controls.

Whole body protein levels increased with development (Fig. 4-3d). In control fish, protein significantly levels increased 1.5-fold from 42 to 65 dpf. At 85 and 112 dpf, protein levels were 3.0-4.0-fold higher than at hatch, while at 140 dpf whole body protein increased by 5.0-fold when compared to hatch. These levels were significantly higher than all the earlier life stages. At 140 dpf, whole body protein concentration in the group whose mothers accumulated 41.3 ng BPA was 1.3-fold higher than in control fish at the same life stage. There was an overall effect of maternal BPA accumulation on whole body protein, as the 41.3 ng group had higher protein levels than controls and the other groups, independent of the life stage.

Figure 4-3. Effects of ancestral exposure to BPA on whole body composition (glycogen, glucose, lactate, protein levels) in F2 individuals.

Changes in whole body (a) glycogen, (b) glucose, (c) lactate and (d) protein contents in F2 generation, at hatch (42 dpf), first feed (65 dpf) and post-feed (85, 112 and 140 dpf), following maternal loading with BPA (for an expanded description of the figure, refer to Fig. 4-1 and Materials and Methods section). Data are expressed as (a-c) μmol g^{-1} ww and (d) mg protein g^{-1} ww, and are shown as mean + S.E.M. (n=4-6 individuals).


Control
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
a)
Glycogen
b)
Glucose
c)
Lactate
d)
Protein
Life stage (dpf)
42
65
85
112
140

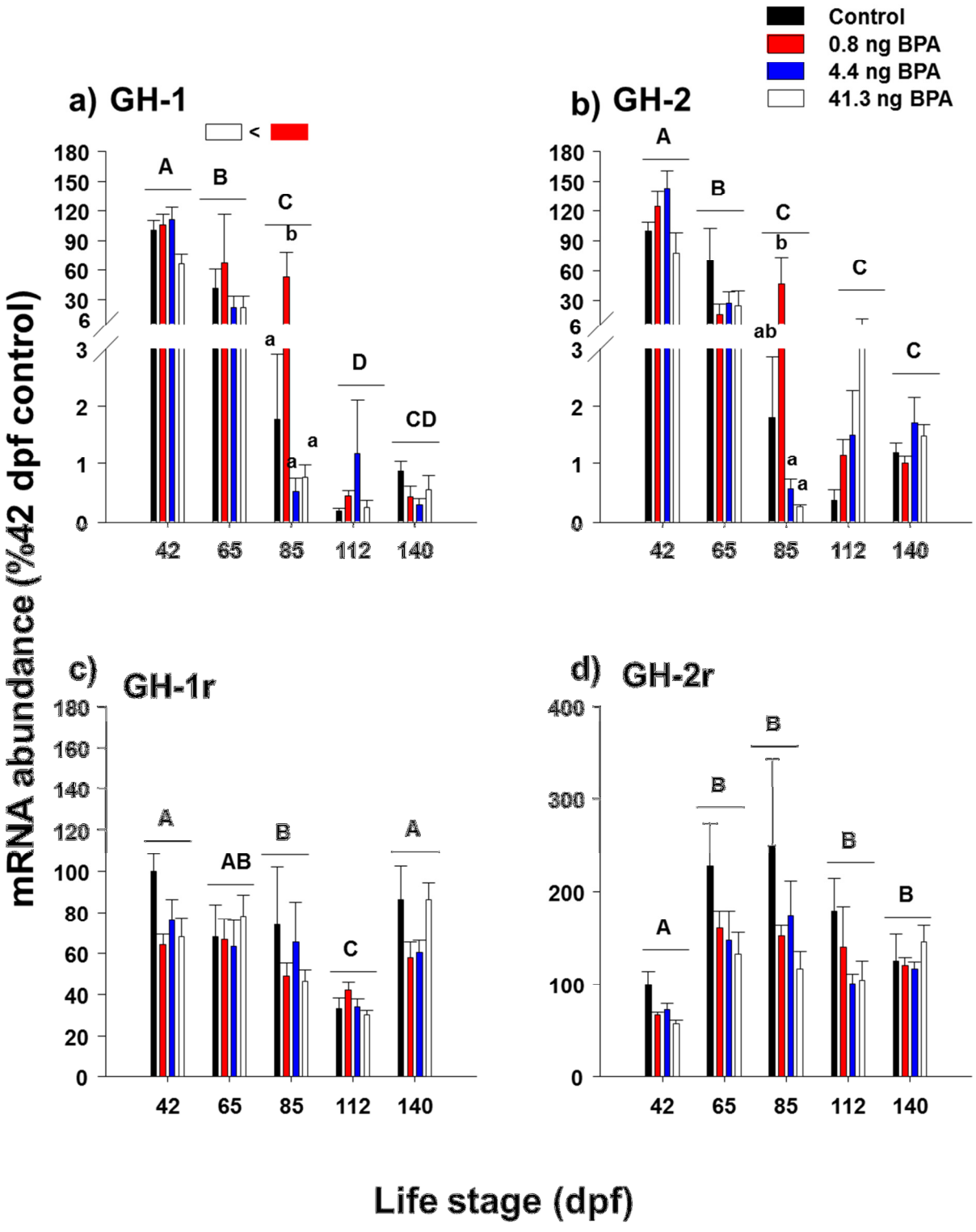
4.4.3 Developmental transcript profiles of growth axis genes

Growth hormone (GH) and its receptor transcript profiles are dependent on life stage (Fig. 4-4). Whole body GH-1 mRNA levels in controls significantly decreased by 2.4-fold, from 42 dpf to 65 dpf (Fig. 4-4a). Post feed, the levels further decreased by 56-fold at 85 dpf and 533-fold at 112 dpf. At 140 dpf, however, GH-1 transcript levels increased when compared to those at 112 dpf, and they were 115-fold lower than 42 dpf controls. The group whose mothers accumulated 0.8 ng BPA had a 30-fold increase in GH-1 transcript numbers when compared to controls at the same life stage. Overall, this group had a higher GH-1 transcript level than the group whose mothers accumulated 41.3 ng BPA. GH-2 mRNA content in control individuals significantly decreased across life stages (Fig. 4-4b). At 65 dpf, levels were 1.4-fold lower than those measured at 42 dpf, while at 85, 112 and 140 dpf, transcript levels further decreased by 56-, 266- and 84-fold, respectively, when compared to 42 dpf. At 85 dpf, significantly higher GH-2 mRNA levels were noted in the 0.8 ng group, where they were 26-fold higher than controls.

GH receptor transcript levels were life stage-dependent. GH-1r control transcripts, although lower by ~1.2-1.4-fold at 65, 85 and 140 dpf, they were not significantly different from those measured at 42 dpf (Fig. 4-4c). At 112 dpf, however, GH-1r mRNA levels significantly decreased by ~3.0-fold when compared to 42 dpf levels. Whole body control GH-2r levels, however, significantly increased after hatch (Fig. 4-4d). From 65 to 85 dpf, the levels were 2.3-2.5 fold greater than those at 42 dpf, while at 112 and 140 dpf, the levels were only 1.8- and 1.2-fold higher, respectively. There were no effects of maternal BPA accumulation on whole body GH-1r or GH-2r transcripts in F2 generation.

Figure 4-4. Effects of ancestral exposure to BPA on growth hormone (GH) and growth hormone receptor (GHr) mRNA levels in F2 individuals.

Changes in whole body developmental profiles of (a) GH-1, (b) GH-2, (c) GH-1r and (d) GH-2r transcripts in F2 individuals, following maternal loading with BPA (for an expanded description of the figure, refer to Fig. 4-1 and Materials and Methods section). Data are expressed as %42 dpf control and are shown as mean ±S.E.M. (n = 5-6 fish).

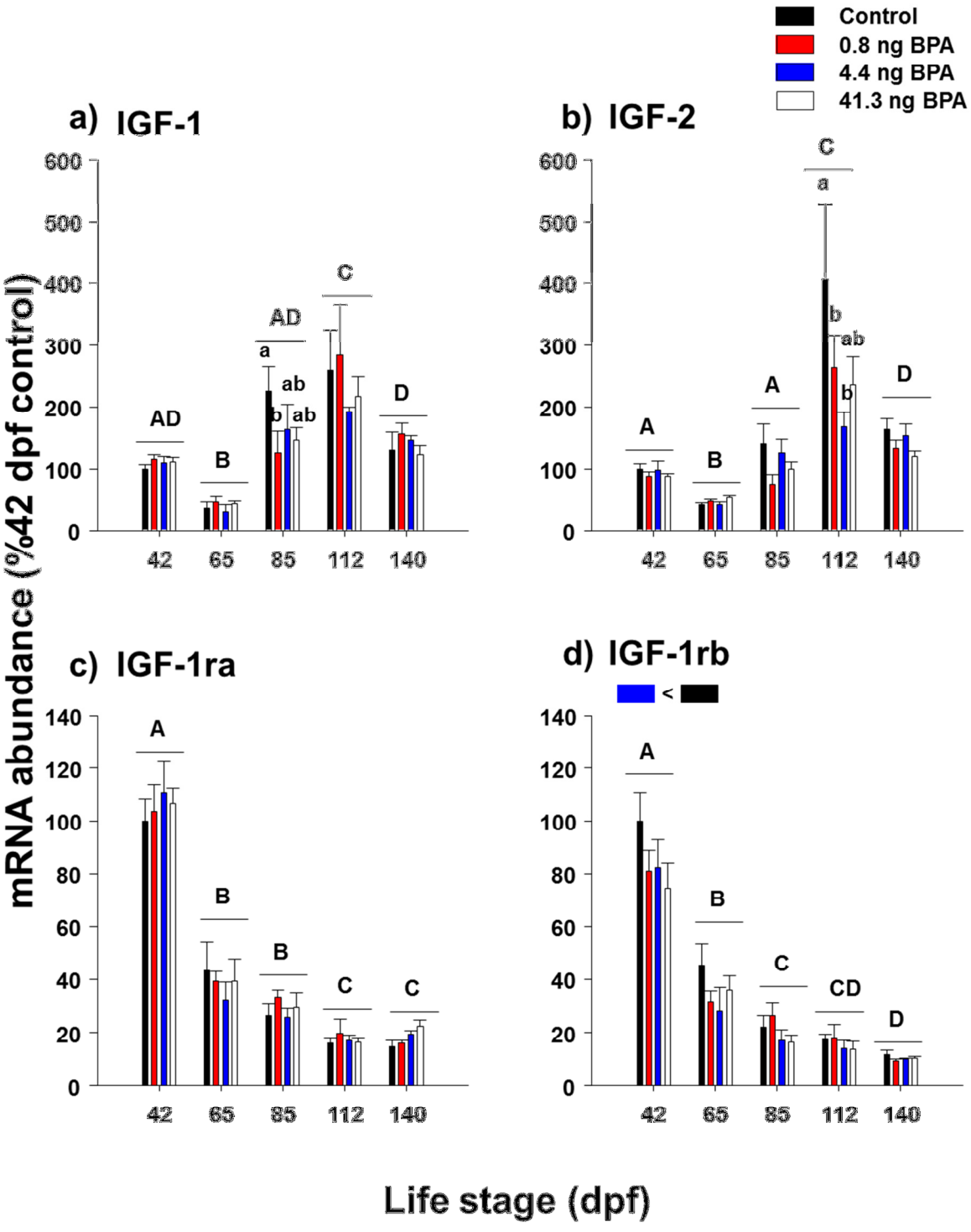

Control
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
a) GH-1
b) GH-2
c) GH-1r
d) GH-2r
mRNA abundance (%42 dpf control)
Life stage (dpf)
42
65
85
112
140

IGF and IGF-receptor mRNA profiles varied across life stages (Fig. 4-5). IGF-1 transcripts significantly decreased by 2.8-fold at 65 dpf when compared to controls, after which they increased by 2.3-fold at 85 dpf (Fig. 4-5a). However, at 112 dpf, IGF-1 transcript levels significantly increased by ~2.6-fold compared to 42 dpf, while at 140 dpf, transcripts were not significantly different from those at hatch. At 85 dpf, the 0.8 ng group had ~1.8-fold lower IGF-1 mRNA levels when compared to the control group at the same life stage. Similar to IGF-1, IGF-2 transcripts in control fish significantly decreased by 2.5-fold at 65 dpf when compared to 42 dpf, only to increase at 85 dpf (Fig. 4-5b). At 112 and 140 dpf, however, transcript levels increased by 4.1 and 1.6-fold, respectively, when compared to their 42 dpf levels. At 112 dpf, the F2 fish groups whose mothers accumulated 0.8 and 4.4 ng BPA showed a 1.6- and 2.4-fold increase, respectively, in IGF-2 transcripts when compared to controls at the same life stage.

IGF-1r mRNA levels decreased across life stages (Fig.4-5c). At first feed (65 dpf), control fish transcript levels significantly decreased by 2.3-fold, while at 85, 112 and 140 dpf, they significantly decreased by 3.8-, 6.2- and 6.8-fold, respectively, when compared to control levels at 42 dpf. There were no effects of maternal accumulation of BPA on IGF-1ra mRNA abundance. IGF-1rb transcripts followed a similar pattern, but the changes were more pronounced (Fig. 4-5d). At 65 dpf, mRNA levels were 2.2-fold lower, while at 85, 112, and 140 dpf, they were 4.5-, 5.8- and 8.5-fold lower than control levels at 42 dpf. There was an overall effect of BPA on IGF-1rb mRNA, with transcript levels being lower overall in the 4.4 ng group than in the control group.

Figure 4-5. Effects of maternal exposure to BPA on insulin-like growth factor (IGF) and IGF receptor (IGFr) mRNA levels in F2 individuals.

Changes in whole body developmental profiles of (a) IGF-1, (b) IGF-2, (c) IGF-1ra and (d) IGF-1rb transcripts in F2 individuals, following maternal loading with BPA (for an expanded description of the figure, refer to Fig. 4-1 and Materials and Methods section). Data are expressed as %42 dpf control and are shown as mean + S.E.M. (n = 5-6 fish).


Control
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
a) IGF-1
b) IGF-2
c) IGF-1ra
d) IGF-1rb
mRNA abundance (%42 dpf control)
Life stage (dpf)
42
65
85
112
140

4.4.4 Developmental profile of cortisol

The basal concentrations of cortisol from fertilization to 140 dpf are shown in Fig. 4-6. Whole body cortisol levels in control embryos steadily decreased to below detection from fertilization (0dpf) to 28 dpf (eyed stage). At 42 dpf (hatch), control cortisol levels significantly increased by ~4.0-fold when compared to fertilization levels, after which they significantly decreased by ~1.3-fold at 65 dpf (first feed). Whole body cortisol levels in controls continued to decrease by ~3.0- and 7.0-fold at 85 and 140 dpf, respectively, when compared to hatch levels. At 65 dpf, whole body cortisol levels in the 0.8 and 4.4 ng groups were significantly lower (3.6- and 2.8-fold, respectively) than control and 41.3 ng levels (Fig. 4-6).

4.4.5 Developmental transcript abundances of MC2R, StAR and P450scc

The developmental profile of key genes involved in cortisol production was examined (Fig. 4-7). MC2R mRNA transcript levels in control fish decreased across life stages (Fig. 4-7a). From 65 to 112 dpf, the levels were significantly lower (1.4-1.9 fold) than those measured at 42 dpf, and remained stable until 140 dpf. The transcript levels significantly decreased by ~5.0-fold at 140 dpf when compared to 42 dpf controls. The 0.8 ng group had significantly higher MC2R mRNA abundance than the 4.4 and the 41.3 ng group. Control StAR transcript levels increased by ~2.8-fold at 65 and 85 dpf when compared to 42 dpf (Fig. 4-7b). At 112 dpf, the levels were ~2.2-fold higher than at 42 dpf, while at 140 dpf, they decreased by ~13-fold when compared to hatch. P450scc transcript abundance in control fish significantly decreased by ~3.0-fold at first feed (65 dpf), while at 85, 112 and 140 dpf, the levels were significantly decreased by ~1.7-, 2.4- and 15-fold, respectively, when compared to controls at 42 dpf. There were not effects of ancestral exposure to BPA on StAR and P450scc mRNA abundances.

Figure 4-6. Effects of ancestral exposure to BPA on the developmental profile of cortisol in F2 individuals.

Changes in whole embryo and whole body developmental profile of cortisol in F2 fish, following maternal loading with BPA at the oocyte stage (for an expanded description of the protocol, refer to Fig. 4-1 and Materials and Methods section). Data are expressed as pg mg^{-1} ww dpf and are shown as mean ± S.E.M. (n = 5-6 individuals). Upper case letters denote a time effect, while asterisks (*) denote a significant change between the marked treatment and controls, within one time point (two-way ANOVA, with Tukey's post-hoc test, $p<0.05$). For simplicity purposes, wherever there was no statistical significance noted between treatments within one time point, no asterisks were used.

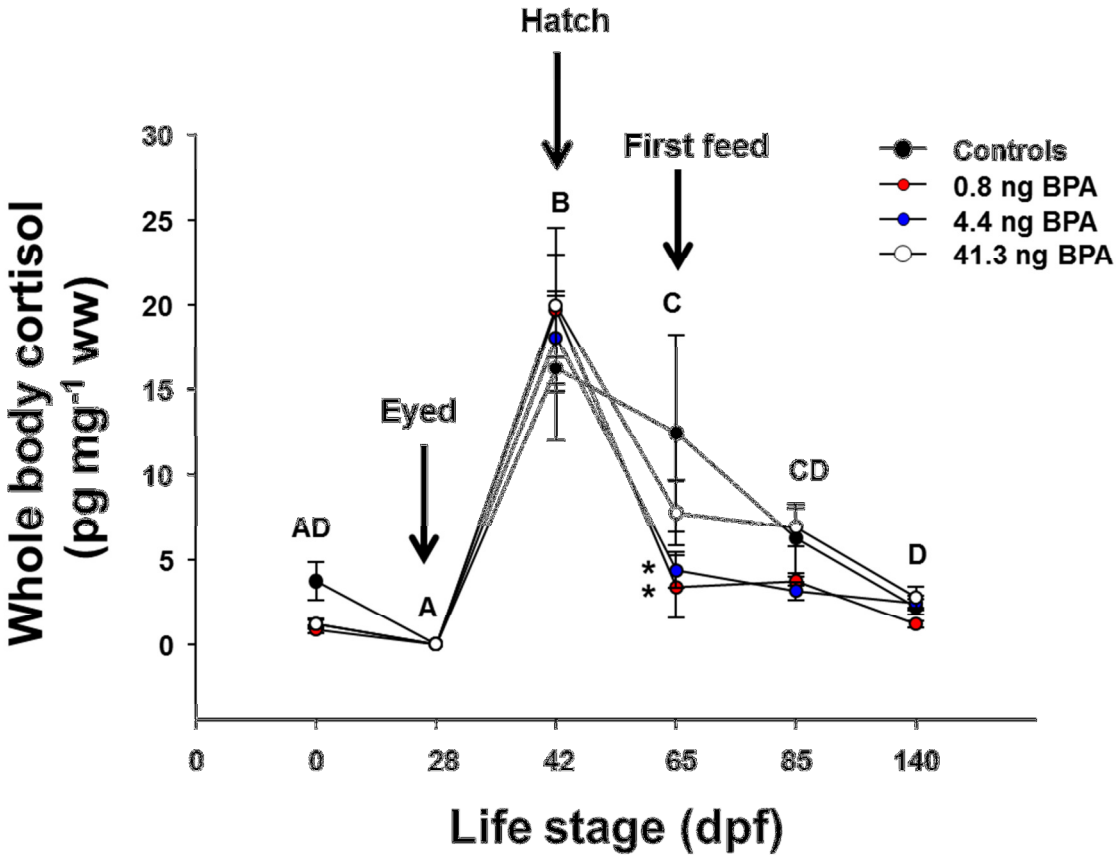
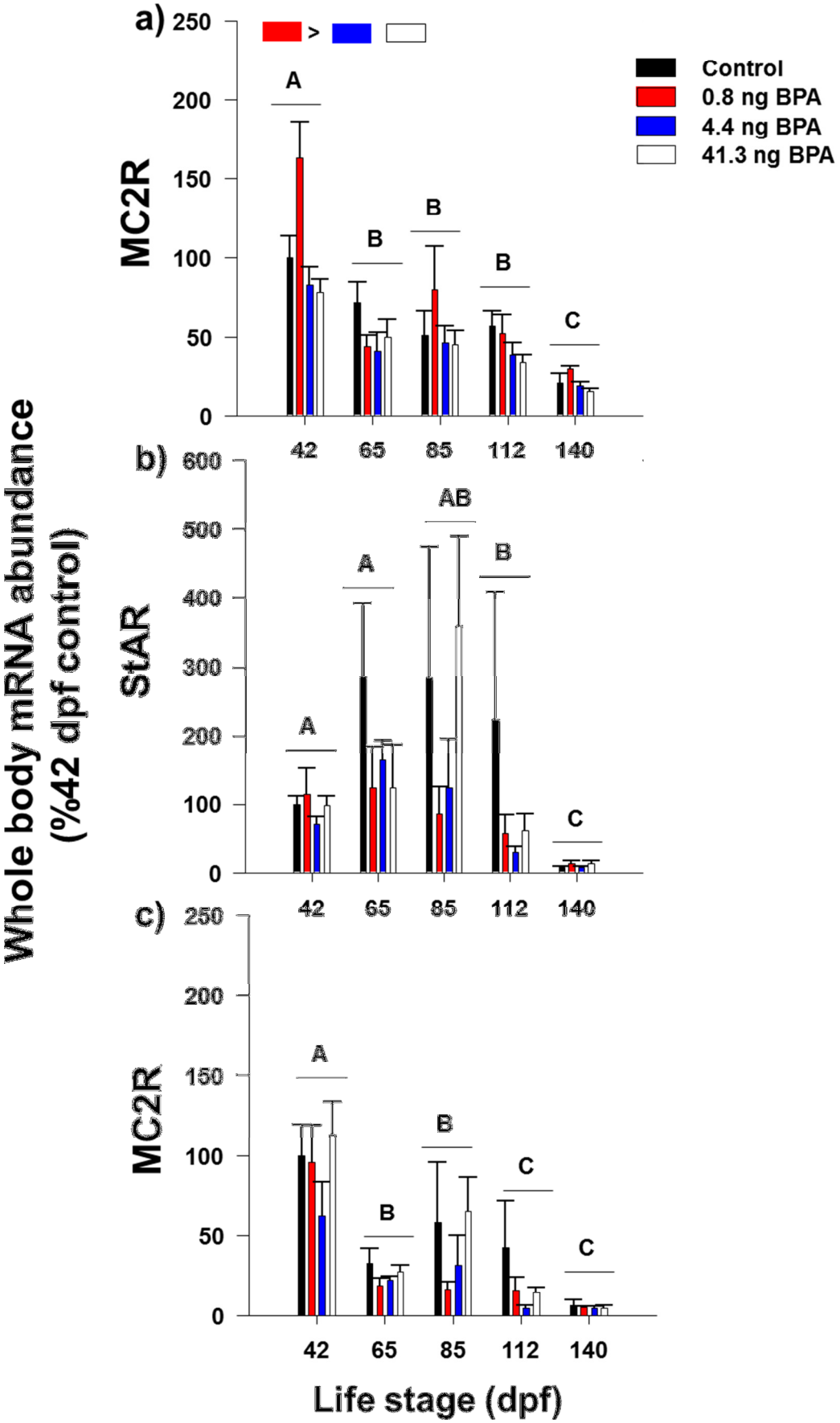

Hatch
First feed
Controls
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
Whole body cortisol (pg mg-1 ww)
30
25
20
15
10
5
0
AD
Eyed
A
B
C
CD
D
* *
0
0
28
42
65
85
140
Life stage (dpf)

Figure 4-7. Effects of ancestral exposure to BPA on the developmental profiles of steroidogenic gene mRNA abundances in F2 generation.

Changes in whole body developmental profiles of (a) MC2R, (b) StAR and (c) P450scc transcript levels in F2 generation (for an expanded description of the figure, refer to Fig. 4-1 and Materials and Methods section). Data are expressed as %42 dpf control and are shown as mean + S.E.M. (n=5-6 individuals).

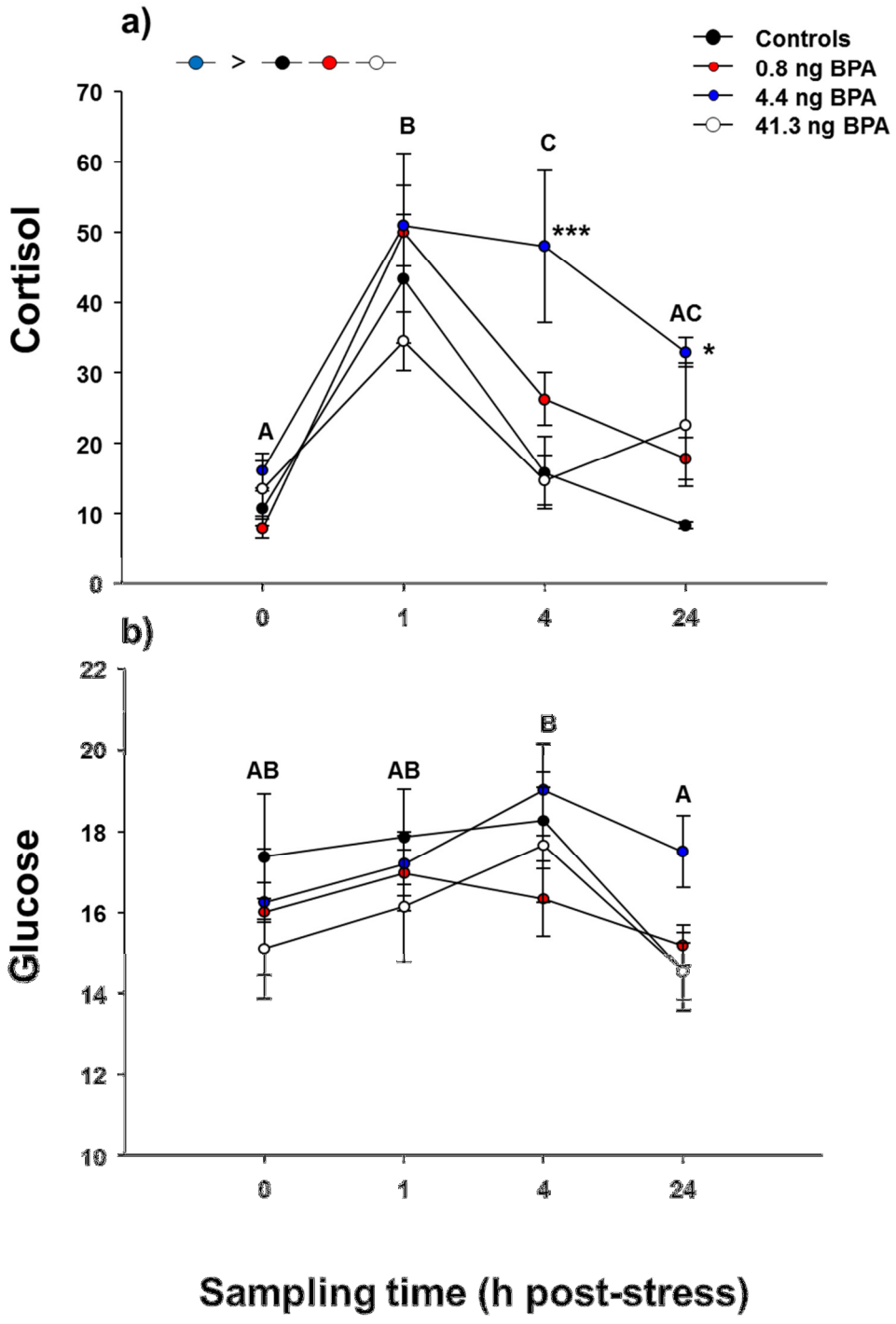

a)
b)
c)
Control
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
MC2R
StAR
MC2R
Whole body mRNA abundance
(%42 dpf control)
Life stage (dpf)
42
65
85
112
140
A
B
B
B
C
A
A
AB
B
C
A
B
C
C

4.4.6 Stress performance

Whole body stress response was assessed at 140 dpf, following exposure to an acute handling stressor (Fig. 4-8). Whole body cortisol levels in control fish significantly increased (3-fold) from 0 h (just prior to stress) to 1 h post-stress (Fig. 4-8a). Cortisol levels then significantly decreased at 4 and 24 h post-stress. In the 4.4 ng BPA group (ancestral exposure), whole body cortisol levels significantly increased 4.5-fold at 1 h post-stress. The cortisol levels remained elevated at 4 h, being significantly higher than the controls and the other two BPA groups. The levels then decreased to at 24 h post-stress, but they were significantly higher than the controls at this time point. Overall, whole body cortisol levels in the 4.4 ng group were significantly higher than the controls and the other two BPA groups independent of sampling time (Fig. 4-8a). Whole body glucose levels in control fish remained relatively unchanged from pre-stress (0 h) to 4 h post-stress (Fig. 4-8b). At 24 h, glucose levels were significantly lower than at 4 h in the control group. There was no effect of ancestral exposure to BPA on whole body glucose profile following an acute stressor exposure.

Figure 4-8. Effects of ancestral exposure to BPA on stress performance in F2 generation.

(a) Cortisol and (b) glucose profiles in F2 individuals, following exposure to an acute handling stressor at 140 dpf (for an expanded description of the figure, refer to Fig. 4-1 and Materials and Methods section). Data are expressed as pg mg^{-1} ww dpf and are shown as mean ± S.E.M. (n = 5-6 individuals). Upper case letters denote a time effect. One asterisk (*) denotes a significant change between the starred treatment and control, while three asterisks (***) denote a significant change between the starred treatment and all other treatments, including control, within one time point (two-way ANOVA, with Tukey's post-hoc test, $p<0.05$).


a)
Controls
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
Cortisol
70
60
50
40
30
20
10
0
A
B
C
AC
***
*
0
1
4
24
b)
Glucose
22
20
18
16
14
12
10
AB
AB
B
A
0
1
4
24
Sampling time (h post-stress)

4.5 Discussion

Traditional multigenerational studies on the effects of toxicants on fish involve standardized exposures from fertilization, through embryogenesis and early juvenile development of several generations of fish. These tests typically use survival, growth and markers of developmental success (condition factor, hepatosomatic and gonadosomatic indices, and fertilization rates) as indicators of long term effects (Staples et al., 2011). To our knowledge, this study is the first to show a multigenerational effect associated with contaminant accumulation in fish eggs, mimicking maternal transfer. Specifically, ancestral exposure to 4.4 ng BPA embryo^{-1} reduced specific growth rate (SGR) and condition factor in the F2 generation in rainbow trout reared in BPA-free water. The lack of an effect at the higher BPA exposure on growth suggests that the generational effect may be non-monotonic. A recent study showed that the BPA responses *in vitro* are indeed non-monotonic (Vandenberg, 2013), leading to the proposal that the generational effect of BPA on growth may involve epigenetic changes to key genes involved in somatotropic axis functioning.

Indeed, ancestral exposure to 4.4 ng BPA disrupted key genes involved in somatotropic axis functioning, including IGF-2 and IGF-1rb, confirming epigenetic changes as a possible mechanism for the generational effect of BPA on growth. Specifically, the significant reduction in IGF-2 (at 112 dpf) and IGF-1rb in response to ancestral exposure to 4.4 BPA supports the reduced SGR in that group, as up-regulation of these genes corresponds with higher growth potential in fish (Reinecke et al., 2005; Reinecke, 2010). Recently studies have shown disruptions in IGFs and their receptors in F1 generation in response to BPA accumulation in eggs (Aluru et al., 2010; Chapter 2). This, along with the results from the present study, clearly implicate the GH-IGF axis as a key target for BPA impact, while the mechanism of action leading to long-term effects remains to be determined.

The developmental stage at 85 dpf is critical, as that time-point is post-feeding in these larvae. Consequently, GH-IGF system may be playing a key role in the energy re-partitioning and allocation for growth during this critical period (Aluru et al., 2010). In the present study, most of the growth-related gene changes were evident at this time point in the BPA groups,

suggesting disturbances in nutrient utilization as proposed previously (Chapter 2). GH is the major hormone involved in directing fish development, growth, osmoregulation and reproduction through stimulation of IGF-1 release from the liver (Reinecke et al., 2005; Reinecke, 2010). Therefore, any changes in the GH-IGF-1 axis would be reflected at the level of the organism. The lower body water content at 85 dpf in the BPA groups suggests disturbances in osmoregulation, but this remains to be determined.

In addition to the 4.4 ng BPA group, ancestral exposure to 0.8 ng BPA also brought about significant changes in growth-related transcript abundances. For instance, growth hormone transcripts (GH-1 and GH-2) were significantly elevated in the 0.8 ng group at 85 dpf, while IGF-1 and IGF-2 mRNA levels were lower in the same group at 85 and 112 dpf, respectively. However, the disruption in growth-related genes seen in the present study with 0.8 ng BPA did not translate to altered growth phenotype in the F2 generation. This, coupled with the lack of an effect of 41.3 ng BPA on either the genes involved in somatotropic axis function or growth, supports our earlier contention that generational effects of BPA on growth may be non-monotonic in trout. The overall decrease in IGF-1rb transcript levels, independent of life stage, seen in the 4.4 ng group leads us to hypothesize that this gene may be a key target for epigenome modification in response to ancestral BPA exposure.

In addition to the growth axis, evidence from our studies suggests that the hypothalamus-pituitary-interrenal (HPI) axis is also a target for BPA toxicity (Aluru et al., 2010; see Chapter 3). At hatch, all fish had increased levels of cortisol, suggesting that there are no delays in interrenal cell development due to ancestral exposure to BPA. Although, interrenal cells have the complement of steroidogenic genes at or around the eyed stage (Barry et al., 1995), activation of the HPI axis does not occur until several days post-hatch, when a cortisol response can be elicited following a stressor challenge (Pillai et al., 1974; Barry et al., 1995; Auperin and Geslin, 2008; see Chapter 3). The whole body cortisol levels at 65 dpf, just prior to first feed, was modified by ancestral exposure to 0.8 and 4.4 ng BPA, but not 41.3 ng BPA. However, these changes were not apparent at the later time-points. Interestingly, the growth changes were also

apparent in the 4.4 ng BPA group, suggesting that this concentration of BPA exposure elicits long-term effects even on HPI axis development in trout.

Further confirmation that 4.4 ng BPA had the most striking generational effect in trout was evident from the stress performance studies carried out at 140 dpf. Whole body cortisol profile was disturbed following a stressor challenge only in the 4.4 ng group. Specifically, the cortisol levels post stressor exposure did not return to basal levels, unlike the other groups, indicating disturbances in the turnover of the hormone. Interestingly, a similar cortisol profile was seen in the F1 generation of fish at 400 dpf raised from BPA-accumulated eggs (Aluru et al., 2010). Cortisol levels in this study increased at 1 h post-stress, but they did not return to pre-stress levels, remaining elevated even at 24 h post-stress when compared to controls. Together, these results highlight for the first time that ancestral exposure to BPA will affect the highly conserved stress performance in rainbow trout, impacting how these animals are able to respond to and adapt to a stressor challenge. The key finding is that the generational effects of BPA on growth and stress performance are not dose-related, but appear to be non-monotonic. Our results suggest that trout eggs containing 4.4 ng BPA will lead to multigenerational effects on growth and stress performances. It can be proposed then that this dosage of BPA will lead to epigenome modifications, leading to generational effects, but the mechanism(s) of action remain(s) to be elucidated.

The current study has shown, for the first time, that exposure to BPA during embryogenesis, mimicking maternal transfer, leads to multigenerational effects on growth and body composition, that are associated with altered developmental profiles of key genes involved in somatotropic axis functioning in F2 fish. In addition, effects of maternal BPA deposition in embryos on the F2 generation HPI axis development and on the developmental profile of cortisol were noted. While several studies have shown that the cortisol stress axis is a target of xenobiotic toxicity, through alterations in cortisol biosynthesis and/or glucocorticoid signaling (Vijayan et al., 2005; Sandhu and Vijayan, 2011; Miller and Hontela, 2011; Aluru et al., 2010; see Chapter 3), none have addressed this aspect by following the effects of maternal transfer of xenobiotics in F2 generation in fish. This study is the first to show that the whole body cortisol profile

following an acute stress challenge was altered in trout post-feed, suggesting that ancestral exposure to contaminants during critical stages of embryonic development can elicit multigenerational effects in the F2 offspring. Whether these effects observed in F2 fish are also transgenerational, remains to be established. The results from this study led to the proposal that evaluations of risk assessment of BPA (and BPA-like compounds) in freshwaters should also take into account maternal transfer of this chemical, in addition to exposure through the water column. Threshold oocyte levels of BPA may be used as a marker to better predict long term and multigenerational effects of this chemical in trout.

4.6 Acknowledgements

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Chapter 5: Multigenerational Effects of Bisphenol A (BPA) on Stress Performance and Cortisol Biosynthesis in Rainbow Trout

5.1 Overview

Early developmental stages are known to be sensitive to chemical exposure. Recent studies on maternally transferred bisphenol A (BPA) in fish oocytes, and the subsequent exposure of the developing embryos, have implicated long term effects on the development and functioning of the hypothalamus-pituitary-interrenal (HPI) axis, but the mechanism are far from clear. However, few studies have examined alterations in HPI axis functioning in multiple generations of fish, following maternal transfer of BPA to oocytes. In this study, two hypotheses were tested: 1) BPA-induced disruptions in the highly conserved acute stress response profile are multigenerational; and 2) these alterations are due to the disruption of ACTH signaling at the interrenal tissue. Oocytes were loaded with either vehicle (control; <0.01% ethanol) or 0.8, 4.4, 41.3 ng BPA embryo^{-1} prior to fertilization, mimicking maternal transfer, and their development was monitored (F1 generation). To obtain the F2 generation, F1 oocytes were fertilized with milt from clean males, and the fish were reared in a BPA-free environment. F1 and F2 juveniles were then exposed to an acute handling stressor and plasma cortisol levels were monitored post-stressor exposure in juveniles. Also, head kidneys pieces from non-stressed juvenile fish from each group, were stimulated with either ACTH or 8-bromo-cyclic AMP, and the cortisol production capacity ascertained. In F1 generation, stressor-induced plasma cortisol was lower in the 4.4 and 41.3 ng BPA groups, while the cortisol stress response was completely abolished in the 41.3 ng group. There was no effect of BPA on plasma glucose levels, but lactate levels were significantly lower in the 41.3 ng BPA group when compared to the control and 0.8 ng BPA group. In the F2, stressor-induced plasma cortisol was lower only in the fish from the 41.3 ng BPA lineage, but not in fish from other BPA lineage groups. There were no effects of BPA on ACTH- or 8-B-cAMP-stimulated cortisol production, while the unstimulated cortisol production was higher in the 4.4 ng BPA group in F1 generation. In the F2, there was no effect of ancestral exposure to BPA on unstimulated cortisol production, while the ACTH-stimulated cortisol production was significantly higher in the 41.3 ng group. Overall, BPA accumulation in eggs leads to endocrine disruption of the cortisol stress axis in trout and the effects are multigenerational.

5.2 Introduction

The physiological response to stressors is highly conserved in vertebrates and it serves an important adaptive role. Elevation of plasma corticosteroid levels occurs in response to an acute stressor and it is an essential process for establishing homeostasis (Sapolsky et al., 2000). While in mammals corticosteroid biosynthesis occurs in the adrenal glands, the steroidogenic cells of teleosts are distributed across the anterior region of the kidney (interrenal tissue; Mommsen et al., 1999). The primary circulating corticosteroid in teleosts is cortisol, and its levels are modulated by the hypothalamus-pituitary-interrenal (HPI) axis activity (Mommsen et al., 1999; Vijayan et al., 2010). Upon perception of a stressor, the hypothalamus releases corticotropin-releasing factor (CRF; Flik et al., 2006; Fuzzen et al., 2011), stimulating the production of proopiomelanocortin (POMC), a precursor of adrenocorticotropic hormone (ACTH). ACTH is produced by post-translational modification and released into the circulation from the anterior pituitary (Sewer and Waterman, 2003; Flick et al., 2006). Circulating levels of ACTH then bind to the melanocortin 2 receptor (MC2R) on the interrenal steroidogenic cells, leading to steroid biosynthesis (Mommsen et al., 1999; Alsop and Vijayan, 2008; Vijayan et al., 2010; Nesan and Vijayan, 2012).

MC2R is a transmebrane G-protein coupled receptor that is present on steroidogenic cells. ACTH binding to MC2R activates the G-protein response pathway (Midzak et al., 2011), leading to production of cyclic adenosine monophosphate (cAMP) from ATP by the adenylyl cyclase. Subsequently, protein kinase A (PKA) is activated, leading to the phosphorylation of the cytoplasmic steroidogenic acute regulatory protein (StAR), which then shuttles cholesterol across the mitochondrial membrane and into the matrix. Cholesterol is then converted to pregnenolone by the cholesterol side chain cleavage enzyme (P450scc), the first rate-limiting step in corticosteroid synthesis. This is followed by a series of reactions catalyzed by hydroxylase and steroid dehydrogenase enzymes, ultimately resulting in cortisol production (Mommsen et al., 1999; Aluru and Vijayan, 2008). As ACTH is the main secretagogue for cortisol synthesis, any impact of MC2R signaling will compromise the cortisol stress

performance either *in vivo* (Vijayan et al., 2005; Sandhu et al., 2014) or *in vitro* (Aluru and Vijayan, 2006; Sandhu and Vijayan, 2011).

Bisphenol A, a persistent chemical that is ubiquitously present in the aquatic environment due to degradation of plastics and epoxy resins (Cao et al., 2008, 2009), has been shown to affect long-term HPI axis functioning when the chemical is present in the embryos during key developmental stages (Aluru et al., 2010; see Chapter 3). Recent studies conducted in our laboratory have proven that exposure of embryos (F1 generation) to BPA causes a delay in HPI axis development by altering the developmental profiles of key genes involved in steroidogenesis (see Chapter 3). This delay in development, along with an altered whole body cortisol stress response profile in larvae was also observed in the F2 generation of fish (see Chapter 4). These findings suggest that the effects of BPA are multigenerational, possibly due to epigenetic modifications of the HPI axis during development.

In this study the hypothesis tested was that BPA accumulation in eggs will lead to longer-term and generational effects in the HPI axis functioning also in juvenile fish. The specific objectives were: 1) Is the cortisol stress response similar in the F1 and F2 generation juvenile fish?, and ii) is the disturbance in cortisol stress response occurring at the level of MC2R. To test the first objective, F1 and F2 juvenile rainbow trout (365 days post-fertilization, dpf) raised from BPA accumulated eggs (see Chapters 2 and 4) were subjected to an acute handling stress and the plasma cortisol, glucose and lactate levels determined post-stressor exposure as described before (Aluru et al., 2010). The second objective was tested by challenging head kidney slices with ACTH- or 8-bromo-cAMP (a cAMP analog) and measuring cortisol production as described before (Sandhu and Vijayan, 2011).

5.3 Materials and Methods

5.3.1 Materials and Chemicals

Unless otherwise specified, all chemicals and enzymes were purchased from Sigma–Aldrich (St. Louis, MO, USA). The scintillation cocktail and the cortisol antibody were purchased from MP Biomedicals (Solon, OH, USA), while the [1,2,6,7-^{3}H] cortisol tracer was

purchased from GE Healthcare (Upsala, Sweden). D-Glucose was purchased from Bioshop (Burlington, ON, CA), while monobasic and dibasic sodium phosphate and sodium bicarbonate were purchased from Fisher Scientific (Ottawa, ON, CA). Multiwell (24-well plates) tissue culture plates for *in vitro* experiments were purchased from Sarstedt (Newton, NC, USA).

5.3.2 Experimental Fish

Experiments were conducted at Alma Aquaculture Research Station (AARS), Alma, Ontario, Canada, in accordance with the Animal Care and Use Committee of the University of Guelph, Guelph, Ontario, Canada and the Animal Care Committee of the University of Waterloo, Waterloo, Ontario, Canada. Oocyte loading with BPA, and the fertilization protocols conducted after to obtain F1 and F2 generation fish, were done exactly as previously described (see Chapters 2 and 4).

5.3.3 Experimental protocols

5.3.3.1 Acute stress exposure

Juvenile rainbow trout at 365 dpf (F1: 82.2 ± 5.0 g; F2: 102.8 ± 8.5 g) were used in the current study.The stress protocol involved a handling and netting disturbance that has been previously described by Aluru et al. (2010).. Briefly, a total of 6 fish were sampled from each of the replicate tanks prior to the application of the stress (0 h group). Fish were food-deprived for 48 h prior to the experiments. Fish were anesthetized with a lethal dose of MS222 (1.0 g l^{-1} buffered with 2.0 g l^{-1} Na_2CO_3). The remaining fish were subjected to a handling disturbance for 3 min, after which they were allowed to recover. Sampling occurred at 1, 4 and 24 h post-stressor exposure and consisted in quickly netting the fish and placing them in the lethal dose of MS222. Fish weight and length were quickly recorded, after which blood was sampled by severing the caudal peduncle and collecting it in 1.5 ml centrifuge tubes containing 40 µl of 5 mmol l^{-1} EDTA (in phosphate buffer saline, pH. 7.4-7.6) as an anticoagulant. Tubes were then gently shaken to ensure thorough mixing of blood with EDTA, after which they were centrifuged for 5.0 min at 5000 × *g* to separate the plasma, which was then transferred into pre-labeled tubes and flash

frozen in dry ice. All samples were stored at -80 ˚C for later analysis of cortisol, glucose and lactate.

5.3.3.2 In vitro cortisol production

Following the acute stress exposure, fish were allowed one week to recover prior to the *in vitro* experiments. Cortisol production was measured as previously described (Aluru and Vijayan, 2006; Sandhu and Vijayan, 2011), with minor modifications. Briefly, fish were food-deprived prior to sampling and anesthetized with MS222, as previously described. Head kidney tissue, containing interrenal steroidogenic cells, was removed from the fish and placed in a Petri dish containing Hank's buffer (containing, in mmol l^{-1}: 136.9 NaCl, 5.4 KCl, 0.8 $MgSO_4 \cdot 7H_2O$, 0.33 $Na_2HPO_4 \cdot 7H_2O$, 0.44 KH_2PO_4, 5.0 each of HEPES, HEPES NA, $NaHCO_3$, and glucose, pH 7.63). The tissue was then thoroughly minced and washed in Hank's buffer three times to remove any blood clots. The tissue was washed once more in Hank's, equally distributed into 24 well plates (3 wells per fish) and then it was incubated for 2 h in 1.0 ml Hank's, 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 to fresh buffer containing either 0.5IU ml^{-1} ACTH or 0.5 mmol l^{-1} 8-bromo-cyclic AMP (8-B-cAMP) for 4 h, at 13 ˚C, with gentle shaking. The concentrations of ACTH and 8-B-cAMP chosen were based on previous work conducted in our laboratory (Aluru and Vijayan, 2006, 2008; Sandhu and Vijayan, 2011). At the end of the exposure, samples were collected, quickly centrifuged at 13,000 × *g* for 1 min, and supernatant was removed and placed in an appropriately labeled centrifuge tube. Pellet and supernatant were frozen on dry ice and then stores at -80 ˚C for later determination of tissue viability and medium cortisol concentration. LDH leakage from tissue to the media was used to measure tissue viability, according to established protocols (Boone & Vijayan, 2002). There was no difference in tissue viability.

5.3.4 Analytical techniques

5.3.4.1 Cortisol measurement

Cortisol measurements were carried out on plasma and media by radioimmunoassay (RIA), as described by Ings et al. (2012). The cortisol antibody was diluted 1:250 prior to use,

while a dilution of 60.0 µl to 11.0 ml RIA buffer (40.0 mmol l^{-1} Na_2HPO_4, 10.0 mmol l^{-1} $NaH_2PO_4 \cdot H_2O$, 1.0 g l^{-1} gelatin and 0.1 g l^{-1} thimerasol) was used for the [1,2,6,7-^{3}H] cortisol tracer. All dilutions were done in RIA buffer and both the antibody and tracer were tittered to ensure 35-50% binding prior to use.

5.3.4.2 LDH assay, plasma glucose and lactate

LDH was measured in the media and head kidney tissue according to Boone & Vijayan (2002), and the ratio of LDH tissue to LDH total (media + tissue) was used to assess tissue viability (ratio < 20% means the tissue is viable). Briefly, LDH was measured over a 5 min period, using a kinetic assay, at 340 nm, with NADH (0.12 mmol l^{-1}) and pyruvate (1.0 mmol l^{-1}) in imidazole buffer (50.0 mmol l^{-1}, pH 7.5). Plasma glucose and lactate were determined enzymatically as previously described (Bergmeyer, 1985; Birceanu et al., 2014; Chapters 2, 3, 4).

5.3.5 Statistical analysis

Statistical analysis was performed using SigmaPot 11.0 software (Systat Software Inc., San Jose, CA, USA). All data are shown as mean + or ± standard error of the mean (S.E.M.). A two-way analysis of variance (ANOVA) was used to determine significance of BPA treatments on plasma cortisol, glucose and lactate levels, while a two-way repeated measures ANOVA was used to determine significance of ACTH and 8-B-cAMP stimulation on head kidney cortisol production. A one-way ANOVA was used for analyzing the effects of either ACTH or 8-B-cAPM on controls and BPA groups in F1 and F2 fish. With both types of ANOVAs, a Tukey's post-hoc test was used whenever interactions were detected, and a probability level of $p < 0.05$ was considered significant. The data was log-transformed wherever necessary to meet the assumptions of normality and equal variance. Only non-transformed data are shown in the figures. For figures showing %change, the S.E.M. is to be used only as a reference and it is not a true reflection of the data.

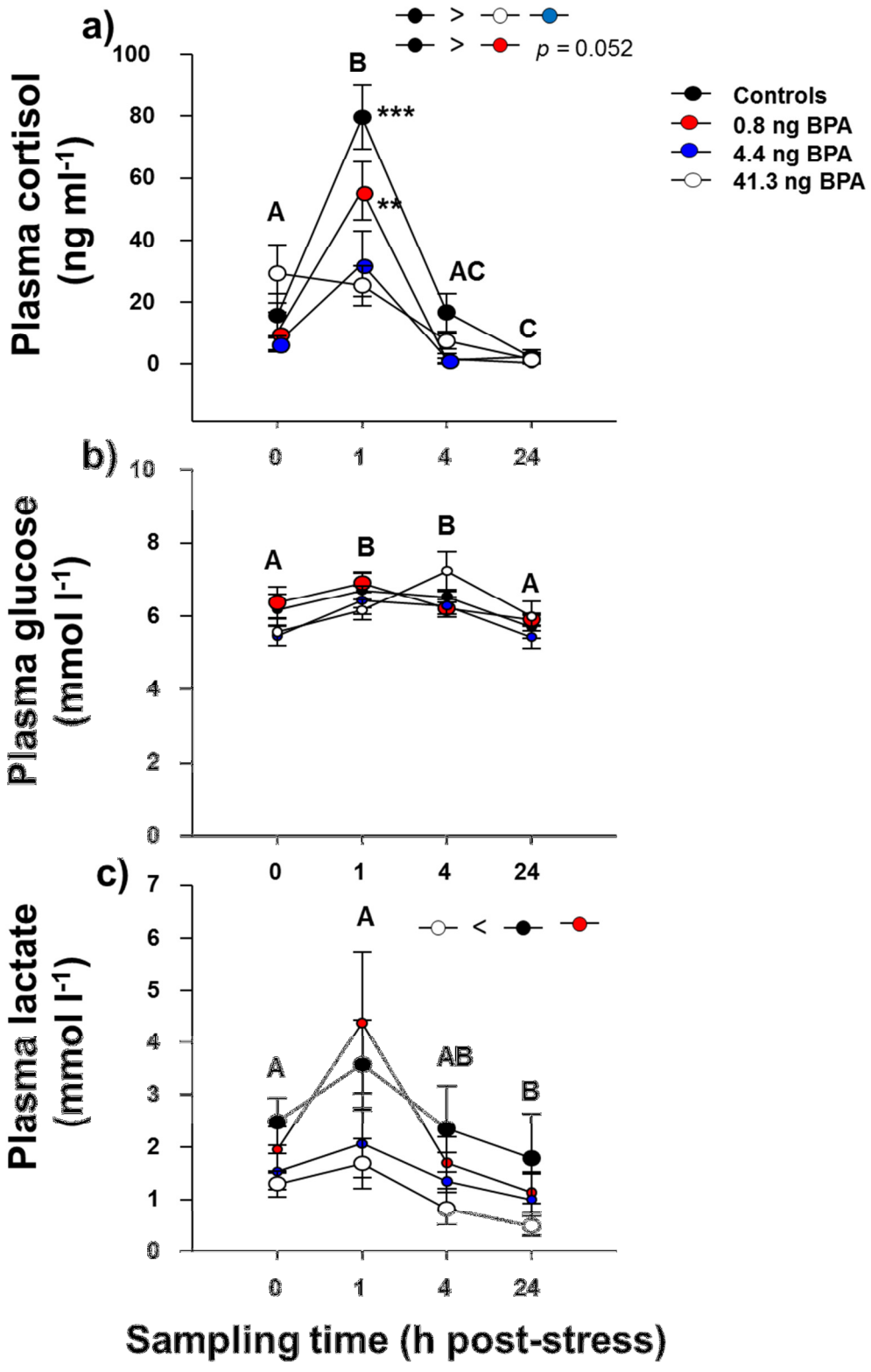
5.4 Results

5.4.1.1 F1 generation – Stress response

Plasma cortisol in control fish significantly increased at 1 h post-stress to ~5.0-fold when compared to pre-stress levels (0 h; Fig. 5-1a). At 4 and 24 h post-stress, cortisol reached basal levels in control fish.. The BPA groups had overall lower plasma cortisol levels compared to the controls, with the stress response profile being completely abolished in the 41.3 ng group. The 0.8 ng group had significantly lower cortisol at 1 h when compared to controls, while in the 4.4 and 41.3 ng groups, the cortisol levels at 1 h were ~2.5- and 3.0-fold, respectively, lower than controls (Fig. 5-1a). Plasma glucose increased at 1 h post-stress in all groups, and remained elevated at 4 h post-stress (Fig. 5-1b). At 24 h, glucose levels returned to pre-stress levels. There was no significant effect of BPA on stressor-induced plasma glucose in F1. Plasma lactate showed a temporal change post-stress, with lower levels at 24 h compared to pre-stress and 1 h post-stress (Fig. 5-1b). Plasma lactate in the 41.3 ng BPA group was significantly lower than controls and 0.8 ng BPA group (Fig. 5-1c).

Figure 5-1. Plasma cortisol, glucose and lactate profiles in response to an acute stress in the F1 generation.

Changes in plasma levels of (a) cortisol, (b) glucose and (c) lactate in F1 generation fish at 365 dpf, following exposure to an acute stressor. Fish were exposed to control (vehicle, <0.01% ethanol), 0.3, 3.0 and 30.0 μg ml^{-1} BPA at the oocyte stage, for 3 h, prior to fertilization. After exposure, oocytes accumulated 0.8, 4.4 and 41.3 ng BPA $embryo^{-1}$ in the three BPA treatments, respectively (after subtracting background levels of ~5.9 ng $embryo^{-1}$ measured in control oocytes). Data shown as mean ± S.E.M. (n=5-6). An outlier test was performed on the data and one data point was eliminated from the 4.4 ng group at 1 h post-stress. Upper case letters denote a time effect. Three asterisks (***) beside treatment groups denote a statistically significant difference from all three treatment groups, while two asterisks (**) next to the 0.8 ng group denote a significant difference from the other BPA treatments. Insets denote an overall treatment effect, independent of sampling time. Time points with different letters are statistically different (two-way ANOVA, Tukey's post-hoc test, $p<0.05$).


a)
b)
c)
Plasma cortisol (ng ml-1)
Plasma glucose (mmol l-1)
Plasma lactate (mmol l-1)
Sampling time (h post-stress)
p = 0.052
***
**
A
B
AC
C
AB
Controls
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA

5.4.1.2 F1 generation – cortisol production in vitro

To determine if BPA accumulation in eggs impacts HPI axis function long-term through disruption of ACTH signaling, cortisol production was monitored in the head kidney of F1 juvenile rainbow trout, following *in vitro* stimulation with either ACTH or 8-B-cAMP (Fig. 5-2). ACTH and 8-B-cAMP stimulation induced a 17- and 6-fold increase in cortisol levels, respectively, following a 4 h incubation of head kidney tissue (Fig. 5-2a). Basal (un-stimulated) cortisol levels were ~2.5-fold higher in the 4.4 ng BPA group when compared to controls and the other BPA groups (Fig. 5-2b). There was no effect of BPA in eggs on either ACTH- or 8-B-cAMP-stimulated cortisol production (Fig. 5-2 c,d).

5.4.1.3 F2 generation – Stress response

Plasma cortisol levels in controls increased by ~4.0-fold at 1 h post-stress, when compared to their pre-stress levels (Fig. 5-3a). After 4 h and 24 h post-stress, cortisol decreased to pre-stress (0 h) levels. Ancestral exposure to 41.3 ng BPA, but not other groups, significantly reduced stressor-induced plasma cortisol levels compared to the control group. At 0 h, cortisol was significantly lower in the 4.4 and 41.3 ng groups when compared to the control and 0.8 ng BPA group (Fig. 5-3a). Plasma glucose steadily increased from 0 to 4 h post-stress, after which this high level was maintained over the 24 h post-stressor-exposure (Fig. 5-3b). This temporal plasma glucose increase post-stress was mainly attributed to the level in the 0.8 ng BPA exposure group, which was significantly higher than all other groups, including the control. Maternal BPA exposure significantly impacted the plasma glucose profiles in the lowest and highest BPA groups. The 0.8 ng group had ~2.0-fold higher glucose levels post-stress when compared to controls, while the 41.3 ng group had ~1.5-fold lower glucose than the controls fish. At 4 h and 24 h post-stress, the 0.8 ng group had significantly higher plasma glucose than the highest BPA group. There was no temporal effect of stress on plasma lactate levels (Fig. 5-3c). Ancestral exposure to 0.8 ng BPA significantly reduced plasma lactate levels compared to the 41.3 ng BPAgroup, but not form the control. While not statistically significant (p=0.065), there appeared to be an overall effect of BPA on lactate levels when compared to controls, as the higher BPA group had ~2.0-fold higher lactate levels than the control fish (Fig, 5-3c).

Figure 5-2. *In vitro* ACTH- and 8-B-cAMP- stimulated cortisol production in the head kidney of F1 generation fish.

(a) The basal, ACTH- and 8-B-cAMP-induced cortisol production in controls. Effect of oocyte BPA loading (0.8, 4.4 and 41.3 ng BPA embryo^{-1}) on (b) basal, (c) ACTH-stimulated and (d) 8-B-cAMP-stimulated cortisol production in rainbow trout head kidney slices. Values are shown as (c) %ACTH-stimulated cortisol production in controls and (d) %8-B-cAMP-stimulated cortisol production in controls. For details on methodology, refer to Fig 5-1. A two-way repeated measures ANOVA was used to calculate statistical differences in controls between no stimulus, ACTH and 8-B-cAMP, while a one-way ANOVA was used for determining differences in basal, ACTH- and 8-B-cAMP-stimulated cortisol production ($p < 0.05$). Data are shown as mean + S.E.M. (n = 5-6). An outlier test was performed on the data and two data points were removed from the 4.4 ng group: one in the basal and the second in the ACTH-stimulated cortisol data sets. Data points sharing the same letter are not statistically different.

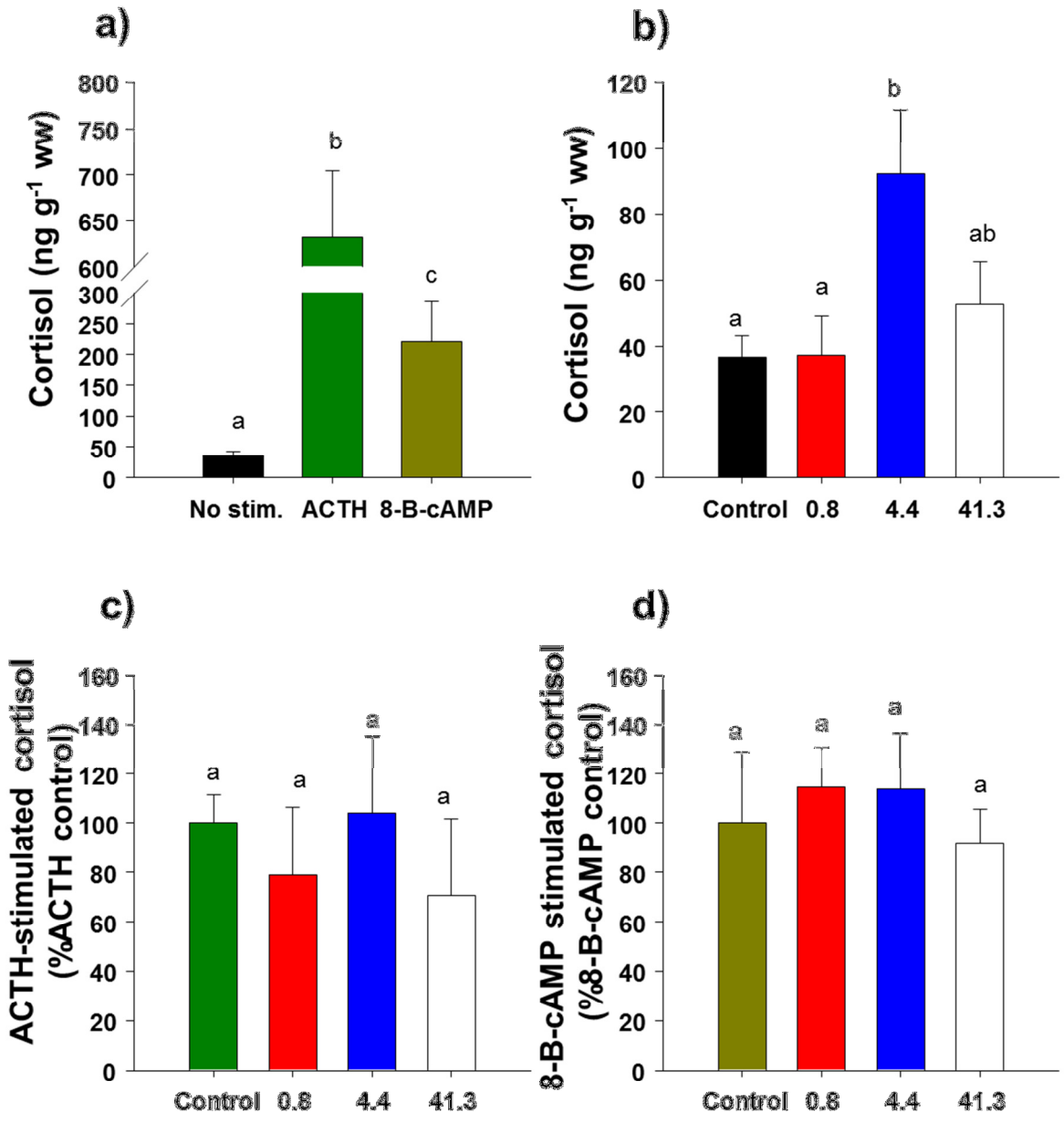
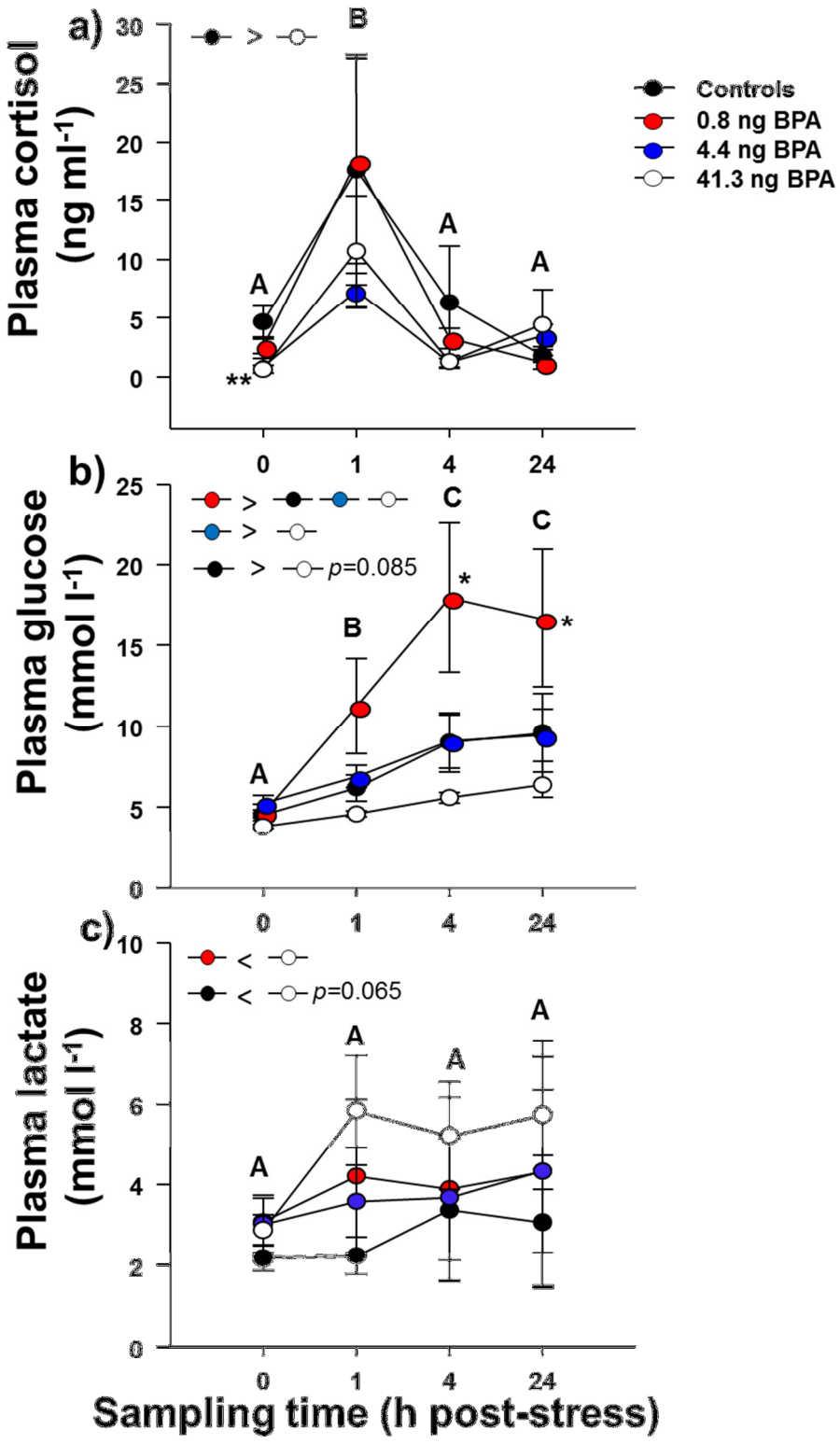

a)
Cortisol (ng g-1 ww)
800
750
700
650
600
300
250
200
150
100
50
0
a
b
c
No stim.
ACTH
8-B-cAMP
b)
Cortisol (ng g-1 ww)
120
100
80
60
40
20
0
a
a
b
ab
Control
0.8
4.4
41.3
c)
ACTH-stimulated cortisol (%ACTH control)
160
140
120
100
80
60
40
20
0
a
a
a
a
Control
0.8
4.4
41.3
d)
8-B-cAMP stimulated cortisol (%8-B-cAMP control)
160
140
120
100
80
60
40
20
0
a
a
a
a
Control
0.8
4.4
41.3

Figure 5-3. Plasma cortisol, glucose and lactate profiles in response to acute stress in the F2 generation.

Changes in plasma levels of (a) cortisol, (b) glucose and (c) lactate in F2 generation fish at 365 dpf, following exposure to an acute stressor. Oocytes from F1 mothers were collected when the females reached reproductive age (3 years) and were fertilized with milt from AARS stock males. At no point were oocytes or embryos treated with BPA during development. The treatments on the x-axis (0.8, 4.4 and 41.3 ng BPA embryo^{-1}) simply denote the BPA accumulated by the mothers immediately following the 3 h incubation with BPA at their respective oocyte stage (see Fig 5-1). Data shown as mean ± S.E.M. (n=5-6). An outlier test was performed on the data and no data points were removed. Upper case letters denote a time effect. Inserts denote an overall treatment effect independent of sampling time. One asterisk (*) denotes a statistically significant difference between the 0.8 ng and the 41.3 ng groups, while two asterisks (**) denote a statistically significant difference from controls and the 0.8 ng groups. Time points showing different letters are statistically different (two-way ANOVA, Tukey's post-hoc test, $p<0.05$).

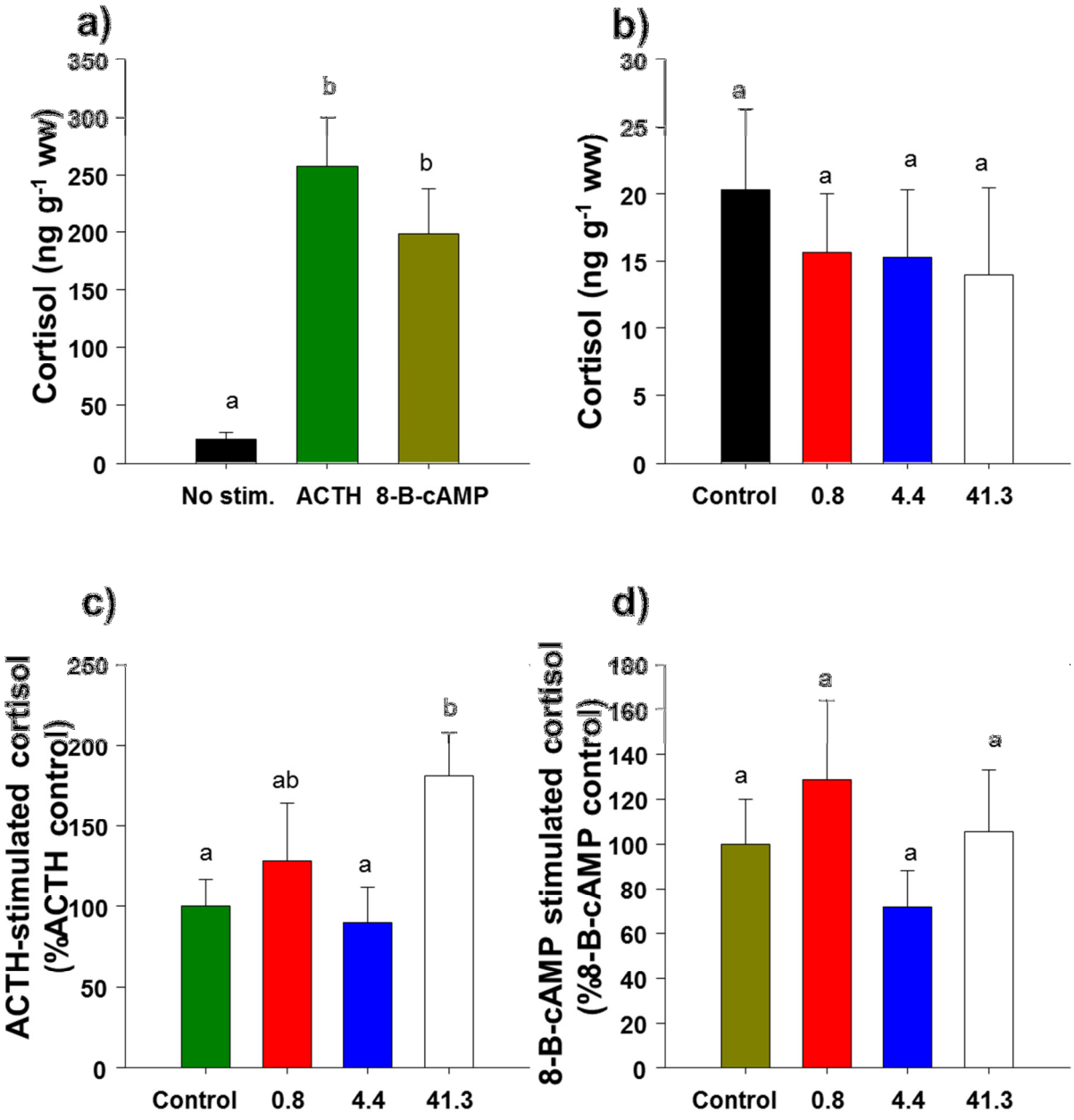

a)
Plasma cortisol (ng ml-1)
Controls
0.8 ng BPA
4.4 ng BPA
41.3 ng BPA
B
A
A
A
**
b)
Plasma glucose (mmol l-1)
p=0.085
C
C
B
A
*
*
c)
Plasma lactate (mmol l-1)
p=0.065
A
A
A
Sampling time (h post-stress)

5.4.1.4 F2 generation – Cortisol production in vitro

To determine if maternal transfer of BPA impacted the long-term HPI axis function by disrupting ACTH signaling, cortisol production was monitored in the head kidney of F2 juvenile rainbow trout, following *in vitro* stimulation with either ACTH or 8-B-cAMP (Fig. 5-4).In control fish, ACTH and 8-B-cAMP induced a 13-fold and 10-fold increase in cortisol levels, respectively, following a 4 h incubation of head kidney slices (Fig. 5-4a). Basal unstimulated cortisol levels were not statistically different in any of the groups (Fig. 5-4b). ACTH-stimulated cortisol levels were 1.5-fold higher in the 41.3 ng group when compared to the control fish, while all other BPA groups were not significantly different from the control (Fig. 5-4c). 8-B-cAMP-stimulated cortisol levels were not significantly different between controls and the BPA groups.

Figure 5-4. *In vitro* ACTH- and 8-B-cAMP- stimulated cortisol production in the head kidney of F2 generation fish.

(a) The basal, ACTH- and 8-B-cAMP-induced cortisol production in controls. Effect of F1 oocyte BPA loading (0.8, 4.4 and 41.3 ng BPA embryo^{-1}) on (b) basal, (c) ACTH-stimulated and (d) 8-B-cAMP-stimulated cortisol production in F2 generation rainbow trout head kidney slices. Values are shown as (c) %ACTH-stimulated cortisol production in controls and (d) %8-B-cAMP-stimulated cortisol production in controls. For details on methodology, refer to Fig 5-2. A two-way repeated measures ANOVA was used to calculate statistical differences in controls between no stimulus, ACTH and 8-B-cAMP, while a one-way ANOVA was used for determining differences in basal, ACTH- and 8-B-cAMP-stimulated cortisol production ($p <$ 0.05). Data are shown as mean + S.E.M. (n = 5-6). An outlier test was performed on the data and no data points were removed. Data points sharing the same letter are not statistically different.


a)
Cortisol (ng g-1 ww)
350
300
250
200
150
100
50
0
a
b
b
No stim.
ACTH
8-B-cAMP
b)
Cortisol (ng g-1 ww)
30
25
20
15
10
5
0
a
a
a
a
Control
0.8
4.4
41.3
c)
ACTH-stimulated cortisol (%ACTH control)
250
200
150
100
50
0
a
ab
a
b
Control
0.8
4.4
41.3
d)
8-B-cAMP stimulated cortisol (%8-B-cAMP control)
180
160
140
120
100
80
60
40
20
0
a
a
a
a
Control
0.8
4.4
41.3

5.5 Discussion

The current study demonstrates, for the first time, that ancestral exposure to BPA disrupts the highly conserved organismal stress response in rainbow trout. The reduced acute plasma cortisol response to stress in F1 and F2 juveniles clearly demonstrates that BPA has long-term and multigenerational effects on HPI axis functioning. While the mechanism is not known, our *in vitro* cortisol release from the head kidney slices suggests that the impact of ancestral exposure to BPA on cortisol response may be occurring upstream of ACTH signaling and may involve reprogramming of HPI axis function.

F1 generation oocytes that were exposed to BPA accumulated 0.8, 4.4 and 41.3 ng BPA embryo^{-1}, and the chemical was present in the embryos during organogenesis (14-21 dpf) and HPI axis programming (see Chapter 2). The measured BPA concentrations were comparable to BPA levels measured in wild fish tissues (Wei et al., 2007; Renz et al., 2013) and in oocytes from previous studies conducted in our laboratory (Aluru et al., 2010). The disturbed acute stress cortisol profile of the 41.3 ng group noted in the current study in the F1 generation was similar to that reported by Aluru et al. (2010) in fish that accumulated comparable levels of BPA following identical exposure conditions. The abolishment of the highly conserved cortisol response to stress in the two studies (this study and Aluru et al., 2010) strengthens the argument that maternal transfer of contaminants will have long-term impact on stress performance. The BPA effect on stress response, however, appears to be dependent on the dosage of the chemical in the oocyte.

Indeed, the HPI axis has been previously shown to be a target of xenobiotic toxicity, leading to alterations in the cortisol and glucose profiles following exposure to an acute stress (Vijayan et al., 2005; Hontela and Vijayan, 2008; Sandhu and Vijayan, 2011; Sandhu et al., 2014). While the acute plasma glucose levels in the current study were not altered in the F1 juveniles, lactate levels were significantly lower in the 41.3 ng group when compared to controls, suggesting a possible metabolic disturbance in the muscle. Milligan (2003) showed that an exercise-induced rise in cortisol levels was associated with lactate production in the muscle, suggesting that cortisol does modulate plasma lactate levels. Recently, Sandhu et al. (2014)

reported lower lactate levels in Cd exposed fish following an acute stress, suggesting that the reduced lactate might be due to the lower cortisol levels and/or a reduction in GR signaling at the level of the target tissues in the exposed fish. Indeed, this could be the case in the current study, as the 41.3 ng group did not show a rise in cortisol levels following an acute stress, and their plasma lactate were lower than controls. However, whether these *in vivo* BPA-induced alterations in stress response are due to reduced GR signaling in the target tissue, needs further investigating.

A key finding from this study is that ancestral exposure to BPA impacts stressor-mediated transiently plasma cortisol, glucose and lactate levels in the F2 generation. The group whose ancestors accumulated 41.3 ng BPA had significantly lower cortisol and glucose levels following an acute stress exposure than controls and other BPA groups. A major role of cortisol in stress adaptation is to increase glucose production via gluconeogenesis, to meet the increased energy demands on the organism due to stress exposure (Mommsen et al., 1999; Aluru and Vijayan, 2009). The lower cortisol levels in the higher BPA group may be playing a role in the lower glucose response seen in this group by reducing gluconeogenesis and/or glycogenolysis. The lower transient glucose levels along with higher plasma lactate levels in the 41.3 ng group, although not statistically different than controls, suggest increased muscle activity, leading to the proposal that ancestral exposure to 41.3 ng BPA may lead to metabolic disturbance in trout. Liver is a key target of cortisol action, and its metabolic activity is increased following an acute stress exposure (Mommsen et al., 1999; Aluru and Vijayan, 2009; Sandhu et al., 2014). Recent studies in our laboratory have shown that chronic exposure to contaminants impacts the glycolytic capacity of the liver, leading to reduced plasma glucose following an acute stressor (Sandhu et al., 2014). It is, therefore, plausible that ancestral exposure to high BPA may increase the metabolic demand of the liver, leading to reduced stressor-mediated plasma glucose levels, possibly through reduction in liver gluconeogenesis, as this pathway is energy demanding and cortisol-responsive.

In contrast to the 41.3 ng group, the 0.8 ng BPA group showed an identical cortisol profile to that of the controls, but their plasma glucose levels were ~2-fold higher at 4 and 24 h

post-stress. This increase in stressor-mediated plasma glucose in this group may be due to either higher production or decreased clearance or a combination of both. Given the abnormally high levels seen in this group compared to the stress levels normally reported in trout (~10 mM; Aluru et al., 2010), it appears likely that the changes may be a combination of increased production and reduced clearance but this needs to be verified. A recent study examining the effects of environmentally relevant BPA concentrations on the metabolism of male goldfish has identified significant metabolic alterations in the liver, particularly in pathways involved in energy metabolism and protein synthesis (Jordan et al., 2012). Further studies examining the liver metabolic capacity is warranted to better understand the pathways that are impacted by ancestral exposure to BPA in trout.

Several studies have reported that xenobiotics exert their effects on the HPI axis by suppressing genes encoding proteins involved in steroidogenesis, either *in vivo* (Levesque et al., 2003; Sandhu et al., 2014) or *in vitro* (Leblond and Hontela, 1999; Sandhu and Vijayan, 2011). To determine whether the alterations in cortisol profile following an acute stress exposure were due to BPA-induced effects on ACTH signaling, head kidney tissue of F1 and F2 juveniles were stimulated with either ACTH or 8-B-cAMP *in vitro*. There was no effect of BPA on ACTH or cAMP-stimulated cortisol production, suggesting that the lack of plasma cortisol response to stress in the 41.3 ng BPA group may not due to the disruption in corticosteroidgenesis. In fact in the F2 generation there was an increase in cortisol production in the 41.3 ng BPA group arguing that the capacity to produce cortisol in response to ACTH stimulation is not compromised. This would suggest that the possible mode of action for the reduced stressor-induced cortisol response in vivo in the BPA group may be either at the level of CRF and/or ACTH production. Indeed, recent studies have reported that exposure to contaminants affects the HPI axis function either by disrupting MC2R signaling (Sandhu and Vijayan, 2011) or by modulating mRNA abundance of genes encoding for POMC, CRF and CRF-binding proteins in the brain, leading to lower circulating ACTH levels (Palermo et al., 2012; Melnyk-Lamont and Vijayan, unpublished findings). However, whether the disturbed cortisol response seen with the BPA groups in the F1 juveniles involves HP axis reprogramming needs further investigation.

In conclusion, ancestral exposure to BPA leads to long term and multigenerational defects in HPI axis functioning in rainbow trout. These findings suggest that BPA may mediate the reprogramming of the HPI axis upstream from the interrenal tissue, leading to the impaired cortisol stress response that persists in multiple generations. My hypothesis is that the BPA effect on HPI axis functioning during development may involve impaired ACTH production and/or release from the pituitary, but this remains to be tested. Overall, ancestral exposure to BPA disrupts cortisol stress axis functioning in rainbow trout and this effect may be dosage-dependent. Given the multigenerational effect, BPA-mediated epigenome modification of key genes involved in the HPI axis functioning may be playing a likely role, but this remains to be tested.

5.6 Acknowledgements

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Chapter 6: Bisphenol A in Eggs Disrupts the Offspring Metabolome in Two Generations of Rainbow Trout

6.1 Overview

Bisphenol A (BPA) accumulation in eggs, and its subsequent presence in the developing embryo, has recently been shown to impact the growth and development of the rainbow trout (*Oncorhynchus mykiss*). However, the mechanisms involved are far from clear. The hypothesis for the current study was that BPA-enrichment of oocytes, mimicking maternal transfer of contaminants, affects the early developmental metabolome profile in two generations of trout. Oocytes were exposed to control (<0.01% ethanol), 3.0 and 30.0 mg l^{-1} BPA for 3 h, prior to fertilization, after which they were fertilized (F1 generation) with milt from clean males, and the larvae sampled at 42 (hatch) and 65 (just prior to first feed) days post-fertilization (dpf) for metabolome analysis by gas chromatography-time of flight-mass spectrometry (GC-TOF-MS). The F2 generation was obtained by fertilizing oocytes from F1 females from controls and the BPA-treatment lineage with clean milt, and collecting larvae at 42 and 65 dpf as described for F1 generation. A bioinformatics approach, along with pathway analysis and specific metabolite profiles, were carried out to test the differences between treatments at each developmental stage in both F1 and F2 generations. There were clear treatment effects associated with BPA accumulation in eggs on offspring metabolome profiles. Specifically, pathways involved in carbohydrate, lipid and amino sugar metabolism, along with amino acid metabolism and protein synthesis were disrupted by BPA. Pathway analysis indicated that nitrogen and amino acid metabolism were impacted in both generations. The consistent multigenerational effect observed in larval trout that either hatched from BPA enriched eggs or from the BPA-treatment lineage suggest that epigenome modification may be involved in the whole body metabolic dysfunction. Overall, this study provides novel insights into multigenerational effects on offspring growth and metabolism associated with maternal transfer of BPA in trout.

6.2 Introduction

Bisphenol A (BPA), a weak estrogen mimicking chemical, is ubiquitously present in the aquatic environment and in fish tissues (Belfroid et al., 2002; Cao et al., 2008, 2009). This chemical accumulates in fish oocytes following maternal exposure and persists in the developing embryo for several days post-fertilization (Takao et al., 2008; Aluru et al., 2010). While the estrogen-like effects of BPA have been the focus of most studies, this chemical also impacts growth and performance in fish (Aluru et al., 2010; McCormick et al., 2010; Hanson et al., 2012, 2014; Chapters 2, 3 and 5). Moreover, early life stage exposure to chemicals in general, and BPA in particular, has recently gained attention given the maternal transfer of contaminants to lipid-rich oocytes in wild fish (Ostrach et al. 2008). This finding raises concern about exposure to contaminants, including BPA, at critical early developmental periods, and its impact on long-term development and growth performances (Aluru et al., 2010; Kundakovic and Champagne, 2013). Few studies have examined the generational effects associated with maternal transfer of contaminants in fish. Studies suggest that the "Omics" responses, including transcriptomics, proteomics and metabolomics, may be more indicative of generational effects compared to phenotypic changes (Anway & Skinner, 2006; Lam et al., 2011; Jordan et al., 2012; Ekman et al., 2013). Quantifying global changes will provide a mechanistic underpinning by identifying and discovery novel pathways that are most sensitive to contaminant impact (Anway et al., 2005; Anway and Skinner, 2006).

Metabolomics is a robust and reproducible quantitative technique that utilizes proton nuclear magnetic resonance (^{1}H NMR) or mass spectrometry (MS) to detect changes in whole body, tissue or body fluid metabolite levels (Keun et al., 2002; Samuelsson et al., 2006). Previous studies on the mode of action of BPA have provided valuable insights on the various tissue-specific metabolites that are impacted by exposing the whole organism to this chemical (Jordan et al., 2012). For instance, acute exposure to BPA has been shown to affect energy and lipid metabolism in the liver of goldfish (*Carassius auratus*; Jordan et al., 2012). Also, a recent study in fathead minnows (*Pimephales promelas*) suggested an antiandrogenic mode of action of BPA by examining the hepatic metabolome (Ekman et al., 2013). While these studies provide

important information on the species- and tissue-specific mode of action of BPA in fish, to our knowledge, no study has examined the metabolome profile over multiple generations in response to contaminant exposure. Such information can provide new insights not only on how conserved the metabolome is across generations, but also on the mode of action of contaminants in impacting this conserved response, which will assist in developing biomarkers to predict generational effects in fish.

Recent studies on the effects of BPA accumulation in trout eggs, and its presence in the developing embryos for several days post-fertilization (Aluru et al., 2010; see Chapter 2), suggest possible metabolic disturbances based on temporal changes in whole body glucose, glycogen and lactate levels in two generations (see Chapters 2, 3, 4 and 5). Clearly, a thorough analysis of the trout metabolome will identify the key pathways that are impacted by this chemical and will provide insights into the mechanism of action of BPA. The hypothesis tested was that BPA accumulation in eggs, mimicking maternal transfer, disrupts metabolic pathways essential for growth and development in trout, and that the changes are persistent in the next generation. This was tested in embryos, at hatch (42 dpf) and just prior to first feed (65 dpf), that were raised from BPA enriched oocytes (0, 4.4 and 41.3 ng BPA embryo^{-1}), mimicking maternal transfer of the contaminant (see Chapter 2). A metabolomics approach with gas chromatography-time of flight-mass spectrometry (GC-TOF-MS), along with bioinformatics and pathway analysis tools, was utilized to quantify metabolic disruption in response to BPA accumulation in eggs. We utilized a metabolomics approach to examine global changes, as opposed to genomics or proteomics, mainly because the trout genome is not fully sequenced yet. A fully sequenced genome is essential for genomics and proteomics in order to identify novel genes and proteins, whereas the sequenced genome is not required for metabolome profile characterization.

6.3 Materials and Methods

6.3.1 Chemicals

Unless otherwise specified, all chemicals and enzymes were purchased from Sigma–Aldrich (St. Louis, MO, USA).

6.3.2 Experimental Fish

Experiments were conducted at Alma Aquaculture Research Station (AARS), Alma, Ontario, Canada, in accordance with the Animal Care and Use Committee of the University of Guelph, Guelph, Ontario, Canada and the Animal Care Committee of the University of Waterloo, Waterloo, Ontario, Canada. F1 and F2 generation fish were obtained and sampled exactly as previously described (see Chapters 2 and 4). Fish were grouped based on the BPA accumulation (control, 4.4 and 41.3 ng BPA embryo^{-1}) in F1 oocytes, immediately following the 3 h exposure to the chemical in maternal fluid (Chapter 2). The BPA-exposed groups accumulated 0 (control), 4.4 and 41.3 ng BPA embryo^{-1} and the metabolome profile was analyzed at 42 (hatch) and 65 dpf (just prior to first feed), in both F1 and F2 generations.

6.3.3 Metabolite extraction and GC-MS Spectrometry

The metabolite extraction protocol was conducted as described by Jordan et al. (2012). Briefly, samples were ground to a fine powder using a mortar and a pestle, under dry ice. A methanol:chloroform solution (2:1, v:v; 600 µl) was then added to the samples and the tubes were placed in a tissue lyser, at a frequency of 20× per second, until homogenized. The samples were sonicated for 15 min, and 400 µl chloroform:water solution (1:1, v:v) was added. The mixture was centrifuged at 13,000 rpm for 20 min at 4 ˚C. Following centrifugation, the supernatant (containing the metabolites) was removed, placed in a clean 1.5 ml tube and dried for at least 24 h in a centrifugal evaporator (Speedvac). For derivatization of aqueous phase, 50 µl of methoxylamine-hydrochloride in pyridine (20 mg l^{-1}) was added to each residue, and then incubated for 2.5 h at 37 ˚C. For trimethylsilylation, 50 µl of N-methyl-N-trimethylsilyltrifluoroacetamide was subsequently added to each sample, followed by incubation at 37 ˚C for 45 min. The total volume was then diluted 6 times with hexane and 200 µl were used for GC-TOF-MS.

6.3.4 GC-TOF-MS data analysis

A Waters GCT premier GC-TOF-MS was used for analysis, under the following conditions: injector temperature was 275 ˚C for aqueous phase and the flow rate of the carrier

gas (helium) was 1.2 ml min^{-1}. Compound information from raw data from GC-MS was extracted by MetaboliteDetector2, a software for targeted and non-targeted GC-MS data analysis. The NIST GC-MS and the Golm metabolome (GMD)3 databases were used as the library for metabolite identification.

6.3.5 Statistical analysis

Data obtained from GC-MS analysis was normalized by median full range (MFC) methods in Microsoft Excel. Normalized data was then imported into SIMCA-P+ 12.0 Software (Umetrics) for multivariate data analysis. Projection method approaches, including principal component analysis (PCA) and orthogonal partial least-square discriminant analysis (OPLS-DA), were used. OPLS-DA significance was assessed based on a cross-validated ANOVA (CV-ANOVA) performed during the model building process. Significance was assessed by setting the threshold for *p* (CV-ANOVA) to 0.05, and any model that had a higher *p*-value would be considered not statistically significant. Metabolites with a Variable Influence on Projection (VIP) score >1 were considered significantly different in the multivariate OPLS-DA model, and were subsequently used to generate the summary tables in Appendix A. These metabolites were also used for pathway analysis using MetaboAnalyst 2.0 (Xia et al., 2012) (http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp).

6.3.6 Pathway analysis

Metabolite set enrichment analysis (MSEA) was performed on the selected metabolites for significant BPA treatment-control pairs for whole body at 42 and 65 dpf, in both F1 and F2 generation, as previously reported (Jordan et al., 2012), but with modifications. Briefly, a list of the selected compounds was inserted into MetaboAnalyst 2.0 quantitative enrichment analysis (QEA) (Xia et al., 2012). Only the pathways that had $p \leq 0.05$ (threshold to eliminate non-significant pathways) were chosen. The samples were then normalized to the median, and log transformed. Data scaling was carried out by selecting Pareto scaling. In addition, Ipath2 (http://pathways.embl.de) was also used for pathway analysis and visualization. This web-based

tool provides a map that summarizes all metabolic pathways derived from the suggested metabolites.

6.4 Results

The exposure of eggs to BPA (controls, 4.4 and 41.3 ng groups) did lead to changes in developmental phenotype as described previously (see Chapters 2, 4 and 5). To identify and measure the abundance of detectable metabolites, the GC-TOF-MS was used to characterize the aqueous phase of whole body extracts from two developmental stages prior to first feed, 42 (hatch) and 65 (just prior to first feed) dpf, from F1 and F2 generation of trout. A total of 80 metabolites were identified as being impacted by in the two generations. In the F1 generation, the BPA-mediated metabolome changes were grouped into 42 metabolic pathways at 42 dpf (Tables A-1 and A-2), and 12 pathways at 65 dpf (Tables A-3 and A-4). In the F2 generation, ancestral exposure BPA impacted 21 pathways at 42 dpf (Tables A-5 and A-6) and 18 at 65 dpf (Tables A-7 and A-8). The marked pathways in the tables denote $p<0.05$. Coefficient plots for metabolites that are up-regulated (increasing) or down-regulated (decreasing) when compared to controls in the F1 and F2 at the two life stages are presented in Figures A1-A4. The CV-ANOVA for each control-treatment pair revealed that BPA significantly altered the whole body metabolic profile in both generations and at both life stages (Table 6-1).

Table 6-1. CV-ANOVA *p*-values for every control to treatment comparison in F1 and F2 generation trout at 42 and 65 dpf.

Generation	Life stage	Variables	p (CV-ANOVA)	R2Y	Q2
FI	42 dpf	Control vs 4.4 ng	0.005	0.856	0.783
		Control vs 41.3 ng	0.041	0.707	0.598
	65 dpf	Control vs 4.4 ng	0.05	0.694	0.559
		Control vs 41.3 ng	0.034	0.838	0.739
FII	42 dpf	Control vs 4.4 ng	0.023	0.964	0.763
		Control vs 41.3 ng	0.044	0.579	0.462
	65 dpf	Control vs 4.4 ng	0.035	0.958	0.732
		Control vs 41.3 ng	0.033	0.692	0.531

6.4.1 BPA effects on metabolome profile in F1 generation

Accumulation of BPA in oocytes significantly altered the whole body metabolome profile when compared to controls in F1 generation (Table 6-1). At 42 dpf and 65 dpf, oocytes that accumulated 4.4 ng BPA had a significantly different metabolic profile ($p = 0.005$ and $p = 0.05$, respectively) than controls at the same life stages. A similar trend was observed in the 41.3 ng group, whose metabolic profile was also different from controls at hatch and first feed ($p = 0.041$ and 0.034, respectively). To further assess the implications of this observation, an OPLS-DA model was constructed for the three conditions (control, 4.4 and 41.3 g BPA) at 42 and 65 dpf (Fig 6-1). At 42 dpf, there was a significant ($p = 0.018$) shift along the median in the 4.4 ng BPA group when compared to controls, and this was even more evident in the 41.3 ng (Fig. 6-1a). At 65 dpf, a similar pattern emerged, where significant shifts ($p = 0.018$) in metabolic profiles were noted in the two BPA groups when compared to controls (Fig. 6-1b).

Analysis of coefficient plots (Figs. A1-A2) for the metabolites that were up-regulated or down-regulated revealed 40 metabolites that were either increasing or decreasing in the 4.4 ng group (Fig A1-a), while only 25 metabolites were identified in the 41.3 ng group at 42 dpf (Fig A1-b). At 65 dpf, there were only 6 metabolites affected in the 4.4 ng group (Fig. A2-a) and 7 in the 41.3 ng group (Fig. A2-b).

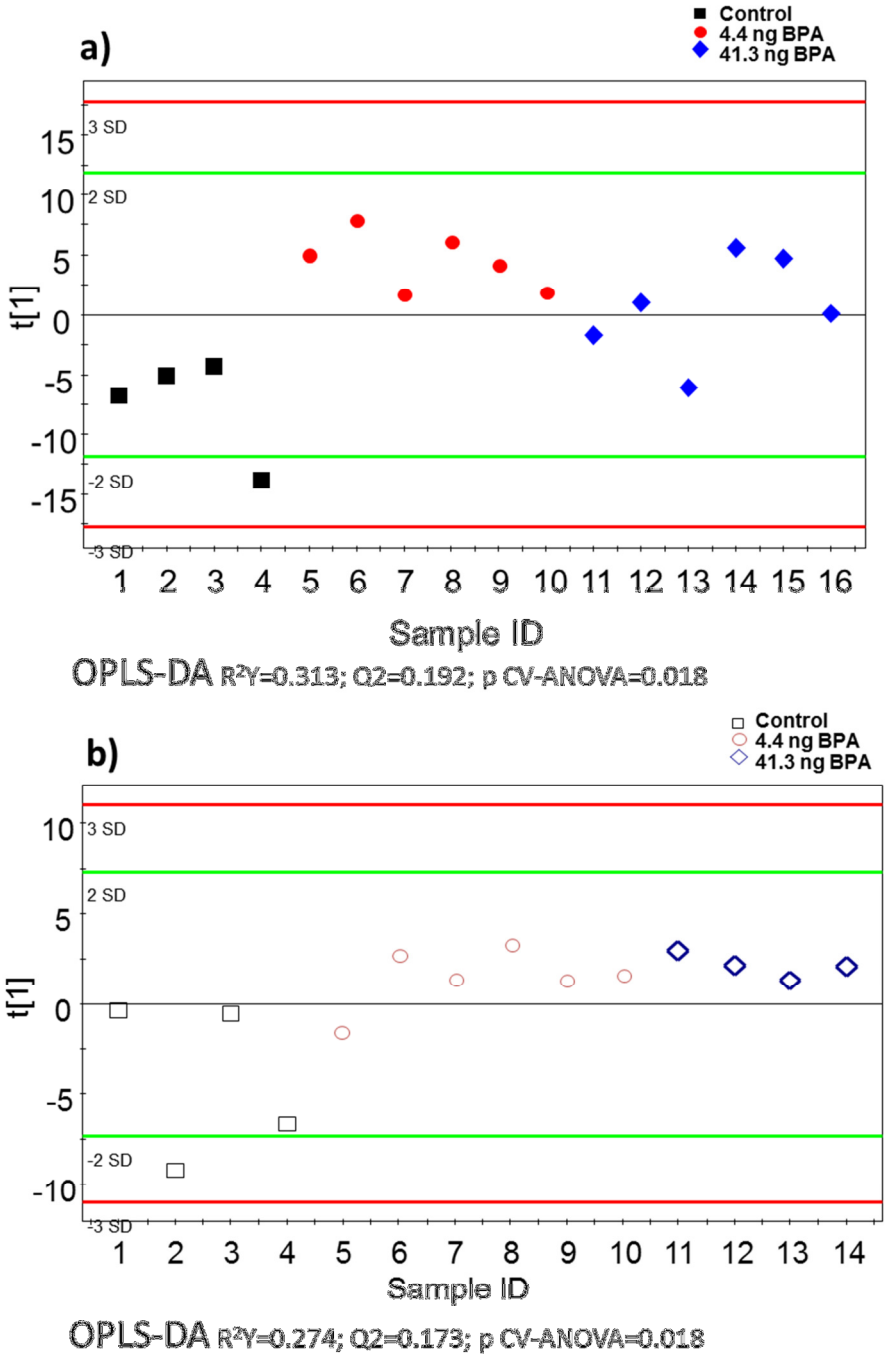

Figure 6-1. F1 whole body metabolome profile.

One component loading plot from orthogonal partial least squares discriminant analysis (OPLS-DA) model of modified GC-TOF-MS peaks from aqueous extracts of whole bodies collected from the controls, 4.4 and 41.3 ng BPA groups at (a) 42 dpf and (b) 65 dpf in F1 generation fish.

6.4.2 Ancestral BPA effects on F2 metabolic profile

Ancestral exposure to BPA significantly altered the whole body metabolome profile in the F2 generation (Table 6-1). At 42 dpf and 65 dpf, individuals that originated from 4.4 ng BPA had a significantly different metabolome profile compared to the controls ($p = 0.023$ and $p = 0.035$, respectively) than controls. A significant BPA effect was also observed in the 41.3 ng group at the two life stages compared to the controls ($p = 0.044$ and 0.033, respectively). To further assess the implications of this observation, an OPLS-DA model was constructed for the three conditions (control, 4.4 and 41.3 g BPA) at 42 and 65 dpf (Fig. 6-2). At 42 dpf, there was an almost significant ($p = 0.055$) shift in the metabolome when compared to the median (Fig. 6-2a). At 65 dpf, however, there appears to be a BPA-induced shift in the metabolome, but this was not statistically significant ($p = 0.073$; Fig. 6-2b).

Analysis of coefficient plots (Figs. A3-A4) for the metabolites that were up-regulated or down-regulated revealed 14 metabolites that were either increasing or decreasing in the 4.4 ng group (Fig A3-a), while only 6 metabolites were identified in the 41.3 ng group at 42 dpf (Fig A3-b). At 65 dpf, there were only 11 metabolites affected in the 4.4 ng group (Fig. A4-a), and 10 in the 41.3 ng group (Fig. A4-b).

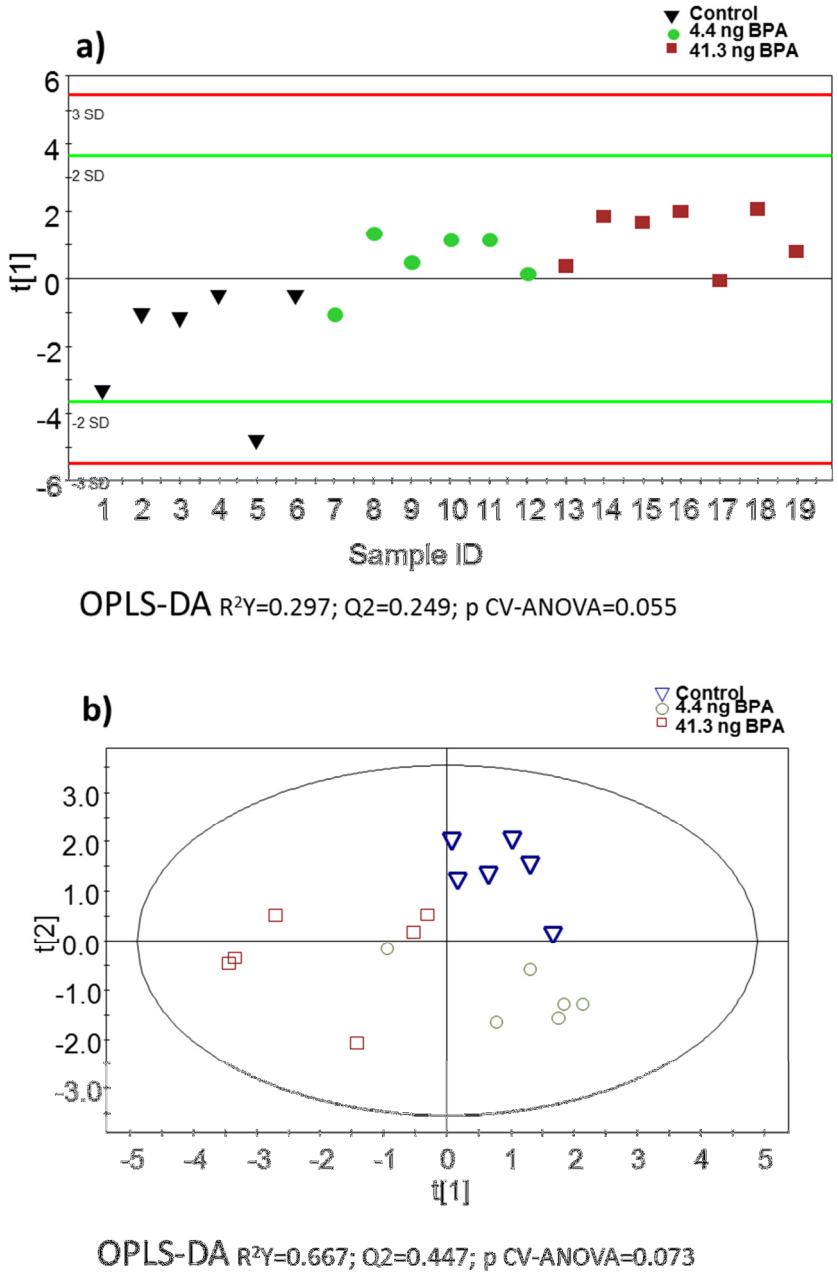

Figure 6-2. F2 whole body metabolome profile.

One component loading plot from orthogonal partial least squares discriminant analysis (OPLS-DA) model (a) and 2-component score plot from PCA (b) of modified GC-TOF-MS peaks from aqueous extracts of whole bodies collected from the controls, 4.4 and 41.3 ng BPA groups at (a) 42 dpf and (b) 65 dpf in F2 generation fish.

6.4.3 Major pathways affected by BPA in the two generations

Metabolite set enrichment analysis (MSEA) identified superpathways that were affected by BPA exposure (Fig. 6-3). In the F1 generation at 42 dpf, there were 13 pathways impacted by oocyte exposure to 4.4 ng BPA, and only 6 in the 41.3 ng group (Fig. 6-3a). No significant alterations were noted in metabolic pathways at 65 dpf in F1 (Fig. 6-3b). In F2 generation, ancestral exposure to 4.4 ng BPA induced alterations in 6 pathways at 42 dpf, but 41.3 ng BPA showed no metabolic pathway changes at hatch (Fig. 6-4a), while at 65 dpf, significantly altered superpathways were noted in the 41.3 ng group, but not in the 4.4 ng group (Fig. 6-4b).

Pathway analysis using MetaboAnalyst 2.0 revealed several major metabolic pathways that were affected by BPA in both F1 and F2 generations (Fig. 6-5). Biological pathways, such as aminoacyl-tRNA biosynthesis, were only impacted in F1 generation at hatch, in both the 4.4 and 41.3 ng groups. BPA-induced alterations in pathways involving carbohydrate, lipid, nitrogen and amino acid metabolism were noted at 42 dpf in both 4.4 and 41.3 ng groups of F1 generation. Alterations in TCA cycle and glutathione metabolism were also noted in the 41.3 and 4.4 ng groups, respectively. At 65 dpf, both BPA groups induced modifications in carbohydrate and lipid metabolism in F1. In F2, the 4.4 ng group showed alterations in sugar metabolism at 42 dpf, while the 41.3 ng group had impacted amino acid and butanoate metabolism at the same life stage. At 65 dpf , 4.4 ng BPA induced changes in amino acid metabolism and TCA cycle, while ancestral exposure to 41.3 ng BPA impacted sugar and glycerophospholipid metabolism in F2.

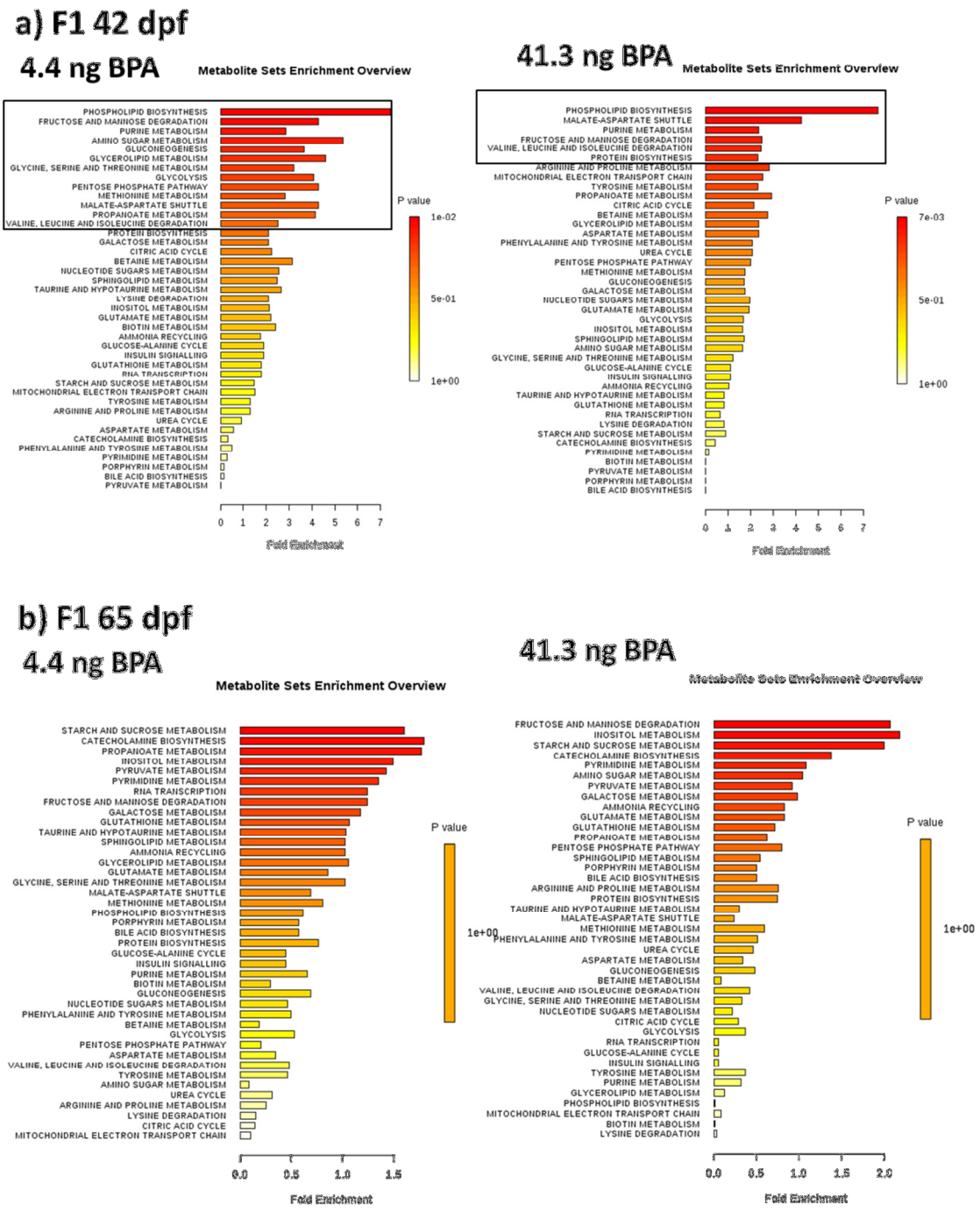

Figure 6-3. Mean set enrichment analysis in F1 generation.

Metabolic pathways disrupted by BPA shown as per fold enrichment (MSEA output) and *p* values. Input: selected differentially abundant metabolites for a pair-wise comparison between controls and treatment. Boxes enclose pathways that are significantly impacted by BPA at (a) 42 dpf and (b) 65 dpf in F1 fish.

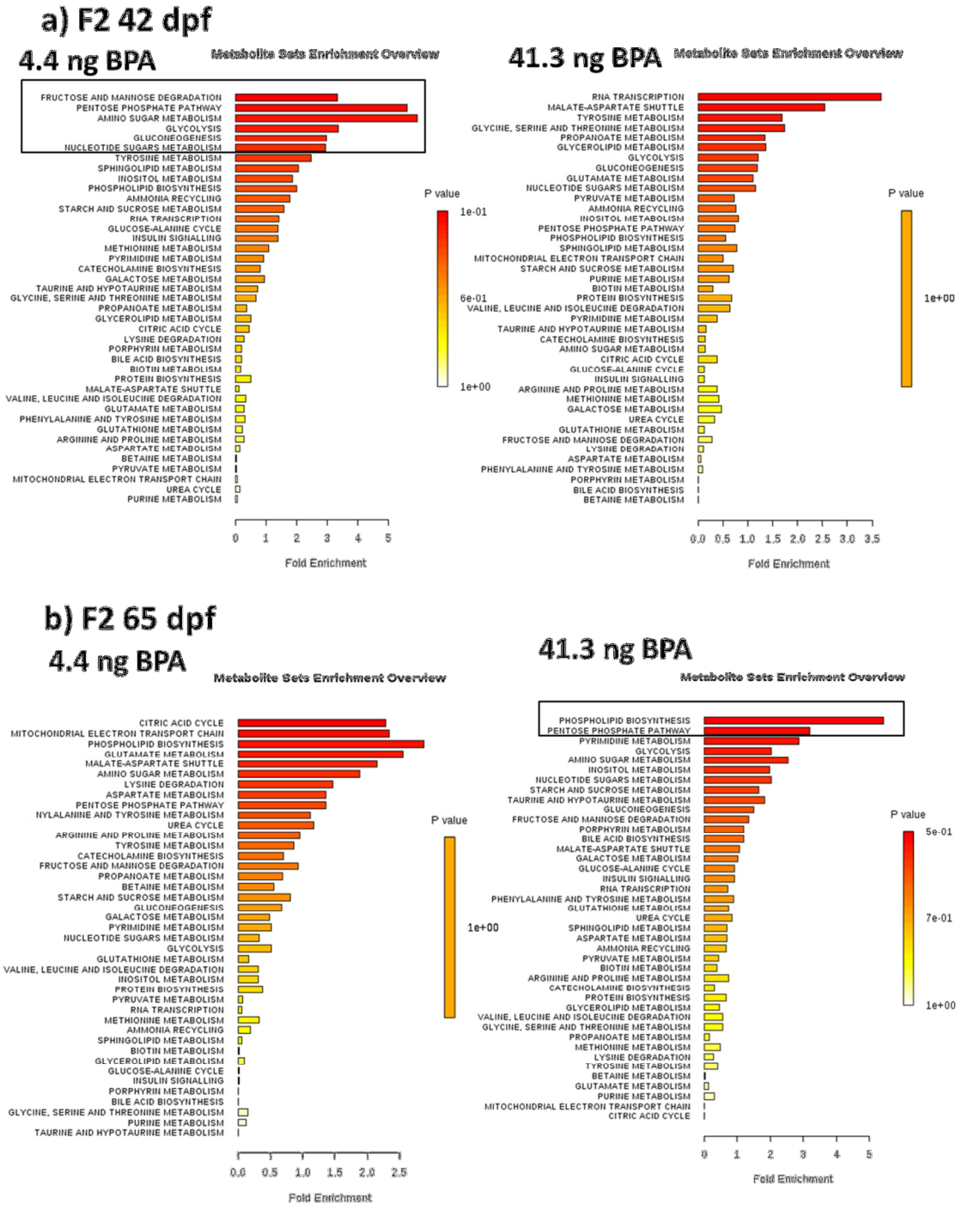

Figure 6-4. Mean set enrichment analysis in F2 generation.

Metabolic pathways disrupted by BPA shown as per fold enrichment (MSEA output) and *p* values. Input: selected differentially abundant metabolites for a pair-wise comparison between controls and treatment. Boxes enclose pathways that are significantly impacted by BPA at (a) 42 dpf and (b) 65 dpf in F2 fish.

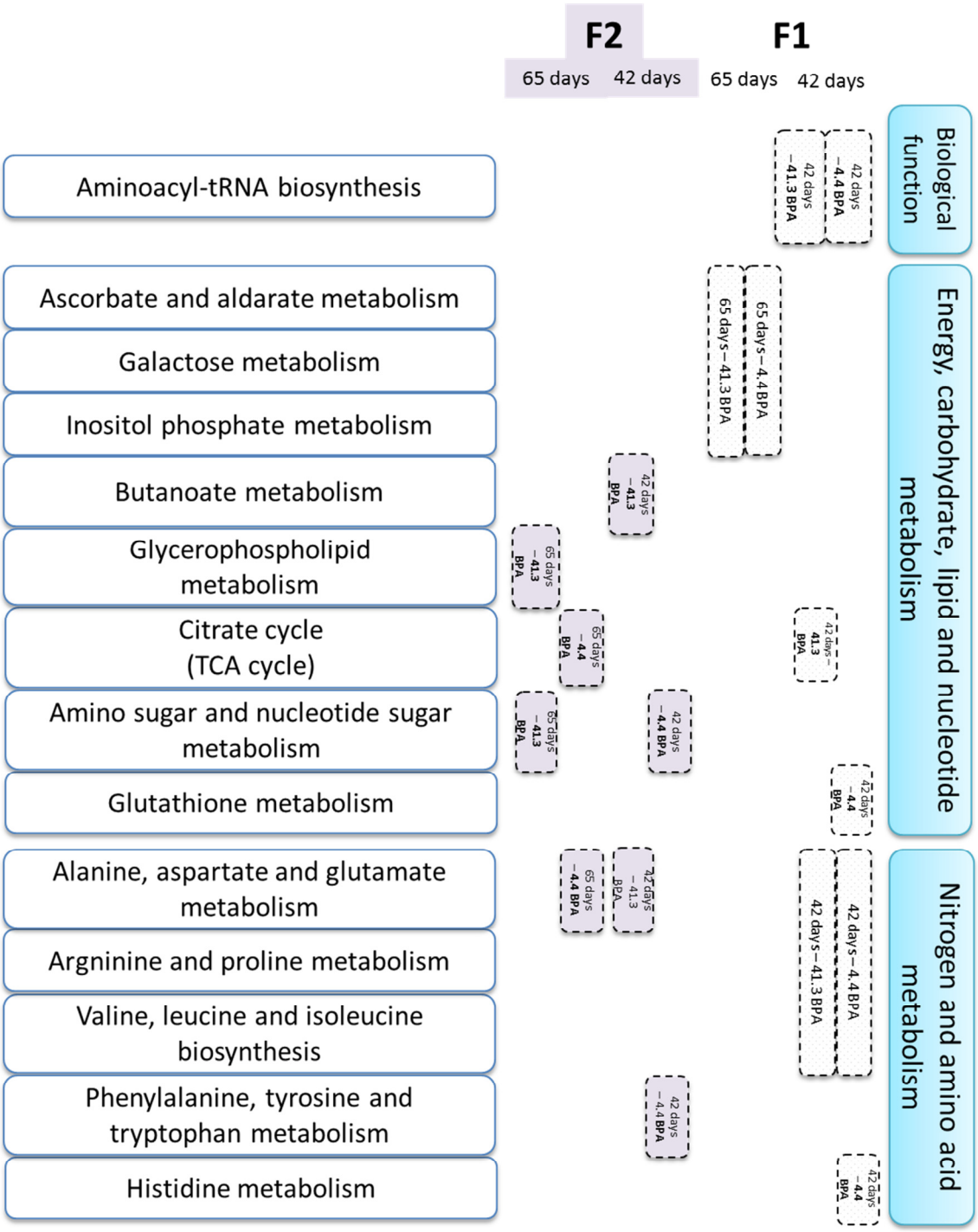

Figure 6-5. Metabolic pathways altered by BPA in F1 and F2.

Metabolic disturbances due to BPA exposure in F1 and F2 generations at 42 and 65 dpf. The figure was constructed based on selected differential metabolites and their respective affected superpathways (p<0.05).

6.5 Discussion

Using a metabolomics approach, our results provide novel insights on BPA-mediated metabolic disruption and, for the first time, demonstrate a generational impact on metabolic pathways in fish. The pathways impacted include carbohydrate (gluconeogenesis, glycogenolysis; Figs. 6-3, 6-4), lipid (phospholipid biosynthesis), nitrogen and amino acid metabolism , and some of these changes were life-stage and dose-specific. The consistent changes in lipid and carbohydrate metabolism (glycolysis and gluconeogenesis) pathways in both the F1 and F2 generations in trout suggest their use as potential biomarkers of BPA-induced toxicity in trout.

F1 generation:

BPA is commonly known as a weak xenoestrogen, but recent research showed that it exerts effects independent of the estrogen receptor (Aluru et al., 2010; Hanson et al., 2012, 2014; Jordan et al., 2012; Ekman et al., 2013; Chapters 2 and 3). Specifically, BPA accumulation in eggs was shown to have long term impact on growth and stress response, pointing to a disruption in somatotropic and stress axis functioning in trout (Aluru et al., 2010). In agreement, our metabolome analysis showed that the protein synthetic pathways were disrupted in embryo hatched from BPA-exposed eggs. Also, amino acids synthesis pathways were impacted by BPA and this may be due to BPA-mediated disruption in aminoacyl-tRNA (aa-tRNA) biosynthesis. AA-tRNA acts as a substrate for translation and it plays a crucial role in determining how the genetic code is translated to amino acids (Ibba & Dieter, 2000). Consequently, it can be proposed that aa-tRNA biosynthesis pathways is a possible target of BPA toxicity, leading to long-term effects on growth and metabolism in trout (Aluru et al., 2010; Chapters 2 and 3).

In addition to protein synthesis, data from MSEA for F1 generation fish identified pathways involved in sugar, lipid and amino acid metabolism as being disrupted by BPA exposure, suggesting possible effects on energy metabolism. The disruption in phospholipid biosynthesis and valine and threonine metabolic pathways at hatch in the BPA exposed trout oocytes would suggest perturbation in membrane and protein synthetic processes during early development. While no study has looked at the metabolome profile during early development,

BPA exposure impacted phospholipid metabolism in the adult goldfish liver and gonad (Jordan et al., 2012). In addition, pathways involved in glycolysis and gluconeogenesis were also impacted in the 4.4 ng, but not in the 41.3 ng group, but this was not reflected in measurable differences in whole body glucose or glycogen at 42 dpf in F1 generation (Chapter 2). Collectively, BPA accumulation in eggs affects progeny metabolome, indicating disturbances in energy metabolism. As the energy substrates during development (42-65 dpf) are derived from yolk stores, any change in yolk content would suggest nutrient deprivation. Delays in yolk absorption were noted in F1 generation in fish exposed to 4.4 and 41.3 ng BPA embryo^{-1} during embryogenesis (Chapter 2). Fish in the two BPA groups had larger yolks at first feed, supporting impaired nutrient utilization. This is further supported by the reduced whole body energy content in the 4.4 and 41.3 ng groups at 65 dpf.

The energy demand of the cells may also be altered, leading to reduced total energy content. The changes in ascorbate, aldarate, galactose and inositol phosphate metabolism pathways in the BPA groups (Fig. 6-5) suggest alterations in energy turnover. For instance, ascorbate and aldarate are low-molecular weight compounds that are part of the antioxidant system in aquatic organisms, and essential to maintain proper energy production within the cell by reducing oxidative stress (Eyckmans et al., 2011; Lushchak, 2011). Galactose metabolism leads to glucose production (gluconeogenesis) in vertebrates (Staehr et al., 2007), while inositol phosphate is known to play a role in osmotic balance in fish (Gardell et al., 2013). Therefore, alterations in these pathways can also lead to disturbances in whole organismal energy budget. Overall, the metabolome profile of the embryos prior to feeding leads us to propose that BPA disrupts pathways involved in energy metabolism and protein synthesis and this may have long-term impact as evident from the reduced specific growth rate seen in the larvae raised from BPA-laden eggs (Chapter 2).

F2 generation:

Data from OPLS-DA suggests that the whole body metabolome profile of F2 generation fish was less impacted relative to the F1 generation. Nevertheless, individuals raised from 4.4 ng BPA mothers had reduced specific growth rate post-feed and altered whole body glycogen and

lactate profiles at 42 dpf, suggesting metabolic disturbances as seen in F1 generation (Chapter 4). Indeed, data from MSEA for F2 identified energy metabolism pathways in the 4.4 ng group at 42 dpf as significantly impacted by ancestral BPA exposure. Glycolysis and gluconeogenesis, two of the major affected processes, play a key role in maintaining whole body energy levels and homeostasis, and is critical for coping with secondary stressors and reestablishing homeostasis (Vijayan et al., 2010). Thus, disruptions in these pathways in the F2 generation may imply long-term alterations in metabolic stress response. Indeed, plasma glucose and lactate profiles in trout following an acute stress in the F2 generation were altered by ancestral BPA exposure in a dose-specific manner (Chapter 5). The 41.3 ng BPA group had lower glucose levels than controls post-stress, suggesting disruption of the adaptive metabolic response. The mechanism involved is not known, but it can be hypothesized that epigenetic modification of genes involved in gluconeogenesis may be playing a role, but this remains to be tested.

Although there was no significant BPA-induced shift in the organismal metabolome according to OPLS-DA, metabolic pathway alterations at 65 dpf were noted in F2 generation fish using MSEA. Two pathways were significantly disrupted by ancestral exposure to 41.3 ng BPA, and these were the phospholipid biosynthesis and pentose phosphate pathways involved in lipid and carbohydrate metabolism, respectively. These findings were also confirmed using pathway analysis (MetaboAnalyst 2.0; Fig. 6-5), but these changes were not reflected in whole body energy and metabolite profiles in these fish at 65 dpf (Chapter 4). Metabolic processes were also altered in the 4.4 ng group, but there was no significant effect of the treatment at 65 dpf ($p>0.05$). However, the whole body glycogen was reduced in this group prior to first feed (Chapter 4), suggesting a possible metabolic disturbance.

Common metabolic pathways associated with ancestral BPA exposure that were present in both F1 and F2 generation fish at hatch (42 dpf) and prior to first feed (65 dpf) were disruptions in citric acid (TCA) cycle functioning, and alanine, aspartate and glutamate metabolism (Fig. 6-5). These are energy generating pathways, and the data supports the notion that energy metabolism is disrupted by exposure to BPA in fish. The TCA cycle in F1 was affected in the 41.3 ng BPA group at 42 dpf, while in F2 it was impacted in the 4.4 ng group at

65 dpf. The metabolism of the above mentioned amino acids was disrupted in F1 in both BPA groups, while in F2 changes occurred in the 41.3 ng group at 42 dpf and in the 4.4 ng group at 65 dpf. These generational effects appear to be dose-specific, suggesting that there might be multiple targets of BPA impact (Vandenberg et al., 2009; Vandenberg et al., 2012).

This study has provided novel insights on the potential long-term effects of BPA deposition in oocytes, by using a metablomics approach to investigate metabolite profiles in two generations of rainbow trout. From the metabolome profile, specific pathways that were disrupted by embryonic exposure to BPA can be identified, therefore confirming that these changes at the level of the metabolome are indeed multigenerational and are reflected in changes in growth and body composition. BPA significantly impacted energy, carbohydrate, lipid and amino sugar metabolism, as well as amino acid metabolism and biosynthesis in both F1 and F2 fish. The generational effects appear to be dose-specific, but processes, such as the TCA cycle and alanine, aspartate and glutamate metabolism were disrupted in both generations. The current study also confirms previously reported work on the BPA-induced alterations in energy pathways from the fish liver and gonad metabolome (Jordan et al., 2012). It appears that carbohydrate and lipid metabolism dysfunctions are commonly induced by BPA, either in specific tissues (liver and gonad; Jordan et al., 2012), or in whole bodies of larvae, following embryonic exposure (current study). The current study has identified protein synthesis, along with amino acid metabolism and amino sugar synthesis pathways as targets for BPA impact during embryogenesis and prior to exogenous feeding. Altogether, the metabolomics approach identified energy metabolism pathways as key targets for developmental effects of BPA in trout. Whether this is direct effect of BPA or indirectly mediated by disruptions in yolk utilization in the F1 generation remains to be determined. The persistence of some of these metabolic changes in the F2 generation suggests that BPA is disrupting metabolism, suggesting that epigenetic modifications may be responsible for the generational effects. Consequently, lipid and carbohydrate metabolism should be further investigated as potential markers of BPA toxicity in fish.

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Chapter 7: General Conclusions

7.1 Chapter conclusions

The overall objective of this thesis was to determine whether BPA accumulation in oocytes, mimicking maternal transfer, has long-term implications on the development and functioning of the growth and stress axes in two generations of rainbow trout (*Oncorhynchus mykiss*). As a whole, the data presented here provides novel insights on the long-term and multigenerational effects of embryonic exposure to BPA in two generations of trout, by establishing for the first time that:

- BPA impacts growth in F1 by disrupting the developmental profiles of genes involved in somatotropic axis and thyroid hormone signaling, leading to alterations in body composition, specific growth rate (SGR) and food conversion ratios that are indicative of a BPA-induced metabolic disturbance in the developing fish (Chapter 2).
- BPA impacts stress response in trout larvae by delaying the onset of endogenous cortisol production and HPI axis maturation in F1 fish (Chapter 3).
- Growth, delays in HPI axis maturation and stress performance defects noted in F1 also persists in F2 fish. Also, lower SGR and altered whole body composition seen in F1 BPA-exposed eggs was also seen in the F2 generation. Together, the results for the first time reveal that the effects of this chemical are multigenerational and dose-specific (Chapter 4).
- BPA impacts the long-term HPI axis functioning in F1 and F2 generation fish. The BPA impact was upstream from the ACTH receptor on the interrenal cells and led to an attenuated plasma cortisol release *in vivo*, but not *in vitro*. This was also accompanied by modifications in metabolic responses, including disrupted plasma glucose and lactate levels post-acute stressor exposure (Chapter 5).
- Whole body metabolome profile is altered by BPA at hatch and prior to first feed in both F1 and F2 generations, identifying energy metabolism pathways, including carbohydrate and lipid metabolism and protein and amino acid synthesis pathways dysfunction as potential biomarkers of BPA toxicity in rainbow trout (Chapter 6).

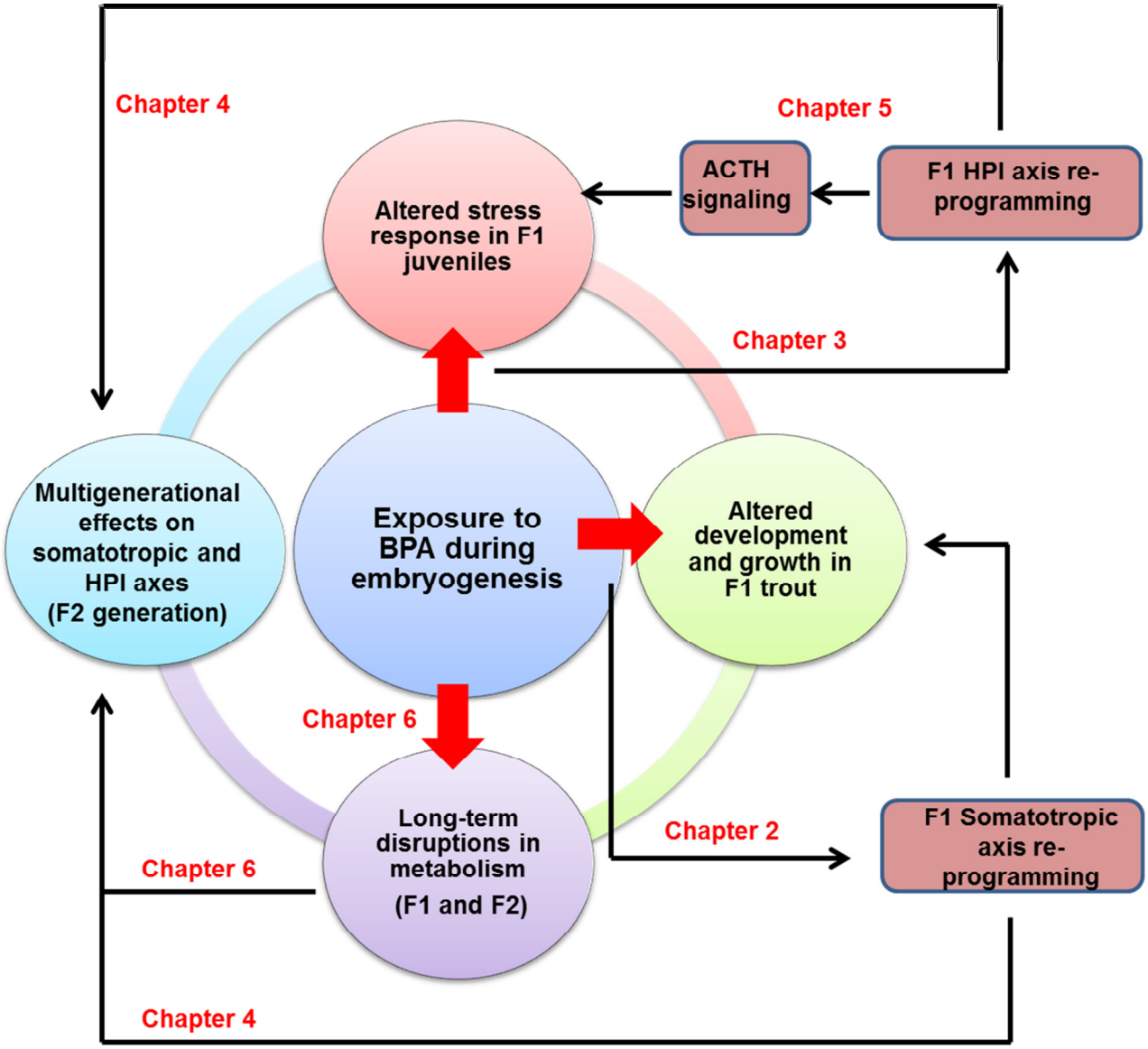
7.2 General conclusions

From a biological perspective, the disruptions in growth and cortisol stress response observed in both F1 and F2 generation fish suggest that exposure to BPA during embryogenesis may affect many aspects of fish physiology, given that cortisol has wide-ranging effects on fish performances. Such changes can ultimately impair the adaptive capacity of the organism to multiple stressors, leading to reduced fitness and population level effects. From an ecological perspective, changes at the biological level in one population can alter the dynamics within an ecosystem, disrupting biodiversity.

Currently, traditional multigenerational studies on the effects of toxicants in fish involve standardized exposures from fertilization, through embryogenesis and early juvenile development of several generations of fish, without considering maternal transfer of contaminants as an important exposure route. These tests typically use survival, growth and markers of developmental success (condition factor, hepatosomatic and gonadosomatic indices, and fertilization rates) as indicators of long term effects (Staples et al., 2011). However, since chemicals have been shown to accumulate in oocytes of wild fish (Ostrach et al., 2008), the current thesis suggests that studies focusing on multigenerational effects of maternal transfer of contaminants are necessary. The work presented in this thesis, for the first time, underscores the various non-estrogenic effects on growth and stress axes programming that BPA elicits when exposure occurs during key embryonic stages in trout (Fig. 7-1). The finding that these alterations are multigenerational and, more importantly, that they are also reflected in the whole body metabolome in the two generations, suggests that BPA-induced epigenetic modifications may be playing a role in growth and stress axis disruptions over two generations. Further research on the transcriptome and proteome of F1 and F2 generation fish can provide new insights on additional pathways that are impacted by maternal deposition of BPA and other organic toxicants in oocytes, and will aid in the discovery and development of biomarkers of (multigenerational) effects in fish.

Figure 7-1. Schematic of the effects of BPA accumulation in oocytes.

Oocyte loading with BPA, mimicking maternal transfer, induced delays in development in the F1 offspring, affecting growth and development in trout (Chapter 2), as previously suggested (Aluru et al., 2010). In addition, juveniles that were exposed to BPA as embryos presented alterations in the highly conserved acute stress response, including cortisol and glucose profiles (Chapter 3). Since BPA was no longer detected in the embryos after hatch, these findings suggest that BPA interferes with somatotropic and HPI axes functioning during development. These BPA-induced alterations were shown to be multigenerational (Chapter 4), as delays in growth and HPI axis maturation were observed in F2 generation. A key finding of the current study is that the cortisol stress response profile was altered in both F1 and F2 fish in the BPA groups (Chapter 5). The disruption in the acute stress response impairs ACTH signaling, along the HP axis. Lastly, loading of oocytes with BPA induced significant disruptions in the whole body metabolome profile at 42 and 65 dpf, in both F1 and F2 fish (Chapter 6), suggesting that the BPA effects on the metabolome are multigenerational, underscoring epigenome modification by BPA as a possible mechanism.


Chapter 4
Chapter 5
ACTH signaling
F1 HPI axis re-programming
Altered stress response in F1 juveniles
Chapter 3
Multigenerational effects on somatotropic and HPI axes (F2 generation)
Exposure to BPA during embryogenesis
Altered development and growth in F1 trout
Chapter 6
Long-term disruptions in metabolism (F1 and F2)
Chapter 2
F1 Somatotropic axis re-programming
Chapter 6
Chapter 4

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Appendix A

Supplemental Information for Chapter 6

Table A-1. Affected pathways of FI – 42 days – 3 BPA vs Control

	Total	Expected	Hits	Raw p	-log(p)	Holm adjust	FDR	Impact
Aminoacyl-tRNA biosynthesis	67	1.70	11	2.48E-07	1.52E+01	2.01E-05	2.01E-05	0.10
Valine, leucine and isoleucine biosynthesis	13	0.33	3	3.60E-03	5.63E+00	2.88E-01	1.01E-01	0.67
Arginine and proline metabolism	43	1.09	5	3.75E-03	5.59E+00	2.96E-01	1.01E-01	0.28
Alanine, aspartate and glutamate metabolism	24	0.61	3	2.10E-02	3.86E+00	1.00E+00	4.23E-01	0.57
Glutathione metabolism	26	0.66	3	2.61E-02	3.65E+00	1.00E+00	4.23E-01	0.05
Histidine metabolism	14	0.36	2	4.71E-02	3.06E+00	1.00E+00	6.35E-01	0.00
Ubiquinone and other terpenoid-quinone biosynthesis	3	0.08	1	7.43E-02	2.60E+00	1.00E+00	7.92E-01	0.00
Phenylalanine, tyrosine and tryptophan biosynthesis	4	0.10	1	9.79E-02	2.32E+00	1.00E+00	7.92E-01	0.50
Butanoate metabolism	22	0.56	2	1.05E-01	2.25E+00	1.00E+00	7.92E-01	0.03
Biotin metabolism	5	0.13	1	1.21E-01	2.11E+00	1.00E+00	7.92E-01	0.00
D-Glutamine and D-glutamate metabolism	5	0.13	1	1.21E-01	2.11E+00	1.00E+00	7.92E-01	1.00
Galactose metabolism	26	0.66	2	1.39E-01	1.97E+00	1.00E+00	7.92E-01	0.04
Ascorbate and aldarate metabolism	6	0.15	1	1.43E-01	1.94E+00	1.00E+00	7.92E-01	0.00
Cyanoamino acid metabolism	6	0.15	1	1.43E-01	1.94E+00	1.00E+00	7.92E-01	0.00
Glycerophospholipid metabolism	28	0.71	2	1.57E-01	1.85E+00	1.00E+00	7.92E-01	0.08
Taurine and hypotaurine metabolism	7	0.18	1	1.65E-01	1.80E+00	1.00E+00	7.92E-01	0.40
Cysteine and methionine metabolism	29	0.74	2	1.66E-01	1.79E+00	1.00E+00	7.92E-01	0.10
Glycine, serine and threonine metabolism	31	0.79	2	1.85E-01	1.69E+00	1.00E+00	8.30E-01	0.26
Nitrogen metabolism	9	0.23	1	2.07E-01	1.57E+00	1.00E+00	8.39E-01	0.00
Methane metabolism	9	0.23	1	2.07E-01	1.57E+00	1.00E+00	8.39E-01	0.40
Purine metabolism	66	1.68	3	2.33E-01	1.46E+00	1.00E+00	8.46E-01	0.02
Amino sugar and nucleotide sugar metabolism	37	0.94	2	2.41E-01	1.42E+00	1.00E+00	8.46E-01	0.09
Phenylalanine metabolism	11	0.28	1	2.47E-01	1.40E+00	1.00E+00	8.46E-01	0.00
Valine, leucine and isoleucine degradation	38	0.96	2	2.51E-01	1.38E+00	1.00E+00	8.46E-01	0.00
Nicotinate and nicotinamide metabolism	14	0.36	1	3.04E-01	1.19E+00	1.00E+00	9.61E-01	0.00
Tyrosine metabolism	44	1.12	2	3.08E-01	1.18E+00	1.00E+00	9.61E-01	0.16
Pantothenate and CoA biosynthesis	15	0.38	1	3.21E-01	1.13E+00	1.00E+00	9.64E-01	0.00
beta-Alanine metabolism	16	0.41	1	3.39E-01	1.08E+00	1.00E+00	9.73E-01	0.00
Lysine degradation	18	0.46	1	3.72E-01	9.88E-01	1.00E+00	9.73E-01	0.00
Glycerolipid metabolism	18	0.46	1	3.72E-01	9.88E-01	1.00E+00	9.73E-01	0.03
Glyoxylate and dicarboxylate metabolism	18	0.46	1	3.72E-01	9.88E-01	1.00E+00	9.73E-01	0.30
Citrate cycle (TCA cycle)	20	0.51	1	4.04E-01	9.05E-01	1.00E+00	1.00E+00	0.05
Sphingolipid metabolism	21	0.53	1	4.20E-01	8.68E-01	1.00E+00	1.00E+00	0.00
Fructose and mannose metabolism	21	0.53	1	4.20E-01	8.68E-01	1.00E+00	1.00E+00	0.16
Starch and sucrose metabolism	22	0.56	1	4.35E-01	8.33E-01	1.00E+00	1.00E+00	0.04
Porphyrin and chlorophyll metabolism	27	0.69	1	5.04E-01	6.85E-01	1.00E+00	1.00E+00	0.00
Inositol phosphate metabolism	27	0.69	1	5.04E-01	6.85E-01	1.00E+00	1.00E+00	0.09
Tryptophan metabolism	39	0.99	1	6.39E-01	4.48E-01	1.00E+00	1.00E+00	0.15

Table A-2. Affected pathways of FI – 42 days – 30 BPA vs Control

	Total	Expected	Hits	Raw p	-log(p)	Holm adjust	FDR	Impact
Aminoacyl-tRNA biosynthesis	67	1.15	6	6.45E-04	7.35E+00	5.23E-02	5.23E-02	0.10
Valine, leucine and isoleucine biosynthesis	13	0.22	2	1.96E-02	3.93E+00	1.00E+00	7.95E-01	0.67
Arginine and proline metabolism	43	0.74	3	3.49E-02	3.35E+00	1.00E+00	8.90E-01	0.17
Citrate cycle (TCA cycle)	20	0.34	2	4.45E-02	3.11E+00	1.00E+00	8.90E-01	0.05
Alanine, aspartate and glutamate metabolism	24	0.41	2	6.20E-02	2.78E+00	1.00E+00	8.90E-01	0.00
Glycerophospholipid metabolism	28	0.48	2	8.14E-02	2.51E+00	1.00E+00	8.90E-01	0.08
Cysteine and methionine metabolism	29	0.50	2	8.66E-02	2.45E+00	1.00E+00	8.90E-01	0.10
Ascorbate and aldarate metabolism	6	0.10	1	9.89E-02	2.31E+00	1.00E+00	8.90E-01	0.00
Cyanoamino acid metabolism	6	0.10	1	9.89E-02	2.31E+00	1.00E+00	8.90E-01	0.00
Amino sugar and nucleotide sugar metabolism	37	0.64	2	1.31E-01	2.03E+00	1.00E+00	9.77E-01	0.09
Valine, leucine and isoleucine degradation	38	0.65	2	1.37E-01	1.99E+00	1.00E+00	9.77E-01	0.00
Methane metabolism	9	0.15	1	1.45E-01	1.93E+00	1.00E+00	9.77E-01	0.40
Tyrosine metabolism	44	0.76	2	1.73E-01	1.75E+00	1.00E+00	1.00E+00	0.03
Lysine degradation	18	0.31	1	2.69E-01	1.31E+00	1.00E+00	1.00E+00	0.00
Glycerolipid metabolism	18	0.31	1	2.69E-01	1.31E+00	1.00E+00	1.00E+00	0.03
Propanoate metabolism	20	0.34	1	2.95E-01	1.22E+00	1.00E+00	1.00E+00	0.00
Sphingolipid metabolism	21	0.36	1	3.07E-01	1.18E+00	1.00E+00	1.00E+00	0.00
Fructose and mannose metabolism	21	0.36	1	3.07E-01	1.18E+00	1.00E+00	1.00E+00	0.16
Purine metabolism	66	1.13	2	3.15E-01	1.16E+00	1.00E+00	1.00E+00	0.01
Butanoate metabolism	22	0.38	1	3.19E-01	1.14E+00	1.00E+00	1.00E+00	0.00
Galactose metabolism	26	0.45	1	3.65E-01	1.01E+00	1.00E+00	1.00E+00	0.00
Glutathione metabolism	26	0.45	1	3.65E-01	1.01E+00	1.00E+00	1.00E+00	0.01
Inositol phosphate metabolism	27	0.46	1	3.77E-01	9.77E-01	1.00E+00	1.00E+00	0.09
Glycine, serine and threonine metabolism	31	0.53	1	4.19E-01	8.70E-01	1.00E+00	1.00E+00	0.23
Tryptophan metabolism	39	0.67	1	4.96E-01	7.01E-01	1.00E+00	1.00E+00	0.15

Table A-3. Affected pathways of FI – 65 days – 3 BPA vs Control

	Total	Expected	Hits	Raw p	-log(p)	Holm adjust	FDR	Impact
Ascorbate and aldarate metabolism	6	0.03	2	2.49E-04	8.30E+00	2.02E-02	2.02E-02	0.50
Galactose metabolism	26	0.12	2	5.19E-03	5.26E+00	4.15E-01	1.51E-01	0.00
Inositol phosphate metabolism	27	0.12	2	5.59E-03	5.19E+00	4.42E-01	1.51E-01	0.09
Pentose and glucuronate interconversions	15	0.07	1	6.55E-02	2.73E+00	1.00E+00	8.34E-01	0.00
Glyoxylate and dicarboxylate metabolism	18	0.08	1	7.81E-02	2.55E+00	1.00E+00	8.34E-01	0.30
Citrate cycle (TCA cycle)	20	0.09	1	8.65E-02	2.45E+00	1.00E+00	8.34E-01	0.05
Fructose and mannose metabolism	21	0.09	1	9.06E-02	2.40E+00	1.00E+00	8.34E-01	0.00
Starch and sucrose metabolism	22	0.10	1	9.48E-02	2.36E+00	1.00E+00	8.34E-01	0.00
Butanoate metabolism	22	0.10	1	9.48E-02	2.36E+00	1.00E+00	8.34E-01	0.03
Alanine, aspartate and glutamate metabolism	24	0.11	1	1.03E-01	2.27E+00	1.00E+00	8.34E-01	0.11
Arginine and proline metabolism	43	0.19	1	1.78E-01	1.73E+00	1.00E+00	1.00E+00	0.01

Table A-4. Affected pathways of FI – 65 days – 30 BPA vs Control

	Total	Expected	Hits	Raw p	-log(p)	Holm adjust	FDR	Impact
Ascorbate and aldarate metabolism	6	0.03	2	2.49E-04	8.30E+00	2.02E-02	2.02E-02	0.50
Galactose metabolism	26	0.12	2	5.19E-03	5.26E+00	4.15E-01	1.51E-01	0.00
Inositol phosphate metabolism	27	0.12	2	5.59E-03	5.19E+00	4.42E-01	1.51E-01	0.09
Pentose and glucuronate interconversions	15	0.07	1	6.55E-02	2.73E+00	1.00E+00	1.00E+00	0.00
Fructose and mannose metabolism	21	0.09	1	9.06E-02	2.40E+00	1.00E+00	1.00E+00	0.00
Starch and sucrose metabolism	22	0.10	1	9.48E-02	2.36E+00	1.00E+00	1.00E+00	0.00
Arginine and proline metabolism	43	0.19	1	1.78E-01	1.73E+00	1.00E+00	1.00E+00	0.00
Purine metabolism	66	0.30	1	2.62E-01	1.34E+00	1.00E+00	1.00E+00	0.00

Table A-3. Affected pathways of FI – 65 days – 3 BPA vs Control

	Total	Expected	Hits	Raw p	-log(p)	Holm adjust	FDR	Impact
Ascorbate and aldarate metabolism	6	0.03	2	2.49E-04	8.30E+00	2.02E-02	2.02E-02	0.50
Galactose metabolism	26	0.12	2	5.19E-03	5.26E+00	4.15E-01	1.51E-01	0.00
Inositol phosphate metabolism	27	0.12	2	5.59E-03	5.19E+00	4.42E-01	1.51E-01	0.09
Pentose and glucuronate interconversions	15	0.07	1	6.55E-02	2.73E+00	1.00E+00	8.34E-01	0.00
Glyoxylate and dicarboxylate metabolism	18	0.08	1	7.81E-02	2.55E+00	1.00E+00	8.34E-01	0.30
Citrate cycle (TCA cycle)	20	0.09	1	8.65E-02	2.45E+00	1.00E+00	8.34E-01	0.05
Fructose and mannose metabolism	21	0.09	1	9.06E-02	2.40E+00	1.00E+00	8.34E-01	0.00
Starch and sucrose metabolism	22	0.10	1	9.48E-02	2.36E+00	1.00E+00	8.34E-01	0.00
Butanoate metabolism	22	0.10	1	9.48E-02	2.36E+00	1.00E+00	8.34E-01	0.03
Alanine, aspartate and glutamate metabolism	24	0.11	1	1.03E-01	2.27E+00	1.00E+00	8.34E-01	0.11
Arginine and proline metabolism	43	0.19	1	1.78E-01	1.73E+00	1.00E+00	1.00E+00	0.01

Table A-4. Affected pathways of FI – 65 days – 30 BPA vs Control

	Total	Expected	Hits	Raw p	-log(p)	Holm adjust	FDR	Impact
Ascorbate and aldarate metabolism	6	0.03	2	2.49E-04	8.30E+00	2.02E-02	2.02E-02	0.50
Galactose metabolism	26	0.12	2	5.19E-03	5.26E+00	4.15E-01	1.51E-01	0.00
Inositol phosphate metabolism	27	0.12	2	5.59E-03	5.19E+00	4.42E-01	1.51E-01	0.09
Pentose and glucuronate interconversions	15	0.07	1	6.55E-02	2.73E+00	1.00E+00	1.00E+00	0.00
Fructose and mannose metabolism	21	0.09	1	9.06E-02	2.40E+00	1.00E+00	1.00E+00	0.00
Starch and sucrose metabolism	22	0.10	1	9.48E-02	2.36E+00	1.00E+00	1.00E+00	0.00
Arginine and proline metabolism	43	0.19	1	1.78E-01	1.73E+00	1.00E+00	1.00E+00	0.00
Purine metabolism	66	0.30	1	2.62E-01	1.34E+00	1.00E+00	1.00E+00	0.00

Table A-7. Affected pathways of FII – 65 days – 3 BPA vs Control

	Total	Expected	Hits	Raw p	-log(p)	Holm adjust	FDR	Impact
Citrate cycle (TCA cycle)	20	0.12	2	5.63E-03	5.18E+00	4.56E-01	3.27E-01	0.05
Alanine, aspartate and glutamate metabolism	24	0.14	2	8.08E-03	4.82E+00	6.46E-01	3.27E-01	0.00
Lysine degradation	18	0.11	1	1.03E-01	2.27E+00	1.00E+00	1.00E+00	0.00
Propanoate metabolism	20	0.12	1	1.14E-01	2.17E+00	1.00E+00	1.00E+00	0.00
Butanoate metabolism	22	0.13	1	1.24E-01	2.08E+00	1.00E+00	1.00E+00	0.00
Starch and sucrose metabolism	22	0.13	1	1.24E-01	2.08E+00	1.00E+00	1.00E+00	0.02
Galactose metabolism	26	0.16	1	1.46E-01	1.93E+00	1.00E+00	1.00E+00	0.04
Glycerophospholipid metabolism	28	0.17	1	1.56E-01	1.86E+00	1.00E+00	1.00E+00	0.00
Amino sugar and nucleotide sugar metabolism	37	0.22	1	2.01E-01	1.60E+00	1.00E+00	1.00E+00	0.00
Arginine and proline metabolism	43	0.26	1	2.30E-01	1.47E+00	1.00E+00	1.00E+00	0.00
Tyrosine metabolism	44	0.26	1	2.35E-01	1.45E+00	1.00E+00	1.00E+00	0.00

Table A-8. Affected pathways of FII – 65 days – 30 BPA vs Control

	Total	Expected	Hits	Raw p	-log(p)	Holm adjust	FDR	Impact
Glycerophospholipid metabolism	28	0.19	2	1.39E-02	4.28E+00	1.00E+00	9.59E-01	0.08
Amino sugar and nucleotide sugar metabolism	37	0.25	2	2.37E-02	3.74E+00	1.00E+00	9.59E-01	0.09
Pantothenate and CoA biosynthesis	15	0.10	1	9.67E-02	2.34E+00	1.00E+00	1.00E+00	0.00
beta-Alanine metabolism	16	0.11	1	1.03E-01	2.27E+00	1.00E+00	1.00E+00	0.40
Glycerolipid metabolism	18	0.12	1	1.15E-01	2.16E+00	1.00E+00	1.00E+00	0.03
Glyoxylate and dicarboxylate metabolism	18	0.12	1	1.15E-01	2.16E+00	1.00E+00	1.00E+00	0.30
Propanoate metabolism	20	0.13	1	1.27E-01	2.06E+00	1.00E+00	1.00E+00	0.00
Citrate cycle (TCA cycle)	20	0.13	1	1.27E-01	2.06E+00	1.00E+00	1.00E+00	0.05
Fructose and mannose metabolism	21	0.14	1	1.33E-01	2.02E+00	1.00E+00	1.00E+00	0.16
Pyrimidine metabolism	41	0.28	1	2.45E-01	1.41E+00	1.00E+00	1.00E+00	0.00

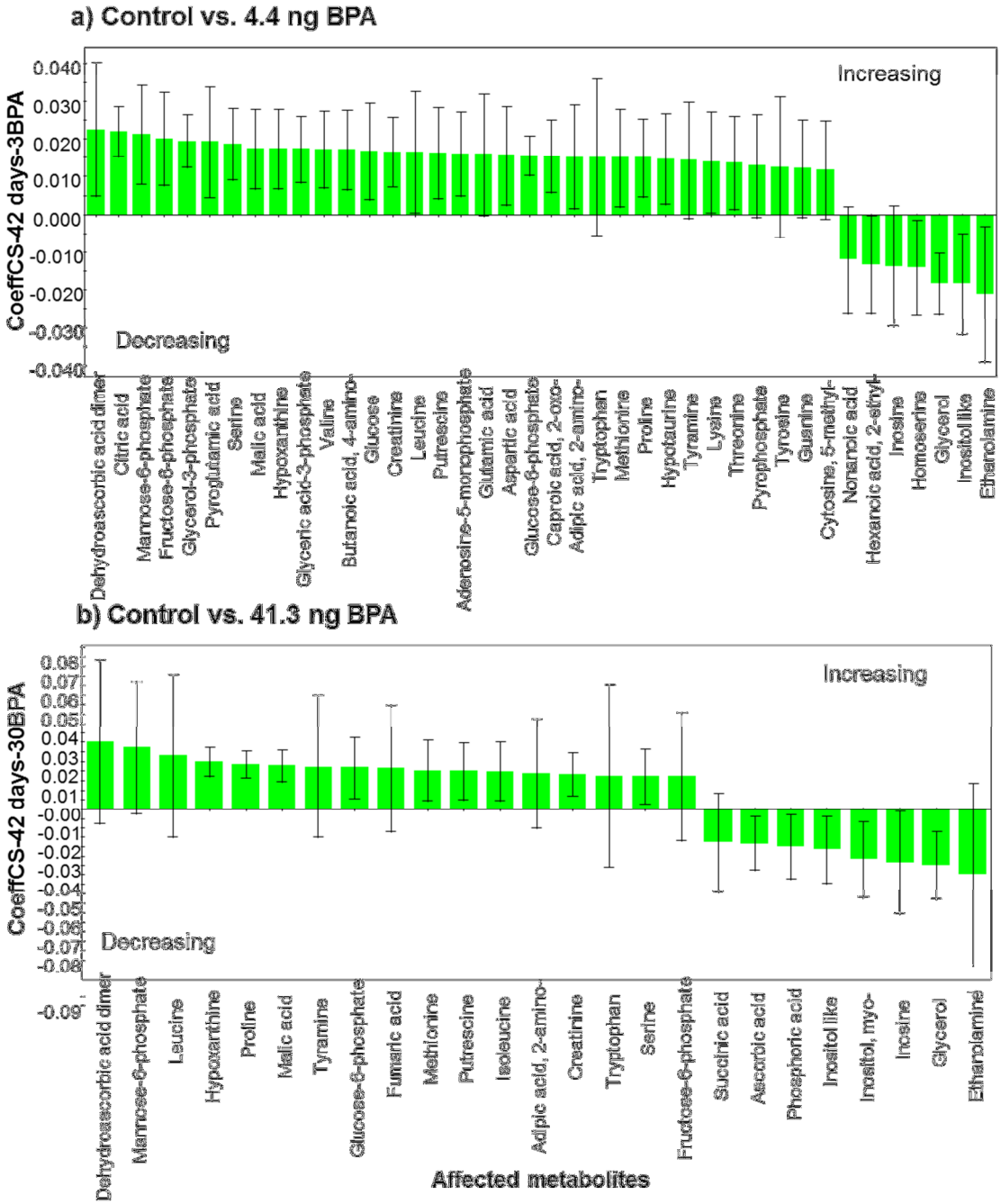

Figure A1. Coefficient plots for F1 generation at 42 dpf. Degree of change in whole body metabolite levels in (a) 4.4 and (b) 41.3 ng BPA groups when compared to controls at 42 dpf.

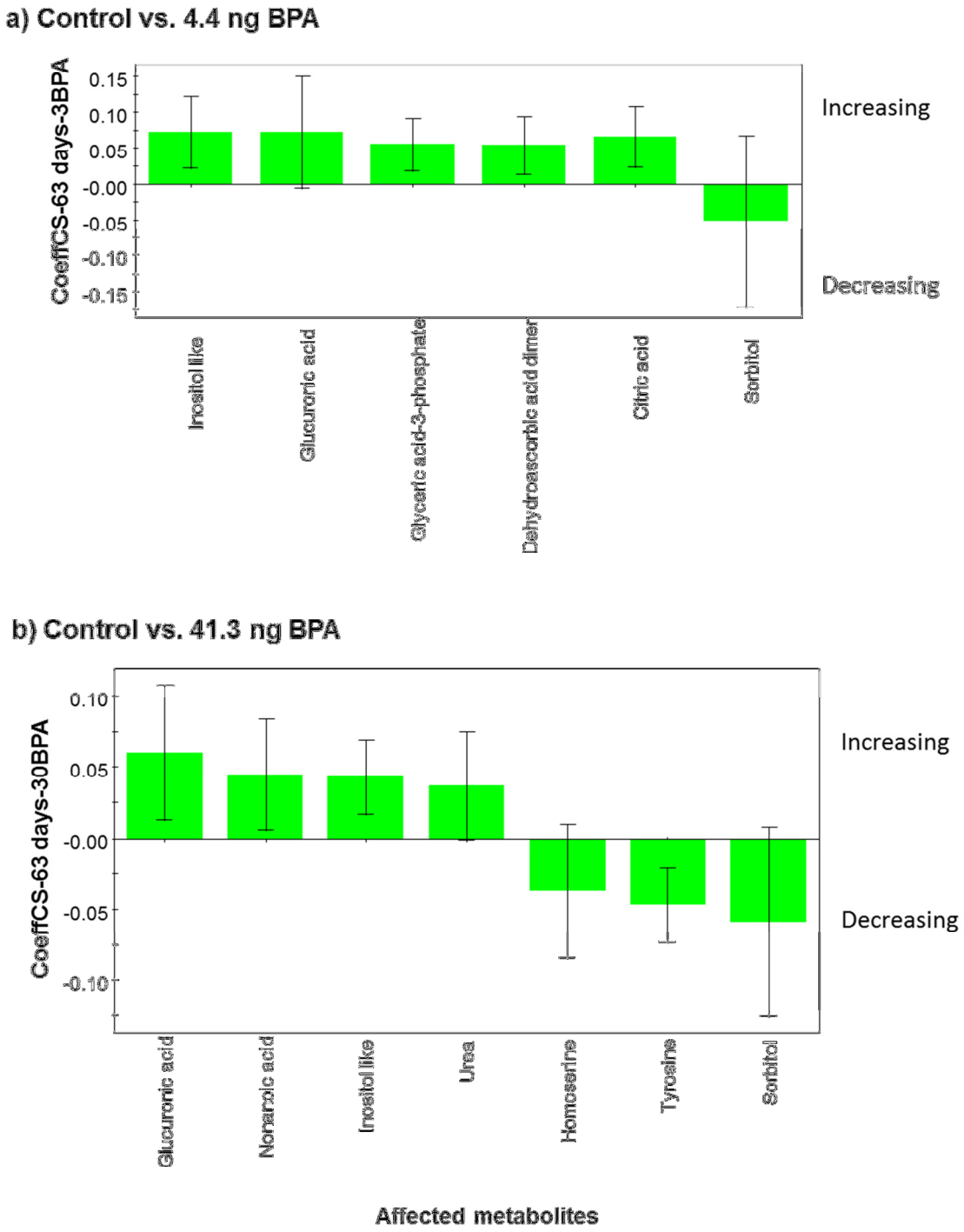

Figure A2. Coefficient plots for F1 generation at 65 dpf. Degree of change in whole body metabolite levels in (a) 4.4 and (b) 41.3 ng BPA groups when compared to controls at 65 dpf.

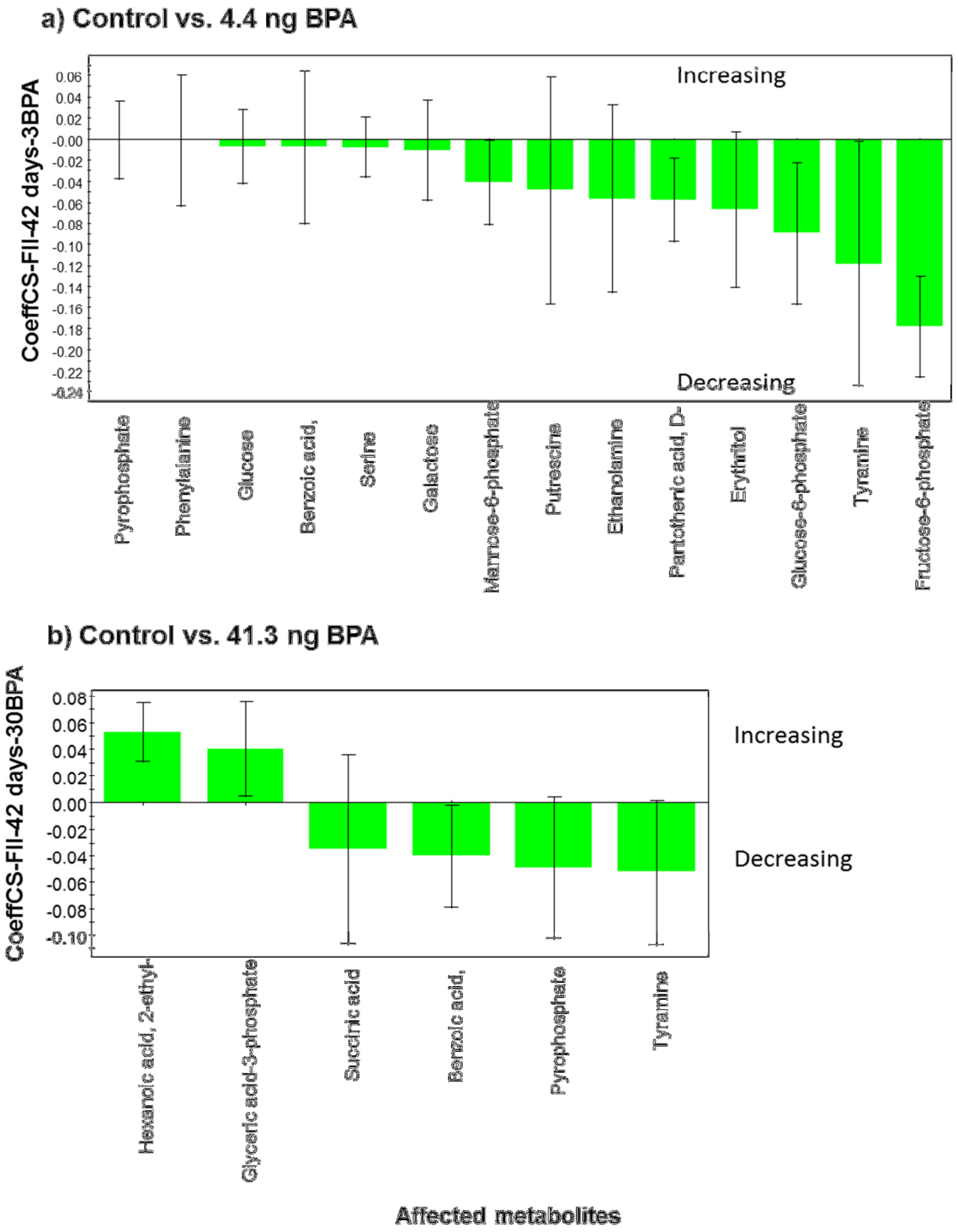

Figure A3. Coefficient plots for F2 generation at 42 dpf. Degree of change in whole body metabolite levels in (a) 4.4 and (b) 41.3 ng BPA groups when compared to controls at 42 dpf.

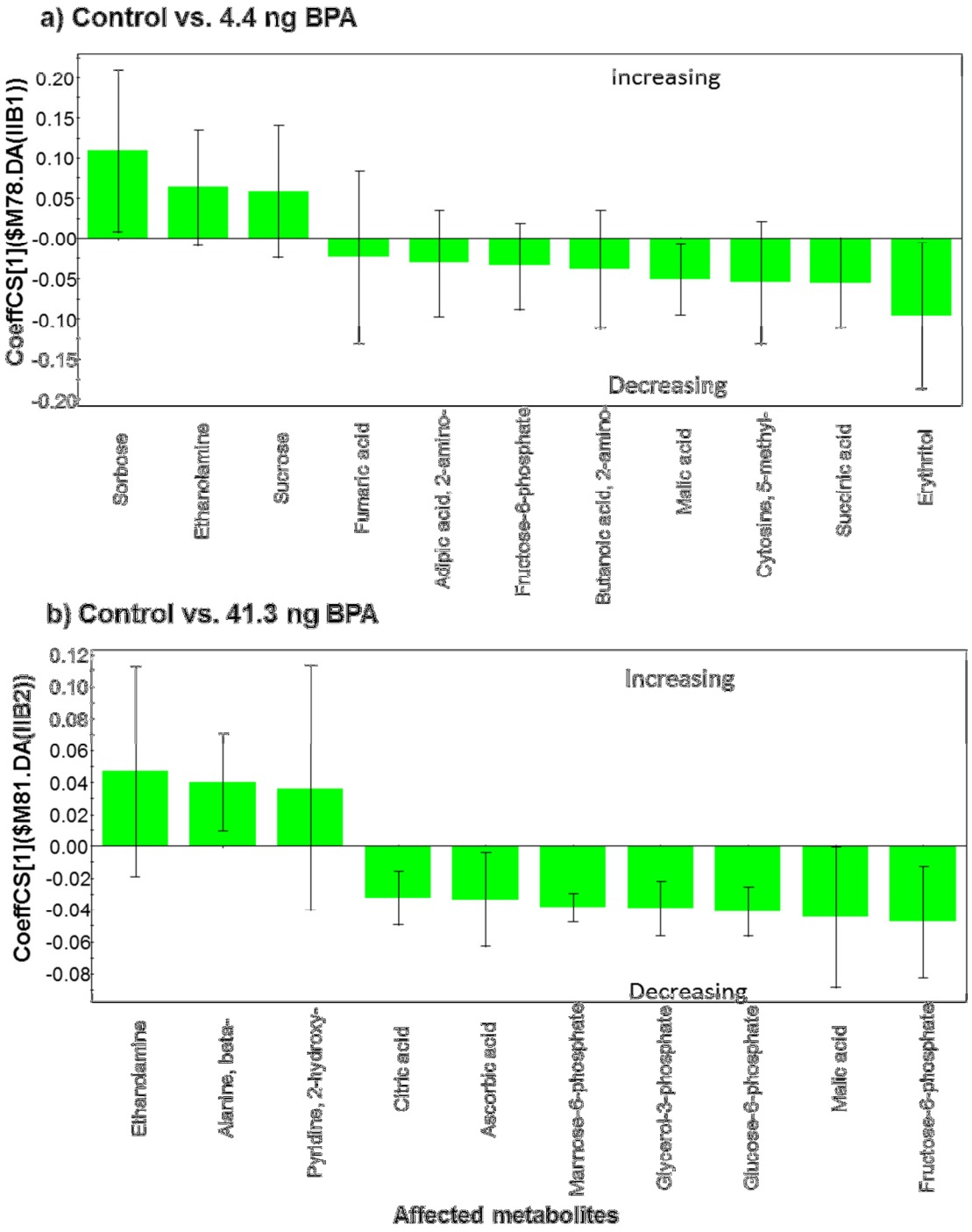

Figure A4. Coefficient plots for F2 generation at 65 dpf. Degree of change in whole body metabolite levels in (a) 4.4 and (b) 41.3 ng BPA groups when compared to controls at 65 dpf.