UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

Ammonia toxicity in zebrafish (*Danio rerio*)

Tese de Doutoramento em Ciências Químicas e Biológicas

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VILA REAL
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DECLARAÇÃO

Declaro que esta tese resulta do trabalho desenvolvido no contexto do meu doutoramento e que, até hoje, não conheço nenhum trabalho semelhante que tenha sido feito, publicado ou escrito anteriormente por outro investigador. Mais declaro que, participei na concepção e na execução de todo o trabalho experimental, cujos resultados se apresentam nesta tese, assim como, na sua análise, interpretação e na escrita dos respectivos capítulos e manuscritos. Além disso, declaro também que tanto a introdução geral como a discussão e a conclusão final foram elaboradas por mim. Para finalizar, reitero que desconheço que os resultados obtidos no decorrer deste trabalho tenham sido aceites para obter outro grau ou diploma, nesta instituição ou em outra instituição.

Esta tese é apresentada para obter o grau de Doutor pela Universidade de Trás-os-Montes e Alto Douro (UTAD; Vila Real, Portugal), tendo a sua componente prática decorrido na maioria no Laboratório de Ecofisiologia do Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR; Porto, Portugal), com exceção das experiências de microarrays (capítulos 3 e 4) que foram desenvolvidas no Departamento de Biologia da Universidade de Waterloo (Canadá), sob a supervisão do Professor Doutor Mathilakath M. Vijayan.

MARIA INÊS A. PÁSCOA

LISTA DE PUBLICAÇÕES

Esta tese encontra-se dividida em capítulos cujos manuscritos já estão ou estarão, assim que possível, submetidos em revistas científicas.

Os capítulos já submetidos são:

CAPÍTULO 2: Páscoa, I.; Fontaínhas-Fernandes, A. and Wilson, J. M. Acute ammonia toxicity in zebrafish (*Danio rerio*): comparison between early development and adults. *Submitted in* Arch Environ Con Tox

CAPÍTULO 5: Páscoa, I.; Castro, L. F. C; Fontaínhas-Fernandes, A. and Wilson, J. M. Regulation of ammonia excretion by prolactin and cortisol in zebrafish (*Danio rerio*) *Submitted in* Comp Biochem Phys A

Apesar dos restantes dois capítulos, necessitarem de trabalho adicional de bancada, nomeadamente a conclusão da validação dos microarrays por *q*PCRs, também se pretende que estes sejam publicados em revistas científicas internacionais. No entanto, e uma vez que ainda não estão concluídos, em seguida apresentam-se os co-autores e os respectivos títulos dos trabalhos:

CAPÍTULO 3: Páscoa, I.; Vijayan, M. M.; Fontaínhas-Fernandes, A., and Wilson, J. M. Transcriptomic response to environmental ammonia exposure in adult zebrafish gill, liver and brain

CAPÍTULO 4: Páscoa, I.; Vijayan, M. M.; Castro, L. F. C; Fontaínhas-Fernandes, A., and Wilson, J. M. Changes in the gene expression of zebrafish following a short-term exposure to ammonia

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RESUMO

Nos peixes, a amónia é um composto tóxico produzido durante o catabolismo dos aminoácidos mas, no meio aquático, a sua acumulação deve-se a várias fontes naturais e antropogénicas. Nos teleósteos, a amónia é produzida no fígado, sendo posteriormente eliminada pelas brânquias através do transporte transcelular realizado pelas glicoproteínas Rh, mas também através do transporte paracelular. Independentemente do mecanismo, a amónia atravessa a membrana branquial seguindo o gradiente de difusão que é mantido pela camada acídica presente junto do epitélio. Devido à sua toxicidade, a acumulação de amónia no organismo desencadeia vários efeitos negativos sendo a sua maioria no sistema nervoso central. Para evitar esses efeitos, os peixes desenvolveram mecanismos para reduzir a intoxicação da amónia e garantir a sua sobrevivência.

O peixe-zebra (*Danio rerio*) é uma espécie modelo com características particulares e cujo genoma se encontra sequenciado, sendo por isso uma espécie muito usada em investigação. Contudo, muito ainda está por se saber sobre a toxicidade e excreção da amónia nesta espécie. Posto isto, os principais objetivos desta tese são determinar 1) as concentrações letais durante o desenvolvimento do peixe-zebra, 2) as alterações no transcriptoma da brânquia, fígado e cérebro deste peixe após exposição a uma concentração sub-letal durante 96 h, 3) quais os mecanismos usados pelos adultos para evitar a intoxicação pela amónia e, finalmente, 4) qual o papel do sistema endócrino na excreção e/ou toxicidade da amónia. Assim, foram realizadas experiências de LC_{50} para todo o ciclo de vida desta espécie, sendo que nos adultos também se testou a sua tolerância perante diferentes condições ambientais. Posteriormente, realizou-se uma experiência de exposição à amónia, durante 96 h, sendo que no final, a brânquia, fígado e cérebro dos peixes foram recolhidos e analisados num 44k *Microarray* da *Agilent*. Os genes com diferenças estatísticas foram utilizados numa análise da ontologia dos genes e das via metabólicas recorrendo a ferramentas on-line, mas também numa análise detalhada da expressão de genes envolvidos em mecanismos que evitam a intoxicação da amónia. Por fim, e considerando a resposta do sistema endócrino à exposição à amónia, experiências sobre a taxa de excreção de amónia após a administração da prolactina e do cortisol foram também realizadas, tanto em condições controlo como após administração de amónia.

Os resultados mostraram que as fases do desenvolvimento embrionário do peixe-zebra são os mais tolerantes à amónia, e que os adultos, quando comparados com os de água salobra

(6 ‰) ou tamponada, apresentam menor sensibilidade do que quando expostos a água doce. Quanto à exposição dos adultos à amónia, verificou-se que estes alteraram o transcriptoma do fígado (2,169 sondas) e cérebro (669 sondas), mas principalmente da brânquia (7,001 sondas). Uma das respostas desta espécie consistiu na alteração das vias de fornecimento de energia, tendo sido os carboidratos preferencialmente usados aos lípidos. Dentro do metabolismo dos carboidratos, a via da glicólise/gluconeogénese foi usada para produzir glucose no fígado e energia na brânquia, o que permitiu a continua excreção de amónia contra gradiente principalmente através de transportadores que consomem energia e cuja expressão também se encontrou aumentada (p.ex. H^+-ATPase e Na^+/K^+-ATPase). Por outro lado, a entrada da amónia no organismo foi impedida, uma vez que se verificou um aumento da expressão dos elementos do transporte paracelular (p.ex. claudinas, ocludinas e *tight junctions*) e, consequentemente, redução da permeabilidade branquial. Para além disto, o ciclo, crescimento, proliferação e diferenciação celular também foram alterados, para reduzir a energia gasta na replicação celular e para eliminar as células danificadas pela amónia. No peixe-zebra, enquanto que o fígado recorreu à incorporação da amónia em novos aminoácidos, a brânquia reduziu a sua produção através da diminuição da expressão das enzimas envolvidas no mesmo metabolismo. Relativamente ao sistema endócrino, a hormona somatolactina e vários receptores hormonais (receptor da prolactina, da hormona do crescimento, da tiroide, da paratiroide e péptido natriurético) responderam à exposição à amónia, o que sugere que este sistema é importante para repor a homeostasia do peixe. No entanto, ao contrário do esperado, a prolactina não induziu taxas de excreção de amónia similares ou inferiores às do cortisol que, por sua vez, permitiu taxas de excreção muito baixas. De facto, e embora ambas as hormonas tenham alterado a permeabilidade da brânquia, aumentando a expressão das ocludinas, apenas o tratamento com cortisol aumentou os níveis de transportadores de amónia. A disparidade destes resultados são discutidos nesta tese.

Concluindo, apesar da amónia não causar nenhum efeito visível nos peixes, ela pode causar efeitos a nível molecular, comprometendo a geral função do organismo e levando, posteriormente, à sua morte. Neste sentido, e tal como tem sido descrito noutras espécies, o peixe-zebra desenvolveu estratégias para ultrapassar a toxicidade da amónia e inclusivé recorreu ao sistema endócrino para garantir a sua homeostase e sobrevivência. Este estudo é portanto relevante especialmente para aquaculturas e para monitorização ambiental.

Palavras chaves: amónia; peixe-zebra; ontogenia; transcriptoma; hormonas

ABSTRACT

In fishes, ammonia is a toxic compound produced endogenously through the catabolism of amino acids, but it can also be taken up from the aquatic environment coming from different natural and anthropogenic sources. In teleosts, ammonia is produced primarily in liver and its elimination occurs directly through the gills. Ammonia excretion is dominated by transcellular transport facilitated by Rh glycoproteins; however, paracellular transport elements may also contribute to its elimination. Regardless of the mechanism, ammonia crosses the branchial membranes following the blood-to-water partial pressure gradient, with the presence of an acidic boundary layer relevant for maintaining its excretion. Due to its toxicity, the internal accumulation of ammonia may cause severe negative effects in several tissues but most profoundly in the central nervous system. In an attempt to avoid these negative effects, fishes have developed mechanisms to reduce ammonia intoxication and guarantee their survival.

Zebrafish (*Danio rerio*) is a vertebrate model species whose particular features and sequenced genome, make it widely used in research; however, much remains unclear about ammonia toxicity and excretion in this species. Hereupon, the main objectives of this thesis were to determine the zebrafish 1) life history stage specific lethal concentrations of ammonia, 2) transcriptomic responses of gill, liver and brain after a 96 h sub-lethal exposure, 3) mechanisms applied by adults to avoid ammonia intoxication and, finally, 4) endocrine roles in ammonia excretion and/or toxicity. To address these goals, LC_{50} experiments were initially performed for all zebrafish life history stages and adult tolerance was also tested under different water conditions. A sub-lethal concentration was then used for an additional 96 h experiment where gill, liver and brain samples were analyzed using an Agilent 44k Zebrafish Microarray. The statistical different genes obtained in the microarray were then used to perform a gene ontology and pathway analysis using online tools, as well as in a detailed gene expression analysis involving mechanisms to avoid ammonia intoxication. In addition, endocrine responses to ammonia exposure were determined, followed by exogenous prolactin and cortisol experiments on ammonia excretion rates under control and ammonia loading conditions.

My results clearly shows that early developmental stages were the most ammonia tolerant among the entire life cycle of zebrafish and adults were less susceptible in freshwater than in 6 ‰ brackish water or Tris buffered freshwater. The 96 h exposure of adults to a sub-

lethal concentration of ammonia resulted in transcriptomic changes in liver (2,169 probes) and brain (669 probes), but most predominately in gill (7,001 probes). Zebrafish increased the energy-demanding pathways, with carbohydrates instead of lipids used as fuel to guarantee the internal homeostasis. Within carbohydrate metabolism, the glycolysis/gluconeogenesis pathway was used to produce glucose in liver, while in gill it was used to produce energy, probably to maintain the excretion of ammonia against the inward gradient, as determined by the upregulation of several branchial transporter proteins that are energy dependent (e.g. H^+-ATPase and Na^+/K^+-ATPase). In addition, branchial permeability was likely altered as indicated by changes in tight junction elements (e.g. claudins, occludins and tight junctions) to impede ammonia entrance. Moreover, changes in pathways involved in cell cycle, growth, proliferation and differentiation were observed, likely in an attempt to reduce energy demanding cell replication and to eliminate ammonia exposure damaged cells. In liver, upregulation of genes involved in the incorporation of ammonia in *de novo* amino acids were also noted, probably to reduce the ammonia intoxication. However, in gill, decreased mRNA of amino acid metabolic enzymes, indicated reduced ammonia production. Regarding the endocrine system response, somatolactin and hormone receptor mRNA levels (e.g. prolactin receptor, growth hormone receptor, thyroid receptor, parathyroid receptor and natriuretic peptide receptor) changed after ammonia exposure, which indicate an endocrine response to overcome the ammonia challenge and to regain the internal homeostasis. However, in contrast to what was expected, exogenous prolactin did not result in lower ammonia excretion rates than cortisol that, in the present study, unexpectedly induced the lowest excretion rates. In fact, although both hormones affected the expression of occludins and thus presumably branchial paracellular permeability, only cortisol enhanced ammonia transporter mRNA levels. The discrepancies in the results are discussed in the thesis.

Overall, although ammonia may not cause any overt effect on fish, it can have impacts at the molecular level, which will compromise the general functions of the organism and consequently impede it performance. However, zebrafish, similarly to other teleosts, developed strategies to overcome ammonia toxicity and mobilized the endocrine system to guarantee their homeostasis and survival. This study is then important, especially for aquaculture and environmental monitoring.

Key words: ammonia; zebrafish; ontogeny; transcriptome; hormones

INDEX OF CONTENTS

INDEX OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

INDEX OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF ABBREVIATIONS

ABBREVIATION	COMPLETE NAME
11βHSD2	11-β hydroxysteroid dehydrogenase 2
ADP	adenosine diphosphate
ALT	alanine aminotransferase
AMP	adenosine monophosphate
AMT	ammonia transporter
AQP	aquaporin
ASL	argininosuccinate lyase
ASS	argininosuccinate synthetase
AST	aspartate aminotransferase
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BBB	blood-brain barrier
CA	carbonic anhydrase
Ca^{2+}	calcium ion
CAM	cell adhesion molecule
cDNA	complementary DNA
CLN	claudin
CNS	central nervous system
CO_2	carbon dioxide
cyp26	cytochrome P450 26
DAG	diacylglycerol
DAVID	Database for Annotation, Visualization and Integrated Discovery
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide
EF1α	elongation factor 1 alpha
F	cortisol
FBP	fructose 1,6-biphosphatase
FC	fold change
GADD45α	growth arrest and DNA damage inducible alpha
GDH	glutamate dehydrogenase
GH	growth hormone
GHR	growth hormone receptor
GLN	glutamine
GLU	glutamate
GLUT	glucose transporter
GO	gene ontology
GR	glucocorticoid receptor
GS	glutamine synthetase
GTP	guanosine triphosphate
H^+	proton ion
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
HE	hepatic encephalopathy
HNO_3	nitric acid
HO^-	hydroxyl radicals
hpf	hours post-fertilization

IP	intraperitoneally (injection)
IP_3	inositol trisphosphate
J_{Amm}	ammonia excretion rates
K^+	potassium ion
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC_{50}	median-lethal concentration for 50 % of the population
LDH	lactate dehydrogenase
$MgCl_2$	magnesium chloride
MK-801	dizocilpine
MPT	mitochondrial permeability transition
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MSO	methionine sulfoximine
Na^+	sodium ion
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NH_3	unionized ammonia
NH_4^+	ammonia ion or ionized ammonia or ammonium
NH_4Cl	ammonium cloride
NH_4HCO_3	ammonium bicarbonate
NHE	Na^+/H^+ exchanger
NKCC	$Na^+:K^+:2Cl^-$ cotransporter
NMDA	N-methyl-D-aspartate
NNA	nitroarginine
NO	nitric oxide
NOS	nitric oxide synthase
NPR	natriuretic peptide receptor
O_2^-	superoxide radical
OCLN	occludin
$ONOO^-$	peroxynitrite ion
oPRL	ovine prolactin
OUC	ornithine urea cycle
p21	cyclin-dependent kinase inhibitor 1
p53	tumor protein p53
PCA	perchloric acid
PCR	polymerase chain reaction
PFK	phosphofructokinase
pK	dissociation constant
PPAR	peroxisome proliferator-activated receptors
PRL	prolactin
PRLR	prolactin receptor
PTHR	parathyroid hormone receptor
***q*PCR**	real-time polymerase chain reaction
Rhag	rhesus glycoprotein a
Rhbg	rhesus glycoprotein b
Rhcg	rhesus glycoprotein c
RNA	ribonucleic acid
ROS	reactive oxygen species

SEM	standard error of the mean
SL	somatolactin
TA-N	total ammonia-nitrogen
TBE	tris-borate-EDTA
TCA	tricarboxylic acid cycle
TER	transepithelial resistance
THR	thyroid hormone receptor
TJ	tight junction
TRIS	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
UIA	unionized ammonia
UO	urate oxidase
UT	urea transporter
αKG	α-ketoglutarate
ΔP_{NH3}	partial pressure gradient of NH_3

CHAPTER 1 |

AMMONIA: A TOXIC COMPOUND PRESENT INSIDE AND OUTSIDE FRESHWATER FISHES

1.1 Abstract

Ammonia is a toxic compound present in the aquatic ecosystems due to natural and anthropogenic factors. Although the environmental concentration and the physicochemical parameters of the water determine the toxicity of ammonia, several factors related with the organism may also contribute for its susceptibility. Inside the organism, ammonia is mainly produced in the liver during the catabolism of amino acids and proteins. However, due to its toxicity, ammonia has to be eliminated from the body, which is done mainly through the gills by simple diffusion of NH_3 or through transporters proteins (especially by Rhesus glycoproteins). Regardless of the excretory mechanism, the presence of an acidic boundary layer close to the gill epithelium is important to maintain the continuous movement of ammonia out of the body. In this sense, a disruption between ammonia production and excretion, or even an uptake of ammonia from the environment, can lead to the accumulation of ammonia inside the organism, which can cause several negative effects in the different organs and tissues but most profoundly in the central nervous system. In this sense, and to avoid the autointoxication of ammonia, aquatic organisms developed several strategies such as the conversion of ammonia to less toxic

compounds (such as urea and glutamine), decrease of ammonia production (reducing the amino acid catabolism) and actively excretion of ammonia (enhancing the mRNA and/or activity of the transporter proteins) even against inward gradients. Hereupon, this chapter reviews the current state of knowledge related with this nitrogenous waste compound, although much it is yet to be known.

1.2 Introduction

Several anthropogenic and natural sources contribute to an increase of ammonia in the water and together, with the physicochemical parameters of the aquatic environment, determine the toxicity of ammonia for the organisms. In freshwater environments, ammonia is inputted by animal excretion, nitrogen fixation by plants, decomposition of organic matter and sewage by microorganisms, industrial processes and emissions, volcanic activity, urban and agricultural run-off and forest fires (Hargreaves, 1998; Randall and Tsui, 2002; Eddy, 2005; USEPA, 2013). In solution, ammonia is present as ionized (NH_4^+) and unionized (NH_3 or UIA) forms and the sum of both is designated as total ammonia-nitrogen (TA-N = NH_4^+ + NH_3) or simply as ammonia. The equilibrium reaction of these two forms is written as NH_3 + $H_3O^+ \Leftrightarrow NH_4^+ + H_2O$, with a dissociation constant (pK) around 9.5 (Wright, 1995; Randall and Tsui, 2002; Ip and Chew, 2010). The NH_3/NH_4^+ equilibrium is highly dependent of pH and, to a lesser degree, on temperature, pressure, and ionic strength. In freshwater, an increase in pH or temperature causes an increase in the environmental NH_3 (the most environmentally toxic form), which will decrease the tolerance of the aquatic organisms. As environmental pH and temperature, salinity is also inversely correlated with ammonia tolerance and, in general, marine species are slightly less tolerant to ammonia than freshwater fishes, which has been associated with the higher branchial ammonia (ion) permeability in seawater species (USEPA, 1999, 2013; Randall and Tsui, 2002; Eddy, 2005).

Although the water physicochemical parameters influence the toxicity of ammonia, several factors related with the organism may also influence the ammonia sensitivity. The tolerance to this nitrogenous waste compound is also dependent on the species, size, age and health condition of the aquatic organism (USEPA, 1999; Randall and Tsui, 2002; Kumar et al., 2007; USEPA, 2013). Furthermore, feeding, swimming and stress increase the level of internal ammonia, which further influence the tolerance of the fish to this toxic compound. Following exercise, the levels of ammonia increase in the white muscle and blood, leading to a decrease in ammonia tolerance from resting to swimming fish. On the other hand, when environmental ammonia is high, fed fish are less sensitive to ammonia than unfed fish

because during feeding, the muscle enzyme glutamine synthetase (GS) is activated to protect the fish from ammonia toxicity, converting ammonia to glutamine (GLN). Stress, in turn, increases the levels of cortisol in fish, which activates glycogenolysis, gluconeogenesis and protein catabolism and consequently raises ammonia production and its release into the blood stream. In this sense, stressed fish are less tolerant to environmental ammonia than unstressed fish. However, this correlation can be overturned if fish are subjected to repeated stresses, given that cortisol also activates the GS pathway and, in this situation, fish become more tolerant to high environmental ammonia in the post stress period (Randall and Tsui, 2002).

The toxicity of ammonia to aquatic organisms occurs because, in addition to the environmental ammonia that may enter the body, this toxic compound can also be produced inside the organism, which causes its accumulation and leads to severe negative effects. Within the organism, ammonia is produced primarily in the liver and, after its release into the blood stream (Ip and Chew, 2010), the gills are the organs responsible for ammonia elimination to the environment (Evans et al., 2005; Wright and Wood, 2009; Ip and Chew, 2010). A rapid increase in ammonia production, a decrease in ammonia excretion and/or uptake from the environment leads to a build-up of ammonia inside the body (Randall and Tsui, 2002; Wright and Wood, 2009; Ip and Chew, 2010), which will cause several deleterious effects in different organs, although its acute toxicity is more directed to the central nervous system (CNS) (Randall and Tsui, 2002). Even if the scientific community has long been interested in ammonia, most of the mechanisms and pathways activated by ammonia exposure are not yet well understood. The following sections present the state of the art regarding ammonia production, excretion and toxic effects inside a freshwater organism but also presents the most common mechanisms used by fish to deal with toxic ammonia.

1.3 Ammonia production inside the organism

In contrast to lipids and carbohydrates that can be stored in the body as glycogen and triglycerides, respectively (Ip and Chew, 2010), amino acids have no direct storage form and have to be catabolized, resulting in a release of nitrogenous wastes products. In fact, amino acids are first used for growth and maintenance of the protein turnover (Wright, 1995; Ip and Chew, 2010) but the excess has to be converted into carbohydrates and lipids to be stored. Hereupon, many amino acids are converted to glucose in the hepatocytes, a process that implies hormonal regulation in fish as in mammals (Ip and Chew, 2010). The amino acids used by the body are mostly dietary (Dosdat et al., 1996) but other important sources are the

proteins present in the muscle tissues (Ip and Chew, 2010). In the red muscle, most processes are aerobic and the major amino acid required for intensive work is leucine. On the other hand, in white muscle, anaerobic processes occur with glucose (stored as glycogen) being the most prominent energy source. Furthermore, although white muscle is an important reservoir of amino acids during periods of starvation or fish migration (Smutná et al., 2002), in general, the proteins in the muscle are primarily needed to produce energy for muscle contraction. However, it is during the catabolism of amino acids, specifically from the α-amino group of the amino acids that ammonia is produced and released (Ip and Chew, 2010).

Inside the organism, the catabolism of proteins, and consequently the ammonia production, occurs mainly in the liver (Wright, 1995; Ip and Chew, 2010) through reactions that take place in the cytosol and mitochondrial matrix (Ip and Chew, 2010). However, besides liver, other organs may contribute for ammonia production and release into the blood stream (Smutná et al., 2002; Wilkie, 2002; Karlsson et al., 2006; Tng et al., 2008; Ip and Chew, 2010). This is the case of, for example, the kidneys that may participate in the ammonia production (Wright, 1995), mostly through the same pathways as in liver (Smutná et al., 2002). As well, the intestine may also support the postprandial amino acid metabolism and produce some ammonia after a meal (Karlsson et al., 2006; Tng et al., 2008; Ip and Chew, 2010). In fact, in organs as liver and intestine, glutamate (GLU) and/or GLN are produced after a feeding and constitute the primary source of energy, especially for intestinal epithelial cell proliferation, somatic growth, protein synthesis and transport of nutrients, ions and water (Smutná et al., 2002; Tng et al., 2008; Ip and Chew, 2010). On the other hand, inside the blood, the circulating erythrocytes may transport amino acids like tyrosine, phenylalanine, tryptophan, histidine, isoleucine and leucine. All these essential amino acids can easily permeate the membranes of the erythrocytes, being able to move in both ways. In fact, enzymes related with the metabolism of amino acids have been detected in the erythrocytes of rainbow trout *Oncorhynchus mykiss* (Ferguson and Storey, 1991). Nevertheless, it is in the liver that most of the ammonia is produced primarily by the direct deamination of amino acids, which occurs in the mitochondrial matrix and in the cytosol of hepatocytes by specific deaminases (histidase, asparaginase, serine dehydratase and threonine dehydratase) or even by the combined action of both cytosolic aminotransferase [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] and mitochondrial glutamate dehydrogenase (GDH) (reactions together known as transdeamination). In fish liver, transdeamination is the main mechanism used to catabolize amino acids, and the enzyme GDH, is exclusively localized in

the matrix of liver mitochondria (Campbell, 1997; Ip and Chew, 2010) (Figure 1.1). This enzyme is also very important for energy metabolism since GDH deaminates GLU to NH_4^+ and α-ketoglutarate (αKG) (Wright, 1995; Randall and Tsui, 2002), with the latter being incorporated into the tricarboxylic acid cycle (TCA) (Figure 1.1), producing nine molecules of ATP (Smutná et al., 2002). The enzyme GDH is stimulated by ADP, AMP and leucine but is inhibited by ATP and GTP. Furthermore, other amino acids present in the mitochondrial matrix, such as GLN, can be directly deaminated by glutaminase, also releasing ammonia (Ip and Chew, 2010) (Figure 1.1).

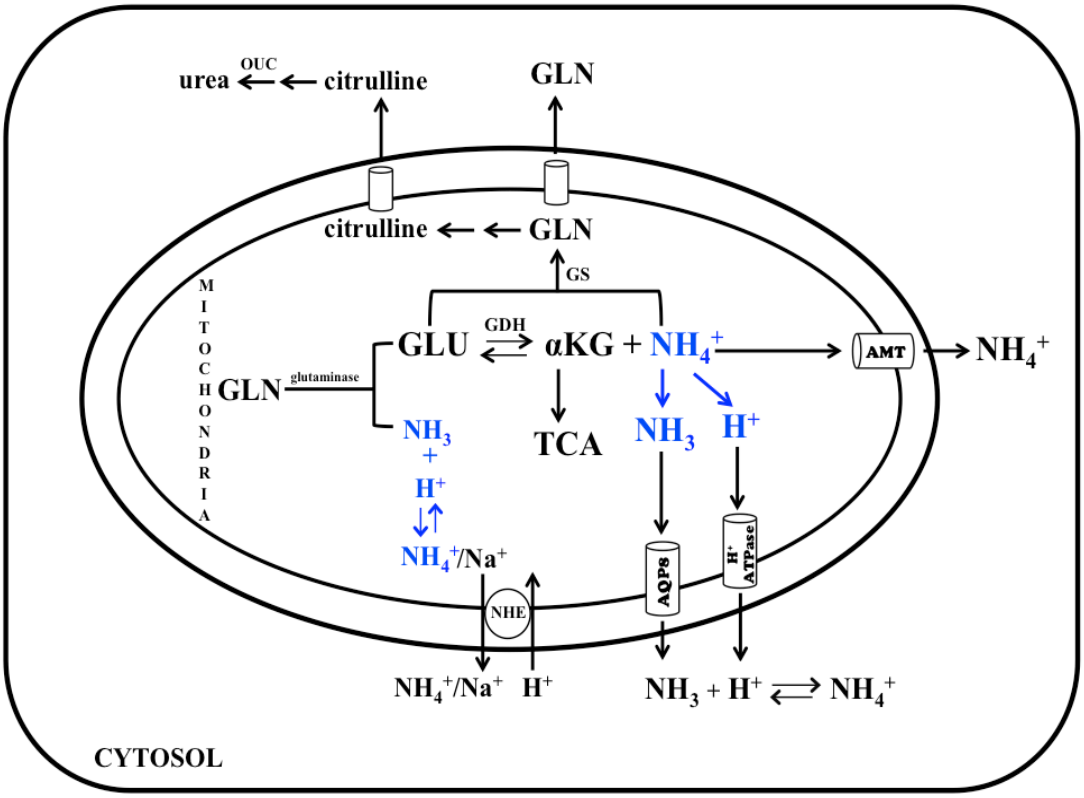


Figure 1.1 Catabolism of amino acids in the hepatocytes. This pathway conducts to the production and release of ammonia ion (NH_4^+) and α-ketoglutarate (αKG) from glutamate (GLU) by the enzyme glutamate dehydrogenase (GDH). After production, ammonia exits the mitochondria matrix by ammonia transporters (AMT) or through Na^+/H^+ exchanger (NHE). Moreover, NH_4^+ may also exit this organelle passing through aquaporins namely aquaporin 8 (AQP8) as unionized ammonia (NH_3), with H^+ crossing through H^+-ATPase, which create a differential pH between the mitochondrial matrix and the inter-membrane region that facilitate the continuous elimination of ammonia. Besides GDH, the enzyme glutamine synthetase (GS) may also detoxify ammonia incorporating this nitrogenous ion into glutamine (GLN). This conversion may be important since directly or by it after conversion to citrulline, may lead to the production of urea through ornithine-urea cycle (OUC); however, both GLN and citrulline products have to firstly pass the mitochondrial membranes to be converted to urea in the cytosol (image done based on Ip and Chew, 2010).

Before being excreted from the body, ammonia must first permeate the liver mitochondrial membranes, a process not yet fully understood. One possible mechanism is the

presence of pH differences between the matrix (alkaline) and the inter-membrane region (acidic) due to the oxidative phosphorylation, which drive the movement of ammonia across the inner mitochondrial membrane through a diffusion gradient (Figure 1.1). However, the movement of ammonia through mitochondrial membranes as NH_4^+ and not as NH_3 may uncouple the oxidative phosphorylation and cause negative effects during its exit from the mitochondrial matrix. In this sense, one other possibility is the existence of transporters, like the Na^+/H^+ exchanger (NHE) (Ip and Chew, 2010), in the inner mitochondrial membrane responsible for ammonia transport without destroying the proton (H^+) gradient established by the electrogenic H^+ pump (Campbell, 1997; Ip and Chew, 2010) (Figure 1.1). On the other hand, in mammals, aquaporin (AQP), specifically AQP8, is present in the inner mitochondrial membrane, facilitating the transport of some neutral NH_3 out of liver mitochondria (Ip and Chew, 2010; Soria et al., 2010) (Figure 1.1). However, no evidence supports that this mechanism is operative in the liver mitochondria of ammonotelic fishes (Ip and Chew, 2010). Furthermore, another mechanism that allows the movement of ammonia through the mitochondrial membranes, without disruption of the H^+ gradient, is the conversion of ammonia into citrulline or GLN, both non-toxic, proton-neutral nitrogenous molecules, before exiting the mitochondria (Campbell, 1997; Ip and Chew, 2010) (Figure 1.1). The production of GLN inside the liver mitochondria implicates the presence of GS in the mitochondrial matrix; however, this conversion can also be important since after crossing the mitochondrial membranes, GLN may serve as a precursor for several biosynthetic pathways that take place in the cytosol (Figure 1.1) (Ip and Chew, 2010).

1.4 Excretion of ammonia through the gills

After production and to avoid toxic effects, ammonia has to be eliminated from the body, which is done mainly through the gills (Wright and Wood, 2009; Ip and Chew, 2010) as ammonia and/or urea. Liver releases the nitrogenous compounds into the blood predominantly as ammonia (Smutná et al., 2002); however, a small fraction is converted to urea through uricolysis and/or arginolysis in the majority of teleosts. Nonetheless, in ammonotelic fishes, the excretion of toxic waste compounds occurs mainly (more than 50 %) as ammonia (Wilkie, 2002; Smutná et al., 2002; Weihrauch et al., 2009; Wright and Wood, 2009; Ip and Chew, 2010). Although organs, such as kidney and skin, may also excrete small amounts of ammonia and urea, the gills present features like high surface area, perfusion by 100 % of cardiac output, ample ventilation rates, reduced diffusion distances, direct contact with water

and respiratory medium, that makes it the primary organ responsible for ammonia excretion (Evans et al., 2005; Ip and Chew, 2010). Moreover, gills are also important for gas exchange and, partially, for NH_3 detoxification. However, it should be noted that, although some *de novo* ammonia can be produced within the gills, most of the excreted ammonia comes from the blood originating from the different parts of the body (Evans et al., 2005).

Based on all data described in the literature during the last decades, Wright and Wood (2009) presented the latest model to explain the movement of ammonia through the gill epithelia in freshwater fish (see Figure 1.2). In this model, known as "Na^+/NH_4^+ exchange complex", NH_3 passes through a member of the ammonia transporter family, the Rhesus (Rh) glycoprotein A (Rhag), out of the erythrocytes and crosses the basolateral membrane of the branchial epithelium through other member of the same family, the Rh glycoprotein B (Rhbg). At the apical membrane of the branchial epithelia, this model admits the existence of several transmembrane transporters [Rh glycoprotein C (Rhcg), V-type H^+-ATPase, NHE and Na^+ channel] that work together to move ammonia across the gill. In detail, the movement of ammonia through Rhcg is done as NH_3, which means that at the intracellular binding site, the NH_4^+ losses the H^+ that, in turn, passes the apical membrane through the V-type H^+-ATPase and/or NHE, with the latter providing the uptake of Na^+. Furthermore, other components of the membranes are considered to have an indirect role in ammonia transport such as, intracellular carbonic anhydrase (CA), basolateral Na^+:HCO_3^- cotransporter and Na^+/K^+-ATPase. Considering this model, ammonia excretion follows the blood-to-water partial pressure gradient (ΔP_{NH3}), which simultaneously drives the influx of Na^+ (Wright and Wood, 2009) (Figure 1.2).

Although this recent model is very important to understand the movement of ammonia through the branchial membranes, it was long known that the movement of this nitrogenous waste compound occurs mostly through facilitated transport proteins (Weihrauch et al., 2009; Wright and Wood, 2009; Ip and Chew, 2010). The NH_4^+ is unable to easily cross the cell membranes therefore its transport is almost obligatory through specialized transporters (Wright, 1995). Since NH_4^+ has the same hydrated ionic radius than K^+, it was suggested that ionized ammonia may displace K^+ in some ion transporter proteins, such as Na^+/K^+-ATPase, Na^+:K^+:$2Cl^-$ cotransporter (NKCC) and K^+ specific channels or pores, to pass the branchial epithelium (Wright, 1995; Wilkie, 1997; Ip et al., 2004a; Ip and Chew, 2010). Furthermore, NH_4^+ might also substitute H^+ in its transport via NHE (Ip et al., 2004a; Ip and Chew, 2010). On the other hand, ammonia ion can also unconventionally pass the cell membranes through

some members of the water and glycerol transporter family known as AQP (Ip and Chew, 2010). However, members of Rh glycoprotein family have also been studied during the last years, being found that they are the most important transporters in ammonia elimination out of the body (Hung et al., 2007, 2008; Braun et al., 2009a, 2009b; Weihrauch et al., 2009; Wright and Wood, 2009; Ip and Chew, 2010). Nakada and collaborators (2007a) were the first to identify members of the Rh glycoprotein family in the gill of the Japanese pufferfish *Takifugu rubripes* and to demonstrate that these proteins were involved in ammonia excretion through the gills. After this finding, the Rh glycoproteins were identified in rainbow trout *O. mykiss* (Nawata et al., 2007; Hung et al., 2008; Nawata and Wood, 2008), mangrove killifish *Kryptolebias marmoratus* (Hung et al., 2007) and zebrafish *Danio rerio* (Braun et al., 2009a, 2009b). In fact, this family comprises several members (Nawata et al., 2007; Hung et al., 2007, 2008; Braun et al., 2009a, 2009b; Wright and Wood, 2009) that exhibit different locations inside the body (Nawata et al., 2007; Nawata and Wood, 2008; Braun et al., 2009b; Wright and Wood, 2009) and inside the cell (Hung et al., 2007; Braun et al., 2009b; Wright and Wood, 2009; Ip and Chew, 2010). In general, and although it was recently described in gill (Braun et al., 2009b; Wright and Wood, 2009), it is considered that Rhag is mainly restricted to the erythrocytes (Nawata et al., 2007; Wright and Wood, 2009); however, the Rhbg and Rhcg are widely expressed in tissues (Nawata et al., 2007; Nawata and Wood, 2008; Braun et al., 2009b; Wright and Wood, 2009). Moreover, these studies highlighted that, in the branchial epithelium cells, the Rhcg is usually localized in the apical membrane whereas the Rhbg is positioned in the basolateral membrane (Hung et al., 2007; Braun et al., 2009b; Wright and Wood, 2009; Ip and Chew, 2010) (Figure 1.2), similarly to what was described in the recent model of Wright and Wood (2009). These particular locations of the different Rh glycoproteins allow the movement of ammonia from blood-to-water, passing from red blood cells to endothelial cells via Rhag and through the epithelium via Rhbg and Rhcg1/Rhcg2a (Wright and Wood, 2009). In fact, several internal and/or external factors may affect the expression of these ammonia transporters, such as, the presence of high ammonia levels in the environment or in the body, aerial exposure and elevated environmental pH, which will increase the expression of Rh glycoproteins (Hung et al., 2007; Nawata and Wood, 2008, 2009; Tsui et al., 2009; Wright and Wood, 2009; Ip and Chew, 2010). Moreover, recent studies also provided evidences that Rh glycoproteins also transport CO_2 or function as CO_2/NH_3 channel (Huang and Peng, 2005; Peng and Huang, 2006; Wright and Wood, 2009; Nawata et al., 2010a; Perry et al., 2010).

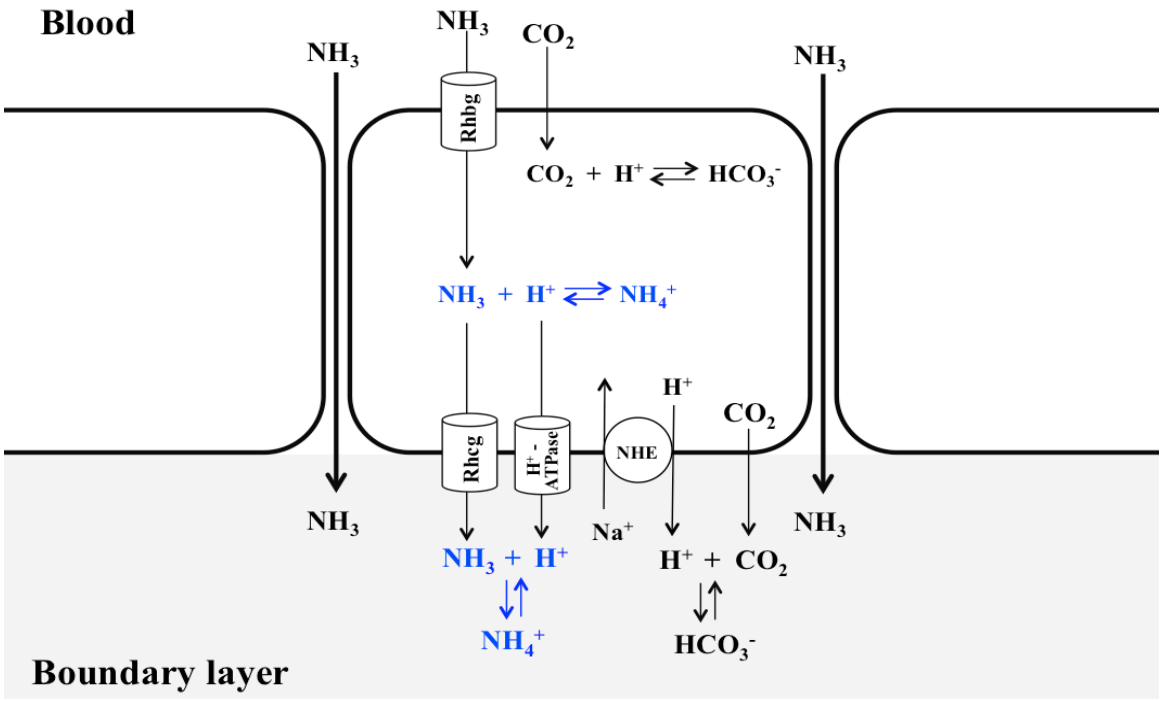


Figure 1.2 Illustration of ammonia movement across the branchial epithelium of freshwater fish based on Wright and Wood (2009) model. Known as "Na^+/NH_4^+ exchange complex", this model consider that Rhesus (Rh) glycoprotein B (Rhbg) is localized in the basolateral membrane allowing the entrance of NH_3 in the branchial epithelium cells being the Rh glycoprotein C (Rhcg), localized apically, responsible for its excretion out of the body. In the boundary layer, NH_3 will then trap a H^+ forming NH_4^+, which guarantee the continuous elimination of ammonia following the blood-to-water diffusion gradient. The H^+ present in the boundary layer results from the hydration of CO_2 and/or H^+ elimination through transporters like V-type H^+-ATPase and Na^+/H^+ exchanger (NHE).

Besides the movement of ammonia through transcellular pathways, this nitrogenous waste compound may also cross the gills through paracellular pathways as NH_3 following its diffusion gradient (Wilkie, 1997, 2002; Evans et al., 2005; Weihrauch et al., 2009; Ip and Chew, 2010; Nawata et al., 2010b). In contrast with the charged NH_4^+ that exhibits low permeation through the phospholipidic membranes, the gaseous NH_3 is a small lipid-soluble uncharged molecule able to cross the cell membranes with relative ease (Wright, 1995; Ip and Chew, 2010). This paracellular movement of NH_3 through the branchial epithelium occurs following the blood-to-water ΔP_{NH3}, which is maintained by the presence of an acidic boundary layer, that allows the excreted NH_3 to be converted into NH_4^+ upon protonation (Wright, 1995; Salama et al., 1999; Weihrauch et al., 2009; Wright and Wood, 2009; Ip and Chew, 2010; Nawata et al., 2010b). The unstirred acidic boundary layer exists close to the

apical surface of the gill epithelium and its acidic pH is partially supported by CO_2 and H^+ excretion (Wilkie, 2002; Wright and Wood, 2009; Ip and Chew, 2010). The CO_2 is a weak acid and since its hydration has a slow half time, the presence of CA on the apical surface and external mucus layers of the gill seems to be relevant for CO_2 hydration and ammonia excretion (Wright et al., 1989; Wilkie, 1997; Wright and Wood, 2009). Furthermore, the acidification of gill boundary layer can also be due to the excretion of H^+ that occurs through the apical V-type H^+-ATPase (Lin et al., 1994; Sullivan et al., 1995; Wright and Wood, 2009; Ip and Chew, 2010) and apical NHE (Ivanis et al., 2008; Wright and Wood, 2009) (Figure 1.2). Regardless the mechanism of acidification, the presence of boundary layer is not obligatory but facilitates the diffusion of NH_3 through CO_2 hydration and H^+ excretion. However, the addition of buffer to the water impairs NH_3 excretion since it destroys the acidic boundary layer (Rahaman-Noronha et al., 1996; Wright et al., 1989; Wilson et al., 1994). Relatively to the charged NH_4^+, the paracellular movement is mostly related with both the concentration and the electrical potential gradient (Wright, 1995; Evans et al., 2005); however, the diffusion of ammonia ion through the paracellular pathway is more relevant in marine fishes (Ip and Chew, 2010).

1.5 When ammonia accumulates inside the body, what happens?

Many conditions may lead to the accumulation of ammonia inside the organism, which can cause negative effects in internal organs, especially in the CNS, liver and gill. Most of the toxic ammonia is present in the body as NH_4^+ (Ip and Chew, 2010) and various endogenous and/or exogenous factors may disrupt the balance between production and excretion of this nitrogenous waste compound (Wilson et al., 1994; Randall and Tsui, 2002; Nawata et al., 2007). This situation may consequently cause an increase of ammonia in blood and tissues, leading to a general ammonia autointoxication (Wright and Wood, 2009). Moreover, when the levels of NH_3 in the water are high (Nawata et al., 2007; Wright and Wood, 2009), due to natural and/or anthropogenic sources (Randall and Tsui, 2002), or even due to high environmental pH (Ip and Chew, 2010), the ΔP_{NH3} is reduced or reversed, which decreases ammonia excretion or causes an uptake of ammonia from the environment, being accumulated in the organism (Randall and Tsui, 2002; Nawata et al., 2007; Wright and Wood, 2009; Ip and Chew, 2010). Elevated concentrations of ammonia inside the body may then cause several negative effects in different tissues (Dhanasiri et al., 2013), being especially severe in the CNS (Randall and Tsui, 2002; Wilkie, 2002; Ip and Chew, 2010). It is already

known that several key metabolic pathways take place in the liver, which makes this organ very vulnerable to intoxication (Benli et al., 2008; Dhanasiri et al., 2013). The presence of high ammonia levels inside the organism causes a decrease in liver weight (Milne et al., 2000; Spencer et al., 2008) due to the use of the energetic compound glycogen (Vijayan et al., 1997; Milne et al., 2000) to maintain ionoregulation, equilibrium and respiration (Spencer et al., 2008). Moreover, some studies described liver damage as a response to high environmental ammonia, such as cloudy swelling and hydropic degeneration (Benli et al., 2008). On the other hand, in gill, despite some inconsistencies, structural damage has been described during ammonia exposure. The observed changes include edema, lamellar fusion, epithelial cell hypertrophy and hyperplasia, telangiectasis on lamella and hyperemia on branchial epithelium (Lease et al., 2003; Benli and Köksal, 2005; Benli et al., 2008; Wright and Wood, 2012), which will cause an increase in lamellar thickness, increase in diffusion distances and decrease in lamellar surface area (Lease et al., 2003) that, consequently, impair oxygen uptake and ionoregulation (Wright and Wood, 2012). The structural damage can be so evident that Lease and collaborators (2003) suggested that gill structure should be considered the most sensitive indicator of ammonia toxicity. Furthermore, at the cell membrane, the presence of elevated internal ammonia concentrations may alter the ionic balance decreasing Na^+ influx (Ip and Chew, 2010) and K^+ efflux since NH_4^+ may substitute K^+ in Na^+/K^+-ATPase and/or NKCC (Wilkie, 2002; Ip and Chew, 2010).

Although ammonia causes all these deleterious effects in liver and gill, most of the toxic effects occur at the CNS where ammonia is a potent neurotoxin (Albrecht, 1998). To better understand the deleterious effects of ammonia in the brain the most updated knowledge of the mechanism activated by ammonia as well as its negative effects on brain, will be detailed separately.

Beyond the effects described, the presence of high levels of ammonia inside the body leads to several negative effects in other cells, tissues and organs, which may, in extreme cases, cause death. In fact, during the last years, several studies described the deleterious effects of ammonia at different biological and physiological levels such as decrease of fish growth (Rasmussen and Korsgaard, 1996; Milne et al., 2000; Björnsson and Ólafsdóttir, 2006; Foss et al., 2007), reduce feeding (Rodrigues et al., 2007), interference on energy metabolism through stimulation of glycolysis in the cytosol and impairment of the TCA cycle in the mitochondria (Tomasso, 1994; Ip and Chew, 2010), alteration of carbohydrate and fatty acid metabolism (Dhanasiri et al., 2013), increase of oxygen consumption rate (Adams et al.,

2001; Barbieri and Doi, 2012) and ventilation frequency (Adams et al., 2001; Benli and Köksal, 2005), affecting swimming performance (Rodrigues et al., 2007), disturbances of the electrochemical gradient, inhibition of the immune system (Connon et al., 2011; Gonçalves et al., 2012; Dhanasiri et al., 2013), change in behavior (Benli and Köksal, 2005; Spencer et al., 2008) and interference in production, chemistry and structure of blood (Abbas, 2006). In fact, all these toxic effects can culmitate in loss of physical equilibrium, convulsions, coma and eventually death (Randall and Tsui, 2002; Benli and Köksal, 2005).

1.6 Mechanisms and effects of toxic ammonia in the central nervous system

In recent years, a number of studies have been published on the effects of ammonia in the brain; however, most of these studies are in mammals and not in aquatic organisms (Brusilow, 2002; Felipo and Butterworth, 2002; Ip and Chew, 2010). In mammals, defects in urea cycle enzymes or transporters or even liver injury, hepatic failure or liver bypass, provoke a decrease in the hepatic capacity to remove diet-derived ammonia, resulting in hyperammonemia and consequently the development of several and complex neuropsychiatric disorders named, in general, as hepatic encephalopathy (HE) (Felipo and Butterworth, 2002; Häussinger and Schliess, 2008; Lemberg and Fernández, 2009; Ip and Chew, 2010; Braissant et al., 2013). Since the mechanisms of acute ammonia neurotoxicity are similar between mammals and fish, aquatic organisms have been used to study complementary treatments to protect the brain from this nitrogenous compound (Feldman et al., 2014).

In the CNS, the conversion of ammonia to GLU and/or GLN is important to control its toxicity; however, the levels of GLN cannot exceed a physiological threshold, since it causes astrocyte and brain swelling. Under normal conditions, ammonia enters the brain by diffusing from the blood (Albrecht and Dolińska, 2001; Felipo and Butterworth, 2002); however, it does not accumulate because GDH produces GLU from αKG and NH_4^+ and GS produces GLN from NH_4^+ and GLU (Randall and Tsui, 2002; Walsh et al., 2003; Norenberg et al., 2007; Ip and Chew, 2010; Braissant et al., 2013), allowing the permanence of ammonia in the CNS but in less toxic forms. On the other hand, glutaminase, which catalyzes the reverse GLN to NH_4^+ and GLU reaction, has also a relevant role in the GLU-GLN cycle localized in the nerve endings of glutamatergic-neurons. In this sense, GDH and GS protect the brain from ammonia toxicity and their activities increase when fish are exposed to environmental ammonia, which leads to an increase in cerebral GLU and GLN content, respectively (Levi et

al., 1974; Arillo et al., 1981; Dabrowska and Wlasow, 1986; Peng et al., 1998; Randall and Tsui, 2002; Veauvy et al., 2005; Wright et al., 2007). In the brain, GS is located in the astrocyte cytosol and usually this enzyme presents a higher activity than GDH (Wright et al., 2007); however, both enzymes allow the detoxification of ammonia that enters in the CNS (Peng et al., 1998; Albrecht and Dolińska, 2001; Veauvy et al., 2005; Wright et al., 2007; Ip and Chew, 2010). Despite this advantage, in mammals as in fish, the excessive activation of both enzymes and the accumulation of GLN in brain, cause several metabolic disorders associated with astrocyte and brain swelling (Albrecht and Dolińska, 2001; Zielińska et al., 2003; Pichili et al., 2007; Cauli et al., 2009), since GLN exerts osmotic effects (Willard-Mack et al., 1996; Donovan et al., 1998; Albrecht and Dolińska, 2001; Ip and Chew, 2010), and/or disruption of the mitochondrial function (Albrecht and Dolińska, 2001). In fact, the inhibition of GS by methionine sulfoximine (MSO) decreases many pathophysiologic and metabolic symptoms caused by hyperammonemia in brain (Ip and Chew, 2010) and even decreases mortality (Feldman et al., 2014), since this compound blocks the production of GLN by GS and impede the accumulation of GLN in astrocytes (Albrecht and Dolińska, 2001; Feldman et al., 2014). This result shows that the neurotoxicity of ammonia may be related with GLN accumulation (Feldman et al., 2014), establishing the first explanation for ammonia toxicity in brain, known as glutamine/osmolyte hypothesis (Zwingmann et al., 2000; Pichili et al., 2007; Ip and Chew, 2010); yet some studies suggest that this cannot be the major cause of death (Veauvy et al., 2005; Ip and Chew, 2010). However, and besides all the available information on astrocyte and brain swelling induced by high levels of ammonia, the explanation of how ammonia increases GLN synthesis in the brain was only recently published. According to this study, the build-up of ammonia in the brain leads to an increase of extracellular K^+, which in turn, causes an increase of the intracellular pH in astrocytes and, consequently, GS activation and GLN synthesis (Brookes, 2000; Albrecht and Dolińska, 2001).

Other explanations for the ammonia toxicity in brain involve glutamatergic dysfunction (Brusilow, 2002; Felipo and Butterworth, 2002; Ip and Chew, 2010) and excessive activation of N-methyl-D-aspartate (NMDA) glutamate receptor (Hermenegildo et al., 1996; Albrecht, 1998; Kosenko et al., 1999; Randall and Tsui, 2002; Ip and Chew, 2010). In fact, the presence of high levels of ammonia in the brain cause an increase of extracellular GLU (an important excitatory neurotransmitter) due to an intensification of its release by neurotransmission and/or decrease in GLU synaptic recycling by astrocytes (Rao et al., 1992; Schmidt et al., 1993; Albrecht, 1998; Randall and Tsui, 2002). This accumulation of GLU in the extracellular

space, causes an excessive activation of its receptor, the NMDA glutamate receptor (Hermenegildo et al., 1996; Albrecht, 1998; Kosenko et al., 1999; Randall and Tsui, 2002; Zielińska et al., 2003), which is neurotoxic and provokes cell swelling, neuronal degeneration and even cell death (Albrecht, 1998; Randall and Tsui, 2002; Zielińska et al., 2003; Ip and Chew, 2010). Indeed, this overactivation of the NMDA glutamate receptor followed the depolarization and hyperpolarization induced by NH_4^+ (Albrecht, 1998; Hermenegildo et al., 2000), which cause an influx of Ca^{2+} and Na^+ (Ratnakumari et al., 1995). Inside the cell, the Ca^{2+} activates Ca^{2+}-dependent enzymes, which in turn activate a cascade of reactions (Randall and Tsui, 2002) that may provoke oxidative stress, neuronal degeneration and even death (Marcaida et al., 1992; Randall and Tsui, 2002; Ip and Chew, 2010). This excessive activation of NMDA glutamate receptor by ammonia can be blocked by MK-801 (a NMDA glutamate receptor antagonist) alone (Albrecht, 1998; Zielińska et al., 2003; Feldman et al., 2014) or administrated with MSO (Feldman et al., 2014), which seems to protect the astrocytes and brain during high levels of ammonia in the CNS. This reinforces the idea that, beyond the accumulation of GLN, the occurrence of cerebral edema may also have other explanation like the overactivation of NMDA glutamate receptor (Veauvy et al., 2005).

Nevertheless, other reason, especially in mammals, for the astrocyte swelling in CNS induced by high ammonia levels include GLN-mediated oxidative stress and/or mitochondrial permeability transition (MPT) (Pichili et al., 2007; Häussinger and Görg, 2010; Ip and Chew, 2010). The overactivation of the NMDA glutamate receptor may also increases the intracellular concentration of arginine (Albrecht, 1998), leading to the production of nitric oxide (NO) by the neuronal nitric oxide synthase (NOS) (Hermenegildo et al., 1996; Albrecht, 1998; Kosenko et al., 1998; Hernández-Fonseca et al., 2008; Braissant et al., 2013). In detail, the increase of arginine concentration in the brain, leads to an upregulation of argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) expression that, in turn, stimulates the citrulline-NO cycle, where neuronal NOS is included (Bachmann et al., 2004). On the other hand, the activation of NMDA glutamate receptors increases the production of superoxide radical (O_2^-) that is though to play a role in the excitotoxic process. The O_2^- and NO may react to produce peroxynitrite ion ($ONOO^-$) that is a high reactive species that generates hydroxyl radicals (HO^-) (Kosenko et al., 1998; Schliess et al., 2002). Moreover, $ONOO^-$ is also a mediator of GLU and NO neurotoxicity. In fact, reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), O_2^- and HO^- may react with biological macromolecules and cause enzyme inactivation, lipid peroxidation and DNA damage,

resulting in oxidative stress. In this sense, after excessive activation of the NMDA glutamate receptor (Kosenko et al., 1998, 1999; Albrecht and Dolińska, 2001) it is the balance between ROS production and the antioxidant defenses of the organism that determines the severity of the oxidative stress that, at the limit, cause neuronal cell death. However, it is important to mention that ammonia also induces oxidative stress in the gills, an organ that lacks NMDA glutamate receptors (Ching et al., 2009). This may indicate that oxidative stress can be stimulated through different pathways in the brain and other tissues (Ching et al., 2009; Ip and Chew, 2010) or even the coexistence of NMDA-dependent and -independent mechanisms of NOS activation (Albrecht, 1998). Moreover, in *in vitro* studies, the administration of an inhibitor of NO synthesis (NNA) reduces the cell swelling caused by the presence of ammonia in the CNS, which may indicate that NO formation may also induce some astrocyte and brain swelling (Zielińska et al., 2003). On the other hand, the MPT consists in the opening of a pore, in the inner mitochondrial membrane, that disrupts the ionic gradient and causes mitochondrial dysfunction (Ip and Chew, 2010). More specifically, after induction of MPT, a continuous movement of GLN occurs through the inner mitochondrial membrane, leading to the production of ammonia in the mitochondrial matrix by glutaminase (Bai et al., 2001; Albrecht and Norenberg, 2006; Ip and Chew, 2010). Factors that induce MPT include free radicals, Ca^{2+}, NO, alkaline pH and GLN, i.e. most of the factors also implicated in the mechanism of HE (Ip and Chew, 2010). In addition to causing imbalance of the ionic gradient, MPT also causes impairment of oxidative phosphorylation, induce ROS production and, ultimately, cell death (Bai et al., 2001; Ip and Chew, 2010). In relation to astrocyte swelling, seems that oxidative stress and MPT are also involved in this process (Ip and Chew, 2010) since they cause the overexpression of AQP4 in the brain tissue (Rao and Norenberg, 2007).

Since ammonia interferes with mitochondria, energy metabolism in the brain is affected by its presence. High levels of ammonia in the brain increase the concentration of glucose by upregulation of glycolysis or by the increase of endothelial cell/astrocytic glucose transporter (GLUT) expression. The increase of glucose uptake is accompanied with an increase in the lactate concentration; however, this seems to be predominantly an astrocytic phenomenon (Desjardins et al., 2001). Although ammonia increases the presence of glucose in brain, it also causes a decrease in ATP, which can be explained by two different mechanisms: 1) inhibition of the TCA cycle and/or 2) a mechanism that includes the NMDA glutamate receptors (Felipo and Butterworth, 2002). McKhann and Tower (1961), favoring the first option, showed that

ammonia inhibited the TCA cycle in brain with the accumulation of αKG and pyruvate. Moreover, Lai and Cooper (1986) showed that α-ketoglutarate dehydrogenase, the rate-limiting TCA cycle enzyme, is inhibited in brain. This situation is consistent with an increase of lactate in brain exposed to high levels of ammonia (Therrien et al., 1991; Mans et al., 1994; Chatauret et al., 2001). On the other hand, the fact that the decrease in ATP is prevented through the administration of NMDA glutamate receptor antagonist (Kosenko et al., 1994) supports the second explanation. However, some studies challenge this hypothesis (Peterson et al., 1990; Marcaida et al., 1995; Hermenegildo et al., 1998). The decrease of ATP and cytochrome C oxidase expression and activity suggest that the depletion of ATP is related with a deceleration of the electron transport chain enzymes (Rao et al., 1997), although, this consequence can also be related with TCA cycle inhibition via α-ketoglutarate dehydrogenase (Hertz and Kala, 2007). Regardless of the mechanism, ATP depletion will disrupt the GLU reuptake (Randall and Tsui, 2002), with the consequences described above. On the other hand, the effects on the cerebral energy metabolism may be related with the accumulation of GLN in the mitochondria that will cause, in turn, mitochondria swelling and activation of the MPT (Albrecht and Dolińska, 2001).

Besides all the effects previously described, ammonia may also be involved in several other negative effects reported in brain. Indeed, the presence of high levels of ammonia in the CNS has been related with the modification of the blood-brain barrier (BBB) properties, alterations of the amino acid transport (Albrecht and Dolińska, 2001), increase of cerebral blood flow (Albrecht and Dolińska, 2001), interference with excitatory amino acid neurotransmitter metabolism namely GLU and aspartate and even cause morphological changes in astrocytes and neurons. In this sense, more studies *in vitro* and *in vivo* will be of high relevance to better understand the mechanisms and consequences of high concentrations of ammonia in brain.

1.7 Natural strategies to avoid ammonia toxicity

Since ammonia causes several deleterious effects inside the organism, aquatic animals developed some strategies to defend themselves against ammonia toxicity. These strategies include the conversion of ammonia to non-toxic compounds, reduction of ammonia production, active excretion of ammonia even against concentration and volatilization of ammonia (Figure 1.3). Considering all these mechanisms it makes sense that some species

may tolerate higher environmental ammonia levels than others (Randall and Tsui, 2002; Ip et al., 2004a).

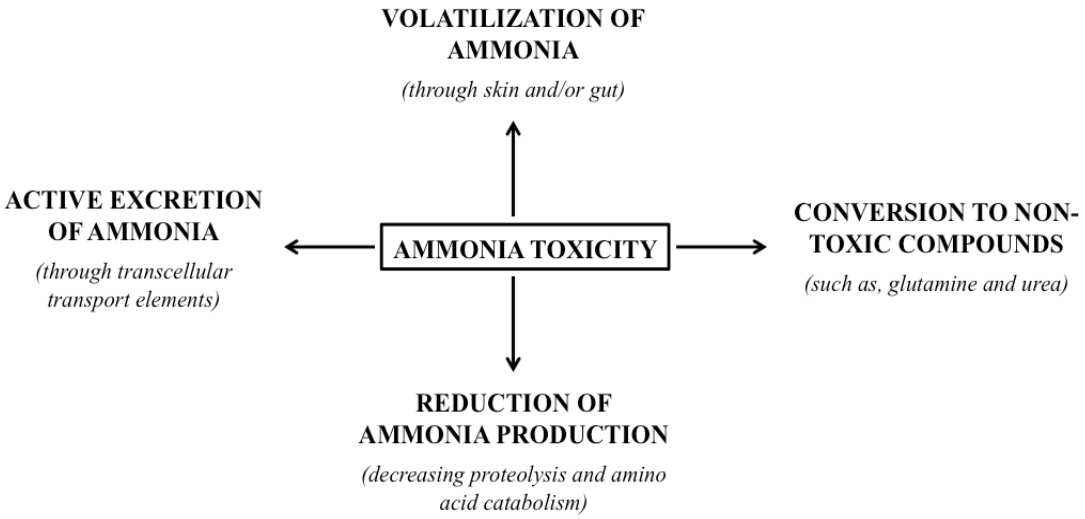


Figure 1.3 Scheme illustrating the different strategies used by teleost fish to avoid ammonia toxicity.

The most common strategy is the conversion of ammonia to non-toxic compounds such as GLN, urea or uric acid (Wright, 1995). As explained above, fish may incorporate ammonia into GLU and GLN through GDH and GS, respectively. In this way, from αKG to GLN, is possible to detoxify two moles of NH_4^+ (Randall and Tsui, 2002). This strategy was developed by fish to deal with ammonia pulses after feeding (Randall and Tsui, 2002; Tng et al., 2008; Ip and Chew, 2010), but it has also been described in cerebral and/or non-cerebral tissues (Peng et al., 1998; Veauvy et al., 2005; Wright et al., 2007). In fact, although this strategy can be very important to deal with exogenous and endogenous high ammonia concentrations (Peng et al., 1998; Veauvy et al., 2005; Wright et al., 2007; Tng et al., 2008; Ip and Chew, 2010), this mechanism may also be used to detoxify ammonia during the aerial exposure, as observed in the sleeper *Bostrychus sinensis* (Anderson et al., 2002), marble goby *Oxyeleotris marmoratus* and Asian swamp eel *Monopterus albus* (Jow et al., 1999; Tay et al., 2003), or even during ammonia-loading (Ip et al., 2004). In species like Asian swamp eel *M. albus* (Tok et al., 2009) and Amazonian freshwater stingray *Potamotrygon motoro* (Ip et al., 2009), the synthetized GLN may function as an osmolyte, regulating cell volume during acclimation to high salinity. The gulf toadfish *Opsanus beta* also uses the conversion of ammonia to GLN as strategy to deal with ammonia, especially during confinement stress

(Walsh and Milligan, 1995; Ip and Chew, 2010). Moreover, GLN can constitute a substrate for the synthesis of urea, which can be important for osmoregulation in hyperosmotic environments (Ip and Chew, 2010). Other organisms such as coelacanths, elasmobranchs, amphibians and specially mammals detoxify ammonia to urea through the ornithine urea cycle (OUC) in the liver (Wright, 1995; Ip and Chew, 2010). However, most invertebrates and teleost lost this pathway and produce urea mostly through uricolysis and argininolysis (Wright, 1995). Although the OUC is absent in the majority of adult teleosts (Wright, 1995; Wright et al., 1995; Kong et al., 1998; Chadwick and Wright, 1999; Randall and Tsui, 2002), adult gulf toadfish *O. beta* (Wood et al., 1995), Lake Magadi tilapia *Alcolapia grahami* (Walsh et al., 2001) and Indian air breathing fish *Heteropneustes fossilis* (Saha et al., 2001), resort to this mechanism to mitigate ammonia toxicity. Moreover, early life stages of teleost species may also present an active OUC (Wright et al., 1995; Randall and Tsui, 2002; Barimo et al., 2004; Braun et al., 2009a, 2009b) to protect them from ammonia toxicity during these sensitive stages of neural development (Wright, 1995). This strategy was described in the early development of Atlantic cod *Gadus morhua* L. (Chadwick and Wright, 1999), rainbow trout *O. mykiss* (Wright et al., 1995; Korte et al., 1997; Pilley and Wright, 2000), pacu *Piaractus mesopotamicus* (Monzani and Moraes, 2008), African catfish *Clarias gariepinus* (Terjesen et al., 1997), gulf toadfish *O. beta* (Barimo et al., 2004), viviparous blenny *Zoarces viviparus* (Korsgaard, 1994) and zebrafish *D. rerio* (Braun et al., 2009a; Bucking et al., 2013; LeMoine and Walsh, 2013). During early development, the catabolism of the yolk proteins results in a high rate of ammonia production but the diffusion is impaired due to undeveloped circulatory system and gills (Griffith, 1991; Randall and Tsui, 2002). Thus, the conversion to urea is a useful mechanism to guarantee the survival of the organism during the early development (Wright et al., 1995; Braun et al., 2009a). However, this detoxification mechanism is partial or completely suppressed in the adult liver (Wright et al., 1995; Korte et al., 1997; Kong et al., 1998; Chadwick and Wright, 1999) since the diffusion of ammonia to the environment has a lower energy cost than the conversion to urea (Randall and Tsui, 2002) [urea synthesis requires five molecules of ATP per urea molecule (Ip and Chew, 2010)].

Another mechanism used by aquatic organisms when exposed to adverse environmental conditions is the reduction of ammonia production (Lim et al., 2001; Randall and Tsui, 2002; Ip and Chew, 2010). This strategy is particularly relevant for fish exposed to high environmental ammonia, high environmental pH or aerial exposure (Wilson et al., 1998; Lim et al., 2001; Randall and Tsui, 2002), since under these conditions, ammonia excretion is

reduced or reversed (Randall and Tsui, 2002; Ip and Chew, 2010). With this strategy, teleosts decreases proteolysis, amino acid catabolism or both and then decrease the total free amino acids content, thus avoiding the build-up of ammonia inside the organism (Lim et al., 2001; Randall and Tsui, 2002; Ip and Chew, 2010). This strategy can be especially important in the brain of teleost fishes (Wee et al., 2007; Tng et al., 2009; Ip and Chew, 2010). However, it is important to mention that one study in rainbow trout *O. mykiss* (Wood, 2004) described, in turn, that exogenous ammonia may stimulante growth since this species incorporates ammonia into amino acids and increases the protein synthesis during exposure to ammonia, which allow an increase in their growth (Wood, 2004).

Another strategy, used by species as weather loach *Misgurnus anguillicaudatus* (Tsui et al., 2002) and mangrove killifish *K. marmoratus* (Wright, 2012), consists on the elimination of ammonia from the body through NH_3 volatilization (Randall and Tsui, 2002; Tsui et al., 2002; Ip et al., 2004a). These species use this mechanism during aerial exposure and/or ammonia loading. When these fishes are out of the water, they accumulate ammonia inside the body and increase the pH of the skin surface and/or gut, enhancing the volatilization of NH_3. In the case of the weather loach *M. anguillicaudatus*, air is swallowed and passed down the intestine to carry the intestinally volatilized NH_3 away (Tsui et al., 2002). In the skin of mangrove killifish *K. marmoratus*, the volatilization of NH_3 during air exposure may be done by Rh glycoproteins present in this tissue and that eliminates NH_3 without occurring its protonation (Hung et al., 2007; Wright, 2012).

The active ammonia excretion, against high environmental ammonia gradients, is another strategy utilized by some aquatic animals (Wilson et al., 2000; Randall and Tsui, 2002; Ip et al., 2004a; Hung et al., 2007; Nawata et al., 2007; Wright and Wood, 2009). In the gill epithelium, the mitochondria rich cells are responsible for the active excretion of ammonia to the environment (Wilson et al., 2000; Randall and Tsui, 2002; Wright and Wood, 2009). More specifically, the NH_4^+ may substitute the transport of some ions, such as Na^+ in seawater and H^+ in freshwater (Wilson and Taylor, 1992; Randall and Tsui, 2002), allowing the movement of ammonia out of the body even when the environmental ammonia is high. Interestingly, in adult zebrafish *D. rerio* the exposure to high environmental ammonia did not increase neither the mRNA nor the protein levels of the ammonia transporters Rh glycoproteins but increased the transcription of the V-type H^+-ATPase. These results may indicate that, under environmental ammonia exposure, zebrafish *D. rerio* may increase the pH gradient to continuously eliminate ammonia *via* acid trapping instead of increasing the

transcription of Rh glycoproteins (Braun et al., 2009b). However, in mangrove killifish *K. marmoratus*, although it is not clear which mechanism is used to excrete ammonia against its gradient, Hung and collaborators (2007) defend that ammonia excretion is done instead by increased Rh glycoproteins expression.

1.8 Zebrafish: a model species to study ammonia toxicity

Figure 1.4 Adult zebrafish (image from the website: https://zfin.org/).

Zebrafish *D. rerio* (Figure 1.4) is a popular model vertebrate species used in several areas of research (Dodd et al., 2000; Briggs, 2002; Hill et al., 2005; Hwang and Chou, 2013) due to their physiological special features but also due to the different types of molecular research that this species allows. The principal advantages of using this species are related with their small size that allows the use of modest aquariums, easy manipulation during all life cycle, high number of offspring (a single pair of adults generates 200 eggs per week), rapidly and external development of transparent embryos and larvae, ontogenic development well characterized and short generation intervals of three months (Kimmel et al., 1995; Westerfield, 2000; Parichy et al., 2009). These features make this species well suited for developmental biology, neurobiology, disease-focused researches (Dodd et al., 2000; Briggs, 2002; Hill et al., 2005; Hwang and Chou, 2013) and for chemical toxicity assays [during embryonic development (teratogenicity) (Voelker et al., 2007; Liedtke et al., 2008; Lammer et al., 2009; Selderslaghs et al., 2009; Brannen et al., 2010; Feldman et al., 2014) and even during adulthood (Liu et al., 2006)]. However, the interest of scientific community in this species is due to the recent sequencing of its genome. In fact, since all the genome is now available, addressing the changes in gene (Dhanasiri et al., 2013; Hagenaars et al., 2013) and protein expression (Hagenaars et al., 2013) is less challenging (Hill et al., 2005) when zebrafish is used. Moreover, and since this species maintains a typical vertebrate systems complexity and shares high genomic similarities with all the vertebrates, the results obtained in this freshwater teleost can serve as basis for effective cross-species extrapolation.

1.9 Thesis Aims

Several studies have been done in zebrafish to determine the molecular responses to ammonia exposure during early life stages of development and during adulthood (Braun et al.,

2009a, 2009b). However, the levels of environmental ammonia tolerated at the different developmental stages of zebrafish are not yet published. To address this gap in our knowledge, the determination of the lethal concentration for 50 % of the population (LC_{50}) during the life cycle of zebrafish constitutes the first objective of this thesis (**chapter 2**).

In addition to ammonia toxicity varying during the life cycle of an aquatic organism, physicochemical parameters of the water, like pH and salinity, are also usually inversely correlated with ammonia tolerance (Randall and Tsui, 2002). Moreover, the presence of buffer in the water can eliminate boundary layer acidification, which would compromise ammonia excretion (Rahaman-Noronha et al., 1996) resulting in high mortality of the aquatic organism. Thus another goal of this thesis was to determine if changing the environmental conditions (pH 8 buffered freshwater or lower ionic strength) would affect the tolerance of adult zebrafish when compared with the LC_{50} value in freshwater (**chapter 2**).

Although the effect on organism survival is an endpoint of extreme relevance for aquaculture, the exposure to sublethal concentrations of ammonia triggers several molecular (Dhanasiri et al., 2013) and physiological (Benli et al., 2008) responses that may compromise the development (Benli and Köksal, 2005), reproduction (Abbas, 2006), growth (Rasmussen and Korsgaard, 1996; Björnsson and Ólafsdóttir, 2006; Foss et al., 2007) and health of cultured fishes (Benli and Köksal, 2005; Gonçalves et al., 2012; Dhanasiri et al., 2013). Consequently, molecular approaches have become a good ecotoxicological method to determine the environmental risk of exposure to toxic levels of ammonia, even because this nitrogenous compound might be at higher concentrations in the environment due to natural and anthropogenic pollution sources (Randall and Tsui, 2002), but also in aquacultures, due to high fish densities, high feeding rates and/or lower water removal (Tomasso, 1994). More recently, the microarray technologies have been used to determine the mode of action of toxic contaminants in aquatic organisms and the fact that these technologies allow monitoring a large number of cellular transcripts at the same time is an advantage (Dhanasiri et al., 2013; Hagenaars et al., 2013). In this sense, the use of microarray together with the already sequenced zebrafish genome is the basis of the goal of **chapter 3**, which consists in the determination of the effects of sublethal ammonia concentrations on the transcriptome of gill, liver and brain of zebrafish after 96 h of exposure. To accomplish this objective, Gene Ontology and pathway analysis were performed after running a 44k microarray of the entire zebrafish genome on control and exposure gill, liver and brain samples.

Although some of the knowledge obtained through the microarray analysis is

completely novel, several studies have been done over the last decades that described some of the effects of ammonia in gill, liver and brain of aquatic organisms (as described above). Considering these studies, the responses and/or mechanisms(s) triggered by high environmental ammonia might be species specific with some species increasing ammonia excretion even against gradient (Randall and Tsui, 2002; Wright and Wood, 2009) and/or reducing ammonia production through the decrease of amino acid catabolism (Randall and Tsui, 2002; Ip and Chew, 2010). The precise mechanism(s) applied by zebrafish to deal with ammonia exposure were analyzed in detail in **chapter 4**. Moreover, and also in **chapter 4**, it was aimed to validate the results obtained in the microarray considering known responsive genes to ammonia exposure, by real-time PCR (*q*PCR).

On the other hand, relatively few studies have been done to determine the effects of hormones on ammonia excretion in aquatic organisms. Alterations in endocrine gene expression and/or its receptors were analyzed, based on the microarrays results, in detail in **chapter 4**, which allowed to better understand which hormone(s) might be involved in the response to ammonia. This was followed up with a hormonal study on ammonia excretion (**chapter 5**). In fact, it is known that prolactin regulates the adaptation of teleost fishes to freshwater (Tang et al., 2001) by modulating the permeability of the branchial epithelium. However, the effect of this hormone on ammonia excretion has not yet been studied. On the other hand, it is better known that cortisol, a hormone that is increased in response to environmental stressors (Mommsen et al., 1999; Gonçalves et al., 2012; Dhanasiri et al., 2013), increases protein and amino acid catabolism, which in turn enhances ammonia production and excretion (Hopkins et al., 1995; Mommsen et al., 1999; Randall and Tsui, 2002). In an attempt to demonstrate that prolactin is an important hormone for ammonia balance, mainly related with its excretion, administration of these hormones exogenously and with an extra ammonia load was performed. Ammonia flux rates and endocrine effects on gene expression of elements of paracellular and transcellular pathways, during ammonia excretion, were analyzed in this chapter (**chapter 5**).

In fact, the relevance of studying ammonia production, excretion and response in teleost fish is related with ecological and environmental questions that might cause mass mortality especially under unfavorable environmental or aquaculture conditions (Tomasso, 1994). At the end of this thesis, it is expected that gaps that still exist in the scientific knowledge, mostly on mechanisms and/or pathways and hormone challenge involved in the response of freshwater fish to environmental ammonia, will be better understood. Moreover, this study

has relevance for medical researchers since HE is mostly caused by the presence of high levels of ammonia in the brain, but also because the mechanism of neuronal toxicity of ammonia is similar between mammals and zebrafish (Feldman et al., 2014).

CHAPTER 2 |

ACUTE AMMONIA TOXICITY IN ZEBRAFISH (*Danio rerio*): COMPARISON BETWEEN EARLY DEVELOPMENT AND ADULTS

2.1 Abstract

Ammonia is a toxic compound present in freshwater ecosystems originating from anthropogenic and natural sources. The toxicity of ammonia depends of its concentration as well as water physicochemical parameters. During the life cycle of an organism, the sensitivity to ammonia may also change. Zebrafish (*Danio rerio*) is a model species and much work has been directed at the molecular mechanisms of ammonia excretion and toxicity; however, basic information on their ammonia sensitivity is lacking. Therefore, 24 h ammonia exposure experiments were performed to determine the lethal effects of NH_3 (un-ionized ammonia; UIA) at different stages of zebrafish development. Moreover, in adults, the median-lethal concentrations of NH_3 during 96 h of exposure were calculated in freshwater, 10 mM Tris-buffered freshwater (pH 8) and brackish water. During early development, the highest 24 h LC_{50} value was obtained for embryos at 19 - 26 hours post-fertilization (hpf) and thereafter the LC_{50} decreased till the most sensitive stage (168 hpf). This early high tolerance to ammonia can be explained by the presence of an active ornithine-urea cycle that converts ammonia to urea, similarly to other teleost species. In adult experiments, the highest tolerance to ammonia was observed in fish exposed to freshwater,

with lower tolerance in buffered freshwater and brackish water probably due to disruption of the acidic boundary layer and difficulties in maintaining the low permeability at the gill surface, respectively. Overall, we concluded that early life stages were the most tolerant in the life cycle of zebrafish and that adults are less susceptible in unbuffered freshwater.

2.2 Introduction

Ammonia is a common toxic compound that, in solution, can be present in two distinct forms, one more related with environmental toxicity and the other causing damages inside the aquatic organisms. In fact, ammonia exists in the aquatic environment as NH_3 (unionized ammonia or UIA) or NH_4^+ (ionized ammonia) and the sum of these two forms are usually referred as total ammonia-nitrogen (TA-N) or simply as ammonia. Environmental ammonia toxicity is related to the ability of NH_3 to pass through the cell membranes (Randall and Tsui, 2002), which is conferred by its moderate lipid solubility (Wilkie, 2002; Ip and Chew, 2010). Considering this, environmental toxicity of ammonia is usually expressed as NH_3 concentration (USEPA, 1999); although, within the organism, the mechanisms of ammonia toxicity are mainly related with NH_4^+ (Wilkie, 2002).

In aqueous solutions, the proportion of NH_3 and NH_4^+ is highly dependent of pH but may also vary with temperature, pressure and ionic strength. The toxic NH_3 proportion increases in alkaline water, which decreases the tolerance of aquatic organisms to ammonia. Moreover, a raise in temperature or salinity results in a higher sensitivity to ammonia (USEPA, 1999, 2013; Randall and Tsui, 2002; Eddy, 2005). However, it is important to keep in mind that, in general, the tolerance towards ammonia varies greatly with species, some being more tolerant to high levels of environmental ammonia than others (Randall and Tsui, 2002; Eddy, 2005).

Ammonia is simultaneously produced within the organism (Ip and Chew, 2010) but also discharged to the aquatic environment from different natural and anthropogenic sources (Randall and Tsui, 2002; Eddy, 2005). In the organism, ammonia is mainly derived from the catabolism of proteins and amino acids (Wilkie, 2002; Ip and Chew, 2010) and, due to its toxicity, it has to be converted into less toxic compounds, such as glutamine (GLN) and/or urea, or eliminated from the body (Randall and Tsui, 2002; Wilkie, 2002; Ip and Chew, 2010). In aquatic animals, the latter is more energetically cost effective (Randall and Tsui, 2002; Wilkie, 2002) and occurs mainly through the gills (Wright and Wood, 2009; Ip and Chew, 2010); being therefore, a concern in high-density fish culture (Tomasso, 1994). On the

other hand, ammonia may also increase in freshwater ecosystems due to different factors like bacterial nitrogen fixation, decomposition of biological wastes and organic detritus by microorganisms, industrial emissions, sewage effluent discharges, urban and agricultural run-offs, animal farming, volcanic activity, release of fertilizers, gas exchange with the atmosphere, forest fires and industrial processes (Hargreaves, 1998; Randall and Tsui, 2002; Wilkie, 2002; USEPA, 1999, 2013).

The accumulation of ammonia outside and/or inside the aquatic organism may cause an imbalance of ammonia homeostasis, which leads to intoxication of the organism. In fact, in teleost fishes, a model that includes several membrane transporters was recently proposed to explain the ammonia excretion (Wright and Wood, 2009); however, it is known that the presence of a passive diffusion gradient from the blood-to-water is also relevant for the branchial ammonia elimination. Nevertheless, in environments with high levels of ammonia or elevated pH, the excretion from the body is reduced or reversed allowing ammonia entrance with relative ease (Randall and Tsui, 2002; Wilkie, 2002; Wright and Wood, 2009). Also, under stress conditions, ammonia production inside the organism can increase, further accumulating in the body (Mommsen et al., 1999; Randall and Tsui, 2002). This overall increase of ammonia may cause several deleterious effects in the organism namely hyperactivity, convulsions, coma and eventually death (Randall and Tsui, 2002; Wilkie, 2002). However, surprisingly, some studies revealed that low levels of environmental ammonia might act as a growth stimulant, as determined in rainbow trout *Oncorhynchus mykiss* (Wood, 2004) and walleye *Sander vitreus* (Madison et al., 2009).

Zebrafish (*Danio rerio*) is a popular model species used in several genomic and toxicological studies that resort the use of early life stages of development as well as adults. Until today, zebrafish has been used in several areas of research (Dodd et al., 2000; Briggs, 2002; Hill et al., 2005; Hwang and Chou, 2013) notably in studies on the molecular basis of ammonia excretion (Braun et al., 2009a, 2009b; Perry et al., 2010; Hwang et al., 2011) and also in studies that evaluate the genetic responses to ammonia exposure (Braun et al., 2009b). However, to our knowledge, no study has addressed the levels of environmental ammonia tolerated by the different stages of zebrafish development; only a few studies have mentioned the lethal dose for some stages of their development (Feldman et al., 2014; Jeffries et al., 2014). In addition, toxicity tests in embryos have been proposed as a valid tool to assess the acute toxicity of chemicals and effluents in fish, especially in zebrafish that presents a reliable correlation between fish embryo test and acute fish test (Braunbeck et al., 2005; Belanger et

al., 2013). Moreover, embryos and larvae of zebrafish are well characterized (Kimmel et al., 1995; Westerfield, 2000; Parichy et al., 2009), which has allowed the use of zebrafish to determine the chemical toxicity during the embryonic development (teratogenicity), considering survival (Lammer et al., 2009; Selderslaghs et al., 2009; Brannen et al., 2010) or molecular endpoints (Voelker et al., 2007; Liedtke et al., 2008). Besides, some studies have even suggested that early stages of aquatic organisms are usually more sensitive to toxicants, such as ammonia, than adults, making these stages important for determining the sensitivity of a species [Nile tilapia *Oreochromis niloticus* L. (Benli and Köksal, 2005)]. The LC_{50} exposure test consists of a quick and inexpensive tool that allows the determination of the concentration at which a toxic compound causes the death of 50 % of the population (USEPA, 1999, 2013). With all this in mind, in this study we pretend to estimate the 24 h acute toxicity levels of UIA at different developmental stages of zebrafish and also to determine the 24, 48, 72 and 96 h susceptibility of adults under different environmental conditions such as freshwater, buffered freshwater (pH 8) and brackish water (salinity 6 ‰).

2.3 Material and Methods

2.3.1 Zebrafish maintenance

Adult zebrafish (*D. rerio*) were obtained in a local pet store in Oporto, Portugal and, once in the laboratory, zebrafish were acclimated to an undisturbed and continuously aerated tank (100 L) for more than one month. This tank was supplied with dechlorinated Oporto city tap water and the water was continuously filtered by an external biological filtration system (Eheim, Germany) supplemented with ultraviolet sterilization (Vectron V2, Tropical Marine Centre, UK). In addition to filtration, 20 % of the water was renewed daily to guarantee that the fecal matter and uneaten food were removed, which ensured low levels of ammonia and other metabolites in the tank. The photoperiod was 16 h Light : 8 h Dark and the temperature was maintained at 28 ± 1 ºC. Adult zebrafish were fed five times per day with commercial flake food (Tetramin, Tetra, Germany) and four times per week with *Artemia* nauplii (Ocean Nutrition, Salt Lake City, UT, USA).

All the experiments done for this study were carried out under the guidance of a Laboratory Animal Science certified supervisor (1005/1092, DGV-Portugal, C category, FELASA), according to European Union directives (2010/63/UE).

2.3.2 Egg production

Zebrafish eggs were obtained in our lab from natural reproduction using mature fish and following the procedures described by Westerfield (2000). For breeding, a group of broodstock was previously transferred to a specific spawning aquarium equipped with a mesh on the bottom to protect the eggs from being eaten. Each spawning tank contained a ratio of one male for two females. The water temperature was kept at 28 ± 1 ºC with a heater, and was continuously aerated. Spawning was induced in the morning when the light was turned on. Two hours after fertilization, eggs were collected, rinsed with freshwater and cleaned in a Petri dish under a dissecting microscope (Leica S4, Germany).

Eggs, embryos and larvae were maintained in a small plastic aquarium (1 L) until they reached to the desired stage for experimentation. The water of the tank was completely renewal every day to ensure high rates of survival. After hatch, larvae were fed with 24 h *Artemia* nauplii (Ocean Nutrition) and powdered commercial flake food (Tetramin).

2.3.3 Acute ammonia exposure at different stages of development

The effects of 24 h of ammonia exposure were evaluated in eggs, embryos and larvae of zebrafish. The choice of the selected endpoints was based on the possibility of an easy measurement and handling but also to cover the higher number of developmental stages. The determination of the stage was according to Kimmel and collaborators (1995).

The static exposure experiments were carried out in 24 well microplates containing 2 ml of ammonia test solution. The nominal TA-N concentrations (mM) and the respective nominal UIA concentrations (mM), the total number of individuals and water pH and temperature during these experiments are present in Table 2.1. The solutions for all experiments were prepared by diluting a stock solution of 1 M of ammonium chloride (NH_4Cl; Merck, Darmstadt, Germany) made up in 1 L of dechlorinated and filtered tap water. One control group (without exogenous ammonia) was run per developmental stage and per experiment. Different batches of eggs, embryos and larvae were used. All exposures were done at natural photoperiod and no food was supplied. It is important to mention that the real concentration of TA-N and UIA present in each control and exposure well were not measured in any exposure experiment.

After 24 h of exposure, embryos or larvae were observed using a stereomicroscope and the absence of heartbeat or no response to mechanical stimulation was used as an indicator of morbidity. At the end of the experiment, embryos and larvae were allowed to recover in

ammonia free water.

Table 2.1 Physicochemical parameters of the water and number of individuals used for each developmental stage of zebrafish during the 24 h LC_{50} experiments. The concentrations of total ammonia-nitrogen (TA-N; mM) and un-ionized ammonia (UIA; mM) were not measured during the experiment. The UIA concentrations were calculated and corrected for pH 8 through the equations present in USEPA (1999). Water pH and temperature (ºC) are present as mean ± coefficient of variation (CV).

Age in hour post fertilization (hpf)	TA-N mM	UIA mM @ pH 8	Total of embryos	pH	Temperature ºC
14 - 19	5, 10, 15, 20, 30, 40, 60, 80, 90, 100	0.250, 0.501, 0.751, 1.001, 1.502, 2.002, 3.004, 4.005, 4.506, 5.006	330	7.914 ± 0.019	26.333 ± 0.026
19 - 26	15, 20, 30, 40, 50, 60, 70, 80, 90, 100	0.751, 1.001, 1.502, 2.002, 2.503, 3.004, 3.504, 4.005, 4.506, 5.006	330		
35 - 48	10, 20, 30, 40, 50, 60, 70, 80, 90, 100	0.501, 1.001, 1.502, 2.002, 2.503, 3.004, 3.504, 4.005, 4.506, 5.006	330		
48 - 60	2.500, 3, 5, 7.500, 10, 15, 20, 30, 40, 50	0.125, 0.150, 0.250, 0.375, 0.501, 0.751, 1.001, 1.502, 2.002, 2.503	330		
60 - 72	2, 2.500, 4, 5, 6, 7.500, 8, 10	0.100, 0.125, 0.200, 0.250, 0.300, 0.375, 0.400, 0.501	270		
168	0.400, 0.800, 1, 1.500, 2, 2.500, 3	0.020, 0.040, 0.050, 0.075, 0.100, 0.125, 0.150	240		

2.3.4 Acute ammonia toxicity in adults

Ten randomly selected fish per tank were acclimatized to the experimental conditions and fasted 48 h before the beginning of the experiment, to minimize the post-prandial variability in fish metabolic state and endogenous ammonia production. After this period, ammonia LC_{50} was determined over 96 h in adult zebrafish subjected to one of the different sets of experimental conditions that are outlined below. In general, the experimental conditions consisted of 5 L tanks with one tank randomly assigned as a control group (no NH_4Cl added), while the remaining tanks were assigned to the respective ammonia concentration used in each experimental set with appropriate pH adjustment (see Table 2.2). The desired nominal TA-N concentration in each tank was prepared by diluting the stock solution of 1 M NH_4Cl in a final volume of 5 L of dechlorinated, filtered and pH adjusted water. The different sets of ammonia exposure experiments were conducted in: *i*) freshwater (control); *ii*) brackish water (6 ‰ of salt) and *iii*) 10 mM Tris buffered freshwater at pH 8. The pre-acclimation to 6 ‰ of salinity (series *ii*) was achieved by progressively increasing salinity by two daily over three days using sea salt and maintained for an additional day until

commencing the experiment (day 4). The experiment with buffered (10 mM Tris base) freshwater (series *iii*) was done using fish pre-acclimated for three days to buffered freshwater with the pH adjusted to 8. During both acclimatization to brackish water and buffered freshwater, zebrafish was fed with commercial flake food (Tetramin), except 48 h before the start of the experiments.

Table 2.2 Physicochemical parameters of the water and weight of individuals during the LC_{50} experiments with adults subjected to freshwater (FW), brackish water (BW) and buffered FW. The concentrations of total ammonia-nitrogen (TA-N; mM) and un-ionized ammonia (UIA; mM) were not measured during the experiment. The UIA concentrations were calculated and corrected for pH 8 through the equations present in USEPA (1999). Water pH, temperature (ºC), salinity, dissolved oxygen (DO) and fish mass are present as mean ± coefficient of variation (CV).

	pH	Temperature ºC	DO	Salinity	Weight g	TA-N mM	UIA mM @ pH 8
FW	7.951 ± 0.016	24.846 ± 0.043	7.008 ± 0.129	-	0.587 ± 0.357	0.500, 1, 2, 3, 4, 6, 8	0.024, 0.049, 0.097, 0.146, 0.194, 0.291, 0.388
BW	7.787 ± 0.022	24.133 ± 0.047	8.545 ± 0.062	5.682 ± 0.019	0.629 ± 0.321	2, 3, 4, 5	0.066, 0.099, 0.132, 0.166
Buffered FW	8.036 ± 0.008	24.149 ± 0.022	8.265 ± 0.027	-	0.537 ± 0.344	0.500, 1.250, 1.500, 2	0.027, 0.068, 0.082, 0.109

The nominal TA-N concentrations used for each set of experiments and the physicochemical characteristics of the water are present in Table 2.2. Water pH, temperature, dissolved oxygen and salinity were monitored daily. Temperature was measured by thermometer, pH with a pH meter (PHM84, Radiometer, Copenhagen, Denmark) and dissolved oxygen and salinity were measured using Hach HQ40d Portable Multi-parameter meter (Hach Company, Colorado, USA). The real concentration of TA-N and UIA present in each control and exposure tank were not measured for any experiment. Feces and residues were siphoned every day and approximately 20 % of the water was renewed with the appropriate concentration of ammonia and pH. Fish were not fed during the experiments. Natural photoperiod was used in all sets of experiments and the water in each tank was gently and continuously aerated.

To determine the LC_{50} values, the moribund fish was verified daily and counted after 24, 48, 72 and 96 h of exposure being removed after. Morbidity was assumed when fish were immobile on the bottom of the aquarium, exhibiting no opercular movement and no response

to mechanical stimuli. At the end of the experiment, all fish were transferred to another tank with freshwater and were allowed to recover. Each animal was only used once and was not included in any further experimentation.

2.3.5 Data analysis

In the adult ammonia exposure, the 24, 48, 72 and 96 h LC_{50} values and the respective 95 % confidence intervals were calculated using the Trimmed Spearman Karber Method (Hamilton et al., 1977). However, in the acute ammonia exposure experiments of eggs, embryos and larvae, only the 24 h LC_{50} value were determined using the same method. The LC_{50} value was considered statistically different if the 95 % confidence intervals did not overlap (Wheeler et al., 2006).

The nominal UIA concentration in buffered and non-buffered freshwater experiments was calculated using the equations described in USEPA (1999) and considering the nominal TA-N concentration, temperature and pH measured during each exposure experiment. Furthermore, in brackish water experiments, the nominal UIA concentration was also calculated but using an additional equation described by Khoo and collaborators (1977) to take salinity into consideration. Since pH is the aquatic parameter that greatly affects ammonia toxicity, all the LC_{50} and 95 % confidence interval values were corrected to pH 8 using the equations present in USEPA (1999). All the results are present as mM of UIA and the graphs were made using Sigma Stat (version 11, SPSS Inc., Chicago, USA).

2.4 Results

2.4.1 Acute ammonia exposure at different stages of development

The 24 h ammonia toxicity experiment was performed in order to determine the LC_{50} values for the different early life stages of zebrafish development. The pH and temperature in the experimental test solutions were 7.914 ± 0.019 and 26.333 ± 0.026 ºC, respectively (Table 2.1).

During the zebrafish development, the 24 h LC_{50} value changed (Figure 2.1), started at 2.224 (1.758 - 2.814) mM UIA in embryos at 14 - 19 hours post fertilization (hpf) but increased to 4.805 (4.603 - 5.016) mM UIA in 19 - 26 hpf embryos. After this transient higher value, the 24 h LC_{50} decreased to 0.449 (0.344 - 0.586) mM UIA in 48 - 60 hpf pro-larvae to 0.270 (0.249 - 0.293) mM UIA in 60 - 72 hpf larvae and finally to 0.094 (0.058 - 0.152) mM UIA in 168 hpf larvae. Embryos with 19 - 26 hpf had the highest 24 h LC_{50} value, which

indicates that this was the most ammonia tolerant embryonic stage. On the other hand, the lowest LC_{50} value was observed at 168 hpf larvae. In all experiments, the highest number of moribund fish was always recorded in the solutions with the highest ammonia concentrations. It is also relevant to mention that it was not possible to determine the 24 h LC_{50} value for 35 - 48 hpf larvae and that the only control groups that presented mortality were 19 - 26 hpf and 168 hpf with 3.333 % and 6.667 %, respectively. Moreover, and since the 95 % confidence intervals did not overlap, the 24 h LC_{50} were significantly different between all the developmental stages analyzed in this study (Figure 2.1).

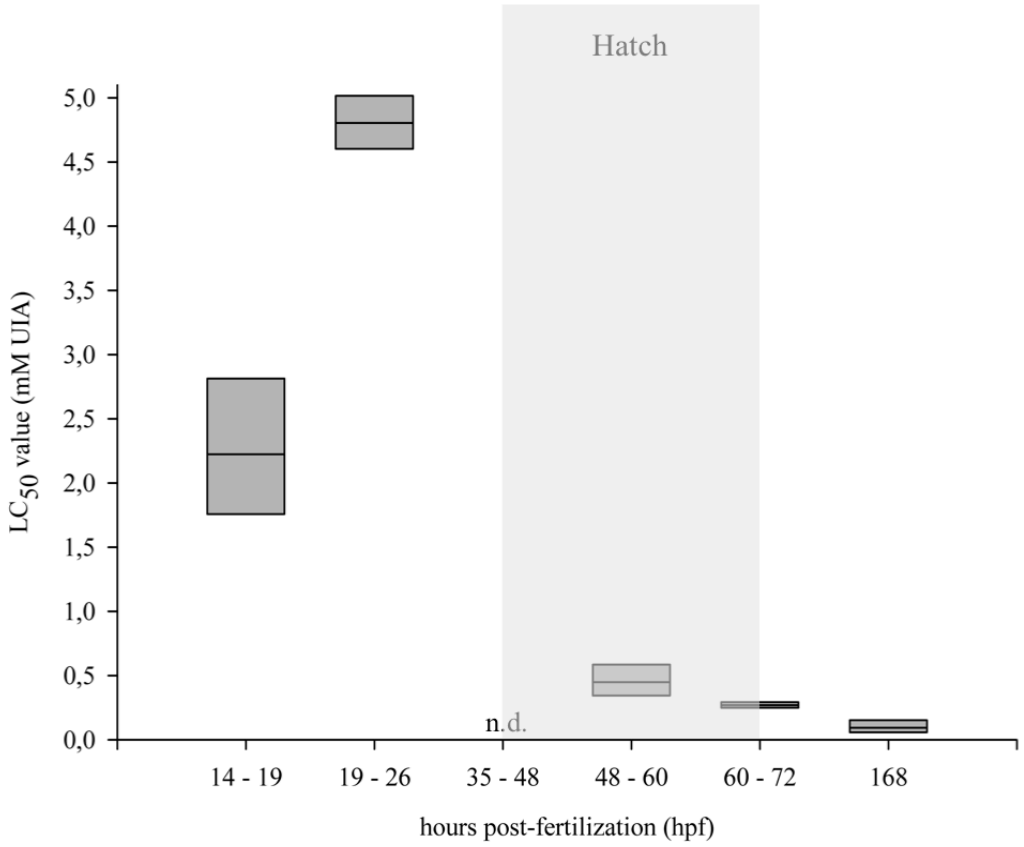


Figure 2.1 LC_{50} values and the respective 95 % confidence intervals for the different early life stages of zebrafish development. The LC_{50} and the confidence intervals were corrected for pH 8 [equation on USEPA (1999)] and are presented as un-ionized ammonia (UIA; mM). The period of zebrafish hatch (48 - 72 hpf) according to Kimmel and collaborators (1995) is also indicated. Legend: n.d. = not determined.

2.4.2 Acute ammonia toxicity in adults

The experiments of ammonia acute toxicity, in adult zebrafish subjected to different environmental conditions, were conducted to determine the 24, 48, 72 and 96 h LC_{50} values (Figure 2.2). In freshwater, the LC_{50} value was 0.170 (0.142 - 0.204) mM UIA at 24 h and 0.144 (0.124 - 0.166) mM UIA at 48 h, remaining in this value until the end of the experiment. In this sense, and since the 95 % confidence intervals overlapped between the

different time points, the LC_{50} values were not statistically different during the time of exposure. On the other hand, ammonia toxicity in zebrafish acclimated to brackish water was 0.118 (0.112 - 0.124) mM UIA at 24 h but slightly changed to 0.108 (0.096 - 0.122) mM UIA at 96 h of exposure (Figure 2.2). As in freshwater, no statistical differences were observed among the times of exposure. However, when we compared the LC_{50} values between freshwater and brackish water, the fish subjected to brackish water were significantly more sensitive to ammonia, during the entire experiment (Figure 2.2).

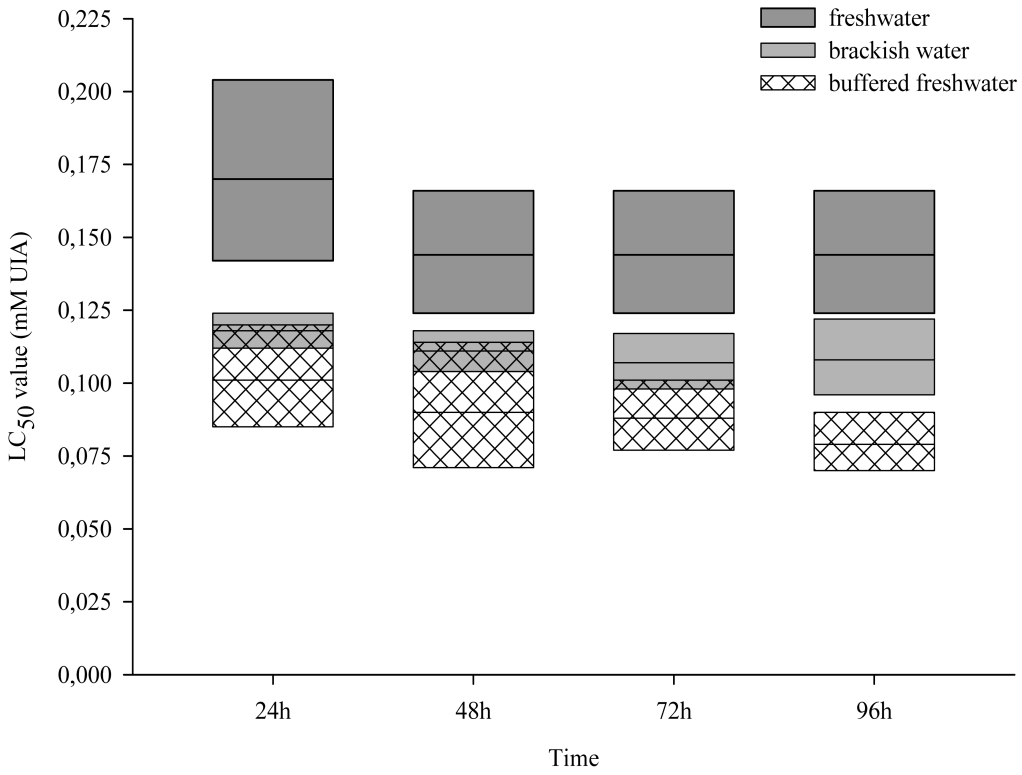


Figure 2.2 LC_{50} values and the respective 95 % confidence intervals for adult zebrafish exposed to ammonia in freshwater, brackish water or buffered freshwater (pH 8). The endpoints were analyzed at 24, 48, 72 and 96 h. The LC_{50} and the 95 % confidence intervals were corrected for pH 8 [equation on USEPA (1999)] and are present as un-ionized ammonia (UIA; mM).

The effects of pH 8 buffered freshwater on the LC_{50} values were also determined being this value initially 0.101 (0.085 - 0.120) mM UIA at 24 h but passing to 0.079 (0.070 - 0.090) mM UIA at 96 h of exposure (Figure 2.2). Similar to the other conditions, no significant differences were associated with the time of exposure. When compared to freshwater, the presence of buffer significantly increased the susceptibility to ammonia during the 96 h of exposure (Figure 2.2).

The water physicochemical parameters in each experiment are present in Table 2.2. In

general, pH, temperature, dissolved oxygen and salinity, were relatively constant over the 96 h of ammonia exposure. No moribund fish were detected in the control groups; however, in all experiments, the higher ammonia concentrations recorded the higher percentage of moribund fish.

2.5 Discussion

Although some studies have suggested that early developmental stages of teleost fishes were less tolerant to ammonia exposure than later stages (Benli and Köksal, 2005), in zebrafish these stages are either more tolerant (embryos) or as tolerant (larvae) as adults. During the endogenous feeding period in embryos and larvae, ammonia can be produced through the catabolism of yolk amino acids (Randall and Tsui, 2002; Kamler, 2008) and despite embryos being able to excrete some ammonia (Wright et al., 1995; Rahaman-Noronha et al., 1996; Hung et al., 2008), most of this nitrogenous waste is accumulated in the egg (Wright et al., 1995; Terjesen et al., 1997). This is likely due to circulatory system and gills undeveloped (Randall and Tsui, 2002) and indirect contact with the external water (Steele et al., 2001). The build up of ammonia inside the egg may therefore contribute for a higher sensitivity to ammonia for some teleost fishes during early developmental stages than in the later post-hatch stages. Species such as Nile tilapia *O. niloticus* L. (Benli and Köksal, 2005) presents an increase in the ammonia tolerance during their development, with a higher resistance in later stages among the entire life cycle. Other species are instead more tolerant to ammonia during early life stages of development when compared with the adult stage including the green sunfish *Lepomis cyanellus* (McCormick et al., 1984), spotted seatrout *Cynoscion nebulosus* (Daniels et al., 1987), gulf toadfish *Opsanus beta* (Barimo and Walsh, 2005) and smallmouth bass *Macropterus dolomieni* Lacepede (Broderius et al., 1985). Our results in zebrafish indicate that embryos are more tolerant to ammonia than later stages since the highest 24 h LC_{50} value was obtained in the initial developmental stage (19 - 26 hpf) (Figure 2.1). However, after this transient higher value, the LC_{50} decreased until values close to the 24 h LC_{50} in adults and since the 95 % confidence intervals at 168 hpf (the last stage studied) and adults overlaps, we may consider that the toxicity of ammonia stabilizes after 168 hours post-fertilization in juvenile and adult stages. In contrast to our results, Jeffries and collaborators (2014) were unable to detect significant differences in ammonia tolerance between embryonic and larval zebrafish stages using LC_{50} tests, which can be explained by differences in the experimental design. However, this decrease in the ammonia tolerance

during the zebrafish development was also mentioned before by Feldman and collaborators (2014); although it was not the main goal of their work determine the LC_{50} values of the different developmental stages.

This transition in ammonia tolerance observed in the present study during zebrafish development is clearly marked by hatching and loss of the protective chorion but it is also related with the conversion of ammonia to non-toxic compounds. In fact, embryos are surrounded by an acellular layer named chorion or egg capsule that may protect them from many environmental contaminants since its permeability is relatively low (Cotelli et al., 1988), making it relevant to toxicity once it increases the tolerance of the embryo (Cotelli et al., 1988; Guadagnolo et al., 2001; Braunbeck et al., 2005; Finn, 2007). However, the presence of the chorion may also restrict the exchange with bulk water (Steele et al., 2001) and together with undeveloped gills and circulatory system (Randall and Tsui, 2002) may lead to an accumulation of toxic ammonia inside the embryos and larvae. In this sense, the fact that, in zebrafish, ammonia excretion increases after hatching (Braun et al., 2009a) may suggest that the chorion is relatively impermeable to ammonia (Smith, 1947), functioning as a physical barrier to ammonia excretion. Nonetheless, some studies have mentioned that some molecules such as oxygen, CO_2 and even ammonia may penetrate the chorion (Rahaman-Noronha et al., 1996; Braun et al., 2009a), which indicate that chorion may not completely explain the lower susceptibility of zebrafish during early development. According to Braun and collaborators (2009a), ammonia excretion in zebrafish embryos and larvae is, instead, limited by a lack of ammonia transporters, which impede its elimination until 3 days post-fertilization and makes relevant the presence of other mechanism(s) to deal with ammonia accumulation during this period (Braun et al., 2009a). In this sense, the higher embryonic tolerance to ammonia observed in this study can also be explained by the presence of a detoxification strategy that protects the fish during this sensitive stage through the conversion of ammonia to urea (Wright et al., 1995; Randall and Tsui, 2002). This mechanism was first proposed and explained by Griffith (1991) and afterwards the enzymes of the ornithine urea cycle (OUC) were detected in the early life stages of rainbow trout *O. mykiss* (Wright et al., 1995; Korte et al., 1997; Steele et al., 2001), Atlantic cod *Gadus morhua* L. (Chadwick and Wright, 1999), gulf toadfish *O. beta* (Barimo et al., 2004), air-breathing walking catfish *Clarias batrachus* (Kharbuli et al., 2006), African catfish *Clarias gariepinus* (Terjesen et al., 1997; Terjesen et al., 2001), Atlantic halibut *Hippoglossus hippoglossus* (Terjesen et al., 2002), pacu *Piaractus mesopotamicus* (Monzani and Moraes, 2008) and also in zebrafish *D.*

rerio (Braun et al., 2009a; Bucking et al., 2013; LeMoine and Walsh, 2013). The presence of an active OUC during early stages of development will then increase the production of urea (Wright et al., 1995), which triggers an increase in the expression of the specific urea transporters (UTs), responsible for urea excretion in embryos and larvae, as already detected in previous studies during this early stages (Pilley and Wright, 2000; Hung et al., 2008; Braun et al., 2009a, 2009b); however, UTs seems to be absent in the chorion, which impairs the urea elimination through this membrane (Wright et al., 1995). It is important to mention that the majority of teleosts that have an active OUC during their early life stages, lose this mechanism during the development, retaining only residual or incomplete hepatic OUC enzyme activities in juveniles and adult (Wright et al., 1995; Korte et al., 1997; Felskie et al., 1998; Kong et al., 1998; Chadwick and Wright, 1999; Randall and Tsui, 2002); nevertheless, some studies suggested that all or some of these enzymes can be active in extra-hepatic tissues (Korte et al., 1997; Felskie et al., 1998; Kong et al., 1998). Moreover, another mechanism that also seems to be active during these stages is the conversion of ammonia to GLN, since the mRNA levels of glutamine synthetase (GS) change during the development of some aquatic organisms (Barimo et al., 2004; Essex-Fraser et al., 2005). In fact, this production of GLN by GS can be relevant since GLN may be stored or shuttled to the pathway of OUC (Essex-Fraser et al., 2005; Monzani and Moraes, 2008). Another explanation for this high tolerance at early life stages of zebrafish development, especially during restricted ammonia excretion, is the upregulation of ammonia transporters, namely the Rhesus (Rh) glycoproteins (Hung et al., 2008; Braun et al., 2009a, 2009b), and also of the V-type H^+-ATPase (Braun et al., 2009b), which allow the continuous excretion of ammonia in zebrafish embryos and larvae even against gradient.

From the experiments in adults, it was possible to determine that zebrafish are relatively tolerant to ammonia in control freshwater, and that sensitivity increased when fish were exposed to brackish water and pH 8 Tris-buffered freshwater (Figure 2.2). After the exposure to ammonia in freshwater over 96 h, zebrafish had a LC_{50} value of 0.144 mM UIA at pH 8. Based on the recent LC_{50}/EC_{50} values published by USEPA (2013) (values standardized for pH 8 in Table 2.3), we verify that zebrafish is relatively tolerant to ammonia with only two other more tolerant species, namely, striped bass *Morone saxatilis* and threespine stickleback *Gasterosteus aculeatus* (USEPA, 2013). However, the LC_{50} value after 96 h of ammonia exposure during ionic strength or pH 8 buffered freshwater decreased significantly to 0.108 and 0.079 mM UIA, respectively (Figure 2.2). It is already known that salinity may modulate

ammonia toxicity (USEPA, 1999, 2013; Randall and Tsui, 2002) and that branchial permeability of seawater fishes (that present a "leaky" gill epithelia) is enhanced for ammonium, which explains the higher toxicity of ammonia in seawater species (Wilson and Taylor, 1992; Wilkie, 2002; Eddy, 2005). Moreover, Altinok and Grizzle (2004) showed that the acclimation of freshwater stenohaline and euryhaline fishes to low salinities (till 9 ‰) might alter the excretion of urea as a percentage of the TA-N excretion (ammonia plus urea), with some species increasing (like the freshwater stenohaline species and the Gulf sturgeon *Acipenser oxyrinchus desotoi*) and other decreasing the urea excretion rates (most of the euryhaline species). In this sense, in a future study, it should be determined if it is the excretion of ammonia or the branchial permeability or even both that cause a decrease on ammonia tolerance of zebrafish. However, it is important to mention that, contrarily to this study, in other species, such as, cobia *Rachycentron canadum* (Barbieri and Doi, 2012), an increase in salinity causes an increase of ammonia tolerance. Water pH is another parameter that highly influences ammonia toxicity (Randall and Tsui, 2002; Eddy, 2005; USEPA, 1999, 2013). Indeed, the excretion of ammonia occurs through a transbranchial NH_3 gradient maintained by an acidic, unstirred gill boundary layer, that traps NH_3 in the water as NH_4^+ (Wilkie, 2002; Weihrauch et al., 2009; Wright and Wood, 2009; Ip and Chew, 2010). The low pH in the boundary layer is maintained by CO_2 hydration and H^+ excretion, which allows the continuous movement of NH_3 following its partial pressure gradient (Wright et al., 1989; Wilkie, 2002; Weihrauch et al., 2009; Wright and Wood, 2009). The presence of buffer in the water impairs the acidification of the boundary layer since H^+ bind to the buffer (Wright et al., 1989; Wilson et al., 1994; Rahaman-Noronha et al., 1996; Wilkie, 2002), resulting in a decrease of the ammonia excretion rate and leading to the accumulation of ammonia inside the organism (Wright et al., 1989; Rahaman-Noronha et al., 1996; Wilkie, 2002; Weihrauch et al., 2009; Wright and Wood, 2009; Ip and Chew, 2010). This decrease in ammonia excretion, due to the presence of buffer in the aquatic environment, was previously observed in weather loach *Misgurnus anguillicaudatus* (Moreira-Silva et al., 2010), zebrafish larvae (Kumai and Perry, 2011) and rainbow trout embryos (Rahaman-Noronha et al., 1996) and adults (Wilson et al., 1994; Salama et al., 1999) and may explain the decrease in the LC_{50} value when Tris buffer were added to freshwater

Table 2.3 Species mean acute value (SMAV) present in USEPA (2013) for 44 freshwater fish species. All values were present as total ammonia-nitrogen (TA-N; $mg \cdot l^{-1}$) but were recalculated for un-ionized ammonia (UIA; mM) at pH 8 [equation on USEPA (1999)] for comparison with the results present in this study.

Species	SMAV mg TAN·l^{-1}	Correct to pH 8 mM UIA
Prosopium williamsoni	51.930	0.027
Micropterus treculii	54.520	0.029
Deltistes luxatus	56.620	0.030
Notemigonus crysoleucas	63.020	0.033
Chasmistes brevirostris	69.360	0.036
Morone saxatilis x chrysops	70.220	0.037
Etheostoma nigrum	71.450	0.037
Hybognathus amarus	72.550	0.038
Poecilia reticulata	74.660	0.039
Etheostoma spectabile	77.170	0.040
Lepomis gibbosus	77.530	0.041
Oncorhynchus clarkii	78.920	0.041
Cyprinella whipplei	80.940	0.042
Oncorhynchus tshawytscha	82.390	0.043
Oncorhynchus mykiss	82.880	0.043
Cyprinella spiloptera	83.800	0.044
Micropterus salmoides	86.020	0.045
Oncorhynchus kisutch	87.050	0.046
Notropis topeka	96.720	0.051
Salmo trutta	102.000	0.053
Lepomis macrochirus	104.500	0.055
Cyprinus carpio	106.300	0.056
Oncorhynchus aguabonita	112.100	0.059
Campostoma anomalum	115.900	0.061
Sander vitreus	117.100	0.061
Morone americana	132.700	0.069
Catostomus platyrhynchus	136.200	0.071
Ictalurus punctatus	142.400	0.075
Morone chrysops	144.000	0.075
Micropterus dolomieu	150.600	0.079
Lepomis cyanellus	150.800	0.079
Salvelinus fontinalis	156.300	0.082
Acipenser brevirostrum	156.700	0.082
Catostomus commersonii	157.500	0.082
Pimephales promelas	159.200	0.083
Salvelinus namaycush	159.300	0.083
Oncorhynchus gorbuscha	180.700	0.095
Salmo salar	183.300	0.096
Oreochromis mossambicus	185.200	0.097
Cyprinella lutrensis	196.100	0.103
Gambusia affinis	219.300	0.115
Cottus bairdii	222.200	0.116
Morone saxatilis	246.200	0.129
Gasterosteus aculeatus	281.500	0.147

2.6 Conclusions

Although several studies have already demonstrated some of the specific effects of ammonia on zebrafish gene expression and foremost that ammonia may constitute a huge problem for fish husbandry, less attention has been given to the basic characterization of ammonia toxicity in this species. With this study, we found that early embryos (until 26 hours post fertilization) are the most ammonia tolerant stage during the life cycle of zebrafish, which might be related with the ability of early-stage embryos to convert ammonia to less toxic compounds and also due to the presence of the chorion. Moreover, zebrafish in the adult stage were more tolerant in control freshwater, whereas increasing ionic strength and water buffering impaired their resistance to ammonia toxicity.

CHAPTER 3 |

TRANSCRIPTOMIC RESPONSE TO ENVIRONMENTAL AMMONIA EXPOSURE IN ADULT ZEBRAFISH GILL, LIVER AND BRAIN

3.1 Abstract

Ammonia is a toxic compound produced in liver during the amino acid catabolism, being after transported to the gills, where it elimination to the environment occur. However, high environmental ammonia compromises it excretion causing ammonia accumulation inside the organism, which trigger several negative effects, mostly at the central nervous system. To better understand the ammonia toxicity mechanisms, zebrafish *Danio rerio* were exposed to a sub-lethal concentration of NH_4Cl during 96 h and the mRNA from gill, liver and brain were profiled with a commercial 44k Agilent Zebrafish Microarray. Gills showed the most pronounced response, which is explained by its direct contact with environmental ammonia but also by its relevance as a protective barrier to contaminants. Moreover, gill and liver tissues had the energy metabolism affected by ammonia exposure being the carbohydrates, instead of lipids, used as energy supplier. On the other hand, in gill and brain, the environmental ammonia decreased the metabolism of vitamins, namely of retinol (vitamin A) and folate (vitamin B_9) vitamins. Beyond this, genes involved in the apoptosis were also activated in these tissues, which together with the previous data, indicate that ammonia affect the proliferation, cycle and death of the cells.

Moreover, although ammonia changed several transcellular transport elements, it also affected elements of paracellular movement, probably altering the epithelial permeability and impeding the entrance of ammonia. In this sense, our results provide further insights of how zebrafish deal with ammonia toxicity, mainly in tissues that are closely related with it production, excretion and toxic action.

3.2 Introduction

Ammonia is a nitrogenous waste compound that can be accumulated inside the aquatic organisms but also in the environment. Aquatic animals produce and excrete toxic ammonia directly to the environment; thus, high animal densities can result in high concentrations of environmental ammonia, which can be a concern in aquacultures (Tomasso, 1994; Eddy, 2005) since ammonia impairs fish growth (Rasmussen and Korsgaard, 1996; Björnsson and Ólafsdóttir, 2006; Foss et al., 2007) and health (Gonçalves et al., 2012). In addition, several natural and anthropogenic sources may also increase the levels of ammonia in the water, such as spills, industrial discharges and emissions, agricultural runoff, urban discharge, mineralization of feces and uneaten food, municipal wastewater, volcanic activity, release of fertilizers, gas exchange with the atmosphere and forest fires (Hargreaves, 1998; Randall and Tsui, 2002; Eddy, 2005; USEPA, 2013). In aquatic solution, ammonia exists in two chemical forms, namely NH_4^+ (ionized ammonia or ammonium) and NH_3 (unionized ammonia or UIA), and the sum of these forms is known as total ammonia-nitrogen (TA-N) or simply as ammonia. The NH_3 form is relatively lipid soluble (Ip and Chew, 2010) and hence, able to cross the lipid membranes directly (Randall and Tsui, 2002) or even through ammonia transporter, known as Rhesus (Rh) glycoproteins (Wright and Wood, 2009). In this sense, NH_3 is often considered the more environmentally toxic form (USEPA, 2013). However, it is the NH_4^+ form that contributes to ammonia toxicity within the animal (Randall and Tsui, 2002) since at physiological pH (7.0 - 7.8), most of the ammonia is present as NH_4^+ and even the NH_3 that enters in the body is converted to the ionized form.

Inside the organism, ammonia production occurs mostly in the liver through the catabolism of dietary and/or structural amino acids and proteins. In fact, the ingestion of proteins by teleost fishes will be used for growth and energy production, with the excess of amino acids converted to carbohydrates and lipids (Ip and Chew, 2010). It is during this catabolism of proteins and amino acids that nitrogenous wastes, as ammonia, are produced and released inside the organism (Mommsen and Walsh, 1992; Wilkie, 2002; Ip and Chew,

2010). More precisely, ammonia results from the transdeamination of amino acids that occur in tissues such as kidney, intestine (Karlsson et al., 2006; Tng et al., 2008), gill and skeletal muscle, but mostly in liver (Karlsson et al., 2006; Tng et al., 2008; Ip and Chew, 2010). The transdeamination inside of the hepatocytes occurs in the cytosol by aminotransferases [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] and in the mitochondria by deaminases, with one of the most active deaminating enzymes in the mitochondria being glutamate dehydrogenase (GDH) (Ip and Chew, 2010). Moreover, within the liver, this catabolism of amino acids is mainly controlled by the concentration of amino acids, rather than the enzymatic activity.

Despite of being produced inside the organism, ammonia is toxic and has to be eliminated to the aquatic environment, which occur by paracellular and/or transcellular movement of ammonia mostly through branchial epithelium (Wright and Wood, 2009). Actually, after its production in the liver, ammonia is released into the hepatic vein and transported by the circulatory system to the gills to be excreted to the environment (Wright and Wood, 2009; Ip and Chew, 2010); however, ammonia may also be converted inside the organism into less toxic compounds such as glutamine (GLN) and/or urea (Randall and Tsui, 2002) thus avoiding the deleterious effects of ammonia. In this sense, ammonia excretion varies greatly with the catabolism of dietary (Dosdat et al., 1996) and/or structural proteins. The excretion of ammonia through the gills is done following the partial pressure gradient of NH_3 (ΔP_{NH3}) from blood-to-water, crossing the branchial membranes by simple diffusion of NH_3 and/or through transporter proteins (Wright and Wood, 2009; Ip and Chew, 2010; Nawata et al., 2010b). Regardless of the pathway, the presence of an acidic boundary layer close to the branchial epithelium has an important role in the maintenance of ammonia excretion because once outside the body, NH_3 will trap a H^+ in the boundary layer and form NH_4^+ (Wilson et al., 1994; Salama et al., 1999; Wright and Wood, 2009). The acidification of the boundary layer is, in turn, ensured by H^+ excretion and CO_2 hydration (Wright et al., 1989; Sullivan et al., 1995; Wilson et al., 2000; Wilkie, 2002; Ivanis et al., 2008; Wright and Wood, 2009). In fact, the excretion of ammonia has been studied extensively in the last years and recently Wright and Wood (2009) presented an updated model to explain the mechanism of ammonia elimination in freshwater fish. This model suggests that ammonia cross basolaterally by a member of the ammonia transporter superfamily, the Rh glycoprotein B (Rhbg), and apically by another member, the Rh glycoprotein C (Rhcg). This movement of ammonia is done as NH_3, resultant from the deprotonation of NH_4^+, with the H^+ able to pass

the branchial epithelium by H^+-ATPase and/or Na^+/H^+ exchanger (NHE), thus contributing to the acidification of the boundary layer (Wright and Wood, 2009).

Besides ammonia production, aquatic organism may also accumulate this toxic compound due to its entrance from the environment, which leads to deleterious effects that, at the limit, may cause the death of the fish (Randall and Tsui, 2002). The presence of ammonia in the environment may interfere with ammonia excretion and even cause an inversion of the gradient diffusion (Wilson et al., 1994; Randall and Tsui, 2002; Nawata et al., 2007; Wright and Wood, 2009; Barbieri and Doi, 2012) allowing the entrance of ammonia and its accumulation inside the body (Randall and Tsui, 2002; Wright and Wood, 2009). This situation may lead to several negative effects in different organs (Dhanasiri et al., 2013) but most notably in the central nervous system (CNS) (Randall and Tsui, 2002). In liver ammonia causes morphological and structural damages of the hepatocytes (Benli et al., 2008) and even a decrease in liver mass (Milne et al., 2000; Spencer et al., 2008). On the other hand, in gill, high levels of ammonia conduct to structural damages of the branchial epithelium (Benli and Köksal, 2005; Benli et al., 2008; Spencer et al., 2008; Wright and Wood, 2012) that impact on the oxygen uptake (Lease et al., 2003; Wright and Wood, 2012) and also causes disturbances in the osmo- and ionoregulation as well as in the acid-base regulation (Lease et al., 2003). In the CNS, ammonia is implicated in astrocyte swelling and cerebral edema, modification of the blood-brain barrier (BBB) properties, alterations of the amino acid transport, imbalance of cerebral blood flow, interfere with excitatory amino acid neurotransmitter metabolism namely glutamate (GLU) and aspartate, morphological changes in the astrocytes and neurons, disruption of cerebral mitochondrial function, ATP depletion and finally neuronal cell death (Albrecht, 1998; Felipo and Butterworth, 2002; Albrecht and Norenberg, 2006; Ip and Chew, 2010; Wilkie et al., 2011; Feldman et al., 2014). Besides the effects in these tissues, ammonia may also causes impairment of energy metabolism (Tomasso, 1994), increase the oxygen consumption (Adams et al., 2001; Barbieri and Doi, 2012) and ventilation frequency (Adams et al., 2001; Benli and Köksal, 2005), disturbances of the electrochemical gradient (Shingles et al., 2001), decrease in the immune system (Connon et al., 2011; Gonçalves et al., 2012; Dhanasiri et al., 2013), decrease of the growth rate (Rasmussen and Korsgaard, 1996; Björnsson and Ólafsdóttir, 2006; Reddy-Lopata et al., 2006; Foss et al., 2007), deterioration of tissues structures (Benli and Köksal, 2005; Benli et al., 2008), compromise the reproductive capacity (Abbas, 2006), behavioral changes (Benli and Köksal, 2005; Spencer et al., 2008), decrease feeding (Rodrigues et al., 2007), change swimming performance (Benli

and Köksal, 2005; Rodrigues et al., 2007), interfere with production, chemistry and structure of blood (Abbas, 2006) and at the limit, ammonia provokes loss of physical equilibrium, convulsions and even death (Randall and Tsui, 2002; Benli and Köksal, 2005).

Beyond the negative physiological effects already described due to ammonia exposure, research has been done to determine the responses at the molecular level of the teleosts to high environmental ammonia (Wright and Wood, 2009; Ip and Chew, 2010; Dhanasiri et al., 2013). During the last years, there has been an investment in the development of microarray technologies that allow a rapid screening of transcriptomic changes caused by a toxicant exposure (Dhanasiri et al., 2013; Hagenaars et al., 2013). In this sense, the use of aquatic model species, namely zebrafish *Danio rerio* (Dhanasiri et al., 2013; Hagenaars et al., 2013; Hussainzada et al., 2014), has been important since its genome is already annotated (ensembl.org) but also because this species is easy to manipulate, has low husbandry costs, small size and a high number of offspring (Kimmel et al., 1995; Westerfield, 2000; Parichy et al., 2009; Hussainzada et al., 2014). With all this in mind, the main objective of this study was to determine the transcriptional response of gill, liver and brain of zebrafish after 96 h of exposure to a sub-lethal dose of ammonia. To achieve this objective, a commercially available 44k Agilent Zebrafish Microarray was applied to each tissue, followed by a Gene Ontology (GO) term and pathway analysis of the genes that responded significantly to ammonia exposure. With this work, the main outcome is a better understanding of the genes and pathways that are affected by this toxic compound in each tissue and also discover the mechanisms developed by zebrafish to avoid the ammonia toxicity, especially at the CNS.

3.3 Material and Methods

3.3.1 Zebrafish maintenance

Adult zebrafish *D. rerio* (body mass of 0.355 ± 0.000 g) were purchased in a local pet store in Toronto, Canada, and were transported to the Biology Department of the University of Waterloo. In the lab, fish were acclimated in tanks (3 L) containing dechlorinated local tap water in a recirculation rack system with filtration, aeration and temperature controlled. During two weeks of acclimation, fish were fed with commercial pellet food twice a day and the tanks were siphoned daily to remove uneatened food and feces. The photoperiod was 14 h Light : 10 h Dark and temperature was 28 ± 1 ºC.

All the experiments done for this study were carried out under the guidance of a Laboratory Animal Science certified supervisor (1005/1092, DGV-Portugal, C category, FELASA), according to European Union directives (2010/63/UE).

3.3.2 Experimental design

Forty-eight hours before the start of the experiment, eight randomly selected fish were transferred to each experimental tank to acclimatize to the exposure holding conditions. Each tank was supplied with artificial water [60 mg of Instant Ocean Sea Salt (Blacksburg, VA) in 1 L of distilled water (Liu et al., 2006; Boyle et al., 2010; Sison and Gerlai, 2011)] with pH previously adjusted to 7 with NaOH (Merck, Darmstadt, Germany). A semi-static system was then used, consisting of 6 tanks of 2.5 L each, partially submerged in a water bath to maintain tank water temperature close to 28 ºC. Each tank was continuously aerated and the water was partially (20 %) renewed every 24 h. The photoperiod during acclimatization and experiment were 14 h Light : 10 h Dark and water temperature and pH were monitored daily. Also during both acclimatization and experimental periods, fish were fasted to avoid changes in the internal ammonia levels due to metabolic ammonia production following feeding.

The experiment started by increasing the ammonia concentration in half of the tanks (exposure tanks; n = 3) while the other tanks were maintained only with artificial water (control tanks; n = 3). A stock solution of 32.5 mM of ammonium cloride (NH_4Cl) (Merck) was prepared in artificial water with the pH adjusted to 7. The final ammonia concentration in each exposure tank was obtained by adding 500 ml of the stock solution, which increased the nominal ammonia concentration to 6.5 mM NH_4Cl (corresponding to 0.099 mM UIA at pH 8). The concentration of ammonia selected for this study corresponded to a sub-lethal dose when compared with the 96 h LC_{50} value [0.144 (0.124 - 0.166) mM UIA at pH 8] previously determined with Trimmed Spearman-Karber Method (see chapter 2). Both control and exposure tanks were present in triplicate and 40 % of the water was daily renewed with the correct concentration of ammonia and pH. Continuous aeration was supplied to guarantee high levels of dissolved oxygen and adequate mixing of ammonia solutions.

Mortality and water pH, temperature and TA-N concentration were monitored daily. Temperature and pH were measured with mercury thermometer and Fisher Scientific Accumet pH meter (Pittsburgh, Pennsylvania, USA), respectively, and TA-N was measured as described below. All these parameters are reported as mean ± coefficient of variation. There were no observed differences in behavior or mortality in any tank.

After 96 h of ammonia exposure, fish were euthanized with buffered tricaine methanesulphonate (MS222; Syndel Laboratories Ltd., Nanaimo, British Columbia, Canada) and weighted. Gill, liver and brain (with pituitary gland) were collected under a microscope, flash frozen in liquid nitrogen and stored at -80 ºC until RNA extraction.

3.3.3 Water ammonia measurements

Water samples were collected to determine the TA-N concentration 1 h prior and 1 h after the water renewal in both control and ammonia exposure tanks. The water samples were immediately acidified after the collection with concentrated nitric acid (HNO_3; Merck) to prevent the loss of the gaseous NH_3 and stored at -20 ºC. The TA-N concentration was measured using a colorimetric assay from Verdouw and collaborators (1978) modified for microplate and were done in triplicate. The UIA concentration was then calculated and corrected to pH 8 (USEPA, 1999). The TA-N and UIA concentrations are both reported as mean ± coefficient of variation and the results are present in mM of UIA.

3.3.4 RNA extraction

The total RNA was isolated from gill, liver and brain (including the pituitary). Gill and liver samples from three individuals, from replicate tanks, were pooled to eliminate the effects of possible differences in ammonia levels between tanks. However, in the case of brain, RNA was extracted from individual fish. The RNA from gill and liver samples were extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and the RNA from brain samples were extracted with the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA, USA), following the respective manufacturer's instructions. To eliminate possible genomic DNA contamination, total RNA was treated with DNase I and stored at -80 ºC until use.

The concentration and A260/A280 ratio of total RNA were measured with a NanoDrop® ND-1000 spectrophometer (Thermo Fisher Scientific, Wilmington, USA) and the RNA integrity were evaluated by gel electrophoresis. Samples containing low quality or low quantity of RNA were rejected from the study.

3.3.5 Microarray

To determine the genomic responses of zebrafish to ammonia exposure, a commercially-available 4 x 44K Zebrafish Gene Expression Microarray (Product No. G2519F, Design ID 019161, version 2.0) manufactured by Agilent Technologies (Santa

Clara, California, USA) was employed. The integrity of the RNA was additionally confirmed using an Agilent 2100 BioAnalyzer (Agilent Technologies) and only the RNA that did not present evidence of degradation was used in the microarray.

Three gill and liver RNA control samples were compared with three gill and liver RNA ammonia exposure samples, respectively. However, for brain, four RNA control samples were compared with four RNA exposure samples. The microarray was processed following Agilent's One-Color Microarray-Based Gene Expression Analysis Protocol (version 6) in the Laboratory for Advanced Genome Analysis in Vancouver Prostate Center (Vancouver, CA), which is an Agilent certified service provider.

3.3.6 Microarray analysis

Each array contains 44,000 oligonucleotides representing 21,904 zebrafish gene targets. All the slide control features (including the positive and negative controls and the landing lights) were excluded from the subsequent analysis. The text outputs were exported to an Excel spread sheet and an update of the list was done using the gene identifying numbers of NCBI and Ensembl. All the probes that were not associated to NCBI or Ensembl numbers or that were identified as unknown following blast analysis were eliminated from future analysis. This reduced the number of probes to 25,764.

Microarray text files were then generated and uploaded to the Subio Platform (version 1.18) (Kagoshima, Japan) and a quantile log 2 normalization followed by a Student's *t*-test was performed for each tissue. The quality of the data was checked with several quality control methods in the platform. Genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05 and an increase or decrease in mRNA level was considered when the fold change (FC) was ≥ 2 and ≤ 0.5, respectively.

Analysis of significantly enriched functional GO categories was carried out using the statistical different genes in each tissue. Enrichment analysis was conducted using the online tools of the Database for Annotation, Visualization and Integrated Discovery (DAVID version 6.7) (Dennis Jr et al., 2003; Huang et al., 2007, 2009) with default settings and the *Danio rerio* Entrez ID as input. These tools were designed to identify functional related gene groups that are significantly enriched with respect to the microarray background (Dennis Jr et al., 2003; Huang et al., 2007, 2009). A GO term over-representation was considered when the *P* was ≤ 0.05. The analyses examined biological process, cellular component and molecular function GO terms. All the individual GO terms enriched in each tissue as well as the number

of genes enriched in each GO term category, are present in supplementary tables (see Supplementary Table 3.3-3.11). The redundant GO classes were deleted to simplify the tables.

Beyond GO term analysis, biological relevant pathways were constructed using DAVID and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa, 1997; Ogata et al., 1998, 1999; Kanehisa and Goto, 2000). As for the GO analysis, the pathways were obtained from the statistical different genes in each tissue. A pathway was considered significantly enriched when the P was ≤ 0.05 and all differentially expressed genes were mapped using the KEGG database (see Supplementary Figures 3.4 - 3.40).

Finally, to integrate the results from the different tissues, a list with the common genes significantly regulated in gill, liver and brain were obtained using Venn diagrams, as well as the list of upregulated and downregulated genes that simultaneously changed between the different tissues. DAVID and KEGG tools were also used to identify the GO terms (see Supplementary Table 3.12-3.15) and pathways, respectively that were commonly affected within these tissues.

3.4 Results

To determine the molecular response of gill, liver and brain to ammonia, the teleost model zebrafish were exposed during 96 h to a sub-lethal dose of this nitrogenous waste compound. During the experiment, no fish died or exhibited abnormal behavior indicative of acute toxic effects. In control groups, the measured TA-N levels were 0.083 ± 0.222 mM, which corresponded to 0.001 ± 0.004 mM UIA at pH 8. However, in the exposure tanks, the measured TA-N concentrations were 9.238 ± 0.086 mM, which correspond to 0.161 ± 0.001 mM UIA at pH 8. It is also important to mention that pH and temperature were 7.128 ± 0.012 and 28.144 ± 0.025 ºC, respectively, remaining relatively stable throughout the experiment.

A two-dimensional hierarchical cluster analysis was performed in the Subio Platform in order to visualize the expression of genes in all tissues and conditions, simultaneously. This analysis takes all the probes into consideration except the stable ones. From Figure 3.1 we may note that the brain profile clustered separately from gill and liver. Moreover, in gill, the samples exposed to ammonia produced a profile that was clearly distinct from the profile produced by the control gill samples. Liver and brain also presented some differences in the hierarchical cluster between exposed and control samples; however, not as pronounced as in gill.

To identify the responsive genes to ammonia, normalization and statistical analyses were performed in the Subio Platform. From a total of 25,764 identifiable probes, gill presented 7,001 (27 %) statistically different and non-stable probes, liver showed 2,169 (8 %) and brain 669 (3 %). In gill 4,206 of these genes were upregulated and 2,795 were downregulated. However, in liver, 1,139 genes were upregulated and 1,030 were downregulated, while in brain, 444 genes were upregulated and 225 were downregulated.

The differentially expressed genes in gill, liver and brain were subjected to GO analysis, using the online DAVID tools, and pathway analysis, using the online KEGG pathway database. In both analyses, $P \leq 0.05$ was used to determine the statistical different categories or pathways. For a better and easy analysis, GO terms and pathways results will be present for each tissue separately.

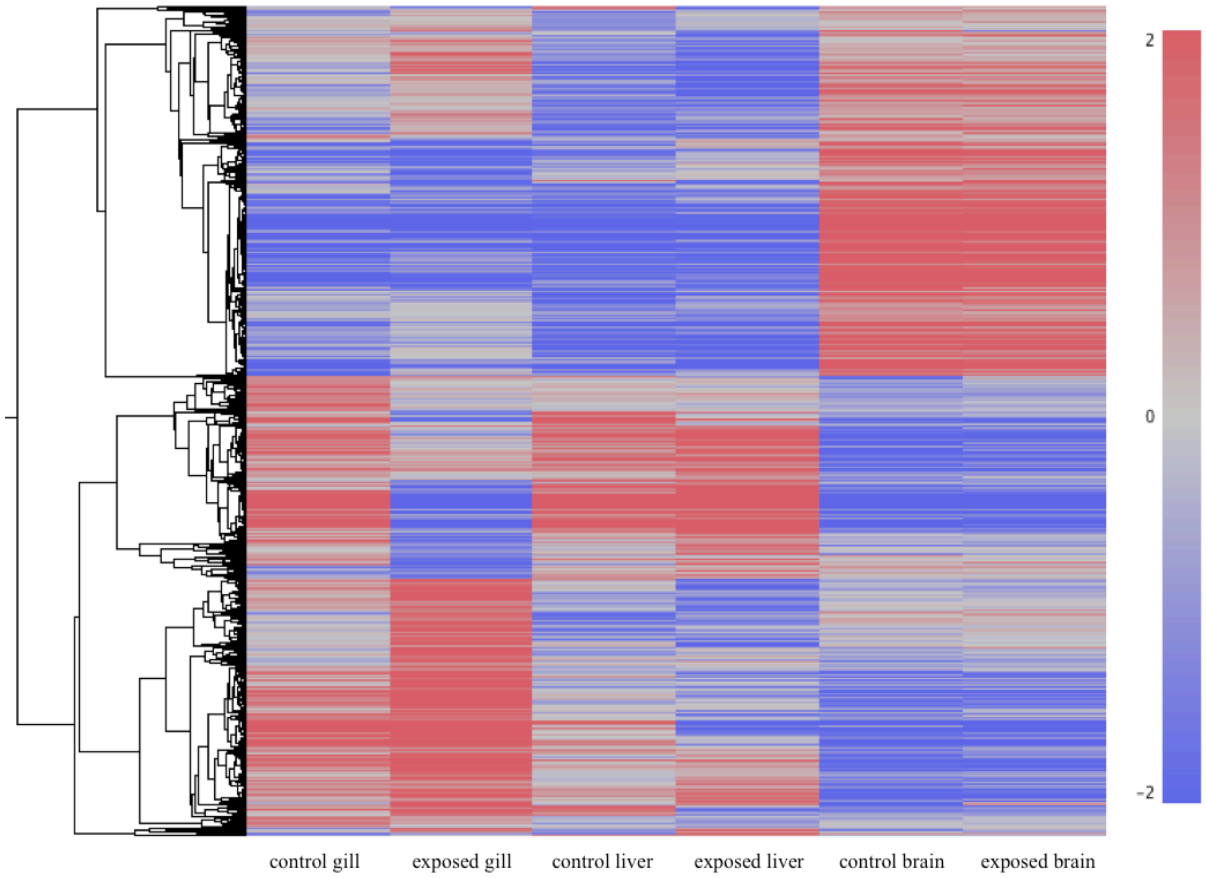


Figure 3.1 Two-dimensional hierarchical cluster analysis designed in Subio Platform (version 1.18). This cluster analysis shows the expression pattern for the control and ammonia exposure samples of gill, liver and brain of zebrafish, previously subject during 96 h to a sub-lethal dose. For this analysis only the stable genes were removed.

3.4.1 GO and pathway analysis for the responsive genes in gill

In gill, a total of 223 GO categories were enriched including 117 biological process terms, 33 cellular component terms and 73 molecular function terms. The GO analysis revealed that in gill, considering the biological process terms, the genes affected by ammonia exposure were mainly involved in metabolic, catabolic and biosynthetic processes, immune response, morphogenesis, response to different biological and chemical stimuli, cellular death, death and apoptosis, cellular organization, cell and tissue regulation, cell growth and tissue development. Moreover, membrane, cytoplasm, extracellular region, cytoskeleton, endoplasmatic reticulum, tight junctions (TJs), peroxisome, microbody, proton-transporting ATPase and intermediate filament were the main cellular component terms enriched. Relative to molecular function terms, genes enriched categories like binding, peptidase, transmembrane transporter, transferase, hydrolase, oxidoreductase, peptidase inhibitor, ATPase and lyase activities (see Supplementary Table 3.3).

Since the biological processes GO terms may reveal further information about the response of zebrafish to environmental ammonia, a more detailed analysis was done. We verified that the majority of the metabolic process GO terms in gill were associated with downregulated genes and only a few categories were related with upregulated genes like regulation of RNA, carbohydrate, alcohol, nucleotides, ribonucleotides, nucleoside, ribonucleoside, ATP and hexose metabolic processes. Moreover, the categories lipid, organic acid, carboxylic acid, coenzyme, vitamins and pyridine nucleotide biosynthetic process GO terms were associated with downregulated genes, while others categories like nucleotide, ribonucleotide, nucleoside, ribonucleoside and ATP biosynthetic processes were related with upregulated genes. However, and although, the genes upregulated dominated the cellular organization GO terms, downregulated genes enriched the peroxisome organization category. On the other hand, the GO terms associated with response to stimulus had categories such as responses to chemical stimulus and oxidative stress enriched by downregulated genes; although, the categories response to biotic stimulus, other organism, inorganic substances and lipopolysaccharide were enriched with upregulated genes. Relatively to the wounding and bacterium response categories, both upregulated and downregulated genes enriched these two categories. The transport GO terms in the categories of ion transmembrane, hydrogen, ATP synthesis coupled proton, organic cation and ammonium transport were enriched by upregulated genes although lipid, mitochondrial and transmembrane transport were associated with downregulated genes. Furthermore, the categories associated with cell and tissue

regulations were mainly enriched with upregulated genes, except the categories regulation of biological quality and hormone levels that were enriched by downregulated genes. Furthermore, the GO terms categories related with morphogenesis, immune response, cellular death, death and apoptosis, tissues development, growth, intracellular signaling cascade, cell cycle, chemical and ion homeostasis, glycolysis and inflammatory response were mainly enriched by upregulated genes. However, the GO term categories related with catabolic processes, oxidation-reduction, proteolysis and blood coagulation were mostly enriched by genes that were downregulated (see Supplementary Table 3.4 and 3.5).

A total of 28 pathways were significantly enriched in gill which belong to 12 different KEGG categories namely carbohydrate metabolism, lipid metabolism, amino acid metabolism, metabolism of cofactors and vitamins, xenobiotics biodegradation and metabolism, global metabolism, transport and catabolism, cell growth and death, cellular community, signaling molecules and interaction, signal transduction and endocrine system. The enriched pathways in each category are present in Table 3.1. It is important to mention that the three most significantly enriched pathways are arachidonic acid metabolism (dre00590; $P = 0.000$), biosynthesis of unsaturated fatty acids (dre01040; $P = 0.000$) and the PPAR signaling pathway (dre03320; $P = 0.001$).

Table 3.1 Pathways enriched by statistical different genes in gill, liver and brain of zebrafish after 96 h of ammonia exposure. This result was obtained after 44k microarray analysis followed by the use of DAVID and KEGG online tools. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. In addition to *P* is possible to see the number of genes differentially expressed (#) and the general regulation (*Reg*) of each pathway (see the Supplementary Figure at the end of the chapter). Legend: ↑↓ - enriched by both downregulated and upregulated genes; ↓↓ - enriched only by downregulated genes; ↑↑ - enriched only by upregulated genes.

KEGG category	Pathway	Gill			Liver			Brain		
		P	**#**	***Reg***	***P***	**#**	***Reg***	***P***	**#**	***Reg***
Metabolism										
Carbohydrate metabolism	dre00520:Amino sugar and nucleotide sugar metabolism	0.003	24	↑↓						
	dre00010:Glycolysis / Gluconeogenesis	0.005	29	↑↓	0.025	11	↑↓			
	dre00051:Fructose and mannose metabolism	0.013	20	↑↓	0.004	10	↑↓			
	dre00630:Glyoxylate and dicarboxylate metabolism	0.017	9	↓↓						
	dre00562:Inositol phosphate metabolism	0.020	27	↑↑						
	dre00640:Propanoate metabolism	0.021	16	↓↓						
	dre00500:Starch and sucrose metabolism	0.029	16	↓↓						
	dre00052:Galactose metabolism	0.040	13	↑↓						

	dre00030:Pentose phosphate pathway				0.011	8	↑↓			
Lipid Metabolism	dre00590:Arachidonic acid metabolism	0.000	21	↑↓						
	dre01040:Biosynthesis of unsaturated fatty acids	0.000	14	↑↓						
	dre00120:Primary bile acid biosynthesis	0.002	10	↓↓						
	dre00592:alpha-Linolenic acid metabolism	0.010	8	↓↓						
	dre00591:Linoleic acid metabolism	0.014	10	↑↓						
	dre00100:Steroid biosynthesis	0.023	10	↓↓						
Amino acid metabolism	dre00260:Glycine, serine and threonine metabolism	0.002	18	↓↓				0.011	5	↑↑
	dre00380:Tryptophan metabolism	0.002	21	↓↓						
Metabolism of cofactors and vitamins	dre00830:Retinol metabolism	0.013	15	↑↓				0.044	4	↓↓
	dre00670:One carbon pool by folate	0.029	9	↓↓						
Xenobiotics biodegradation and metabolism	dre00980:Metabolism of xenobiotics by cytochrome P450	0.012	13	↑↓						
	dre00982:Drug metabolism - cytochrome P450	0.035	12	↑↓						
Global and overview maps	dre00071:Fatty acid metabolism	0.007	18	↓↓						
Cellular Processes										
Transport and catabolism	dre04142:Lysosome	0.007	52	↑↑						
Cell growth and death	dre04210:Apoptosis	0.007	40	↑↑						
	dre04115:p53 signaling pathway	0.032	28	↑↑				0.013	7	↑↑
Cellular community	dre04530:Tight junction	0.002	60	↑↓						
Environmental Information Processing										
Signaling molecules and interaction	dre04514:Cell adhesion molecules	0.001	47	↑↓	0.023	16	↑↓			
Signal transduction	dre04070:Phosphatidylinositol signaling system	0.041	32	↑↓						
Organismal Systems										
Endocrine system	dre03320:PPAR signaling pathway	0.001	30	↓↓						
Circulatory system	dre04260:Cardiac muscle contraction				0.013	14	↑↓			
Genetic Information Processing										
Translation	dre00970:Aminoacyl-tRNA biosynthesis							0.001	7	↑↑

3.4.2 GO and pathway analysis for the responsive genes in liver

In liver, the differentially expressed genes enriched 134 GO terms categories namely 87 biological process terms, 15 cellular component terms and 32 molecular function terms. These differentially expressed genes in liver were mostly involved in metabolic processes,

biosynthetic processes, morphogenesis, tissue development, transport, cell and tissue regulation and muscle cell differentiation and contraction according to biological process. On the other hand, membrane, extracellular region, cytoskeleton, intermediate filament and TJs were the main terms enriched related with cellular components terms. According to molecular function, the main terms enriched were binding, transmembrane transport, transferase, ATPase, lyase and transcription activities (see Supplementary Table 3.6).

In a more detailed analysis of the biological process GO terms, we verified that the upregulated genes enriched the category oxidation-reduction; however, the downregulated genes enriched several categories related with cellular and tissue development, morphogenesis, biosynthetic processes, homeostasis and cytoskeleton organization. Moreover, downregulated genes also enriched all the biological process GO terms associated with muscle, such as, development, cell differentiation and contraction. Metabolic process GO terms in the categories of amino acid and amine metabolic processes were enriched by upregulated genes while nucleotide, ribonucleotide, nucleoside and ribonucleoside metabolic processes were enriched by downregulated genes. Furthermore, the transport GO terms related with the categories ion and metal ion transport were enriched by both upregulated and downregulated genes, while the other categories of transport GO terms were enriched by downregulated genes. The different regulation GO terms, had enrichment of both upregulated and downregulated genes (see Supplementary Table 3.7 and 3.8).

In liver, a total of five pathways were significantly enriched, belonging to carbohydrate metabolism, signaling molecules and interactions and circulatory system (see Table 3.1). The pathways fructose and mannose metabolism (dre00051; $P = 0.004$), pentose phosphate pathway (dre00030; $P = 0.011$) and cardiac muscle contraction (dre04260; $P = 0.013$) were the most statistical different pathways in this tissue.

3.4.3 GO and pathway analysis for the responsive genes in brain

Brain presented the lowest number of statistically different genes and consequently the lowest number of enriched GO terms categories and pathways. The statistical different genes in brain enriched a total of 59 categories including 40 biological process terms, six cellular component terms and 13 molecular function terms. The major biological process terms enriched in brain were metabolic process, biosynthetic process, cellular death, death and apoptosis and response to different biological and chemical stimulus. The cellular component terms enriched were endoplasmic reticulum, membrane-enclosed lumen, organelle lumen,

nuclear lumen and nucleolus, while the main molecular function terms were binding, oxidoreductase and ligase activities (see Supplementary Table 3.9).

The detailed analysis of the biological process terms showed that upregulated genes significantly enriched the GO terms biosynthetic processes, cellular death, death and apoptosis, response to chemical and biological stimulus, oxidation-reduction and tRNA aminoacylation. However, the downregulated genes enriched categories like morphogenesis and tissue development. Of the metabolic process GO terms, almost all enriched categories were associated with upregulated genes, including the category of nitrogen compound; however, the category related with lipid metabolic processes were enriched by downregulated genes (see Supplementary Table 3.10 and 3.11).

In respect to the pathways, only four pathways were statistical different in brain that included the KEGG categories amino acid metabolism, metabolism of cofactors and vitamins, cell growth and death and translation (see Table 3.1). Among these pathways, aminoacyl-tRNA biosynthesis (dre00970; $P = 0.001$), glycine, serine and threonine metabolism (dre00260; $P = 0.011$) and p53 signaling pathway (dre04115; $P = 0.013$) were the most significantly enriched pathways in brain.

3.4.4 Integration of the results from the different tissues

To integrate and better understand the results from the different tissues, Venn diagrams were constructed (Figure 3.2). From their analysis it was possible to determine that 43 genes were significantly and simultaneously altered in gill, liver and brain (Figure 3.2A). Moreover, from these genes, 12 were upregulated in all tissues (Figure 3.2B) and none were simultaneously downregulated in gill, liver and brain (Figure 3.2C). A two-dimensional hierarchical cluster analysis of these 43 genes was also performed, as shown in Figure 3.3. The two GO terms categories enriched by these 43 genes were lipid metabolic processes as biological process term and calcium-dependent phospholipid binding as molecular function term (see Supplementary Table 3.12). Regarding pathway analysis, none was enriched.

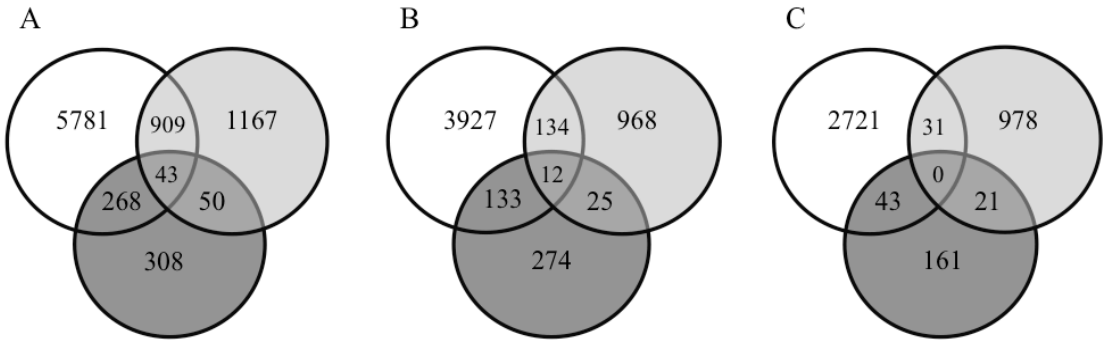


Figure 3.2 Integrative response of gill (white), liver (light grey) and brain (dark grey) of zebrafish after 96 h of exposure to environmental ammonia. The Venn diagrams were obtained through the Subio Platform (version 1.18) and considered all the differentially expressed genes (A) or only the upregulated (B) or the downregulated (C) genes, in each tissue.

When considering gill and liver, a total of 952 (909 + 43) genes were differently expressed in both tissues with 146 (134 + 12) upregulated and only 31 (31 + 0) downregulated simultaneously in gill and liver (Figure 3.2). These 952 genes enriched 93 GO terms including 60 biological processes, 15 cellular components and 18 molecular functions. The main biological process GO terms enriched were cellular and tissue regulation, tissue development, morphogenesis, lipid and steroid metabolic process, cell death and apoptosis, transport of molecules and ions and oxidation-reduction. However, the cellular component GO terms enriched were related with membranes, cell junction, intermediate filament and cytoskeleton, while the molecular function GO terms were related with binding, peptidase, transporter, transferase, hydrolase, oxidoreductase, transcription factor and regulator and transaminase activities (see Supplementary Table 3.13). From the KEGG pathway analysis it was possible to determine that only six pathways were enriched namely, cell adhesion molecules (CAMs) (dre04514; P = 0.021), tight junction (dre04530; P = 0.023), glycolysis/gluconeogenesis (dre00010; P = 0.031), pentose phosphate pathway (dre00030; P = 0.031), phosphatidylinositol signaling system (dre04070; P = 0.034) and drug metabolism (dre00983; P = 0.038) (Table 3.2).

Table 3.2 KEGG pathway analysis for the 952 differentially expressed genes simultaneously altered in gill and liver tissues of zebrafish after 96 h of ammonia exposure. This result was obtained using the DAVID and KEGG online tools. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. In addition to *P* is possible to see the number of genes differentially expressed (#) in each pathway.

KEGG category	Pathway	*P*	# genes
Metabolism			
Carbohydrate metabolism	dre00010:Glycolysis / Gluconeogenesis	0.031	7
	dre00030:Pentose phosphate pathway	0.031	5
Xenobiotics biodegradation and metabolism	dre00983:Drug metabolism	0.038	5
Environmental Information Processing			
Signaling molecules and interaction	dre04514:Cell adhesion molecules	0.021	10
Signal transduction	dre04070:Phosphatidylinositol signaling system	0.034	8
Cellular Processes			
Cellular community	dre04530:Tight junction	0.023	12

Between gill and brain, 311 (268 + 43) genes were commonly altered with 145 (133 + 12) upregulated and only 43 (43 + 0) downregulated in both tissues simultaneously (Figure 3.2). The 311 genes enriched a total of 34 GO terms including 21 biological processes, one cellular component and 12 molecular function terms. The biological process GO terms were associated mainly with metabolic processes, response to chemical stimulus and bacterium, oxidation-reduction and iron ion homeostasis. Although the endoplasmic reticulum was the only cellular component GO term enriched, the 12 categories of molecular functions were associated with binding, oxidoreductase, catalytic, electron carrier, monooxygenase and carbohydrate kinase activities (see Supplementary Table 3.14). From the KEGG analysis, no pathway was enriched when considering the 311 genes commonly altered in gill and brain.

Between liver and brain only 93 (50 + 43) genes were commonly altered with 37 (25 + 12) upregulated and 21 (21 + 0) downregulated in both tissues (Figure 3.2). The GO term analysis showed that only two categories of the biological process terms were enriched, namely, lipid and steroid metabolic processes (see Supplementary Table 3.15). Moreover, the KEGG analysis revealed that these 93 genes enriched no pathway.

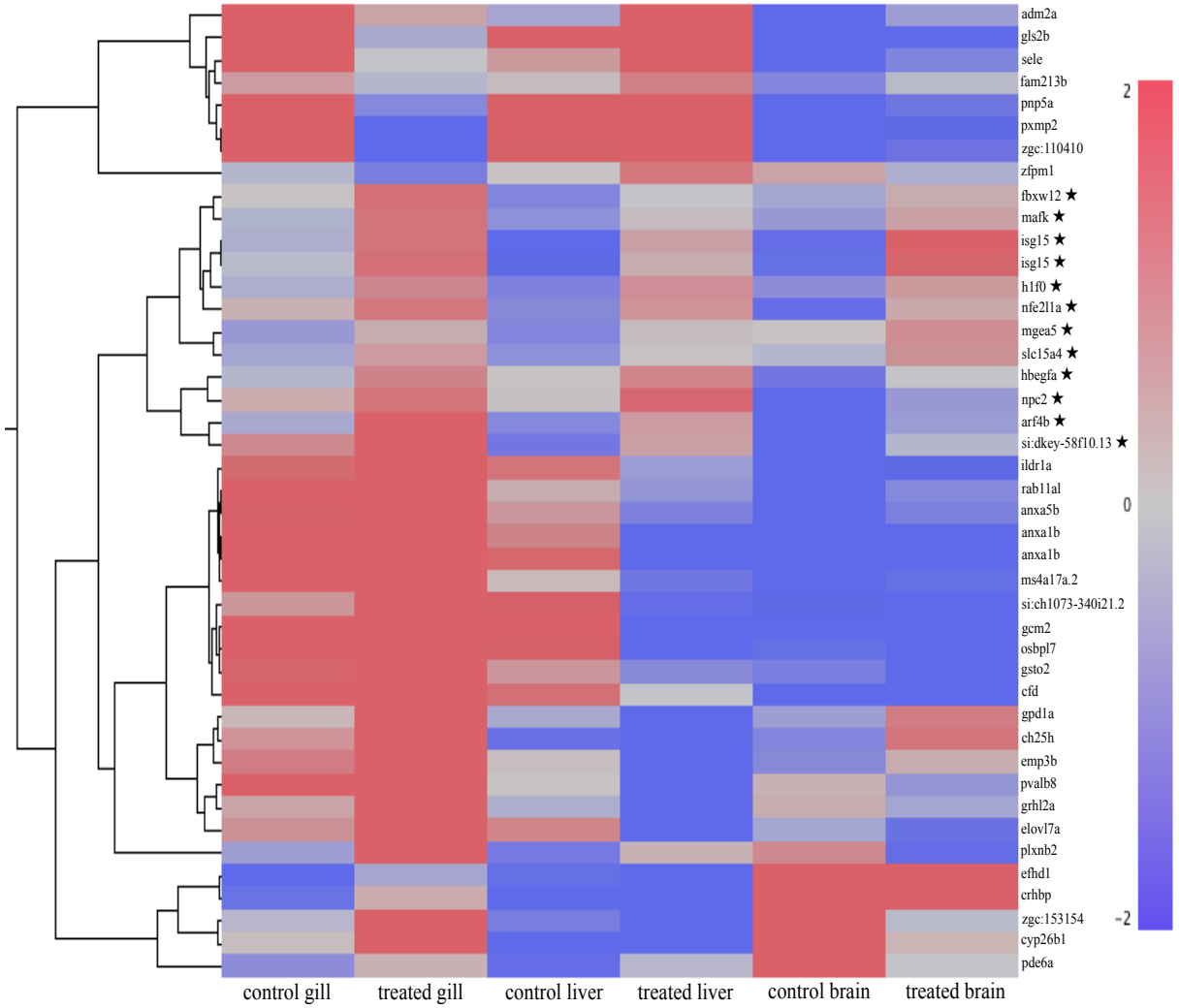


Figure 3.3 Expression pattern for the 43 genes simultaneously altered in gill, liver and brain of zebrafish exposed to ammonia. Legend: *adm2a* - adrenomedullin 2a; *anxa1b* - annexin A1b; *anxa5b* - annexin A5b; *arf4b* - ADP-ribosylation factor 4b; *cfd* - complement factor D (adipsin); *ch25h* - cholesterol 25-hydroxylase; *crhbp* - corticotropin releasing hormone binding protein; *cyp26b1* - cytochrome P450, family 26, subfamily b, polypeptide 1; *efhd1* - EF-hand domain family, member D1; *elovl7a* - ELOVL family member 7, elongation of long chain fatty acids a; *emp3b* - epithelial membrane protein 3b; *fam213b* - family with sequence similarity 213, member B; *fbxw12* - F-box and WD repeat domain containing 12; *gcm2* - glial cells missing homolog 2; *gls2b* - glutaminase 2b (liver, mitochondrial); *gpd1a* - glycerol-3-phosphate dehydrogenase 1a; *grhl2a* - grainyhead-like 2a; *gsto2* - glutathione S-transferase omega 2; *h1f0* - H1 histone family, member 0; *hbegfa* - heparin-binding EGF-like growth factor a; *ildr1a* - immunoglobulin-like domain containing receptor 1a; *isg15* - ISG15 ubiquitin-like modifier; *mafk* - v-maf musculoaponeurotic fibrosarcoma oncogene homolog K; *mgea5* - meningioma expressed antigen 5 (hyaluronidase); *ms4a17a.2* - membrane-spanning 4-domains, subfamily A, member 17a.2; *nfe2l1a* - nuclear factor, erythroid derived 2, -like 1a; *npc2* - Niemann-Pick disease, type C2; *osbpl7* - oxysterol binding protein-like 7; *pde6a* - phosphodiesterase 6A, cGMP-specific. rod. alpha; *plxnb2* - plexin B2; *pnp5a* - purine nucleoside phosphorylase 5a; *pvalb8* - parvalbumin 8; *pxmp2* - peroxisomal membrane protein 2.22 kDa; *rab11al* - RAB11a, member RAS oncogene family, like; *sele* - selectin E; *slc15a4* - solute carrier family 15, member 4; *zfpm1* - zinc finger protein, multitype 1; ⋆ - genes that were simultaneously upregulated in gill, liver and brain tissues of exposed fish.

3.5 Discussion

Although the ammonia level used in this study did not cause any abnormal behavioral or morphological effect in zebrafish, the presence of ammonia in the water induced alterations in the transcriptome of liver and brain, and most dramatically in gill. During the experiment, although adults zebrafish were exposed to a sub-lethal concentration of ammonia over 96 h, the dosage of ammonia was slightly higher (0.161 ± 0.001 mM UIA at pH 8) than the 96 h LC_{50} value previously determined in the lab [0.144 (0.124 – 0.166) mM UIA at pH 8, chapter 2]; however, this concentration was within the 95 % confidence interval. When control and ammonia exposed samples were clustered, brain presented a distinct profile from gill and liver tissues (Figure 3.1), which can be explained by the specific genes and mechanisms activated in this tissue and that control and protect this vital organ. On the other hand, gill presented the most extensive responses in gene expression to environmental ammonia (Figure 3.1). These effects can be immediately noted by the differences in the cluster of control and exposed gill samples and even by the higher number of differentially expressed genes recorded in gill after ammonia exposure. In this sense, these results reinforce the idea that gills are the primary barrier and/or responsive organ to unfavorable environmental conditions (Benli et al., 2008). This is most likely because of the gill's direct contact with the aquatic environment and consequently, greater exposure to elevated ammonia concentrations (Evans et al., 2005; Ip and Chew, 2010). Furthermore, gills have a relevant role in the maintenance of ion regulation and homeostasis of the aquatic organism (Evans et al., 2005). In fact, gills present a high surface area, perfusion by 100 % of cardiac output, ample ventilation rates and reduced diffusion distances (Evans et al., 2005; Ip and Chew, 2010), which also make them an important organ for ammonia excretion (Wright and Wood, 2009). However, the presence of high levels of ammonia in the aquatic environment from natural or anthropogenic sources (Randall and Tsui, 2002) can cause structural damage to the gills (Benli and Köksal, 2005; Benli et al., 2008), which can impair the ammonia excretion and allow its accumulation inside the organism (Randall and Tsui, 2002; Wright and Wood, 2009). Due to this response to environmental ammonia, it was suggested that gill should be considered the most sensitive indicator of ammonia toxicity (Lease et al., 2003). Liver, in turn, is an organ where various key metabolic pathways take place (Benli et al., 2008), with ammonia entering through the hepatic portal vein and being incorporated in *de novo* amino acids (Wood, 2004; Madison et al., 2009) and/or produced in the metabolic pathways that occur in this organ. In this sense, the toxic effects of ammonia also appear primarily in the liver (Benli et al., 2008; Dhanasiri et

al., 2013). In contrast with gill and liver, the fact that the brain presented the lowest number of differentially expressed genes can be indicative of a biological mechanism where brain is the least organ affected by elevated environmental ammonia.

During stress situations, like exposure to contaminants, an imbalance in energy homeostasis can result from an increase in energy-demanding pathways (Dhanasiri et al., 2013). Lipid and carbohydrate metabolism constitute two very important pathways to control the energy inside the body. From the analysis of GO terms and KEGG pathways, it is possible to verify that both types of metabolism were affected by ammonia exposure, especially in gill, where carbohydrate metabolism was enriched by upregulated and downregulated genes and the lipid metabolism was enriched only by downregulated genes. This regulation of the branchial energy metabolism may indicate the relevance of carbohydrates as fuel for the different energy demands of the gill (Soengas et al., 1995; Perry and Walsh, 1989). Similarly, brain presented enrichment of lipid metabolism by downregulated genes while the carbohydrate metabolism was enriched by upregulated genes, which indicate that brain regulates the energy metabolism in the same manner as gill, favoring the use of carbohydrates (Soengas et al., 2002). In turn, liver presented three carbohydrate metabolism pathways enriched by both upregulated and downregulated genes; however, any GO term or pathway related with lipid metabolism, was enriched in this tissue. In this sense and considering all these results, it is quite obvious that, at 96 h of ammonia exposure, zebrafish control the energy metabolism, generally, downregulating the lipid metabolism and favoring the carbohydrate metabolism. The differential use of carbohydrates or lipids as fuels was previously described (Finn et al., 1995; Dhanasiri et al., 2013; Hagenaars et al., 2013), during the endogenous feeding of early developmental stages (Finn et al., 1995) as well as during adulthood (Dhanasiri et al., 2013; Hagenaars et al., 2013). However, although liver results contrast with the results of Dhanasiri and collaborators (2013) that showed that genes related with lipid metabolism were downregulated in zebrafish liver after exposure to ammonia during a simulated transport stress, the use of carbohydrates instead of lipids as energy supplier, also occurred in that study (Dhanasiri et al., 2013). The discrepancy between these results may be explained by the differences in the levels of environmental ammonia, which are higher in the present study, as well as other experimental design conditions. In fact, this increase in energy demand, to maintain the internal homeostasis of the organism, especially during a stressful environment, may explain the lower growth rate already described in other

studies (Pickering, 1993; Rasmussen and Korsgaard, 1996; Björnsson and Ólafsdóttir, 2006; Foss et al., 2007).

Within carbohydrate metabolism, the glycolysis/gluconeogenesis pathway is responsible for the production of energy needed to maintain osmo- and iono-regulation inside the fish (Perry and Walsh, 1989; Dhanasiri et al., 2013). Indeed, glycolysis/gluconeogenesis pathway was enriched in both gill (Supplementary Figure 3.5) and liver (Supplementary Figure 3.32) tissues, through the fructose and mannose pathway (Supplementary Figure 3.33) and pentose phosphate pathway (Supplementary Figure 3.34), in liver, and by fructose and mannose pathway (Supplementary Figure 3.6) and galactose pathway (Supplementary Figure 3.11), in gill. These different pathways that support glycolysis/gluconeogenesis are very important since they use the same enzymes, allowing the continuous production of intermediate substrates to be used for the glycolysis/gluconeogenesis pathway, which guarantee the continuous production of energy. In a more detailed analysis it is possible to determine that glycolysis/gluconeogenesis pathways are probably operating in different directions between these tissues: in liver gluconeogenesis while in gill mostly glycolysis was activated. Gluconeogenesis allows the production of glucose through pyruvate whereas glycolysis allows the production of energy (ATP) from glucose. The direction of the pathway is mostly determined by the activation of the enzyme fructose 1,6-biphosphatase (FBP) or by phosphofructokinase (PFK) at the same limiting step of the pathway, resulting in gluconeogenesis or glycolysis, respectively. Since these enzymes are usually not activated at the same time, the upregulation of one determines the direction of the pathway. In liver is the FBP upregulated, while in gill is the PFK. Hence, it is possible to say that during this stress situation, liver is able to produce glucose through gluconeogenesis (Vijayan et al., 1997; Dhanasiri et al., 2013) that, in turn, can be used by extra-hepatic tissues for energy production via glycolysis thus guarantying homeostasis (Dhanasiri et al., 2013). It is already known that the exposure to toxicants increases the levels of plasma glucose (Vijayan et al., 1997; Abbas and Authman, 2009; Dhanasiri et al., 2013); however, in the particular case of the gill, the energy produced from glucose, most likely from the liver, is of high relevance in supporting the energy dependent ion transport and even for the excretion of ammonia against its concentration gradients (Perry and Walsh, 1989; Soengas et al., 1995). Similarly to our study, Dhanasiri and collaborators (2013) have also observed the activation of gluconeogenesis in zebrafish liver after 72 h of ammonia exposure during a transport stress; however, they did not analyze the response of gill to the same stress.

Under stress conditions, the concentration of plasma lactate increases being used for gluconeogenesis in fish; however, in this study, the lactate may be produced in gill to allow the continued production of glucose (Dhanasiri et al., 2013) that will converted to energy by glycolysis (Supplementary Figure 3.5). In fish, lactate and amino acids are primarily used as substrates for gluconeogenesis (Polakof et al., 2012). Immediately after stress, plasma lactate concentrations increase largely from glycolysis in muscle and may be used for glucose production (Dhanasiri et al., 2013) and/or glycogen repletion in the liver of stressed fish (Mommsen et al., 1999). Lactate is also a metabolic fuel used by tissues such as gill through lactate dehydrogenase (LDH) working in the oxidative direction (Perry and Walsh, 1989; Soengas et al., 1995). In this study, gill LDH changed, which indicate that lactate would be an important fuel for producing energy. In contrast to the gill, liver did not present any changes in enzymes involved in the production of lactate (Supplementary Figure 3.32). This result contrasts with the ones obtained in Mozambique tilapia *Oreochromis mossambicus* (Vijayan et al., 1997) and zebrafish *D. rerio* (Dhanasiri et al., 2013), since both presented increased liver LDH levels during confinement stress.

In addition to the mobilization of glucose from the different parts of the body, the branchial cells also activated other pathways that released secondary messengers to mobilize glucose. In the GO term analysis, the category intracellular signaling cascade was enriched in gill by upregulated genes. This GO term enrichment is possibly related with the inositol phosphate metabolism and phosphatidylinositol signaling pathways that were enriched in the gill pathway analysis (Supplementary Figure 3.8 and 3.30, respectively). Inositol is a sugar alcohol that can be produced from glucose and then converted to phosphatidylinositol, a membrane phospholipid. The cleavage of phosphatidylinositol produces the diacylglycerol (DAG) and inositol trisphosphate (IP_3) that will act inside the cell as a secondary messenger, activating protein kinase C and releasing Ca^{2+} into the cytosol. In turn, the increase of intracellular Ca^{2+} in response to stress, can be very important since it induces several cascade reactions such as the mobilization of glucose (Yone et al., 1971; McClelland, 2011).

Pathways related with the metabolism of vitamins were also affected by ammonia exposure, leading to a disruption of branchial cell growth, proliferation and differentiation. In fact, the GO term analysis in gill revealed that downregulated genes enriched metabolic and biosynthetic processes related with vitamins. These data were reinforced by the pathway analysis, where retinol (Supplementary Figure 3.20) and folate (Supplementary Figure 3.21) pathways were downregulated. Retinol is one form of a fat-soluble vitamin, known as vitamin

A, and this pathway leads to the production of different forms of this vitamin such as retinol, retinal and retinoic acid that are generally termed as retinoids. Retinoids are very important in the regulation of cell proliferation, growth and differentiation (Hinds et al., 1997). Moreover, the fact that in the retinol pathway the enzyme *cyp26* was upregulated may indicate that this pathway follows on the degradation of retinol. On the other hand, folate (also known as folic acid or vitamin B_9) is a water-soluble vitamin important for DNA synthesis. In this sense, the downregulation of these two pathways may impede the proliferation, differentiation and replication of branchial epithelial cells, which would involve energy consumption (in the proliferation). Besides gills, brain also presented retinol metabolism pathway (Supplementary Figure 3.38) enriched by downregulated genes, which may also indicate a decrease in the cell proliferation probably to control the energy consumption.

It is already known that ammonia interferes in the cell cycle and even causes cell death; however, the activation of p53 pathway in response to ammonia exposure was only recently described (e.g. Ching et al., 2009). The GO term analysis revealed that in gill the categories cell death, apoptosis, growth and regulation of cellular component size, were enriched by upregulated genes. Furthermore, from the pathway analysis it was possible to verify that in the same tissue, upregulated genes enriched p53 signaling (Supplementary Figure 3.27), apoptosis (Supplementary Figure 3.26) and lysosome (Supplementary Figure 3.25) pathways after 96 h of ammonia exposure. The induction of these pathways in response to an aquatic toxicant may indicate a disruption of the cell cycle and even cellular death. The apoptosis of branchial cells may reflect a remodelling of the gill to respond to osmo- and ionoregulatory needs during ammonia exposure and/or compromised oxygen uptake. Beyond the gill, the GO term analysis also revealed that brain presented categories related with cell death, apoptosis and death enriched by upregulated genes. Again this result was reinforced by the pathway analysis since the p53 signaling pathway (Supplementary Figure 3.39) was also enriched in the brain by upregulated genes. Although in brain this pathway is not so profoundly studied in the context of a response to toxic contaminants, Görg and collaborators (2015) found changes in astrocytic expression of genes belonging to this pathway, namely p21, GADD45α and p53 in response to ammonia exposure. The authors explained these results as a mechanism to control the proliferation since they also showed that ammonia inhibits the proliferation of astrocyte cells (Görg et al., 2015). At normal conditions, p53 protein is kept at low levels mainly due to its half-life. However, in response to cellular stress, the half-life of p53 increases and consequently the p53 protein levels increase (Banin et al., 1998; Canman et al.,

1998). The activated p53 mediates cell cycle arrest or programmed cell death (apoptosis), which allows the elimination of cells that contain mutations or prevent high mutation rate by blocking the duplication of damaged DNA (Levine, 1997; Prives and Hall, 1999). Moreover, several target genes contain p53-responsive elements and are induced by the activated p53; however, the p53 transcriptional response depends of the nature of the DNA damage (Zhao et al., 2000). Reactive oxygen species (ROS) that are induced during ammonia exposure, are factors that may regulate the p53 protein and consequently apoptosis (Polyak et al., 1997).

The presence of a stressor in the environment may affect the degradation of xenobiotics in fish (Christen et al., 2010). In fact, in the branchial pathway analysis it was noted that two different pathways related with the biodegradation and metabolism of xenobiotics, namely the metabolism of xenobiotics by cytochrome P450 (Supplementary Figure 3.22) and the drug metabolism by cytochrome P450 (Supplementary Figure 3.23) were affected by ammonia exposure. The presence of contaminants in the environment might alter the genes related with the detoxification of xenobiotics in fish (Christen et al., 2010), which indicate a decrease in the capacity of the organism to respond to an additional chemical or disease stressor.

It is well documented that ammonia interferes in the transcellular movement of several ions (Wright and Wood, 2009; Ip and Chew, 2010); however, from the microarray analysis it is possible to confirm that the paracellular movement may also be affected. Ions and molecules may pass the epithelial membranes by one of the two different pathways: the paracellular pathway that occurs between cells, or through the cells, that is named as transcellular pathway. Most studies have been focused on the effects of ammonia on elements involved on transcellular pathway, namely, the ammonia transporters Rh glycoproteins (Hung et al., 2007), the V-type H^+ ATPase and the Na^+/K^+-ATPase (Wright and Wood, 2009). Although a more detailed analysis of the effects of ammonia on the expression of elements related with transcellular movement will be done in the next chapter, the GO term and pathway analysis also showed that ammonia affected the expression of many elements of TJs (Supplementary Figure 3.28) and CAMs (Supplementary Figure 3.29), which may indicate that the permeability of the gill epithelium might be altered in response to environmental ammonia. In fact, some studies and reviews have mentioned that the permeability of epithelia may increase or decrease in response to the aquatic environment, especially during the adaptation of fish to different salinities (Chasiotis et al., 2012; Kolosov et al., 2014). Indeed, fish are able to remodel the epithelia from “leaky” (in seawater) to “tight” (in freshwater), which is important to guarantee the homeostasis and survival of the aquatic organism (Evans

et al., 1995). Considering this and the results obtained in the microarray, it seems that the branchial epithelia may respond to environmental ammonia altering its permeability, probably in an attempt to impede the entrance of ammonia from the environment.

3.6 Conclusion

Although organism survival can be an important endpoint of ammonia toxicity in aquacultures and in natural environment, the presence of a sub-lethal concentration in the water may lead to several molecular and physiological effects that, in turn, may comprise the normal function of the body and eventually cause impairment of performance and mortality in cultured and wild fishes. In this respect, this study shows that, although no obvious effects were observed in fish, sub-lethal concentrations of ammonia induce changes in the transcriptome of three important organs in this freshwater teleost. Moreover, the results show that very complex networks involving multiple physiological and metabolic pathways are affected by the exposure to ammonia, including energy metabolism, cell cycle and death and paracellular transport. This knowledge improves our understanding of the biological impact of ammonia in gill, liver and brain, which can be of relevance for aquacultures and for environmental monitoring.

3.7 Supplementary tables

Supplementary Table 3.3 Gene Ontology (GO) terms significantly enriched by the responsive genes in the gill of zebrafish after exposure to ammonia during 96 h. This analysis was performed in the DAVID online tool after a 44k microarray in the gill exposed and control samples and included the categories biological processes, cellular component and molecular function. In this table is also possible to check the number of probes (#) altered in each category term.

GO category	Term	#
Biological process	GO:0050896~response to stimulus	246
	GO:0055114~oxidation reduction	213
	GO:0009653~anatomical structure morphogenesis	197
	GO:0006508~proteolysis	180
	GO:0007242~intracellular signaling cascade	142
	GO:0009056~catabolic process	138
	GO:0005975~carbohydrate metabolic process	131
	GO:0006629~lipid metabolic process	114
	GO:0065008~regulation of biological quality	106
	GO:0006082~organic acid metabolic process	105
	GO:0019752~carboxylic acid metabolic process	105
	GO:0042180~cellular ketone metabolic process	105
	GO:0043436~oxoacid metabolic process	105
	GO:0002376~immune system process	98
	GO:0042221~response to chemical stimulus	88
	GO:0009308~amine metabolic process	87
	GO:0044262~cellular carbohydrate metabolic process	86
	GO:0006519~cellular amino acid and derivative metabolic process	78
	GO:0046483~heterocycle metabolic process	77
	GO:0055086~nucleobase, nucleoside and nucleotide metabolic process	75
	GO:0007264~small GTPase mediated signal transduction	71
	GO:0006066~alcohol metabolic process	70
	GO:0044106~cellular amine metabolic process	70
	GO:0006955~immune response	67
	GO:0006520~cellular amino acid metabolic process	65
	GO:0044255~cellular lipid metabolic process	64
	GO:0009792~embryonic development ending in birth or egg hatching	56
	GO:0032989~cellular component morphogenesis	56
	GO:0043009~chordate embryonic development	56
	GO:0000902~cell morphogenesis	48
	GO:0009605~response to external stimulus	47
	GO:0005996~monosaccharide metabolic process	45
	GO:0001944~vasculature development	43
	GO:0007010~cytoskeleton organization	43
	GO:0010941~regulation of cell death	43
	GO:0043067~regulation of programmed cell death	43
	GO:0001568~blood vessel development	42
	GO:0019318~hexose metabolic process	42

	GO:0042981~regulation of apoptosis	42
	GO:0008610~lipid biosynthetic process	37
	GO:0016052~carbohydrate catabolic process	36
	GO:0048858~cell projection morphogenesis	36
	GO:0009101~glycoprotein biosynthetic process	35
	GO:0001501~skeletal system development	33
	GO:0006725~cellular aromatic compound metabolic process	33
	GO:0009611~response to wounding	33
	GO:0008219~cell death	32
	GO:0016265~death	32
	GO:0019637~organophosphate metabolic process	32
	GO:0032787~monocarboxylic acid metabolic process	32
	GO:0006915~apoptosis	30
	GO:0012501~programmed cell death	30
	GO:0006006~glucose metabolic process	29
	GO:0009607~response to biotic stimulus	29
	GO:0044275~cellular carbohydrate catabolic process	29
	GO:0046164~alcohol catabolic process	29
	GO:0048878~chemical homeostasis	28
	GO:0006952~defense response	26
	GO:0016053~organic acid biosynthetic process	26
	GO:0042060~wound healing	26
	GO:0046394~carboxylic acid biosynthetic process	26
	GO:0051704~multi-organism process	26
	GO:0051707~response to other organism	26
	GO:0006007~glucose catabolic process	25
	GO:0019320~hexose catabolic process	25
	GO:0046365~monosaccharide catabolic process	25
	GO:0006631~fatty acid metabolic process	23
	GO:0009617~response to bacterium	23
	GO:0016042~lipid catabolic process	23
	GO:0006096~glycolysis	22
	GO:0051216~cartilage development	22
	GO:0008202~steroid metabolic process	21
	GO:0040008~regulation of growth	21
	GO:0001558~regulation of cell growth	20
	GO:0016054~organic acid catabolic process	20
	GO:0046395~carboxylic acid catabolic process	20
	GO:0006575~cellular amino acid derivative metabolic process	19
	GO:0010035~response to inorganic substance	19
	GO:0031016~pancreas development	19
	GO:0007411~axon guidance	18
	GO:0009310~amine catabolic process	18
	GO:0001708~cell fate specification	17
	GO:0009063~cellular amino acid catabolic process	17
	GO:0007596~blood coagulation	15

	GO:0007599~hemostasis	15
	GO:0050817~coagulation	15
	GO:0050878~regulation of body fluid levels	15
	GO:0042127~regulation of cell proliferation	14
	GO:0006766~vitamin metabolic process	13
	GO:0009072~aromatic amino acid family metabolic process	13
	GO:0042742~defense response to bacterium	11
	GO:0016125~sterol metabolic process	10
	GO:0043281~regulation of caspase activity	10
	GO:0048701~embryonic cranial skeleton morphogenesis	10
	GO:0048704~embryonic skeletal system morphogenesis	10
	GO:0048706~embryonic skeletal system development	10
	GO:0052547~regulation of peptidase activity	10
	GO:0052548~regulation of endopeptidase activity	10
	GO:0008064~regulation of actin polymerization or depolymerization	9
	GO:0030832~regulation of actin filament length	9
	GO:0030833~regulation of actin filament polymerization	9
	GO:0032271~regulation of protein polymerization	9
	GO:0032956~regulation of actin cytoskeleton organization	9
	GO:0032970~regulation of actin filament-based process	9
	GO:0043254~regulation of protein complex assembly	9
	GO:0048264~determination of ventral identity	9
	GO:0048547~gut morphogenesis	9
	GO:0006544~glycine metabolic process	8
	GO:0007031~peroxisome organization	8
	GO:0031290~retinal ganglion cell axon guidance	8
	GO:0032101~regulation of response to external stimulus	8
	GO:0046688~response to copper ion	8
	GO:0016126~sterol biosynthetic process	7
	GO:0009074~aromatic amino acid family catabolic process	6
	GO:0019439~aromatic compound catabolic process	6
	GO:0002474~antigen processing and presentation of peptide antigen via MHC class I	5
	GO:0048002~antigen processing and presentation of peptide antigen	5
Celular component	GO:0016020~membrane	953
	GO:0044425~membrane part	760
	GO:0031224~intrinsic to membrane	669
	GO:0016021~integral to membrane	658
	GO:0005737~cytoplasm	566
	GO:0044444~cytoplasmic part	380
	GO:0005576~extracellular region	167
	GO:0005856~cytoskeleton	119
	GO:0005783~endoplasmic reticulum	104
	GO:0044430~cytoskeletal part	77
	GO:0030054~cell junction	60
	GO:0005911~cell-cell junction	45

	GO:0019898~extrinsic to membrane	35
	GO:0016327~apicolateral plasma membrane	33
	GO:0043296~apical junction complex	33
	GO:0005615~extracellular space	31
	GO:0005923~tight junction	29
	GO:0070160~occluding junction	29
	GO:0015629~actin cytoskeleton	21
	GO:0005882~intermediate filament	20
	GO:0045111~intermediate filament cytoskeleton	20
	GO:0005777~peroxisome	16
	GO:0042579~microbody	16
	GO:0005839~proteasome core complex	13
	GO:0033178~proton-transporting two-sector ATPase complex, catalytic domain	13
	GO:0019897~extrinsic to plasma membrane	12
	GO:0033176~proton-transporting V-type ATPase complex	10
	GO:0033180~proton-transporting V-type ATPase, V1 domain	7
	GO:0005778~peroxisomal membrane	6
	GO:0031903~microbody membrane	6
	GO:0044438~microbody part	6
	GO:0044439~peroxisomal part	6
	GO:0016342~catenin complex	5
Molecular function	GO:0003824~catalytic activity	1319
	GO:0016787~hydrolase activity	502
	GO:0016740~transferase activity	435
	GO:0016491~oxidoreductase activity	255
	GO:0008233~peptidase activity	176
	GO:0070011~peptidase activity, acting on L-amino acid peptides	168
	GO:0005509~calcium ion binding	158
	GO:0019001~guanyl nucleotide binding	133
	GO:0032561~guanyl ribonucleotide binding	132
	GO:0005525~GTP binding	130
	GO:0004175~endopeptidase activity	126
	GO:0030234~enzyme regulator activity	112
	GO:0005506~iron ion binding	99
	GO:0048037~cofactor binding	84
	GO:0016757~transferase activity, transferring glycosyl groups	83
	GO:0009055~electron carrier activity	76
	GO:0022804~active transmembrane transporter activity	71
	GO:0008289~lipid binding	70
	GO:0008236~serine-type peptidase activity	64
	GO:0017171~serine hydrolase activity	64
	GO:0008092~cytoskeletal protein binding	60
	GO:0050662~coenzyme binding	59
	GO:0004252~serine-type endopeptidase activity	58
	GO:0046906~tetrapyrrole binding	55

	GO:0004857~enzyme inhibitor activity	52
	GO:0020037~heme binding	52
	GO:0016758~transferase activity, transferring hexosyl groups	50
	GO:0003779~actin binding	49
	GO:0004497~monooxygenase activity	49
	GO:0016829~lyase activity	45
	GO:0019842~vitamin binding	44
	GO:0030414~peptidase inhibitor activity	44
	GO:0004866~endopeptidase inhibitor activity	41
	GO:0016810~hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	37
	GO:0016705~oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	33
	GO:0004197~cysteine-type endopeptidase activity	32
	GO:0016614~oxidoreductase activity, acting on CH-OH group of donors	31
	GO:0016616~oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	31
	GO:0031406~carboxylic acid binding	30
	GO:0004867~serine-type endopeptidase inhibitor activity	27
	GO:0030170~pyridoxal phosphate binding	24
	GO:0070279~vitamin B6 binding	24
	GO:0042625~ATPase activity, coupled to transmembrane movement of ions	22
	GO:0008238~exopeptidase activity	21
	GO:0042802~identical protein binding	21
	GO:0004091~carboxylesterase activity	20
	GO:0016298~lipase activity	20
	GO:0016701~oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	20
	GO:0016811~hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides	20
	GO:0016627~oxidoreductase activity, acting on the CH-CH group of donors	19
	GO:0016702~oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	19
	GO:0016835~carbon-oxygen lyase activity	19
	GO:0051213~dioxygenase activity	19
	GO:0019955~cytokine binding	18
	GO:0016836~hydro-lyase activity	17
	GO:0016769~transferase activity, transferring nitrogenous groups	16
	GO:0019904~protein domain specific binding	16
	GO:0004298~threonine-type endopeptidase activity	13
	GO:0005520~insulin-like growth factor binding	13
	GO:0016860~intramolecular oxidoreductase activity	13
	GO:0019829~cation-transporting ATPase activity	13
	GO:0070003~threonine-type peptidase activity	13
	GO:0004180~carboxypeptidase activity	12

	GO:0005544~calcium-dependent phospholipid binding	12
	GO:0008483~transaminase activity	12
	GO:0033293~monocarboxylic acid binding	11
	GO:0005504~fatty acid binding	9
	GO:0019200~carbohydrate kinase activity	9
	GO:0019992~diacylglycerol binding	9
	GO:0004536~deoxyribonuclease activity	8
	GO:0004435~phosphoinositide phospholipase C activity	7
	GO:0008519~ammonium transmembrane transporter activity	6
	GO:0015101~organic cation transmembrane transporter activity	6

Supplementary Table 3.4 Biological process terms significantly enriched by the upregulated genes in the gill of zebrafish after 96 h of ammonia exposure. This analysis was performed in the DAVID online tool after a 44k microarray in gill exposed and control samples. In this table is also possible to check the number of probes (#) altered in each category term.

Term	#
GO:0065007~biological regulation	590
GO:0050789~regulation of biological process	567
GO:0050794~regulation of cellular process	547
GO:0032501~multicellular organismal process	290
GO:0032502~developmental process	282
GO:0007275~multicellular organismal development	273
GO:0007165~signal transduction	243
GO:0048856~anatomical structure development	221
GO:0048731~system development	197
GO:0048513~organ development	178
GO:0051252~regulation of RNA metabolic process	171
GO:0006355~regulation of transcription, DNA-dependent	170
GO:0009653~anatomical structure morphogenesis	140
GO:0007242~intracellular signaling cascade	116
GO:0009790~embryonic development	104
GO:0002376~immune system process	77
GO:0005975~carbohydrate metabolic process	77
GO:0009888~tissue development	76
GO:0009887~organ morphogenesis	67
GO:0048598~embryonic morphogenesis	59
GO:0007389~pattern specification process	58
GO:0007264~small GTPase mediated signal transduction	54
GO:0006955~immune response	53
GO:0044262~cellular carbohydrate metabolic process	51
GO:0009966~regulation of signal transduction	48
GO:0048518~positive regulation of biological process	47
GO:0003002~regionalization	45
GO:0007049~cell cycle	45

GO:0009792~embryonic development ending in birth or egg hatching	45
GO:0043009~chordate embryonic development	45
GO:0006928~cell motion	44
GO:0048522~positive regulation of cellular process	43
GO:0048523~negative regulation of cellular process	42
GO:0010941~regulation of cell death	39
GO:0040011~locomotion	39
GO:0043067~regulation of programmed cell death	39
GO:0048870~cell motility	39
GO:0051674~localization of cell	39
GO:0007010~cytoskeleton organization	38
GO:0032989~cellular component morphogenesis	38
GO:0042981~regulation of apoptosis	38
GO:0034404~nucleobase, nucleoside and nucleotide biosynthetic process	37
GO:0009165~nucleotide biosynthetic process	36
GO:0048568~embryonic organ development	36
GO:0006066~alcohol metabolic process	35
GO:0006091~generation of precursor metabolites and energy	35
GO:0016477~cell migration	34
GO:0000902~cell morphogenesis	33
GO:0006163~purine nucleotide metabolic process	33
GO:0006164~purine nucleotide biosynthetic process	32
GO:0009150~purine ribonucleotide metabolic process	32
GO:0009259~ribonucleotide metabolic process	32
GO:0001568~blood vessel development	31
GO:0001944~vasculature development	31
GO:0009152~purine ribonucleotide biosynthetic process	31
GO:0009260~ribonucleotide biosynthetic process	31
GO:0007369~gastrulation	29
GO:0009141~nucleoside triphosphate metabolic process	29
GO:0009952~anterior/posterior pattern formation	29
GO:0048729~tissue morphogenesis	29
GO:0065009~regulation of molecular function	29
GO:0001667~ameboidal cell migration	28
GO:0005996~monosaccharide metabolic process	28
GO:0009144~purine nucleoside triphosphate metabolic process	28
GO:0009199~ribonucleoside triphosphate metabolic process	28
GO:0009205~purine ribonucleoside triphosphate metabolic process	28
GO:0051239~regulation of multicellular organismal process	28
GO:0009142~nucleoside triphosphate biosynthetic process	27
GO:0009145~purine nucleoside triphosphate biosynthetic process	27
GO:0009201~ribonucleoside triphosphate biosynthetic process	27
GO:0009206~purine ribonucleoside triphosphate biosynthetic process	27
GO:0016055~Wnt receptor signaling pathway	27
GO:0043010~camera-type eye development	27
GO:0046034~ATP metabolic process	27

GO:0001501~skeletal system development	26
GO:0006754~ATP biosynthetic process	26
GO:0019318~hexose metabolic process	26
GO:0051056~regulation of small GTPase mediated signal transduction	26
GO:0060429~epithelium development	26
GO:0008219~cell death	25
GO:0016265~death	25
GO:0050793~regulation of developmental process	25
GO:0002009~morphogenesis of an epithelium	24
GO:0007017~microtubule-based process	24
GO:0006915~apoptosis	23
GO:0012501~programmed cell death	23
GO:0040007~growth	23
GO:0048514~blood vessel morphogenesis	23
GO:0048562~embryonic organ morphogenesis	23
GO:0007167~enzyme linked receptor protein signaling pathway	22
GO:0009607~response to biotic stimulus	21
GO:0035282~segmentation	21
GO:0042074~cell migration involved in gastrulation	21
GO:0001756~somitogenesis	20
GO:0030029~actin filament-based process	20
GO:0030036~actin cytoskeleton organization	20
GO:0048878~chemical homeostasis	20
GO:0009611~response to wounding	19
GO:0051336~regulation of hydrolase activity	19
GO:0050801~ion homeostasis	18
GO:0051128~regulation of cellular component organization	18
GO:0051216~cartilage development	18
GO:0051704~multi-organism process	18
GO:0051707~response to other organism	18
GO:0019882~antigen processing and presentation	17
GO:0034220~ion transmembrane transport	17
GO:0006818~hydrogen transport	16
GO:0010942~positive regulation of cell death	16
GO:0015985~energy coupled proton transport, down electrochemical gradient	16
GO:0015986~ATP synthesis coupled proton transport	16
GO:0015992~proton transport	16
GO:0030902~hindbrain development	16
GO:0032535~regulation of cellular component size	16
GO:0043065~positive regulation of apoptosis	16
GO:0043068~positive regulation of programmed cell death	16
GO:0009617~response to bacterium	15
GO:0001558~regulation of cell growth	14
GO:0006096~glycolysis	14
GO:0007219~Notch signaling pathway	14
GO:0010035~response to inorganic substance	14

GO:0001708~cell fate specification	13
GO:0014033~neural crest cell differentiation	12
GO:0033043~regulation of organelle organization	12
GO:0000226~microtubule cytoskeleton organization	11
GO:0051493~regulation of cytoskeleton organization	11
GO:0001570~vasculogenesis	10
GO:0007059~chromosome segregation	9
GO:0008064~regulation of actin polymerization or depolymerization	9
GO:0030832~regulation of actin filament length	9
GO:0030833~regulation of actin filament polymerization	9
GO:0032271~regulation of protein polymerization	9
GO:0032956~regulation of actin cytoskeleton organization	9
GO:0032970~regulation of actin filament-based process	9
GO:0043254~regulation of protein complex assembly	9
GO:0043281~regulation of caspase activity	9
GO:0044087~regulation of cellular component biogenesis	9
GO:0052547~regulation of peptidase activity	9
GO:0052548~regulation of endopeptidase activity	9
GO:0007398~ectoderm development	8
GO:0048701~embryonic cranial skeleton morphogenesis	8
GO:0048704~embryonic skeletal system morphogenesis	8
GO:0048706~embryonic skeletal system development	8
GO:0048264~determination of ventral identity	7
GO:0006954~inflammatory response	6
GO:0008544~epidermis development	6
GO:0002474~antigen processing and presentation of peptide antigen via MHC class I	5
GO:0030010~establishment of cell polarity	5
GO:0048002~antigen processing and presentation of peptide antigen	5
GO:0010466~negative regulation of peptidase activity	4
GO:0015695~organic cation transport	4
GO:0015696~ammonium transport	4
GO:0032496~response to lipopolysaccharide	4
GO:0043154~negative regulation of caspase activity	4
GO:0051346~negative regulation of hydrolase activity	4

Supplementary Table 3.5 Biological process terms significantly enriched by the downregulated genes in the gill of zebrafish after 96 h of ammonia exposure. This analysis was performed in the DAVID online tool after a 44k microarray in gill exposed and control samples. It is also possible to check in this table the number of probes (#) altered in each category term.

Term	**#**
GO:0008152~metabolic process	637
GO:0051234~establishment of localization	191
GO:0006810~transport	190
GO:0055114~oxidation reduction	150
GO:0050896~response to stimulus	110

GO:0006082~organic acid metabolic process	77
GO:0019752~carboxylic acid metabolic process	77
GO:0042180~cellular ketone metabolic process	77
GO:0043436~oxoacid metabolic process	77
GO:0006508~proteolysis	75
GO:0006629~lipid metabolic process	68
GO:0009308~amine metabolic process	65
GO:0009056~catabolic process	64
GO:0006519~cellular amino acid and derivative metabolic process	61
GO:0033036~macromolecule localization	61
GO:0055085~transmembrane transport	60
GO:0005975~carbohydrate metabolic process	54
GO:0044106~cellular amine metabolic process	53
GO:0042221~response to chemical stimulus	52
GO:0006520~cellular amino acid metabolic process	49
GO:0065008~regulation of biological quality	49
GO:0006412~translation	41
GO:0006066~alcohol metabolic process	35
GO:0044262~cellular carbohydrate metabolic process	35
GO:0044255~cellular lipid metabolic process	34
GO:0006725~cellular aromatic compound metabolic process	27
GO:0008610~lipid biosynthetic process	27
GO:0032787~monocarboxylic acid metabolic process	23
GO:0051186~cofactor metabolic process	22
GO:0006732~coenzyme metabolic process	20
GO:0016053~organic acid biosynthetic process	18
GO:0046394~carboxylic acid biosynthetic process	18
GO:0005996~monosaccharide metabolic process	17
GO:0016052~carbohydrate catabolic process	17
GO:0006631~fatty acid metabolic process	16
GO:0008202~steroid metabolic process	16
GO:0016054~organic acid catabolic process	16
GO:0019318~hexose metabolic process	16
GO:0046395~carboxylic acid catabolic process	16
GO:0006006~glucose metabolic process	15
GO:0006869~lipid transport	15
GO:0009310~amine catabolic process	15
GO:0009611~response to wounding	15
GO:0010876~lipid localization	15
GO:0006575~cellular amino acid derivative metabolic process	14
GO:0009063~cellular amino acid catabolic process	14
GO:0009116~nucleoside metabolic process	14
GO:0042060~wound healing	14
GO:0006605~protein targeting	13
GO:0044275~cellular carbohydrate catabolic process	13
GO:0046164~alcohol catabolic process	13

GO:0007596~blood coagulation	12
GO:0007599~hemostasis	12
GO:0016042~lipid catabolic process	12
GO:0050817~coagulation	12
GO:0050878~regulation of body fluid levels	12
GO:0006766~vitamin metabolic process	10
GO:0009072~aromatic amino acid family metabolic process	10
GO:0009108~coenzyme biosynthetic process	10
GO:0009617~response to bacterium	10
GO:0019748~secondary metabolic process	10
GO:0006633~fatty acid biosynthetic process	9
GO:0006790~sulfur metabolic process	9
GO:0009119~ribonucleoside metabolic process	9
GO:0033365~protein localization in organelle	9
GO:0006694~steroid biosynthetic process	8
GO:0007031~peroxisome organization	8
GO:0009069~serine family amino acid metabolic process	8
GO:0016125~sterol metabolic process	8
GO:0046700~heterocycle catabolic process	8
GO:0006544~glycine metabolic process	7
GO:0006576~biogenic amine metabolic process	7
GO:0006612~protein targeting to membrane	7
GO:0006720~isoprenoid metabolic process	7
GO:0006839~mitochondrial transport	7
GO:0006979~response to oxidative stress	7
GO:0019362~pyridine nucleotide metabolic process	7
GO:0043603~cellular amide metabolic process	7
GO:0006767~water-soluble vitamin metabolic process	6
GO:0009066~aspartate family amino acid metabolic process	6
GO:0009074~aromatic amino acid family catabolic process	6
GO:0009110~vitamin biosynthetic process	6
GO:0010817~regulation of hormone levels	6
GO:0019439~aromatic compound catabolic process	6
GO:0042364~water-soluble vitamin biosynthetic process	6
GO:0042445~hormone metabolic process	6
GO:0044270~nitrogen compound catabolic process	6
GO:0000096~sulfur amino acid metabolic process	5
GO:0006081~cellular aldehyde metabolic process	5
GO:0016126~sterol biosynthetic process	5
GO:0034655~nucleobase, nucleoside, nucleotide and nucleic acid catabolic process	5
GO:0034656~nucleobase, nucleoside and nucleotide catabolic process	5
GO:0042219~cellular amino acid derivative catabolic process	5
GO:0001523~retinoid metabolic process	4
GO:0006546~glycine catabolic process	4
GO:0006555~methionine metabolic process	4
GO:0006721~terpenoid metabolic process	4

GO:0008203~cholesterol metabolic process	4
GO:0009071~serine family amino acid catabolic process	4
GO:0009166~nucleotide catabolic process	4
GO:0016101~diterpenoid metabolic process	4
GO:0019363~pyridine nucleotide biosynthetic process	4
GO:0030168~platelet activation	4
GO:0042402~biogenic amine catabolic process	4
GO:0006558~L-phenylalanine metabolic process	3
GO:0006559~L-phenylalanine catabolic process	3
GO:0006568~tryptophan metabolic process	3
GO:0006569~tryptophan catabolic process	3
GO:0016559~peroxisome fission	3
GO:0019321~pentose metabolic process	3
GO:0042436~indole derivative catabolic process	3
GO:0042574~retinal metabolic process	3
GO:0043288~apocarotenoid metabolic process	3
GO:0046218~indolalkylamine catabolic process	3

Supplementary Table 3.6 Gene Ontology (GO) terms significantly enriched by the responsive genes in the liver of zebrafish after exposure to ammonia during 96 h. This analysis was performed in the DAVID online tool after a 44k microarray in the liver exposed and control samples and included the categories biological processes, cellular component and molecular function. In this table is also possible to check the number of probes (#) altered in each category term.

GO category	Term	#
Biological process	GO:0065007~biological regulation	304
	GO:0050789~regulation of biological process	289
	GO:0050794~regulation of cellular process	277
	GO:0032501~multicellular organismal process	176
	GO:0032502~developmental process	168
	GO:0007275~multicellular organismal development	162
	GO:0051179~localization	154
	GO:0051234~establishment of localization	140
	GO:0006810~transport	138
	GO:0031323~regulation of cellular metabolic process	136
	GO:0080090~regulation of primary metabolic process	131
	GO:0031326~regulation of cellular biosynthetic process	129
	GO:0009889~regulation of biosynthetic process	129
	GO:0051171~regulation of nitrogen compound metabolic process	128
	GO:0048856~anatomical structure development	128
	GO:0019219~regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	128
	GO:0010556~regulation of macromolecule biosynthetic process	127
	GO:0045449~regulation of transcription	126
	GO:0048731~system development	116
	GO:0051252~regulation of RNA metabolic process	108

	GO:0006355~regulation of transcription, DNA-dependent	108
	GO:0048513~organ development	106
	GO:0007166~cell surface receptor linked signal transduction	85
	GO:0009653~anatomical structure morphogenesis	77
	GO:0009790~embryonic development	61
	GO:0006811~ion transport	60
	GO:0006812~cation transport	48
	GO:0005975~carbohydrate metabolic process	42
	GO:0009888~tissue development	40
	GO:0009887~organ morphogenesis	40
	GO:0030001~metal ion transport	38
	GO:0048598~embryonic morphogenesis	35
	GO:0007417~central nervous system development	29
	GO:0048518~positive regulation of biological process	28
	GO:0007423~sensory organ development	28
	GO:0015672~monovalent inorganic cation transport	27
	GO:0048568~embryonic organ development	24
	GO:0007420~brain development	24
	GO:0051239~regulation of multicellular organismal process	23
	GO:0001501~skeletal system development	21
	GO:0007507~heart development	20
	GO:0006066~alcohol metabolic process	20
	GO:0048562~embryonic organ morphogenesis	19
	GO:0009259~ribonucleotide metabolic process	18
	GO:0009150~purine ribonucleotide metabolic process	18
	GO:0048839~inner ear development	17
	GO:0043583~ear development	17
	GO:0009152~purine ribonucleotide biosynthetic process	17
	GO:0050793~regulation of developmental process	16
	GO:0006814~sodium ion transport	15
	GO:0051216~cartilage development	14
	GO:0040008~regulation of growth	13
	GO:0007517~muscle organ development	13
	GO:0048878~chemical homeostasis	12
	GO:0042692~muscle cell differentiation	12
	GO:0042472~inner ear morphogenesis	12
	GO:0042471~ear morphogenesis	12
	GO:0015674~di-, tri-valent inorganic cation transport	12
	GO:0001558~regulation of cell growth	12
	GO:0051146~striated muscle cell differentiation	11
	GO:0045941~positive regulation of transcription	11
	GO:0010628~positive regulation of gene expression	11
	GO:0006816~calcium ion transport	11
	GO:0033333~fin development	10
	GO:0010648~negative regulation of cell communication	9
	GO:0008202~steroid metabolic process	9

	GO:0001822~kidney development	9
	GO:0001655~urogenital system development	9
	GO:0048793~pronephros development	8
	GO:0001708~cell fate specification	8
	GO:0042127~regulation of cell proliferation	7
	GO:0030239~myofibril assembly	7
	GO:0010927~cellular component assembly involved in morphogenesis	7
	GO:0006821~chloride transport	7
	GO:0006694~steroid biosynthetic process	7
	GO:0044057~regulation of system process	6
	GO:0030916~otic vesicle formation	6
	GO:0009798~axis specification	6
	GO:0031290~retinal ganglion cell axon guidance	5
	GO:0008284~positive regulation of cell proliferation	5
	GO:0006941~striated muscle contraction	5
	GO:0006936~muscle contraction	5
	GO:0006029~proteoglycan metabolic process	5
	GO:0003012~muscle system process	5
	GO:0015696~ammonium transport	3
	GO:0015695~organic cation transport	3
	GO:0009950~dorsal/ventral axis specification	3
Cellular component	GO:0016020~membrane	320
	GO:0044425~membrane part	263
	GO:0031224~intrinsic to membrane	251
	GO:0016021~integral to membrane	242
	GO:0005886~plasma membrane	79
	GO:0005576~extracellular region	55
	GO:0044430~cytoskeletal part	30
	GO:0031226~intrinsic to plasma membrane	22
	GO:0005882~intermediate filament	12
	GO:0005923~tight junction	12
	GO:0016327~apicolateral plasma membrane	12
	GO:0043296~apical junction complex	12
	GO:0045111~intermediate filament cytoskeleton	12
	GO:0070160~occluding junction	12
	GO:0015629~actin cytoskeleton	11
Molecular function	GO:0046872~metal ion binding	246
	GO:0003677~DNA binding	134
	GO:0030528~transcription regulator activity	108
	GO:0005215~transporter activity	99
	GO:0003700~transcription factor activity	94
	GO:0022892~substrate-specific transporter activity	75
	GO:0043565~sequence-specific DNA binding	75
	GO:0005509~calcium ion binding	70
	GO:0022857~transmembrane transporter activity	69
	GO:0022891~substrate-specific transmembrane transporter activity	67

	GO:0015075~ion transmembrane transporter activity	60
	GO:0008324~cation transmembrane transporter activity	45
	GO:0022804~active transmembrane transporter activity	30
	GO:0048037~cofactor binding	27
	GO:0046873~metal ion transmembrane transporter activity	25
	GO:0016829~lyase activity	18
	GO:0019842~vitamin binding	17
	GO:0031420~alkali metal ion binding	13
	GO:0030170~pyridoxal phosphate binding	12
	GO:0070279~vitamin B6 binding	12
	GO:0042625~ATPase activity, coupled to transmembrane movement of ions	10
	GO:0015662~ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	9
	GO:0016830~carbon-carbon lyase activity	9
	GO:0019838~growth factor binding	9
	GO:0042802~identical protein binding	9
	GO:0005520~insulin-like growth factor binding	8
	GO:0016769~transferase activity, transferring nitrogenous groups	8
	GO:0019205~nucleobase, nucleoside, nucleotide kinase activity	8
	GO:0008483~transaminase activity	7
	GO:0031402~sodium ion binding	7
	GO:0005544~calcium-dependent phospholipid binding	6
	GO:0005007~fibroblast growth factor receptor activity	3

Supplementary Table 3.7 Biological process terms significantly enriched by the upregulated genes in the liver of zebrafish after 96 h of ammonia exposure. This analysis was performed in the DAVID online tool after a 44k microarray in liver exposed and control samples. In this table is also possible to check the number of probes (#) altered in each category term.

Term	#
GO:0007165~signal transduction	66
GO:0055114~oxidation reduction	38
GO:0006811~ion transport	32
GO:0030001~metal ion transport	21
GO:0009308~amine metabolic process	19
GO:0048518~positive regulation of biological process	18
GO:0006519~cellular amino acid and derivative metabolic process	17
GO:0048522~positive regulation of cellular process	16
GO:0044106~cellular amine metabolic process	15
GO:0006520~cellular amino acid metabolic process	14
GO:0051239~regulation of multicellular organismal process	11
GO:0009891~positive regulation of biosynthetic process	9
GO:0009893~positive regulation of metabolic process	9
GO:0010557~positive regulation of macromolecule biosynthetic process	9
GO:0010604~positive regulation of macromolecule metabolic process	9
GO:0010628~positive regulation of gene expression	9

GO:0031325~positive regulation of cellular metabolic process	9
GO:0031328~positive regulation of cellular biosynthetic process	9
GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	9
GO:0045941~positive regulation of transcription	9
GO:0051173~positive regulation of nitrogen compound metabolic process	9
GO:0007218~neuropeptide signaling pathway	6

Supplementary Table 3.8 Biological process terms significantly enriched by the downregulated genes in the liver of zebrafish after 96 h of ammonia exposure. This analysis was performed in the DAVID online tool after a 44k microarray in liver exposed and control samples It is also possible to check in this table the number of probes (#) altered in each category term.

Term	#
GO:0065007~biological regulation	156
GO:0050789~regulation of biological process	147
GO:0050794~regulation of cellular process	139
GO:0032501~multicellular organismal process	112
GO:0032502~developmental process	111
GO:0007275~multicellular organismal development	107
GO:0048856~anatomical structure development	84
GO:0019222~regulation of metabolic process	80
GO:0031323~regulation of cellular metabolic process	78
GO:0010468~regulation of gene expression	77
GO:0060255~regulation of macromolecule metabolic process	77
GO:0009889~regulation of biosynthetic process	76
GO:0019219~regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	76
GO:0031326~regulation of cellular biosynthetic process	76
GO:0048731~system development	76
GO:0051171~regulation of nitrogen compound metabolic process	76
GO:0080090~regulation of primary metabolic process	76
GO:0010556~regulation of macromolecule biosynthetic process	75
GO:0045449~regulation of transcription	75
GO:0051179~localization	75
GO:0048513~organ development	70
GO:0006355~regulation of transcription, DNA-dependent	63
GO:0051252~regulation of RNA metabolic process	63
GO:0009653~anatomical structure morphogenesis	50
GO:0009790~embryonic development	38
GO:0009888~tissue development	31
GO:0048869~cellular developmental process	31
GO:0006811~ion transport	29
GO:0009887~organ morphogenesis	29
GO:0030154~cell differentiation	29
GO:0006812~cation transport	26
GO:0048598~embryonic morphogenesis	25
GO:0065008~regulation of biological quality	21

GO:0007423~sensory organ development	19
GO:0030001~metal ion transport	18
GO:0048568~embryonic organ development	17
GO:0048646~anatomical structure formation involved in morphogenesis	17
GO:0007417~central nervous system development	16
GO:0048468~cell development	16
GO:0001501~skeletal system development	15
GO:0042592~homeostatic process	15
GO:0032989~cellular component morphogenesis	14
GO:0043583~ear development	14
GO:0048562~embryonic organ morphogenesis	14
GO:0048839~inner ear development	14
GO:0007420~brain development	13
GO:0006163~purine nucleotide metabolic process	12
GO:0007507~heart development	12
GO:0009150~purine ribonucleotide metabolic process	12
GO:0009259~ribonucleotide metabolic process	12
GO:0051239~regulation of multicellular organismal process	12
GO:0006164~purine nucleotide biosynthetic process	11
GO:0009152~purine ribonucleotide biosynthetic process	11
GO:0009260~ribonucleotide biosynthetic process	11
GO:0048729~tissue morphogenesis	11
GO:0051216~cartilage development	11
GO:0007010~cytoskeleton organization	10
GO:0016055~Wnt receptor signaling pathway	10
GO:0030029~actin filament-based process	10
GO:0030036~actin cytoskeleton organization	10
GO:0042471~ear morphogenesis	10
GO:0042472~inner ear morphogenesis	10
GO:0001667~ameboidal cell migration	9
GO:0007517~muscle organ development	9
GO:0009141~nucleoside triphosphate metabolic process	9
GO:0009144~purine nucleoside triphosphate metabolic process	9
GO:0009199~ribonucleoside triphosphate metabolic process	9
GO:0009205~purine ribonucleoside triphosphate metabolic process	9
GO:0040008~regulation of growth	9
GO:0042692~muscle cell differentiation	9
GO:0048878~chemical homeostasis	9
GO:0050801~ion homeostasis	9
GO:0051146~striated muscle cell differentiation	9
GO:0001558~regulation of cell growth	8
GO:0015674~di-, tri-valent inorganic cation transport	8
GO:0055080~cation homeostasis	8
GO:0010927~cellular component assembly involved in morphogenesis	7
GO:0030239~myofibril assembly	7
GO:0030902~hindbrain development	7

GO:0031032~actomyosin structure organization	7
GO:0033333~fin development	7
GO:0033334~fin morphogenesis	7
GO:0035107~appendage morphogenesis	7
GO:0035270~endocrine system development	7
GO:0048589~developmental growth	7
GO:0048736~appendage development	7
GO:0055001~muscle cell development	7
GO:0055002~striated muscle cell development	7
GO:0030003~cellular cation homeostasis	6
GO:0003012~muscle system process	5
GO:0006936~muscle contraction	5
GO:0006941~striated muscle contraction	5
GO:0048732~gland development	5
GO:0048793~pronephros development	5
GO:0031101~fin regeneration	4
GO:0043049~otic placode formation	4
GO:0060560~developmental growth involved in morphogenesis	4
GO:0015695~organic cation transport	3
GO:0015696~ammonium transport	3
GO:0045214~sarcomere organization	3
GO:0060037~pharyngeal system development	3

Supplementary Table 3.9 Gene Ontology (GO) terms significantly enriched by the responsive genes in the brain of zebrafish after exposure to ammonia during 96 h. This analysis was performed in the DAVID online tool after a 44k microarray in the brain exposed and control samples and included the categories biological processes, cellular component and molecular function. In this table is also possible to check the number of probes (#) altered in each category term.

GO category	Term	#
Biological process	GO:0050896~response to stimulus	35
	GO:0055114~oxidation reduction	31
	GO:0006082~organic acid metabolic process	25
	GO:0019752~carboxylic acid metabolic process	25
	GO:0042180~cellular ketone metabolic process	25
	GO:0043436~oxoacid metabolic process	25
	GO:0009308~amine metabolic process	18
	GO:0006519~cellular amino acid and derivative metabolic process	17
	GO:0006520~cellular amino acid metabolic process	16
	GO:0042221~response to chemical stimulus	16
	GO:0044106~cellular amine metabolic process	16
	GO:0044271~nitrogen compound biosynthetic process	14
	GO:0006066~alcohol metabolic process	11
	GO:0034660~ncRNA metabolic process	11
	GO:0009607~response to biotic stimulus	9
	GO:0032787~monocarboxylic acid metabolic process	9

	GO:0005996~monosaccharide metabolic process	8
	GO:0051704~multi-organism process	8
	GO:0051707~response to other organism	8
	GO:0006915~apoptosis	7
	GO:0008219~cell death	7
	GO:0010035~response to inorganic substance	7
	GO:0012501~programmed cell death	7
	GO:0016053~organic acid biosynthetic process	7
	GO:0016265~death	7
	GO:0046394~carboxylic acid biosynthetic process	7
	GO:0006418~tRNA aminoacylation for protein translation	6
	GO:0009617~response to bacterium	6
	GO:0022613~ribonucleoprotein complex biogenesis	6
	GO:0042254~ribosome biogenesis	6
	GO:0043038~amino acid activation	6
	GO:0043039~tRNA aminoacylation	6
	GO:0008652~cellular amino acid biosynthetic process	5
	GO:0009309~amine biosynthetic process	5
	GO:0006563~L-serine metabolic process	4
	GO:0009069~serine family amino acid metabolic process	4
	GO:0000096~sulfur amino acid metabolic process	3
	GO:0009070~serine family amino acid biosynthetic process	3
	GO:0009396~folic acid and derivative biosynthetic process	3
	GO:0051597~response to methylmercury	3
Cellular component	GO:0005783~endoplasmic reticulum	13
	GO:0031974~membrane-enclosed lumen	12
	GO:0043233~organelle lumen	12
	GO:0070013~intracellular organelle lumen	12
	GO:0031981~nuclear lumen	11
	GO:0005730~nucleolus	7
Molecular function	GO:0003824~catalytic activity	147
	GO:0016491~oxidoreductase activity	36
	GO:0005506~iron ion binding	22
	GO:0016874~ligase activity	13
	GO:0046906~tetrapyrrole binding	13
	GO:0020037~heme binding	12
	GO:0048037~cofactor binding	12
	GO:0019842~vitamin binding	10
	GO:0004497~monooxygenase activity	9
	GO:0016705~oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	7
	GO:0004812~aminoacyl-tRNA ligase activity	6
	GO:0016875~ligase activity, forming carbon-oxygen bonds	6
	GO:0016876~ligase activity, forming aminoacyl-tRNA and related compounds	6

Supplementary Table 3.10 Biological process terms significantly enriched by the upregulated genes in the brain of zebrafish after 96 h of ammonia exposure. This analysis was performed in the DAVID online tool after a 44k microarray in brain exposed and control samples. In this table is also possible to check the number of probes (#) altered in each category term.

Term	#
GO:0008152~metabolic process	111
GO:0006807~nitrogen compound metabolic process	42
GO:0034641~cellular nitrogen compound metabolic process	40
GO:0050896~response to stimulus	24
GO:0055114~oxidation reduction	23
GO:0006082~organic acid metabolic process	20
GO:0019752~carboxylic acid metabolic process	20
GO:0042180~cellular ketone metabolic process	20
GO:0043436~oxoacid metabolic process	20
GO:0009308~amine metabolic process	16
GO:0005975~carbohydrate metabolic process	15
GO:0006519~cellular amino acid and derivative metabolic process	15
GO:0016070~RNA metabolic process	15
GO:0006520~cellular amino acid metabolic process	14
GO:0044106~cellular amine metabolic process	14
GO:0044085~cellular component biogenesis	12
GO:0044271~nitrogen compound biosynthetic process	12
GO:0046483~heterocycle metabolic process	12
GO:0034660~ncRNA metabolic process	11
GO:0042221~response to chemical stimulus	11
GO:0006066~alcohol metabolic process	10
GO:0055086~nucleobase, nucleoside and nucleotide metabolic process	10
GO:0009607~response to biotic stimulus	9
GO:0005996~monosaccharide metabolic process	8
GO:0051704~multi-organism process	8
GO:0051707~response to other organism	8
GO:0006163~purine nucleotide metabolic process	7
GO:0006164~purine nucleotide biosynthetic process	7
GO:0019318~hexose metabolic process	7
GO:0006399~tRNA metabolic process	6
GO:0006418~tRNA aminoacylation for protein translation	6
GO:0006725~cellular aromatic compound metabolic process	6
GO:0006915~apoptosis	6
GO:0008219~cell death	6
GO:0009617~response to bacterium	6
GO:0012501~programmed cell death	6
GO:0016265~death	6
GO:0022613~ribonucleoprotein complex biogenesis	6
GO:0032787~monocarboxylic acid metabolic process	6
GO:0042254~ribosome biogenesis	6

GO:0043038~amino acid activation	6
GO:0043039~tRNA aminoacylation	6
GO:0010033~response to organic substance	5
GO:0010035~response to inorganic substance	5
GO:0016053~organic acid biosynthetic process	5
GO:0046394~carboxylic acid biosynthetic process	5
GO:0006364~rRNA processing	4
GO:0006563~L-serine metabolic process	4
GO:0008652~cellular amino acid biosynthetic process	4
GO:0009069~serine family amino acid metabolic process	4
GO:0010942~positive regulation of cell death	4
GO:0043065~positive regulation of apoptosis	4
GO:0043068~positive regulation of programmed cell death	4
GO:0000096~sulfur amino acid metabolic process	3
GO:0006760~folic acid and derivative metabolic process	3
GO:0009070~serine family amino acid biosynthetic process	3
GO:0009396~folic acid and derivative biosynthetic process	3
GO:0009615~response to virus	3
GO:0051597~response to methylmercury	3

Supplementary Table 3.11 Biological process terms significantly enriched by the downregulated genes in the brain of zebrafish after 96 h of ammonia exposure. This analysis was performed in the DAVID online tool after a 44k microarray in brain exposed and control samples. It is also possible to check in this table the number of probes (#) altered in each category term.

Term	#
GO:0032501~multicellular organismal process	31
GO:0032502~developmental process	28
GO:0007275~multicellular organismal development	27
GO:0051179~localization	27
GO:0048856~anatomical structure development	26
GO:0048731~system development	22
GO:0048513~organ development	18
GO:0009653~anatomical structure morphogenesis	16
GO:0007399~nervous system development	11
GO:0009887~organ morphogenesis	10
GO:0006629~lipid metabolic process	9
GO:0009888~tissue development	8
GO:0048598~embryonic morphogenesis	7
GO:0048646~anatomical structure formation involved in morphogenesis	7
GO:0009792~embryonic development ending in birth or egg hatching	6
GO:0016477~cell migration	6
GO:0040011~locomotion	6
GO:0043009~chordate embryonic development	6
GO:0048870~cell motility	6
GO:0051674~localization of cell	6

GO:0001501~skeletal system development	5
GO:0001667~ameboidal cell migration	5
GO:0050877~neurological system process	5
GO:0051216~cartilage development	4

Supplementary Table 3.12 Gene Ontology (GO) terms significantly enriched by the responsive gene that were simultaneously altered in gill, liver and brain of zebrafish after exposure to ammonia during 96 h. This analysis was performed in the DAVID online tool after a 44k microarray and included the categories biological processes, cellular component and molecular function. In this table is also possible to check the number of probes (#) altered in each category term.

GO category	Term	#
Biological process	GO:0006629~lipid metabolic process	4
Molecular function	GO:0005544~calcium-dependent phospholipid binding	2

Supplementary Table 3.13 Gene Ontology (GO) terms significantly enriched by the responsive gene that were simultaneously altered in gill and liver of zebrafish after exposure to ammonia during 96 h. This analysis was performed in the DAVID online tool after a 44k microarray and included the categories biological processes, cellular component and molecular function. In this table is also possible to check the number of probes (#) altered in each category term.

GO category	Term	#
Biological process	GO:0065007~biological regulation	155
	GO:0050789~regulation of biological process	146
	GO:0050794~regulation of cellular process	139
	GO:0032501~multicellular organismal process	82
	GO:0032502~developmental process	81
	GO:0007275~multicellular organismal development	79
	GO:0031323~regulation of cellular metabolic process	72
	GO:0080090~regulation of primary metabolic process	69
	GO:0051171~regulation of nitrogen compound metabolic process	69
	GO:0031326~regulation of cellular biosynthetic process	69
	GO:0019219~regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	69
	GO:0009889~regulation of biosynthetic process	69
	GO:0045449~regulation of transcription	68
	GO:0010556~regulation of macromolecule biosynthetic process	68
	GO:0048856~anatomical structure development	63
	GO:0051252~regulation of RNA metabolic process	62
	GO:0006355~regulation of transcription, DNA-dependent	62
	GO:0048731~system development	56
	GO:0048513~organ development	52
	GO:0009653~anatomical structure morphogenesis	42
	GO:0055114~oxidation reduction	33
	GO:0009790~embryonic development	33
	GO:0006811~ion transport	32
	GO:0006812~cation transport	24

	GO:0009888~tissue development	21
	GO:0009887~organ morphogenesis	20
	GO:0006629~lipid metabolic process	20
	GO:0048598~embryonic morphogenesis	19
	GO:0048518~positive regulation of biological process	19
	GO:0048522~positive regulation of cellular process	16
	GO:0048568~embryonic organ development	13
	GO:0043009~chordate embryonic development	13
	GO:0009792~embryonic development ending in birth or egg hatching	13
	GO:0051239~regulation of multicellular organismal process	11
	GO:0001501~skeletal system development	11
	GO:0048562~embryonic organ morphogenesis	10
	GO:0048839~inner ear development	9
	GO:0043583~ear development	9
	GO:0012501~programmed cell death	8
	GO:0010604~positive regulation of macromolecule metabolic process	8
	GO:0006915~apoptosis	8
	GO:0051216~cartilage development	7
	GO:0045941~positive regulation of transcription	7
	GO:0045165~cell fate commitment	7
	GO:0042472~inner ear morphogenesis	7
	GO:0042471~ear morphogenesis	7
	GO:0030902~hindbrain development	7
	GO:0010628~positive regulation of gene expression	7
	GO:0006820~anion transport	7
	GO:0060485~mesenchyme development	6
	GO:0048762~mesenchymal cell differentiation	6
	GO:0015698~inorganic anion transport	6
	GO:0008202~steroid metabolic process	6
	GO:0001708~cell fate specification	6
	GO:0006821~chloride transport	5
	GO:0031290~retinal ganglion cell axon guidance	4
	GO:0008284~positive regulation of cell proliferation	4
	GO:0060037~pharyngeal system development	3
	GO:0015696~ammonium transport	3
	GO:0015695~organic cation transport	3
Celular component	GO:0016020~membrane	166
	GO:0044425~membrane part	139
	GO:0031224~intrinsic to membrane	132
	GO:0016021~integral to membrane	131
	GO:0005886~plasma membrane	48
	GO:0044459~plasma membrane part	33
	GO:0030054~cell junction	17
	GO:0005911~cell-cell junction	15
	GO:0070160~occluding junction	12
	GO:0043296~apical junction complex	12

	GO:0016327~apicolateral plasma membrane	12
	GO:0005923~tight junction	12
	GO:0045111~intermediate filament cytoskeleton	7
	GO:0015629~actin cytoskeleton	7
	GO:0005882~intermediate filament	7
Molecular function	GO:0003677~DNA binding	69
	GO:0030528~transcription regulator activity	60
	GO:0003700~transcription factor activity	54
	GO:0005215~transporter activity	50
	GO:0043565~sequence-specific DNA binding	44
	GO:0022891~substrate-specific transmembrane transporter activity	33
	GO:0004252~serine-type endopeptidase activity	13
	GO:0008236~serine-type peptidase activity	13
	GO:0017171~serine hydrolase activity	13
	GO:0005543~phospholipid binding	8
	GO:0016614~oxidoreductase activity, acting on CH-OH group of donors	8
	GO:0016616~oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	8
	GO:0042802~identical protein binding	8
	GO:0005544~calcium-dependent phospholipid binding	6
	GO:0016769~transferase activity, transferring nitrogenous groups	6
	GO:0008483~transaminase activity	5
	GO:0008519~ammonium transmembrane transporter activity	3

Supplementary Table 3.14 Gene Ontology (GO) terms significantly enriched by the responsive gene that were simultaneously altered in gill and brain of zebrafish after exposure to ammonia during 96 h. This analysis was performed in the DAVID online tool after a 44k microarray and included the categories biological processes, cellular component and molecular function. In this table is also possible to check the number of probes (#) altered in each category term.

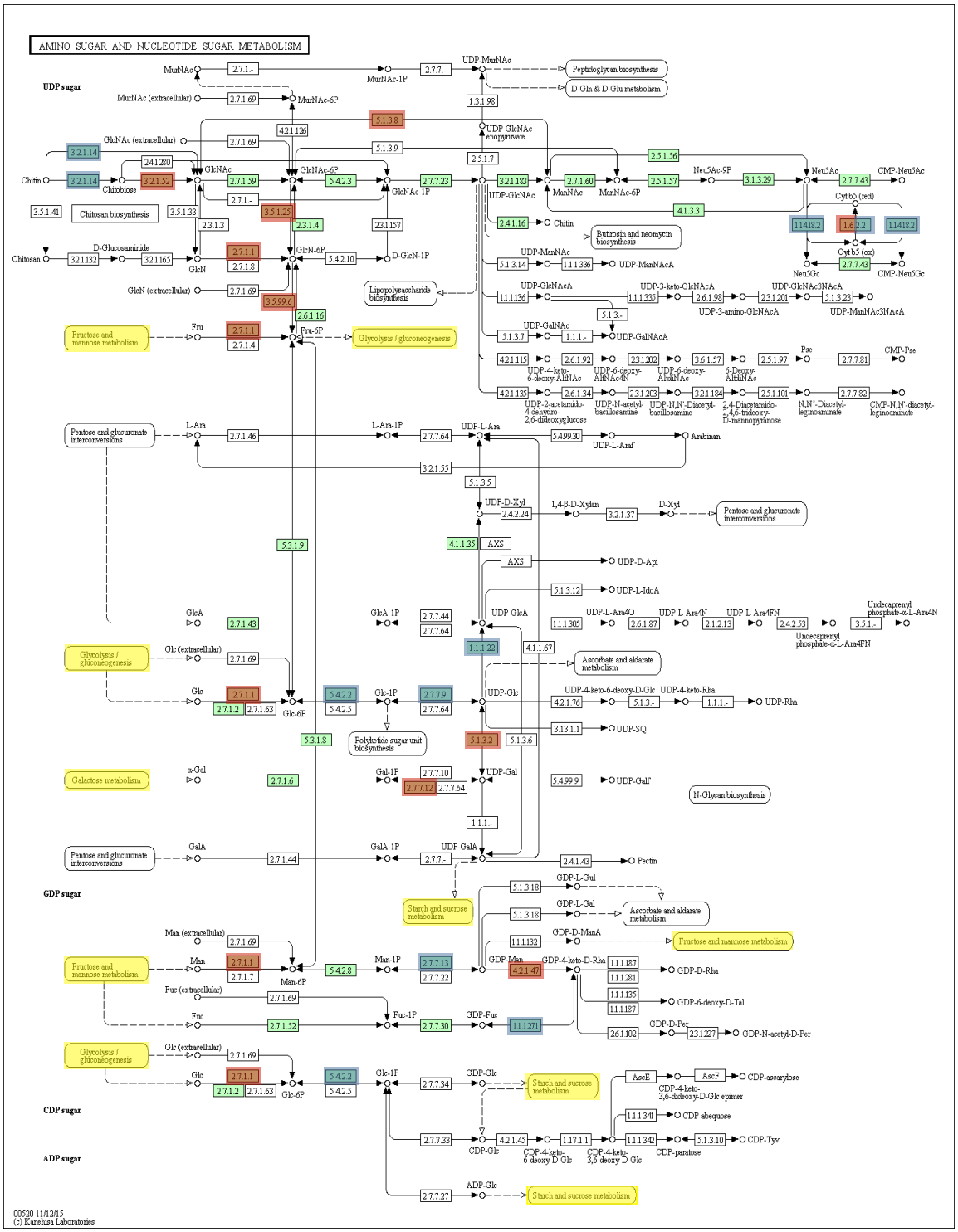
GO category	Term	#
Biological process	GO:0008152~metabolic process	91
	GO:0055114~oxidation reduction	25
	GO:0043436~oxoacid metabolic process	18
	GO:0042180~cellular ketone metabolic process	18
	GO:0019752~carboxylic acid metabolic process	18
	GO:0006082~organic acid metabolic process	18
	GO:0009308~amine metabolic process	11
	GO:0006629~lipid metabolic process	11
	GO:0006519~cellular amino acid and derivative metabolic process	11
	GO:0044106~cellular amine metabolic process	10
	GO:0042221~response to chemical stimulus	10
	GO:0006520~cellular amino acid metabolic process	10
	GO:0006066~alcohol metabolic process	9
	GO:0032787~monocarboxylic acid metabolic process	8
	GO:0005996~monosaccharide metabolic process	6

	GO:0019318~hexose metabolic process	5
	GO:0009617~response to bacterium	4
	GO:0055072~iron ion homeostasis	3
	GO:0006879~cellular iron ion homeostasis	3
	GO:0006720~isoprenoid metabolic process	3
	GO:0000096~sulfur amino acid metabolic process	3
Celular component	GO:0005783~endoplasmic reticulum	9
Molecular function	GO:0003824~catalytic activity	91
	GO:0016491~oxidoreductase activity	27
	GO:0005506~iron ion binding	19
	GO:0046906~tetrapyrrole binding	11
	GO:0020037~heme binding	10
	GO:0009055~electron carrier activity	8
	GO:0048037~cofactor binding	8
	GO:0004497~monooxygenase activity	7
	GO:0019842~vitamin binding	7
	GO:0016705~oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	6
	GO:0005543~phospholipid binding	5
	GO:0019200~carbohydrate kinase activity	3

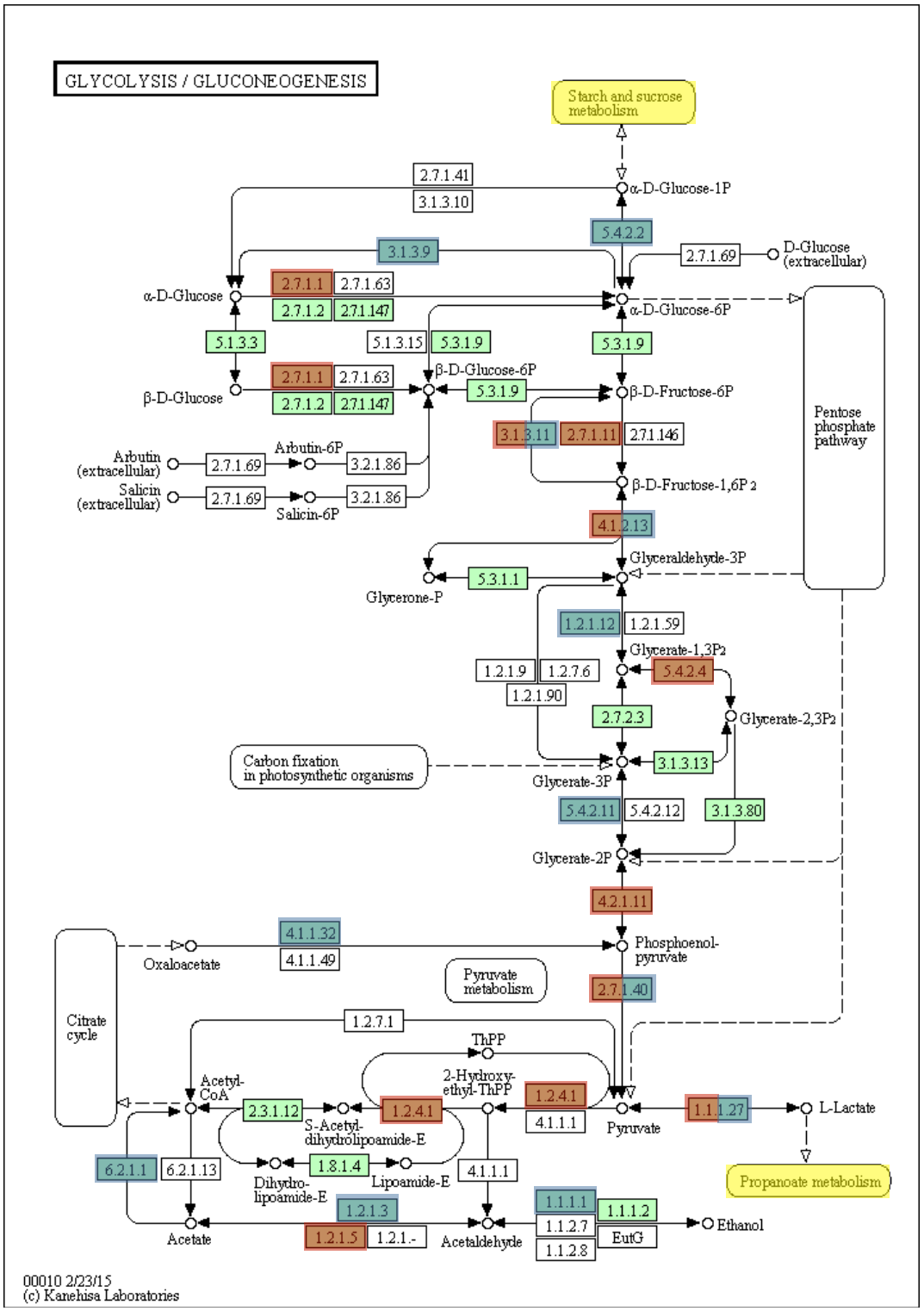
Supplementary Table 3.15 Gene Ontology (GO) terms significantly enriched by the responsive gene that were simultaneously altered in brain and liver of zebrafish after exposure to ammonia during 96 h. This analysis was performed in the DAVID online tool after a 44k microarray and included the categories biological processes, cellular component and molecular function. In this table is also possible to check the number of probes (#) altered in each category term.

GO category	Term	#
Biological process	GO:0006629~lipid metabolic process	6
	GO:0008202~steroid metabolic process	3

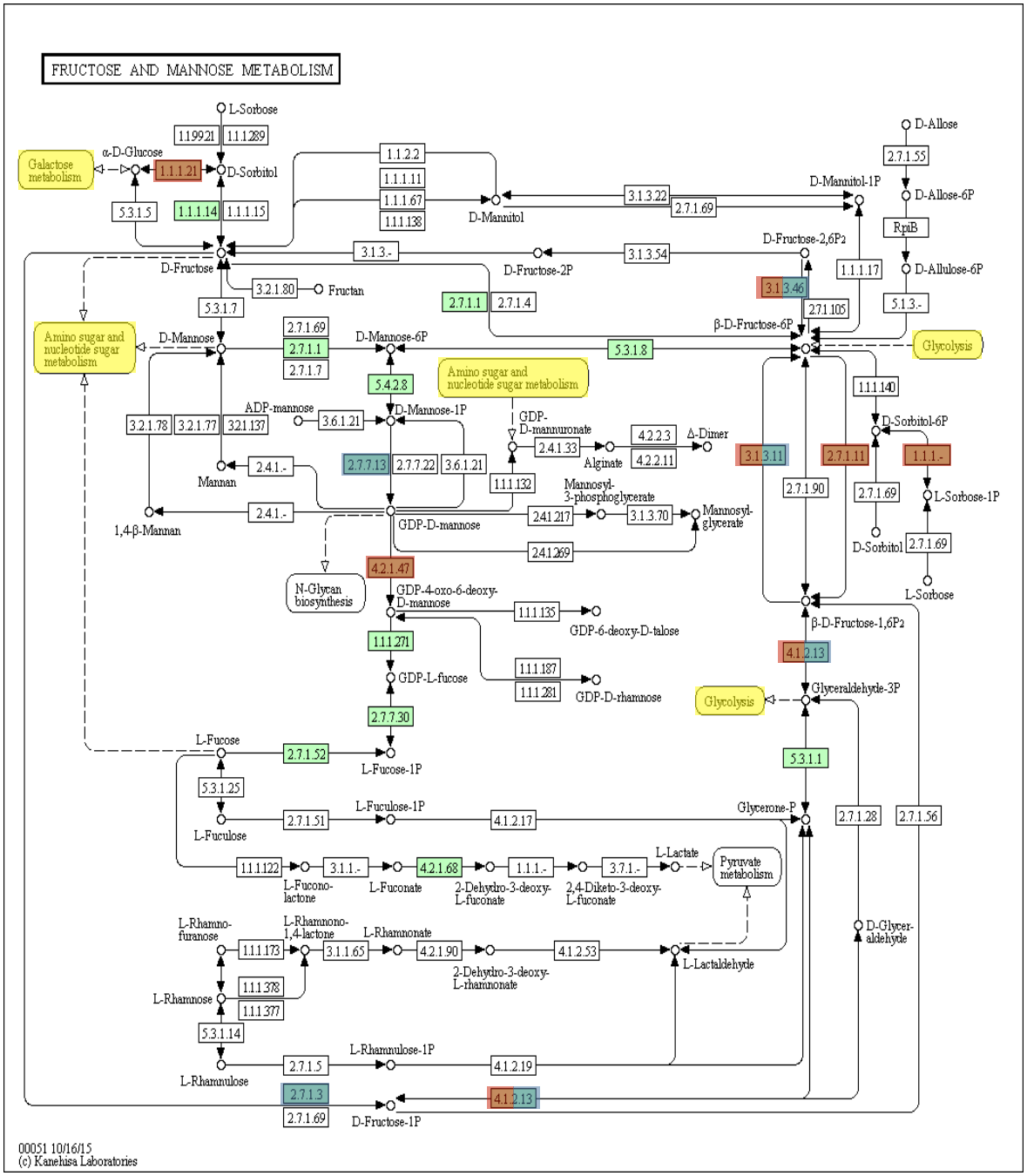
3.8 Supplementary figures



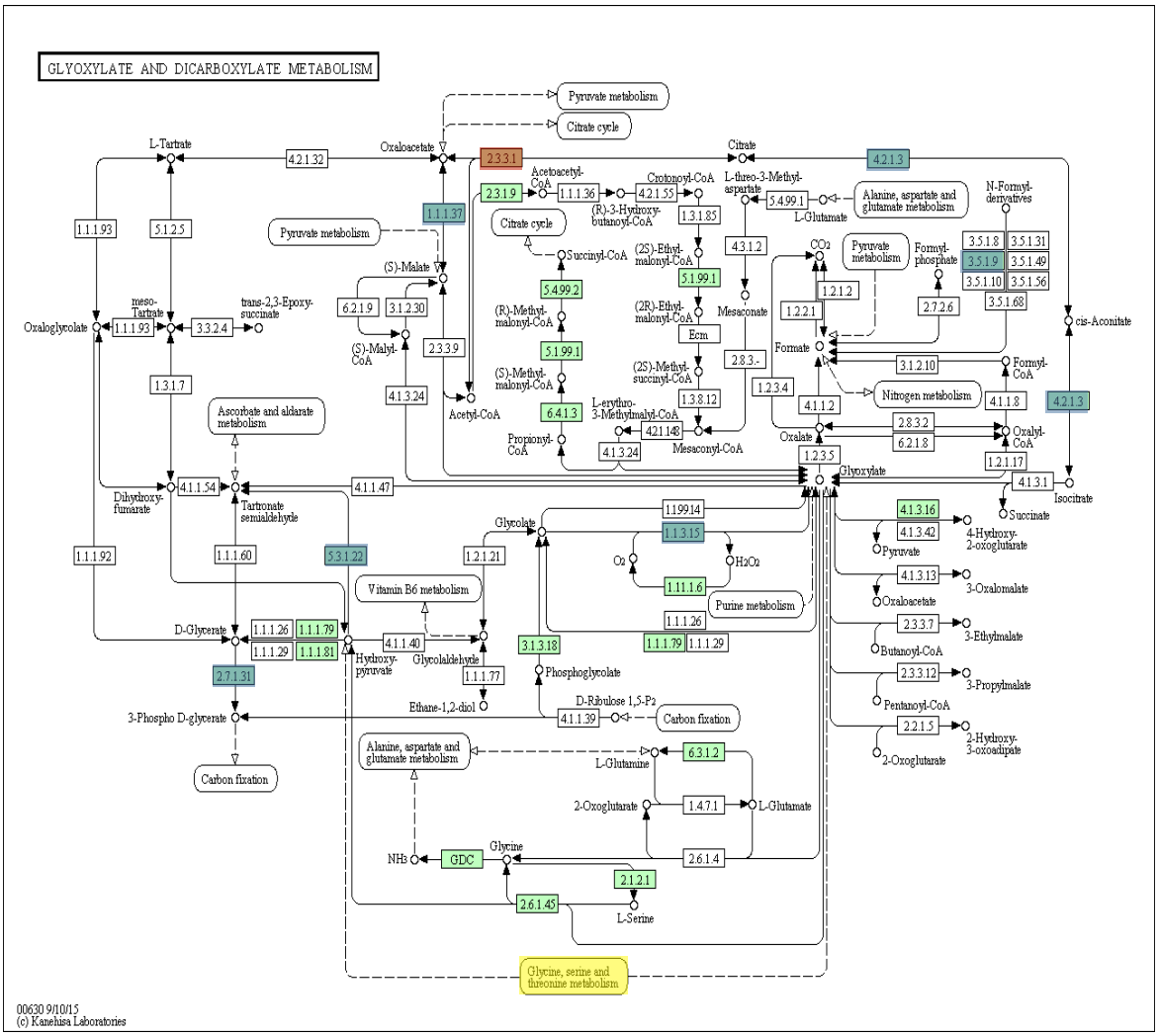
Supplementary Figure 3.4 Amino sugar and nucleotide sugar metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in gill.



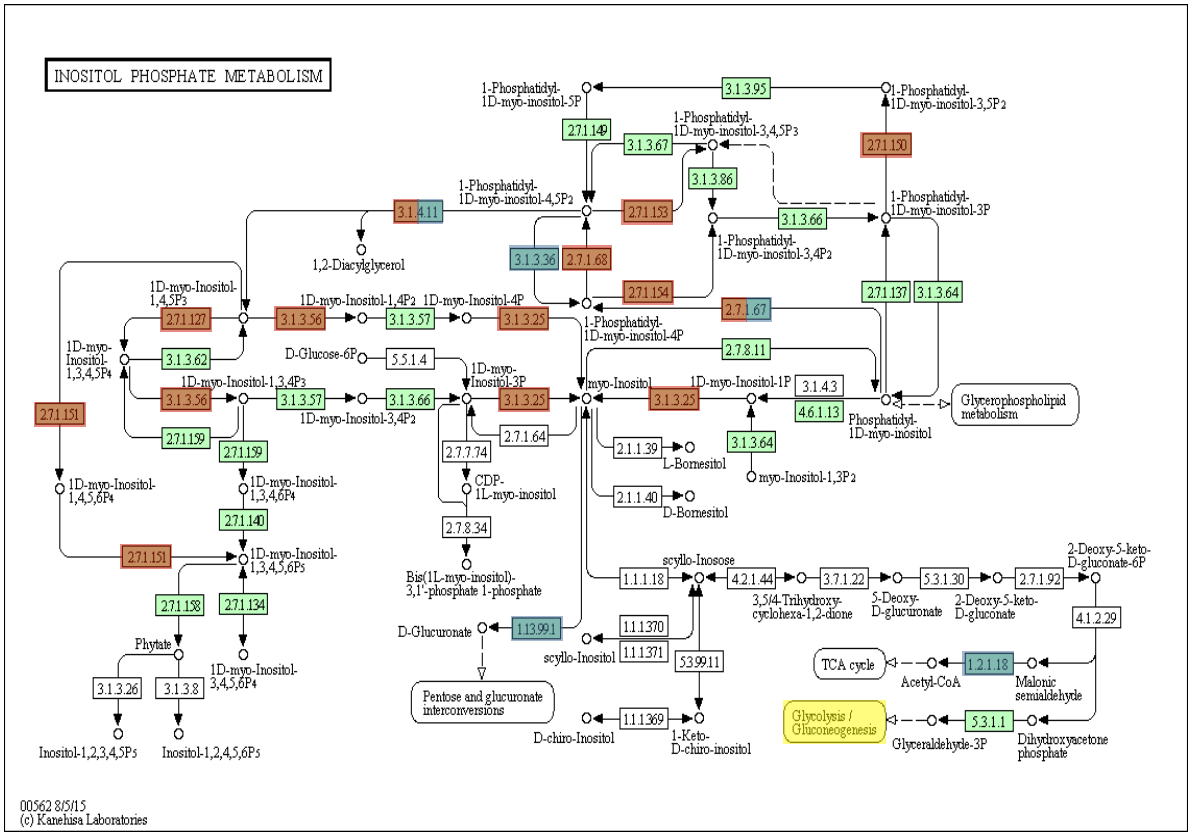
Supplementary Figure 3.5 Glycolysis/gluconeogenesis pathway in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in gill.



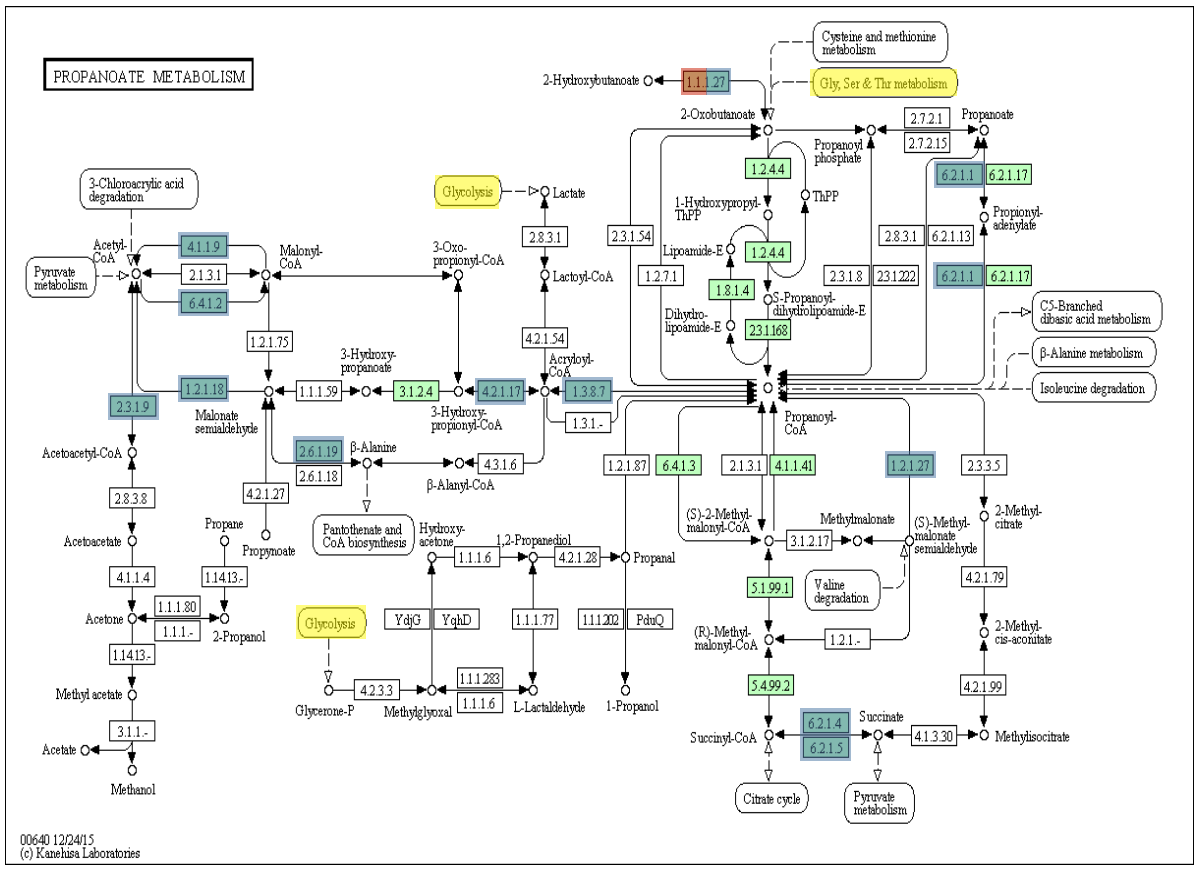
Supplementary Figure 3.6 Fructose and mannose metabolic pathway in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in gill.



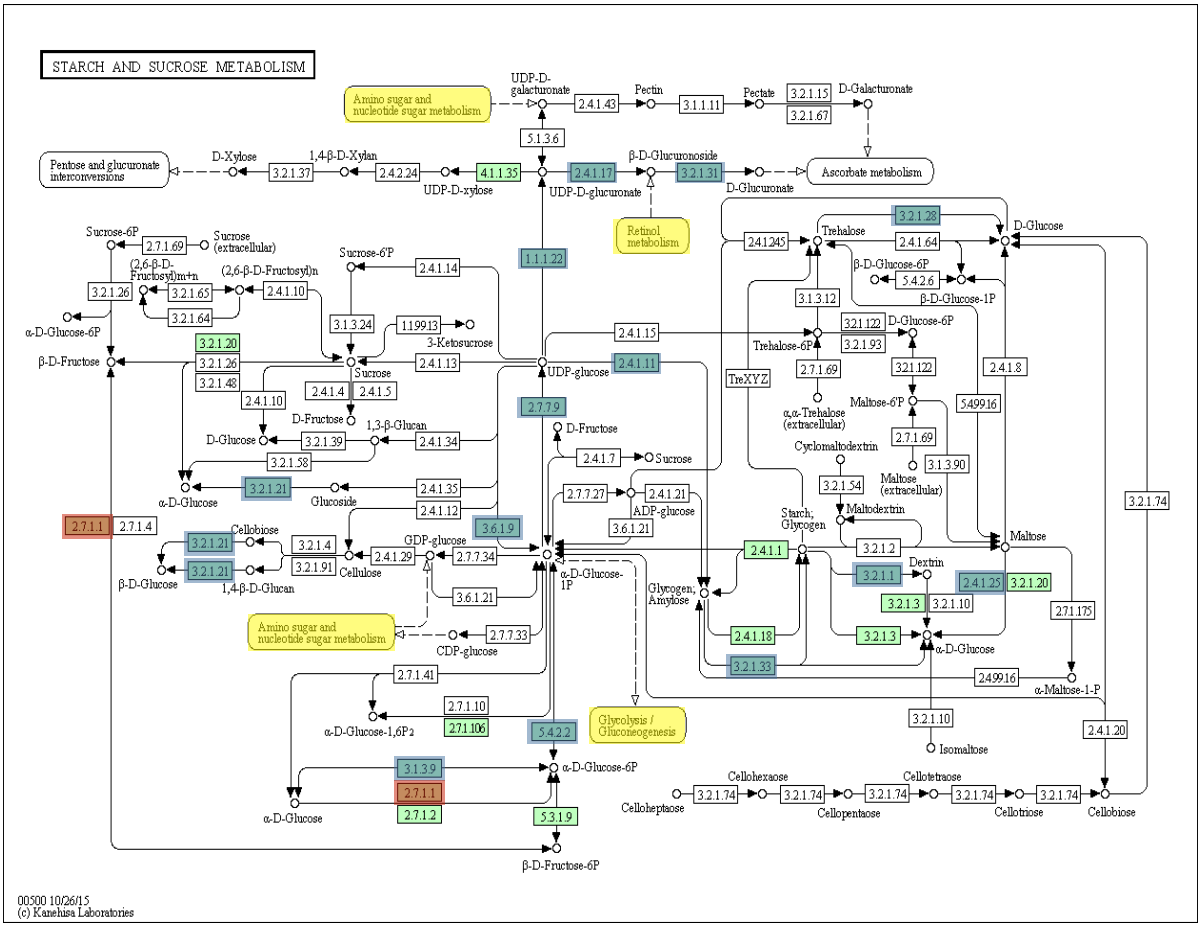
Supplementary Figure 3.7 Glyoxylate and dicarboxylate metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; yellow - pathway also responsive in gill.



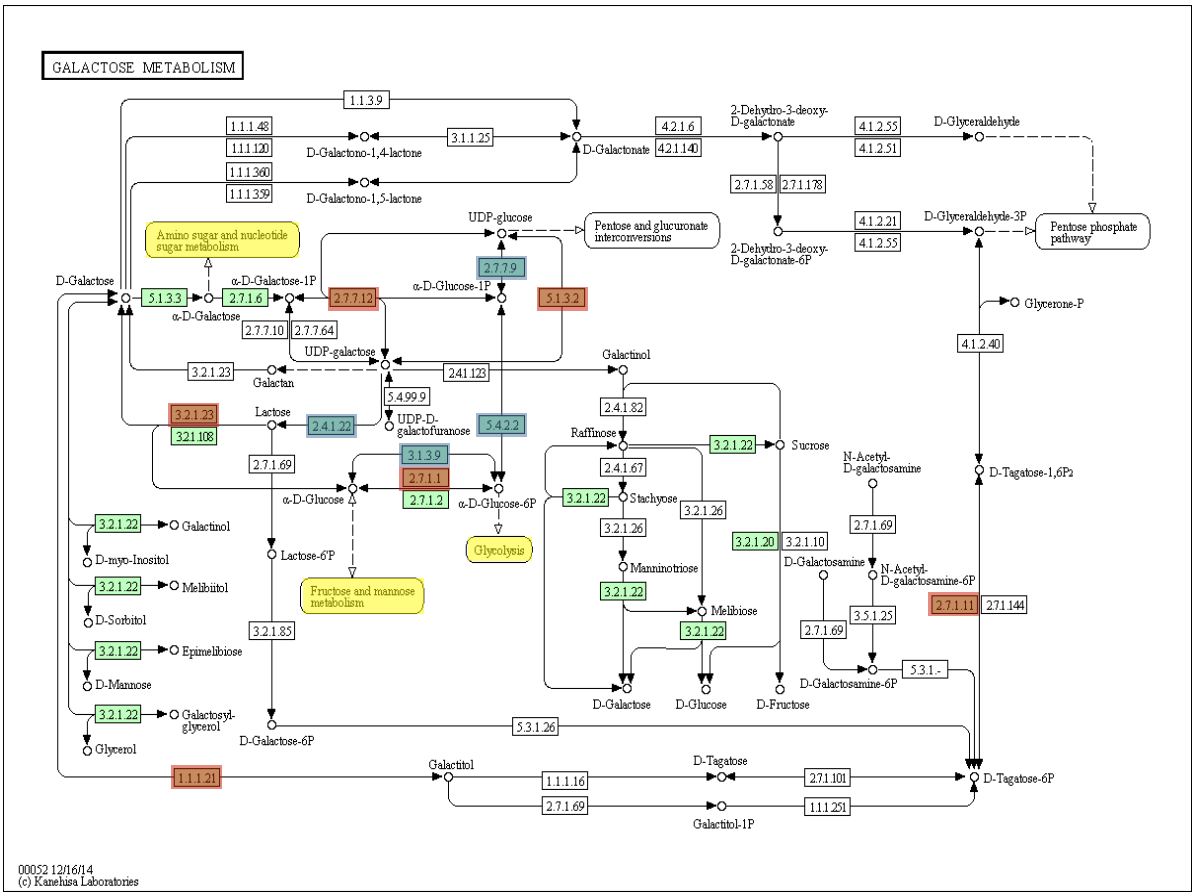
Supplementary Figure 3.8 Inositol phosphate metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in gill.



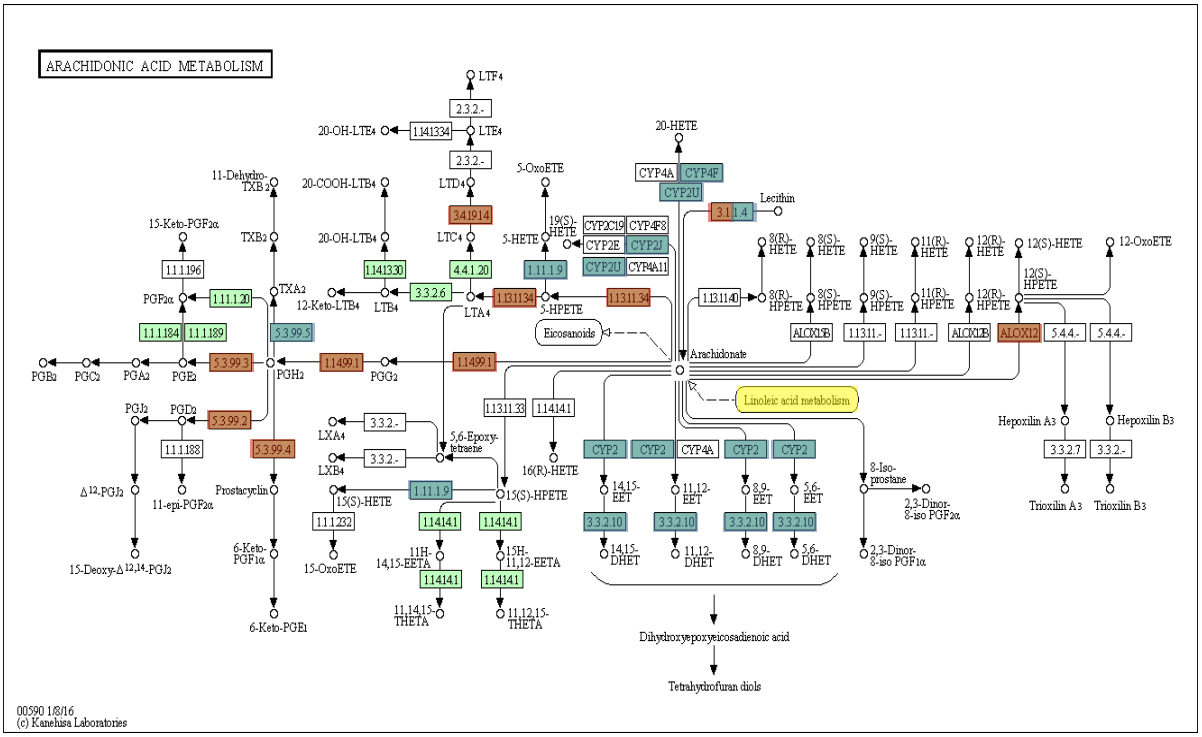
Supplementary Figure 3.9 Propanoate metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in gill.



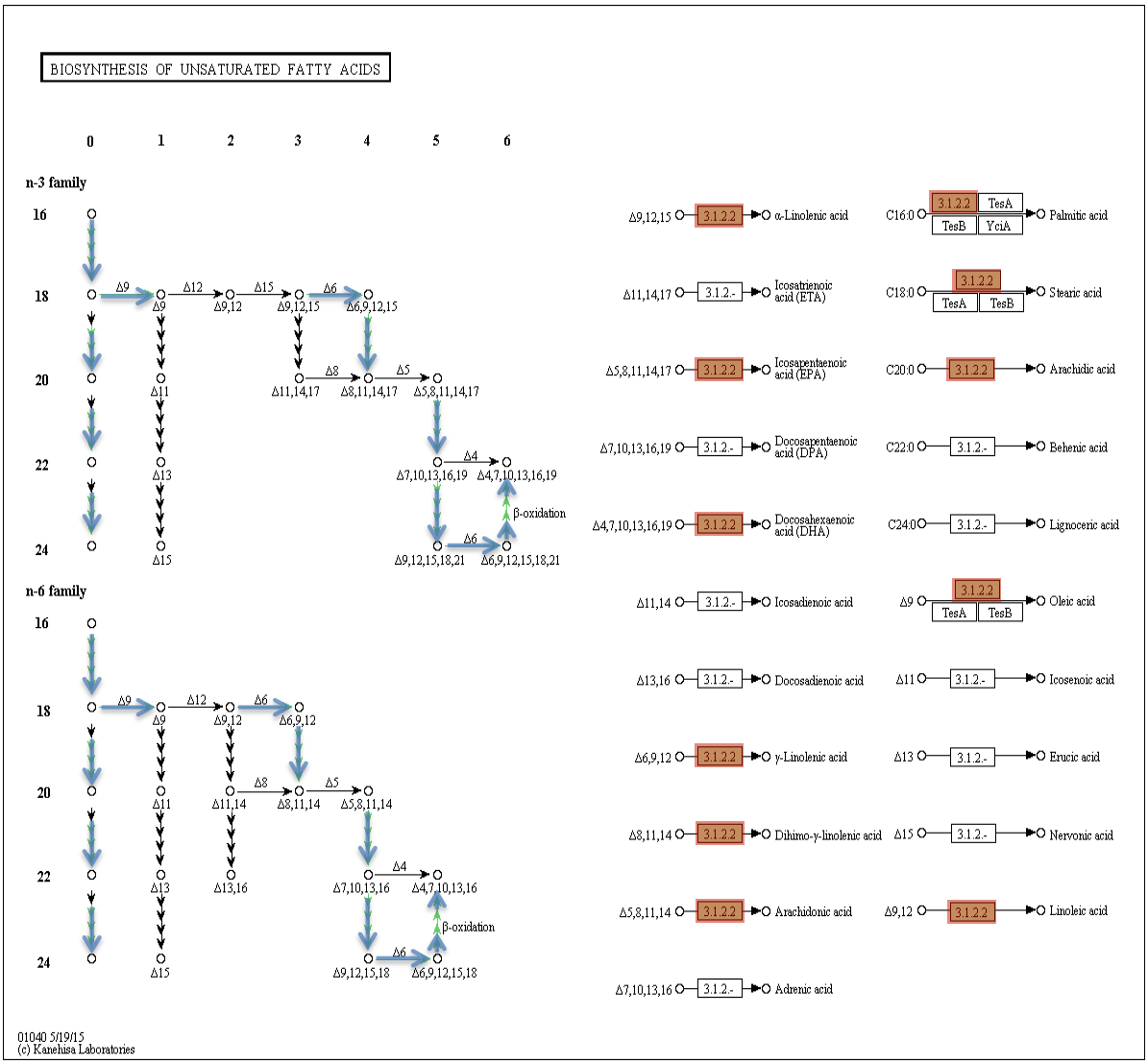
Supplementary Figure 3.10 Starch and sucrose metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; yellow - pathway also responsive in gill.



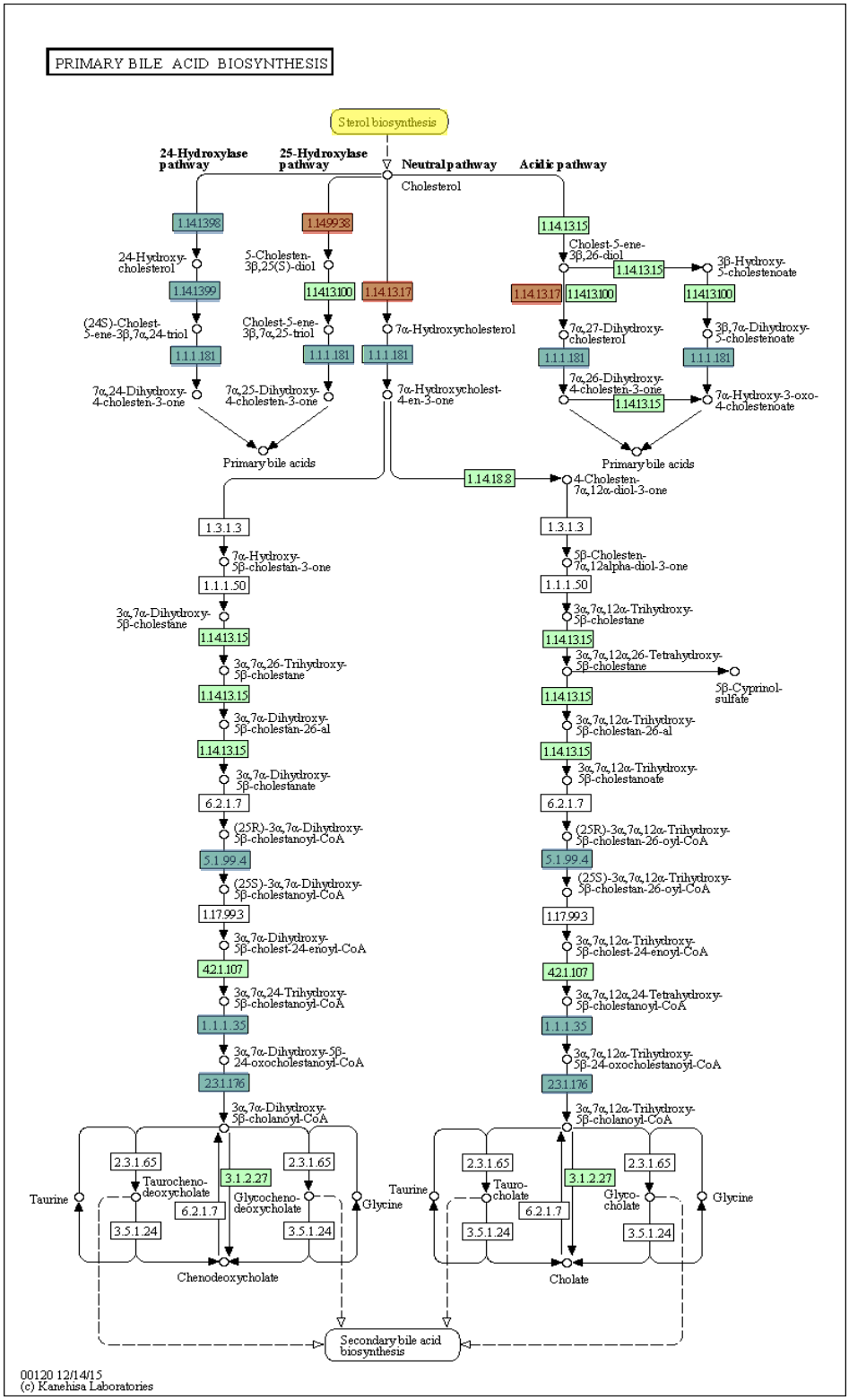
Supplementary Figure 3.11 Galactose metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; yellow - pathway also responsive in gill.



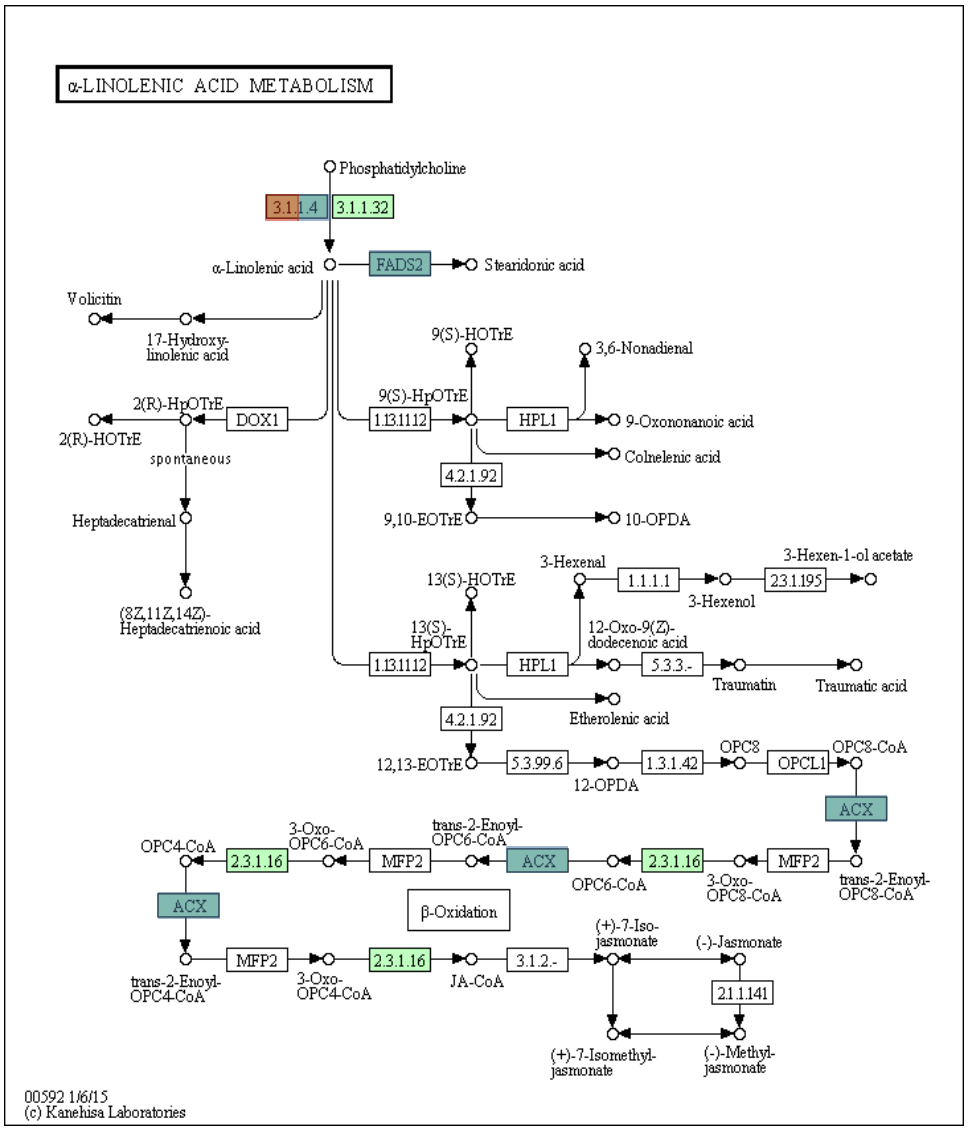
Supplementary Figure 3.12 Arachidonic acid metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in gill.



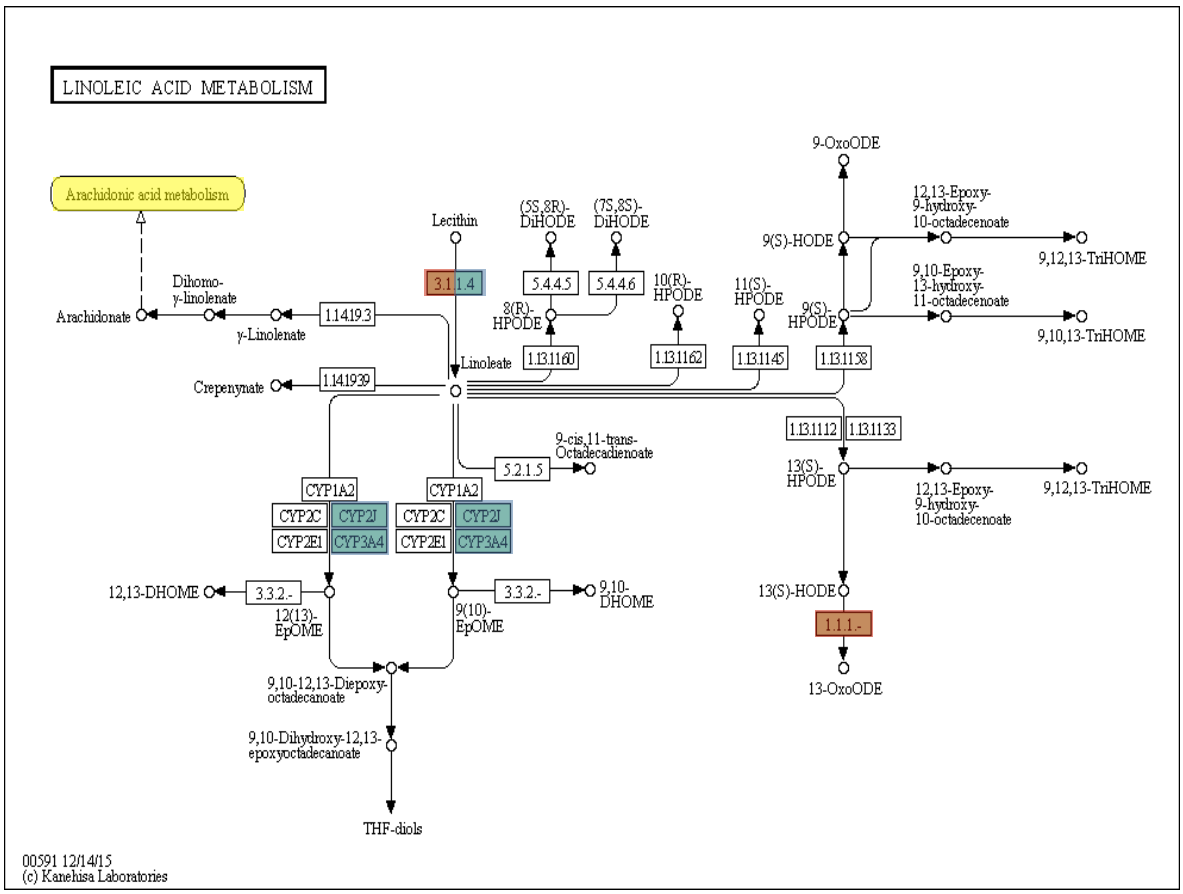
Supplementary Figure 3.13 Unsaturated fatty acids biosynthesis pathway in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated.



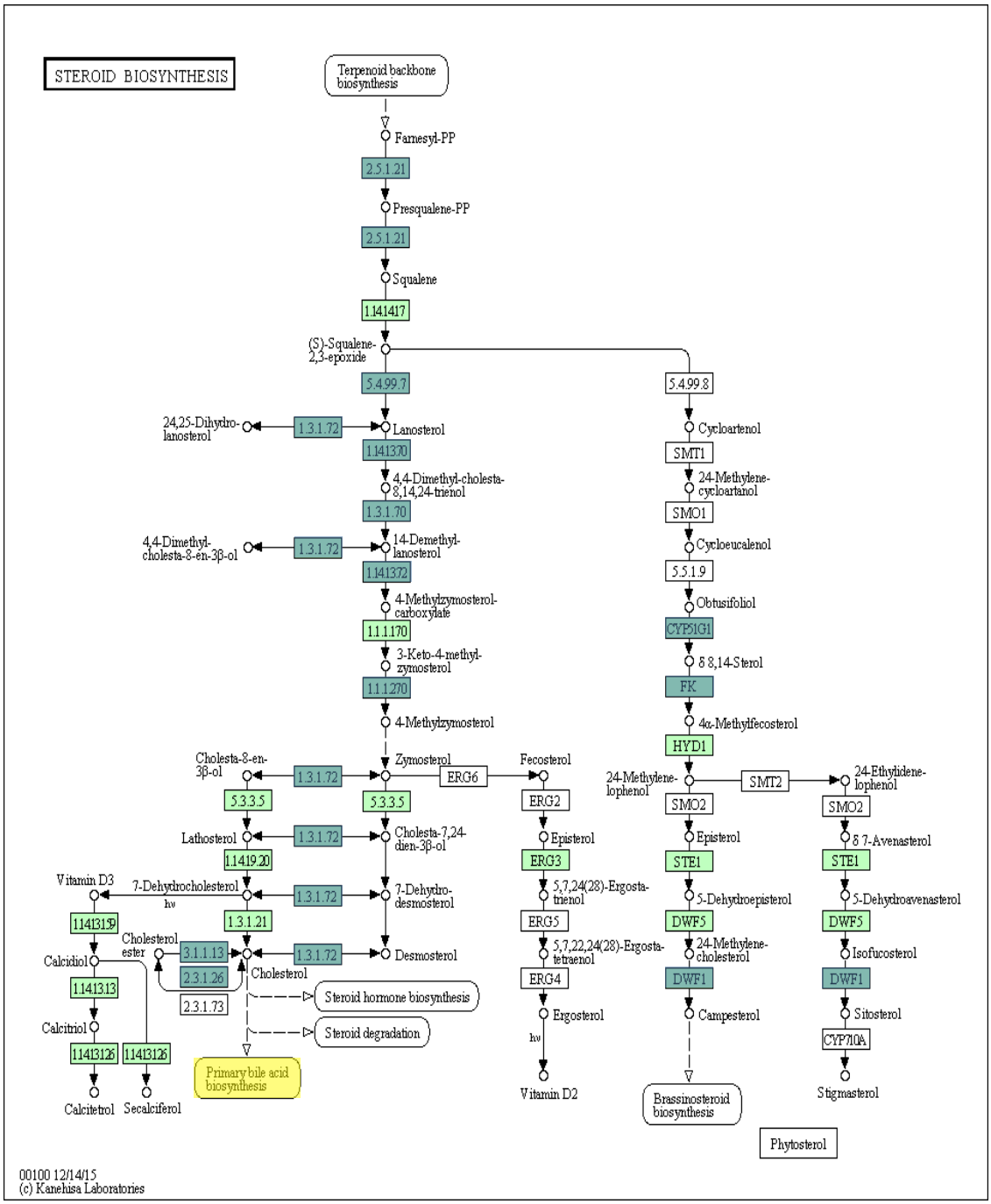
Supplementary Figure 3.14 Primary bile acid biosynthesis in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - dow-regulated; red - upregulated; yellow - pathway also responsive in gill.



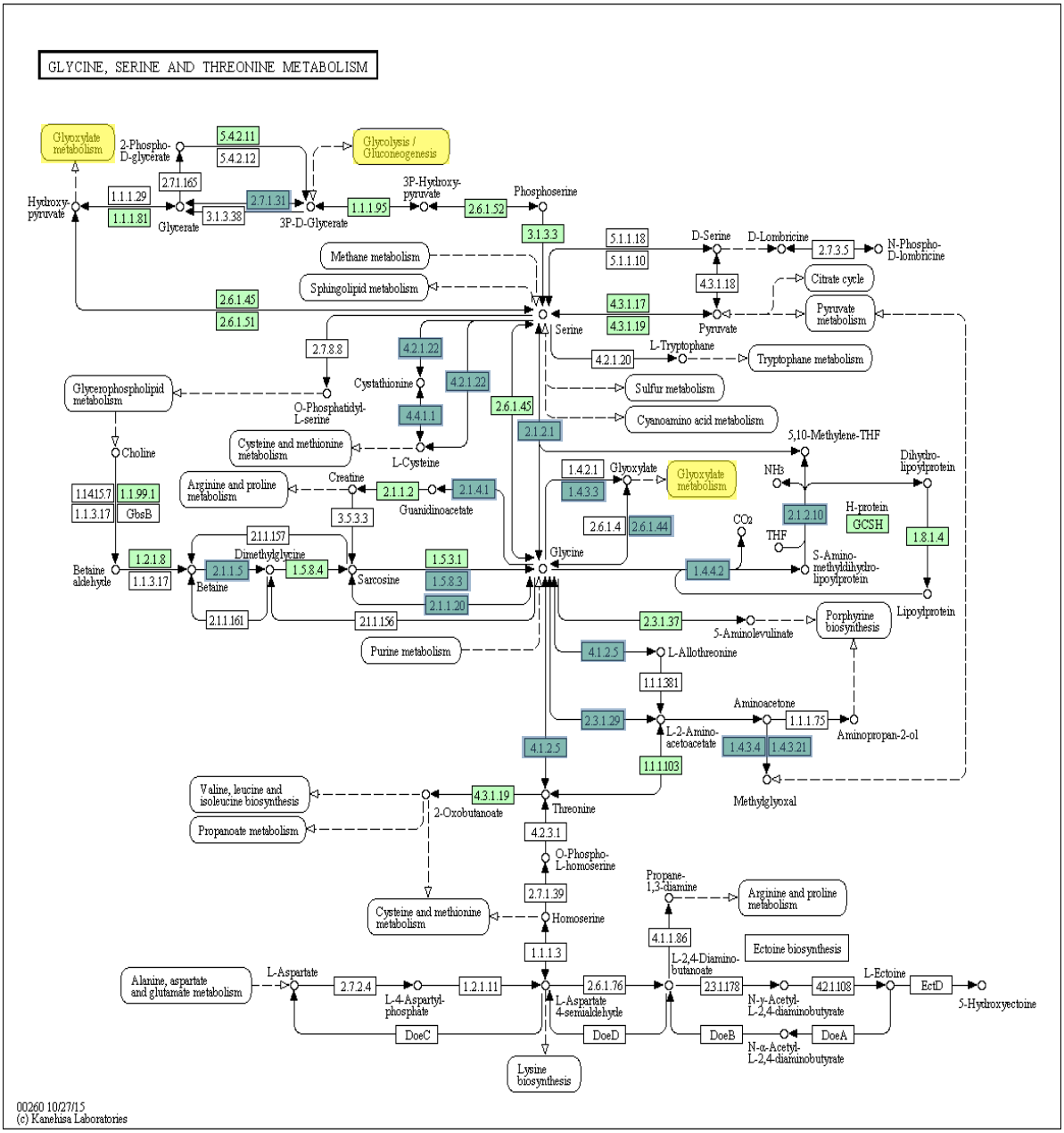
Supplementary Figure 3.15 Alpha-linolenic acid metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; blue and red - downregulated and upregulated.



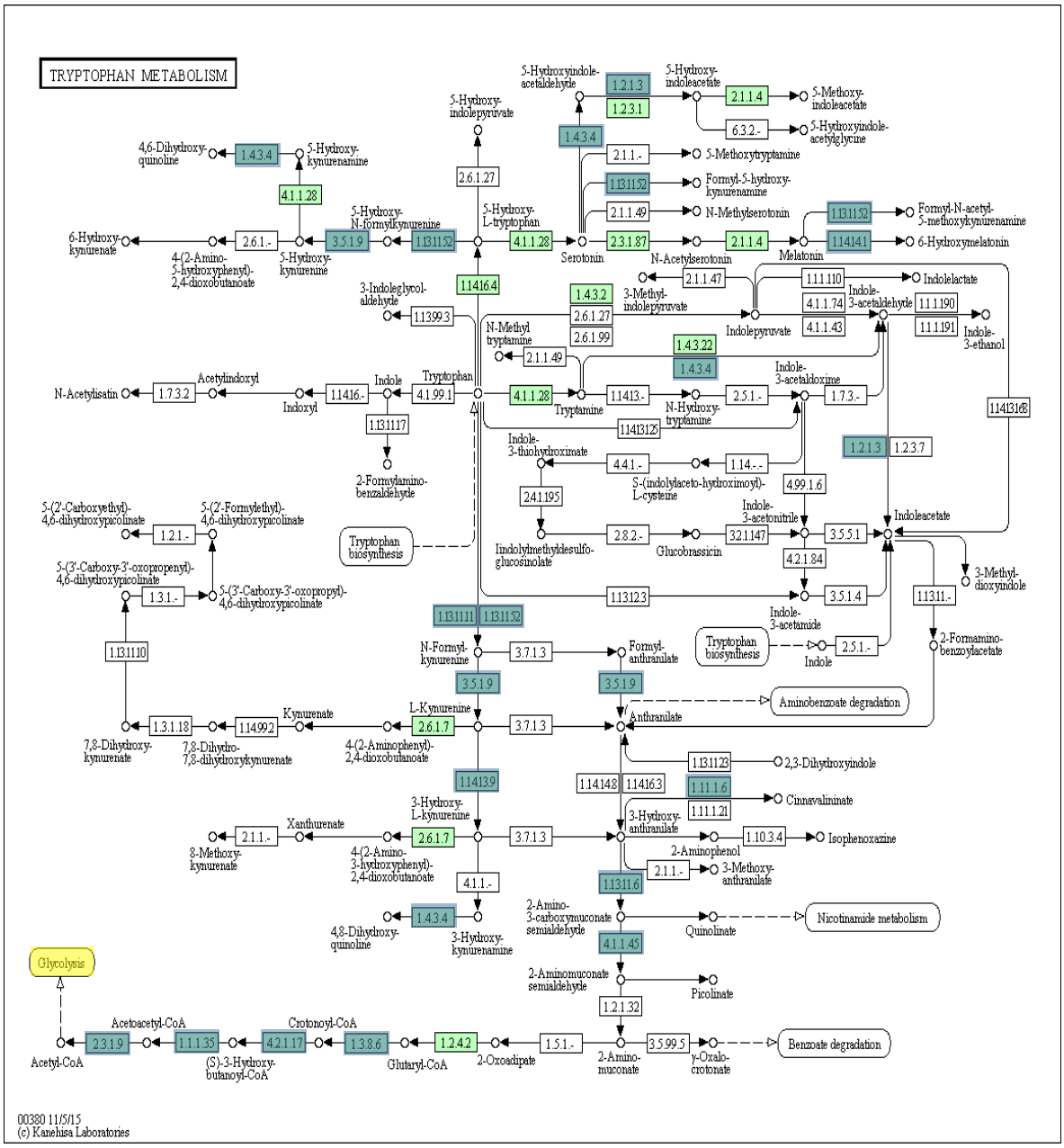
Supplementary Figure 3.16 Linoleic acid metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in gill.



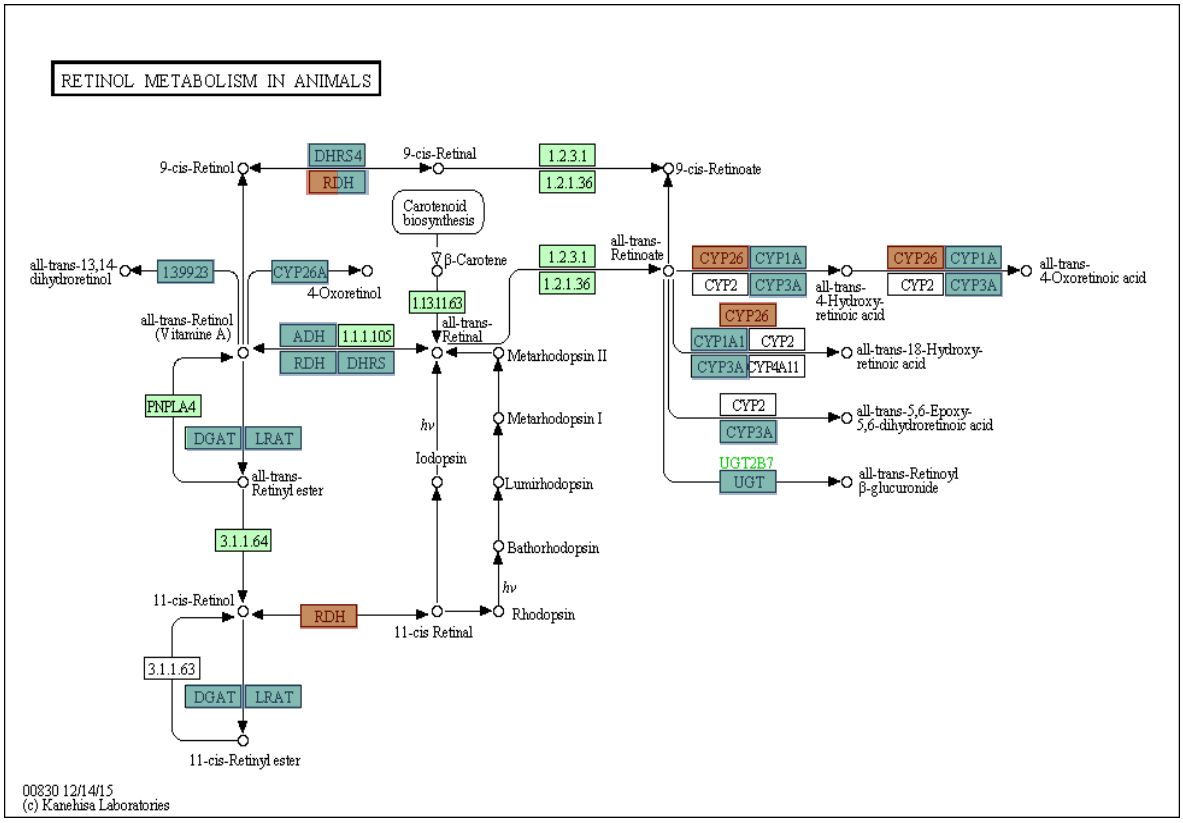
Supplementary Figure 3.17 Steroid biosynthesis pathway in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; yellow - pathway also responsive in gill.



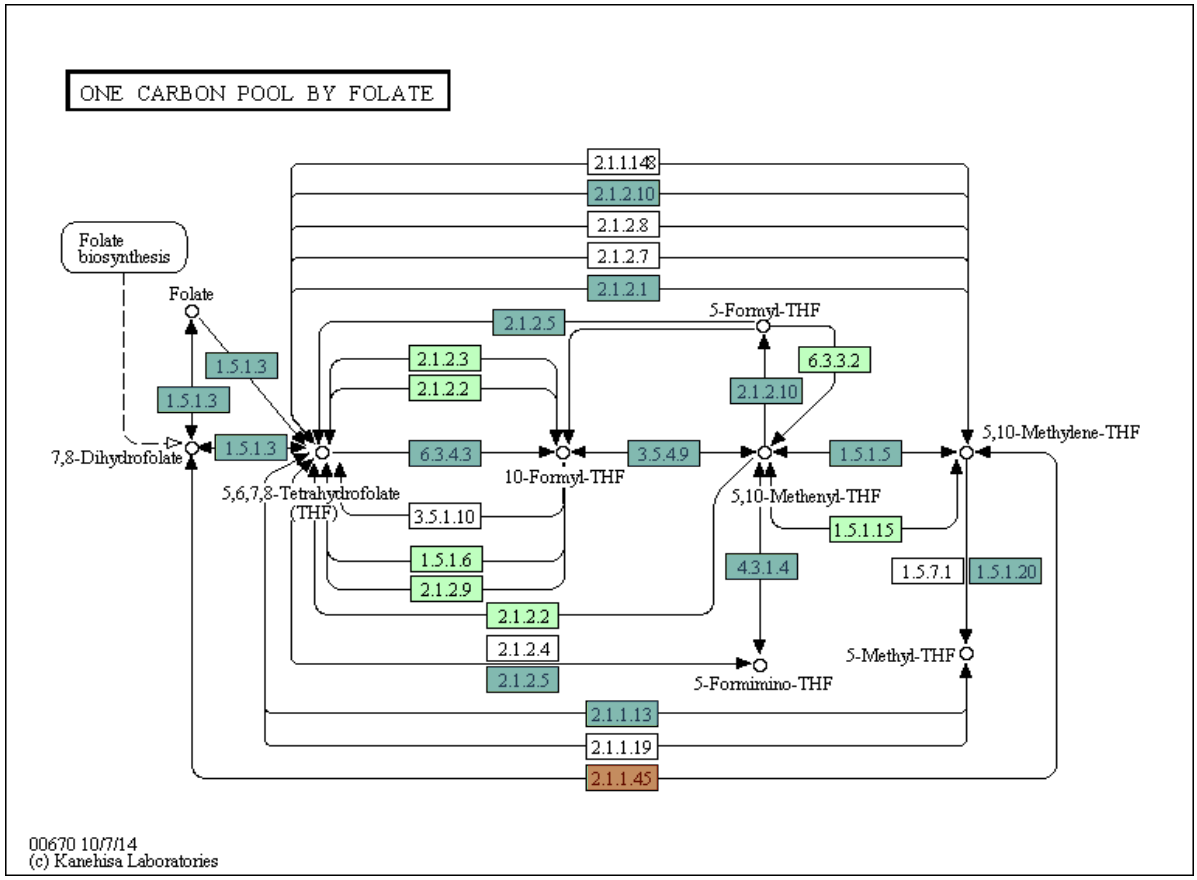
Supplementary Figure 3.18 Glycine, serine and threonine metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; yellow - pathway also responsive in gill.



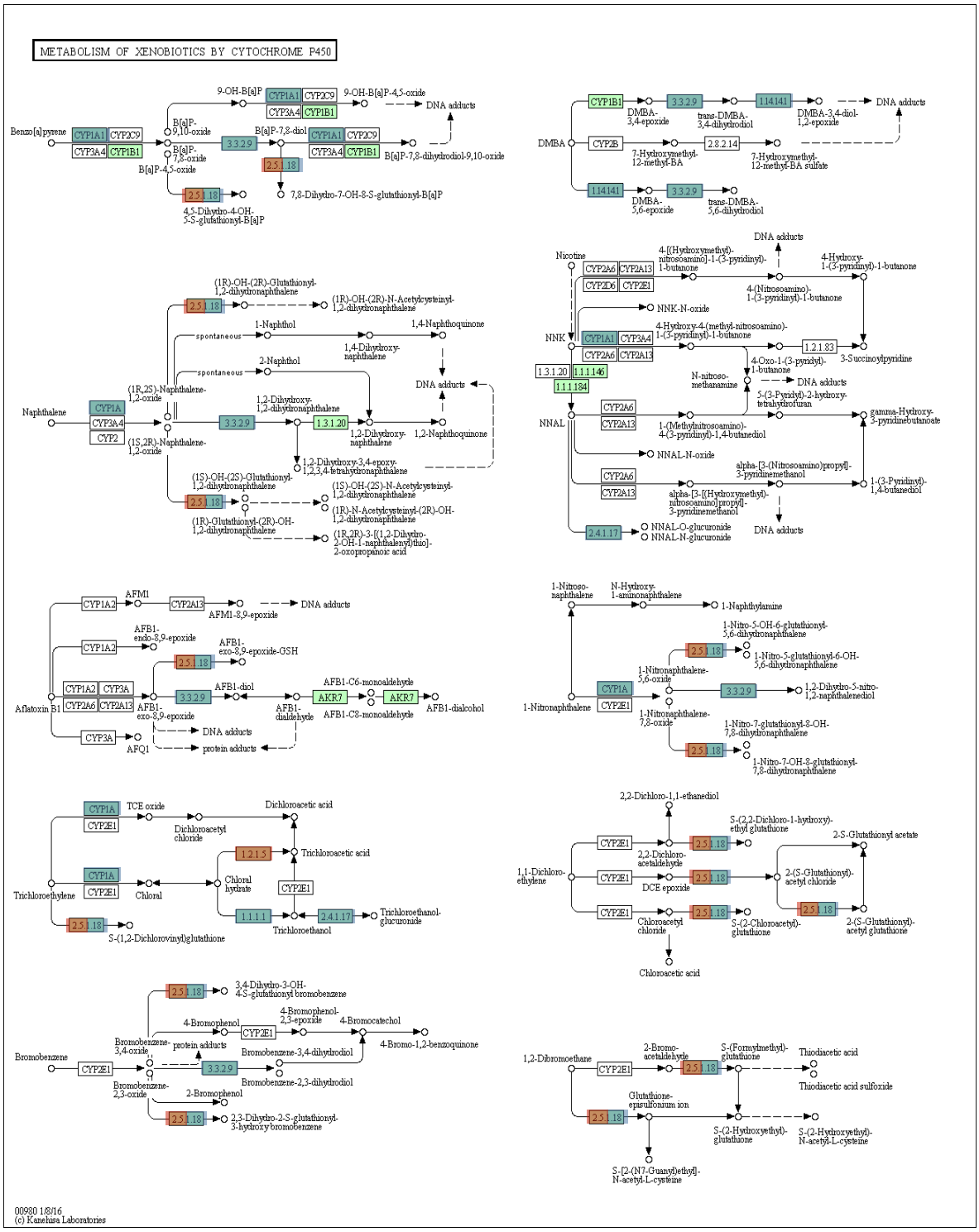
Supplementary Figure 3.19 Tryptophan metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; yellow - pathway also responsive in gill.



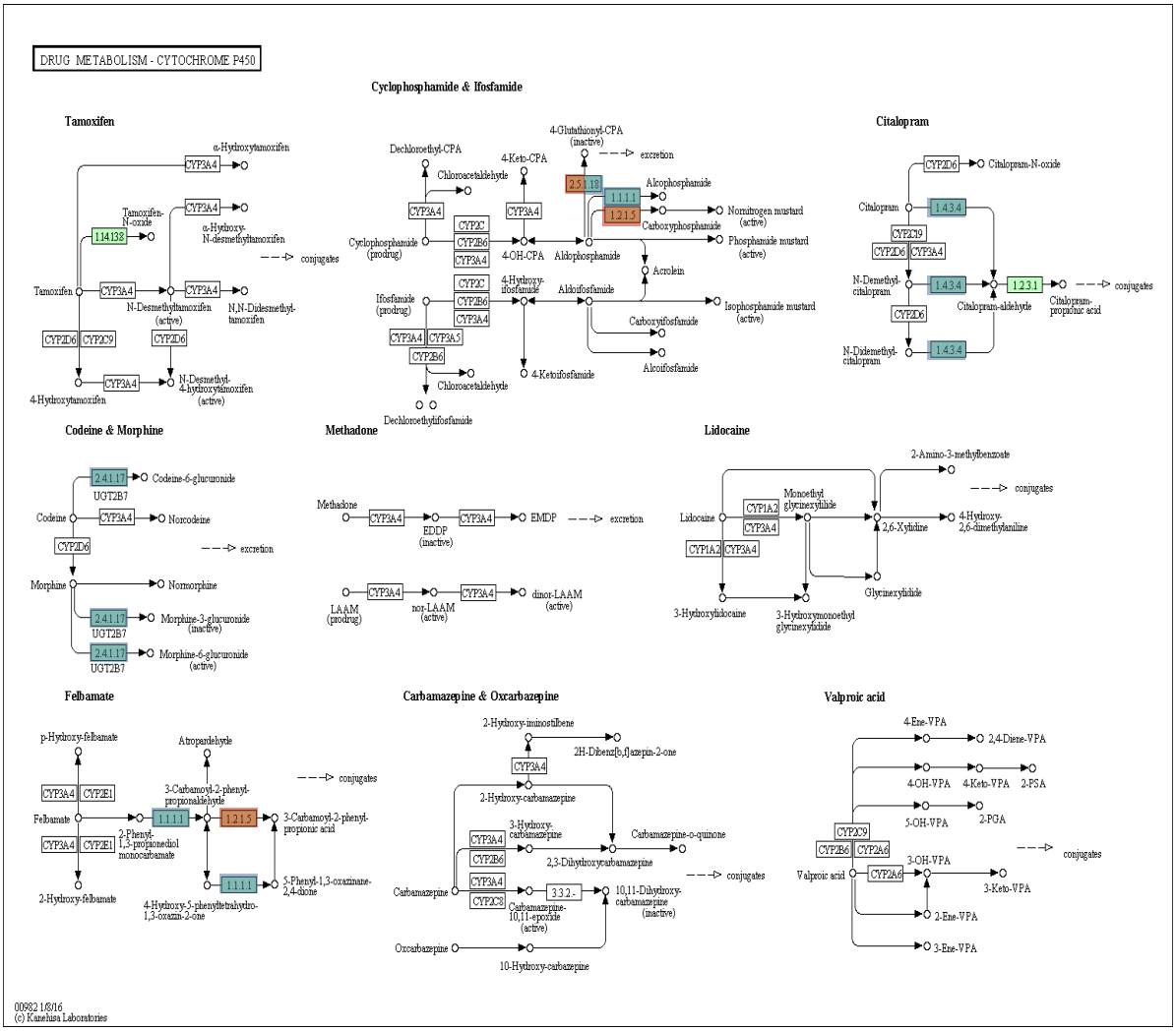
Supplementary Figure 3.20 Retinol metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated.



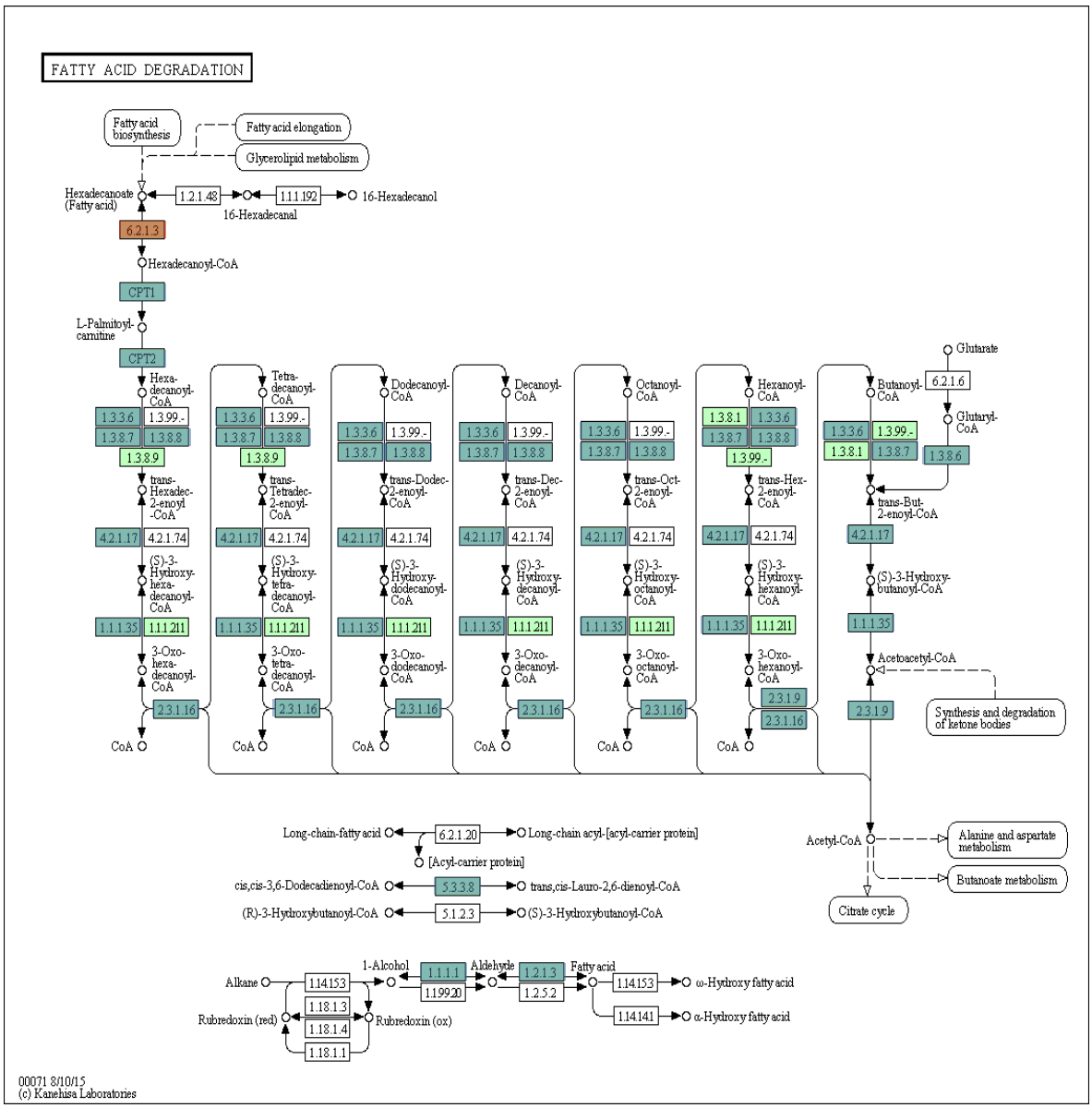
Supplementary Figure 3.21 Folate pathway one carbon metabolism in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated.



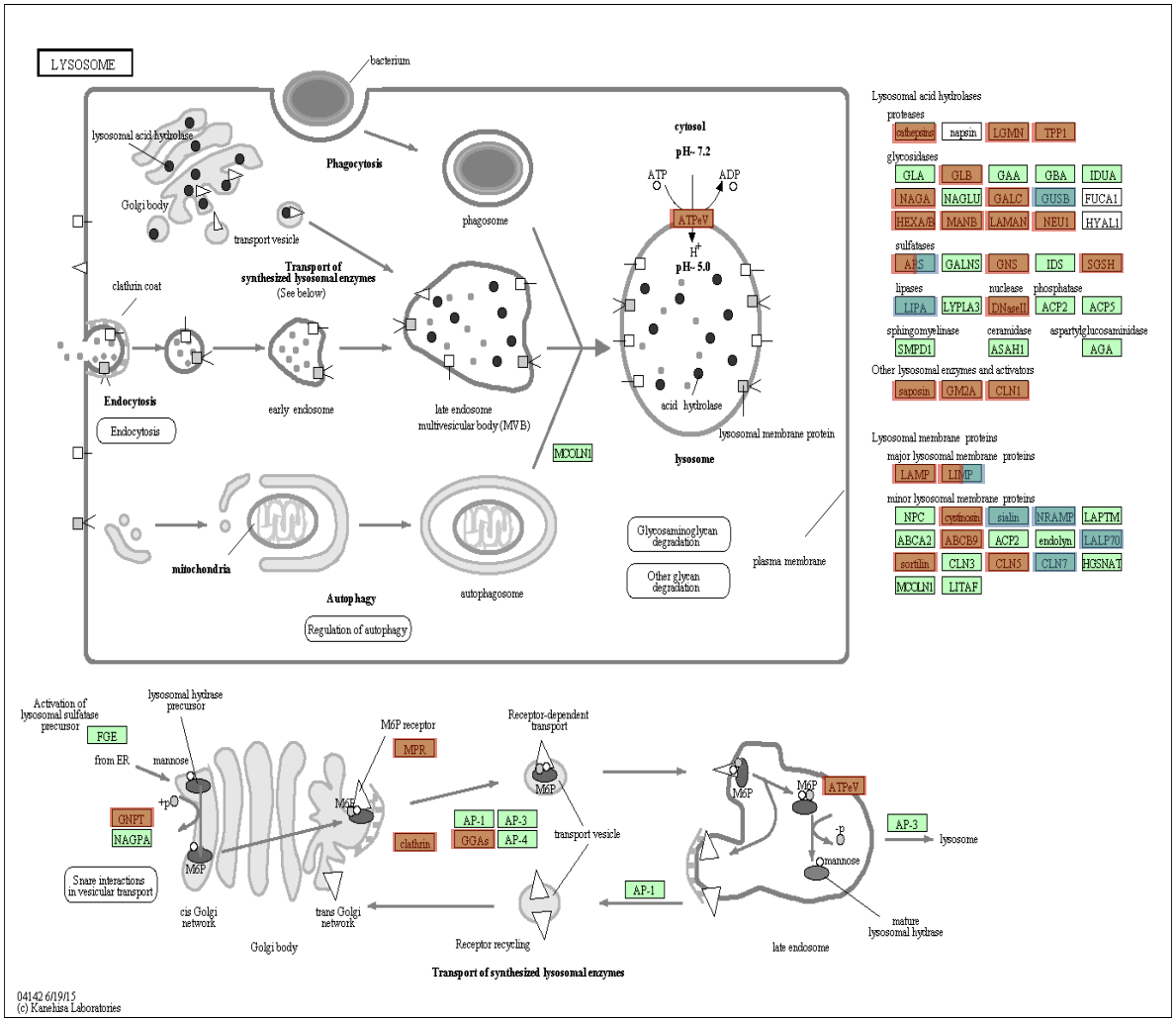
Supplementary Figure 3.22 Metabolism of xenobiotics by cytochrome P450 in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated.



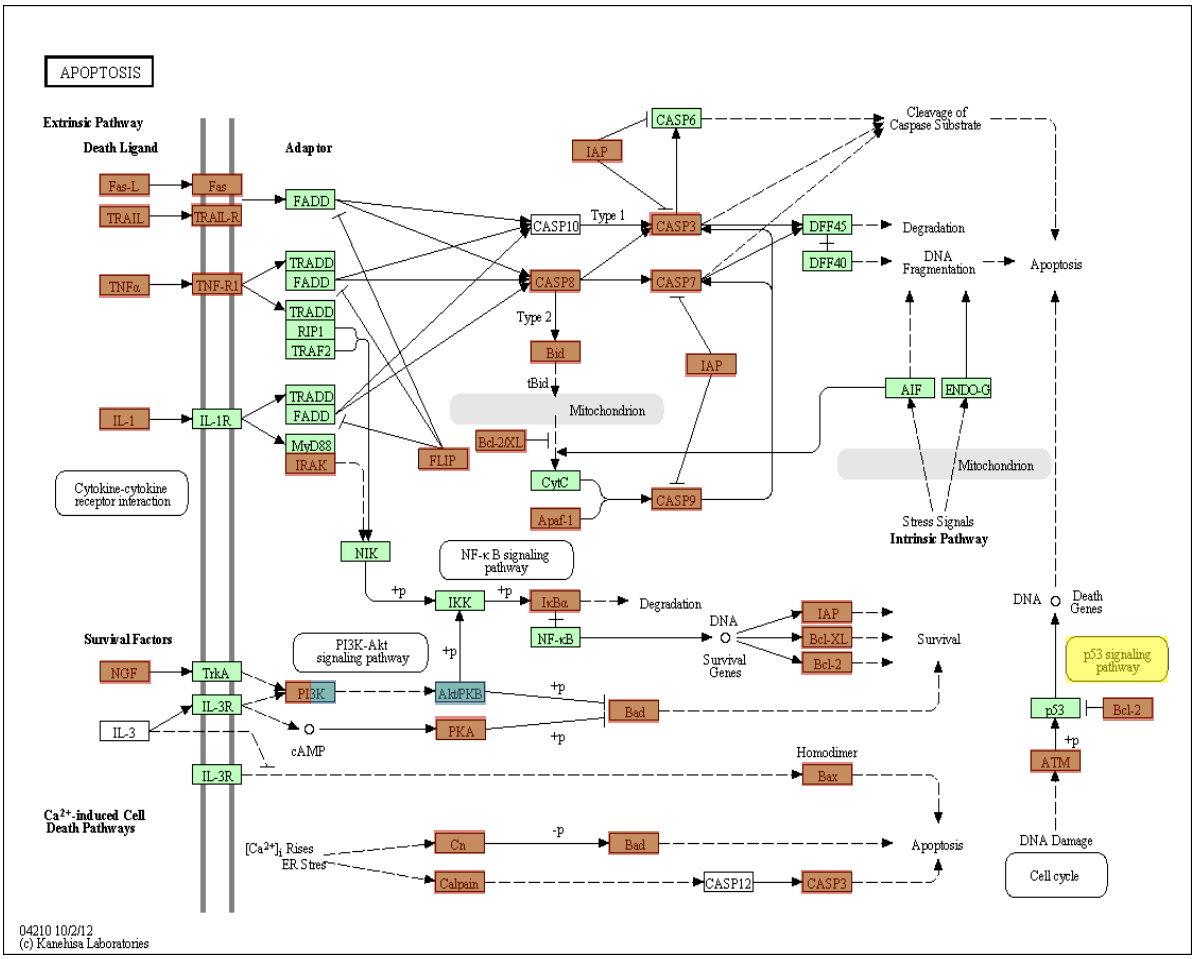
Supplementary Figure 3.23 Drug metabolism - cytochrome P450 in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated.



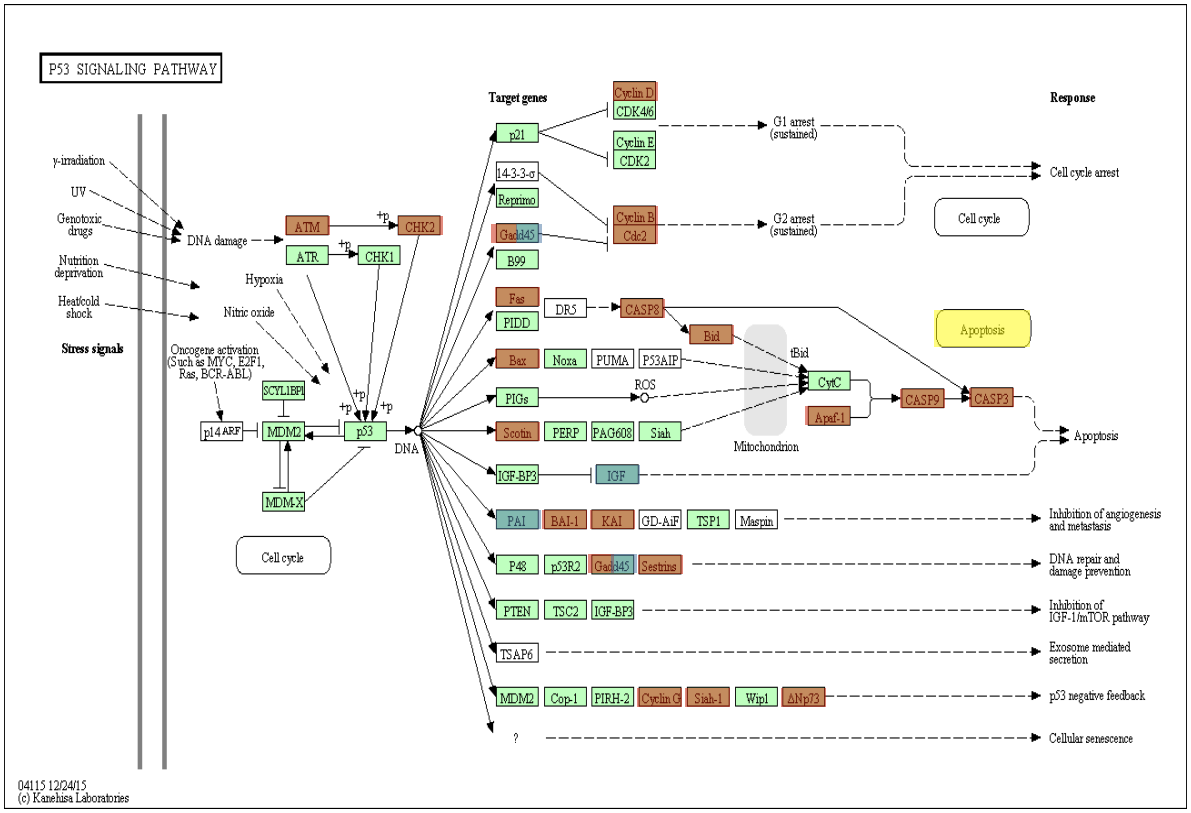
Supplementary Figure 3.24 Fatty acid degradation pathway in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated.



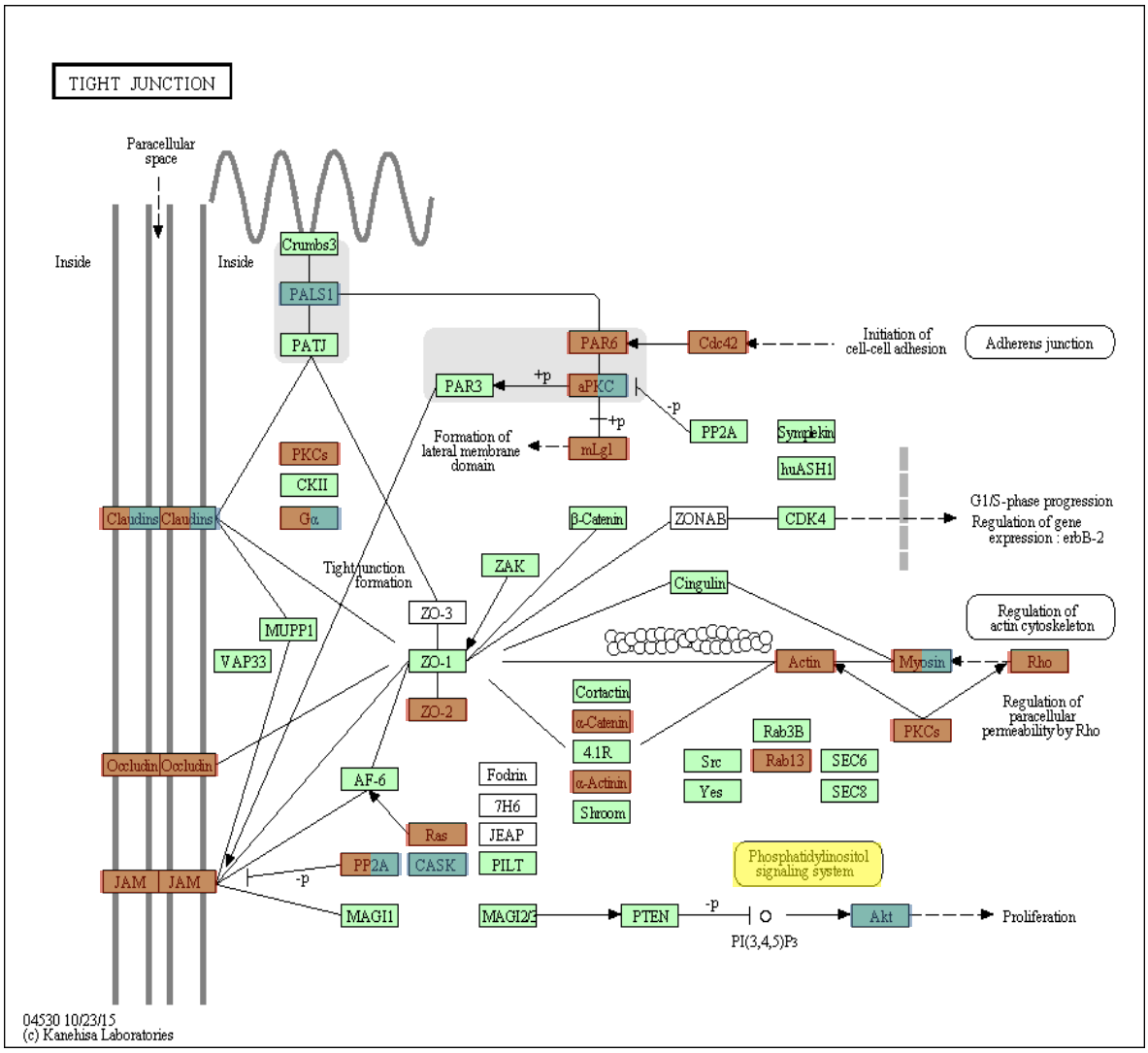
Supplementary Figure 3.25 Lysosome pathway in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated.



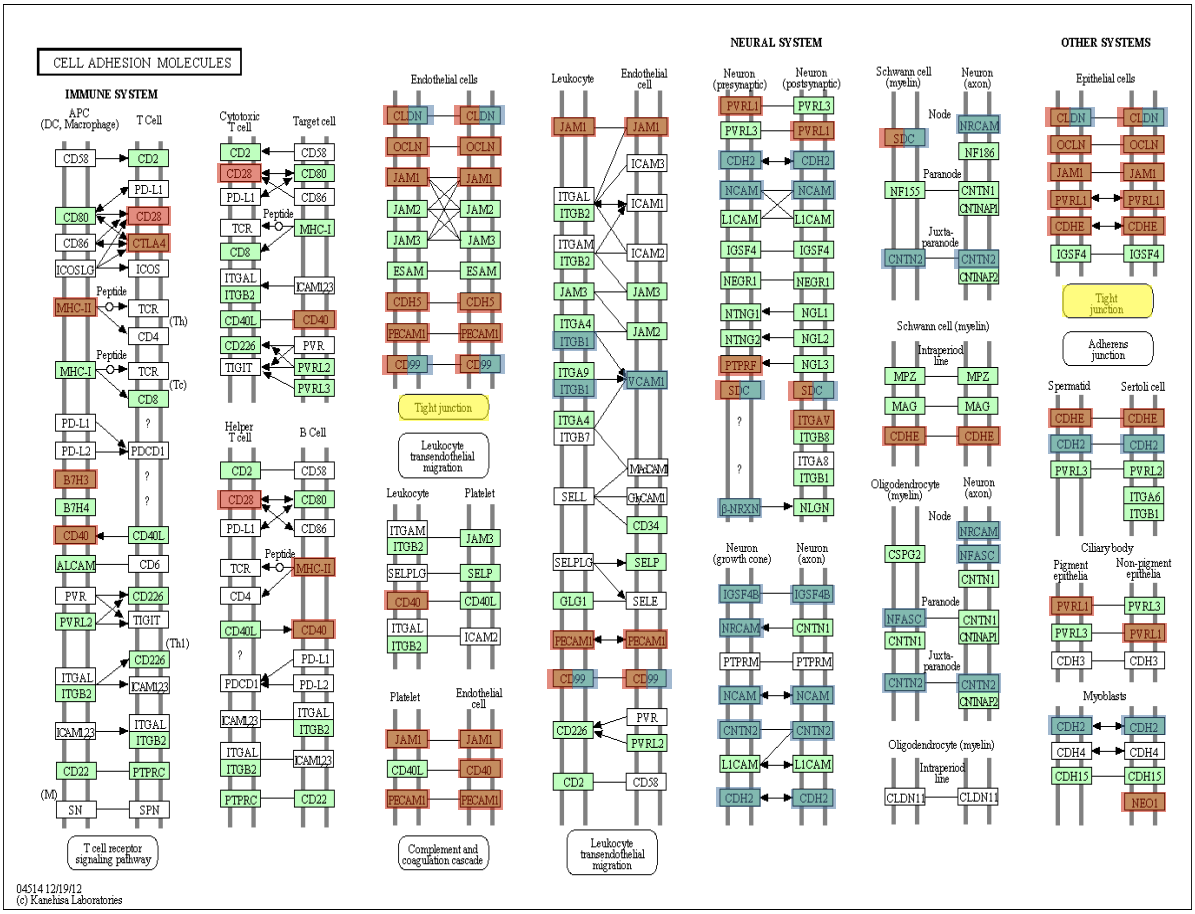
Supplementary Figure 3.26 Apoptosis pathway in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in gill.



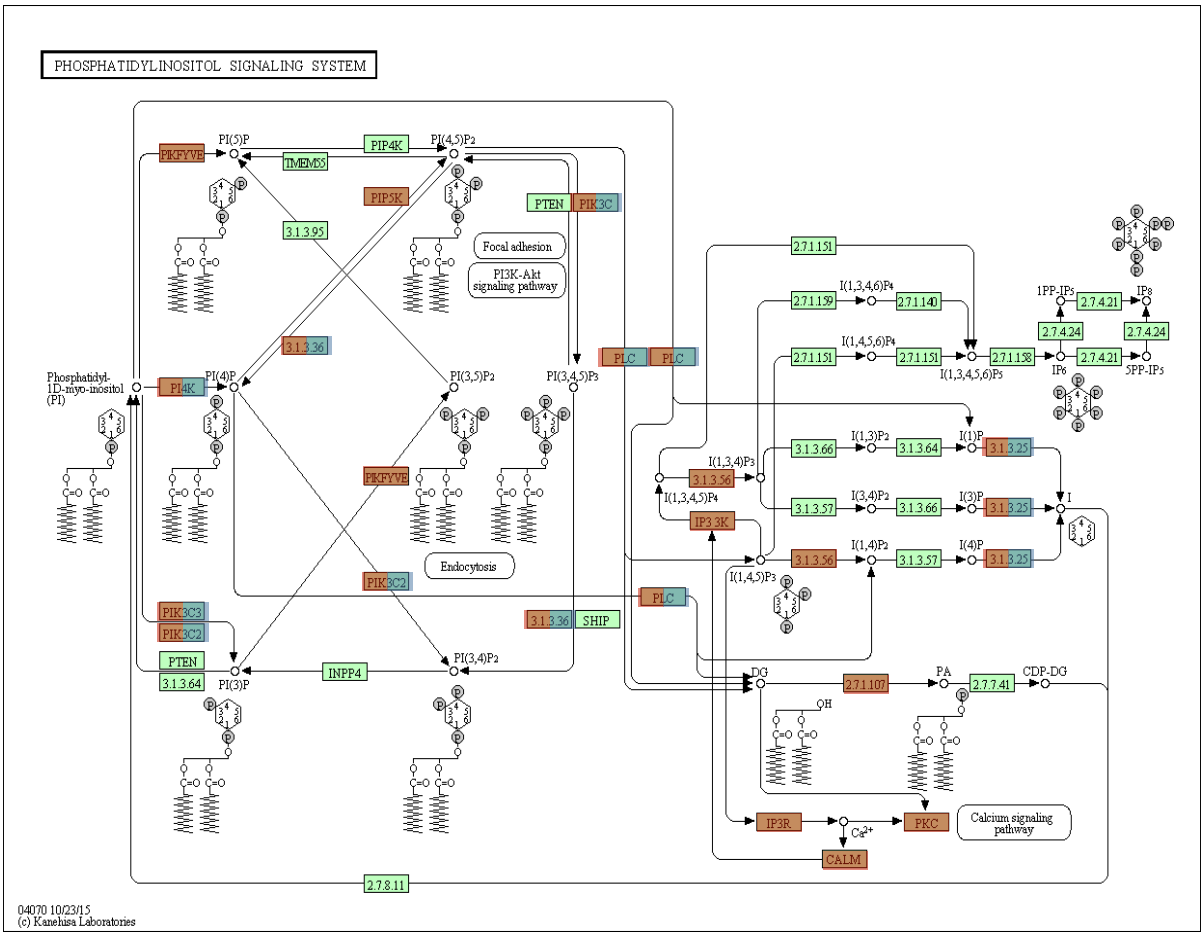
Supplementary Figure 3.27 The p53 signaling pathway in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in gill.



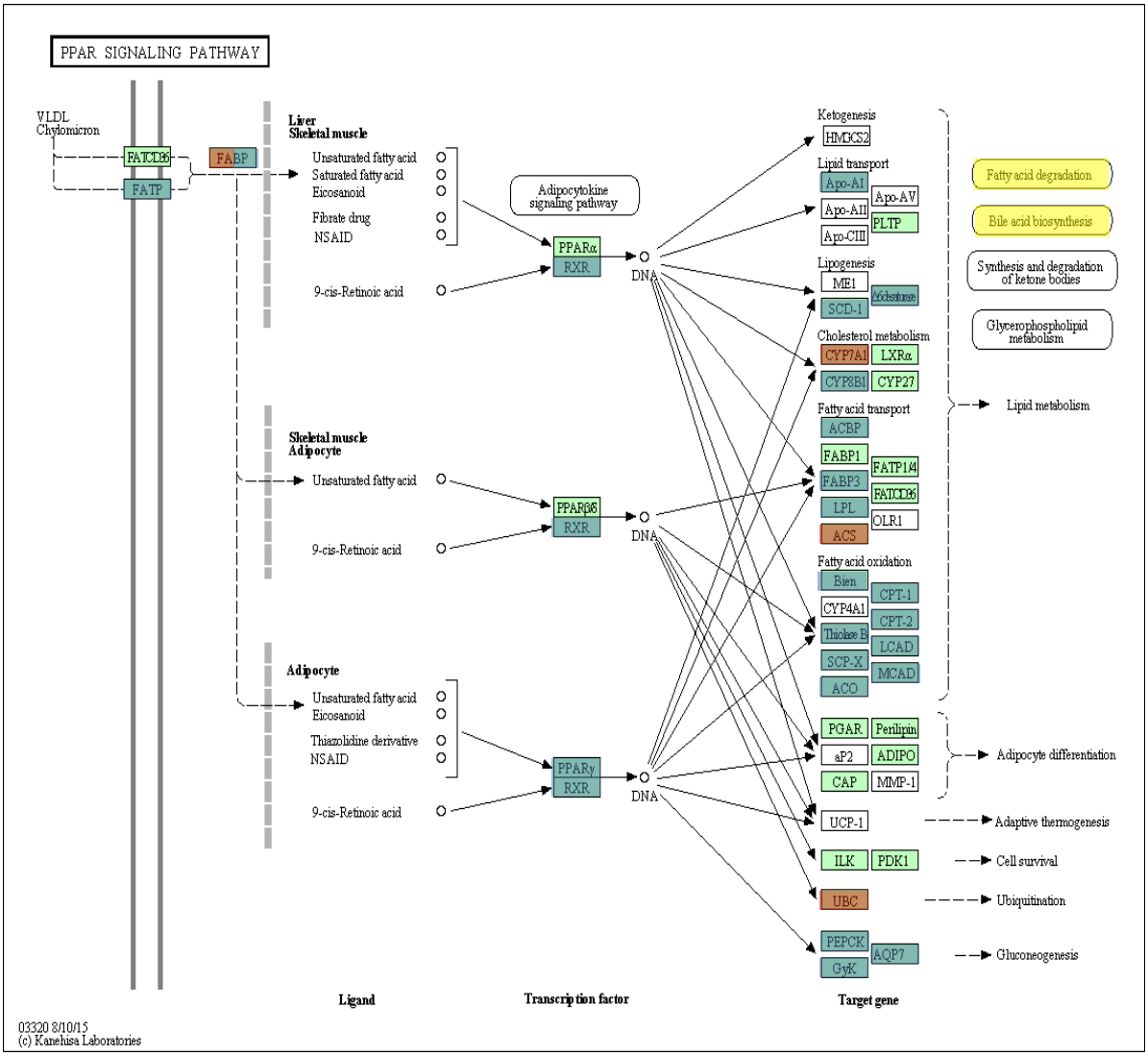
Supplementary Figure 3.28 Tight junction pathway in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in gill.



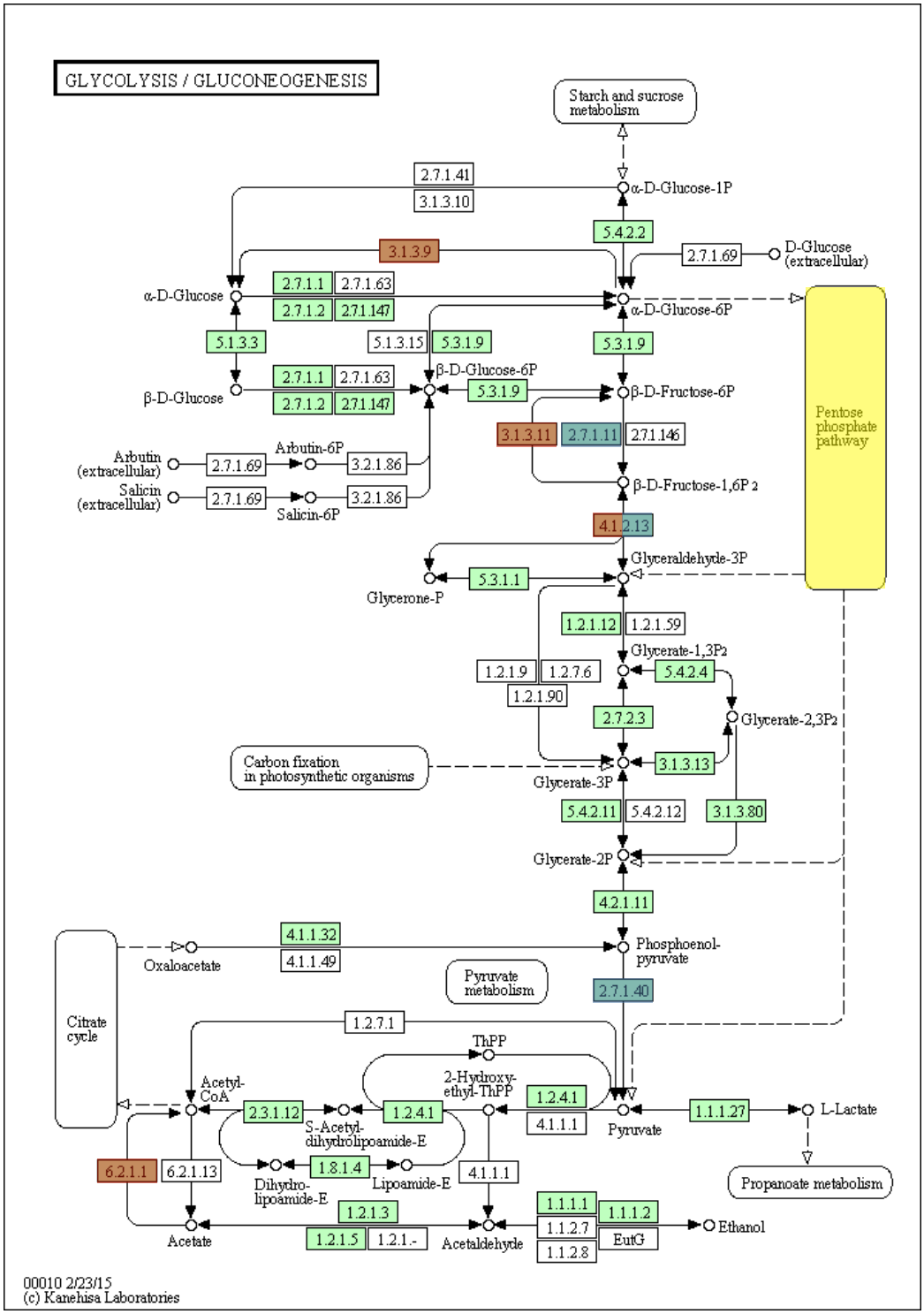
Supplementary Figure 3.29 Cell adhesion molecules pathway in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in gill.



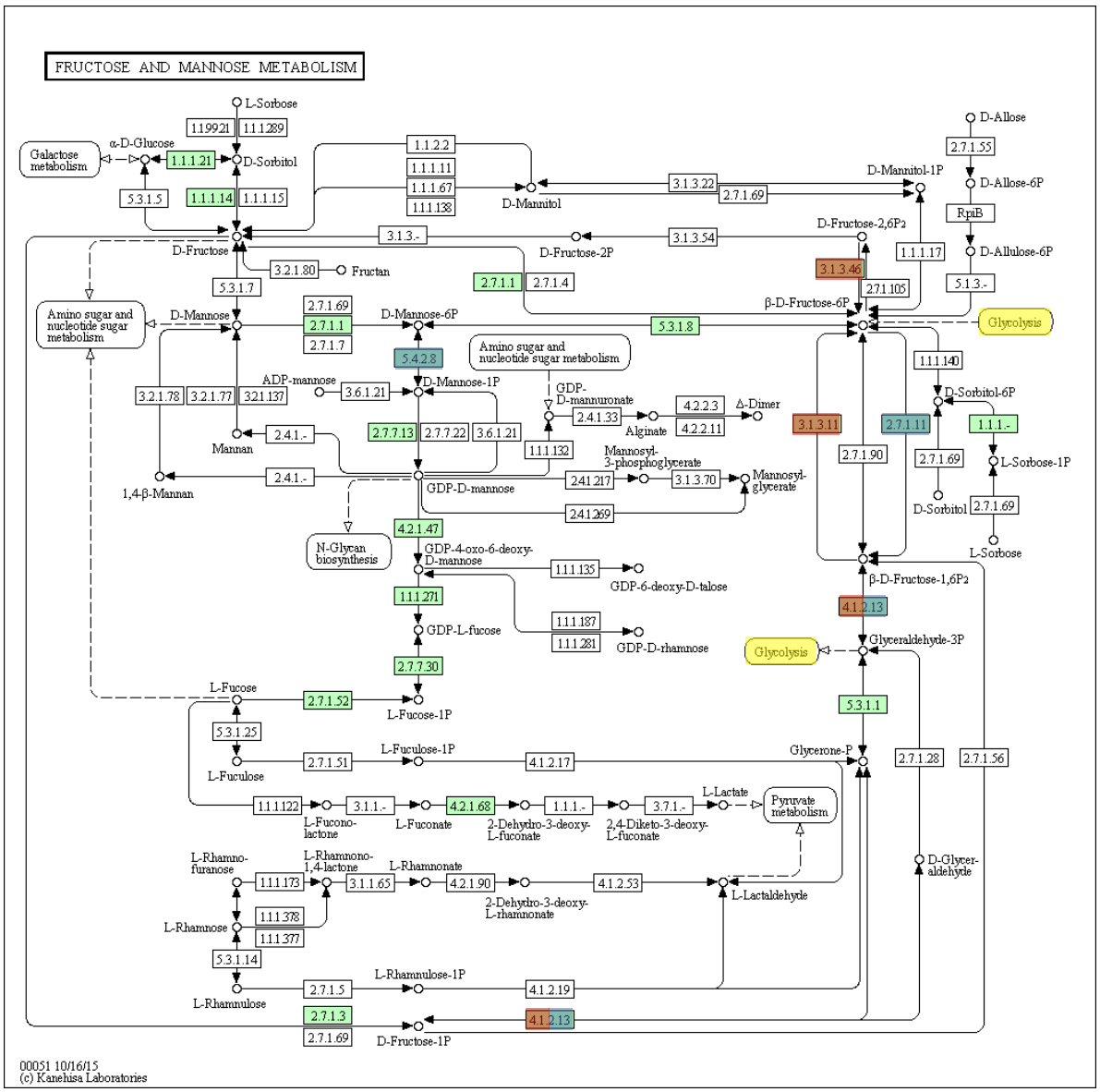
Supplementary Figure 3.30 Phosphatidylinositol signaling system pathway in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: red - upregulated; blue and red - downregulated and upregulated.



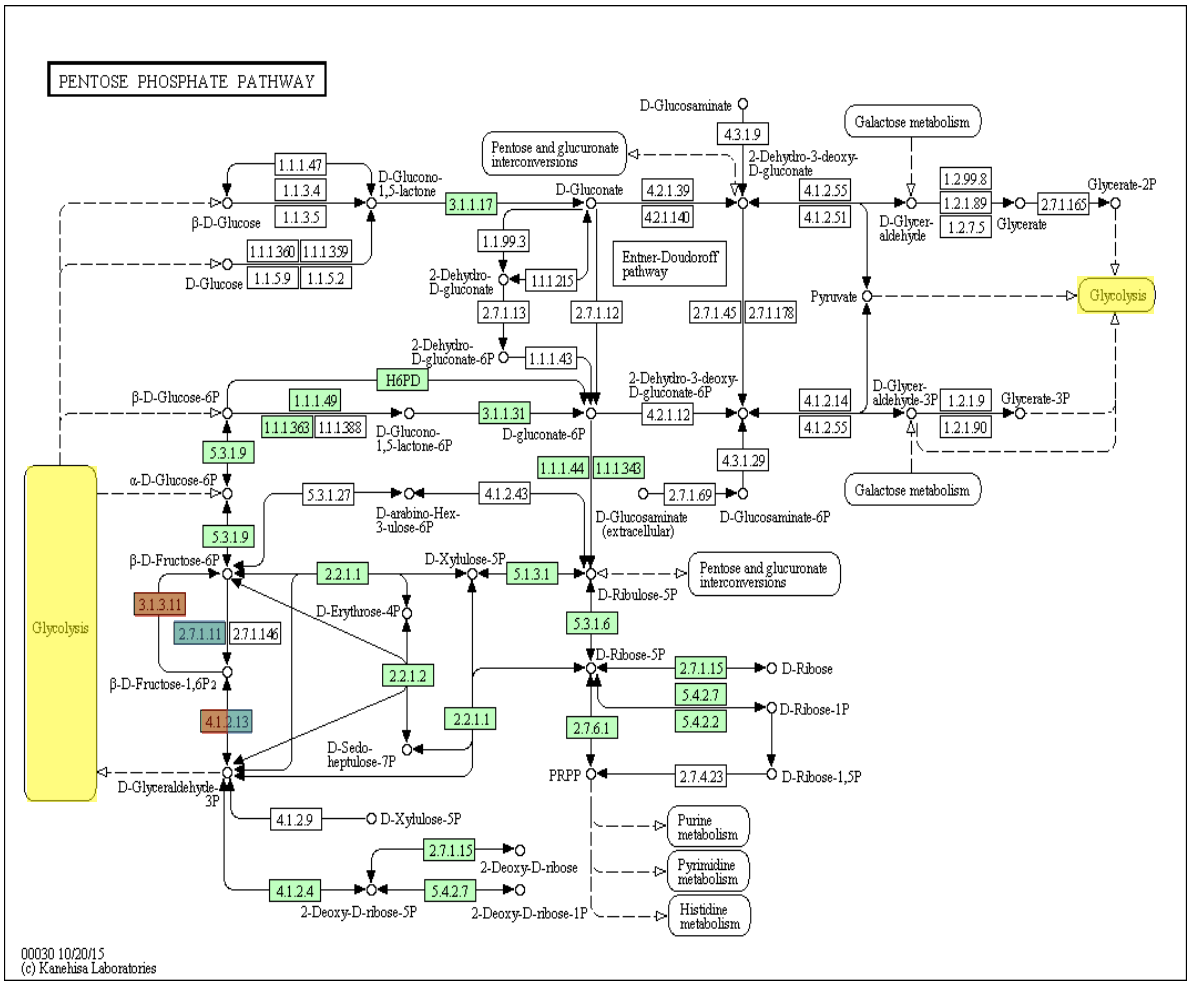
Supplementary Figure 3.31 The PPAR signaling pathway in gill after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in gill.



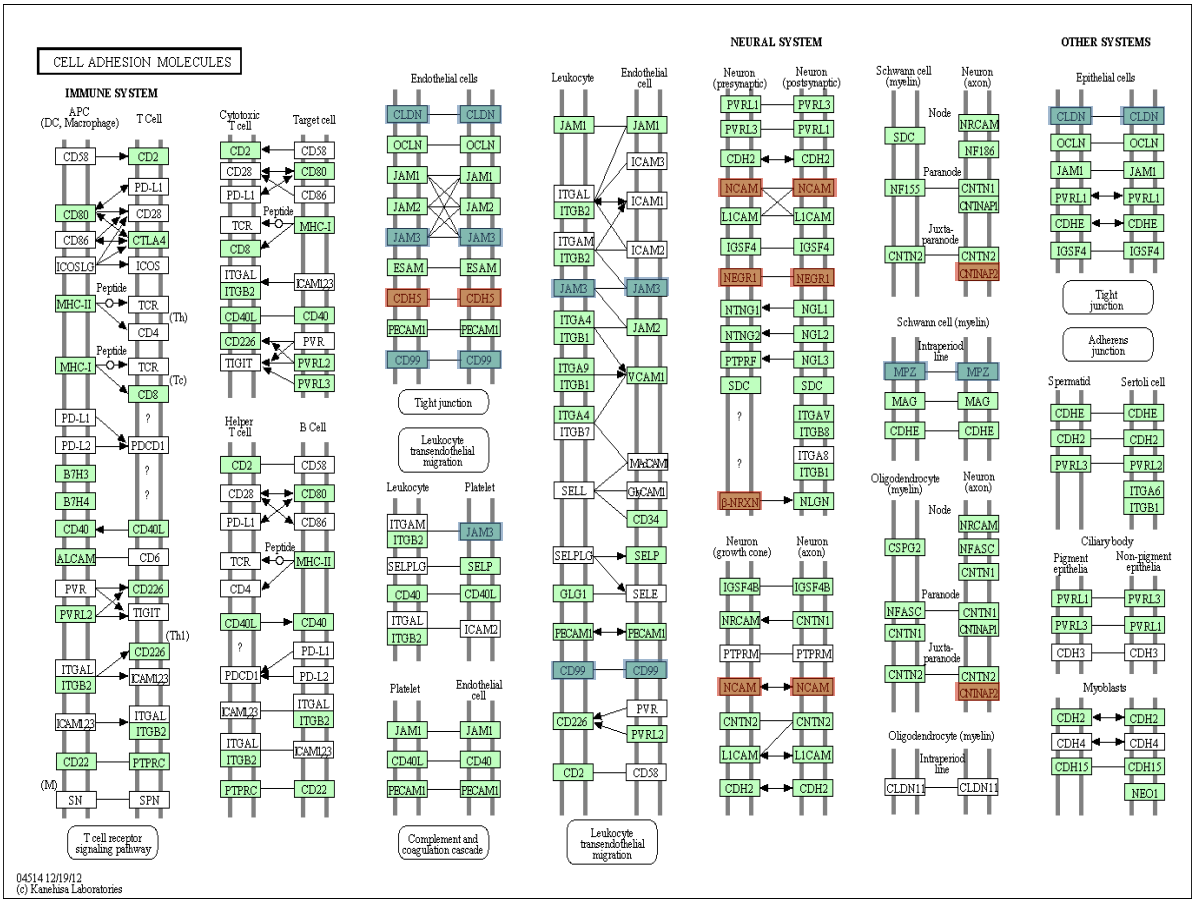
Supplementary Figure 3.32 Glycolysis/gluconeogenesis pathway in liver after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in liver.



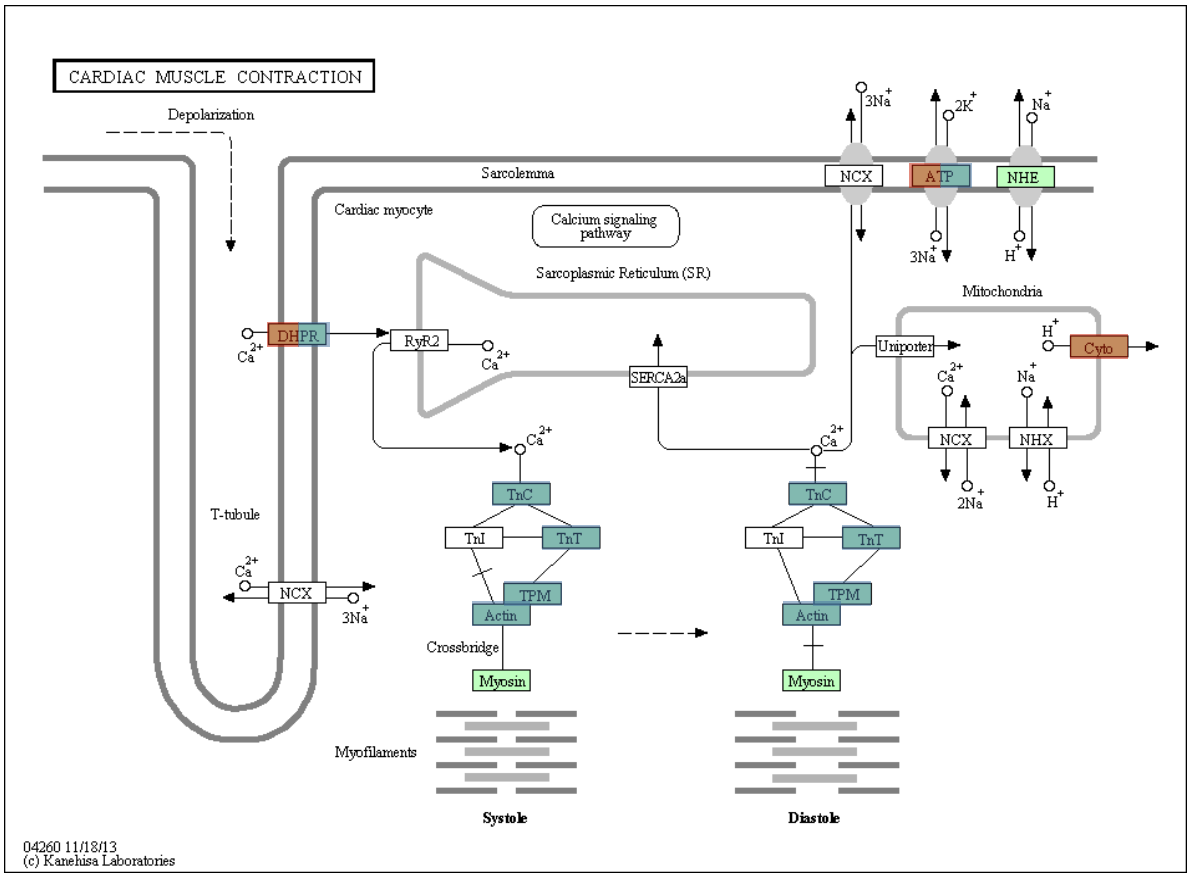
Supplementary Figure 3.33 Fructose and mannose metabolism in liver after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in liver.



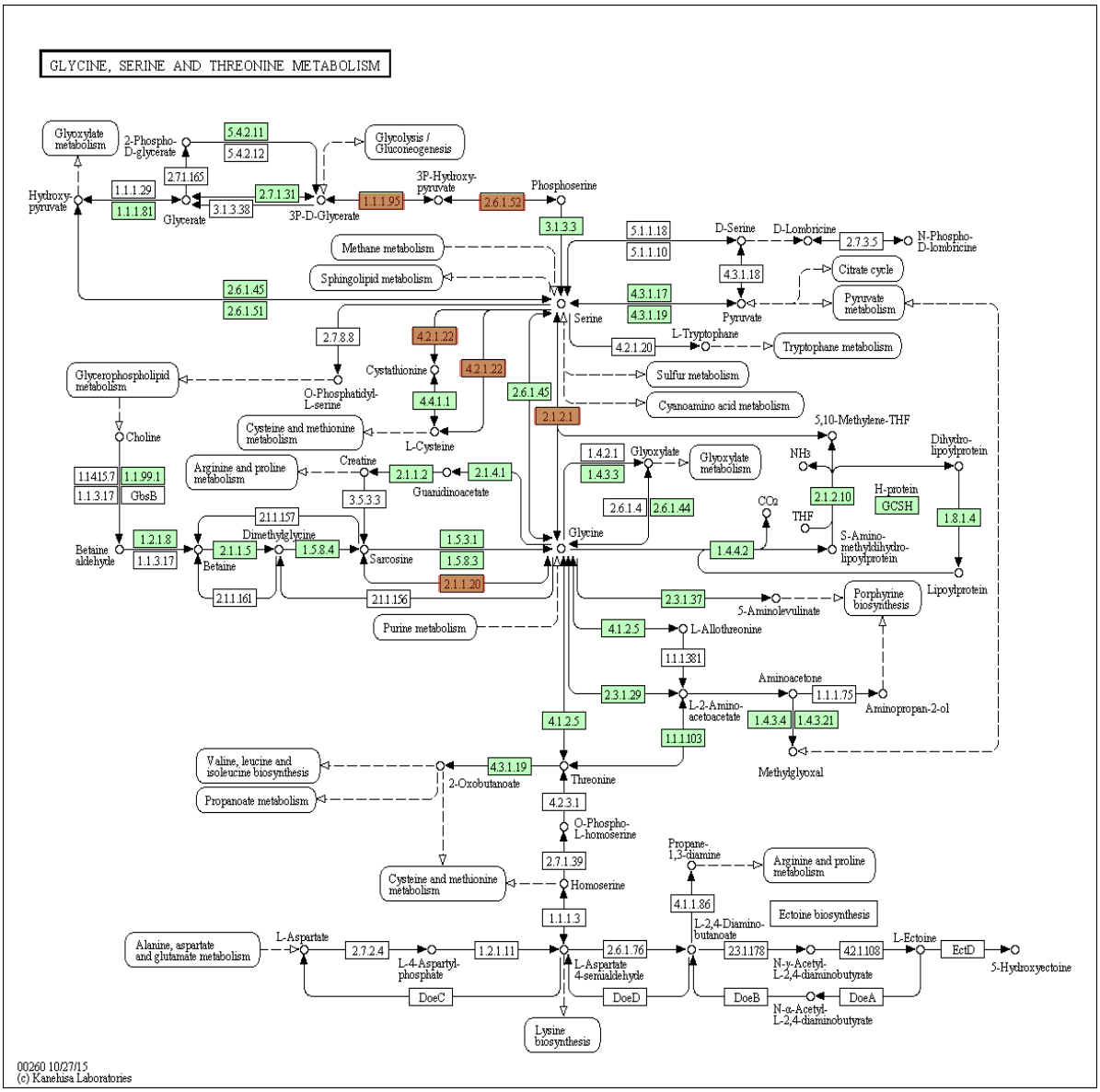
Supplementary Figure 3.34 Pentose phosphate pathway in liver after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated; yellow - pathway also responsive in liver.



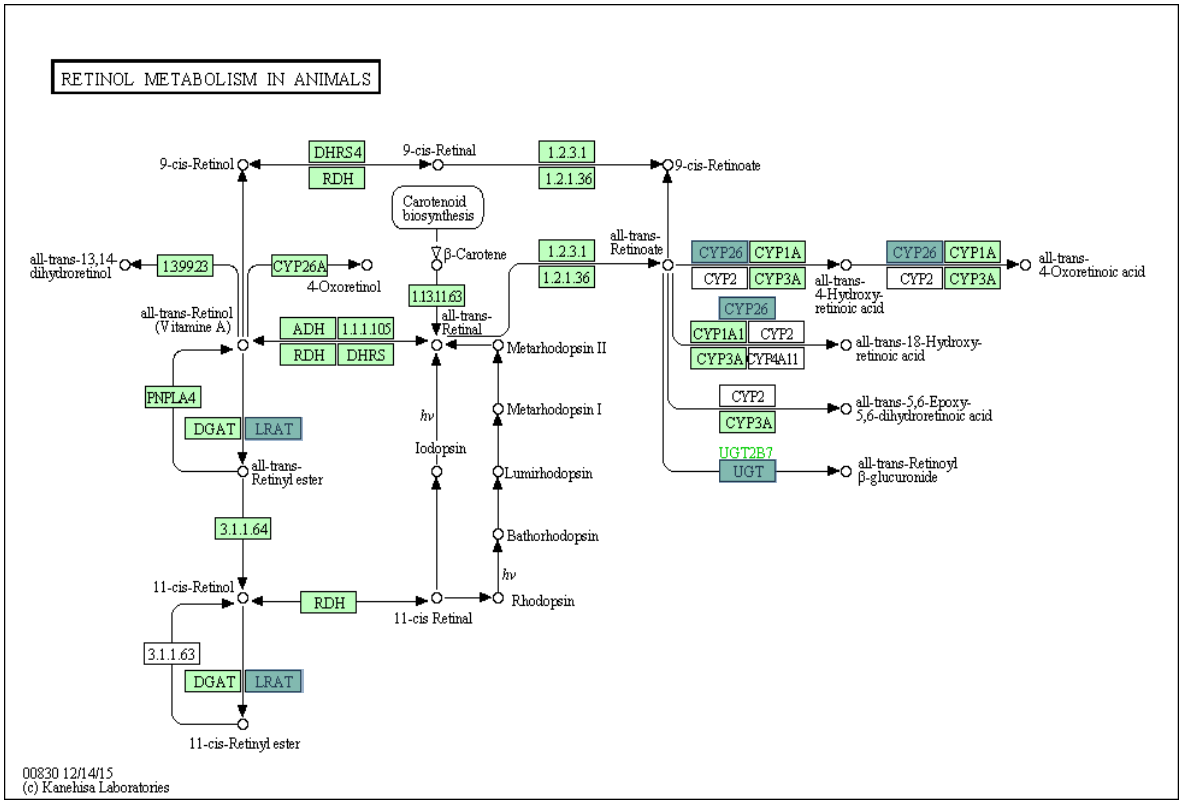
Supplementary Figure 3.35 Cell adhesion molecules pathway in liver after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated.



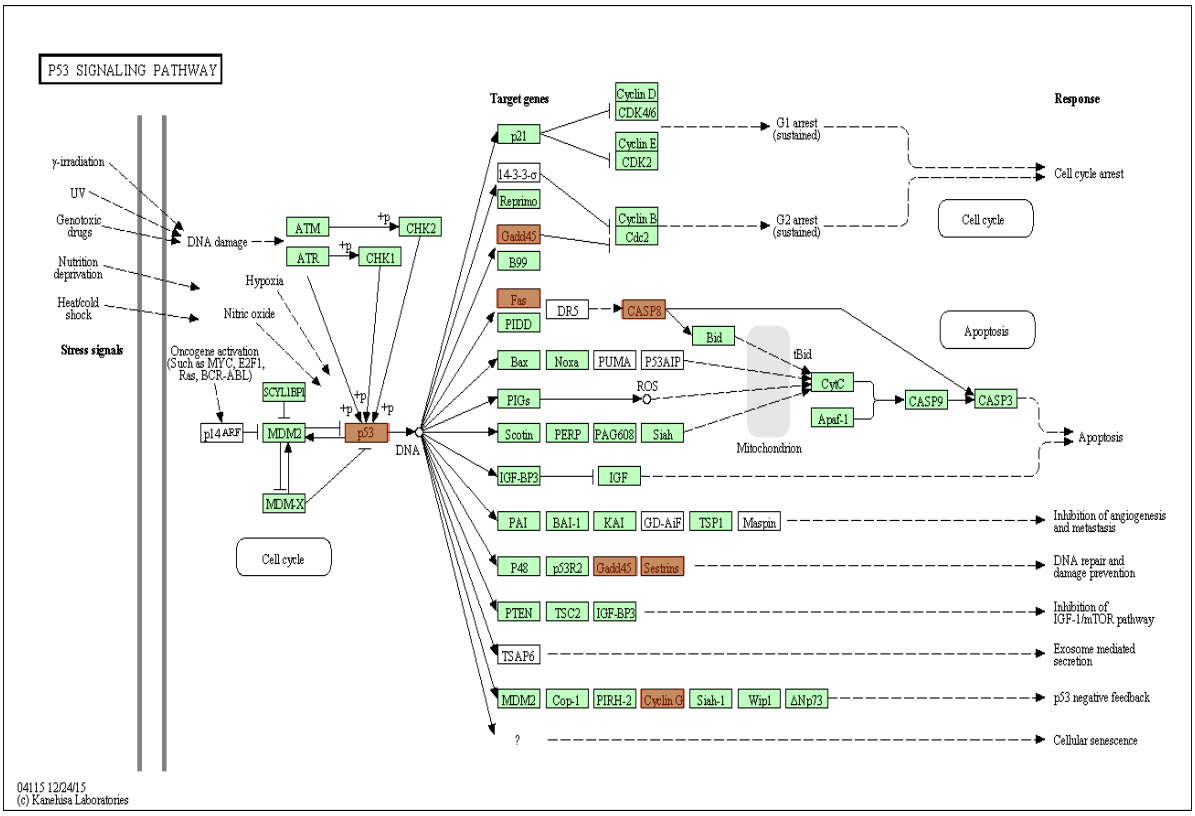
Supplementary Figure 3.36 Cardiac muscle contraction pathway in liver after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated; red - upregulated; blue and red - downregulated and upregulated.



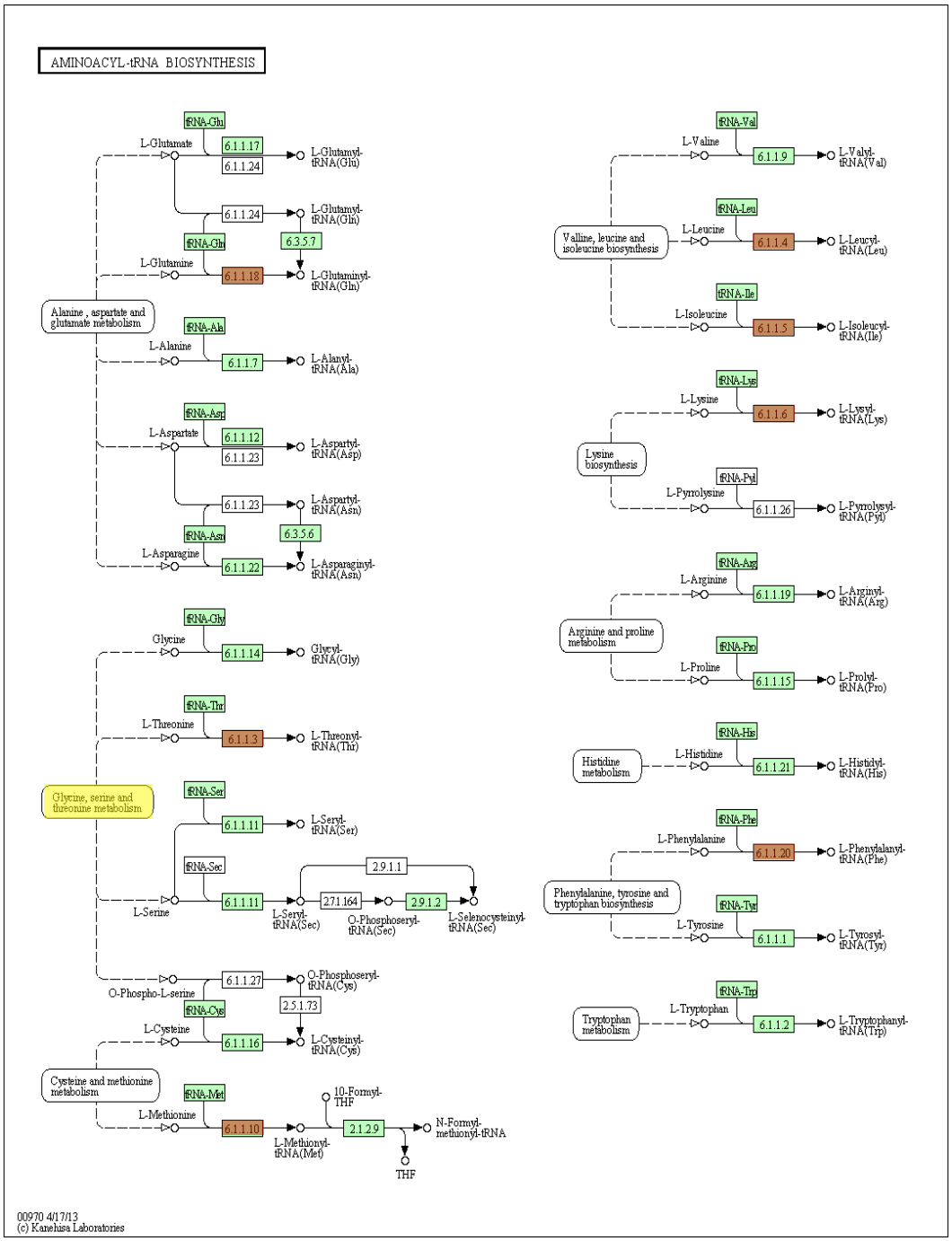
Supplementary Figure 3.37 Glycine, serine and threonine metabolism in brain after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: red - upregulated.



Supplementary Figure 3.38 Retinol metabolism in brain after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: blue - downregulated.



Supplementary Figure 3.39 The p53 signaling pathway in brain after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: red - upregulated.



Supplementary Figure 3.40 Aminoacyl-tRNA biosynthesis pathway in brain after 96 h of exposure to a sub-lethal dose of ammonia. This transcriptomic response of zebrafish to environmental ammonia was obtained using a 44k microarray analysis and the pathway diagram was acquired through the KEGG website. The pathways and genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05. The genes were considered upregulated when the fold change (FC) was ≥ 2 and downregulated when the FC was ≤ 0.5. Legend: red - upregulated; yellow - pathway also responsive in brain.

CHAPTER 4 |

CHANGES IN THE GENE EXPRESSION OF ZEBRAFISH FOLLOWING A SHORT-TERM EXPOSURE TO AMMONIA

4.1 Abstract

Ammonia is a toxic compound that may be increased in fishes. To avoid intoxication, species developed mechanisms such as it conversion to non-toxic compounds, decrease of it production or even it excretion against gradient. Hereupon, the aim of this study was to determine which mechanisms are applied by zebrafish *Danio rerio* to avoid ammonia intoxication, as well as, which hormones are involved in ammonia response. After exposure to a sub-lethal concentration of ammonia for 96 h, a 44k Agilent Zebrafish Microarray was performed. The results showed that ammonia transporters, H^+-ATPase, carbonic anhydrase and Na^+/K^+-ATPase increased their mRNA expression, suggesting that zebrafish maintains the excretion of ammonia against gradient. Moreover, the expression of paracellular transport elements changed, indicating an alteration of branchial permeability and regulation of ammonia movement through intercellular spaces. To avoid the internal accumulation of ammonia, zebrafish decreased the expression of enzymes of amino acid metabolism in gill; however, in liver, some of these enzymes were upregulated probably to incorporate ammonia in *de novo* amino acids. Another strategy in gill consisted in the convertion of ammonia to glutamine through glutamine synthetase; however, in

brain, this enzyme was downregulated to avoid brain swelling. Although no evidence indicates that cortisol was enhanced, the mRNA of a hormone and five hormone receptors were affected in gill by ammonia exposure, which indicate that endocrine system might be relevant for internal homeostasis. Overall, it was evident that zebrafish resort to several mechanisms to avoid ammonia intoxication; however, the endocrine regulation can be crucial in response to ammonia.

4.2 Introduction

Ammonia is a toxic end product of amino acid catabolism (Ip and Chew, 2010) and although the permanence of ammonia inside the body in non-toxic forms is possible, its excretion to the environment is more favourable (Randall and Tsui, 2002), occurring mainly through the gills (Wright and Wood, 2009). In fact, ammonia is a natural metabolic by-product of the catabolism of dietary and structural proteins that occurs mostly in the liver (Wilkie, 1997; Ip and Chew, 2010). As its accumulation cause negative effects to the organism (Ip and Chew, 2010), ammonia might be converted to non-toxic compounds [such as urea or glutamine (GLN)] (Randall and Tsui, 2002) or directly eliminated out of the body (Wright and Wood, 2009; Ip and Chew, 2010). Since the latter requires less energy, it occurs preferentially (Randall and Tsui, 2002) taking place in the gills, by simple diffusion or crossing transporter proteins (Wright and Wood, 2009; Ip and Chew, 2010).

In the last decade, the excretion of ammonia through the gills has been extensively studied, with a family of transporter proteins, known as Rhesus (Rh) glycoproteins, being directly implicated in ammonia movement (Wright and Wood, 2009). In fact, several studies showed that the non-erythroid Rh glycoproteins members are able to transport NH_3 across the branchial membranes, facilitating its elimination out of the organism (Hung et al., 2007, 2008; Wright and Wood, 2009; Ip and Chew, 2010). This family of ammonia transporters comprises several members (Nawata et al., 2007; Hung et al., 2007, 2008; Braun et al., 2009a, 2009b; Wright and Wood, 2009) that are present in different organs (Hung et al., 2007; Nawata and Wood, 2008; Braun et al., 2009b; Wright and Wood, 2009) and have different locations inside the cell (Verlander et al., 2003; Hung et al., 2007; Braun et al., 2009b; Wright and Wood, 2009; Ip and Chew, 2010). In the branchial epithelium cells, the Rh glycoprotein C (Rhcg) is usually localized in the apical membrane whereas the Rh glycoprotein B (Rhbg) is positioned in the basolateral membrane (Verlander et al., 2003; Hung et al., 2007; Braun et al., 2009b; Wright and Wood, 2009; Ip and Chew, 2010). Wright and Wood (2009) recently published a model describing the excretion of ammonia through the branchial epithelium to the aquatic

environment, comprising both Rh glycoproteins, but also other transporters like the V-type H^+-ATPase, the Na^+/H^+ exchanger (NHE) and Na^+ channels, all functioning together to facilitate the elimination of ammonia.

Beyond this excretory mechanism, ammonia may cross the branchial epithelium by simple diffusion of NH_3 and following the blood-to-water partial pressure gradient of NH_3 (ΔP_{NH3}); however, both types of excretion depend of the acidic boundary layer to maintain the continuous excretion of ammonia (Wright and Wood, 2009; Nawata et al., 2010b). In solution, ammonia may exist in two chemical forms: the unionized ammonia (UIA, NH_3) and the ionized ammonia (NH_4^+) (Randall and Tsui, 2002; Ip and Chew, 2010). Although at physiological pH most of the ammonia is present as NH_4^+ being considered the most toxic form inside the organism (Randall and Tsui, 2002; Wilkie, 2002; Ip and Chew, 2010), the NH_3 is able to diffuse passively through the gill epithelium, due its lipophilic nature and lack of charge, which makes the UIA the most environmental toxic form (Randall and Tsui, 2002; Weihrauch et al., 2009; Ip and Chew, 2010). In fact, in normal conditions, the movement of NH_3 follows the blood-to-water ΔP_{NH3}; however, the presence of high environmental ammonia concentrations or pH may impede and reverse this gradient, allowing ammonia entrance and accumulation (Randall and Tsui, 2002; Wright and Wood, 2009). Nonetheless, regardless of whether the movement of ammonia is by simple diffusion of NH_3 or through transporter proteins, the presence of an acidic boundary layer close to the gill epithelium has an important role, facilitating the continuous movement of NH_3 out of the body since it would be trapped as NH_4^+. The acidification of boundary layer occurs by H^+ excretion through the transporter protein V-type H^+-ATPase and also by CO_2 hydration catalyzed by the enzyme carbonic anhydrase (CA). Although the presence of boundary layer is not obligatory, it guarantees the continuous excretion of ammonia following the ΔP_{NH3} and that NH_3 will be trapped as NH_4^+ out of the organism (Wright and Wood, 2009).

Several factors may imbalance the ammonia production and excretion such as the presence of high levels of ammonia in the aquatic environment, which contributes for its accumulation inside the body reaching concentrations that may cause severe negative effects and even death of the organism. In fact, fish produces ammonia inside the body, but it presence in the aquatic environment due to natural and/or anthropogenic sources (Randall and Tsui, 2002), may impede ammonia excretion or even cause an uptake of ammonia from the environment (Wilson et al., 1994; Randall and Tsui, 2002; Wilkie, 2002; Eddy, 2005; Wright and Wood, 2009). This may lead to the accumulation of ammonia internally and cause several

negative effects inside the organism (Randall and Tsui, 2002) such as deterioration of gill structure (Benli and Köksal, 2005; Benli et al., 2008; Spencer et al., 2008), decreased immune system (Gonçalves et al., 2012), compromise the reproductive capacity (Abbas, 2006), imbalance of osmoregulation, decreased growth rate (Rasmussen and Korsgaard, 1996; Björnsson and Ólafsdóttir, 2006; Foss et al., 2007), reduced feeding (Rodrigues et al., 2007), cell dysfunction, increased oxygen consumption (Adams et al., 2001; Barbieri and Doi, 2012) and ventilation frequency (Adams et al., 2001; Benli and Köksal, 2005), unbalance of blood chemistry and production (Abbas, 2006), altering swimming performance (Benli and Köksal, 2005; Rodrigues et al., 2007) and even cause mortality (Randall and Tsui, 2002). Among all organs that suffer the toxicity of ammonia, the central nervous system (CNS) is the principal target (Randall and Tsui, 2002). To date, several deleterious effects were described in this vital organ namely, astrocyte and brain swelling, modification of the blood-brain barrier (BBB) properties, alterations of the amino acid transport, increase of cerebral blood flow, oxidative stress (Ching et al., 2009), development of neuronal degeneration, interference with excitatory amino acid neurotransmitter metabolism and even morphological changes in astrocytes and neurons and may lead to neuronal cell death (Albrecht, 1998; Feldman et al., 2014)

Since ammonia may cause these effects on organism, that can culminate in fish death, different teleost fishes developed mechanisms to avoid ammonia intoxication, including the conversion of ammonia to non-toxic compounds such as urea or GLN (Randall and Tsui, 2002), decrease of proteolysis and amino acid catabolism (Lim et al., 2001; Randall and Tsui, 2002) and excretion of ammonia even against a concentration gradient (Randall and Tsui, 2002; Wright and Wood, 2009). The mechanism used by each species to deal with the presence of ammonia in the environment, may result in a varying sensitivity among species, with some being more tolerant to ammonia than others (Randall and Tsui, 2002).

In addition to detoxification mechanisms, high environmental ammonia induce stress response in fish (Gonçalves et al., 2012), activating physiological pathways regulated by endocrine system, that will guarantee the internal homeostasis and fish survival. This physiological stress response has been associated mostly to the corticosteroid hormone cortisol (Mommsen et al., 1999), although, other hormones have also responded to stress, such as prolactin (PRL) (Auperin et al., 1997; Tang et al., 2001). Cortisol is a seawater adapting hormone that also regulates growth, reproduction, immunity and osmoregulation (Mommsen et al., 1999) of fishes. Although cortisol controls the stress response, it also

induces the endogenous production of ammonia, activating proteolysis and amino acid catabolism to produce energy (ATP) (Mommsen et al., 1999; Randall and Tsui, 2002). On the other hand, PRL is considered a freshwater adapting hormone (Tang et al., 2001; Manzon, 2002) and, despite its involvement in stress response might be species specific, the levels of this hormone in plasma may also be enhanced during a stress response (Auperin et al., 1997; Tang et al., 2001). Beyond these hormones, it is still not known if other hormones may be also altered in fish, controlling their response to ammonia toxicity. Hereupon, the principal goal of this chapter was to determine which mechanisms are used by zebrafish to deal with ammonia toxicity and also to characterize the endocrine response of this vertebrate model species to high environmental ammonia. Moreover, is also an objective of this chapter to validate the microarray, resorting to the use of real-time PCRs (*q*PCRs) and confirming the mRNA changes of some genes that are already known to respond to ammonia exposure from previous studies.

4.3 Material and Methods

4.3.1 Animal maintenance and experimental design

Zebrafish *Danio rerio* (body mass of 0.355 ± 0.000 g) were acquired in a local pet store in Toronto, Canada, and were transported to the Biology Department of the University of Waterloo. During two weeks, fish were acclimated in tanks, in a recirculate-controlled system, supplied with dechlorinated local tap water and fed with commercial pellet food twice a day. In addition to the filtration system, each tank was siphoned (20 %) daily, avoiding the accumulation of uneaten food and fecal matter. The photoperiod was controlled for 14 h Light : 10 h Dark and the temperature were maintained at 28 ± 1 ºC. It is important to mention that all the experiments done for this study were carried out under the guidance of a Laboratory Animal Science certified supervisor (1005/1092, DGV-Portugal, C category, FELASA), according to European Union directives (2010/63/UE).

A detailed description of the experiment was done in the last chapter. However, briefly, eight adult zebrafish per tank were transferred to a semi-static system and during 48 h they were acclimated and fasted. All tanks were partially submerged in a water bath to maintain the water temperature at 28 ºC. After the acclimation period, zebrafish of half of the tanks (n = 3) were exposed during 96 h to 9.238 ± 0.086 mM of total ammonia-nitrogen (TA-N) (corresponding to 0.161 ± 0.001 mM UIA at pH 8) prepared in artificial water [60 mg of Instant Ocean Sea Salt (Blacksburg, VA) in 1 L of distilled water] (Liu et al., 2006; Boyle et

al., 2010; Sison and Gerlai, 2011) and pH previously adjusted to 7. The other half of the tanks (n = 3) were maintained only in artificial water (no ammonia were added) with pH adjusted to 7, serving as a control. The precise concentration of TA-N and UIA during the experiment was determined daily using the colorimetric assay modified for microplates from Verdouw and collaborators (1978). The physicochemical parameters of the water, namely pH and temperature, were monitored daily (7.128 ± 0.012 and 28.144 ± 0.025 ºC, respectively) and the photoperiod was 14 h Light : 10 h Dark. The calculation and correction for pH 8 was done based on the equations present in USEPA (1999) and considered the pH and temperature measured in the tanks, and also the TA-N determined through the colorimetric assay, during the experiment. Aeration was continuously supplied and there were no reports of different behavior or mortality in any tank. At the end of the 96 h of ammonia exposure, fish were euthanized with buffered tricaine methanesulphonate (MS222; Syndel Laboratories Ltd., Nanaimo, British Columbia, Canada) and gill, liver and brain (with pituitary gland) were collected under a dissecting microscope, flash frozen in liquid nitrogen and stored at -80 ºC.

4.3.2 RNA extraction and microarray analysis

The RNA extraction and microarray analysis were described in detail in chapter 3. In summary, pooled samples (n = 3) of gill and liver and individual samples of brain (with pituitary gland) were used to extract RNA from control and exposed fish. After treatment with DNase I, RNA quantity and quality were verified with NanoDrop® ND-1000 spectrophometer (Thermo Fisher Scientific, Wilmington, USA) and electrophoresis gel, respectively. Samples containing low quality or low quantity of RNA were rejected from the study.

The DNase-treated total RNA from gill, liver and brain samples were then used to perform a commercially 4 x 44K Zebrafish Gene Expression Microarray (Product No. G2519F, Design ID 019161, version 2.0) manufactured by Agilent Technologies (Santa Clara, California, USA). After a further confirmation of the quality and quantity of RNA using an Agilent 2100 BioAnalyzer (Agilent Technologies), three gill and liver RNA control samples were compared with three gill and liver RNA ammonia exposure samples, respectively. However, for brain, four RNA control samples were compared with four RNA exposed samples. The microarray was performed in the certified Laboratory for Advanced Genome Analysis in Vancouver Prostate Center (Vancouver, CA) following all the manufacturer's protocols.

The microarray analysis started with an update of the gene list considering the ID number of NCBI and Ensembl, which allowed the reduction of the number of probes from 44,000 to 25,764 since some probes were not associated with NCBI or Ensembl ID numbers or were identified as unknown. Text files of these probes were generated and uploaded into the Subio Platform (version 1.18) (Kagoshima, Japan) and a quantile log 2 normalization followed by Student's *t*-test were performed. The quality of the data was checked with several quality control methods in the platform. Genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05 and an increase or decrease in mRNA expression level was considered when the fold change (FC) was ≥ 2 and ≤ 0.5, respectively.

After a Gene Ontology (GO) and pathway analysis of the microarray (see the previous chapter), the mRNA expression of several genes already known, from the literature, that respond to ammonia toxicity were analyzed in the microarray. These genes are associated with amino acids metabolism, transcellular and paracellular transport of ammonia through the cells and ammonia detoxification. Moreover, endocrine regulation and/or ammonia responsive hormone receptors were also analyzed in the microarray.

4.3.3 cDNA synthesis and real-time PCR

To confirm and validate the results obtained in the microarray for some genes responsive to ammonia toxicity, cDNA synthesis and *q*PCRs were performed. For cDNA synthesis, 1 µg of DNase-treated total RNA from gill and liver tissues were reversed-transcribed using the iScript kit (Bio-Rad, Hercules, CA, USA) in a 20 µl reaction volume and then stored at -20 ºC. The cDNA quality was evaluated performing a semi-quantitative PCR of the zebrafish elongation factor 1 alpha (EF1α, *eef1al1*) under the following cycling conditions: 2 min at 94 ºC; 30x of 94 ºC during 30 s, 60 ºC during 30 s and 72 ºC during 30 s and an additional step of 5 min at 72 ºC. The PCR results were verified by agarose gel electrophoresis. Since this cDNA were made from the same DNase-treated RNA used in the microarray, the *q*PCR provides an additional measure for both control and ammonia exposure samples.

The primers used in the *q*PCR were chosen from previous papers or were designed using Primer 3 (Rozen and Skaletsky, 2000) (see Table 4.1). The efficiency of each primer pair was analyzed from the slope of a linear regression of the quantification cycle versus the relative concentration of cDNA. The linear regression was achieved using a serial dilution of a pooled sample done with 1 µl of each cDNA from the tissue samples. The *q*PCR efficiency

was deemed satisfactory if it was between 90 % and 105 % with $R^2 > 0.980$. The relative dilution of a given sample was extrapolated by the linear regression of the standard curve. The *q*PCR fragments from the standard curve were later analyzed by agarose gel electrophoresis to confirm that only a single product of the desired size was obtained from each reaction. Primer sequences, annealing temperature and cDNA dilution are listed in Table 4.1. Samples were assayed in duplicate with only one target gene assayed per well. Water-only controls were run in all *q*PCRs to ensure that reagents were not contaminated.

Table 4.1 Primer sequences, size of the product, annealing temperature (ºC) and cDNA dilution for each gene analyzed by *q*PCR. These genes were selected considering the results obtained in the microarray analysis and also due to it previous response to ammonia exposure, in this or other species, and described in the literature. Most of the primers pairs were obtained from published studied, except the primers for prolactin receptor a (*prlra*) that were designed with Primer 3 [labeled with asterisk (*)].

Gene	Primer sequences	size	ºC	cDNA dilution	Reference
EF1α (*eef1al1*)	F-TGGGTGTTGGACAAACTGAA	190	60	1:500	Gonçalves et al., 2012
	R-CAACACCACCAGCAACAATC				
Rhbg (*rhbg*)	F-TCAATGGAGCGCTCAAACAAAGGGT	233	59	1:1000	Nakada et al., 2007b
	R-TGAGTTTCTCCACCTCGTTTTGCGT				
Rhcga (*rhcga*)	F-GGGAGGTACCTGAAGATGAAGAGAG	237	58	1:100	Nakada et al., 2007b
	R-TGAGCGCAGACTTGGTCTGAACATT				
Rhcgb (*rhcgb*)	F-CTGTTTGGGATCACGCTGTA	127	58	1:100	Braun et al., 2009a
	R-CCCTTGAGATGGAGAGACCA				
occludin a (*oclna*)	F-ACCATTACTGCGTGGTGGA	199	58	1:100	Clelland and Kelly, 2010
	R-ACCCAGTCCTCCACATCTTG				
occludin b (*oclnb*)	F-CAAAATCAGGCAAAGGCTTC	176	58	1:100	Clelland and Kelly, 2010
	R-AACAATAGTGGCGATGAGCA				
claudin b (*cldnb*)	F-ACCAACCAACAAGGAAAACG	214	59	1:100	Clelland and Kelly, 2010
	R-GCATCTGTCCAGTGCTTTGA				
claudin 2 (*cldn2*)	F-CCACAAACCCCAACATTACC	211	58	1:100	Clelland and Kelly, 2010
	R-GAGCCTTTCAGCAATCCAAG				
aquaporin 8a (*aqp8a*)	F-GCTGCCACTATGGCTGAGATGA	349	58	1:100	Tingaud-Sequeira et al., 2010
	R-AAAAGCTTGCAATCCTCTTCA				
prolactin receptor a (*prlra*)	F- GGGACAAACCACGGACCGCA	179	58	1:100	*
	R- TGGTTTACACCGCACCTGCACC				

The *q*PCR reactions were run in iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) and were performed with: 1x SYBR Green Super Mix (Bio-Rad), 200 nM of each primer pair and 4 µl of diluted cDNA, for a final reaction volume of 20 µl. The cDNAs were previously diluted to 1:100, 1:500 and 1:1000 for target and reference genes according to earlier optimizations (Table 4.1). The expression levels of these genes were assessed using the

following program: 95 ºC for 4 min; 40 cycles of denaturation at 95 ºC for 10 s, annealing temperature optimized for each primer pair (Table 4.1) for 30 s, and elongation at 72 ºC for 30 s. All programs included a melting curve at the end of each *q*PCR run to ensure that no primer dimers were amplified.

To account for differences in cDNA loading and RNA reverse transcriptase efficiency, each sample was normalized to the expression level of the reference gene EF1α (*eef1al1*). The expression of EF1α was unchanged between both treatments and tissues. For the biological replicates, the average of the replicates was considered a single point and the mean and standard error were calculated. The copy number of each mRNA of interest was expressed as a fold induction or repression based on the expression in the control samples. All data were analyzed using the ΔΔCt method (Livak Method; Livak and Schmittgen, 2001) and statistical differences were analyzed through Student's *t*-test or Mann-Whitney Rank Sum Test when normality or equal variance failed, using SigmaPlot (version 11, SPSS Inc., Chicago, IL, USA). The *q*PCR results were considered statistically different when *p*-value (*P*) was ≤ 0.05. Results are present as mean ± coefficient of variation for fold change (FC) for each gene.

4.4 Results

4.4.1 Expression of genes in the microarray

Among genes involved in the transepithelial movement of ions and molecules, 27 responsive genes were analyzed in detail in the microarray results. Nineteen of these genes are involved in transcellular transport and seven in paracellular transport. The mRNA of the enzyme CA was also evaluated due to it relevance on ammonia movement. The number of gene subtypes that were upregulated or downregulated after ammonia exposure is shown in Figure 4.1. In the gill, the highest number of subtypes that responded to ammonia exposure were CA, H^+-ATPase, Na^+/K^+-ATPase, K^+ channel, Cl^- channel and Rh glycoproteins, with most of the subtype genes being upregulated. In contrast, in response to ammonia exposure, the aquaporin (AQP) family presented five subtypes that were downregulated in gill, one subtype downregulated in liver and no subtype responding in brain. In liver, although five K^+ channels were upregulated, most of the subtypes genes of CA, Na^+/K^+-ATPase, Ca^{2+}-ATPase, Ca^{2+} channels and Rh glycoproteins were downregulated. Moreover, it is also important to mention that the urea transporter (UT) was downregulated in both gill and liver. In the brain, only a few subtype genes related with the transcellular movement of ions [H^+-ATPase, Na^+/K^+-ATPase, Na^+/HCO_3^- cotransporter and facilitated glucose transporter (GLUT)]

responded to ammonia exposure.

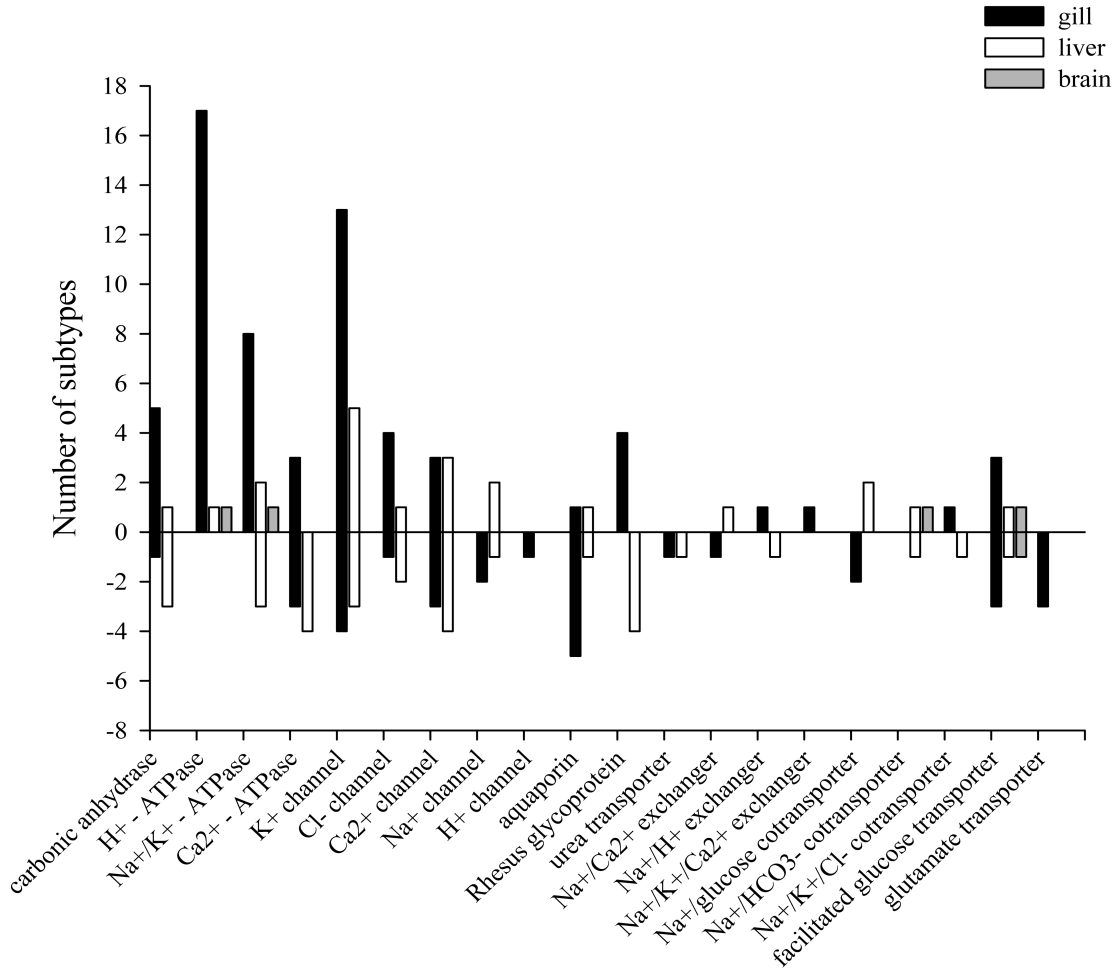


Figure 4.1 Genes that responded to ammonia exposure in the microarray and that are related with the transcellular movement of ammonia and ions. The positive numbers corresponded to upregulated subtypes while the negative numbers corresponded to downregulated subtypes. The genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05, which was determined in the Subio Plataform (version 1.18).

In addition to the previous genes that are involved with the transcellular movement, seven genes that regulate the paracellular movement of ions and molecules through the epithelia were also analyzed (Figure 4.2). Within this group of genes, claudins (CLNs) presented the highest number of subtypes affected by ammonia exposure in gill tissue, with most of them being upregulated. In addition to CLNs, subtypes of occludins (OCLNs), tight junctions (TJs), cadherins, catenins and cell adhesion molecules (CAMs) were also upregulated in gill. However, in contrast to gill, liver and brain, only presented a few subtypes of CLNs affected by ammonia exposure being all downregulated. Moreover, liver presented subtypes of cadherin, protocadherin and CAMs that were upregulated, while brain presented

subtypes of cadherin and CAMs that were downregulated.

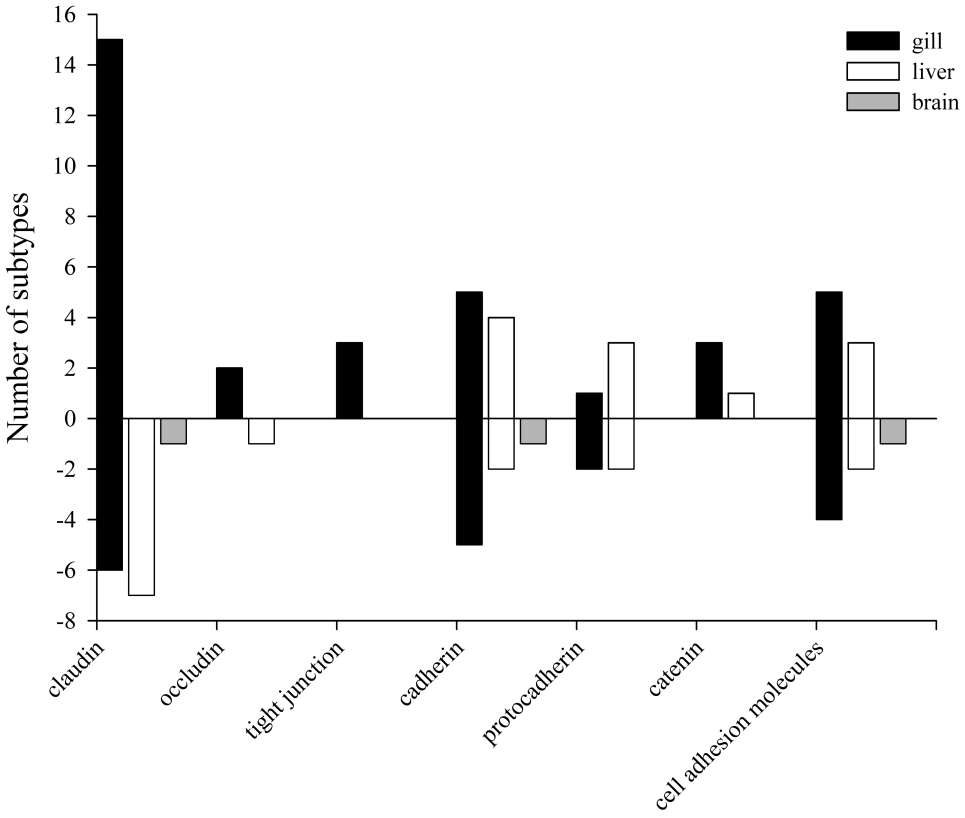


Figure 4.2 Genes that responded to ammonia exposure in the microarray and that are related with the paracellular movement of ammonia and ions. The positive numbers corresponded to upregulated subtypes while the negative numbers corresponded to downregulated subtypes. The genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05, which was determined in the Subio Plataform (version 1.18).

Due to the relevance of the amino acid catabolism for ammonia production, 39 enzymes belonging to this metabolism and that responded to ammonia exposure, were also analyzed in detail in the microarray (Figure 4.3). In gill, most of the subtype genes associated with amino acid metabolism were downregulated with the highest number of decreased subtypes noted for tryptophan 2.3-dioxygenase, creatine kinase, enoyl Coenzyme A hydratase and cystathionase. However, the enzyme gamma-glutamyltransferase presented two subtypes that were upregulated in gill. In contrast to the gill, only a few amino acid metabolism subtype genes responded in liver and brain tissues, being mostly upregulated.

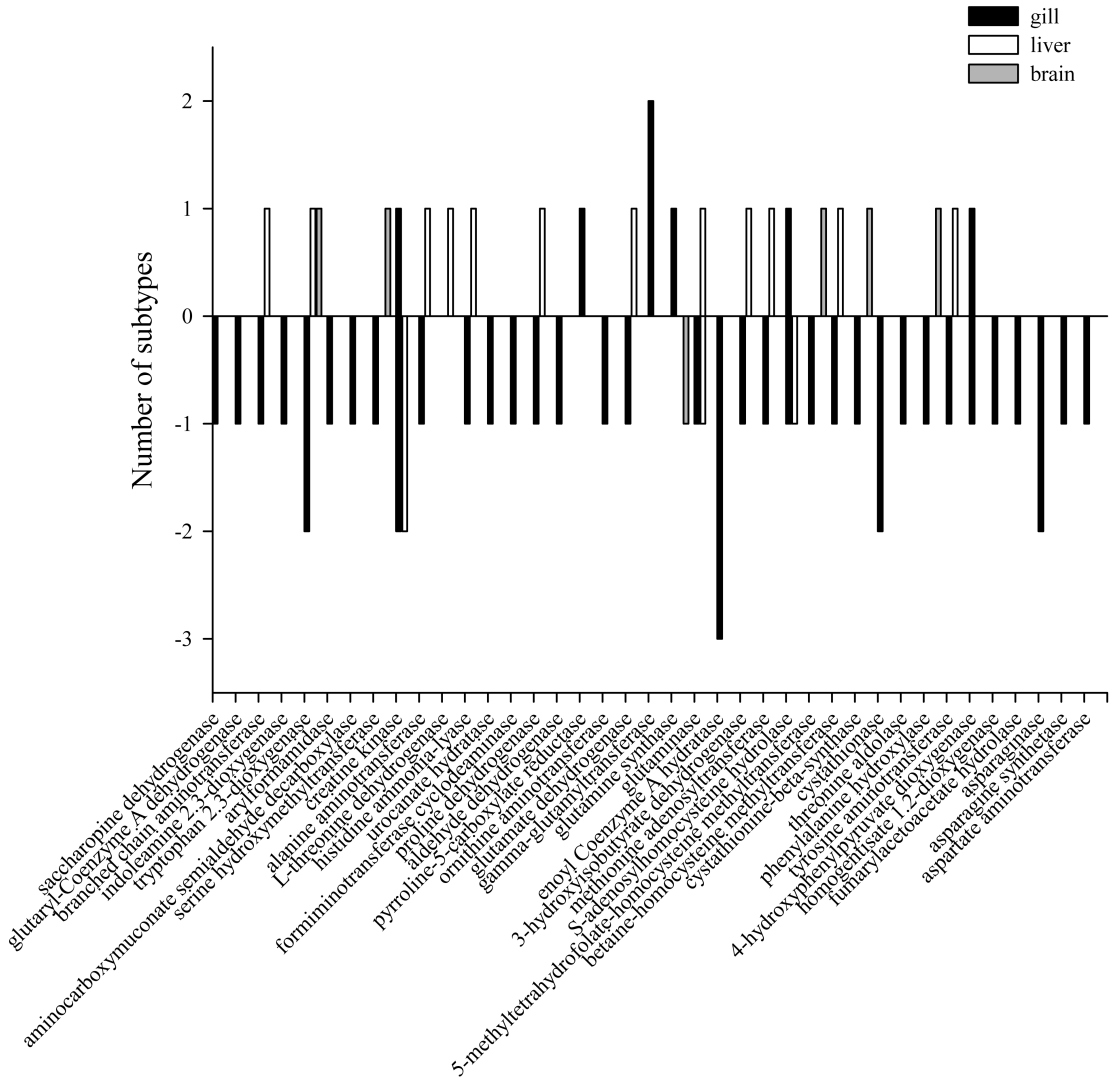


Figure 4.3 Genes that responded to ammonia exposure in the microarray and that are involved in the amino acid metabolism. The positive numbers corresponded to upregulated subtypes while the negative numbers corresponded to downregulated subtypes. The genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05, which was determined in the Subio Plataform (version 1.18).

Among the enzymes involved in amino acid metabolism, some are known to be directly related with ammonia metabolism, namely, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutaminase (Figure 4.3). In gill, only GS was upregulated, with all the other enzymes (ALT, AST, GDH and glutaminase) downregulated. However, in liver, ALT and GDH were upregulated and glutaminase had one subtype upregulated and other subtype downregulated. The other enzymes (AST and GS) were not affected in liver by ammonia exposure. In brain, only one subtype of the enzyme GS responded to ammonia exposure and was downregulated.

One of the strategies to deal with ammonia toxicity is converting ammonia to less toxic compounds like GLN or urea. The conversion to GLN implies the enzymes GDH, GS and glutaminase that were previously analyzed; however, the conversion to urea occurs through the ornithine-urea cycle (OUC) that includes several enzymes that may respond to ammonia exposure. In fact, among all the enzymes of OUC, only argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) presented subtypes increased in gill after ammonia exposure (Figure 4.4). However, none of the enzymes of OUC were affected in the other two organs. On the other hand, another pathway to produce urea, in fish, is through uricolysis. However, the only enzymes of this pathway that responded to ammonia exposure were allantoicase and urate oxidase (UO), with their expression downregulated in gill tissue (Figure 4.4).

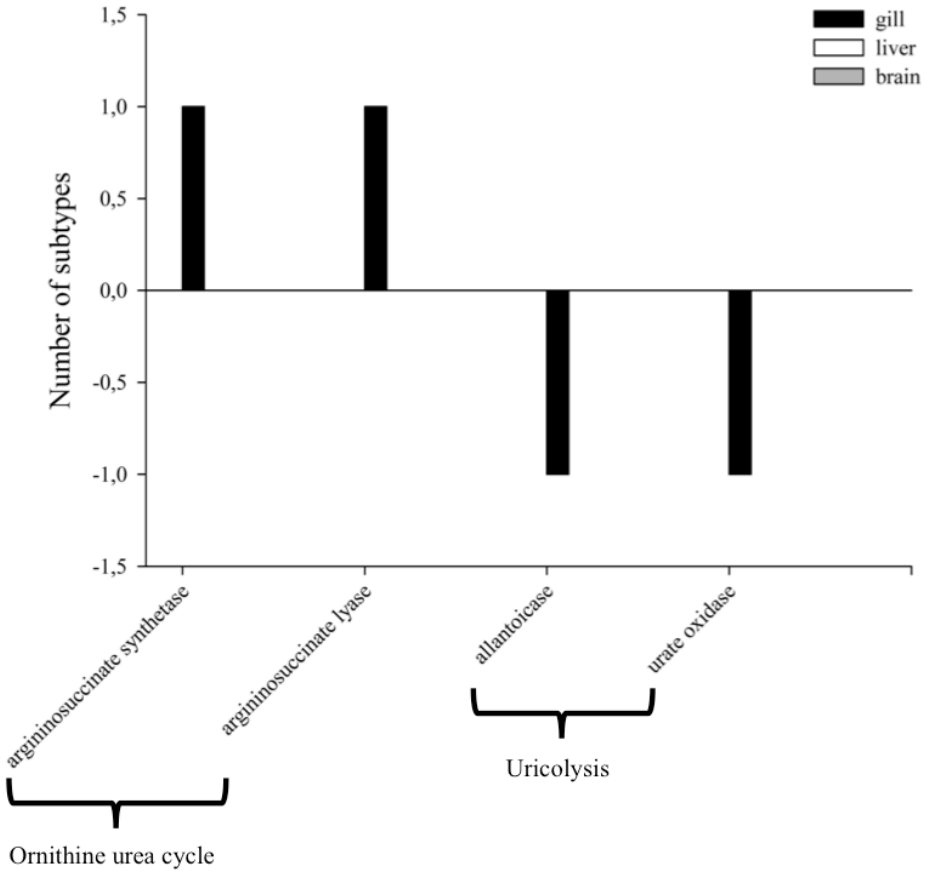


Figure 4.4 Genes that responded to ammonia exposure in the microarray and that are involved in the ornithine urea cycle (OUC) and uricolysis. The positive numbers corresponded to upregulated subtypes while the negative numbers corresponded to downregulated subtypes. The genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05, which was determined in the Subio Plataform (version 1.18).

Beyond all these genes that are involved in the different mechanisms to avoid ammonia intoxication, the endocrine system may also be important to regulate the response to environmental ammonia. Indeed, the expression of one hormone and five hormone receptors changed in response to ammonia exposure (Figure 4.5). The mRNA of the hormone somatolactin (SL) was downregulated in gill but upregulated in liver. The responsive hormone receptors were the prolactin receptor (PRLR), the growth hormone receptor (GHR), the thyroid hormone receptor (THR), the parathyroid hormone receptor (PTHR) and the natriuretic peptide receptor (NPR), that presented different expression levels in gill and liver. Specifically, two subtypes of PRLR and one of NPR were upregulated in gill but downregulated in liver. Also in gill, the THR and the two subtypes of GHR were downregulated, while the PTHR was upregulated. However, none of these receptors altered their expression in liver. It is also important to mention that no hormone or receptor responded to ammonia in the brain tissue.

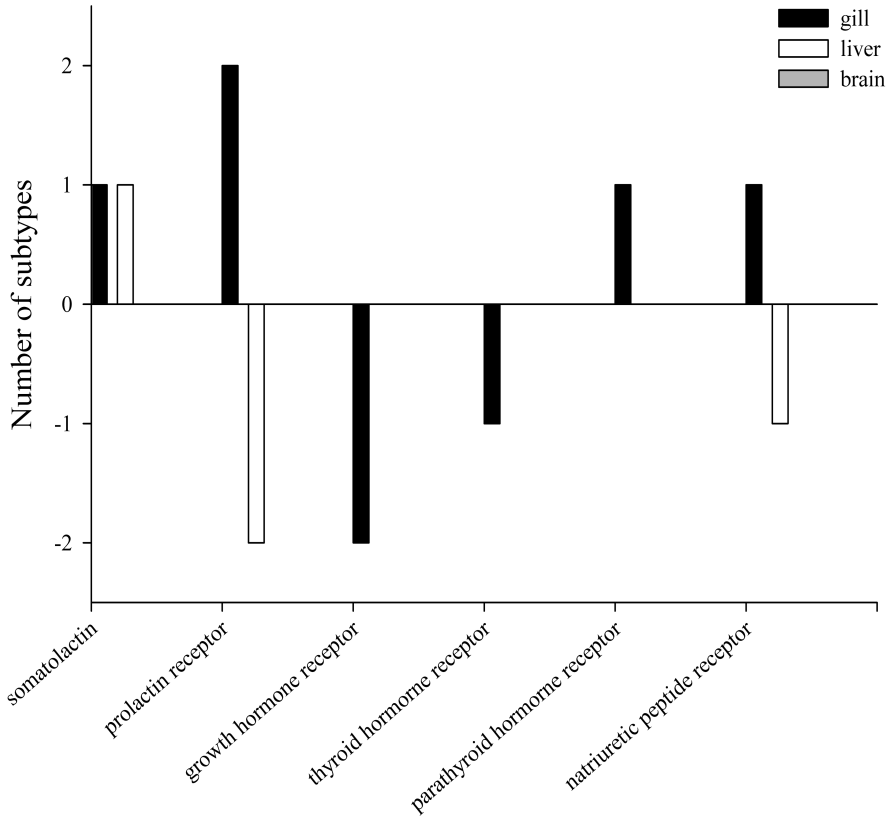


Figure 4.5 Genes of hormones and hormones receptors significantly altered in the microarray of gill, liver and brain of zebrafish after 96 h of ammonia exposure. The positive numbers corresponded to upregulated subtypes while the negative numbers corresponded to downregulated subtypes. The genes were considered statistically different when the *p*-value (*P*) was ≤ 0.05, which was determined in the Subio Plataform (version 1.18).

*4.4.2 Validation of microarray by real-time PCR (*q*PCR)*

Some of the results obtained in the microarray were validated by *q*PCR; although only for gill and liver. In gill, from a total of nine genes, five presented the same result as the microarray, namely, the *rhcga*, *rhcgb*, *oclna*, *oclnb* and *cldnb* (Figure 4.6). In liver, the results were more consistent with eight out of nine genes presenting the same results in both microarray and *q*PCR. In fact, in this organ, only the *cldnb* presented different results (Figure 4.7).

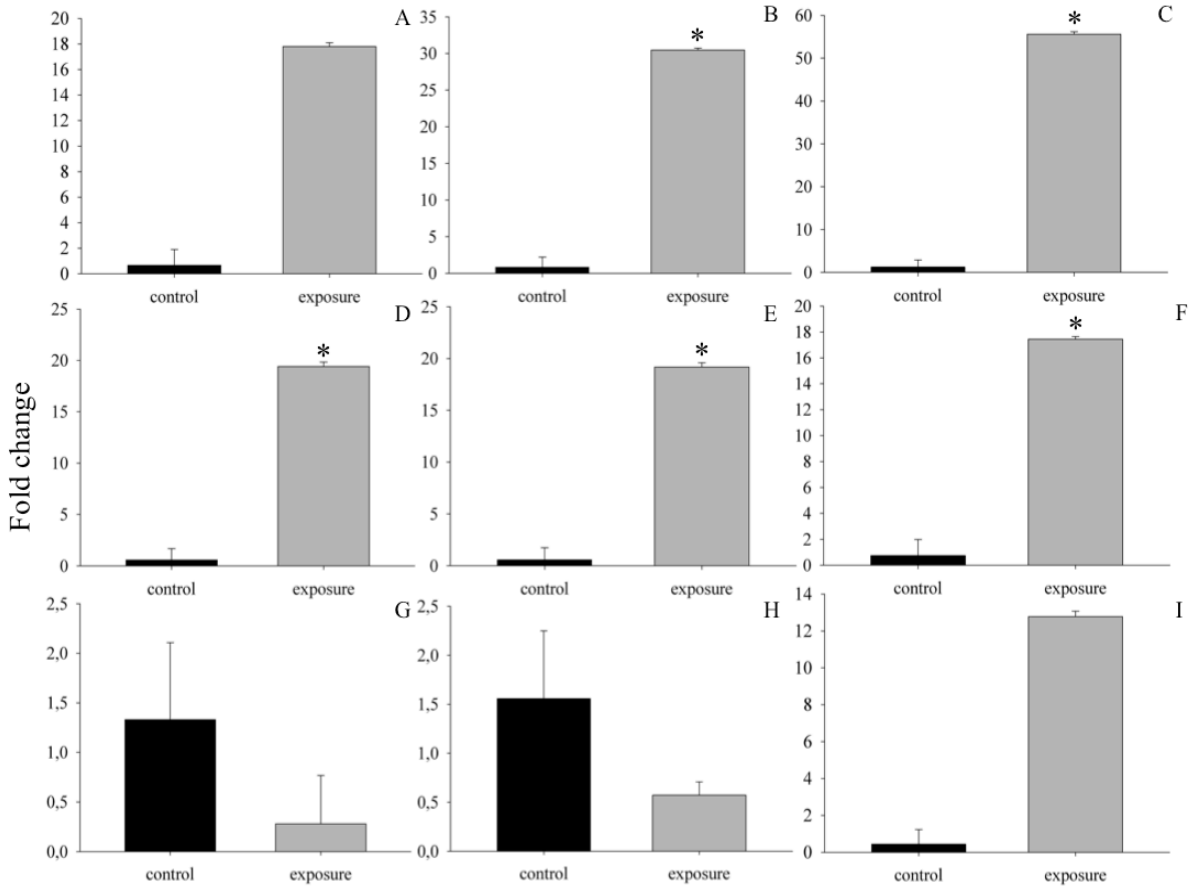


Figure 4.6 The *q*PCR results, in gill, of some responsive genes after 96 h of ammonia exposure. Gene expression was considered statistically different (*) when the *p*-value (*P*) was ≤ 0.05. Legend: A – Rhesus glycoprotein B (*rhbg*); B - Rhesus glycoprotein Cb (*rhcgb*); C - Rhesus glycoprotein Ca (*rhcga*); D - occludin a (*oclna*); E - occludin b (*oclnb*); F - claudin b (*cldnb*); G - claudin 2 (*cldn2*); H - aquaporin 8a (*aqp8a*); I - prolactin receptor a (*prlra*).

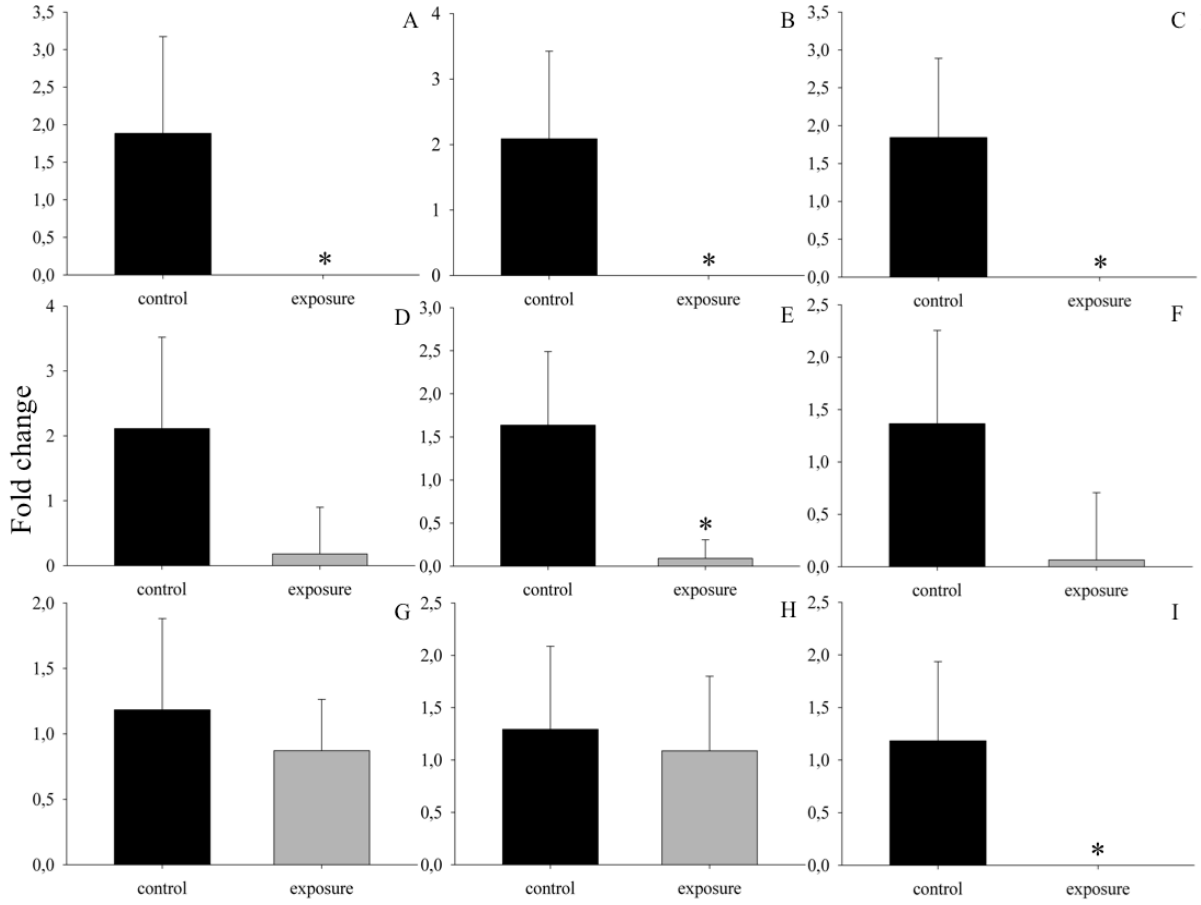


Figure 4.7 The *q*PCR results, in liver, of some responsive genes after 96 h of ammonia exposure. Gene expression was considered statistically different (*) when the *p*-value (*P*) was ≤ 0.05. Legend: A - Rhesus (Rh) glycoprotein B (*rhbg*); B - Rhesus glycoprotein Cb (*rhcgb*); C - Rhesus glycoprotein Ca (*rhcga*); D - occludin a (*oclna*); E - occludin b (*oclnb*); F - claudin b (*cldnb*); G - claudin 2 (*cldn2*); H - aquaporin 8a (*aqp8a*); I - prolactin receptor a (*prlra*).

4.5 Discussion

During exposure to high environmental ammonia, zebrafish increased the branchial mRNA expression of several transporter proteins (Figure 4.1), which allow the excretion of ammonia against its concentration gradient (Wright and Wood, 2009). In addition, there were changes in the expression of elements of the paracellular transport (Figure 4.2), altering the paracellular permeability of the gill epithelia to presumably impede ammonia entrance. Since ammonia is a toxic compound, its elimination out of the body is very important to guarantee the survival of the aquatic organism. As explained in the introduction, in the gill, ammonia may pass the epithelium through Rh glycoproteins following the ΔP_{NH3} (Wright and Wood, 2009; Ip and Chew, 2010; Nawata et al., 2010b). An increase in environmental ammonia or pH, increases the mRNA expression of these ammonia transporters (Hung et al., 2007; Wright and Wood, 2009), potentially favoring the continuous excretion of ammonia even against a

concentration gradient (Wright and Wood, 2009). In fact, the upregulation of the four subtypes of ammonia transporters (Figure 4.1) in zebrafish gill, suggests that this teleost fish is able to excrete ammonia against a concentration gradient, similar to other fishes (Hung et al., 2007; Nawata and Wood, 2008, 2009; Wright and Wood, 2009; Ip and Chew, 2010; Diricx et al., 2013). Moreover, the upregulation of 17 subtypes of H^+-ATPases (Figure 4.1) is also important, since the movement of ammonia out of the body depends on the acidification of the boundary layer to protonate the NH_3 to NH_4^+, ensuring the continuous elimination of ammonia (Wright and Wood, 2009; Nawata et al., 2010b). This increase of H^+ excretion was previously described by Braun and collaborators (2009b), in adult zebrafish; however they did not detect any change in the mRNA expression or protein levels of Rh glycoproteins. Also important for boundary layer acidification is the enzyme CA that catalyzes the CO_2 hydration, releasing H^+ for ammonia excretion (Wright and Wood, 2009). In this sense, the upregulation of five subtypes of CA (Figure 4.1) may also be related with the acidification of the boundary layer and excretion of ammonia against gradient. Another relevant result in this study is the downregulation of five subtypes of AQPs (Figure 4.1) in the gill. In fact, although AQPs are transmembrane transporters of water, they may also transport ammonia (Ip and Chew, 2010). In this sense, this downregulation of AQPs may serve as a way to prevent the movement of ammonia together with water. Although ammonia passes the cell membranes via transporter proteins (Wright and Wood, 2009), the simple diffusion through the intercellular space (paracellular transport) is also possible. The charge selectivity and even the type of molecules able to cross the paracellular space is controlled by proteins that belong to the tight junction complex, such as CLNs, OCLNs, TJ proteins, cadherin, protocadherin, catenin and CAMs (Chasiotis et al., 2012). The most important function of these proteins is to control the paracellular permeability of the epithelia; however, only CLNs and OCLNs have been recently studied, mostly in response to environmental challenges (Kumai et al., 2011), namely salinity (Bagherie-Lachidan et al., 2009), and during hormonal regulation (Clelland and Kelly, 2010) and treatment (Bui et al., 2010; Chasiotis et al., 2010; Chasiotis et al., 2012). From Figure 4.2, several proteins related with the paracellular movement of molecules and ions, responded in gill, to ammonia exposure, generally increasing in mRNA expression. The increase of OCLNs has been associated with the "tightness" of the epithelia; however, the response of CLNs varies with the subtype, the species and the environment. With this in mind, the results obtained in zebrafish after 96 h of ammonia exposure indicate that this species is able to change the expression of elements of paracellular pathway, probably to

decrease the branchial paracellular permeability and impede the entrance of ammonia from the environment.

In contrast to the gill, liver decreased the mRNA expression of most transporter proteins (Figure 4.1) and paracellular transport elements (Figure 4.2), while in brain very few had altered it expression. In liver, the four ammonia transporter proteins belonging to the Rh glycoproteins family were downregulated, which may be related with a decrease in the hepatic production that, in turn, decreased the flux rate and/or retention of ammonia inside the liver probably to be incorporated in other amino acids (Wood, 2004; Madison et al., 2009) avoiding the ammonia toxicity (Ip and Chew, 2010). Through this mechanism, ammonia is maintained inside the body but in less toxic forms, making possible for later utilization by the organism, without causing negative effects. On the other hand, the presence of high levels of ammonia in the environment did not cause relevant alteration in the mRNA expression of transcellular (Figure 4.1) and paracellular (Figure 4.2) transport elements in brain, which may indicate that ammonia had not reached to high concentrations in this organ and did not trigger its negative effects. It is also important to mention that, in brain, AQPs did not responded to ammonia exposure, which may indicate that it is not producing cerebral and/or astrocytic swelling. In fact, brain swelling is a described negative effect of high internal ammonia concentrations in some fishes but mostly in mammals (Ip and Chew, 2010; Wilkie et al., 2015).

Since ammonia is produced inside the organism, one of the mechanisms to deal with high concentration in the environment is reducing its production (Randall and Tsui, 2002); however, the incorporation of ammonia in *de novo* amino acids may also be an advantage (Wood, 2004; Madison et al., 2009). In fact, ammonia is produced in the liver of teleost fishes during the catabolism of amino acids and proteins being after released to the blood stream to be transported to the gills, where it will be excreted (Ip and Chew, 2010). In this sense, most of the ammonia eliminated through the gills is derived from the different parts of the body; although some may be produced inside the gill as well. In the present study, to avoid the production of ammonia inside the gill, all the enzymes involved in the metabolism of amino acids were downregulated (Figure 4.3), which constitutes a strategy used by several fishes to impede ammonia intoxication (Lim et al., 2001; Randall and Tsui, 2002). Moreover, the use of this mechanism may vary according to the species and tissue, since giant mudskipper *Periophthalmodon schlosseri* decreased the total free amino acids content in liver and blood, while mudskipper *Boleophthalmus boddaerti* reduced it in muscle (Lim et al., 2001).

However, in contrast to the mechanism applied by the gills, liver increased the mRNA expression of some enzymes involved in the metabolism of amino acid (Figure 4.3), which may be explained by the incorporation of ammonia in *de novo* amino acids and its maintenance inside the body but in forms that are not toxic and that may later be used by the body (Wood, 2004; Madison et al., 2009). In fact, Wood (2004) explained the increased growth of rainbow trout *Oncorhynchus mykiss* during an exposure to low environmental ammonia levels as being due to the incorporation of ammonia into amino acids and increased protein synthesis, which stimulated growth without altering the food consumption.

Similarly to other teleosts fishes, zebrafish resort to the conversion of ammonia to less toxic compounds, namely glutamate (GLU) and GLN, to detoxify ammonia (Randall and Tsui, 2002). Several fishes [e.g. sleeper *Bostrichthys sinensis* (Anderson et al., 2002), rainbow trout *O. mykiss* (Arillo et al., 1981), goldfish *Carassius auratus* (Levi et al., 1974) and common carp *Cyprinus carpio* (Dabrowska and Wlasow, 1986)] utilize the enzymes GDH to produce GLU from NH_4^+ and α-ketoglutarate (αKG) and then, utilize the enzyme GS to produce GLN from GLU and NH_4^+. Therefore, from αKG to GLN it is possible to detoxify two moles of NH_4^+ (Randall and Tsui, 2002) and produce molecules that are non-toxic and that can be accumulated in the organism avoiding the deleterious effects of ammonia (Randall and Tsui, 2002; Ip and Chew, 2010). In this sense, the fact that this enzyme's mRNA was upregulated in gill (Figure 4.3) indicates the resorting to this detoxification mechanism to avoid ammonia intoxication in this tissue. Moreover, probably to avoid the swelling of branchial cells due to GLN accumulation, most of the branchial AQPs were, as mentioned before, downregulated (Figure 4.1), which may constitute another explanation for these latter results. On the other hand, in brain, although GS may, at first, protect this organ from ammonia toxicity (Levi et al., 1974; Arillo et al., 1981; Dabrowska and Wlasow, 1986; Peng et al., 1998; Randall and Tsui, 2002; Veauvy et al., 2005; Wright et al., 2007), the accumulation of high levels of GLN may function as an osmolyte, which causes an increase of water content inside the astrocytes and neurons inducing astrocyte and brain swelling (Zwingmann et al., 2000; Albrecht and Dolińska, 2001; Pichili et al., 2007; Ip and Chew, 2010) that, in turn, cause several metabolic disorders (Albrecht and Dolińska, 2001; Pichili et al., 2007; Cauli et al., 2009) and, at the limit, neuronal cell death (Willard-Mack et al., 1996; Albrecht and Dolińska, 2001; Feldman et al., 2014). In this sense, probably to avoid this situation, contrarily to what happens in gill, GS, in brain, was downregulated (Figure 4.3), protecting the brain of this negative effect of GLN. These results contrast with those obtained

in African catfish *Clarias gariepinus*, rainbow trout *O. mykiss* (Arillo et al., 1981; Wright et al., 2007), mudskippers *B. boddaerti* (Peng et al., 1998), goldfish *Carassius auratus* (Levi et al., 1974), common carp *Cyprinus carpio* (Dabrowska and Wlasow, 1986) and gulf toadfish *Opsanus beta* (Veauvy et al., 2005) that increased the mRNA and/or activity of GS and enhance the GLN concentration in the brain during their immersion in water containing a sub-lethal concentration of ammonia (Peng et al., 1998; Veauvy et al., 2005; Wright et al., 2007; Ip and Chew, 2010). In addition to GLU and GLN, some teleost fishes may convert ammonia to urea (Randall and Tsui, 2002), especially during high environmental pH, high environmental ammonia and/or aerial exposure. For example, gulf toadfish *O. beta* and oyster toadfish *Opsanus tau* are able to produce urea during adverse environmental conditions, mostly through an active OUC. However, although zebrafish exhibit an active OUC during their early development (see chapter 2; Braun et al., 2009a; Bucking et al., 2013; LeMoine and Walsh, 2013), in adult it is not able to produce urea in this manner. In this sense, among all the enzymes of the OUC, only ASS and ASL were affected by the exposure to ammonia (Figure 4.4); however, their upregulation is probably related with their function in other metabolic pathway and not with OUC since all the remaining enzymes were lacking.

Contrarily to what was expected, in zebrafish, cortisol may not be involved in the response to ammonia; however, hormones that have not previously been involved in ammonia toxicity appear, in this study, probably playing a protective role. The response of cortisol to high levels of environmental ammonia has been well documented (Wood and Nawata, 2011; Sinha et al., 2012, 2013; Gonçalves et al., 2012); however, in this study, there are no evidences that this hormone is involved in the response of zebrafish to ammonia exposure. During a stress situation, the levels of cortisol increase inside the body (Auperin et al., 1997; Mommsen et al., 1999; Gonçalves et al., 2012), leading to an increase in amino acid catabolism and enhancement of ammonia excretion (Hopkins et al., 1995; Mommsen et al., 1999; Randall and Tsui, 2002). Since cortisol stimulates the production of ammonia, stressed fish have lower tolerance to ammonia when it is accumulated in the environment (Randall and Tsui, 2002). However, for cortisol to exert its effect, this hormone has to bind to the glucocorticoid receptor (GR) and/or mineralocorticoid receptor (MR) usually increasing in expression and/or activity (Sinha et al., 2012). However, in this study, GR and MR changes were not detected in the microarray analysis, suggesting that cortisol was not enhanced in zebrafish in response to ammonia exposure. Although these results contrast with the ones from Gonçalves and collaborators (2012) that presented elevated plasma cortisol levels in

zebrafish during acute (15 min, 1 h and 24 h) and chronic (14 d) ammonia exposure, the results described in this work are in agreement with those obtained by Diricx and collaborators (2013), since they did not find any relationship between plasma cortisol and ammonia excretion in common carp *C. carpio*. In fact, the authors explained their results considering that cortisol is not the only ion-regulatory hormone that responds to ammonia exposure, with other hormones, such as PRL and thyroid hormones, being important to control the ion balance during a stressful condition (Tang et al., 2001; Diricx and collaborators, 2013). In this sense, and agreeing with Diricx and collaborators (2013), the present study also showed that hormones such as PRL, SL, growth hormone (GH), thyroid hormone, parathyroid hormone and natriuretic peptide, are potentially involved in the regulation of osmoregulation and homeostasis in fish after 96 h of ammonia exposure (Figure 4.5). Interestingly, PRL, SL and GH belong all to the same family, sharing a common ancestral molecule between them (Ono et al., 1990; Rand-Weaver et al., 1993). However, it is also important to mention that, the regulation of PRLR and GHR were opposite in the gill tissue, similar to previous studies (Tang et al., 2001), which can be explained by the important role of PRL in osmoregulation, while growth is suppressed by GH, during a stress situation.

4.6 Conclusion

The tolerance of the organism to ammonia varies with the water physicochemical parameters, the concentration of environmental ammonia, and also with the mechanisms used by the organism to avoid ammonia intoxication. Zebrafish increase the mRNA expression of all specific ammonia transporters, which suggests the continuous excretion of ammonia to the environment. Furthermore, ammonia exposure also increase the mRNA levels of several subtypes of H^+-ATPase and CA, enhancing the acidic properties of the boundary layer and trapping NH_3 as NH_4^+ after bind an H^+. In addition to the changes of the expression of transcellular transport elements, zebrafish also change the expression of proteins present in the TJs, altering the paracellular permeability of the branchial epithelium. Besides this, zebrafish may also decrease the expression of enzymes involved in amino acid metabolism in the gill, reducing the production of ammonia in this organ. On the other hand, in the liver, some of the enzymes involved in this metabolism were upregulated probably to incorporate some of the ammonia in the *de novo* amino acids synthesis.

That hormones are affected by the presence of toxicants is already known; however, contrary to what was expected, neither GR nor MR responded to ammonia exposure,

suggesting that cortisol was not altered at this time point. However, the mRNA levels of other hormones and/or their receptors changed in response to high environmental ammonia, such as, the different elements of PRL/SL/GH family, as well as, thyroid and parathyroid hormones, which indicate that zebrafish may alter their endocrine system to maintain the homeostasis.

CHAPTER 5 |

REGULATION OF AMMONIA EXCRETION BY PROLACTIN AND CORTISOL IN ZEBRAFISH (*Danio rerio*)

5.1 Abstract

In teleosts, ammonia excretion can be done directly through Rhesus (Rh) glycoproteins, but also regulated by tight junctions elements [like claudins (CLNs) and occludins (OCLNs)] located between epithelial cells and that control banchial permeability. The hormonal regulation of the transcellular transporters has been extensively studied; however, the relationship between paracellular transport and endocrine system was only recently provided but under salinity challenge. In this study, the objectives were to determine the effects of both cortisol and prolactin (PRL) on ammonia excretion rates (J_{Amm}) and also in the mRNA levels of elements of transcellular and paracellular transport. After 24 h in a bath of cortisol, fishes presented the lowest J_{Amm}, which occurred with an increase of Rh glycoproteins mRNA and a decrease of branchial permeability, caused by upregulation of OCLN. The lower J_{Amm} in this hormonal treatment can be explained by cortisol dynamics since plasma cortisol levels may increase from a few minutes till hours after injections or induction, later returning to basal levels. Unexpectedly the injection of PRL resulted in higher J_{Amm}; however, this was not reflected in mRNA levels of ammonia transporters. These results, in turn, may be explained by the direct or indirect effect of

PRL in zebrafish metabolism. An extra load of ammonia caused, in turn, an increase in J_{Amm}, with PRL administration inducing again higher J_{Amm} than cortisol treatment. Overall, this study shows that PRL affects the branchial permeability mostly through upregulation of OCLNs and that cortisol modulates the expression of both paracellular and transcellular transport elements.

5.2 Introduction

Ammonia is an end product of the catabolism of amino acids, whose excretion by trancellular and paracellular transport is vital to guarantee the survival and good health of the fish. In fact, ammonia is produced in the liver and after its release into the blood stream (Ip and Chew, 2010), this nitrogenous waste compound has to be eliminated from the body, which occurs mostly through the gills (Wright and Wood, 2009; Ip and Chew, 2010). At the branchial epithelium, ammonia is either able to permeate the membranes crossing through the cells (transcellular movement) (Wright and Wood, 2009) or through the tight junctions (TJs) that exist between the epithelial cells (paracellular movement). The first type of transport, implicates the presence of several transporter proteins that directly or indirectly favor the movement of ammonia out of the body (Wright and Wood, 2009). To explain this movement, Wright and Wood (2009) have reviewed the literature and described the "Na^+/NH_4^+ exchange complex" model where NH_3 exits the erythrocytes and endothelial cells through the Rhesus (Rh) glycoprotein A (Rhag) and passes the basolateral membrane of the branchial epithelium by another member of the same family, the Rh glycoprotein B (Rhbg). In turn, in the apical membrane, the NH_3 is excreted to the environment via the Rh glycoprotein C (Rhcg). The relevance of the different non-erythroid Rh glycoproteins has long been demonstrated in ammonia transport (Hung et al., 2007; Wright and Wood, 2009); however, in this model, several other transporters are also considered, such as the V-type H^+-ATPase and Na^+/H^+ exchanger (NHE), contributing to the continuous excretion of ammonia to the water through the maintenance of a partial pressure gradient of NH_3 (ΔP_{NH3}) between the gill and the environment (Wright and Wood, 2009). Due to their function as ammonia transporters, several internal and/or external conditions may affect the mRNA expression of Rh glycoproteins in the gills, such as, high levels of ammonia or pH in the aquatic environment or in the body (Hung et al., 2007; Nawata and Wood, 2008, 2009; Tsui et al., 2009; Wright and Wood, 2009; Ip and Chew, 2010). Besides these carriers, ammonia may pass unduly the branchial membranes through water channels named as aquaporins (AQPs). On the other hand, the movement of ammonia across the branchial epithelium may also occur through

paracellular transport that is, in turn, regulated by elements of the TJs, such as claudins (CLNs) and occludins (OCLNs). In general, TJs are a continuous circumferential cell-cell contact localized at the apical and lateral borders of the epithelial cells and, although they confer several properties to the epithelia, the most important is the regulation of ions, solutes and water movement through the branchial membranes. Both CLNs and OCLNs are elements of the TJ architectural complex (Van Itallie and Anderson, 2006) and although each one confers specific properties to the epithelia, both regulates the paracellular permeability (Fujita et al., 2006; Van Itallie and Anderson, 2006) determining if an epithelium is “tight" or "leaky".

In response to environmental challenges, several properties of the branchial epithelium may alter under the control of hormones of the endocrine system. Prolactin (PRL) is a hormone involved in the adaptation of aquatic organisms to freshwater (Tang et al., 2001; Manzon, 2002), decreasing the membrane permeability and maintaining the sodium balance. In addition, this hormone presents several other physiological functions related with cellular growth and differentiation, metabolism, growth, development, immunity, reproduction and behavior (Manzon, 2002; Power, 2005). In contrast to PRL, the glucocorticoid hormone cortisol increases in fish during a seawater challenge, which shows its importance for the adaptation to hyperosmotic environments (Mommsen et al., 1999), mostly increasing the branchial Na^+/K^+-ATPase activity and reducing the passive ion loss (Kelly and Wood, 2001).

Besides their relevance during environmental challenges, both cortisol and PRL may also be involved in the response to a stress situation (Auperin et al., 1997; Mommsen et al., 1999; Tang et al., 2001). The presence of a stressor in the water induces an increase of plasma cortisol concentration (Pickering, 1993; Hopkins et al., 1995; Mommsen et al., 1999; Gonçalves et al., 2012), which may affect several physiological functions such as growth, reproduction, immunity and osmoregulation (Mommsen et al., 1999; Gonçalves et al., 2012). Moreover, stress leads to respiratory, metabolic and circulatory adjustments that increase the release of oxygen to the tissues and mobilize energy storage (Mommsen et al., 1999). Similar to cortisol, PRL may also respond to stress, increasing its concentration in plasma (Auperin et al., 1997; Tang et al., 2001); however, different results have been described, which may indicate that PRL response might be species specific.

The effects of PRL and cortisol are only induced after its binding to a specific receptor; however, cortisol may also bind to another receptor regulating other functions. In fact, cortisol is able to bind to two different corticosteroid receptors, the glucocorticoid receptor (GR) and

the mineralocorticoid receptor (MR) (Bonga, 1997; Cruz et al., 2013). Although MR in mammals binds to aldosterone, in fish this hormone is not present (Jiang et al., 1998) and cortisol is the major ligand of MR. The branchial epithelial cells of fish contains high levels of the enzyme 11-β hydroxysteroid dehydrogenase 2 (11βHSD2) that is able to convert cortisol to an inactive state, thus avoiding the overactivation of MR and the induction of its effects thereafter (Pippal and Fuller, 2008). Although both GR and MR belong to the same nuclear receptor superfamily and regulate similar physiological functions, the activation of each corticosteroid receptor may also affect distinct and specific functions: GR affects the cardiovascular function, immune/stress response, cell cycle, growth, reproduction and neuronal activity, whereas MR affects osmoregulation and acid-base homeostasis (Bonga, 1997). In turn, PRL binds to a prolactin receptor (PRLR), with fish presenting two different subtypes (α and β) (Bole-Feysot et al., 1998; Manzon, 2002).

The effects on the transcellular and paracellular movement of ions as well as changes on the plasma hormone concentrations during environmental challenge has been described in several studies (Kumai et al., 2011); however, to our knowledge, no study, depicting the molecular changes of ammonia transcellular and paracellular excretion, induced by cortisol and PRL, was conducted. The transcellular movement of ammonia through the branchial epithelia has been extensively studied (Wright and Wood, 2009), while the paracellular passage remains unclear and has received less attention. In fact, both CLNs and OCLNs are relevant in response to salinity challenges (Bagherie-Lachidan et al., 2009; Tipsmark et al., 2008); although, it is not known if these elements of TJs are able to change their expression to modulate ammonia excretion rates (J_{Amm}). On the other hand, although it is known that cortisol modulates, at cellular and molecular level, the paracellular and the transcellular transport through branchial epithelia (McCormick and Bradshaw, 2006; Bui et al., 2010; Chasiotis et al., 2010), recent studies described the effects of PRL on CLNs and OCLNs expression but only during a salinity challenge (Breves et al., 2014). Hereupon, the aim of this chapter was to determine the hormonal effects of PRL and cortisol on J_{Amm} and also to determine if the expression of components of the transcellular (Rh glycoproteins and aquaporin 8) and/or paracellular (CLNs and OCLNs) transport respond to these hormones during ammonia excretion and in response to ammonia loading. Since PRL is a freshwater-adapting hormone (Manzon, 2002; Power, 2005), and considering the previous results obtained in our lab during ammonia exposure (see the previous chapter), our expectation is that PRL induces a higher or similar J_{Amm} than cortisol. In addition, with the intention to

better understand the role of PRL on ammonia elimination, the inhibitor (bromocriptine) and promoter (sulpiride) of PRL production and release (Deane et al., 2000; Freeman et al., 2000) were also analyzed. To answer these questions, the use of the vertebrate model zebrafish *Danio rerio* was advantageous mostly due to its small size, which allows the use of a small experimental space (Westerfield, 2000), but also because this species allows resort to techniques of molecular biology, such as real-time PCR (*q*PCR) since its genome is already sequenced and annotated (ensembl.org).

5.3 Material and Methods

5.3.1 Experimental animals and holding conditions

Adult zebrafish (*D. rerio*) (0.677 ± 0.017 g) were purchased in a local aquarium fish supplier in Oporto (Portugal) and maintained at CIIMAR (Interdisciplinary Center of Marine and Environmental Research) for at least two weeks to acclimate to the laboratory conditions. During this period, fish were kept in 100 L glass aquarium supplied with aerated and dechlorinated local tap water that was continuously filtered using an external biological filtration system (Eheim, Germany) and supplemented with ultraviolet sterilization (Vectron V2, Tropical Marine Centre, UK). In addition to filtration, 20 % of the water was changed daily to guarantee low concentration of toxic metabolites in the tank. Fish were fed five times per day with commercial flake food (Tetramin, Tetra, Germany) using an automatic feeder (Eheim) and supplemented with *Artemia* nauplii (Ocean Nutrition, Salt Lake City, UT, USA) four times per week. The room air and water temperature were maintained constant at approximately 28 ± 1 ºC and the photoperiod was 16 h Light : 8 h Dark.

All the experiments done for this study were carried out under the guidance of a Laboratory Animal Science certified supervisor (1005/1092, DGV-Portugal, C category, FELASA), according to European Union directives (2010/63/UE).

5.3.2 Experiment design

One week before the start of the experiments, fish for all sets of experiments were transferred to a smaller glass tank (25 L) supplemented with internal filtration system. In addition to filtration, 20 % of the water was changed daily. During this period, natural photoperiod was used and feeding was suspended 24 h prior to the start of the experiments, reducing the influence of feeding history on ammonia excretion.

Four experimental series were conducted with the same basic injection and ammonia flux measurement procedure. Briefly, each zebrafish (n = 8 per treatment) was anaesthetized with buffered tricaine methanesulfonate [MS-222 1:10 000 (w/v) (Pharmaq, UK)], weighed and injected intraperitoneally (IP) with pre-prepared solutions described in the following subsections (Series *i-iv*). After injection, each fish was allowed to recover in an individual experimental flux chamber (65 ml) provided with flowing freshwater delivered by a multichannel peristaltic pump (16 channel BVP, Ismatec, Germany) at a nominal flow rate of 9.5 ml·min^{-1}. Experimental flux chambers were 90 % submerged in a water bath to maintain the water temperature inside the chamber close to 28 ºC. The water bath/reservoir also had a submersible heater and mechanical filtration to maintain the water circulation and quality. Moreover, the water that circulated through the experimental chambers came from this large water bath/reservoir and the water exiting the chambers drained directly back into the reservoir. The experimental flux chambers were cylindrical containers that had sealable lids and were fitted with internal aeration making it possible for the chambers to operate as closed systems for flux measurements when the peristaltic pump was switched off. Moreover, the internal aeration also guaranteed good levels of oxygen inside the experimental chamber and an efficient mixing for the water sampling.

After injection, each fish was maintained in the experimental flux chamber for approximately 24 h to recover and after, the water flow was stopped and the flux experiments started. During all of the experiments the aeration inside the flux chambers was maintained. To measure the J_{Amm}, water samples (1 ml) were collected at 0, 30, 60, 90 and 120 min after the start of the flux experiments. A replacement volume of 1 ml of water was added to the chamber immediately after each sampling. Water samples were immediately acidified with concentrated HNO_3 (Merck, Darmstadt, Germany) and frozen at -20 ºC for later determination of total ammonia-nitrogen (TA-N) concentration.

In the experimental series, different hormones [ovine prolactin (oPRL) and cortisol (F)] or pharmacological inhibitors or stimulants of PRL synthesis (bromocriptine and sulpiride, respectively) and/or different doses of oPRL were IP injected. These four series are:

Series i: dose response to prolactin

Adult zebrafish (0.631 ± 0.025 g) were injected IP (5 µl·g^{-1} wet body mass) with 0.8 % NaCl (w/v) (Merck) (vehicle) or different doses of oPRL. The oPRL was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (National Hormone and

Peptide Program; USA) and the dosages used were 5, 50 and 100 $\mu g \cdot g^{-1}$ of wet body mass. The different dosages were obtained by serial dilutions. The use of mammalian hormones such as oPRL, in teleosts is well established (Manzon, 2002). Ammonia flux measurements were conducted as described above 24 h after injection. No mortality was observed during the experiment and after the ammonia fluxes no fish sampling was done.

Series ii: effects of prolactin and cortisol on ammonia fluxes

Zebrafish (0.647 ± 0.036 g) were injected IP (5 $\mu l \cdot g^{-1}$ wet body mass) with 0.8 % NaCl (w/v) (vehicle) or 100 $\mu g \cdot g^{-1}$ wet body mass of oPRL (dosage that presented the highest ammonia excretion rate during the series *i*) or placed in a bath of cortisol (10 $\mu g \cdot ml^{-1}$) (Sigma Aldrich, St. Louis, USA). The cortisol exposure was conducted in a separate water bath/reservoir; although, the cortisol treated fish were also vehicle injected. No mortality was observed during the experiment. After collection of the last water sample to determine the ammonia fluxes, fish were sampled (described below).

Series iii: effects of prolactin and cortisol plus ammonia load on ammonia fluxes

All the hormone treatment procedure in series *ii* was repeated in this third series. However, 24 h after the first injection, zebrafish (0.689 ± 0.033 g) were anaesthetized (as described above) and injected IP (10 $\mu l \cdot g^{-1}$ wet body mass) with 0.8 % NaCl (w/v) (vehicle) or NH_4HCO_3 (0.5 M; 5 $\mu mol \cdot g^{-1}$ wet body mass) (Merck). After a 30 min recovery in the experimental flux chamber, the water flow was stopped and water samples were collected for ammonia flux determination (as described above). Four different treatments were made in this experimental series: 1) vehicle; 2) vehicle + NH_4HCO_3; 3) oPRL + NH_4HCO_3 and 4) cortisol bath + NH_4HCO_3. No mortality was observed during the experiment and all fish were sampled at the end of the experiment (described below).

Series vi: effects of prolactin, bromocriptine and sulpiride on ammonia fluxes

Zebrafish (0.723 ± 0.034 g) received an IP injection (5 $\mu l \cdot g^{-1}$ wet body mass) of either vehicle (saline or acidic solution) or vehicle plus one of the next treatments: 100 $\mu g \cdot g^{-1}$ wet body mass of oPRL, 1 $\mu g \cdot g^{-1}$ of bromo-α-ergocriptine (Sigma Aldrich) or 5 $\mu g \cdot g^{-1}$ of (-)-sulpiride (Sigma Aldrich). The oPRL and bromocriptine were suspended in 0.8 % NaCl (w/v) (vehicle), and since sulpiride is more soluble in acidic solutions, this stimulant of PRL synthesis was suspended in a solution containing 0.1 N HCl (Merck) and neutralized with 0.1 M $NaHCO_3$ (Merck) (acidic vehicle). No mortality was observed during the experiment and fish were not sampled after the experiment.

5.3.3 Sampling procedures

At the end of the experiments in series *ii* and *iii*, fish were over dosed with buffered MS-222 [1:5 000 (w/v); Pharmaq] and were blotted dry on paper towels, weighted and the standard length was measured. After cervical transection, blood samples were quickly taken by cardiac puncture with heparinized (Lithium heparin, 100 000 U; Sigma Aldrich) 10 µl capillary tubes. The blood was not centrifuged and was stored at -20 ºC until use. After blood sampling, gill arches were excised under a dissecting microscope, immediately frozen in liquid nitrogen and stored at -80 ºC for total RNA extraction. The remaining carcass was saved in 1.5 ml tube, frozen in liquid nitrogen and then stored at -80 ºC for later analysis.

5.3.4 Ammonia excretion determination

Water samples (1 ml) were taken from the eight replicates of each treatment and control groups and immediately acidified to convert the gaseous ammonia (NH_3) to ionized ammonia (NH_4^+). The TA-N concentration in the water samples was then determined using the colorimetric salicylate-hypochlorite assay from Verdouw and colleagues (1978) modified for microplates. In the 96 well microplates, all samples and ammonia standards were run in triplicate on BioTek PowerWave 340 microplate reader (BioTek, Vermont, USA) and the absorbance was obtained using KC Jr software (BioTek). A non-linear regression (ammonia concentration of the standards versus absorbance at 650 nm) was performed and the TA-N concentration (mM) of each sample was calculated and corrected for volume changes during the flux period. The TA-N concentrations in $\mu mol \cdot g^{-1}$ were calculated multiplying the TA-N concentration (mM) by the volume of the experimental flux chamber (65 ml) and dividing by the mass of the fish (g). Whole-animal J_{Amm} are expressed as µmol of TA-N $\cdot$ g^{-1} of body mass $\cdot$ h^{-1} calculated over each 30 min flux interval.

5.3.5 Blood cortisol levels

Blood samples from the experiments of series *ii* and *iii* were collected as explained above. Blood cortisol levels were measured using an indirect enzymatic immunoassay (ELISA) (Neogen, Lansing, USA) following the manufacturer's recommendations. Blood samples were prepared by dilution in 100 µl of distilled water and then subsamples of 50 µl were transferred to clean 1.5 ml tubes. After adding 500 µl of ethyl ether to the 1.5 ml tube and vortexing for 30 s, the organic phase was transferred to a new 1.5 ml tube that was then

dried. The samples were after resuspended in 100 µl of extraction buffer. Each sample was measured in duplicate using a BioTek PowerWave 340 microplate reader (BioTek) and the absorbance (650 nm) was obtained using KC Jr software (BioTek). The concentration of cortisol in the blood was expressed in $ng \cdot ml^{-1}$ taking into account the sample dilutions.

5.3.6 Carcass ammonia concentration

To measure whole body ammonia levels, we used an enzymatic microplate technique modified from Bergmeyer and Beutler (1985) and described by Gonçalves and collaborators (2012). Briefly, the carcass samples were weighted and a ratio of 1:5 (w/v) of 8 % Perchloric Acid (PCA) (Merck) was added to the tissue sample. After 10 min in PCA, samples were homogenized with a bead homogenizer for 30 s at 6800 rpm (Precellys 24 homogenizer, Bertin, Montigny-le-Bretonneus, France). The homogenate was then centrifuged at 10,000 *g* for 15 min at 4 ºC (3K30, Sartorius, Göttingen, Germany) and the deproteinized samples were neutralized by adding a predetermined quantity of saturated Tris(hydroxymethyl)aminomethane (TRIS) (Sigma Aldrich) solution, vortexed and centrifuged again at 10,000 *g* for 5 min at 4 ºC (3K30, Sartorius). The neutralized product was then saved at -20 ºC until later analysis of ammonia content. In the 96 well microplates, all samples and ammonia standards were run in triplicate. All measurements were done using BioTek PowerWave 340 microplate reader (BioTek) at an absorbance of 340 nm. A linear regression (ammonia concentration of the standards versus the absorbance at 340 nm of the samples) was performed and the ammonia concentration of each sample was calculated. These values were corrected for the various dilutions and expressed as $\mu mol \cdot g^{-1}$ wet mass.

5.3.7 RNA extraction and cDNA synthesis

For RNA extraction, gills were collected and flash frozen in RNase-free 1.5 ml tubes. Initially, the gill samples were homogenized in 700 µl of lysis solution with two ceramic beads for 30 s at 6800 rpm using the Precellys 24 homogenizer (Bertin). Total RNA from individual gills was then extracted using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA), following the manufacturer's protocol including on-column of DNase I treatment to minimize genomic DNA contamination. After extraction, the final concentration and the quality of the total RNA were assessed using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) at 260 nm and 280 nm and by 1.2 %

formaldehyde-agarose gel electrophoresis, respectively. Total RNA was stored at -80 ºC for later use.

The cDNA was synthesized from 1 µg of total RNA (20 µl final volume) using the iScript cDNA Synthesis Kit (Bio-Rad), following the manufacturer's recommendations. Reverse transcription was conducted using iScript reverse transcriptase and the following program in MJ Mini Personal Thermocycler (Bio-Rad) was applied: 25 ºC for 5 min; 42 ºC for 30 min and 85 ºC for 5 min.

Polymerase chain reaction (PCR) amplifications were performed using DyNAzyme II DNA polymerase (Finnzymes; Thermo Fisher Scientific) according to the manufacturer's instruction. The PCR was done with elongation factor 1 alpha (EF1α, *eef1al1*) primers (see Table 5.1) to verify the cDNA quality for each sample. The PCR reactions had a final volume of 20 µl and contained: 10.9 µl H_2O, 1x buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 250 nM of each primer, 0.5 U Taq and 0.8 µl cDNA. The PCR amplification was performed in MJ Mini Personal Thermocycler (Bio-Rad) and the program was 1 cycle of denaturation at 94 ºC for 2 min, 30 cycles of denaturation at 94 ºC for 30 s, annealing at 60 ºC for 30 s and extension at 72 ºC for 30 s, followed by 1 cycle of 5 min at 72 ºC for the final extension. Amplified PCR products were processed by electrophoresis using 2 % agarose gel in TBE (Tris-borate-EDTA) buffer. Gels were stained with GelRed (Biotium, Fremont, CA) and the images were acquired with LAS-4000 Mini Luminescent Image Analyser (FujiFilm, Tokyo, Japan).

*5.3.8 Quantitative real-time PCR (q*PCR*)*

The mRNA expression of target genes was measured by *q*PCR. An extensive validation of the *q*PCR methods was previously performed to guarantee that samples were all present in the exponential phase of the amplification reaction. Standard curves for each specific primer pairs (Table 5.1) were constructed to ensure the linearity of the amplification, to determine the *q*PCR efficiency and the dilution of the cDNA. This standard curve consisted of a serial dilution from a pooled sample done with 1 µl of different gill cDNA samples. The *q*PCR efficiency was considered satisfactory when between 90 % and 105 % and with $R^2 > 0.980$. From the standard curve analysis, the cDNA products were diluted 1:100, 1:500 and 1:1000 for reference and target genes in sterile water. The *q*PCR products of the standard curve were subjected to a melting curve analysis and, after they were electrophoresed in 2 % agarose gels to verify that only single products were present.

Table 5.1 Gene-specific primer pair sequences, predicted product size, annealing temperature (ºC), dilution of the cDNA, and reference source, for the different primers used in the present study. Asterisk (*) was used to identify the primer pair that was designed with Primer 3.

Gene	Primer sequences	size	ºC	cDNA dilution	Reference
EF1α (*eef1al1*)	F-TGGGTGTTGGACAAACTGAA	190	60	1:500	Gonçalves et al., 2012
	R-CAACACCACCAGCAACAATC				
Rhbg (*rhbg*)	F-TCAATGGAGCGCTCAAACAAAGGGT	233	59	1:1000	Nakada et al., 2007b
	R-TGAGTTTCTCCACCTCGTTTTGCGT				
Rhcga (*rhcga*)	F-GGGAGGTACCTGAAGATGAAGAGAG	237	58	1:100	Nakada et al., 2007b
	R-TGAGCGCAGACTTGGTCTGAACATT				
Rhcgb (*rhcgb*)	F-CTGTTTGGGATCACGCTGTA	127	58	1:100	Braun et al., 2009a
	R-CCCTTGAGATGGAGAGACCA				
occludin a (*oclna*)	F-ACCATTACTGCGTGGTGGA	199	58	1:100	Clelland and Kelly, 2010
	R-ACCCAGTCCTCCACATCTTG				
occludin b (*oclnb*)	F-CAAAATCAGGCAAAGGCTTC	176	58	1:100	Clelland and Kelly, 2010
	R-AACAATAGTGGCGATGAGCA				
claudin a (*cldna*)	F- TGCTTGATTCCTGTTTGCTG	231	59	1:100	Clelland and Kelly, 2010
	R- TCCTCAGACATACCCCTTGG				
claudin b (*cldnb*)	F-ACCAACCAACAAGGAAAACG	214	59	1:100	Clelland and Kelly, 2010
	R-GCATCTGTCCAGTGCTTTGA				
claudin c (*cldnc*)	F- GTACCCTCCGCAAAGTCGTA	130	59	1:100	Kumai et al., 2011
	R- CTTTCAAGGAAAGACTGACAGC				
claudin d (*cldnd*)	F- CTCCATGCTGGCTCTACCTC	154	59	1:100	Clelland and Kelly, 2010
	R- GACCACGACTTTAGCCTTCG				
claudin e (*cldne*)	F- ATTGCTGGAGTTCTGGTGC	219	59	1:100	Clelland and Kelly, 2010
	R- CGGCTGGGAGTATTTCATG				
claudin f (*cldnf*)	F- TGCTTCTGATTCCTGTGTGC	200	59	1:100	Clelland and Kelly, 2010
	R- GCCTGGGTACTGTGGGTAGA				
claudin h (*cldnh*)	F- TAGCATTGTGGCTTGTGCTC	197	59	1:100	Clelland and Kelly, 2010
	R- TGACAGTCATGGCTCTGGAC				
claudin i (*cldni*)	F- GTGGATGCAGTGTGTGGTTC	195	59	1:100	Clelland and Kelly, 2010
	R- GGCTTGACGTCTTCGTTTTC				
claudin 2 (*cldn2*)	F-CCACAAACCCCAACATTACC	211	58	1:100	Clelland and Kelly, 2010
	R-GAGCCTTTCAGCAATCCAAG				
aquaporin 8a (*aqp8a*)	F-GCTGCCACTATGGCTGAGATGA	349	58	1:100	Tingaud-Sequeira et al., 2010
	R-AAAAGCTTGCAATCCTCTTCA				
11βHSD2 (*hsd11b2*)	F- TGCTGCTGGCTGTACTTCAC	123	59	1:1000	Alsop and Vijayan, 2008
	R- TGCATCCAACTTCTTTGCTG				
MR (*nr3c2*)	F- CCCATTGAGGACCAAATCAC	106	59	1:1000	Alsop and Vijayan, 2008
	R- AGTAGAGCATTTGGGCGTTG				
GR (*nr3c1*)	F- ACAGCTTCTTCCAGCCTCAG	116	59	1:1000	Alsop and Vijayan, 2008
	R- CCGGTGTTCTCCTGTTTGAT				
prolactin receptor a (*prlra*)	F- GGGACAAACCACGGACCGCA	179	58	1:100	*
	R- TGGTTTACACCGCACCTGCACC				

The primers used for *q*PCR were chosen from published papers or were designed using Primer 3 (Rozen and Skaletsky, 2000; see Table 5.1). Gene sequences to design the primers were obtained from Ensembl (www.ensemble.org) and/or GenBank (www.ncbi.nlm.nih.gov/). The *q*PCR amplifications were performed with iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) and each reaction had a final volume of 20 µl containing 1x SYBR Green Super Mix (Bio-Rad), 200 nM of each primer pair and 4 µl of diluted cDNA; however, an exception was the optimized reactions for *hsd11b2* that contained instead, 320 nM of each primer pair. The cycling profile was: 95 ºC for 4 min, 40 cycles of 95 ºC for 10 s, annealing temperature optimized for each primer pair (see Table 5.1) for 30 s and 72 ºC for 30 s. To ensure that the primers amplified a specific product, a melting curve was performed at the end of each *q*PCR run. Control reactions were conducted with sterile water to determine the levels of background and the degree of contamination. Each sample and control was run in duplicate. The housekeeping gene used was the zebrafish EF1α (*eef1al1*). The effects of the experimental treatments on the expression of EF1α were tested and no changes were observed.

After each *q*PCR run, the data were analyzed with the software of the system (Bio-Rad) and expressed as a fold change (FC). All analyses were based on the Ct values of the *q*PCR products using the Livak Method (Livak and Schmittgen, 2001) and data were normalized using the EF1α expression.

5.3.9 Data presentation and statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). All statistical analyses and graphs were performed using Sigma Stat (version 11, SPSS Inc., Chicago, IL, USA). Ammonia fluxes were assessed for statistical significance using a Two-Way ANOVA and blood cortisol, whole-body ammonia and *q*PCR results were analyzed by One-Way ANOVA. The Post-hoc Fisher LSD Method test was run when significances were detected by ANOVA. In all cases, significance was set at *p*-value (P) ≤ 0.05.

5.4 Results

5.4.1 Effects of prolactin and cortisol on ammonia excretion

The excretion of ammonia in control and hormone treated groups were measured in water samples collected over 120 min in intervals of 30 min. Four different sets of experiments were done with different hormones and/or pharmacological agents, in addition to

a set with an extra load of ammonia. There was no tank effect in the different experiments, as were assessed with replicate tanks.

In series *i*, the effects of three different doses of ovine prolactin (oPRL; 5, 50 and 100 $\mu g \cdot g^{-1}$) on ammonia excretion were assessed, with the results shown in Figure 5.1. The three doses of oPRL induced an increase in J_{Amm} when compared with the vehicle alone. Moreover, fishes treated with 100 $\mu g \cdot g^{-1}$ of oPRL had the highest level of ammonia excretion; however, no differences were found over time within each treatment group.

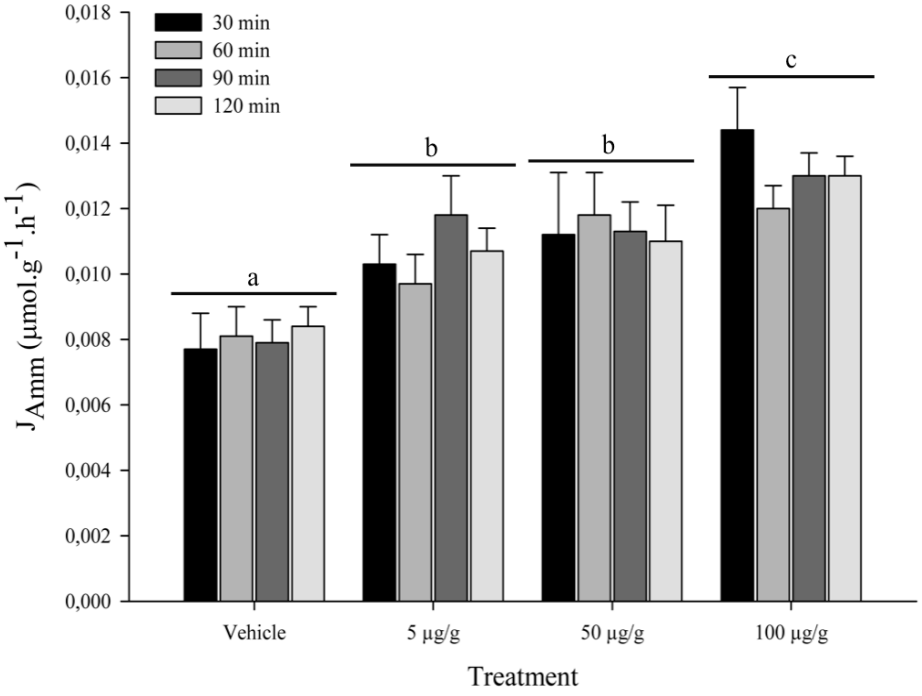


Figure 5.1 Effects of different doses of ovine prolactin (oPRL; 5, 50 and 100 $\mu g \cdot g^{-1}$), injected intraperitoneally (IP) on ammonia excretion rate (J_{Amm}; $\mu mol \cdot g^{-1} \cdot h^{-1}$). In the vehicle group, fish were injected with 0.8 % NaCl (w/v). Ammonia flux measurements were made over 120 min in intervals of 30 min. Different letters indicates a significant difference (Two-way ANOVA; Post-hoc Fisher LSD Method; $P \leq 0.05$).

In series *ii*, fish were injected with vehicle or vehicle plus 100 $\mu g \cdot g^{-1}$of oPRL or were placed in a bath of cortisol (10 $\mu g \cdot ml^{-1}$). The results showed higher J_{Amm} in fish injected with oPRL while lower J_{Amm} were present in cortisol treated fish (Figure 5.2). As before, no differences were present over time within each group.

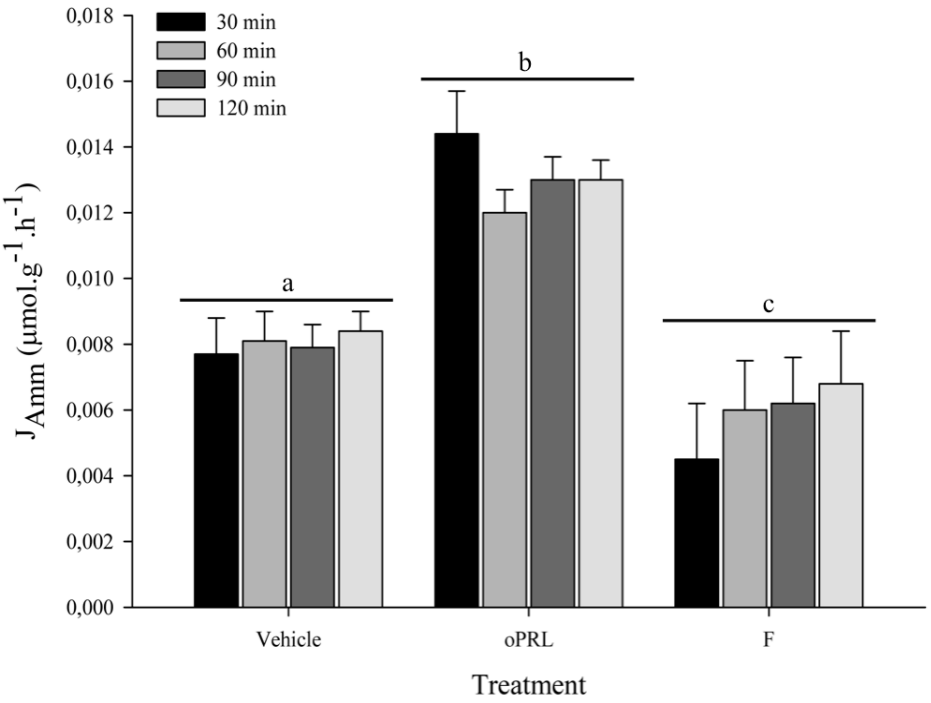


Figure 5.2 Total ammonia excretion (J_{Amm}; µmol·g^{-1}·h^{-1}) in zebrafish injected intraperitoneally (IP) with 0.8 % NaCl (w/v) (vehicle) or vehicle plus ovine prolactin (oPRL; 100 µg·g^{-1}) or placed in cortisol bath (F; 10 µg·ml^{-1}). Ammonia flux measurements were made over 120 min in intervals of 30 min. Different letters indicates a significant difference (Two-way ANOVA; Post-hoc Fisher LSD Method; $P \leq 0.05$).

After obtaining the results on the effects of PRL and cortisol on J_{Amm} in series *ii*, series *iii* was performed to examine the clearance of ammonia loaded 24 h later of the oPRL injection and cortisol bath treatment. The results showed (Figure 5.3) that fish injected with ammonia, presented an increase of J_{Amm}. Moreover, ammonia loaded fish firstly injected with vehicle alone or vehicle plus oPRL, had higher J_{Amm} than fish treated with cortisol. On the other hand, during the time series, the results showed that within each group, the first 30 min had the highest J_{Amm} and, over time, occurred a decrease in the ammonia excretion.

The effects of PRL release inhibitor (bromocriptine) and promoter (sulpiride) on ammonia excretion (series *iv*) are shown in Figure 5.4. Fish injected with sulpiride presented the highest J_{Amm}, in contrast with fish injected with vehicle that had the lowest J_{Amm}. Fish injected with acidic vehicle, oPRL and bromocriptine had no significant differences between them; however, they had higher J_{Amm} than fishes injected only with vehicle but lower J_{Amm} than fish loaded with sulpiride. In relation to their respective vehicle controls, all treatments increased J_{Amm}. Consistent with series *i* and *ii*, no differences were noted over time within each treatment.

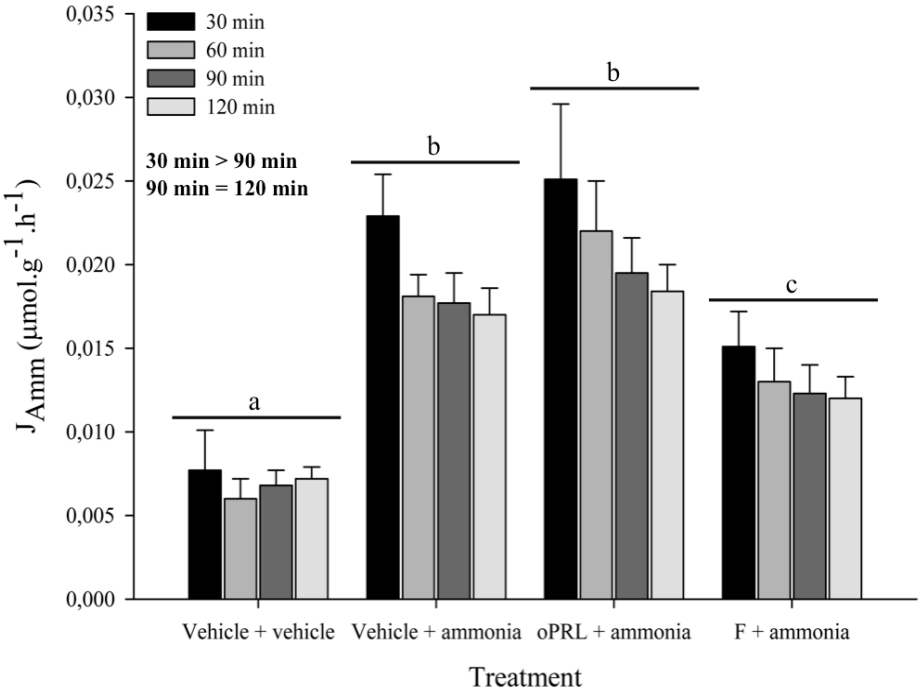


Figure 5.3 Excretion of ammonia in response to an intraperitoneally (IP) injection of 0.8 % NaCl (w/v) (vehicle) or vehicle plus ovine prolactin (oPRL; 100 $\mu g \cdot g^{-1}$) or cortisol bath (F; 10 $\mu g \cdot ml^{-1}$), followed of an IP injection of vehicle or vehicle plus NH_4HCO_3 (0.5 M), 24 h after the first hormonal treatment. Ammonia flux measurements (J_{Amm}; $\mu mol \cdot g^{-1} \cdot h^{-1}$) were made over 120 min in intervals of 30 min. Different letters indicates a significant difference (Two-way ANOVA; Post-hoc Fisher LSD Method; $P \leq 0.05$).

5.4.2 Levels of ammonia in whole-body and cortisol in blood

At the end of the series *ii* and *iii*, the cortisol levels in the blood and ammonia concentration in whole-body were measured and the results are present in Table 5.2 and 5.3. There were no significant differences in either cortisol in the blood or ammonia in the body.

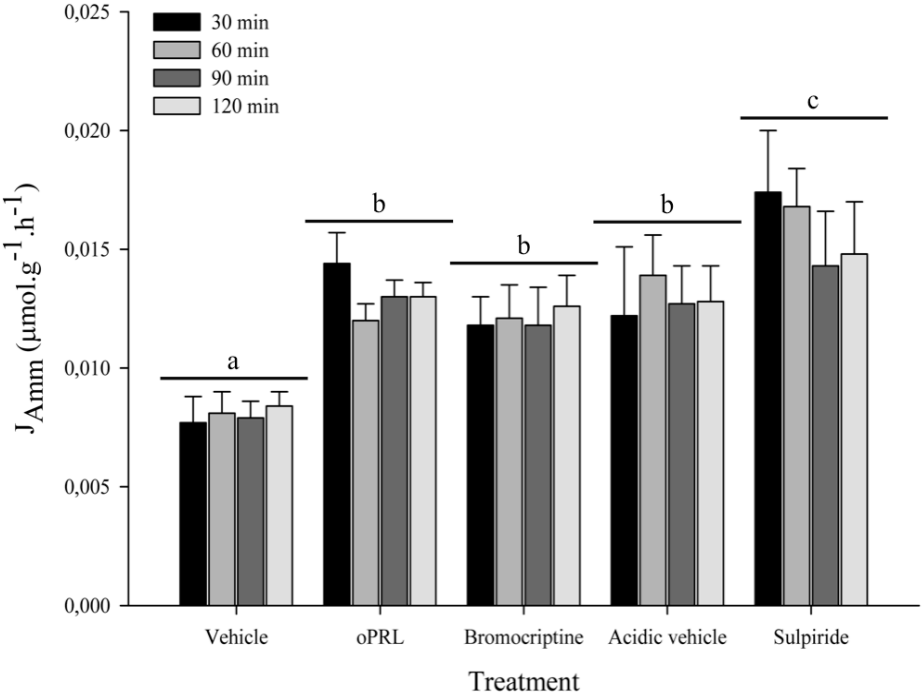


Figure 5.4 Response of zebrafish to an intraperitoneally (IP) injection of 0.8 % NaCl (w/v) (vehicle) or vehicle plus ovine prolactin (oPRL; 100 μg·g^{-1}) or plus bromocriptine (1 μg·g^{-1}). Considering the higher solubility of sulpiride in acidic solutions, and acidic vehicle (containing 0.1 N HCl and neutralized with 0.1 M $NaHCO_3$) group and acidic vehicle plus sulpiride (5 μg·g^{-1}) were also performed. Ammonia flux measurements (J_{Amm}; μmol·g^{-1}·h^{-1}) were made over 120 min in intervals of 30 min. Different letters indicate a significant difference (Two-way ANOVA; Post-hoc Fisher LSD Method; $P \leq 0.05$).

Table 5.2 Whole-body ammonia content (μmol·g^{-1}) and cortisol concentration in blood (ng·ml^{-1}) in zebrafish injected intraperitoneally (IP) with 0.8 % NaCl (w/v) (vehicle) or vehicle plus ovine prolactin (oPRL; 100 μg·g^{-1}) or placed in a bath of cortisol (F; 10 μg·ml^{-1}). Values are present as mean ± standard error of the mean (SEM) (n = 8). Data were analyzed by One-Way ANOVA but no statistical differences were achieved.

Treatment	Ammonia in body μmol·g^{-1}	Cortisol in blood ng·ml^{-1}
Vehicle	4.824 ± 0.454	105.911 ± 26.151
oPRL	5.620 ± 0.071	177.221 ± 48.531
F	5.558 ± 0.165	121.553 ± 26.174

Table 5.3 Concentration of ammonia in body (μmol·g^{-1}) and cortisol in blood (ng·ml^{-1}) in zebrafish injected intraperitoneally (IP) with 0.8 % NaCl (w/v) (vehicle) or vehicle plus ovine prolactin (oPRL; 100 μg·g^{-1}) or placed in a bath of cortisol (F; 10 μg·ml^{-1}) followed of an extra IP injection of vehicle or vehicle plus NH_4HCO_3 (0.5 M), 24 h after of the first injection. Values are present as mean ± standard error of the mean (SEM) (n = 8). Data were analyzed by One-Way ANOVA but no statistical differences were achieved.

Treatment	Ammonia in body μmol·g^{-1}	Cortisol in blood ng·ml^{-1}
Vehicle + vehicle	3.305 ± 0.564	56.391 ± 16.452
Vehicle + NH_4HCO_3	4.806 ± 0.400	54.775 ± 20.462
oPRL + NH_4HCO_3	4.493 ± 0.439	35.663 ± 10.446
F + NH_4HCO_3	4.132 ± 0.487	142.850 ± 90.313

5.4.3 Effects of hormone treatment in the mRNA expression of genes

The expression levels of Rh glycoproteins (*rhbg*, *rhcga* and *rhcgb*), claudins (*cldna*, *cldnb*, *cldnc*, *cldnd*, *cldne*, *cldnf*, *cldnh*, *cldni* and *cldn2*), occludins (*oclna* and *oclnb*), prolactin receptor a (*prlra*), aquaporin 8a (*aqp8a*), glucocorticoid receptor (*nr3c1*), mineralocorticoid receptor (*nr3c2*) and 11-β hydroxysteroid dehydrogenase 2 (*hsd11b2*) were analyzed in the gill of fish from series *ii* and *iii*. In series *ii*, only *hsd11b2*, *rhcgb*, *oclna*, *oclnb* and *prlra* changed their mRNA levels (Figure 5.5). Within these genes, *oclnb* and *prlra* increased in oPRL injected fish, while *hsd11b2*, *rhcgb* and *oclna* mRNA levels increased in cortisol treated fish. None of the other genes were statistically different in series *ii* (see the Supplementary Figures 5.7 and 5.8).

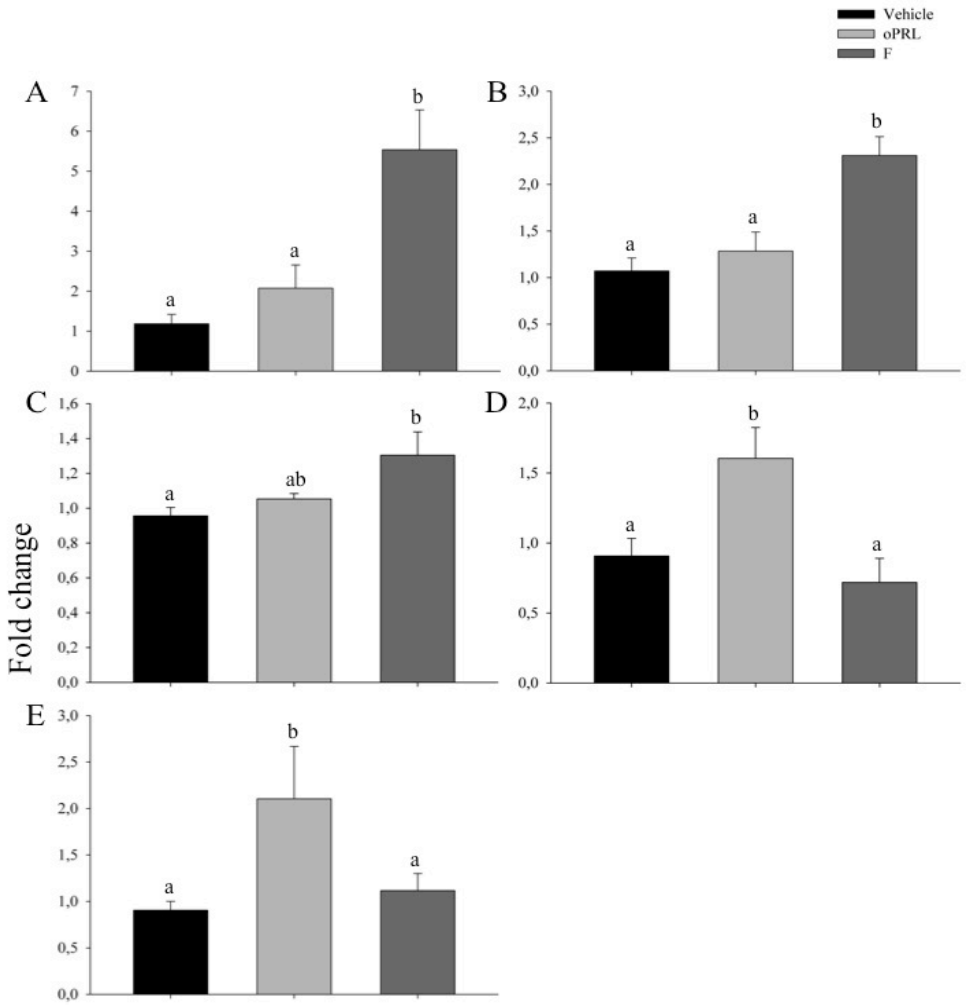


Figure 5.5 The mRNA expression levels of 11-β hydroxysteroid dehydrogenase 2 (*hsd11b2*) (**A**), Rhesus glycoprotein Cb (*rhcgb*) (**B**), occludin a (*oclna*) (**C**), occludin b (*oclnb*) (**D**) and prolactin receptor a (*prlrα*) (**E**) in the gill of zebrafish intraperitoneally (IP) injected with 0.8 % NaCl (w/v) (vehicle) or vehicle plus ovine prolactin (oPRL; 100 μg·g^{-1}) or placed in a cortisol bath (F; 10 μg·ml^{-1}). Results are expressed as normalized fold change (FC) with respect to the elongation factor 1 alpha (*eef1al1*). Data are present as mean ± standard error of the mean (SEM). Different letters indicate a significant difference (One-way ANOVA; Post-hoc Fisher LSD Method; $P \leq 0.05$).

On the other hand, in series *iii*, only *hsd11b2*, *rhcga*, *rhcgb* and *oclna*, were statistically different (Figure 5.6) with the higher expression observed in fish that were first treated with cortisol and that received an extra load of ammonia. Neither ammonia loading nor oPRL treatment significantly affected gene expression. Relative to the other genes, no statistical differences were noted in this series (see the Supplementary Figures 5.9 and 5.10).

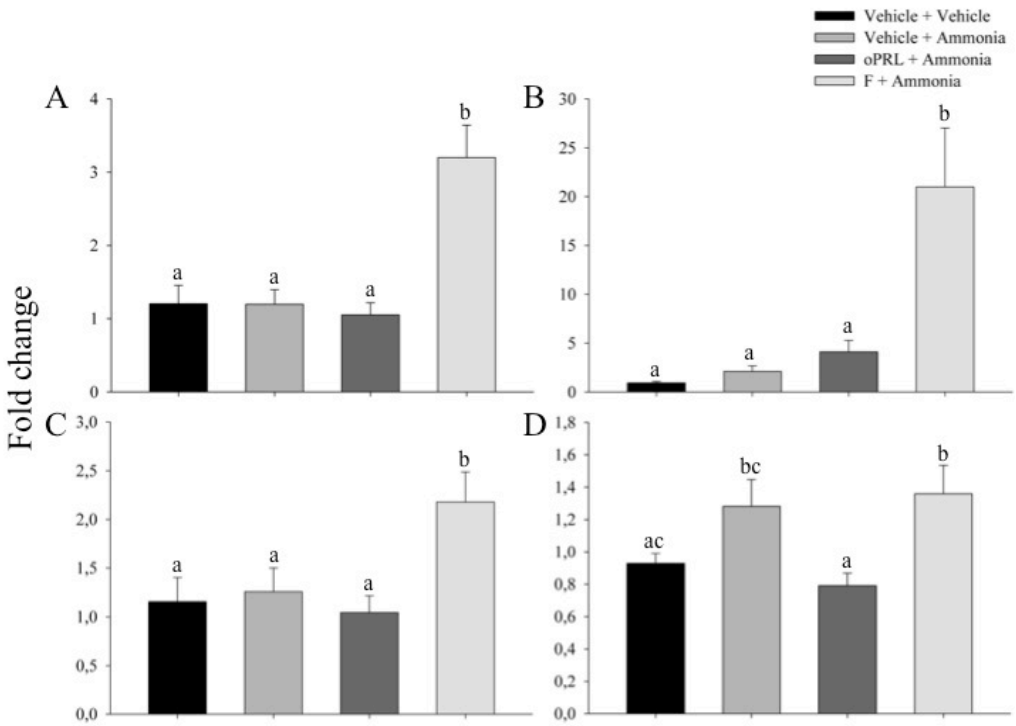


Figure 5.6 Expression of statistically different genes [11-β hydroxysteroid dehydrogenase 2 (*hsd11b2*) (**A**), Rhesus glycoprotein Ca (*rhcga*) (**B**), Rhesus glycoprotein Cb (*rhcgb*) (**C**) and occludin a (*oclna*) (**D**)] in the gill of zebrafish injected intraperitoneally (IP) with 0.8 % NaCl (w/v) (vehicle) or vehicle plus ovine prolactin (oPRL; 100 $\mu g \cdot g^{-1}$) or immersed on a cortisol bath (F; 10 $\mu g \cdot ml^{-1}$) and followed by an IP injection of vehicle or vehicle plus NH_4HCO_3 (0.5 M), 24 h after the first hormonal treatment. Results are expressed as normalized fold change (FC) with respect to the elongation factor 1 alpha (*eef1al1*). Data are present as mean ± standard error of the mean (SEM). Different letters indicate a significant difference (One-way ANOVA; Post-hoc Fisher LSD Method; $P \leq 0.05$).

5.5 Discussion

Cortisol is a stress hormone that is positively correlated with an increase of ammonia inside the fish but also with elevated J_{Amm} (Hopkins et al., 1995; Mommsen et al., 1999); however, in the present study, fish submerged in a cortisol bath had the lowest J_{Amm}. During a stress situation, teleost fishes produce and release cortisol to the blood, which will directly or indirectly cause several genetic, morphological and physiological changes in many tissues (Bonga, 1997; Vijayan et al., 1997; Mommsen et al., 1999; Gonçalves et al., 2012). One of

the mechanisms activated by cortisol is the mobilization of glycogen that will be catabolized to glucose that, in turn, will be used to produce energy (ATP) to maintain the homeostasis of the organism (Bonga, 1997; Vijayan et al., 1997; Mommsen et al., 1999). However, during this process, ammonia is also produced and released (Mommsen et al., 1999; Randall and Tsui, 2002), being accumulated inside the fish till values that may be toxic and even cause death (Randall and Tsui, 2002). In this sense, to avoid the autointoxication of ammonia, fish increase it elimination to the environment (Hopkins et al., 1995). After induction (Hopkins et al., 1995) or injection (de Boeck et al., 2001) of cortisol, high levels of this hormone appear in the blood in a few minutes and it may remain at this elevated concentrations during hours (Auperin et al., 1997) and even days (Gonçalves et al., 2012); however, after this peak, the levels of cortisol decrease till values close or even lower to the basal line. In fact, in the literature, an increase of plasma cortisol levels corresponded to higher J_{Amm} (Hopkins et al., 1995) and when the cortisol returns no basal levels, the excretion of ammonia also decreased substantially. This may explain the results obtained in the present study (Figure 5.2) since the ammonia fluxes were measured 24 h after the cortisol challenge. Moreover, although the predictions were an increase of plasma cortisol due to the continuous exposure in a cortisol bath, the levels of this hormone in the blood were not statistically different between the treatments of the series *ii* (Table 5.2). Similarly, Balm and collaborators (1994) found that Mozambique tilapia *Oreochromis mossambicus* had the ability to decrease the plasma cortisol levels during the continuous presence of a stressor, which was explained as a mechanism to avoid the deleterious effects caused by the prolonged elevated cortisol levels.

The lowest J_{Amm} during cortisol treatment occurs due to alterations of the mRNA expression of genes involved in both transcellular and paracellular transport of ammonia (Figure 5.5). In general, cortisol increases the ion-transporting capacity of the gills through the proliferation of chloride cells and through the increase of the mRNA and/or activity of the ion-transporting enzymes (Lin and Randall, 1993; Shrimpton and McCormick, 1999). Among all the ions whose transport is affected by cortisol, ammonia movement through the gills may also be enhanced, which occurs mainly through an increase of its transporter proteins elements that belongs to the Rh glycoprotein family (Tsui et al., 2009). In this sense, and although the J_{Amm} were lower at the end of the cortisol treatment (Figure 5.2), the mRNA expression of *rhcgb* was still upregulated (Figure 5.5), which may indicate that ammonia was excreted through Rh glycoprotein elements during the 24 h of cortisol exposure. Moreover, and to better understand the relationship between plasma cortisol concentrations, the mRNA

of Rh glycoproteins elements and J_{Amm}, a 24 h time-response of all these parameters should be analyzed in a future study. However, and in addition to these effects on the transcellular transport of ammonia, cortisol also has a "tightening" effect of the branchial membrane since it increases the OCLN expression (Figure 5.5), which in turn may lead to a reduction of the paracellular permeability and increase of the transepithelial resistance (TER) (Chasiotis et al., 2010). In fact, in the present study, the branchial mRNA expression of *oclna* increased in zebrafish exposed to cortisol, which indicates that, zebrafish decreased the paracellular permeability of the gill to probably impede the passive reuptake of ammonia. Claudins are other proteins present in the TJs that may regulate the branchial permeability and respond to cortisol (Bui et al., 2010). In fact, in a cultured gill epithelia study with spotted green pufferfish *Tetraodon nigroviridis*, some isoforms of CLNs were unresponsive, while others increased and others decreased its expression in response to cortisol treatment (Bui et al., 2010). Moreover, Bui and collaborators (2010) determined that the response of some isoforms of CLNs to cortisol treatment were dosage-dependent. Considering all this, the unresponsiveness of all the isoforms analyzed in the present study (Supplementary Figures 5.7 and 5.8) may be explained by the dosage and/or type of administration of the cortisol.

As mentioned before, the effects of cortisol are mediated by its binding to both GR and/or MR (Bonga, 1997; Cruz et al., 2013); however, to avoid the overactivation and the effects triggered by these receptors (Pippal and Fuller, 2008), the enzyme 11βHSD2 might be enhanced protecting the organism. In fact, although cortisol binds to the specific receptor GR, it is also possible to activate MR, regulating several and vital functions inside the organism (Bonga, 1997). However, their overactivation has to be avoided which is guaranteed by the enzyme 11βHSD2 that convert cortisol in its inactive forms. In this sense, the upregulation of *hsd11b2* (Figure 5.5) and the lack of mRNA changes in both receptors (*nr3c1* and *nr3c2*) (Supplementary Figure 5.7), in the present study, may indicate an avoidance of the overactivation of GR and/or MR.

Contrarily to what was initial predicted, the injection of PRL did not induce a similar or even lower J_{Amm} than fish subjected to cortisol treatment, which may be explained by the direct or indirect effect of PRL in fish metabolism. Prolactin is known as a freshwater-adapting hormone (Bole-Feysot et al., 1998; Tang et al., 2001) playing an essential role for the regulation of salt and water homeostasis in freshwater fish (Bole-Feysot et al., 1998); however, this hormone either does not change or decreases after seawater challenge (Avella et al., 1990; Tang et al., 2001). Despite all the attempts to understand the effects of this hormone

on branchial physiology, no studies have been done to determine if PRL has an important role during ammonia loading and/or during ammonia excretion. Surprisingly, in the previous study (chapter 4) the upregulation of PRLR after 96 h of ammonia exposure, suggests that this hormone has some role during ammonia toxicity; however, it was not clear if PRL affects the active excretion of ammonia and/or alter the branchial permeability impeding the ammonia entrance in the organism. With this in mind, this study was done to determine if J_{Amm} were impacted, in a dose-dependent manner, 24 h after PRL injection (Figure 5.1). Moreover, since the levels of ammonia in zebrafish carcass were not statistically different between the different hormonal groups (Table 5.2), this indicates that, besides the different excretion rates, ammonia were also not accumulated inside the body. These results may be explained taking into consideration the effects of PRL on the metabolism of the teleosts. In the Pacific bluefin tuna *Thunnus orientalis*, high levels of PRL mRNA expression were found at 6 am, when fish are the most active, according to the authors (i.e., higher metabolism) (Adachi et al., 2009). Moreover, the fact that a 3 h of confinement stress was necessary to obtain a significant increase in plasma PRL concentration in the FW-adapted coho salmon *Oncorhynchus kisutch* (Avella et al., 1991) indicates that this hormone does not respond rapidly to stress, in agreement with some studies describing an inverse correlation between plasma PRL and cortisol concentrations (Avella et al., 1990); however, this response of PRL might be species specific since other responses were described (Auperin et al., 1997). Furthermore, studies in Mozambique tilapia *O. mossambicus* (Borski et al., 1991) and in coho salmon *O. kisutch* (Kelley et al., 1990) showed that cortisol has an inhibitory effect on PRL release and synthesis. However, other authors showed that during a stress occurs an elevation of plasma cortisol levels simultaneously with enhanced plasma PRL levels. Taken together, PRL and cortisol effects might be dependent on the species, the type and degree of the environmental stress (Auperin et al., 1997).

Since fish injected with PRL maintain higher levels of ammonia excretion even 24 h after the administration, the effects of a stimulant and inhibitor of PRL were also studied and the results were consistent with the previous findings (Figure 5.4). The production and release of PRL may be induced by sulpiride but also inhibited by bromocriptine (Deane et al., 2000; Freeman et al., 2000). The effects of these compounds on J_{Amm} were also analyzed in an attempt to clarify the role of PRL on ammonia fluxes. The fish injected with acidic vehicle plus sulpiride presented the highest J_{Amm}, which reinforces the idea that PRL leads to metabolic changes increasing the production of ammonia. However, contrarily to sulpiride,

bromocriptine did not significantly reduce the J_{Amm}, which may be explained by the injected dose. Surprisingly, fish injected with acidic vehicle and fish injected with vehicle plus PRL were not statistically different between them. In fact, the injection of acidic vehicle possibly stimulates proton excretion through proton pumps that are present in the branchial membrane, which acidifies the boundary layer that exists close to the gill epithelium contributing for the continuous excretion of ammonia due to the protonation of NH_3 to NH_4^+ form (Wright and Wood, 2009).

Although PRL-injected fish presented higher levels of ammonia excretion 24 h after the administration, this hormone did not cause any change in the expression of ammonia transporters but induced a decrease on branchial permeability. From the *q*PCR results of series *ii* (Figure 5.5), it is possible to verify that *prlra* mRNA was upregulated, which indicates that the oPRL injected IP were able to activate the PRLR mediating its effects in the zebrafish gill. In fact, for PRL triggering its effects, this hormone has to bind to its receptor and usually this binding also leads to an increase of mRNA expression of PRLR. On the other hand, the results in this study indicate that OCLNs, instead of CLNs, are regulated by hormones, with *oclnb* regulated by PRL and *oclna* by cortisol. However, since the expression of Rh glycoproteins were not affected by PRL injection, the increased J_{Amm} are likely to be related with other mechanisms and/or transporters. Hereupon, we may infer that, although ammonia fluxes were higher 24 h after PRL injection, PRL did not change the expression of ammonia transporters and even decrease the branchial paracellular permeability; however, the relevance of these findings needs to be clarified.

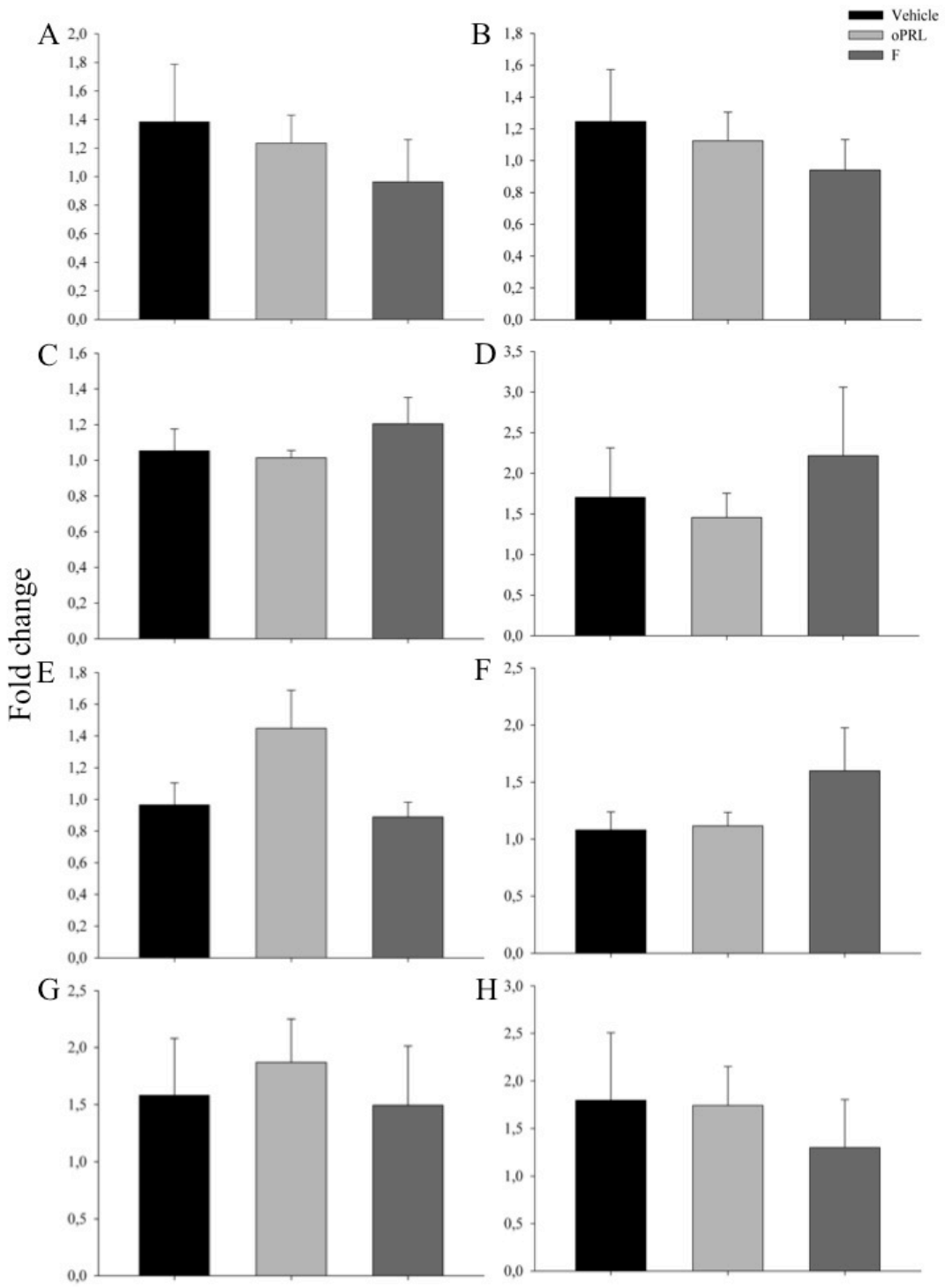
The extra load of ammonia (series *iii*) induced a predictable increase in J_{Amm} that was time dependent and, consistently with the previous results, higher in PRL than in cortisol treated fish (Figure 5.3). This observation, although it was again unexpected, can be explained by a more rapid response of cortisol-treated fish to a second injection stress period. In fact, during an exposure to a second stress, fish are able to activate the enzyme glutamine synthetase (GS) converting ammonia to glutamine (GLN) during the recovery period (Randall and Tsui, 2002); however, the mRNA expression of this enzyme was not analyzed. Moreover, this second injection may also deal to a much rapid upregulation of ammonia transporters in cortisol treated fish, leading to a faster excretion of ammonia. In fact, in this study, the load of ammonia in cortisol treated fishes induces the expression of both Rh glycoproteins (*rhcga* and *rhcgb*), similarly to what was previously described; however, no differences were noted in the expression of these ammonia transporters in the PRL-injected fishes (Figure 5.6). Beyond

these genes, cortisol treatment also induced the expression of *hsd11b2* and *oclna*, both already upregulated during the series *ii*. On the other hand, the injection of vehicle or ammonia (Figure 5.3) led to a decrease in the J_{Amm} over time, which may be related with the handling during the second injection procedure. Moreover, although fish sequentially injected with PRL and ammonia, had a slightly higher ammonia flux, this measured fluxes were not statistically different from the fish injected firstly with vehicle and after with ammonia. These results were not expected, being necessary new approaches to better understand the role of PRL on ammonia excretion. Moreover, and similarly to series *ii*, in series *iii* the blood cortisol and whole body ammonia were not statistically different between treatments (Table 5.3); although, these results were not expected, they can be explained by the higher fluxes of ammonia and/or possibly due to the conversion of ammonia to other compounds (Randall and Tsui, 2002).

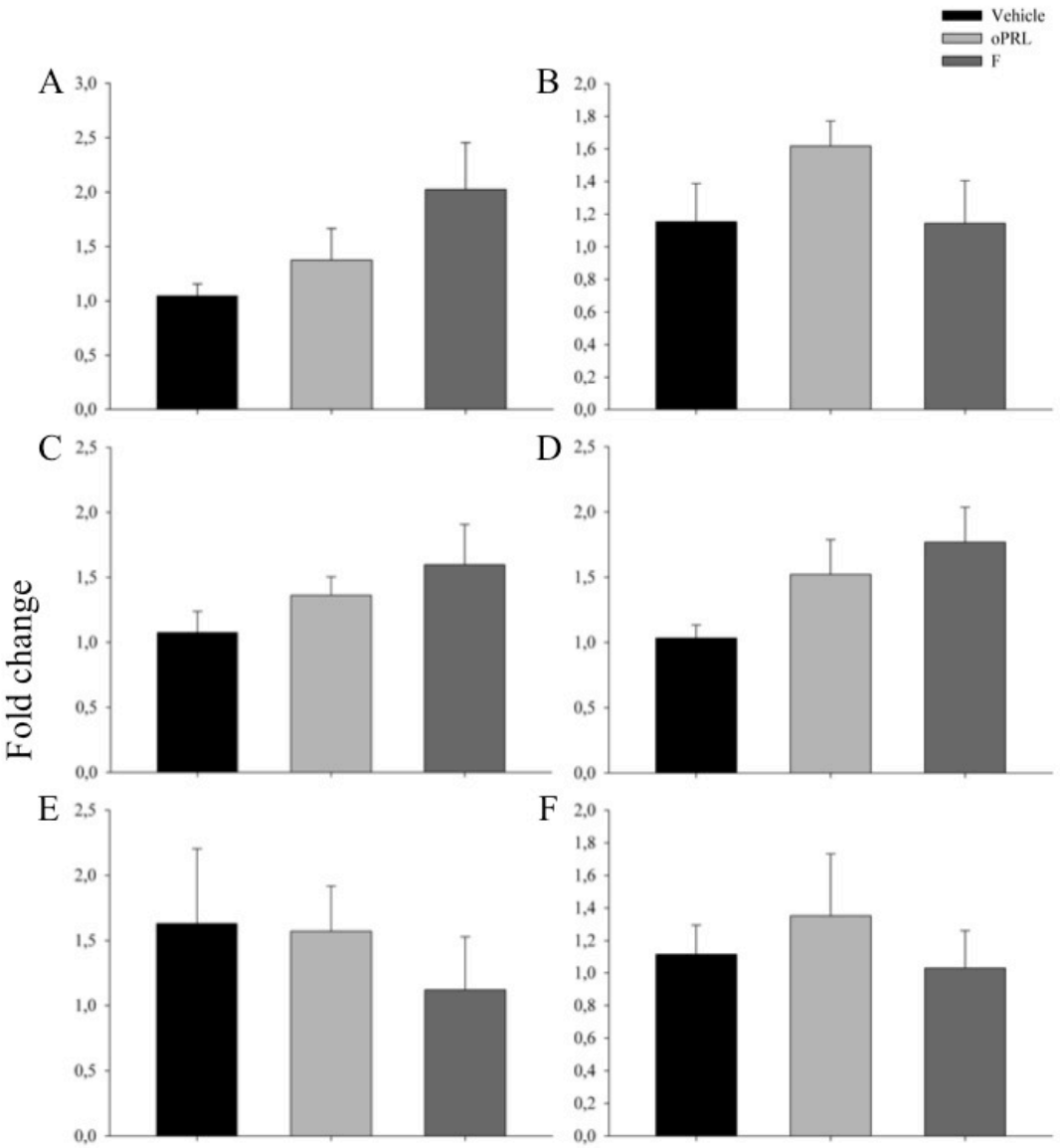
5.6 Conclusion

Against expectations, exogenous oPRL produced high rates of ammonia excretion; however, this is not correlated with transcript expression levels of ammonia transporter but with a predicted decrease in paracellular permeability based on an increased expression of *oclnb*. In this sense, this study suggests that PRL might affect fish metabolism that in turn will alter ammonia production and excretion. Moreover, this study shows that after 24 h of cortisol exposure, the excretion of ammonia was the lowest among the treatments probably due to enhance of ammonia transporter, especially *rhcgb*, and potential decrease in paracellular permeability through increase of *oclna* mRNA expression.

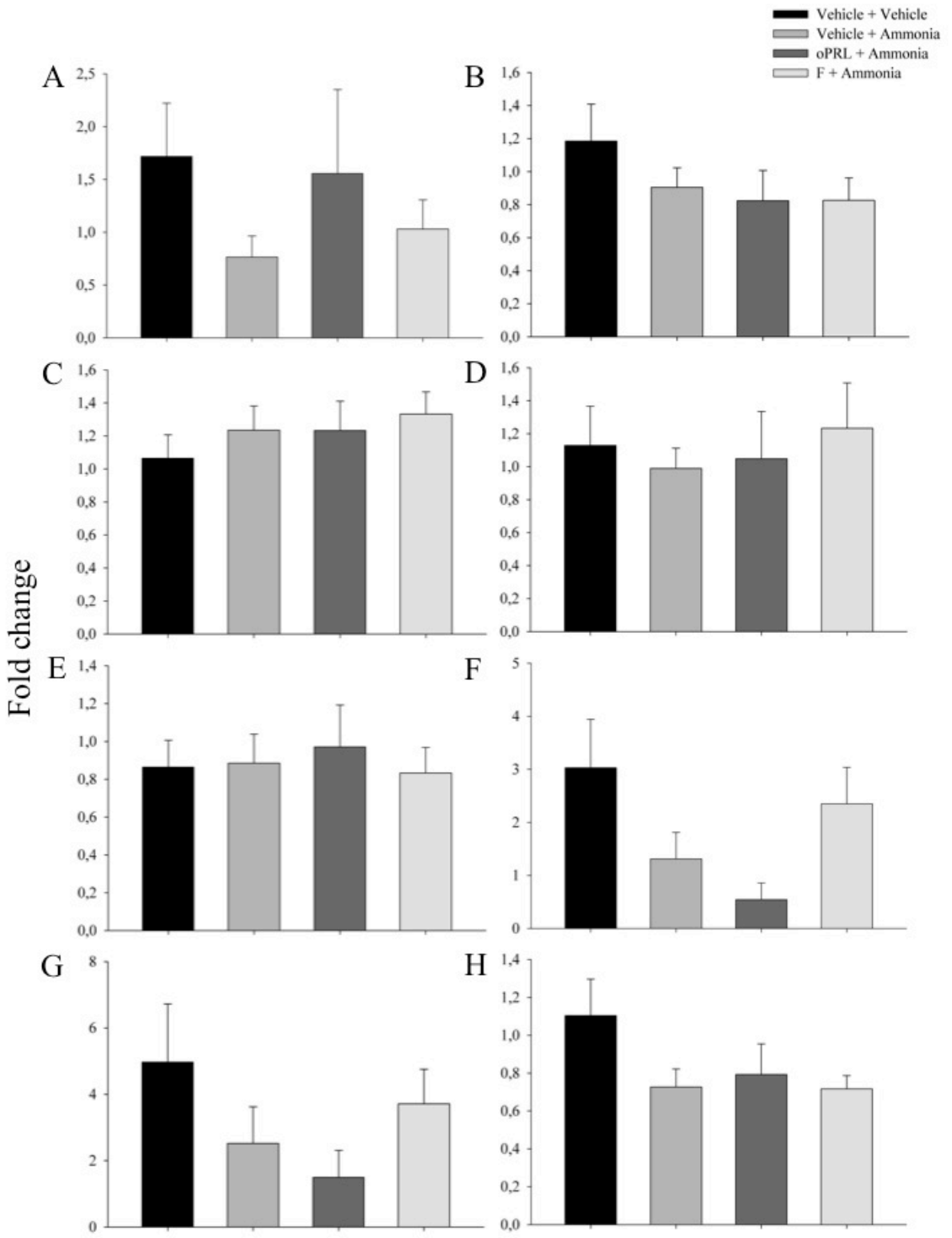
5.7 Supplementary figures



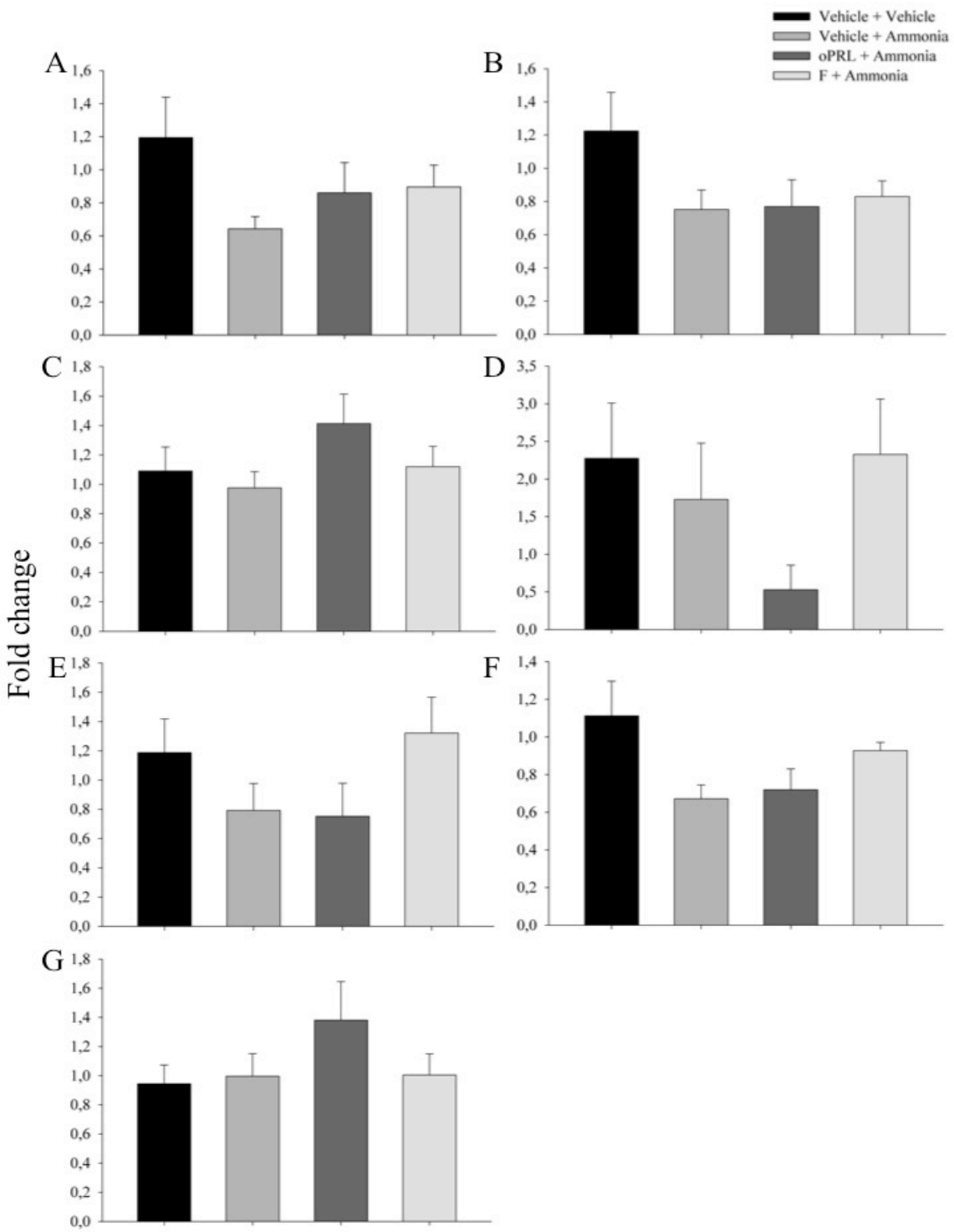
Supplementary Figure 5.7 Expression of mineralocorticoid receptor (*nr3c2*) (**A**), glucocorticoid receptor (*nr3c1*) (**B**), Rhesus glycoprotein B (*rhbg*) (**C**), Rhesus glycoprotein Ca (*rhcga*) (**D**), claudin a (*cldna*) (**E**), claudin b (*cldnb*) (**F**), claudin c (*cldnc*) (**G**) and claudin d (*cldnd*) (**H**) in the gill of zebrafish 24 h after of an intraperitoneally (IP) injection of 0.8 % NaCl (w/v) (vehicle) or vehicle plus ovine prolactin (oPRL; 100 $\mu g \cdot g^{-1}$) or immersed on a cortisol bath (F; 10 $\mu g \cdot ml^{-1}$). Results are expressed as normalized fold change (FC) with respect to the elongation factor 1 alpha (*eef1al1*) levels for the same samples. Data are present as mean ± standard error of the mean (SEM). No statistically differences were found in any of these genes (One-way ANOVA; Fisher LSD Method; $P \leq 0.05$).



Supplementary Figure 5.8 Expression of claudin e (*cldne*) (**A**), claudin f (*cldnf*) (**B**), claudin h (*cldnh*) (**C**), claudin i (*cldni*) (**D**), claudin 2 (*cldn2*) (**E**) and aquaporin 8a (*aqp8a*) (**F**) in the gill of zebrafish 24 h after of an intraperitoneally (IP) injection of 0.8 % NaCl (w/v) (vehicle) or vehicle plus ovine prolactin (oPRL; 100 $\mu g \cdot g^{-1}$) or immersed on a cortisol bath (F; 10 $\mu g \cdot ml^{-1}$). Results are expressed as normalized fold change (FC) with respect to the elongation factor 1 alpha (*eef1al1*) levels for the same samples. Data are present as mean ± standard error of the mean (SEM). No statistically differences were found in any of these genes (One-way ANOVA; Fisher LSD Method; $P \leq 0.05$).



Supplementary Figure 5.9 Expression of mineralocorticoid receptor (*nr3c2*) (**A**), glucocorticoid receptor (*nr3c1*) (**B**), Rhesus glycoprotein B (*rhbg*) (**C**), claudin a (*cldna*) (**D**), claudin b (*cldnb*) (**E**), claudin c (*cldnc*) (**F**), claudin d (*cldnd*) (**G**) and claudin e (*cldne*) (**H**) in the gill of zebrafish injected intraperitoneally (IP) with 0.8 % NaCl (w/v) (vehicle) or vehicle plus ovine prolactin (oPRL; 100 $\mu g \cdot g^{-1}$) or immersed on a cortisol bath (F; 10 $\mu g \cdot ml^{-1}$) and followed by an IP injection of vehicle or vehicle plus NH_4HCO_3 (0.5 M), 24 h after the first hormonal treatment. Results are expressed as normalized fold change (FC) with respect to the elongation factor 1 alpha (*eef1al1*) levels for the same samples. Data are present as mean ± standard error of the mean (SEM). No statistically differences were found in any of these genes (One-way ANOVA; Fisher LSD Method; $P \leq 0.05$).



Supplementary Figure 5.10 Expression of claudin f (*cldnf*) (**A**), claudin h (*cldnh*) (**B**), claudin i (*cldni*) (**C**), claudin 2 (*cldn2*) (**D**), occludin b (*oclnb*) (**E**), aquaporin 8a (*aqp8a*) (**F**) and prolactin receptor a (*prlrα*) (**G**) in the gill of zebrafish injected intraperitoneally (IP) with 0.8 % NaCl (w/v) (vehicle) or vehicle plus ovine prolactin (oPRL; 100 $\mu g \cdot g^{-1}$) or immersed on a cortisol bath (F; 10 $\mu g \cdot ml^{-1}$) and followed by an IP injection of vehicle or vehicle plus NH_4HCO_3 (0.5 M), 24 h after the first hormonal treatment. Results are expressed as normalized fold change (FC) with respect to the elongation factor 1 alpha (*eef1al1*) levels for the same samples. Data are present as mean ± standard error of the mean (SEM). No statistically differences were found in any of these genes (One-way ANOVA; Fisher LSD Method; $P \leq 0.05$).

CHAPTER 6 |

GENERAL DISCUSSION

6.1 General discussion

Ammonia is a toxic compound able to cause impaired performance and even mortality in teleosts; however, zebrafish *Danio rerio* is relatively tolerant to acute ammonia toxicity especially during early development. The presence of ammonia in freshwater ecosystems may occur due to different natural and anthropogenic sources (Randall and Tsui, 2002; USEPA, 2013). However, in aquaculture systems, high concentrations of ammonia may also occur due to high fish densities, high feeding rates and/or lower water removal rates (Tomasso, 1994). Although ammonia ion (NH_4^+) to be less able to cross the branchial membranes due to it charge, unionized ammonia (NH_3) may pass freely (Randall and Tsui, 2002), usually following the blood-to-water partial pressure gradient of NH_3 (ΔP_{NH3}), through simple diffusion or carrier (Wright and Wood, 2009; Nawata et al., 2010b). However, when environmental ammonia levels increase, the ΔP_{NH3} is reduced or reversed allowing ammonia uptake, which may culminate in fish death (Randall and Tsui, 2002). Since ammonia has been described as the cause of impaired performance and mortality in natural and aquaculture environments (Tomasso, 1994), the toxicity of this nitrogenous compound has been studied

over the years for a variety of aquatic species (Tomasso, 1994; USEPA, 1999, 2013); however, it is still little known about the toxicity of ammonia in zebrafish. In fact, zebrafish is a vertebrate model species highly used in scientific research (Dodd et al., 2000; Briggs, 2002; Hill et al., 2005; Hwang and Chou, 2013) due to it particular features such as small size, easy manipulation, high number of offspring, rapid and external development of transparent embryos and larvae, ontogenic development well characterized and short generation intervals (Kimmel et al., 1995; Westerfield, 2000; Parichy et al., 2009). These features make this species well suited for different areas of research, namely, for chemical toxicity, resorting to the use of early development (teratogenicity) (Voelker et al., 2007; Liedtke et al., 2008; Lammer et al., 2009; Selderslaghs et al., 2009; Brannen et al., 2010) as well as adulthood (Liu et al., 2006). However, to my knowledge, the levels of toxic ammonia for the different stages of zebrafish development have not previously been determined, with only a few studies mentioning the lethal dose for some stages of their development (Feldman et al., 2014; Jeffries et al., 2014). It was determined in my thesis, that zebrafish embryos were the most tolerant within the life cycle of this species and that larvae were as tolerant as adult (Figure 2.1). This result is similar to other species and is explained by the presence of the protective chorion (Braunbeck et al., 2005), but mostly by the conversion of ammonia to the less toxic urea (Randall and Tsui, 2002). In fact, although the majority of adult teleost do not present an active ornithine urea cycle (OUC), most of them resort to this detoxification mechanism during early development (Chadwick and Wright, 1999; Randall and Tsui, 2002; Barimo et al., 2004), which is important to protect these species during sensitive stages of neural development (Wright, 1995). The conversion of ammonia to urea through OUC was previously described in the early development of several different species (Chadwick and Wright, 1999; Barimo et al., 2004; Monzani and Moraes, 2008) and even in zebrafish (Braun et al., 2009a; Bucking et al., 2013; LeMoine and Walsh, 2013), constituting the main explanation for the high tolerance of embryos to ammonia during the life cycle of zebrafish. In this study it was also determined that adults (Figure 2.2) were very tolerant to ammonia when compared with other freshwater fish (USEPA, 2013); however, this susceptibility increased when adults were exposed to brackish water (6 ‰) and Tris buffered freshwater, which was explained by the disruption of branchial permeability and boundary layer acidification, respectively.

At sublethal concentrations, ammonia usually causes several molecular and morphological effects in several tissues; however, although in zebrafish the exposure to

ammonia did not cause any abnormal behavioral or mortality, changes in the transcriptome of gill, liver and brain were readily detected. As mentioned above, the presence of high levels of ammonia in the environment may impede or even reverse the movement of ammonia from blood-to-water, which contributes to it accumulation inside the fish (Randall and Tsui, 2002). On the other hand, since ammonia is continuously produced inside the fish during the catabolism of amino acids and proteins and then, transported by the blood stream (Ip and Chew, 2010) to the gills to be eliminated (Wright and Wood, 2009), any endogenous and/or exogenous factors that interfere with the balance between production and excretion of ammonia, can also contribute to its accumulation within the fish (Randall and Tsui, 2002; Wright and Wood, 2009). In fact, high internal ammonia concentrations have been associated with several negative effects in many tissues, as well as decreased on growth (Rasmussen and Korsgaard, 1996; Björnsson and Ólafsdóttir, 2006; Foss et al., 2007), swimming ability, reproductive capacity (Abbas, 2006), immune system and even with the death of a large number of fish (Tomasso, 1994; Randall and Tsui, 2002). In aquaculture and environmental monitoring, these endpoints would want to be avoided and it would be important to utilize other endpoints and new technologies to impede ammonia toxicity and thus potential economic losses. In this sense, molecular approaches have become good ecotoxicological method and although changes in gene expression has been determined mostly through real-time PCR (*q*PCR) (Braun et al., 2009a; Ip and Chew, 2010), the development of microarray technologies was a great improvement for scientific community since allow to monitor a large number of cellular transcripts all at the same time (Dhanasiri et al., 2013; Hagenaars et al., 2013). The use of the latter technique to determine the molecular responses after exposure to a toxic compound (Hagenaars et al., 2013) or other types of stress (Dhanasiri et al., 2013) has been high and even some studies involving ammonia have been conducted (Dhanasiri et al., 2013); however, no previous study has analyzed the transcriptome changes of gill, liver and brain simultaneously after 96 h of ammonia exposure. In this sense, the use of zebrafish was an advantage since its genome is now openly available to the scientific community. From the microarray results it was clear that gill was the tissue that strongly responded to ammonia exposure; although, liver and brain also exhibited altered transcriptomes (Figure 3.1). This result was expected since gill is in direct contact with the aquatic environment and thus directly exposed to environmental ammonia (Evans et al., 2005; Ip and Chew, 2010), but also because this tissue is responsible for ammonia excretion and for the maintenance of osmo- and ionoregulation of fishes (Evans et al., 2005). In this sense, regulation of the molecular and

physiological machinery in gill is necessary for guarantee the homeostasis of the organism. Moreover, gill constitutes the primary barrier and/or responsive organ to unfavorable environmental conditions namely during ammonia exposure (Benli et al., 2008). On the other hand, several pathways to produce and detoxify ammonia take place in liver (Ip and Chew, 2010), which make this organ also responsive to ammonia exposure as previously demonstrated in other studies (Benli et al., 2008; Dhanasiri et al., 2013). In addition to the effects on gill and liver, several studies have described the consequences of high internal ammonia levels in brain (Wilkie et al., 2007; Feldman et al., 2014); however, in this work, ammonia caused fewer effects on the transcriptome of brain, when compared with the other two tissues (Figure 3.1), which may be related with the high tolerance of adult to ammonia. In fact, before the death of the organism, there usually occurs several negative effects in the central nervous system (CNS) that may lead to neuronal cell death (Feldman et al., 2014); however, since the molecular effects on brain were lower, it seems that this tissue had not yet been strongly affected by ammonia exposure. Alternatively, the effects may be more regionalized and studying whole brain may have masked those effects.

During exposure to a stressful condition like the presence of high environmental ammonia (Gonçalves et al., 2012), similar to other teleost fish, zebrafish altered their energy-demanding pathways, resorting to carbohydrates instead of lipids as energy supplier (Dhanasiri et al., 2013; Hagenaars et al., 2013), to maintain their internal homeostasis. In response to a stress, fish may alter their energy metabolism (Dhanasiri et al., 2013) mostly by increasing the production of glucose to compensate the high energy demand needed to regain homeostasis. In my thesis, zebrafish increased the production of glucose in liver through enhancement of gluconeogenesis (Supplementary Figure 3.32); however, most of this glucose is to be used by tissues other than the liver. In fact, in gill, the glycolysis/gluconeogensis pathway was also be affected (Supplementary Figure 3.5), and although some glucose may also be produced through gluconeogenesis, most of it is used to produce energy through glycolysis. Moreover, several other pathways in these organs were altered in response to ammonia exposure to increase the production of glucose, namely, fructose and mannose pathway (Supplementary Figure 3.33) and pentose phosphate pathway (Supplementary Figure 3.34), in liver, and fructose and mannose pathway (Supplementary Figure 3.6) and galactose pathway (Supplementary Figure 3.11), in gill. In fact, similar results were obtained in the liver of zebrafish during the induced-stress of transportation (Dhanasiri et al., 2013); however, in this study the transcriptome changes in the gill tissue were not determined. Moreover, in

agreement with previous studies (Dhanasiri et al., 2013; Hagenaars et al., 2013), lipid metabolism was downregulated in the organs analyzed in the present study, which indicates that in contrast to carbohydrates, lipids were not preferentially used as an energy supply by zebrafish.

In fact, the mobilization of energy by gill tissue during ammonia exposure may be relevant for zebrafish to maintain the excretion of ammonia even against inward concentration gradients. The branchial elimination of ammonia may be regulated by transporter proteins, inserted within the membranes (Wright and Wood, 2009), but also by proteins present between cells and that control the paracellular permeability. Among all the transporter proteins present in the branchial membranes, the members of the Rhesus (Rh) glycoproteins family have been widely studied for their relevance in the transport of ammonia out of the body (Hung et al., 2007; Wright and Wood, 2009). The most up-to-date review of the excretion of ammonia by the gills of freshwater fish was presented in the model of Wright and Wood (2009) named "Na^+/NH_4^+ exchange complex". In this model, NH_3 cross the endothelial cells through the Rh glycoprotein A (Rhag) and enters in the branchial epithelial cell by the basolateral Rh glycoprotein B (Rhbg). At the apical membrane, NH_3 is in turn eliminated to the aquatic environment passing through the Rh glycoprotein C (Rhcg). Although this model considered that the excretion of ammonia occurs through members of Rh glycoprotein, it also presents several other transporter proteins and enzymes with an important roles for ammonia excretion, namely the H^+-ATPase, Na^+/K^+-ATPase, Na^+/H^+ exchanger (NHE), carbonic anhydrase (CA), amongst others. In this sense, since some of these transport proteins require energy to move ions, gill needs to increase the energy demands to excrete ammonia against its concentration gradient namely when the environmental ammonia levels are high. From the microarray results (Figure 4.1), this strategy to impede accumulation of ammonia was applied by zebrafish since the majority of subtypes of Rh glycoproteins, H^+-ATPase, Na^+/K^+-ATPase, NHE as well as other transporter proteins, were upregulated. In addition, together with the H^+-ATPase, zebrafish increased the expression of several subtypes of CA, to ensure that the boundary layer remains acidified thereby allowing the continuous excretion of ammonia through protonation of NH_3 (Wright and Wood, 2009). In fact, this response of zebrafish was also described in other species during exposure to high environmental ammonia (Hung et al., 2007), as well as in environments with elevated pH and even during aerial exposure (Hung et al., 2007). However, beyond this strategy, zebrafish also changed the expression of several elements of the paracellular transport pathway [movement of ions and/or molecules through

the space that exist between cells], namely the proteins claudins (CLNs) and occludins (OCLNs) (Figure 4.2). Although this has been mentioned and considered by several researchers, few studies have shown that these elements might alter their expression to respond to environmental challenges; however, most of these studies have been during salinity acclimation. In fact, these changes in the expression of paracellular transport elements should be important for the regulation of the paracellular permeability of branchial epithelia, mainly by impeding the entrance of ammonia.

The endocrine system is important to maintain the internal homeostasis and the survival of the organism and, in this sense, several hormones involved with osmo- and ionoregulation as well as branchial permeability, responded to ammonia exposure in zebrafish. One of the first hormones to respond to a stress is the glucocorticoid hormone cortisol (Mommsen et al., 1999); however, its action enhances the production of ammonia inside the fish since it induce protein and amino acid catabolism (Mommsen et al., 1999; Randall and Tsui, 2002). In this sense, under normal conditions, increased plasma cortisol concentrations lead to an increased ammonia excretion (Hopkins et al., 1995; Mommsen et al., 1999) and, in turn, when fish is exposed to high environmental ammonia, stressed fish presents lower tolerance to ammonia (Randall and Tsui, 2002). Beyond this, cortisol is known as a seawater adapting hormone that may regulate growth, reproduction, immunity and osmoregulation (Mommsen et al., 1999) of fishes. Since cortisol level were not measured in zebrafish and considering that the gene expression of both of its receptors [the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR)] was not altered after ammonia exposure, we may not say that cortisol is enhanced in zebrafish. However, some fish are able to reduce plasma cortisol levels even when continues to be exposed to the stressor, to impede the overactivation of corticosteroid receptor and avoid the effects triggered thereafter in the organism. In contrast to cortisol, other hormones responded to ammonia exposure namely somatolactin (SL), growth hormone (GH), prolactin (PRL), thyroid, parathyroid and natriuretic peptide (Figure 4.5). Among all the hormones, PRL, GH, SL and thyroid are responsible for osmo- and ionoregulation and also of the permeability of the epithelia, which may explain why zebrafish altered these hormones.

During stressful conditions, plasma cortisol levels increase (Mommsen et al., 1999; Gonçalves et al., 2012) which trigger different physiological responses (Mommsen et al., 1999) that allow the body regains the homeostasis. As mentioned before, one of the mechanisms activated by cortisol is the catabolism of proteins and amino acids (Mommsen et

al., 1999; Randall and Tsui, 2002), which will lead to higher ammonia excretion rates (J_{Amm}) (Hopkins et al., 1995; Mommsen et al., 1999); however, in the present work, the J_{Amm} were the lowest in the cortisol treatment group (Figure 5.2), which can be explained by the increased expression of the ammonia transporters Rh glycoproteins and also by the reduction of the branchial permeability, mostly through increased expression of occludin a (*oclna*) (Figure 5.5). These results appeared, however, even with no significant differences in the blood cortisol levels, between treatments (Table 5.2). This result is similar to the ones obtained in others species being explained by their capacity to reduce the plasma cortisol levels, even during the continuous exposure to stressor, in an attempt to avoid the overactivation of its receptors and continuous response. Another hormone that respond to stress in some fish species (Tang et al., 2001) as well as in zebrafish (see chapter 4), is PRL, however, this hormone is more studied as a freshwater adapting-hormone (Tang et al., 2001) being important to regulate osmo- and ionoregulation as well as the branchial permeability (Manzon, 2002). Surprisingly, and in contrast to cortisol, PRL-treated fishes presented the highest J_{Amm} (Figure 5.1) but did not alter the expression of Rh glycoproteins (Figure 5.5). However, in agreement with previous studies, PRL decreased the branchial permeability (Breves et al., 2014), since increased the expression of occludin b (*oclnb*) (Figure 5.5). In this sense, although it was not complete understood why PRL was induced during ammonia exposure, after this hormonal experiment, it appears that PRL was not involved directly in ammonia excretion.

6.2 Conclusion

In conclusion, with this study, it was possible to increase our knowledge about how ammonia affects the teleost zebrafish and which mechanisms can be used by this species to avoid ammonia intoxication. Moreover, new data related with the involvement of the endocrine system during ammonia toxicity and excretion, were also obtained. Since this species maintains typical vertebrate system complexity and shares high genomic similarities with other vertebrates, the results obtained in this freshwater teleost can serve as the basis for effective cross-species extrapolation. In this sense, this study is of high relevance for the scientific community, although so many questions remains to be answered. Moreover, the relevance of studying ammonia production, excretion and response in teleost fishes is related with ecological and environmental questions since this nitrogenous compound may cause mass mortality especially under unfavorable environmental or aquaculture conditions. In this

respect, this study presents several molecular and physiological consequences of ammonia exposure that might be used hereinafter to study the avoidance of the negative effects of high environmental ammonia.

6.3 Future directs

Although this work has successfully addressed several key issues, yet others remained puzzling and also new questions emerged. With this is mind, some of the future approaches include:

- The water physicochemical parameters affect ammonia toxicity; however, is it the branchial permeability or the J_{Amm}, or even both, that are modulated by the increased ionic strength?

- Since several hormones responded to ammonia exposure, and most of them are involved with osmo- and ionoregulation and/or branchial permeability, can these hormones, individually or synergistically, affect the J_{Amm}? And, with regards to gill permeability, can these hormones, individually or synergistically, be involved in the regulation of transcellular and paracellular transport elements and, consequently, involved in the movement of ammonia through the gill?

- This work highlighted that PRL response to ammonia toxicity, however, this hormone does not appear to be related with its excretion. On the other hand, beyond other functions, PRL regulates fish energy metabolism and immune system. Hereupon, will PRL respond to ammonia exposure by increasing the mobilization of energy reserves, helping fish regain homeostasis? Or, since ammonia compromised the immune system, is this response related to the strengthening of immune defenses?

- Yet, it is known that PRL regulates the Na^+ concentrations inside the fish, being therefore thoroughly studied during salinity challenges. In this sense, can the effect on the J_{Amm} after PRL administration, be related to the movement/regulation of the internal concentration of Na^+? Although PRL did not induce changes in the expression of ammonia transporters, can this hormone affect the expression of other carriers involved in the movement of Na^+ and thus affect one or more elements of the Wright and Wood (2009) model?

Taken together these issues should provide further insight into the crosstalk between hormonal systems and ammonia metabolism and excretion.

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