

Reprotoxic effects of microcystins and secondary metabolites produced by cyanobacteria Microcystis in adult medaka fish

Qin Qiao

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Reprotoxic effects of microcystins and secondary metabolites produced by cyanobacteria *Microcystis* in adult medaka fish

Sous la direction de : Prof. Cécile Bernard et Dr. Benjamin Marie, MNHN

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Abstract

Cyanobacterial blooms threaten human health as well as other living organisms of the aquatic environment, particularly due to the production of natural toxic components (called cyanotoxins). So far, one of the most studied cyanotoxins is the microcystin (MC). This thesis evaluated the potential reproductive toxicity of microcystin-LR (MC-LR) and the extract of one *Microcystis* strain (MC-producing) by investigating their toxic effects on the liver and gonad of adult medaka fish with one acute and one chronic study.

An investigation of the metabolic specificities of the liver in two genders of medaka fish was performed prior to the MC-containing exposure by using histological, metabolomic, proteomic and transcriptomic approaches, which attested to a strong sexual dimorphism of medaka liver, and highlights the importance of metabolic adjustments of the liver for maintaining the reproductive competency in adult medaka fish.

In the acute investigation, adult medaka fish were administered with $10 \ \mu g.g^{-1}$ body weight (bw) of pure MC-LR for 1 hour by gavage. The histological examination and immunolocalization of MC-LR in fish liver revealed a severe liver lesion along with an intense distribution of MC-LR in the liver, being particularly localized in the cytoplasm and nucleus of hepatocytes. In the gonad of MC-treated fish, MC-LR was shown to be present in the connective tissue of ovary and testis. Additionally, immunogold electron microscopy, for the first time, revealed that MC-LR was also localized in the chorion, cytoplasm and yolk vesicles of oocytes.

In the chronic study, adult medaka fish were exposed to 1 and 5 μ g.L⁻¹ of MC-LR and to the extract of MC-producing *Microcystis aeruginosa* PCC 7820 containing 5 μ g.L⁻¹ of equivalent MC-LR by balneation for 28 days. The results revealed that both MC-LR and the *Microcystis* extract exert adverse effects on reproductive parameters including fecundity and egg hatchability. The main cause of the observed reproduction impairment is believed to be an overall liver dysfunction induced by MC-containing treatments, rather than their directly deleterious effects on gonad. The histological observation indicated that hepatocytes of female fish under toxic treatments present noticeable glycogen storage loss along with severe cellular damages. The quantitative proteomics analysis revealed that the quantities of 150 hepatic proteins were dysregulated under at least one of

the three toxic treatments. In particular, a notable decrease in protein quantities of vitellogenin and choriogenin was observed, which could explain the decrease in reproductive output. In addition, our toxic treatments did not induce any characteristic estrogenic effects. Liver transcriptome analysis through Illumina RNA-seq revealed that over 100 to 400 genes were differentially expressed under 5 μ g.L⁻¹ of MC-LR and *Microcystis* extract treatments, respectively. Ingenuity Pathway Analysis highlighted that various metabolic pathways, such as energy production, protein biosynthesis and lipid metabolism, were disturbed by both MC-LR and the *Microcystis* extract, which could be provoking the observed reproductive impairment. The transcriptomics analysis also constituted the first report of the impairment of hepatic circadian-rhythm-related genes induced by MCs. This study contributes to a better understanding of the potential consequences of chronic exposure of fish to environmental concentrations of cyanotoxins, suggesting that *Microcystis* extract could impact a wider range of biological pathways compared with pure MC-LR, and even 1 μ g.L⁻¹ of MC-LR potentially induces a health risk for aquatic organisms.

Overall, the results of this thesis demonstrated that MC might directly impact gonadal function by inducing cytotoxicity in gonadal somatic cells and reproductive cells, and it could also impact the reproductive function indirectly by disturbing the general liver function. This improves our understanding of the potential reproductive toxicity of cyanotoxins in model fish, and advances our current knowledge on the protection of aquatic organism populations as well as human health from cyanotoxin issues.

Keywords

Microcystin; *Microcystis aeruginosa* PCC 7820; Reproduction; Fish liver sexual dimorphism; Liver dysfunction; Gonad; Immunolocalization; Transcriptomics; Proteomics

Résumé

Les efflorescences de cyanobactéries sont susceptibles d'avoir des effets néfastes sur les organismes des écosystèmes aquatiques, ainsi que sur les populations environnantes, notamment à travers la production de nombreuses molécules potentiellement toxiques (appelées cyanotoxines). Jusqu'à présent, une des cyanotoxines les plus étudiées est la microcystine (MC). Cette thèse a pour objectif d'évaluer la toxicité potentielle sur la reproduction de la MC-LR et de l'extrait d'une souche de *Microcystis* productrice de MCs en étudiant leurs effets toxiques sur le foie et les gonades de poissons medaka adultes exposés de manière aiguë ou chronique.

Une étude complète du foie des poissons médaka deux sexes a été menés par ailleurs avec des approches histologiques, métabolomiques, protéomiques et transcriptomiques, attestant d'un fort dimorphisme sexuel aussi bien au niveau cellulaire que moléculaire et souligne les importantes spécificités métaboliques du foie entre les deux sexes, notamment pour le maintien de la compétence de reproduction chez les poissons medaka adultes femelles.

Dans l'étude des effets induits par une exposition aiguë, les poissons medaka adultes ont été exposés par gavage à 10 μ g.g⁻¹ bw de MC-LR pure pendant 1 heure. L'examen histologique et l'immunolocalisation des MCs ont révélé des lésions hépatiques sévères ainsi qu'une distribution intense de la MC-LR dans le foie de poisson traité par la MC-LR, localisée particulièrement dans le cytoplasme et dans le noyau des hépatocytes. Dans la gonade des poissons traités, la MC-LR a été détectée dans les tissus conjonctifs de l'ovaire et des testicules. De plus, l'observation par microscopie électronique couplé à la technique d'immunogold a révélé, pour la première fois, que la MC-LR était également détectable dans le chorion, le cytoplasme et le vitellus des ovocytes matures.

Au cours des études des effets induits par l'exposition chronique, les poissons medaka adultes ont été exposés durant 28 jours par balnéation à 1 et 5 μ g.L⁻¹ de MC-LR et à un extrait de la souche de *Microcystis aeruginosa* (PCC 7820) productrice de microcystines (5 μ g.L⁻¹ équivalent MC-LR). Ces résultats ont révélé que la MC-LR et l'extrait de *Microcystis* induisent des effets délétères sur différents paramètres de reproduction, tels la fécondité et le taux d'éclosion des embryons. La cause principale de ces perturbations de la reproduction semblent principalement résulté d'un dysfonction hépatique globale induite par les traitements aux MCs (hépatotoxiques), plutôt qu'à des effets

directs sur les gonades. L'observation histologique a montrée que les hépatocytes des poissons femelles exposées aux toxines présentent une perte notable du stockage de glycogène intracellulaire ainsi que des dommages cellulaires sévères. L'analyse par protéomique quantitative a révélé que les quantités de 150 protéines hépatiques sont dérégulées par au moins un des trois traitements aux cyanotoxines. En particulier, il a été observé une diminution notable de la quantité protéique de plusieurs formes de vitellogénines et de choriogénines, pouvant expliquer ainsi directement la diminution de certains paramètres de la production, tel la fécondité. En outre, les traitements toxiques n'induisent pas d'effet oestrogénique spécifique. L'analyse du transcriptome global du foie par séquençage des ARN messager par Illumina révèle que plus de 100 à 400 gènes sont exprimés différemment suite aux traitements avec 5 µg.L⁻¹ de MC-LR et d'extrait de Microcystis, respectivement. L'intégration de ces données à l'aide du logiciel « Ingenuity Pathway Analysis » souligne le fait que diverses voies métaboliques, telles que la production d'énergie, la biosynthèse des protéines et le métabolisme lipidique, seraient perturbées à la fois par la MC-LR et également par l'extrait de Microcystis, ce qui pourrait également expliquer les perturbations observées du système reproductif. Ces analyses par transcriptomique constituent également le premier rapport de l'induction de la dérégulation de différents gènes liés à la régulation du rythme circadien hépatique par les MCs. Cette étude contribue à une meilleure compréhension des conséquences potentielles de l'exposition chronique des poissons à des concentrations environnementales de cyanotoxines, suggérant ainsi que, par rapport à la MC-LR pure, l'extrait de Microcystis pourrait avoir un impact sur un plus large éventail de mécanismes biologiques et que même la plus faible concentration testée dans notre étude (1 µg.L⁻¹ MC-LR, correspondant au seuil de potabilité préconisé par l'OMS) semble induire un risque potentiel pour la santé des organismes aquatiques.

Dans l'ensemble, les résultats démontrent que même si les microcystines pourraient avoir un impact direct, mais modéré, sur la fonction gonadique en induisant une cytotoxicité dans les cellules somatiques gonadiques et les cellules reproductrices, elle semble avoir principalement avoir un impact indirect sur la fonction reproductrice en perturbant la fonction hépatique générale. Ces données améliorent notre compréhension des processus liés à la toxicité potentielle des cyanotoxines pour la reproduction chez un poisson modèle, et fait d'une manière générale progresser questionnement quant à la protection des populations exposées à ces cyanotoxines.

Mots clés

Microcystine; *Microcystis aeruginosa* PCC 7820; Reproduction; Dimorphisme sexuel du foie de poisson ; Dysfonction hépatique; Gonade; Immunolocalisation; Transcriptomique; Protéomique

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Abbreviation

ALS	amyotrophic lateral sclerosis
APX	aplysiatoxin
ATX-a	anatoxin-a
BMAA	β-N-methylamino-L-alanine
bmal1	brain and muscle ARNT-like 1
bw	body weight
CaMKII	calcium-calmodulin-dependent multifunctional protein kinase II
cat	catalase
CHG	choriogenin
Clock	circadian locomotor output cycles protein kaput
CNS	central nervous system
cry	cryptochrome
CSN	central suprachiasmatic nucleus
CYN	cylindrospermopsin
cyp17a	cytochrome P450 hydroxylase/lyase A
CYPs	cytochrome P450
d.w.	dry weight
DAB	diaminobenzidine
DMEs	drug-metabolizing enzymes
DNA-PK	protein kinase activity of the DNA-dependent protein
D_{ow}	distribution ratio
E2	17β -estradiol
EDCs	endocrine disrupting chemicals
EEQ	estrogenic equivalents
ELISA	enzyme-linked immunosorbent assay
ETC	electron transport chain
FSH	follicle-stimulating hormone
GSH	glutathione
GV	gas vesicles
HepG2	hepatoma cell line
HES	hematoxylin-eosin-saffron
HPG	hypothalamic-pituitary-gonadal
HPLC	high-performance liquid chromatogram
i.p.	intraperitoneal
IPA	Ingenuity Pathway Analysis
IPCS	International Programme on Chemical Safety

LAs	lyngbyatoxins
LC-ESI-	liquid chromatography-electrospray ionization-mass spectrum
MS	system
LH	luteinizing hormone
MAPKs	mitogen-activated protein kinases
MC	microcystin
MC-LR	microcystin-LR
MMTS	methyl methane-thiosulfonate
MPD	membrane potential depolarization
MPT	mitochondrial permeability transition
Mya	mililion yeas
Ν	nitrogen
NHEJ	nonhomologous end joining
NODs	nodularins
OATP	organic anion transporting polypeptides
P value	partition-coefficient
Р	phosphate
PAS	periodic acid-Schiff/alcian blue
PCA	Principal Component Analysis
per	period
PP1 and	
PP2A	protein serine/threonine phosphatases1 and 2A
PP3	protein phosphatase type 3
PSTs	paralytic shellfish toxins
QC	Quality Check
rp17	ribosomal protein 17
SD	standard deviation
SLS	segment long-spacing
sod	superoxide dismutase
STX	saxitoxin
TB	toluidine blue
TCA	tricarboxylic acid
TCEP	tris-[2-carboxyethyl] phosphine
TEAB	triethylammonium bicarbonate buffer
TMSP	sodium-3-tri-methylsilylpropionate
TNF-α	tumor necrosis factor-α
VTG	vitellogenin
WHO	World Health Organization

Publication and Conference

Article 1: <u>Oin Qiao</u>, Séverine Le Manach, Benoît Sotton, Hélène Huet, Evelyne Duvernois-Berthet, Alain Paris, Charlotte Duval, Loïc Ponger, Arul Marie, Alain Blond, Lucrèce Mathéron, Joelle Vinh, Gérard Bolbach, Chakib Djediat, Cécile Bernard, Marc Edery, Benjamin Marie, 2016. Deep sexual dimorphism in adult medaka fish liver highlighted by multi-omic approach, Scientific Reports 6: 32459. (Collaborative work)

Article 2: <u>**Oin Oiao**</u>, Séverine Le Manach, Hélène Huet, Evelyne Duvernois-Berthet, Soraya Chaouch, Charlotte Duval, Benoît Sotton, Loïc Ponger, Arul Marie, Lucrèce Mathéron, Sarah Lennon, Gérard Bolbach, Chakib Djediat, Cécile Bernard, Marc Edery, Benjamin Marie, 2016. An integrated omics analysis of hepatic alteration in medaka fish chronically exposed to cyanotoxins with possible mechanisms of reproductive toxicity. Environmental Pollution 219: 119-131.

Article 3: Séverine Le Manach, Nour Khenfech, Hélène Huet, <u>Qin Qiao</u>, Charlotte Duval, Arul Marie, Gérard Bolbach, Gilles Clodic, Chakib Djediat, Cécile Bernard, Marc Edery, and Benjamin Marie, 2016. Gender-specific toxicological effects of chronic exposure to pure microcystin-LR or complex *microcystis aeruginosa* extracts on adult medaka Fish, Environmental Science and Technology 2;50(15):8324-34. (Collaborative work)

Article 4: Benoît Sotton, Alain Paris, Séverine Le Manach, Alain Blond, Gérard Lacroix, Alexis Millot, Hélène Huet, <u>**Oin Qiao**</u>, Sophie Labrut, Giovanni Chiapetta, Joelle Vinh, Arnaud Catherine, Benjamin Marie, 2016. Metabolic changes in Medaka (*Oryzia latipes*) fish induced by cyanobacterial exposures: an integrative approach combining proteomic and metabolomics analysis. (Submitted to Scientific Reports, collaborative work)

Article 5: Benoît Sotton, Alain Paris, Séverine Le Manach, Alain Blond, Gérard Lacroix, Alexis Millot, Charlotte Duvall, <u>**Oin Qiao**</u>, Arnaud Catherine, Benjamin Marie, 2016. Global metabolome changes induce by cyanobacterial blooms in three representative fish species. (Submitted to Science of the Total Environment, collaborative work)

Poster:

Qin Qiao, Séverine Le Manach, Hélène Huet, Evelyne Duvernois-Berthet, Charlotte Duval, Loïc Ponger, Lucrèce Mathéron, Gérard Bolbach, Chakib Djediat, Cécile Bernard, Marc Edery, Benjamin Marie, Reprotoxic effects of microcysin-LR and microcystin-producing *Microcystis* extract on adult medaka. Societe d'ecotoxicologie fondamentale et appliquee, Bordeaux, France, 2015.

<u>**Qin Qiao**</u>, Séverine Le Manach, Hélène Huet, Evelyne Duvernois-Berthet, Charlotte Duval, Loïc Ponger, Lucrèce Mathéron, Gérard Bolbach, Chakib Djediat, Cécile Bernard, Marc Edery, Benjamin Marie, Reprotoxic effects of microcysin-LR and microcystin-producing *Microcystis* extract on adult medaka. Phycotox and GIS cyano, Brest, 2015

<u>**Qin Qiao**</u>, Cécile Bernard, Marc Edery, Benjamin Marie, Reprotoxic effects of microcysin-LR and microcystin-producing *Microcystis* PCC 7820 extract on medaka. Poster. Societe d'ecotoxicologie fondamentale et appliquee, Reims, France, 2016

Oral presentation:

<u>**Qin Qiao**</u>, Cécile Bernard, Marc Edery, Benjamin Marie, Reprotoxic effects of microcysin-LR and microcystin-producing *Microcystis* PCC 7820 extract on medaka. Phycotox and GIS cyano, 2016

<u>**Qin Qiao**</u>, Cécile Bernard, Marc Edery, Benjamin Marie, Reprotoxic effects of microcysin-LR and microcystin-producing *Microcystis* PCC 7820 extract on medaka. Spotlight presentation. SETAC Europe 26th Annual Meeting, Nantes, France, 2016

<u>**Qin Qiao**</u>, Cécile Bernard, Marc Edery, Benjamin Marie, Hepatic dysfunction is responsible for reproduction impairment in medaka fish upon chronic exposure to MC-LR and the extract of Microcystis aeruginosa PCC7820. The 10th international conference on toxic cyanobacteria, Wuhan, China, 2016

Chapter 1 Introduction

1.1 General introduction

Water is one of the most important resources globally for humans and for aquatic resources. Emerging environmental pollution due to the expansion of anthropogenic activities has profoundly altered the quality and availability of water resources. The release of phosphates and nitrogen from septic system effluents, fertilizer runoff, fossil fuel, animal waste, and industrial discharge has been largely responsible for water eutrophication, which results in recurrent and massive cyanobacterial blooms. Besides, global warming also promotes the occurrence of blooms. Cyanobacterial blooms bring many deleterious impacts on the aquatic ecosystem, including the disruption of hydrochemistry and sunlight condition in water bodies, the devastation of water quality, and a loss of biodiversity. Moreover, the formation of cyanobacterial blooms can be a threat to human as well as any living organisms in the aquatic environment particularly due to the production of toxic components, so-called cyanotoxins.

Among the cyanotoxin diversity, the microcystin (MC) is one of the most toxic and notorious groups that exhibit severe hepatotoxicity. Besides, MCs have been also revealed to cause cardiotoxicity, nephrotoxicity, and embryotoxicity (G. Li et al., 2011a; Milutinović et al., 2003; Qiu et al., 2009). In recent years, more research attention has been specially put on the reproductive toxicity of MCs. It has been reported that MCs accumulate in the gonad of freshwater shrimp, snail, water bird, duck and fish (Chen et al., 2009, 2005; Chen and Xie, 2005). Fish is one of the key components of biotic communities in the aquatic ecosystem, and in the aquatic ecosystem, fish can be easily exposed to cyanotoxins either by directly ingestion or indirectly ingestion through the food chain, or by breathing when toxins pass through the gill. Therefore, fish are very good vertebrate models to investigate the potential reproductive toxicity of MCs in the aquatic environment. However little is still known regarding the directly adverse effects of MCs on the fish gonad or about the interplay between reproductive impairment and general liver dysfunction induced by this potent hepatotoxin in oviparous fish. In addition, *Microcystis*, one of most common bloom generating cyanobacteria, is a cause of concern in many continental water reserves. They produce not only MCs but also a wide range of currently unknown compounds that may also cause

deleterious effects alone on the organism, or lead to synergistic, antagonistic or additive effects together with MCs.

1.2 Cyanobacteria

Cyanobacteria are a group of oxygenic photosynthetic prokaryotes that live in a wide variety of environments, from terrestrial environment to fresh and marine waters. Like all plants and algae, cyanobacteria synthesize the greenish pigment *chlorophyll* α , necessary for performing photosynthesis. Most cyanobacteria also produce the bluish pigments phycobilin and phycocyanin, which make the cyanobacteria appear blue-green, and they are therefore often called blue-green algae.

The earliest evidence of cyanobacteria extends back to ~3.5 billion years ago, while oxygenproducing photoautotrophic cyanobacteria originate at ~2.45 billion years ago (Schopf, 2012). These photoautotrophic cyanobacteria are believed to have a great impact on the formation of early oxidizing atmosphere and on the subsequent change of the composition of life forms on Earth by providing biologically useable oxygen. Nowadays, cyanobacteria still play a crucial role in the photosynthetic primary production and nitrogen fixation in various ecosystems (Whitton, 2012), contributing to the balance of energy conversion and nutrition transmission on Earth. However, freshwater cyanobacteria have become a big concern in recent decades due to frequent occurrences of harmful cyanobacterial blooms throughout the world, which disrupt the aquatic ecosystem and threaten the population of aquatic organisms and human health as well.

1.2.1 Classification, morphology and physiology

The classification of *cyanobacteria* has long remained an enigma due to their long and arguably complex evolutionary history. A modern systematic classification was recommended by Hoffmann and his colleagues, who classified *cyanobacteria* into 4 subfamilies, 6 orders and 32 families (Hoffmann et al., 2005), based on the morphology of cells, ultrastructural properties, the type of cell division and molecular phylogenetics. In 2014, a more phylogenetically-based system of higher level taxonomy and classification of the cyanobacteria was proposed (Komárek et al., 2014). The recognized and accepted cyanobacterial genera were classified into 8 orders comprised of *Gloeobacterales*, *Synechococcales*, *Spirulinales*, *Chroococcales*, *Pleurocapsales*, *Oscillatoriales*,

Chroococcidiopsidales and *Nostocales* (Komárek et al., 2014), based on a combination of definable molecular, morphological and ecological criteria.

Cyanobacteria are morphologically diverse, comprising unicellular, colonial and multicellular filamentous forms with branched and unbranched trichomes (Mur et al., 1999), as seen in Figure 1.1. Unicellular cyanobacteria may present spherical, cylindrical or ovoid shapes, such as in the order *Chroococcales* (Casamatta and Hašler, 2016). The cells may aggregate in irregular or ordered colonies during different life periods. Filamentous morphology is formed from repeated cell divisions occurring in a single plane (Mur et al., 1999). A large variability of the filamentous organization has been observed. Some species in the order *Oscillatoriales* are composed of identical cells that are characterized by uniseriate and unbranched trichomes (Mur et al., 1999). Some orders are composed of heterogeneous cells (orders *Nostocales*). Some of them form heterocysts that function for nitrogen fixation. Some filamentous cyanobacteria contain akinetes that contain reserve materials and enable survival under unfavorable environments (Mur et al., 1999).



Figure 1. 1 Basic morphology of cyanobacteria (Mur et al., 1999).

The phylum *Cyanobacteria* belongs to the superkingdom *Eubacteria*. The gram-negative cyanobacteria have some commonalities with bacteria, such as the absence of membrane-bound subcellular organelles and the presence of a wall structure based upon a peptidoglycan layer. On the

other hand, cyanobacteria perform oxygenic photosynthesis in a manner similar to that found in the chloroplast, where photosynthesis takes place in eukaryotic plants or microalgae (Whitton, 2012). It is widely believed that chloroplasts of microalgae and plants evolved from an endosymbiotic cyanobacterium living within a mitochondria-containing eukaryotic host cell (Falcón et al., 2010).

The photosynthesis of cyanobacteria occurs in the photosynthetic pigments located in thylakoids, and the thylakoids are freely present in the cytoplasm (Whitton, 2012). The basic features of photosynthesis in cyanobacteria have been described previously (Whitton, 2012). Two reaction centers of Photosystem I and Photosystem II are present in the photosynthetic apparatus of cyanobacteria, which is similar to that in microalgae. Cyanobacteria capture light effectively with various photosynthetic pigments, such as chlorophyll *a*, carotenoids, phycocyanin, allophycocyanin and phycoerythrin, and convert light energy into chemical energy by using water as an electron donor (Whitton, 2012). Oxygen is released as a byproduct.

Certain groups of cyanobacteria equipped with the nitrogenase enzyme complex can fix atmospheric nitrogen, providing them this fundamental nutrient (Berman-Frank et al., 2007). Specialized cells called heterocysts are thought to be responsible for the nitrogen fixation (Whitton, 2012). Filamentous heterocyst-forming genera, such as *Anabaena and Nostoc* are well-documented examples of nitrogen-fixing cyanobacteria (Casamatta and Hašler, 2016). Other species of cyanobacteria with unusual nitrogen fixation properties (heterocyst-independent) have also been reported, such as the genus *Trichodesmium* (Casamatta and Hašler, 2016). Overall, this important metabolic machinery provides them with a major advantage when sources of combined inorganic nitrogen are depleted in the water.

Cyanobacteria can be found in nearly any given habitat in the world, like wetlands, arid deserts, hot springs and glaciers. The diverse cell morphology corresponding to various functional requirements and the variations in metabolic strategies, motility and cell division together contribute to their high adaptability and wide-spreading.

1.2.2 Cyanobacterial blooms

Cyanobacteria gain a competitive advantage over other photoautotrophic organisms thanks to their highly adapted physiological feature. Under optimal environmental conditions, such as high temperature and pH values, low turbulence and high nutrients input, cyanobacteria can grow rapidly and reach the dense population in the water body (Paerl and Otten, 2013). The excessive cyanobacteria turn clear, transparent water to soupy green in appearance, or even blue, gray, red or purple in color (Whitton, 2012). Some cyanobacteria can congregate near the water's surface forming a thin "oily" looking film or a blue-green scum, as shown in Figure 1.2. This proliferative phenomenon is called cyanobacterial bloom.



Figure 1. 2 Cyanobacteria blooms in different sites.

(a) the small scale of Lake Mendota, Wisconsin, USA (C. Spillman); (b) the whole-lake scale of Karori Reservoir, Wellington, New Zealand; (c) aerial photo of Lake Rotorua, Nelson, New Zealand (S.Wood) (Whitton, 2012).

A cyanobacterial bloom may be dominated by a single species or be contemporaneously composed of several species. Gas-vacuolate species are principally responsible for forming blooms. A number of genera that vary in form and size are involved, such as filamentous genera, Anabaena, Nodularia, Gloeotrichia and Spirulina, or non-filamentous ones, Microcystis and Coelosphaerium (Whitton and Malcolm, 2002). Different genera or species of cyanobacteria have own optimal temperature and light intensity which vary between temperate and tropical regions. Planktothrix agardhii is one of the typical bloom-forming species in temperate lakes (Scheffer et al., 1997). Within tropics, blooms are usually caused by genera of Microcystis, Cylindrospermopsis and Anabaena (Mowe et al., 2015). However, blooms of these tropical cyanobacteria are becoming more and more frequent in temperate regions nowadays (Harke et al., 2016; Mehnert et al., 2010; Sukenik et al., 2012). Tropical species Cylindrospermopsis raciborskii (Nostocales) was found in temperate, subtropical and tropical regions with a similar contribution to a total biovolume, which revealed its expansion to temperate latitudes (Bonilla et al., 2012). Anabaena Bergii, another tropical species of genus Anabaena, was also reported in some temperate regions, e.g Greman (Stüken et al., 2006), North Carolina, USA (Moisander et al., 2002) and Slovakia (Hindák, 2000) in the recent decade. Globally, Microcystis, Anabaena, Aphanizomenon, Cylindrospermopsis and *Planktothrix (Oscillatoria)* are the main genera of bloom-forming cyanobacteria in a large range of countries or areas, including Europe, Eastern Asia and Nouth America (Codd et al., 2005).

Cyanobacteria blooms display a range of temporal dynamics. The temporal pattern of a cyanobacterial bloom in a particular ecosystem is based on multiple environmental factors including nutrient availability, water temperatures, sunlight and wind velocity. Some blooms follow a seasonal pattern, some encompass all seasons, and some may also occur as extreme peaks and crash rapidly lasting just days or weeks (Havens, 2008). In temperate regions, blooms normally start in summer and last into autumn. This regular seasonal pattern also occurs in subtropical waters, including coastal systems (Murrell and Lores, 2004). In tropical regions, blooms can occur at any time of the year and usually last for a few weeks at a time, and wet/dry seasonality can influence the occurrence of cyanobacterial blooms (Mowe et al., 2015).

Cyanobacterial blooms exert detrimental effects on the aquatic environment. On one hand, blooms have negative impacts on ecosystem functioning, e.g., disturbances of the food web, changes of biodiversity, light conditions and oxygen levels. On the other hand, cyanobacterial blooms bring a significant water quality problem, especially as specific cyanobacterial species are capable of producing a wide range of potent toxins, so called cyanotoxins, with important implications for health risks associated with the human exploitation of recreational and drinking waters (Sivonen and Gary Jones, 1999).

The increase of anthropogenic activities along with deficient water management has exacerbated water eutrophication (increased input of nutrients, especially phosphorous and nitrogen) worldwide. In Europe, Asia and America, more than 40% of lakes and reservoirs are eutrophic, which offers favorable nutrients for massive proliferations of cyanobacteria (Bláha et al., 2009). Climatic changes, such as global warming, increased atmospheric concentrations of carbon dioxide, elevated UV fluxes, also favor the growth of cyanobacteria (Paul, 2008). Recurring blooms are found in various inland freshwater ecosystems worldwide, for instance, Lake Victoria (Africa), Lake Erie and Lake Michigan (USA-Canada), Lake Okeechobee (Florida, USA), Lake Kasumigaura (Japan), Lake Taihu (China), and estuarine and coastal waters, e.g., the Baltic Sea, Caspian Sea, tributaries of Chesapeake Bay, North Carolina's Albemarle-Pamlico Sound, Florida Bay, the Swan River Estuary in Australia, the Patos, and other coastal lagoonal estuaries in Brazil (Paerl et al., 2011; Paerl and Otten, 2013).

1.2.3 Microcystis, the frequently bloom-forming genera

Microcystis is a genus of freshwater cyanobacteria which belongs to the order *Chroococcales* and the family *Microcystaceae*. It is characterized by spherical cells with a diameter of a few micrometers. The protoplast of the cell is light blue-green, but overall the cells appear dark or brownish under light microscopy due to optical effects of the gas vesicles (one of the diagnostic features in the genus *Microcystis*) (Van Vuuren et al., 2006). The cells are usually irregularly grouped into colonies that may be bounded by colorless mucilage composed of complex polysaccharide compounds (Whitton, 2012).

The classification of species in genus *Microcystis* is under large revision. Traditional morphological criteria including colony form, mucilage structure, cell diameter, the density and organization of cells within the colony, pigment content and life cycles are used for morphospecies recognition. Five dominant morphospecies (*M. aeruginosa*, *M. ichthyoblabe*, *M. viridis*, *M. novacekii*, *M. wesenbergii*) of the genus *Microcystis* were suggested by Otsuka et al. (2001). However, with the development of molecular and biochemical markers, some contradictory results of *Microcystis* taxonomy have been found. For instance, 16S rRNA analysis revealed no differences among the morphospecies (Otsuka et al., 1998). In consideration of both morphological and molecular markers, it has been suggested to classify *Microcystis* into three clusters: the small cell-size group composed of *M. ichthyoblabe* and *M. flos-aquae*, the middle cell-size group based on *M. aeruginosa* (incl. *M. novacekii*) and the large cell-size group represented by *M. wesenbergii* (Whitton, 2012).

Some important features of *Microcystis*, such as buoyancy regulation, storage strategy at the bottom of the water column, phosphate (P) and nitrogen (N) uptake capacities and resistance to zooplankton grazing, favor its worldwide spread (Whitton, 2012). *Microcystis* possess oval hollow gas-filled structures, called gas vesicles (GV), which provides cells with buoyancy. Long-term buoyancy regulation in *Microcystis* is associated with changes in GV volume whereas short-term buoyancy regulation is mainly related to carbohydrate accumulation and consumption (Chu et al., 2007). The buoyancy regulation of *Microcystis* is key to their dominance in eutrophic waters, by positioning themselves optimally in response to different environmental conditions in the water column. Furthermore, *Microcystis* overwinters as vegetative colonies at the bottom of water column and sediments in response to decreased light intensity and nutrient abundance, favoring its perennial dominance in eutrophic waters (Whitton, 2012). In the aquatic ecosystem, P and N are limiting

nutrients for the growth and replication of cyanobacteria and other microalgae. *Microcystis* has competitive advantages over other cyanobacteria or microalgae in response to nutrient limitation (Sommer, 1985). For instance, *Microcystis* is able to adapt to P limitation by increasing its P uptake and storage capacity, decreasing its light-harvesting capacity, and changing the colony size (Whitton, 2012). NO₃, NO₂ or NH₄+ can be used as N resources by *Microcystis* (Whitton, 2012). In addition, various secondary metabolites produced by *Microcystis* have been suggested to inhibit zooplankton grazing (Ger et al., 2016). MCs are the most studied potential cyanotoxin produced by a large proportion of *Microcystis* species as a response to grazing. Beyond MCs, other toxic compounds or potential secondary metabolites produced by *Microcystis* have also been reported as chemical warfare against grazing of herbivores (Whitton, 2012).



Figure 1. 3 The global occurrence of *Microcystis* blooms and MCs as determined through literature searches from 257 countries and territories (Harke et al., 2016).

Microcystis is one of the most pervasive bloom-forming cyanobacteria in freshwater ecosystems (Harke et al., 2016). Recent literature has reported that *Microcystis* blooms were found

in 108 countries, including United States, China, Colombia and so on. For 79 countries the blooms were associated with MCs (Figure 1.3). This report has revealed that *Microcystis* proliferate and dominate phytoplankton communities in a wide range of freshwater ecosystems in both temperate and tropical climates (Harke et al., 2016).

1.3 Microcystins

Cyanobacteria produce various potent toxic compounds (cyanotoxins) and other potentially bioactive molecules. Among cyanotoxins, MCs are one of the most common and notorious hepatotoxins. Term microcystin was first used when describing cyclic peptide hepatotoxins produced by the species *Microcystis aeruginosa* in 1965 (Konst et al., 1965). The toxicities and structures of these hepatotoxic peptides were also summarized in that study (Konst et al., 1965). These hepatotoxic peptides of cyanobacteria were first isolated from the genus *Microcystis* and therefore the toxins were named the microcystin (Carmichael et al., 1988). Since 1965, MCs have been studied globally by chemists, pharmacologists, biologists, and ecologists for decades. Nowadays, they are a cause of environmental health concern, due to their widespread presence in freshwater bodies throughout the world.

1.3.1 Chemical structure and properties

MCs are a group of monocyclic heptapeptides and they have a molecular weight of 800-1100 g.mole⁻¹ (EPA, 2015). The chemical structure of MCs (Figure 1.4) is cyclo-(D-Ala¹-L-X²-D-isoMeAsp³-L-Z⁴-Adda⁵-D-Glu⁶-M-Dha⁷-), where D-isoMeAsp is D-erythro- β -methyl-aspartic acid, M-Dha is N-methyl-dehydro-alanine, and Adda is an unusual C20 amino acid which is found only in cyanobacterial peptides and that is thought to be responsible for the bioactivity of the molecule (Welker and Von Döhren, 2006). X and Z are highly variable L-amino acids that contribute to the variability of structural variants and determine the suffix in the nomenclature of MCs (Carmichael et al., 1988). More than 100 different structural variants have been reported to data (Puddick et al., 2014). One of the most studied variants is MC-LR characterized by the presence of leucine (L₂) and arginine (R₄) as the variable amino acids.



Figure 1. 4 Chemical structure of microcystins (MCs).

X and Z in positions two and four are highly variable L-amino acids (L. Chen et al., 2016).

Microcystins are amphipathic molecules containing some hydrophilic functions, such as the carboxyl groups and the guanidino group in the frequently present arginine residue, and some hydrophobic parts such as the Adda residue (which contains two conjugated double bonds) (Vesterkvist and Meriluoto, 2003). The l-octanol/water partition coefficients (log P) of MC-LR, - LW and -LF have been estimated by HPLC to be 2.16, 3.46 and 3.56, respectively (Ward and Codd, 1999), which reveals that MC-LR is a more hydrophilic compound possessing arginine residue, compared with MC-LW and MC-LF which contain tryptophan and phenylalanine residue, respectively (Vesterkvist et al., 2012). Moreover, the n-octanol/water distribution ratio (D_{ow}) of MC-LR was reported to be pH dependent, and the log D_{ow} decreased from 2.18 at pH=1 to -1.76 at pH=10, especially in the pH range 6±9 at which cyanobacteria may flourish lower log D_{ow} ranging from 0 to -1 was determined (De Maagd et al., 1999).

MCs are soluble in water (the solubility of MC-LR in water is >1 g.L⁻¹) (Rivasseau et al., 1998), ethanol and acetone. MCs are relatively stable due to their double bonds and cyclic structure, they are also nonvolatile, resistant to high temperatures and changes in pH. They can not be denatured by boiling, nor can they be effectively removed by conventional drinking water treatment processes involving coagulation and filtration (Himberg et al., 1989). Other methods, such as chlorination and oxidation are also not recommended due to the formation of harmful by-products (Lawton and Robertson, 1999). The application of powdered or granulated activated carbon in

association with coagulation or filtration is an emerging effective technology for the removal of MCs from drinking water (Albuquerque Júnior et al., 2008; Lee and Walker, 2006).

In the laboratory studies, the decimal reduction time for dissolved MCs in water varied from 3 to 40 days, being usually less than 14 days (Lahti et al., 1997; A. K.-Y. Lam et al., 1995; a Lam et al., 1995). While the persistence of MC-LR in the aquatic environment was longer, the decimal reduction time for dissolved MC-LR in the lake Tuusulanjärvi (Finland) was 30 days (Lahti et al., 1997). At an enclosed site in the southwest corner of the Lake Centenary (Australia), MCs persisted at high levels (1300-1800 μ g.L⁻¹) for 9 days before significant degradation (Jones and Orr, 1994).

1.3.2 Microcystin-producing genera, biosynthesis, release and degradation in aquatic environment

MCs are produced by several genera of cyanobacteria (Figure 1.5), including *Microcystis, Oscillatoria, Nostoc, Anabaena, Planktothrix, Fischerella, Rivularia* and *Synechococcus*, but most frequently by the various species belonging to the genera *Microcystis* and *Anabaena* (Aboal and Puig, 2009; Fiore et al., 2009; Liu et al., 2011; Mohamed, 2008; Oksanen et al., 2004; Sivonen et al., 1992).



Figure 1. 5 Several representative genera of MC-producing cyanobacteria.

From left to right: *Microcystis* and *Planktothrix* (immunolabeling of MCs, indicated in brown, by Q. Qiao, 2015); *Oscillatoria* (by J. Kinross, 2002), *Nostoc* (by Loch an Eilein, 2005), and *Anabaena* (by Ardnave Loch, 2003), from http://algalweb.net/indext-no.htm.

The biosynthesis of MCs has been elucidated in *Microcystis aeruginosa* strains PCC7806 (Tillett et al., 2000) and K-139 (Nishizawa et al., 2000), and in Planktothrix agardhii (Christiansen et al., 2003). MCs are synthesized by giant multi-enzyme complexes composed of non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs) and additional modifying enzymes (Nishizawa et al., 2000; Tillett et al., 2000). The multi-enzyme complexes are organized into coordinate enzymatic sites, termed modules, where each module is responsible for one cycle of polyketide or polypeptide chain elongation (Cane et al., 1998). The MC synthesis gene (mcy) is organized in a cluster of above 55 kb encoding 10 open reading frames (ORFs), mcyA-mcyC encode five non-ribosomal peptide synthetase (NRPS) modules, mcyD encodes two type I PKS modules, and mcvE and mcvG encode hybrid NRPS-PKS modules (Moffitt and Neilan, 2004). The genes mcvF, mcvH, mcvI, and mcvJ encode for a racemase, an ABC transporter, D-3-phosphoglycerate dehydrogenase, and O-methyltransferase, respectively (Moffitt and Neilan, 2004). The assembly of MC starts with the activation of a phenylalanine-derived phenyl propionate starter unit at the NRPS-PKS hybrid enzyme McyG (Hicks et al., 2006). Catalytic domains of McyA to McyE and McyG are responsible for incorporation of precursors phenyl propionate, malonyl coenzymeA, S-adenosyl-Lmethionine, glutamate, serine, alanine, leucine, D-methyl-isoaspartate, and arginine (Rastogi et al., 2014). The elongated peptide is released from the enzyme complex by the thioesterase domain of McyC (Dittmann et al., 2013).

Typically, MCs are produced within the cyanobacterial cells and only released into the surrounding environment during cell senescence (death and lysis) (Whitton, 2012). For pure cultures of MC-producing strains, MCs apparently increase in concentration in the late logarithmic growth phase and the release of toxin occurs when the cultures shift from growth phase to stationary and death phases (Chorus and Bartram, 1999). In the field, MCs are released into the aquatic environment when the concentration of cyanobacteria reaches the peak and the release by cell lysis starts. The concentration of MCs in most freshwater bodies is generally below 10 μ g.L⁻¹, with high concentrations being only occasionally reported e.g. MC-LW (up to 98 μ g.L⁻¹) in the Lake Kovada (Turkey) (Gurbuz et al., 2009) and 7300 μ g.g⁻¹ dry weight MCs in a *Microcystis* bloom from the Lake Taihu (China) (Sivonen and Gary Jones, 1999).

Photodegradation is the main way to reduce the concentration of MCs in natural waters. The purified MCs are easily decomposed by UV light at wavelengths around the absorption maxima of

the toxins and the half-life of MC-LR by 147 μ W.cm⁻² UV irradiation is 10 min (Tsuji et al., 1995). UV irradiation isomerized MCs to the corresponding geometrical 4(*Z*) and 6(*Z*) -Adda MCs which showed much weaker toxicity (Tsuji et al., 1995). However, in a natural system, the rate of photodegradation is rather low, the half-life of MC-LR is estimated at 90–120 days per meter of water depth (Welker and Steinberg, 2000). Moreover, biodegradation is also one essential process for the reduction of MCs in the aquatic environment. Certain aquatic bacteria of different genera (e.g. *Actinobacteria, Sphingomonas, Sphingopyxis, Pseudomonas* and *Paucibacter*) have been reported to degrade different variants of MCs (Nybom, 2013). Additionally, bioaccumulation and adsorption on the particulate matter also facilitate the maintenance of low concentrations of bioavailable toxins.

1.3.3 Exposure, bioaccumulation and transmission of MCs

MCs exert harmful effects on humans either through direct contact or by means of consumption of toxin-contaminated water or food. Few studies on human have previously described exposure to MCs via drinking water, food, cyanobacterial dietary supplements, recreation activities and renal dialysis. Oral route mainly through drinking water, or by eating contaminated foods is a key MC exposure way. Contact with water during recreational activities is also one unneglectable exposure route. One survey of MCs in recreational lakes in Brittany (France) between 2004 and 2011 showed that the MC concentrations appeared higher than 13 μ g.L⁻¹ (chronic exposition guideline under current French regulation) in 15.5% of all analyzed samples, which poses a health hazard to children and young adults (Pitois et al., 2016). For aquatic organisms, it is even more frequent to be affected by MCs either through ingestion of cyanobacterial cells as food or through direct contact with toxins or consumed organisms, which have been contaminated. For example, several fish species including roach (Rutilus rutilus) (Kamjunke et al., 2002), silver carp (Hypophthalmichthys molitrix) (Chen et al., 2006), and bighead carp (H. nobilis) (Xia Zhang et al., 2008) are able to graze cyanobacteria cells in eutrophic lakes. Although there is no enough evidence to implicate MCs in occurrences of fish deaths, it has been reported that the presence of MCs in the diet of fish is potentially harmful to their physiology, morphology, and behavior, according to experimental data (Malbrouck and Kestemont, 2006).

In natural waters, MCs accumulate in a wide range of aquatic animals, including fish (Magalhães et al., 2003), shrimps (Chen and Xie, 2005), gastropods (J. Zhang et al., 2012) and bivalves (Paldavičienė et al., 2015). Fish, standing at the top of the aquatic food chain, are more
likely to accumulate a higher concentration of MCs when biomagnification occurs. To date, there have been extensive studies on the bioaccumulation of MCs in multiple organs and tissues of fish under laboratory condition and in the field (Table 1.1). Mezhoud and his team (2008) studied the distribution of labeled MC-LR in multiple tissues of medaka fish via gavage and their result showed a significantly high quantity of toxin accumulated in liver and gut tube after 3 and 24 h exposures. Djediat and his colleagues (2010) observed the presence of MC-LR in liver, spleen, and intestine of medaka fish after acute exposure by using immunohistochemistry. In the field, Xie and his team (2005) measured MC concentration in 8 species of fish in Lake Chaohu of China subjected to heavy cyanobacterial blooms, and their result indicated that fish were severely contaminated with a high content of toxin in intestines, blood, liver, bile, kidney, and muscle. Cazenave and his colleagues (2005) examined MC content in liver, gill, muscle and brain of Odontesthes bonariensis collected from a reservoir of Argentina, showing relative high bioaccumulation of MC-RR in liver and muscle. One survey of MC concentration in fish of 33 species from several tropical (Ugandan) and temperate (North American) lakes showed levels of up to 10-30 µg.kg⁻¹ wet weight in fish muscle (Poste et al., 2011). The report of EFSA (European Food Safety Authority) presented the extensive literature search conducted on the occurrence of cyanotoxins in food, and showed the broad occurrence of MCs in aquatic edible fish species throughout the world (European countries, North America, South America, African countries and Asian countries) (Testai et al., 2016).

Species	Toxin	Laboratory /Field study	Sampling	Method	Toxin distribution and concentration	Reference
Oryzias latipes	MC-LR	Gavage, 5 µg.g ⁻¹ body weight (bw)	24 h	Radiotraci ng	Mainly in liver and gut tube	(Mezhoud et al., 2008)
Oryzias latipes	MC-LR	Gavage, 5 µg.g ⁻¹ bw	2 h	Immunohi stochemist ry	Mainly in liver and intestine, also in spleen	(Djediat et al., 2010)
Hypophthalmichthys molitrix, Parabramis pekinensis, Cyprinus carpio, Carassius auratus, Culter ilishaeformis, Culter erythropterus, Pseudobagrus fulvidraco, Coilia	MC-LR MC- RR	Lake Chaohu (China)	Sep. 2003	High- performan ce liquid chromatog ram (HPLC)	Average content of MC-RR + MC-LR: 22.0, 14.5, 7.77, 6.32, 5.81 and 1.81 μ g.g ⁻¹ d.w. (dry weight) in intestines, blood, liver, bile, kidney and muscle, respectively	(Xie et al., 2005)

Table 1. 1 Some studies regarding the bioaccumulation of MCs in fish under laboratory condition and in the field.

ectenes

Laboratory: Jenynsia multidentata, Corydoras paleatus Field: Odontesthes bonariensis	MC- RR	Lab: balneation, 50 µg.L ⁻¹ Field: San Roque reservoir (Argentina)	Lab: 24 h Field: once in wet season and once in dry season of 2004	HPLC and LC-ESI- TOF-MS spectromet ry	Lab: 1.62, <below detectable limit, 0.56, 0.11 (<i>J. multidentata</i>) and 19.63, 2.09, 1.40, 0.04 (<i>C. paleatus</i>) μg.g⁻¹ f.w. (fresh weight) in liver, intestine, gills and muscle, respectively Field: 0.16, 0.03 and 0.05 μg.g⁻¹ f.w. in liver, gills and muscle, respectively (year average)</below 	(Cazenave et al., 2005)
33 species of fish	MC	Lake Erie and Ontario (temperate), Lake Victoria, Albert and Edward (tropical), and other smaller Ugandan lakes (George, Mburo, Nkuruba, Saka)	July-Sep. of 2006 and 2007	Anti-Adda enzyme- linked immunoso rbent assay (ELISA)	0.5-1917 and 4.5-215.2 μg.kg ⁻¹ of f.w. in muscle tissue and whole fish, respectively	(Poste et al., 2011)
Cyprinus carpio	MC	Lake Karla (Greece)	June and Aug. of 2010	ELISA	Toxin concentration: liver (181.91 ng.g ⁻¹) > kidney > brain > intestine > muscles (29.83 ng.g ⁻¹ f.w.)	(Papadimit riou et al., 2013)
Pomoxis nigromaculatus, Cyprinus carpio	MC-LR	Lake St. Marys (USA)	Summer of 2011 and 2012	LC- MS/MS	1.0 -70 μ g.kg ⁻¹ f.w. in <i>Pomoxis</i> 3.5 μ g.kg ⁻¹ f.w. in <i>Cyprinus</i>	(Schmidt et al., 2013)

MCs poorly penetrate vertebrate cell via passive transport through their membranes due to their hydrophilic nature, and therefore imply uptake via active transport for the tissue that presents a higher tropism for this molecule. Organic anion transporting polypeptides (OATP) are indeed known to transport MCs through cell membranes. Once within the cell, MC-LR is known to covalently binding to proteins phosphatases PP1 and PP2A (Campos and Vasconcelos, 2010), or to be biotransformed into intermediate products, which are excreted out of the organism and released back into the environment. The biotransformation of MCs starts with the formation of glutathione conjugates, which is catalyzed by glutathione S-transferase enzymes. These conjugates are further broken down to cysteine conjugates. Glutathione and cysteine conjugates of MC-LR still present a certain toxicity, although they are less toxic than free MC-LR (Campos and Vasconcelos, 2010). Therefore, organisms from the environment could also be secondarily affected by the conjugated MC-LR excreted from organisms.

In the aquatic food chain, zooplankton is known to be able to graze cyanobacteria (Davis et al., 2012; Oberholster et al., 2006), and being also a major part of the diet of zooplanktivorous fish.

Therefore, zooplankton has been proposed to be a potential vector for the trophic transfer of MCs to zooplanktivorous fish (Sotton et al., 2014). Additionally, MCs can be also transmitted to cultivated plants through irrigation with contaminated water (Crush et al., 2008) potentially impacting widely human populations.

1.3.4 Mechanisms of toxicity at molecular and tissue levels

The toxicity of MCs predominantly relies on their potent inhibition of eukaryotic protein serine/threonine phosphatases1 (PP1) and 2A (PP2A) by interacting with the catalytic subunits of these enzymes (Campos and Vasconcelos, 2010). It has been documented that MC-LR binds to the PP-1c α isoform through interactions at three sites of the enzyme, the hydrophobic groove, the Cterminal groove and the catalytic site (Goldberg et al., 1995). Another study reported that MC-LR binds to the surface pocket of PP2A through interactions with the two manganese atoms and the active site of the enzyme (Xing et al., 2006). The interaction between MCs and phosphatases comprises a two-step mechanism in which the first bind inactivate enzymes and covalent adducts are subsequently formed to render the binding irreversible (Campos and Vasconcelos, 2010). Moreover, MC-LR was found to inhibit protein phosphatase type 3 (PP3), and the order of inhibition potency is PP2A>PP3 ≈ PP1 (Honkanen et al., 1991). PP1, PP2A and PP3 are important regulatory enzymes by modifying the phosphorylation state of key control proteins and their protein substrates that function in multiple physiological reactions via phosphorylation cascades (Campos and Vasconcelos, 2010; Rastogi et al., 2014). The phosphatase inhibition effect of MCs leads to the hyperphosphorylation of these key control molecules. disrupting the normal phosphorylation/dephosphorylation regulation mechanism. Therefore, a variety of cellular processes is affected by exposure to MCs, such as DNA repair, cell apoptosis and death, the viability of cells and cytoskeleton organization (Rastogi et al., 2014) (Figure 1.6).



Figure 1. 6 Schematic representation of cellular and molecular effects by exposure of MCs (Rastogi et al., 2014).

MCs could impact DNA repair mechanisms in cell lines by affecting the phosphorylation mediated repair pathways (Lankoff et al., 2006a, 2006b). Toxin-induced phosphorylation leads to the loss of the protein kinase activity of the DNA-dependent protein (DNA-PK), which functions in the nonhomologous end joining (NHEJ) repair pathway (Lankoff et al., 2006b). MCs have been also suggested to induce cell apoptosis, necrosis and death by impacting various corresponding signal molecules and modifying certain upstream pathways. Through the inhibition of PP1 and PP2A, MC could activate the calcium-calmodulin-dependent multifunctional protein kinase II (CaMKII) and consequently induce cell apoptosis/death. Moreover, the expression of mitogenactivated protein kinases (MAPKs), which associates with the expression of genes (e.g. *c-jun*) involved in the cell growth and differentiation (Gehringer, 2004), is mediated by PP2A (Gehringer, 2004). This suggests that MC is implicated in the regulation of cell growth and differentiation

through modifying MAPKs expression levels. Another important molecule involved in MCmediated cellular toxicity is the nuclear phosphoprotein P53, which plays a role as a transcriptional trans-activator in DNA repair, apoptosis and tumor suppression pathways (Fu et al., 2005). It has been demonstrated that MC leads to an increase in the expression of P53 gene/protein in apoptotic hepatoma cell line (HepG2) cells, human amniotic cells, cultured hepatocytes, and rat liver tissues (Fu et al., 2005; Xing et al., 2009; Žegura et al., 2008).

MC-induced inhibition of PP1 and PP2A has been shown to also cause cytoskeletal disruption, as the structure and function of the cytoskeleton are highly influenced by regulated phenomena of phosphorylation and dephosphorylation (Toivola and Eriksson, 1999). In addition, multiple cytoskeletal and cytoskeleton-associated proteins (e.g. b-actin, tubulin, Desmin, Ezrin and Moesin) appear to be affected by MC-LR at transcriptional or protein levels (Chen et al., 2012; Zeng et al., 2014). A number of *in vivo* and *in vitro* studies have reported that MC-LR exposure induces the rearrangement or collapse of the three components of the cytoskeleton, namely microfilaments, microtubules and intermediate filaments in a variety type of cells, including the hepatocyte of human, mouse and rat, human epidermal skin cells, carp immune cells, and rat testes (M. Zhou et al., 2015). Since the cytoskeleton plays an important role in cellular structural stability, intracellular transport and signal transduction (Wickstead and Gull, 2011), the cytoskeletal rearrangement or collapse induced by MCs could lead to the hepatocyte deformation, plasma membrane blebbing, affect the proliferation, differentiation and apoptosis of hepatocytes, and consequently disrupt liver architecture that has been visually observed in several *in vivo* studies with light and fluorescence microscopy (Hooser et al., 1991; Jiang et al., 2013).

A biochemical feature of MC toxicity is the induction of oxidative stress through the production of ROS, leading to an increase in lipid peroxidation, mitochondrial damage such as membrane potential depolarization (MPD) and mitochondrial permeability transition (MPT), disruption of the antioxidant system and genome damage. ROS-mediated MC-LR toxicity has been detected in various cell lines using *in vitro* systems such as a human HepG2 (Nong et al., 2007), fish cell lines RTG-2 and PLHC-1 (Pichardo et al., 2007), and primary cultured rat hepatocytes (Ding et al., 2000), as well as in multiple organs of fish (Amado and Monserrat, 2010; Jos et al., 2005; Zhang et al., 2009), mice and rat (Chen et al., 2014; Li et al., 2008; Sun et al., 2011) with *in vivo* studies. The underlying mechanisms of MC-induced ROS production have been suggested to be related to the depletion of cytosolic glutathione (GSH) and the disruption of mitochondrial electron transport chain (ETC) (Rastogi et al., 2014). Disruption of ETC subsequently leads to

mitochondrial permeability transition and the release of apoptotic factors (e.g. Bcl-2, Bax and Bid) from mitochondria. The up-regulation of pro-apoptotic protein Bax and down-regulation of anti-apoptotic Bcl-2 are detected in both *in vivo* and *in vitro* studies upon exposure to MC-LR, and the consequent release of cytochrome C from the mitochondrion into the cytosol, and caspase cascade activation are also reported, resulting in apoptosis (Fu et al., 2005; Zhang et al., 2013a, 2013b).

MCs are mainly known for their hepatotoxicity properties. Reported LD_{50} values of MCs in fish, obtained by using intraperitoneal (i.p.) injections of extracted MCs, range from 25 to 1500 μ g.kg⁻¹ body weight (bw) (Malbrouck and Kestemont, 2006). Like small mammals, acute studies using i.p. injections or oral gavage of MCs in fish often observed a severe liver damage characterized by massive intrahepatic haemorrhage, disruption of the parenchymal architecture, hepatocyte swelling, cytoplasmic vacuolization and necrosis (Bury et al., 1997; Kotak et al., 1996; Li et al., 2005; Råbergh et al., 1991; W. Fournie and Countrney, 2002).

In the aquatic environment, fish are most likely subject to MCs over days or weeks. Chronic effects in fish exposed to environmentally relevant concentrations of MCs have been investigated under laboratory condition by balneation or diet administration, as well as in the field. A severe liver damage was observed in carp (*Cyprinus carpio*) following a diet containing greater than 130-2500 μ g.kg⁻¹ diet weight wet of MCs for 2 or more weeks (Carbis et al., 1996; Fischer and Dietrich, 2000; Li et al., 2004). A widespread liver damage was also observed in the majority of common carp (*Cyprinus carpio* L.) sampled from Lake Mokoan (Australia) with 22000-40000 μ g.kg⁻¹ bloom material (w.w) of MC-LR (Carbis et al., 1997). Besides, additional effects of MCs on kidney (Fischer et al., 2000), gill (C. Chen et al., 2016), growth (Bury et al., 1995), immune status (Qiao et al., 2013a) and reproductive performance (Qiao et al., 2013b; Zhao et al., 2015) have been reported in various species of fish following the chronic exposure.

Chronic exposure to low doses of MCs has been shown to promote tumors in humans and animals. The suppression manner of MCs on protein phosphatase PP1 and PP2A is similar to that of the known tumor promoter okadaic acid and therefore MCs were believed to act as a tumor promoter through the okadaic acid pathway (Nishiwakimatsushima et al., 1992). Furthermore, another study revealed that tumor necrosis factor- α (TNF- α) is an endogenous tumor promoter, and MC-LR and okadaic acid act as tumor promoters due to their TNF- α inducing function (Fujiki and Suganuma, 2011). Since MCs are preferentially incorporated by hepatocytes, the main organ where MCs may promote tumors is the liver. In a two-stage carcinogenesis study, MC-LR (below the acute toxicity level) dose-dependently increased the occurrence of positive foci for the placental form of glutathione *S*-transferase (a marker protein for preneoplastic liver foci) in rat liver initiated

with diethylnitrosamine (Nishiwakimatsushima et al., 1992). It was reported that the high incidence of primary liver cancer in Haimen and Fusui (China) might be resulted from drinking the MC contaminated water in some ponds and ditches (Ueno et al., 1996).

1.3.5 Reproductive toxicity of MCs

In addition to liver, MCs can also affect other organs, such as the brain, the heart, the intestine, the kidney, and the gonad. Among these organs, the toxic effect on reproductive organs has gained a large attention in the recent decade. Several field studies showed that MCs did not only accumulate in the liver, but also in the gonads of freshwater shrimp (Chen and Xie, 2005), snails (Chen et al., 2005), water bird, duck and fish (Chen et al., 2009), suggesting that gonad could be the second important target of MCs.

The potential reproductive toxicity of MCs has been investigated in different animal species, mainly in mouse, rat, and fish (Table 1.2). Microstructural and ultrastructural damages, apoptosis, oxidative stress in testes, sperm abnormality, and alteration of hormone levels have been extensively reported in male mice or rats acutely or chronically exposed to pure MC-LR (L. Chen et al., 2013; Chen et al., 2011; Dong et al., 2008; Li et al., 2008; Saad et al., 2012; Wang et al., 2012; X. Wang et al., 2013; Xiong et al., 2014; Zhou et al., 2013). Additionally, crude extracts of cyanobacterial blooms mainly containing *Microcystis aeruginosa* were used to conduct several acute and chronic studies, showing a similar toxic effect in the testis of male animals (Ding et al., 2006; D. Li et al., 2011; G. Li et al., 2011b, 2011c; Li et al., 2009; Xiong et al., 2010, 2009). These results showed that the reproductive system of male mammalian is sensitive to MCs. One study has also reported the reproductive toxicity of MCs on female mice, in which the apparent pathomorphological changes of the ovary including reduced primordial follicles and the unaltered follicle-stimulating hormone (FSH) or luteinizing hormone (LH) levels revealed that MCs seem to exert direct effect on the ovary, too (Wu et al., 2014).

Compared with rat and mouse, fish are potentially often exposed to the MCs either by natural ingestion of contaminated water or food, or to a lesser extent by breathing when the toxins pass through gills in the aquatic environment. The studies referring to the reproductive toxicity of MCs in fish have been paid more attention in the past five years. Trinchet and her colleagues (2011) observed numerous lytic areas in the seminiferous tubules and the destruction of the gonadal somatic tissue in the ovary of medaka chronically exposed to 5 μ g.L⁻¹ of MC-LR. In another field study, they suggest the presence of MCs in the gonadal somatic tissue of fish by using

immunohistochemical method (Trinchet et al., 2013). Qiao and her colleagues (2013) reported a decline in reproduction output, remarkable ovarian lesions and high apoptosis rates in female zebrafish chronically exposed to 5 μ g.L⁻¹ of MC-LR. Other *in vivo* studies reported more toxic effects induced by MCs in male and female fish, with regard to the reproductive function, including the alteration of oogenesis and spermatogenesis, oxidative stress in reproductive organs and the disturbance of hypothalamic-pituitary-gonadal (HPG) axis (Hou et al., 2016, 2014; Liu et al., 2016; Su et al., 2016; Zhao et al., 2012, 2015). These findings indicate that MC exposures could cause significant reproductive impairment in fish by directly affecting gonadal function or indirectly disturbing HPG axis. However, more information regarding HPG axis-involved modulation, clear proofs of the presence of MCs in gonads, and chronic effects with low doses of the toxin is needed. For example, the implication of MC-induced liver impairment on reproduction parameters, via general metabolic and more specific mechanisms needs to be further investigated.

Test	Toxin	Exposure	Exposure	Main results	References
organism		route and does	time		
Male zebrafish	MC-RR	i.p. 2 mg.kg ⁻¹ bw	2, 6, 24 h	Testes: ultrastructural damage, showing widened intercellular junction, distention of mitochondria oxidative stress proteomic results, alteration of 24 proteins involved in cytoskeleton assembly, oxidative stress, glycolysis metabolism, calcium ion binding	(Zhao et al., 2012)
Female zebrafish	MC-LR	i.p. 50, 200 μg.kg ⁻¹ bw	1, 3, 12, 24, 48, 168 h,	In ovary: pathological lesions, oxidative stress, MDA, CAT, GPx↑, GST, GSH↓	(Hou et al., 2014)
Female and male zebrafish	MC-LR	Balneation 0.5, 5, 15, 50 μg.L ⁻¹	6 d	The spawning activity and success↓	(Baganz et al., 1998)
Female zebrafish	MC-LR	Balneation 2, 10, 50 μg.L ⁻¹	21 d	Abnormal previtellogenic oocytes growth and maturation eggs production, fertilization and hatching rates↓, modulation of E2, testosterone, and VTG, modulation of oogenesis related gene expression including gnrh3, gnrhr1, fshb, fshr, lhr, bmp15, mrpb, ptgs2 and vtg1	(Zhao et al., 2015)
Female and male zebrafish	MC-LR	Balneation 5 μg.L ⁻¹	30 d	In ovary: vitellus storage↓, lysis of the gonadosomatic tissue, disruption of the relationships between the follicular cells and the oocytes In testis: spermatogenesis was disrupted.	(Trinchet et al., 2011)
Female and male zebrafish	MC-LR	Balneation 1, 5, 10, 20 μg.L ⁻¹	30 d	The hatchability and E2↓ Whole body VTG levels ↑ in females,↓ in males VTG1 transcriptional level ↑ in liver	(Qiao et al., 2013b)

Table 1. 2 Summary of *in vivo* studies regarding the reproductive toxicity of MCs in fish.

Marked histological lesions in livers,

ovaries and testes

Female and male zebrafish	MC-LR	Balneation 1, 5, 20 μg.L ⁻¹	30 d	Females: serum E2, T, 11-KT and FSH↑ Males: modulation of T, FSH and LH retarded oogenesis and spermatogenesis transcriptional changes of 22 genes of the HPG axis	(Liu et al., 2016)
Zebrafish larvae (5 d post- fertilization)	MC-LR	Balneation 0.3, 3, 30 μg.L ⁻¹	90 d	Males: growth inhibition, testicular damage, delayed sperm maturation T/E ₂ ratio ↓hepatic vtg1 mRNA expression↑ changes in transcriptional responses of HPG-axis related genes	(Su et al., 2016)
Zebrafish larvae (5 d post- fertilization)	MC-LR	Balneation 0.3, 3, 30 μg.L ⁻¹	90 d	Females: growth inhibition, ovarian ultra- pathological lesions, ovary weight and ovarian $T\downarrow$ changes in transcriptional responses of HPG-axis related genes, brain <i>FSH</i> β , <i>LH</i> β , and ovarian <i>ERa</i> , <i>FSHR</i> , <i>LHR</i> \uparrow hepatic <i>vtg1,ERa</i> , and ovarian VTG content \downarrow	(Hou et al., 2016)
Bream and roach	MC- producing blooms	Field sampling		lesions in the ovary Immunohistochemistry: MCs present in the gonadosomatic tissue, but not in the oocytes	(Trinchet et al., 2013)

The reproductive toxicity of MCs has been also explored with *in vitro* studies by using isolated reproductive cells of the male mouse and rat. Declined cell viability, cell apoptosis and oxidative stress were usually found in spermatogonia, Sertoli and Leydig cells acutely exposed to MC-LR (Yu Chen et al., 2013; Li et al., 2008; Li and Han, 2012; Wang et al., 2012; Zhang et al., 2011; Y. Zhou et al., 2015; Zhou et al., 2014). It is noteworthy that at least 5 types of OATP transporter can be expressed in isolated rat spermatogonia, and MC-LR was found to be able to immigrate into spermatogonia with *in vitro* study (Zhou et al., 2012). However, to date, according to *in vivo* studies, no evidence regarding the incorporation of MCs into reproductive cells has been reported yet.

Previous studies showed a strong association between reproductive toxicity and MC exposure, at multiple doses and routes of exposures, and in different organisms. The underlying mechanisms have been proposed previously (L. Chen et al., 2016), as shown in Figure 1.7. MCs are transferred into various organs and tissues, and they mainly accumulate in the liver but also in the gonad. Within the gonad, MCs potently inhibit protein serine/threonine phosphatases, causing a series of cytotoxic response, e.g. apoptosis, cytoskeleton disruption, cellular proliferation perturbation. MCs also disrupt the gonadal structure and function through the induction of oxidative stress, leading to tissue lesion, mitochondrial dysfunction and DNA damage. In addition to this direct effect on

gonads, MCs indirectly affect sex hormones by disturbing the HPG axis. Moreover, liver dysfunction caused by MCs also contributes to the reproductive impairment by mediating the global organism's metabolism, and especially for oviparous vertebrates since the liver is essential for reproductive process linked to oogenesis.



Figure 1. 7 Schematic review of the reproductive toxicity of MCs (L. Chen et al., 2016).

1.4 Other secondary metabolites of cyanobacteria

Cyanobacteria produce a multitude of secondary metabolites that are structurally and biochemically diverse. According to their structural classification, cyanobacterial secondary metabolites can be divided into fatty acid derivatives, terpenes, saccharides and glycosides, peptides, polyketides and lipopeptides (Voloshko et al., 2008). Some of these compounds were revealed to be toxic towards aquatic and terrestrial organisms. According to their toxicity mechanisms and/or to their organ tropism, these toxic metabolites, so called cyanotoxins, are classified as hepatotoxins (e.g. MCs and nodularins), cytotoxins (e.g. cylindrospermopsins and lipopolysaccharides), neurotoxins (e.g. saxitoxins, anatoxins and BMAA) and dermatotoxins (e.g. lyngbyatoxin-A and aplysiatoxin). Other compounds frequently produced by bloom-forming cyanobacteria include cyanopeptolins, anabaenopeptins, microviridins, microginins and aeroginosins, whose physiological functions remain predominantly unknown to date. Some of them were considered as inhibitors of key enzymes in eukaryotic cells, but the toxic effects are far less potent.

1.4.1 Other hepatotoxins

Nodularins (NODs) are a class of hepatotoxic cyclic pentapeptides that are mainly produced by *Nodularia spumigena*. Structures of NODs are closely related with MCs, possessing a ring structure built up by 5 amino acids (one is variable amino acid). The structure of nodularin (Figure 1.8) is cyclo-(D-MeAsp-L-Arg-Adda-D-Glu-Mdhb), sharing following amino acids Adda, D-Glu, D-MeAsp and L-Arg in common with MCs (Yun Chen et al., 2013). The primary target organ of NODs is the liver and the toxicity of NODs is based on the inhibition of protein phosphatases. In contrast to MCs, NODs do not bind covalently to protein phosphatases (Bagu et al., 1997). NODs are present prominently in blooms from brackish waters, such as the Baltic Sea, Brackish water estuaries and coastal lakes of Australia and New Zealand, and some lakes with low salinities that might be still suitable for drinking (Chorus and Bartram, 1999). However, NODs have not been studied as extensively as MCs due to their limited distribution.



Figure 1.8 Chemical structures of representative hepatotoxins produced by cyanobacteria (Dittmann et al., 2013).

Apart from MCs and NODs, two typical hepatotoxins, cylindrospermopsin (CYN), a cyclic sulfated guanidine alkaloid produced by various species of freshwater cyanobacteria, has also been classified as a hepatotoxin, but CYN is also known as cytotoxin and neurotoxin. CYN is a highly water-soluble zwitterion consisted of a sulphated and methylated tricyclic guanidine moiety that is linked to uracil via a hydroxylated carbon (Figure 1.8) (Dittmann et al., 2013). Several species of cyanobacteria, including *Cylindrospermopsis raciborskii*, *Umezakia natans, Aphanizomenon* sp., *Raphidiopsis curvata, Lyngbya wollei* and *Anabaena bergii*, have been implicated in the production of CYN (B-Béres et al., 2015). CYN is present in the bodies of freshwater worldwide, being identified in Antarctica, Australia, South and North America as well as Europe (Dittmann et al., 2013). Unlike MCs, over 90% of total CYN quota is present in the extracellular form, which makes CYN to be primarily considered an extracellular toxin. High concentrations of CYN have often been observed in freshwater, exceeding 500 μ g.L⁻¹ in Australia (Saker and Eaglesham, 1999) and 100 μ g.L⁻¹ in drinking water.

1.4.2 Non-hepatotoxins

Cyanobacteria are well known to produce certain hepatotoxins (e.g. microcystins, nodularins and cylindrospermopsins). However, the toxic compounds produced by cyanobacteria are far more abundant than these hepatotoxins. Some compounds that induce neurotoxic, cytotoxic and other toxic effects, also cause a serious threat to domestic and wild animal life as well as for human health.





Saxitoxin (STX) is one of the most potent cyanobacterial neurotoxins. It is produced by eukaryotic dinoflagellates in marine waters and by freshwater cyanobacteria of the genera *Anabaena* (Negri and Jones, 1995), *Cylindrospermopsis* (Lagos et al., 1999), *Aphanizomenon* (Pereira et al., 2004), *Planktothrix* (Pomati et al., 2000) and *Lyngbya* (Onodera et al., 1997). Its basic structure is a trialkyl tetrahydropurine (Llewellyn, 2006) with the NH₂ groups at the positions 2 and 8 of the purine ring (Figure 1.9). STX and its more than 50 naturally occurring derivatives (Wiese et al., 2010) that differ structurally at four positions around the ring, are known as paralytic shellfish toxins (PSTs), also collectively termed as saxitoxins (STXs). STX is more toxic than its derivatives with the i.p. LD_{50} value of 10 µg.kg⁻¹ bw for mice (Chorus and Bartram, 1999).

In addition to STX, anatoxin-a (ATX-a) is another important neurotoxic alkaloid, isolated for the first time from the cyanobacterium *Anabaena flos aquae* (Devlin et al., 1977). Its structure is a bicyclic secondary amine with a ketone group (Figure 1.9). ATX-a and its derivatives are known to be produced by a broad spectrum of cyanobacterial genera including *Anabaena* (Harada et al., 1989), *Aphanizomenon* (Selwood et al., 2007), *Cylindrospermum* (Sivonen et al., 1989), *Oscillatoria* (Sivonen et al., 1989), *Microcystis* (Park et al., 1993), *Raphidiopsis* (Namikoshi et al., 2004), *Planktothrix* (Viaggiu et al., 2004), or *Nostoc* (Ghassempour et al., 2005).

 β -*N*-methylamino-L-alanine (BMAA), a cyanobacterial neurotoxin, has gained increased attention in recent years. Its structure consists of the amino acid alanine with a methylamino group on the side chain (Figure 1.9). BMAA is a non-proteinogenic amino acid, which has been proposed to be produced by all known groups of cyanobacteria, including cyanobacterial symbionts and free-living cyanobacteria (Cox et al., 2005). It may accumulate through higher trophic orders of the food chain resulting in neurodegenerative disease in animals and humans (Pearson et al., 2016). BMAA has been connected with incidents of amyotrophic lateral sclerosis (ALS) and dementia on the island of Guam (Cox et al., 2003). The observation of high BMAA levels in relevant central nervous system (CNS) areas in Alzheimer's disease and ALS patients in North America (Pablo et al., 2009) further suggested that BMAA acts as a possible pathogenic factor in human neurodegenerative disease.



Figure 1. 10 Chemical structures of representative dermatotoxins produced by cyanobacteria (Dittmann et al., 2013).

Some species of cyanobacteria are able to produce several toxic substances that target human skin, these substances are usually called dermatotoxins. Lyngbyatoxins (LAs) are indole alkaloids, produced by filamentous cyanobacteria, marine *Lyngbya majuscule* and freshwater *Lyngbya wollei* (Rzymski and Poniedziałek, 2012). Three isoforms of lyngbyatoxin have been identified and the structure of lyngbyatoxin A is identical to an isomer of teleocidin A (Figure 1.10). LAs are the causative agent of blistering dermatitis that was frequently reported by surfers in Hawaii (Cardellina et al., 1979). Lyngbyatoxin A also has a skin tumor promoting activity. The i.p. LD_{50} of lyngbyatoxin A for mice is 250 µg.kg⁻¹ bw.

Like LAs, aplysiatoxin (APX), first isolated from marine mollusks feeding upon *Cyanobacteria* species from *Lyngbya*, *Schizothrix* and *Planktothrix* genus, is capable of inducing contact dermatitis through the activation of protein kinase C. The i.p. LD_{50} value for mice is 107 and 117 μ g.kg⁻¹ bw for 3- and 5-week-old mice, respectively (Ito and Nagai, 1998). Environmental concentrations of aplysiatoxin and bioaccumulation in aquatic organisms have not yet been assessed.

1.4.3 Other bioactive compounds

In addition to the potent toxins described above, cyanobacteria also produce a wide variety of linear (e.g. aerugenosins and microginins) and cyclic (e.g. anabaenopeptins, anabaenopeptilides, microviridins, nostopeptilides) peptides, which are not apparently toxic but have other bioactivities, some of which can be considered as deleterious.

Cyanobacterial peptides possessing serine protease inhibition activities were classified into four groups consisting of cyclic depsipeptides, tricyclic depsipeptides, linear peptides, and cyclic peptides by Nanikoshi and Rinehart (Sivonen and Börner, 2008). Aeruginosins was assigned into the group of the linear peptide, along with microcins. Aeruginosins was firstly isolated from *Microcystis aeruginosa* NIES-298 (aeruginosin 298-A, Figure 1.11) (Murakami et al., 1994). Subsequently, aeruginosins have been isolated from different genus and species of cyanobacteria, including *Microcystis viridis* and *Oscillatoria sp.*, and also from marine sponges of the family Dysideidae. The general structure of aeruginosins is composed of an N- terminalhydroxy or acidic group, a bulky hydrophobic amino acid, a 2-carboxyperhydroindole core, and a C-terminal guanidine-containing group (Ersmark et al., 2008). Aeruginosins have gained attention due to their activities as inhibitors of trypsin, chymotrypsin, and other serine proteases. These enzymes are involved in a number of important physiological processes, particularly associated with complex blood coagulation cascade.



Figure 1. 11 Chemical structures of representative aeruginosins, cyanopeptolins and cyclamides produced by *Microcystis* (Voloshko et al., 2008).

The group of cyclic depsipeptides characterized by the unique amino acid 3-amino-6-hydroxy-2-piperidone (Ahp) is composed of variants of micropeptins, cyanopeptolins, oscillapeptins, nostopeptins, aeruginopeptins, and anabaenopeptilides. Cyanopeptolins are one of the most common compounds in this structural group. The structure of cyanopeptolins An isolated from *Microcystis* sp. PCC7806 (Martin et al., 1993) is shown in Figure 1.11. Cyanopeptolin is a 19membered cyclic depsipeptide possessing the Ahp moiety, which is essential for its protease inhibition activity. Cyanopeptolin 1020 isolated from cyanobacterium *Microcystis* was reported to potently inhibit trypsin with an IC₅₀ value of 0.67 nM (Gademann et al., 2010). Interestingly, exposure of zebrafish embryos to cyanopeptolin 1020 resulted in differentially transcriptional alteration of genes related to various important biological and physiological pathways concerning DNA damage recognition and repair, circadian rhythm, response to light, and to some extent metabolic activities (Faltermann et al., 2014).

Cyclamides are small cyclic peptides that characteristically contain multiple thiazole, thiazoline, oxazole, and oxazoline rings. They are produced by the symbiotic cyanobacterium *Prochloron* sp. Some compounds from this class were also documented to be produced by *Microcystis aeruginosa* (e.g. microcyclamide, Figure 1.11) (Ishida et al., 2000). Although the biological function or molecular action of cyclamides are not fully understood, many compounds of this class possess noticeable cytotoxic properties (Voloshko et al., 2008).

1.4.4 Potential estrogenic compounds

Numerous studies have documented hazardous potential of toxic compounds produced by cyanobacteria. However, there are still a lot of unidentified, biologically active cyanobacterial secondary metabolites, as it has been highlighted in various toxicological investigations with complex mixtures of cyanobacterial extract (Lecoz et al., 2008; Marie et al., 2012). It has been hypothesized that some of these compounds might possess adverse effects on endocrine system in organisms, namely, they act as endocrine disruptors. The definition of endocrine disrupting chemicals (EDCs) has been presented in the document of International Programme on Chemical Safety (IPCS) and the World Health Organization (WHO): "An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (IPCS and WHO, 2002).

To date, compounds produced by cyanobacteria have been hypothesized to exhibit one specific endocrine disrupting effect, the estrogenicity, by binding to the estrogen receptors and mimicking the action of endogenous estrogen, which consequently disturbs the normal functioning of the endocrine system. A restricted number of publications suggested that MCs could exhibit such estrogenic bioactivity. However, there has been only one *in vitro* study which showed an activation of the estrogen-regulated luciferase gene in stably transfected cell line following MC-LR exposure, implying that MC-LR possesses estrogenicity (Oziol and Bouaïcha, 2010). In contrary, in another *in vivo* study, a significant up-regulation of the estrogenicity biomarker, *vtg* gene, was detected by using microarray in larval zebrafish exposed to MC-producing cyanobacterial extracts, but not to the pure MC-LR alone (Rogers et al., 2011).

More studies have focused on other potential estrogenic compounds from cyanobacteria. The extract of *Planktothrix agardhii* (intracellular metabolites) was reported to have a potency of estrogenic equivalents (EEQ) of 3.8 ng 17β-estradiol.g⁻¹ d.w. with *in vitro* reporter gene transactivation assays (Štěpánková et al., 2011). A great estrogenic potency for the exudates of Microcystis aerigunosa was also attested in the same team by using the same in vitro assay (Sychrová et al., 2012). Additionally, the presence of some sterol-like compounds in the exudates of some cyanobacterial cultures (Nostoc ellipsosporum, Nostoc muscorum, Anabaena oryzae and Anabaena sp.) was confirmed, and these compounds could be correlated with the disruption of the sex hormonal levels of mice exposed to the exudates (Essa and Fathy, 2014). Indeed, 8 different isoflavones, which have the capacity to bind to estrogen receptors and induce vitellogenin synthesis, were detected and quantified in the cyanobacterium Nostoc (Klejdus et al., 2010). All these findings further support the idea that the extracts or exudates of certain strains of cyanobacteria could induce estrogenic effects, suggesting that the presence of some still-undetermined compounds produced by cyanobacteria might be the cause of estrogenic response in organisms. However, to date, no specific compound concerning estrogenic effects has been identified in the cyanobacterial extracts or exudates, and further studies are urgently needed.

1.5 Medaka fish, a model organism of aquatic vertebrates in ecotoxicological studies

Japanese medaka (*Oryzias latipes*) is a member of the *Adrianichthyidae* family of the *Beloniformes* order. It is a small, egg-laying freshwater fish that is native to East Asian countries, primarily Japan, Korea, and China. In Japan, it can be usually found in small rivers, creeks and rice fields (Figure 1.12).

1.5.1 History and features of medaka

Medaka has been reared as an ornamental fish since the Edo period of the seventeenth to the nineteenth centuries in Japan. It was first described scientifically in Siebold's *Fauna Japonica* in 1850 (Temminck and Schlegel, 1850) and, in 1906, Jordan and Snyder named this species *Oryzias latipes* (Jordan and Snyder, 1906). In 1921, the systematic genetic analysis of medaka showed the occurrence of crossing over between X and Y chromosomes (Aida, 1921). After that, genetic studies in medaka have focused on pigmentation and sex determination. Aida (1921) found the sex-

limited inheritance of the r locus that controls the expression of orange pigment cells (xanthophores). Based on this discovery, the d-rR strain that showed body-color dimorphism with the male orange-red and female white was established in 1953 (Yamamoto, 1953). The key reference for medaka as a genetic model system was published in 1975 (Yamamoto, 1975), and subsequent important advances included the establishment of an efficient method for making transgenic medaka in1986 (Ozato et al., 1986), and the establishment of several inbred lines from genetically different natural populations in 1985 (Hyodo-Taguchi and Egami, 1985). From around 2000, the establishment of genetic/genomic resources of medaka has gained a great development. The draft genome sequence of medaka was published in 2007 (Kasahara et al., 2007). All genetic/genomic data are now available online through the UT genome browser (http://medaka.utgenome.org/), Ensembl genome browser (http://www.ensembl.org/Oryzias latipes/index.html), and UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway). Medaka possesses a relatively small genome (700 Mb) that is around half the size of the one for zebrafish (1800 Mb) (Naruse et al., 2004). It also has 24 chromosome pairs that largely maintain ancestral vertebrate syntenic relationships present throughout the vertebrate classes, including humans (Naruse et al., 2004). Apart from these genome resources, isolation of mutants with a specific phenotype for different research purposes has also been conducted in several laboratories and about 500 mutants have been established (Furutani-Seiki et al., 2004; Yokoi et al., 2007).



Figure 1. 12 The Japanese medaka Oryzias latipes.

(A) Medaka lives in small rivers and creeks, such as irrigation systems of rice fields. (B) The pigmentation of wild medakas is shown. Note also the sexual dimorphism of the dorsal fin. The male dorsal fin has a slit (arrowhead in inset) (Kirchmaier et al., 2015).

Japanese medaka is a teleost model fish. The teleost group includes other model organisms, such as zebrafish (*Danio rerio*), tilapia (*Oreochromis niloticus*), fugu (*Takifugu rebripes*),

Tetraodon (*Tetraodon nigroviridis*), and the three-spined stickleback (*Gasterosteus aculeatus*). The evolutionary relationship of medaka and other teleost model is shown in Figure 1.13 (Kirchmaier et al., 2015). Medaka is closely related to fugu, tetraodon and stickleback, while it is separated from zebrafish by ~150 million yeas (Mya) of divergent evolution (Kirchmaier et al., 2015).



Figure 1.13 Evolutionary relationships of medaka and other teleost model systems.

Only teleost model systems with publicly available genome sequence data on Ensembl are shown (Kirchmaier et al., 2015).

Adult medaka fish reach 3-4 cm in body length. Male and female fish can be distinguished easily by observing the size and shape of the dorsal fin and anal fin from the side. The dorsal fin of the male is longer than that of the female and has a saw-toothed distal edge with a cleft at the posterior margin. In the female, the anal fin is smaller than that of the male; it has a right-angled triangle shape, and narrowed posterior edge ends (Figure 1.12) (Shinomiya et al., 2009).

Medaka is a favorite experimental animal for researchers in Japan and other countries. It can tolerate a wide range of temperatures (4-40 °C) (Naruse et al., 2009) and adapt to high environmental salinities (Inoue and Takei, 2002). Like zebrafish, it is oviparous and its embryonic development occurs externally and embryos, particularly the pigment-less mutants, are completely transparent allowing easy visualization of all stages of early development (Wittbrodt et al., 2002).

1.5.2 Reproduction and embryonic development

The gonad development of medaka is under the control of hypothalamic-pituitary-gonadal (HPG) axis. One form of estrogen, 17β -estradiol (E2), plays critical roles in oocyte growth. E2 synthesized in the follicular layer is transported to the liver and stimulates the synthesis of a specific glycolipophosphoprotein, vitellogenin, which is the precursor protein of egg yolk (Okubo et al., 2009). The liver also synthesizes choriogenin, the precursor protein of egg envelope, in response to E2 (Okubo et al., 2009). For males, two types of testicular somatic cells, Sertoli and Leydig cells, play critical roles in steroidogenesis and spermatogenesis. Medaka becomes sexually mature at around 6 months of age under the laboratory condition (Ding et al., 2010). Spawning tightly correlates with light cycles and the appropriate reproductive condition is 14 h light/10 h dark at 25-28 °C (Wittbrodt et al., 2002). Medaka fish usually spawn within the first hour after the light is turned on. Female fish spawn between 30 and 50 eggs per day during the reproductive period (Wittbrodt et al., 2002).

The appropriate temperature for the growth of medaka embryos is 25-30 °C, but the embryonic development can be arrested at 10 °C and resumes at 25 °C (applicable until the blastula stage), which makes it possible to control the embryonic development by adjusting temperatures in the laboratory (Okubo et al., 2009). The embryonic development follows the typical teleostean pattern. The process of development can be divided into 39 stages based on diagnostic features under light microscopy observation (Iwamatsu, 2004). Embryos hatch after 7-8 days at 28 °C.

1.5.3 Histology of the gonad

In most teleosts, the ovary is a hollow bilaterally paired structure attached to the dorsal peritoneum. However, only one ovary develops in medaka (Dietrich and Krieger, 2009a). Medaka possesses an asynchronous-type ovary, in which various stages of oocytes are present simultaneously. The oocyte development of medaka can be classified into four different phases: previtellogenic, vitellogenic, postvitellogenic, and ovulation phases (Okubo et al., 2009). In previtellogenic phase, oocytes with a diameter of 20-150 µm are located adjacent to the ovarian epithelium. The oocyte cytoplasm is stained with hematoxylin uniformly (perinucleolar oocyte), although late previtellogenic oocytes may have vacuoles in the cytoplasm (cortical–alveolar oocyte). The oocyte surface is extended to numerous microvilli, around which the chorion precursor material begins to accumulate in patches. Entering vitellogenic phase, the oocytes increase in size from a diameter of around 150 µm to 800 µm. Yolk nucleus disappears, and the yolk vesicle is developed

in the cytoplasm. Yolk globules, which are stained strongly with eosin, appear around yolk vesicles. In the late vitellogenic phase, yolk globules become larger in the center of the oocyte. Yolk vesicles are pushed out to outer parts in the oocyte cytoplasm. In late vitellogenic phase, oocytes are 800-1200 μ m in diameter. The yolk mass occupies most of the oocyte, and a well-developed egg envelope is still attached with the follicular layer, and is weakly stained with eosin. In ovulation phase, mature oocytes are separated from the follicle layer and ovulated into the ovarian cavity (Figure 1.14) (Dietrich and Krieger, 2009a; Okubo et al., 2009).



Figure 1. 14 Various stages of reproductive cells in the ovary and testis of adult medaka fish.

Ovary (left), PO, perinucleolar oocyte; CO, cortical–alveolar oocyte; EV, early vitellogenic oocyte; LV, late vitellogenic oocyte; M, mature oocyte (by Q. Qiao, 2015). Testis (right), 1: spermatogonia; 2: early primary spermatocyte; 3: late primary spermatocytes (dividing diplotene) or secondary spermatocyte; 4: spermatids; 5: spermatozoa. (by Courtesy of J.W. Fournie, U.S.EPA, Gulf Breeze, Florida.) (Dietrich and Krieger, 2009a).

In male fish, spermatogenesis takes place following the head-tail and periphery-central lumen direction. Primary spermatogonia are individually surrounded by Sertoli cell processes, and secondary spermatogonia are smaller than primary spermatogonia, with large, lightly basophilic nuclei and little cytoplasm. Spermatocytes are smaller and have increasingly dense basophilic nuclei. After undergoing the second meiotic division, spermatocytes enter to spermatid stage, which have condensed, intensely basophilic nuclei and very little cytoplasm. Spermatids undergo maturation events that include nuclear shape changes, development of a flagellum, loss of cytoplasmic volume, and phagocytosis of cytoplasmic remnants prior to their release into the lobular lumen as spermatozoa (Figure 1.14) (Dietrich and Krieger, 2009b; Okubo et al., 2009).

1.6 Structure and Objectives of the thesis

Based on the previous researches, the present study was conducted in order to describe the toxic effects of MCs and of the complex metabolite extract of one species of *Microcystis* on fish liver and gonad, using both in-depth cellular and molecular approaches providing an important basis for further exploration of the reproductive toxicity of MCs and other compounds produced by cyanobacteria.

In the present study, adult medaka fish *Oryzias latipes*, aquatic vertebrate model organism, were used as the experimental animals. MC-LR was selected as the model of MC, because of its wide distribution and high toxicity. The study comprised the investigation of toxicological parameters on one acute and one chronic exposure, as shown in Figure 1.15.



Figure 1. 15 Flow chart of the acute and chronic exposure.

The objectives of this study are:

1) to describe the sexual dimorphism in adult medaka fish liver through multi-omic approach, emphasizing the importance of liver function in the reproductive process for oviparous vertebrates.

2) to assess presence and accumulation of MC-LR in gonadal tissue by immunohistochemistry upon acute and chronic exposure, respectively.

3) to evaluate the possible adverse effects on gonad function of chronic exposure to MC-LR and to the cyanobacterial extracts.

4) to investigate the possible liver dysfunction and the consequence on the biosynthesis of oviparous reproduction-related proteins vitellogenin (VTG) and choriogenin (CHG) upon chronic exposure to MC-LR and to the cyanobacterial extracts.

5) to determine the possible estrogenic potency of MC-LR and the cyanobacterial extracts.

6) to compare the effects induced by MC-LR and the cyanobacterial extracts.

The structure of the thesis comprises five chapters.

Chapter 1: the current knowledge about cyanobacteria, cyanobacterial blooms and cyanotoxins is summarized. The big part of the introduction concerns on genus *Microcystis* and MCs.

Chapter 2 (article 1): the sexual dimorphism in adult medaka fish liver is investigated by using histological, metabolomic, proteomic and transcriptomic approaches, which provides important information for further investigation of hepatotoxic effects in fish. The metabolomic and proteomic parts are collaborative work with other people in my team. For metabolomics part, the data acquisition and analysis were conducted by Dr. Benoît Sotton (post-doc). For the proteomic part, the data acquisition and preliminary analysis were performed by Séverine Le Manach (Ph.D. student), the post-processing of data treatment was conducted by myself.

Chapter 3 (article in preparation): the histopathological change and immunolocalization of MC are investigated in the adult medaka fish acutely exposed to 10 μ g.g⁻¹ b.w. of pure MC-LR for 1 hour. All the experimental processes were conducted by myself.

Chapter 4 (article 2): the potential reproductive toxicity of MC is evaluated in the adult medaka fish chronically exposed to environmentally relevant concentrations of cyanotoxins (1 and 5 μ g.L⁻¹ MC-LR, and extract of MC-producing *Microcystis aeruginosa* PCC 7820 with 5 μ g.L⁻¹ of equivalent MC-LR) for 28 days. For the proteomic part, the data acquisition and preliminary analysis were performed by Séverine Le Manach, the post-processing of data treatment was conducted by myself. Other parts of this experiment were performed by myself.

Chapter 5: the summary, general discussion, perspectives and conclusion of the study is presented.

Chapter 2 Sexual dimorphism in adult medaka fish liver highlighted by multi-omic approach

2.1 Introduction

The liver is a key organ that plays fundamental roles in various physiological processes, including digestion, energetic metabolism, xenobiotic detoxification, biosynthesis of serum proteins, and also in endocrine or immune responses. Because of the different metabolic needs between sexes, especially during the reproductive phase, the liver is one of the most sexually dimorphic organs in terms of gene expression (Roy and Chatterjee, 1983). The first evidence of a sex-related difference in the rat hepatic steroid metabolism was published in 1953 (Hübener and Amelung, 1953). Based on these initial observations, five decades of research have since then established the existence of an HPG-liver axis that determines the differences between male and female liver. Moreover, the importance of hormone secretion patterns has been revealed and the understanding of hepatic gene regulation at the molecular level has advanced in mammals (Mode and Gustafsson, 2006). For example, various studies have shown that many hepatic genes associated with xenobiotic metabolisms, such as cytochrome P450 (CYPs), are expressed in a sex-dependent manner during the detoxification process (Waxman and Holloway, 2009). Particularly, the sex-dimorphism of the liver is obvious in oviparous animals, as the female liver is the main organ for the synthesis of oocyte constituents, such as the yolk protein precursors (vitellogenins) and the zona pellucida proteins (choriogenins) (Arukwe and Goksøyr, 2003).

As continental aquatic environments are threatened by a large spectrum of xenobiotics and pollutants, freshwater oviparous organisms such as fish are especially impacted by these potential toxicants, and their liver detoxification capabilities constitute essential defenses for the fitness of these organisms. In this context, one can suppose that the various sexual dimorphisms of oviparous organisms, concerning energetic metabolism, detoxification and reproduction processes may drastically influence the hepatic responses of different sexes.

In this work, we were interested in identifying the molecular sexual dimorphism in the liver of adult medaka fish. Small fish such as the Japanese medaka (*Oryzias latipes*) have emerged as useful

vertebrate model organisms, suitable for studying various physiological processes (Fujisawa et al., 2016), toxicological mechanisms (Marie et al., 2012) and also ecotoxicological effects (Deng et al., 2010). Medaka fish presents the advantages of small size, established models produced from inbred lines, rapid development and growth, high fecundity, omnivorousness, and also shows sugar and lipid metabolic profiles similar to those of mammals (Fujisawa et al., 2016). By developing an integrative approach comprising histology and different high-throughput omic investigations (i.e. metabolomics, proteomics and transcriptomics), we are now able to globally describe the sex-dimorphism in the medaka liver. To our knowledge, this constitutes the first systematic investigation of the liver sex-dimorphism in this model organism, providing important information for further investigation of potential different repercussions on the biology of fish environmentally exposed to chemical issues.

2.2 Material and methods

2.2.1 Medaka fish

Medaka fish (*Oryzias latipes*) of the inbred Cab strain were used for this experiment. The animals were handled and experiments were performed in accordance with European Union regulations concerning the protection of experimental animals and the experimental procedures were approved (N°68-040 for 2013-18) by the "Cuvier's ethical committee" of the Muséum National d'Histoire Naturelle (French national number C2EA-68). All fish used in this study (during summer 2014) originate from the same broodstock (F0 from genitors provided in November 2013 by the Amagen CNRS/INRA platform - Gif-sur-Yvette, France).

All histopathology, metabolomics, proteomic and transcriptomic analyses were performed on the untreated fish breed and kept under control conditions, described as follow. Six-month-old adult fish (around 0.55 ± 0.08 and 0.59 ± 0.10 g, for males and females, respectively; n = 39), mature and sexually active (with secondary sexual character well developed; sex determination was confirmed by further histology of the gonads), were maintained at 25 ± 1 °C, with 15 h:9 h light/dark cycle (in reproductive cycle). Fish were raised in 20 L glass aquaria (in triplicate tanks, containing above 13 male and 13 female per aquarium) filled with a continuously-aerated mixture of tap water and reverse osmosis filtered water (1/3–2/3, respectively), which was renewed once a week. Fish were fed three times a day with commercial food for juvenile salmon, supplemented once a day with fresh artemia, and were inspected three times daily. No abnormal behavior nor mortality was observed. Fish were randomly selected, briefly anesthetized in buffered 0.1% MS-222, sacrificed. The liver samples were collected and prepared for further analysis, as described below.

2.2.2 Histopathology

The half liver of each individual (9 females and 9 males, 3 from each triplicate aquarium) were fixed in cold 10% buffered formalin (4 °C, 48 h), then transferred into 70% ethanol, dehydrated in successive baths of ethanol (from 70 to 95%), and then embedded in paraffin. Blocks were cut in 3-5 μ m-thick sections, and slides were stained with hematoxylin-eosin-saffron (HES) or periodic acid-Schiff/alcian blue (PAS), according to the standard histological procedure. Alternatively, liver samples were fixed with a mixture of paraformaldehyde (2%), glutaraldehyde (0.5%), picric acid (0.5%) and sucrose (0.18 M) in 0.1 M pH 7.4 Sørensen buffer prior to post-fixation in osmium tetroxide (1%). Samples were then dehydrated in ethanol, embedded in the epoxy mixture (Spurr's resin), and cut in semi-thin 0.5 μ m-thick sections and stained with toluidine blue (TB).

2.2.3 Metabolome ¹H-NMR spectra

Liver extraction was carried out using the methanol/chloroform/water method (ratio 2/2/1.8). Fresh frozen livers of 18 individuals for each gender (6 individuals from each triplicate aquarium) were weighted and then homogenized in the ice cold methanol (8 mL.g⁻¹ of tissue; AnalaR Normapur, 99.8%) and ice cold milliQ water (2.5 mL.g⁻¹) and then vortexed for 1 min. Subsequently, ice cold chloroform (4 mL.g⁻¹; Normapur, 99.3%) and milliQ water (4 mL.g⁻¹) were added. Then, the mixture was vortexed for 1 min and incubated on ice for 10 min to partition. The supernatant was then centrifuged at 4 °C for 10 min at 2,000 g. The upper polar fraction was then transferred to 2 mL Eppendorf tubes, dried under Speed-vac device and then kept at -80 °C until NMR analysis. The extracts were dissolved in 550 µL of 0.1 M sodium phosphate buffer prepared in D₂O (10% v/v) containing 0.25 mM sodium-3-tri-methylsilylpropionate (TMSP) as an internal standard, then were transferred to a 5-mm NMR tube (Norell, France) and analyzed immediately by ¹H-NMR.

All NMR data were recorded at 298 K on a 600 MHz Bruker AVANCE III HD spectrometer equipped with a 5 mm TCI CryoProbe ($^{1}H-^{13}C-^{15}N$) with Z-gradient. One-dimensional ^{1}H NMR spectra were acquired using a standard Bruker noesygppr1d pulse sequence to suppress water resonance. Each spectrum consisted of 512 scans of 32,768 data points with a spectra width of 7.2 kHz, a relaxation delay of 3 s and an acquisition time of 2.3 s. A Quality Check (QC) sample was

injected every 6 samples, in order to verify that no significant drift of the analysis occurs, according to expected reference. Spectra were then processed with Topspin software (Bruker) for alignment and noise reduction, and analyzed for bucketing, annotation and quantification with the Batman R package. Individual metabolite intensities were compared according to sex groups using Metaboanalyst 3.0 online tool (Xia et al., 2015) for Principal Component Analysis (PCA), PLS-DA, volcano plot reconstruction and metabolite pathway enrichment analyses.

2.2.4 Proteomic analysis

Liver samples from 9 males and 9 females were randomly pooled to get triplicate pooled samples (one liver from each triplicate aquarium in each triplicate pool), and homogenized on ice with a Dounce homogenizer in 500 µL of a solution of 6 M guanidine hydrochloride, 500 mM triethylammonium bicarbonate buffer (TEAB, pH 8.3), 0.1% Triton X-100 and 10 µg of protease inhibitor mixture (Roche, Switzerland). The homogenates were centrifuged at 4 °C (12,000 g; 10 min), and then the supernatants were collected. Proteins were precipitated with cold acetone (-20 °C; overnight), centrifuged (2,000 g; 4 °C), and then resuspended in 500 mM TEAB with 6 M urea and 0.1% SDS. The protein concentration was measured using a micro-BCA kit (Sigma-Aldrich, USA), with BSA as a protein standard.

One hundred µg of each liver protein pool was used for digestion with 5 µg of proteomic-grade trypsin (Sigma-Aldrich, USA), reduced with 2 mM tris-(2-carboxyethyl) phosphine (TCEP) and cysteine-blocked with 10 mM methyl methane-thiosulfonate (MMTS), prior to analyses with a Q ExactiveTM Hybrid Quadrupole-OrbitrapTM mass spectrometer (Thermofisher Scientific). Liver protein digests were concentrated on C18 stages tips, recovered in 40 µL 2% aqueous TFA, 2% ACN before injection in triplicates (6 µL injected). NanoLC was performed on an Ultimate 3000 RSLCnano System (Thermofisher Scientific): digests were desalted on a trap column (Pepmap, C₁₈ 300 µm x 5 mm, 5 µm 100 Å, Dionex) with water containing 2% ACN with 0.1% formic acid (solvent A) for 6 min, and the peptides were finally eluted from a separation column (Pepmap, C₁₈ 75 µm, x 500 mm, 3 µm 100 Å, Dionex). The separation gradient as optimized for the samples is divided into 3 successive slopes: 2-20% in 120 min, 20-35% in 45 min and 35-80% ACN + 0.1% formic acid (solvent B) at a flow rate of 300 nL.min⁻¹. Each MS spectrum acquisition (*m/z* 400–2000, 70,000 Res.) was followed by up to ten data dependent HCD MS/MS spectra (first fixed mass *m/z* 90, 17,500 Res., 30 normalized collision energy) with a dynamic exclusion window of 30 s.

All MS/MS-analyzed samples were analyzed using Mascot 2.4.1 (Matrix Science, UK) and X!Tandem with Scaffold software (version 4.5.1; Proteome Software, USA) to search Uniprot

databases of Teleostei (downloaded in December 2015). The ion mass tolerance and the parent ion tolerance were set to 20 mDa and 10 ppm, respectively. The methyl methanethiosulfonate of cysteine was specified as fixed modifications. Oxidation of methionine and deamination of N and Q were specified as variable modifications. The Scaffold was used to probabilistically validate the protein identifications derived from MS/MS sequencing results. Normalized semi-quantifications and identification probability of male and female identified proteins were estimated using Scaffold+ default parameters from MS and MS/MS data for proteins presenting at least two peptides.

2.2.5 RNA-seq analysis

The half liver of female fish (2-3 individuals per pool, 4 pools) and male fish (1-2 individuals per pool, 3 pools) were pooled, respectively, adjusting according to the size of tissue. Total RNA of the 4 pooled female samples and 3 pooled male samples was isolated and purified using RNeasy Plus Mini Kit with gDNA eliminator spin (Qiagen). RNA quantity and quality were evaluated using Qubit RNA Assay Kit in Qubit®2.0 Fluorometer (Life Technologies, USA) and an Agilent Bioanalyzer 2100 eukaryote total RNA Pico series II chip (Agilent Technologies Inc., USA), respectively. The transcriptome libraries were prepared from total RNA samples with RIN value over 7.7 using Illumina TruSeq Stranded mRNA Sample Preparation kits (Illumina Inc., USA) following the manufacturer's handbook. The libraries were sequenced on an Illumina Hi-Seq1000 instrument using the TruSeq SBS kit V3-HS 50-cycles (Illumina Inc., USA), and 30-40 million of 51 bp-long single-end reads per library were generated. CASAVA-1.8.2 software was used for demultiplexing.

Raw reads were first cleaned by removing adaptors and only 51 bp-long reads were kept. The global quality of the reads was checked using the FastQC 0.10.1 and good global Phred scores (>30) were obtained in all the libraries. A step of duplicated reads removal was conducted that analyzes the quality of reads and keeps the one with the best global quality score. Tophat2 (v2.0.10) (Kim et al., 2013) was used to map the clean unique reads on the medaka genome (release 81) downloaded from Ensembl. Multiple hits were removed by samtools (v0.1.18) and read counting on gene exons was accomplished by HTSeq-count (v0.6.1p1) (Anders et al., 2015) in union mode against the annotation of medaka genomes downloaded from Ensembl. DESeq2 (v1.8.1) (Love et al., 2014) was used to do differential expressed gene analysis on the raw count data. Genes were considered differently expressed when the p-value below 0.001, using the control group as reference. Furthermore, we only included genes with the expression level of at least 4 |FC| in order to capture

more physiologically relevant genes. Then, we identified 375 female- and 147 male-enriched transcripts (p < 0.001 and |FC| > 4) (Annex 2.4).

2.2.6 Molecular network analysis

Molecular pathway was determined for our merged transcriptome and metabolome data using the Ingenuity Pathway Analysis (IPA) software (V01-04; Qiagen) with the Human orthologous of medaka proteins available from Ensembl online platform (http://www.ensembl.org), according to specific Ingenuity Knowledge Database (Genes and Endogenous Chemicals), which is a repository of biological interactions and functional annotations. The fold change values (females *vs* males) and *p* values calculated according to the quantifications of all replicates for 2214 gene expressions (FDR<0.05) and 245 metabolites (HMDB numbers) were imported into IPA, then "Core Analysis" was performed, with default setting on liver tissue and relaxed filters, including both direct and indirect relationship between our dataset and the reference annotations, in order to interpret data in the context of biological pathways, molecular functions and networks.

2.3 Results

2.3.1 Histology

The liver of organisms under undisturbed lab condition presents in both sexes a characteristic architectural organization with polyhedral hepatocytes organized around the capillary sinusoids and the bile canaliculi, appearing in characteristic cord-like parenchymal structures. As shown in Figure 2.1, medaka fish liver presents a sexual dimorphism at the cellular level. Indeed, male and female hepatocytes present obvious differences in their global cytoplasm appearance with a clear distribution of vesicles that are revealed with HES, PAS or TB stainings. Whereas female hepatocytes present large isolated reserve vesicles (mostly one per hepatocyte) with dense contents of glycoprotein and/or glycogen (Figure 2.1 C, D, G & H), male hepatocytes exhibit more diffuse small vesicles (Figure 2.1 A, B, E & F).



Figure 2. 1 Histological investigation of male and female medaka livers.

Representative histological observations under a light microscope of thick or thin sections of adult medaka liver stained with HES (A-D), PAS (E & G), and toluidine blue (F & H), for males (A-B & E-F) and females (C-D & G-H). Scale bars represent 20 µm. g, glycogen reserve; n, nucleus; m, membrane; c, cytosol; v, vesicle.

2.3.2 NMR metabolomics

The hydrophilic fraction of medaka liver is investigated in 18 males and 18 females by ¹H NMR analysis. Up to 237 different potential metabolites have been detected and relatively quantified. The global analysis of the molecular pathway involved in liver metabolism reveals that the medaka liver metabolome presents a significant enrichment in a very wide diversity of processes, comprising principally glutathione, taurine, amino acid, carbohydrate, lipid, steroid hormone and tricarboxylic acid (TCA) cycle metabolisms (Annex 2.2). Although our metabolomic analysis was performed on the hydrophilic fraction of the liver, we are able to observe various mostly hydrophobic metabolites, such as steroidal compounds, which testify of the intense lipid metabolism, especially in males. PCA clearly discriminates specific liver metabolome between males and females according to its component 1 that comprises 76% of the total sample variation (Figure 2.2 A).

Α		PCA			В	Vol	cona plot		
Dimension 2 (7.4%)	·***	••	11. A.		6 and + 4 - COg10 A - 2 -	1		•	
szoo _o	-0.002 -0.001 Dir	0.000 mension 1 (7	0.001 76.3%)	e Female Male	0 -6 D	-2 Log	g ₂ FC ² (F/M)	6 10)
Metabolites	HMDB	PubChem	Fold Change	p value	Metabolites	HMDB	PubChem	Fold Change	p value
Hypotaurine	HMDB00965	107812	-50.0	0.000001	Maltotetraose	HMDB01296	439639	385.0	0.000001
Methylamine	HMDB00164	6329	-33.2	0.000001	Taurine	HMDB00251	1123	327.2	0.000001
Carnosine	HMDB00033	439224	-31.6	0.000001	Rhamnose	HMDB00849	25310	210.5	0.000001
Cortisone	HMDB02802	222786	-30.3	0.000001	cis-Aconitic acid	HMDB00072	643757	178.6	0.000001
Tetrahydrocortisone	HMDB00903	12444617	-29.8	0.000001	1,3-Dimethyluric acid	HMDB40177	70278	113.0	0.000001
Mevalonic acid	HMDB00227	449	-28.6	0.000001	Glucose 6-phosphate	HMDB01401	5958	107.0	0.000001
S-Adenosylhomocysteine	HMDB00939	439155	-23.1	0.000001	D-Glucose	HMDB00122	5793	44.6	0.00042
Scyllitol	HMDB06088	17	-22.5	0.000001	Argininosuccinic acid	HMDB00052	16950	41.8	0.00002
Gamma-Butyrolactone	HMDB00549	7302	-20.4	0.000001	Glycerol	HMDB00131	753	41.6	0.000001
Uridine 5'-diphosphate	HMDB00295	6031	-18.9	0.000001	Pantothenic acid	HMDB00210	988	41.2	0.000001
3,7-Dimethyluric acid	HMDB07021	53477956	-16.9	0.000001	Myoinositol	HMDB00211	121	17.0	0.000001
Nicotinuric acid	HMDB03269	68499	-16.4	0.000001	1,5-Anhydrosorbitol	HMDB03911	64956	16.4	0.00003
9-Methyluric acid	HMDB01973	108714	-13.3	0.000001	Alpha-Lactose	HMDB00186	84571	15.1	0.000001
Quinone	HMDB03364	4650	-13.3	0.000001	Sucrose	HMDB00258	5988	14.8	0.00001
Hydrocinnamic acid	HMDB00764	107	-13.1	0.000001	L-Arabitol	HMDB01851	439255	12.9	0.000001
Retinal	HMDB01358	638015	-12.8	0.000001	D-Galactose	HMDB00143	439357	12.3	0.00002
Ethanolamine	HMDB00149	700	-12.2	0.000001	Glucosamine 6-sulfate	HMDB00592	72361	10.9	0.000001
2-Hydroxyestrone	HMDB00343	440623	-11.6	0.000001	Allocystathionine	HMDB00455	10104953	10.7	0.000001
Guanine	HMDB00132	764	-11.5	0.000001	L-Cystathionine	HMDB00099	439258	9.9	0.000001
Tiglylglycine	HMDB00959	6441567	-11.3	0.000001	Acetylcholine	HMDB00895	187	9.8	0.00011
Succinic acid	HMDB00254	1110	-11.2	0.000001	Guaiacol	HMDB01398	460	9.4	0.00185
Estrone	HMDB00145	5870	-11.1	0.000001	L-Homoserine	HMDB00719	12647	8.6	0.00005
L-Aspartyl-L-phenylalanine	HMDB00706	93078	-10.9	0.000001	L-Cystine	HMDB00192	67678	8.6	0.000001
Malonic acid	HMDB00691	867	-10.9	0.000001	7-Methylxanthine	HMDB01991	68374	8.3	0.00005

Figure 2. 2 Metabolomics of the male and female medaka liver by ¹H NMR.

Principal component analysis (PCA) performed with the quantification values of the 237 metabolites detected by Batman' R package algorithm from the 18 males and 18 female individual NMR spectra (A); Volcano plot representation of the 237 metabolites according to female/male fold change average and significance of the differences ($|log_2FC|>1$ and $log_{10}p$ -value<1.3) Female and male over-represented metabolites are determined with positive and negative significant FC (F/M) values and are shown in red and blue, respectively, and are represented with darker colours when metabolite presenting VIP values are superior to 1, according to PLS-DA analysis (B); Top-25 lists of the putative annotations of male- and female-representative metabolites (C & D, respectively).

Significantly over-represented metabolites in the liver of female (compared with males) and male (compared with females) fish (|FC|>2 and p<0.05) are highlighted in a volcano plot (Figure 2.2 B). Whereas 59 molecules are over-represented in females, 103 appear to be over-represented in male metabolome (Annex 2.1). According to their putative annotation provided by the Batman algorithm, a pathway enrichment analysis indicates that female-enriched metabolomes would rather

be implicated in some saccharide and amino acid metabolic processes, whereas male-enriched metabolomes seem to exhibit signatures of steroid hormone biosynthesis and energy (TCA, nitrogen) processes, when the common metabolite set is more relevant to other amino acid and saccharide metabolism (Annex 2.1). Another interesting noticeable specificity of the medaka fish metabolome concerns the relative quantity of taurine and hypotaurine that are strongly over-represented in females and males, with 327 and 50 [FC], respectively (Figure 2.2 C and D).

2.3.3 Proteomics

Among the 820 proteins identified in the 3 male pools, 64 appear to be potentially male-biased, because they were detected in both 3 male pools, and not in any of the three female pools. For females, 178 seem to be similarly biased of the 934 total proteins identified in the 3 female pools, and not in any male pool (Figure 2.3 A). This large protein sex-biased composition of the liver is also illustrated with semi-quantification of the proteins according to both MS and MS/MS data, represented in a volcano plot (Figure 2.3 B). Among 1241 identified proteins, 25 and 125 are significantly over-represented in males and females, respectively (|FC|>3 and p<0.01, Annex 2.3). A global analysis of these sex-biased proteins are significantly related to tRNA and nucleotide sugar metabolism, which might be related with intense gene expression and synthesis processes, whereas male-biased proteins are related to bile and amino acid metabolisms, and common proteins to other TCA, sugar, lipid and amino acid metabolisms, characteristic of classical liver metabolic pathways (Annex 2.3).

Among the highly abundant proteins in female livers, the predominant ones are various isoforms of vitellogenins and choriogenins, together with fatty acid-biding, CYPs and various isoforms of ribosomal and translation-related proteins, whereas male-enriched liver proteins interestingly present other CYPs isoforms, complement proteins, glutathione S-transferases (GSTs) and various TCA metabolism-related proteins, together with wap65, a protein of unknown function whose transcript over-expression appears characteristic of the male Gulf pipe *Syngnathus scovelli* (Rose et al., 2015), highlighting important singularities of the liver proteome in both sexes (Figure 2.3 C and D).



Figure 2. 3 Proteomics of male and female medaka livers.

Unscaled Venn's diagram of the protein identified with at least 95% protein identification certainty in all of the 3 different 3-individual pools of male and/or female medaka livers (A). Volcano plot representation of the 1241 proteins according to female/male fold change average and significance of the differences ($|log_2FC|>1.5$ and log_{10} *p*-value<2) determined according to Scaffold 4.5.1 semi-quantitative values based on both MS and MS/MS data (B). Female and male over-represented proteins are determined with positive and negative significant FC (F/M) values and are shown in red and blue, respectively. Top-25 lists of the male- and female-superabundant proteins (C and D, respectively).

2.3.4 Transcriptomics

PCA clearly discriminates between all male and female liver transcriptomes (Figure 2.4 A), and the volcano plot representation indicates a colossal over-expression of some genes in females compared with males (Figure 2.4 B). Indeed, some genes such as *vitellogenin 1, 3* or *6* reach above 15 to 19 $|\log_2 FC|$ variations (namely up to 500,000 |FC|, Annex 2.4), representing a large portion of the total female liver transcriptome. In contrast, in male livers the most over-expressed gene,

hydroxysteroid dehydrogenase 3, exhibits only up to 8 $|\log_2 FC|$ (250 |FC|) variation in comparison with females. Additional pathway analyses, performed with the 375 and 147 significantly enriched transcripts in females and males, considering their human orthologs (Annex 2.4), indicate global enrichments (*p*<0.1) of steroid and amino acid processes in both sexes when males also exhibit terpenoid-quinone and fatty acid biosynthesis over-representations.

The list of the over-expressed genes in females comprises, along with some genes whose expressions are well known to be female-specific, such as *vitellogenins*, *choriogenins* and *chorionic protease inhibitors*, various forms of *FAM20C* genes, belonging to the serine-threonine kinase 20c-like family. Various isoforms of the FAM20C protein family were similarly over-expressed in the female liver of the Gulf pipefish (Rose et al., 2015). Although the biological function of the proteins of this family remains poorly documented, they are interestingly annotated in GO library as "cellular response to estrogen stimulus" genes, and may constitute female-specific markers in fish livers. With some agreement with our proteomic observation, other genes of interest belong to the *cytochrome-P450 2w1* appear clearly over-expressed in male and female medaka livers, respectively (Figure 2.4 C and D).



Figure 2. 4 Transcriptomics of the male and female medaka liver investigated by RNA-seq approach.

Principal component analysis (PCA) performed according to the transcript count for the 16,523 genes encoding medaka liver proteins from the 3 male and 4 female pooled cDNA sequenced by Hiseq 1000 comprising at least 30 million reads per libraries (A). Volcano plot representation of the gene expression according to female/male fold change average and significance of the differences ($|log_2FC|>2$ and $log_{10}p$ -value<3) (B). Female and male over-expressed genes are determined with positive and negative significant FC (F/M) values and are shown in red and blue, respectively. Top-25 lists of the male- and female-over-expressed genes (C and D, respectively).

2.3.5 Integrated pathway analysis

To investigate and visualize the biological connectivity of the sex-enriched metabolites and transcripts, the network-generating algorithm of IPA was used to maximize the interconnectedness of molecules based on all known connectivity in the database developed from Human molecular knowledge in the liver. The results of the IPA biological function analysis (Annex 2.5), represented

as a bar chart and a heatmap, are shown in Figure 2.5 A and B, respectively. Lipid metabolism, molecular transport, small molecule biochemistry, inflammatory response, organismal development, vitamin and mineral metabolism, and free radical scavenging appear to be the most significantly represented functional categories according to the combined liver transcriptome and metabolome dataset. The sex-specificity of the molecules involved in these processes is indicated in the heatmap representation (Figure 2.5 B) that clearly shows a global up-regulation of molecules involved in small molecule biochemistry, lipid metabolism, tissue development, vitamin and mineral metabolism, energy production and carbohydrate metabolism in males, whereas in females most of the molecules involved in cellular movement, haematological system, inflammatory response, or immune cell trafficking appear largely up-regulated.

The IPA network search shows that 2 of the top networks consisted predominantly of only female- and male-enriched molecules are related respectively to RNA post-transcriptional modification and lipid metabolism processes, as shown in Figure 2.5 C and D. These molecular network representations clearly illustrate the selected massive induction of some genes and metabolites related to RNA post-translational modification in females (Figure 2.5 C), and specific lipid metabolism processes connected with cholesterol metabolism and steroidogenesis, in males (Figure 2.5 D).


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Figure 2. 5 Ingenuity pathway analysis performed on male *versus* female fold change values (M/F FC) of both transcriptomic and metabolomic data.

Top dysregulated molecular pathways represented in the bar chart (A) and heatmap (B). Molecular functions that are specifically activated in males and females are indicated in blue and red, respectively. Examples of significant molecular networks related to RNA post-translational modification (C) and lipid metabolism (D) processes (score =38 and 28). Relative up-regulation of transcripts and metabolites in males or in females are indicated in blue and red, respectively.

2.4 Discussion and conclusion

The liver is a key organ in vertebrates performing a large diversity of vital functions, including processing and storage of nutrients, maintenance of serum composition, bile production, and xenobiotic detoxification. It is primarily an exocrine gland, secreting bile into the intestine, but it is also an endocrine organ and a blood filter. The liver is a metabolic factory, which synthesizes and breaks down a variety of substances, comprising the production of bile salt anions, the synthesis of urea and many plasma proteins, the metabolism of glycogen, cholesterol and fatty acids, the detoxification of many drugs, and the processing of steroid hormones and vitamin D. Studies carried out in rodents have established that sex-based differences in liver function also characterize many drug-metabolizing enzymes (DMEs), including sulfotransferases, glutathione S-transferases, P450s and other steroid metabolizing enzymes (Waxman and Holloway, 2009). The sexual dimorphism of liver gene expression is not confined to DMEs, and it concerns more than 1000 genes in these organisms (Yang et al., 2010), including plasma lipoproteins, pheromone binding proteins, regulators of fatty acid homeostasis, nuclear receptors, and other transcription factors (Conforto and Waxman, 2012).

Similar to the mammalian liver, the teleost liver plays an important role in the metabolic homeostasis of the whole organism, in addition to that, oviparous vertebrate-specific processes related to the synthesis of various oocyte protein precursors (i.e. mainly vitellogenins, choriogenins, and other minor vitamin-binding proteins) are synthesized in females under the direct control of estrogens, which bind to estrogen receptor complex and activate the translation of messenger RNA via *cis*-regulation mechanisms (Arukwe and Goksøyr, 2003). The massive rate of synthesis of vitellogenin in the egg-laying animal causes considerable ultrastructural changes in liver cells, which are characterized by extensive proliferation of the rough endoplasmic reticulum and the Golgi apparatus (Braunbeck et al., 1992). In mature female medaka, a remarkable part of the liver metabolism might be dedicated to these reproduction-related processes, as each female can spawn above 30 mature oocytes daily. This massive synthesis is known to induce large cellular and molecular modifications, as it can also increase the lipid synthesis of hepatocytes (Roy and Chatterjee, 1983). This metabolic adjustment to maintain the reproductive competency of the female constitutes one of the physiological bases for the extended sexual dimorphism in fish livers.

Our histological observations of the male and female medaka liver clearly showed differences of reserve vesicle within hepatocytes, which is consistent with ultrastructural modifications in the liver cells between males and females. Interestingly, previous observation of mature medaka liver under transmission electron microscope showed that, in the perinuclear region, granular endoplasmic reticulum, mitochondria, and peroxisomes appear largely increased in number in female hepatocytes (Braunbeck et al., 1992). These hepatocyte sexual differences in cellular organization and content might be related to the intense activity of protein synthesis and consequently the high energy requirement of female hepatocytes. Indeed, in sexually mature fish, as in other oviparous vertebrates, livers globally present morphological, molecular and functional sexual-dimorphisms (Braunbeck et al., 1992; Viitaniemi and Leder, 2011; X. Zhang et al., 2012). The liver of female performs an important function in the synthesis of a large set of proteins involved in the oocyte envelop and vitellogenin reserves, whereas male liver hepatocytes do not exhibit such activity.

Although the precise function of taurine and hypotaurine, which were highly abundant in female and male livers, respectively, and the balance between the two remains poorly documented in fish liver (Salze and Davis, 2015), one previous study has reported the influence of taurine on egg maturation (Matsunari et al., 2006). To date, only one investigation performed in adult zebrafish has attempted to compare male and female metabolomes, according to various analytical approaches, including GC-MS, LC-MS and NMR, and has observed a significant up-regulation in various fatty acids, together with valine, acetate, glutamate, glutamine, creatinine and betaine in female liver (Ong et al., 2009).

The sex-biased pattern depicted in our transcriptomic analyses appears acutely congruent and even more contrasted than our global proteome investigation. These strong sex-biases testify to the intensity of the female liver efforts for the gene expression and the synthesis of the oocyte precursor proteins (Arukwe and Goksøyr, 2003; Van Der Ven et al., 2003), and the involvement of the male liver in steroid hormone and metabolism processes (Xiaowei Zhang et al., 2008), such as urea and energy cycles (Fujisawa et al., 2016), respectively.

Previous investigation of medaka fish has observed that liver transcriptome globally exhibits significant enrichment in the expression of genes related to macromolecule, RNA, and nitrogen compound metabolic processes with regard to the gene expression in other tissues, but it has not considered the differences between sexes (Lai et al., 2015; Murata et al., 2015). Previous works performed on zebrafish and Gulf pipefish have highlighted large sets of genes whose expression appears to be driven by sex-dependent processes (Zheng et al., 2013). On one side, zebrafish

transcriptomic approach reveals that the female-over-expressed gene list included vitellogenins and zona pellucida glycoproteins, many ribosomal proteins, and estrogen receptor 1, in contrast, the list of male-over-expressed genes contains fatty acid-binding protein2, apolipoprotein 4, and also genes that are supposed to be involved in anti-inflammatory processes, such as complement factors 9b and 3b, together with several chitinases (Zheng et al., 2013). On the other side, transcriptomic investigation performed on Gulf pipefish shows a quite similarly high over-expression in characteristic genes of females, such as vitellogenin b and c, choriogenin h, and zpc 4-like, in addition to estrogen receptor 1 and various fam20c isoforms, whereas males exhibit less intense over-expression of specific genes, comprising various metabolism related genes such as hint3, ctl, nsun3 and wap65 (Rose et al., 2015). Moreover, primary investigation of the medaka fish liver transcriptome by microarray analysis indicates that the female-specific transcript list comprises some previously characterized female-specific genes such as vitellogenins, choriogenins, ZP family genes, cyclins B and 42S nucleoprotein, whereas most of the male-specific transcripts have not yet been assigned or characterized (Kishi et al., 2006).

Similarly, previous proteinaceous investigations have highlighted that female liver proteome moreover contains massive amounts of oocyte precursor proteins (i.e. vitellogenins, choriogenins and fatty acid-binding proteins) that are being secreted by the hepatocytes (Viitaniemi and Leder, 2011), together with variations between sexes in drug metabolism capabilities (Waxman and Holloway, 2009). CYPs constitute a diverse group of enzymes that are potentially involved in key reactions of oxidation of organic substances, such as drug detoxification (Viitaniemi and Leder, 2011) and steroid hormone metabolism (Mode and Gustafsson, 2006; Rose et al., 2015; Waxman and Holloway, 2009). The sexual polymorphism in the expression of these enzymes may have fundamental repercussion on liver physiology such as drug-metabolism processes.

In addition to our transcriptomic data that gives a congruent view with previously published observations, our systematic investigation constitutes an unprecedented opportunity to globally depict the medaka liver sexual dimorphism at different molecular and cellular levels. By developing an integrative approach combining high output proteomic, RNA-Seq transcriptomic and non-targeted metabolomics outputs, together with histological examinations, we are able to appreciate the wideness and the deepness of the sexual dimorphism, in terms of both number and intensity of the sex-dependent dysregulations. At the mRNA level, twice more sex-over-expressed transcripts appear in females, comprising some genes involved in ovogenesis (e.g. *vitellogenins* and *choriogenins*), and reach very high fold change values (up to 50,000 and 250 FC in females and

males, respectively). Proteomic investigations also show more proteins that appear to be specific of female proteomes, with higher fold change too (up to 750 and 50 FC in females and males, respectively). The quantitative metabolome analysis performed by NMR indicates that the differences in fold changes between female- and male-enriched metabolites appear to be much higher in female (up to 350 and 50 FC in females and males, respectively). The observations of globally more intensive molecular up-regulations in female livers are in agreement with the conception of an oviparous female liver that is in charge of extra metabolic activity, according to their massive production of oocyte yolk stocks and chorion precursors, which have substantial impacts on both amino acid, saccharide and fatty acid metabolism of the global liver activities (Arukwe and Goksøyr, 2003).

Overall, the present study provides the first insight into the molecular mechanism underlying the sex-specificity of the liver of oviparous organisms, and concerning important liver processes, such as energetic metabolism, detoxification and reproduction.

Chapter 3 Immunolocalization of microcystin in the liver and the gonad of medaka fish acutely exposed to microcystin-LR

3.1 Introduction

Recurrent cyanobacterial blooms frequently occur in eutrophic freshwaters worldwide. Various bloom-generating species of cyanobacteria can produce natural toxic components (cyanotoxins), and their blooms threaten human health as well as any living organisms in the aquatic environment. Among all cyanotoxins, microcystins (MCs) are the most prevalent cyanobacterial hepatotoxins, with more than 100 structural variants, being produced by at least six genera of cyanobacteria (Puddick et al., 2014). Among all these known variants, microcystin-LR (MC-LR) is considered to be the most common and potently toxic (Puddick et al., 2014).

MCs are transported through cell membranes by a few specific organic anion transporting polypeptides (OATP) then specifically inhibit eukaryotic serine/threonine protein phosphatases 1 and 2A, which causes the disruption of numerous cellular signals and processes (Fischer et al., 2005; MacKintosh et al., 1990). Although there have been 70 members of OATP superfamily identified in the database from humans, rodents and some additional species (Hagenbuch and Stieger, 2013), not all OATPs/Oatps are capable of transporting MCs. The capability of MC-transport have been demonstrated for OATP1A2 (mainly expressed in the brain), liver-specific OATP1BA and OATP1B3 in humans (Fischer et al., 2005). In fish, liver-specific Oatps, such as Oatp1d1 in little skate (Meier-Abt et al., 2007) and rtOatp1d1in rainbow trout (Steiner et al., 2014), have been reported to mediate MC-transport. Different types of Oatp/OATP distributed in different tissue or organs possess varying levels of affinities and capacities for different MC variant (Fischer et al., 2010, 2005; Steiner et al., 2016). Liver is the organ that presents the highest tropism for MCs (socalled "target"), since it is rich in a few liver-specific OATP members possessing high affinities and capacities to MC-LR. Several studies have reported the acute hepatotoxicity of MCs characterized by hepatocellular apoptosis (Zhang et al., 2013a), necrosis (Mattos et al., 2014), intrahepatic hemorrhaging (Hou et al., 2015), and cytoskeleton disruption (M. Zhou et al., 2015). Additionally,

the distribution of MCs in the liver has been detected using immunohistochemical methods in mice (Guzman and Solter, 2002; Yoshida et al., 1998) and fish (Djediat et al., 2011; Fischer and Dietrich, 2000; Marie et al., 2012).

In addition to liver, MCs have been documented to distribute and accumulate in various fish organs including intestine, kidney, gill and gonad (Acuña et al., 2012; Djediat et al., 2010; Mezhoud et al., 2008; Trinchet et al., 2013, 2011). Among these organs, the gonad could be considered the second important target of MCs. Several field studies reported the presence of MCs in the gonad of fish. For instance, in Lake Pamvotis (Greece), the gonad of common carp (Cyprinus caprio) was reported to contain about 50 ng eq. MC-LR g⁻¹ bw using ELISA test (Papadimitriou et al., 2012), and in Lake Taihu (China), three variants of MC (MC-LR, -YR and -LR) were determined in the gonad of various fish species using a liquid chromatography-electrospray ionization-mass spectrum system (LC-ESI-MS). Particularly, silver carp (Hypophthalmichthys molitrix) and goldfish (Carassius auratus) were observed to contain high concentrations of MCs in the gonad (60 and 150 ng MCs g⁻¹DW, respectively) (Chen et al., 2009). In laboratories, the distribution of MC-LR in the gonad of medaka fish administered with pure toxin was also detected using a radiotracing method (Mezhoud et al., 2008). Although the accumulation of MCs in the gonad has been documented previously through different methods, little is known about the localization of MCs within the gonad tissue, or about the intracellular distribution of MCs. Only one field study reported the presence of MCs in the gonadal somatic tissue, but apparently not in the oocytes in common bream (Abramis brama) using immunohistochemical method (Trinchet et al., 2013). To date, it is still hard to know whether MC could enter the reproductive cells or only accumulate in the conjunctive tissue of gonad. Although not the most sensitive detection method, the immunohistochemical examination is a well-established way of providing the precise subcellular localization of MCs in the gonad, which could largely advance our current knowledge of the directly toxic effects of MCs on the gonad.

In order to identify and localize MC in fish gonads, an immunolocalization study was conducted using medaka fish that were gavaged with acute doses of MC-LR (10 μ g MC-LR g⁻¹ bw, 1 h exposure). Localization of the toxin into the liver and gonad was shown by MC-LR specific antibodies MC10E7. In addition, the microcystin-induced histopathological change was investigated.

3.2 Material and methods

3.2.1 Chemical and reagent

Five hundred μ g MC-LR purchased from Novakit® (Nantes, France) was dissolved in 500 μ L ethanol and 500 μ L water. The ethanol was evaporated with Speedvac. The concentration of MC-LR in the obtained solution was tested by ELISA, and then adjusted to be 1 μ g. μ L⁻¹.

3.2.2 Fish maintenance, exposure and sampling

Five-month-old medaka fish (*Oryzias latipes*) of the inbred Cab strain maintained in the lab was used for this experiment. Female and male fish were maintained in 2 glass aquaria, respectively, filled with a mixture of tap water and reverse osmosis filtered water (1:2) in a flow-through system for aeration and filtration, in a temperature controlled room (25 ± 1 °C), with a 12 h:12 h light:dark cycle. Fish were fed three times a day with commercial dry bait for juvenile salmons.

The process of the experiment was briefly shown in the flow chart (Figure 3.1). Eight females and eight males were randomly selected from the aquaria. The fish were anesthetized with tricaine (150 mg.L⁻¹) before gavage. Five females and five males were gavaged with 5 μ L of MC-LR solution (1 μ g. μ L⁻¹) per fish, representing about 10 μ g MC-LR g⁻¹ bw (average body weight is 0.52 g, the body weight of individual fish is shown in Table 3.1). This concentration was modified from previous studies in which medaka fish exhibited noticeable tissue damage and toxin presence in liver upon exposure to 5 μ g.g⁻¹ bw of MC-LR (Djediat et al., 2011, 2010). Two females and two males were gavaged with 5 μ L water as the non-toxin control, and 1 female and 1 male without any treatment were used as the non-gavage control. After 1 h oral exposure, fish were anesthetized with tricaine, sacrificed, and the liver and gonad were collected on ice. One tissue was cut into 2 parts, one part was immediately fixed with fixing solution for paraffin sections (Annex 3.1) and the other part was fixed for resin sections (Annex 3.2).

Group	Fish	Gender	Body weight
Ĩ	No.		(g)
MC-LR	1	Female	0.45
	2	Male	0.43
	3	Female	0.44
	4	Female	0.55
	5	Male	0.60
	6	Male	0.52
	7	Female	0.43
	8	Male	0.64
	9	Male	0.62
	10	Female	0.55
Non-toxin control	11	Male	0.43
	12	Female	0.55
	13	Male	0.59
	14	Female	0.50
Non-gavage control	15	Male	0.60
	16	Female	0.44

Table 3. 1 The body weight of individual fish in different treatment groups



Figure 3.1 Flow chart of the sample treatment procedure

3.2.3 Histopathological observation

The liver and gonad sample for paraffin sections were fixed in formaldehyde fixing solution (Annex 3.1) at 4 °C for 48 h, then dehydrated in successive baths of ethanol (from 70 to 100%), and embedded in paraffin. Blocks were cut into 4 μ m thick sections. The sections of each sample were deposited on 3 slides. One slide was stained with HES staining (Annex 3.3) for histopathological observation. Another slide (only liver sample) was stained with PAS staining (Annex 3.4) for assessment of hepatic glycogen reserve. The glycogen reserve analysis was performed by ImageJ software (version 1.51d), which was estimated as a surface percentage of the purple-red pixels on 5 randomly selected views/photos (200 × magnification, photo size 1388×1040 pixels, seen Figure 3.4) per individual. Statistical analysis was conducted by one-way ANOVA with post hoc Tukey HSD test for p<0.05 or p<0.01 using R software. The last slide was treated for immunolocalization of microcystin as described below.

The liver and gonad samples for resin sections were fixed at 4 \circ C overnight. The samples were then dehydrated in successive baths of ethanol (from 30 to 100%), and embedded into the Unicryl resin. Blocks were cut in 0.5 µm think semi-thin sections that were stained with toluidine blue staining for histopathological observation.

3.2.4 Immunolocalization of microcystins

One slide of the liver and gonad sections from paraffin-embedded blocks were incubated with a monoclonal antibody to MC-LR (MC10E7, Alexis) that recognizes all MCs with Arg in position 4 (dilution 1:4000). The immunolabeling was routinely performed with an automated Module Discovery XT (Ventana, Tuscon, USA) using a colorimetric peroxidase-specific staining with diaminobenzidine (DAB), a substrate that produces a highly insoluble brown precipitate. The slides were counterstained with hematoxylin. The secondary antibody control sections were prepared by skipping the first antibody MC10E7 incubation step. One MC-contaminated medaka liver sample from an acute high dose exposure in the lab previously was included as the technically positive control.

The resin block was cut in 60 nm thick ultrathin sections that were collected by the gold grid. Then the sections were incubated with the same primary antibodies (MC10E7) at 4 \circ C overnight and rinsed, then incubated with a secondary antibody coupled to gold nanoballs (6 and 10 nm \emptyset) at room temperature for 1 h. After being well rinsed with distilled water, the sections were stained

with a saturated solution of uranyl acetate in 50% ethanol and then observed under an H-7100 Hitachi (Tokyo, Japan) transmission electron microscope.

3.3 Results

3.3.1 Histopathological effects with light microscopy

For each individual, the observation result with light microscopy has been shown in Table 3.2. In the liver of all control fish, the hepatocytes present compact and characteristic cord-like parenchymal organization, and a distinctive central nucleus with a prominent nucleolus (Figure 3.2 A and B, Figure 3.3 A and B). The cytoplasm of hepatocytes contains mainly glycoprotein and/or glycogen stores that are intensively stained in red-purple with PAS (Figure 3.4 A and B). In contrast, the livers of MC-LR treated fish exhibit noticeable tissue lesions characterized by a disintegration of the parenchymal organization, significant cell lysis and disjunctions. All the hepatocytes lose their polyhedral shape and become rounded and swollen (Figure 3.2 C and D, Figure 3.3 C-F). Within the hepatocytes, the reserve vesicles disappear, the cytoplasm presents various small bubbles, and the nuclear chromatin becomes dense. Some hepatocytes exhibit a breakdown of the nuclear membrane, mitosis arrest, and dilation of endoplasmic reticulum. In addition, the hepatic intracellular glycoprotein and/or glycogen content are showed to significantly decrease by PAS staining in the female fish exposed to MC-LR (Figure 3.4 C-E). For the gonad, there is no apparent cellular difference between the toxin-treated fish and the control ones.

Group	Fish ^a	Exposure time	Light microscopy observation			
			Liver			Gonad
			Round hepatocytes, disjunctive cells, mitosis arrest ^b	Glycogen reserve (%) ^c	MC-LR labeling	MC-LR labeling ^d
	F01	1 h	+	22.3	++	+
	F03	1 h	++	21.9	++	+
	F04	1 h	+	15.1	++	+
	F07	1 h	++	12.4	++	+
5 μg	F10	1 h	+	0.7	++	+
MC-LR per fish	M02	1 h	+	0.3	++	0
per non	M05	1 h	++	20.3	++	+
	M06	1 h	+	1.9	++	+
	M08	1 h	+	25.9	++	0
	M09	1 h	+	6.0	++	+
	F12	1 h	0	21.9	0	0
Non-toxin	F14	1 h	0	50.1	0	0
Control	M13	1 h	0	15.5	0	0
	M11	1 h	0	25.4	0	0
Non-	M16	/	0	48.9	0	0
gavage Control	M15	/	0	47.2	0	0

Table 3. 2 Light microsco	pe observation	of each ind	ividual live	r and gonad.
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^a F and M indicate female and male fish, respectively. ^b "++" indicates a severe liver damage, characterized by a large proportion of round hepatocytes, significant mitosis arrest, and obvious cell lysis and disjunction. "+" indicates a moderate liver damage, characterized by a large proportion of round hepatocytes, and a small area of cell lysis and disjunction. "0" indicates the normal liver structure. ^c indicates the percentage of glycogen reserve in the liver, the calculation formula: the area of the purple-red pixels \div the area of the whole image (1388×1040 pixels) × 100%. ^d "++" indicates strong and widely distributed immunolabelings, "+" indicates only a few immunolabeling spots, and "0" indicates no immunolabeling.



Figure 3. 2 Representative photos of paraffin sections (4 µm thick) of medaka liver with HES staining under a light microscope.

Female control (A); Females exposed to 10 μ g.g⁻¹ bw of MC-LR (C): disintegration of the cord-like parenchymal organization, significant cell lysis (l), rounded hepatocytes and loss of nuclei (n); Male control (B); Males exposed to 10 μ g.g⁻¹ bw of MC-LR (D): disintegration of the cord-like parenchymal organization, rounded hepatocytes containing granulated cytoplasm (g).



Figure 3. 3 Representative photos of resin sections (0.5 µm thick) of medaka liver with toluidine blue staining under a light microscope.

Female control (A): abundant and distinct distribution of reserve vesicles (v); Females exposed to $10 \ \mu g.g^{-1}$ bw of MC-LR, (C): disintegration of the cord-like parenchymal organization, rounded hepatocytes containing various small bubbles (b), loss of reserve vesicles, nuclear membrane breakdown, chromatin concentration (c), (E):

mitosis arrest in some hepatocytes (m), (F): cell disjunctions (d); Male control (B): abundant and distinct distribution of reserve vesicles (v); Males exposed to $10 \ \mu g.g^{-1}$ bw of MC-LR (D): disintegration of the cord-like parenchymal organization, swollen and rounded hepatocytes, nuclear chromatin concentration (c), and increase in endoplasmic reticulum (e) and loss of nuclei (n).



Figure 3. 4 Glycogen reserve in the liver.

(A-D) Representative photos of paraffin sections (4 μ m thick) of medaka liver with PAS staining under a light microscope. Female (A) and male (B) control: abundant and distinct distribution of glycogen-reserve vesicles (red-purple); Females (C) and males (D) exposed to 10 μ g.g⁻¹ bw of MC-LR: less glycogen-reserve vesicles compared with the control group; E: Percentage of glycogen content determined through imageJ software (mean ± SD, n=3 or 5), * indicates significant differences at *p*<0.05.

3.3.2 Immunolocalization of MC in the liver and the gonad

Through light microscopy, all livers of MC-LR treated fish exhibit a strong positive signal of MC-LR specific antibodies whereas secondary antibody controls do not display any positive signal (Figure 3.5 B), being similar to non toxin-control fish in which no immunolabeling is observed (Figure 3.5 G, Table 3.1). For the toxin treated fish, the brown immunolabelings are evenly distributed in the hepatocytes, except for the erythrocytes which are only stained in blue with hematoxylin. Within the hepatocytes, the immunolabeling of MCs is found in the cytoplasm and more intense staining is observed in nuclei (Figure 3.5 A). Immunogold electron microscopy also shows clear labelings localized in the cytoplasmic inclusion and the nucleus of hepatocytes of MC-LR treated fish, being particularly intense in the lysis area (Figure 3.6 C and D). MC-LR is also distributed intensely in residual bodies of the macrophage (Figure 3.6 E), whereas no immunolabeling is observed in either non toxin-control fish (Figure 3.6 A) or secondary antibody control sections (Figure 3.6 B).

By light microscopy observation of the ovary of toxin-treated fish, a clear immunolabeling is detected in the connective tissue, and some round cells with above 10 µm in diameter surrounded by the connective tissue, being probably the perinucleolar oocyte, also present positive labeling, as shown in Figure 3.5 C, whereas secondary antibody controls do not display any positive signal (Figure 3.5, D), being similar to control fish in which no immunolabeling is observed (Figure 3.5 H, Table 3.1). Moreover, electron microscopy shows that the immunogold labelings are not only present in the connective tissue, but also distributed in the chorion and cytoplasm of oocytes (Figure 3.7 C), being less intense in the yolk vesicles (Figure 3.7 D), whereas no immunolabeling is observed in either non toxin-control fish (Figure 3.7 A) or secondary antibody control sections (Figure 3.7 B).

For the testis, a weaker immunolabeling is observed in the connective tissue in some toxintreated male fish (Figure 3.5 E), whereas secondary antibody controls do not display any positive signal (Figure 3.5 F), being similar to control fish in which no immunolabeling is observed (Figure 3.5 I, Table 3.1). Immunogold electron microscopy shows a remarkable labeling in the connective tissue (SLS fibers are visible), being particularly strong in some round identified structures (Figure 3.7 G-H), whereas no immunolabeling is observed in either non toxin-control fish (Figure 3.7 E) or secondary antibody control sections (Figure 3.7 F).





Figure 3. 5 Representative photos of immunolocalization of MC-LR in the liver and gonad of adult medaka fish.

Sections of the toxin-treated female liver (A), the ovary (C), and the testis (E), stained with both toluidine blue and MC-LR immunolabeling (MC10E7), revealed with peroxidase specific reaction of DAB. n, nucleus; c, cytoplasm; h, hematoxylin; The secondary antibody control sections of the toxin-treated female liver (B), the ovary (D), and the testis (F) stained with toluidine blue; Sections of non-toxin control female liver (G), the ovary (H), and the testis (I) stained with toluidine blue.



Figure 3. 6 Representative photos of immunolocalization of MC-LR in the liver of toxin-treated fish through immunogold electron microscopy.

The liver of non-toxin control fish (A) and the secondary antibody control sections of the toxin-treated liver (B); The immunolabeling of MC is indicated by the white arrow in the liver (C-E) of the fish exposed to $10 \ \mu g.g^{-1}$ bw

of MC-LR. (C): hepatocyte, immunolabeling in the nucleus (n), (D): lysis area, immunolabeling localized in the cytoplasmic inclusions of the lytic hepatocyte, (E) macrophage in the liver, intense immunolabeling observed in the residual bodies (r).





Figure 3. 7 Representative photos of immunolocalization of MC-LR in the gonad of toxin-treated fish through immunogold electron microscopy

In non-toxin control fish, the ovary (A) and the testis (E), and the secondary antibody control sections of the toxin-treated ovary (B) and testis (F); The immunolabeling of MC is indicated by the white arrow in the ovary (C-D) and the testis (G-H) of the fish exposed to 10 μ g.g⁻¹ bw of MC-LR. (C) connective tissue and oocyte, immunolabeling found in the connective tissue (left) and the chorion (c) of the oocyte (right), (D) inside the oocyte, few labeling observed in the yolk (y) and the cytoplasm (cy); (G) labeling observed in connective tissue of testis, (H) labeling localized in the unidentified round structures surrounded by SLS (segment long-spacing) fibers (f).

3.4 Discussion and conclusion

In the present study, histopathological evaluation on the different sickness of liver sections showed a significant alteration including disruption of the normal structure and depletion of glycogen reserve in the medaka fish exposed to high concentration of MC-LR in a short time. The

observed characteristics of liver damage are in accordance with those observed previously in fish (Djediat et al., 2011; Fischer et al., 2000; Fischer and Dietrich, 2000; Marie et al., 2012; Mezhoud et al., 2008) and mice (Guzman and Solter, 2002; Yoshida et al., 2001, 1998) acutely administered with MCs or MC-containing cyanobacteria extracts. For instance, disorganization of the hepatic parenchyma, significant cell lysis, massive rounded hepatocytes and decreased glycogen reserves were reported in the medaka fish gavaged with a similar quantity (5 and 10 µg.g⁻¹ bw) of MC-LR (Marie et al., 2012; Mezhoud et al., 2008). Apoptotic nuclei characterized by highly condensed chromatin were observed in the liver of carp gavaged with 0.4 μ g.g⁻¹ bw of MC-LR equivalent from Microcystis extract (Fischer and Dietrich, 2000). In fact, the histological modifications observed in the present study were also similar to some of those described in the fish after chronic intoxication with MCs (Acuña et al., 2012; Trinchet et al., 2011). Indeed, Trinchet and her colleagues observed an increase in rough endoplasmic reticulum in hepatocytes of medaka fish chronically exposed to MC-LR (5 µg.L⁻¹, 30 d). In the present study, the increase in hepatic endoplasmic reticulum was also found in the male toxin-treated fish. This could be associated with the induction of detoxification process. In addition, increased energy needs for detoxification process might interpret for the significant liver glycogen depletion observed in the present study.

In the acute and chronic studies described above, severe liver damages were often accumulation of MCs in accompanied by remarkable the liver demonstrated by immunohistochemistry. The present study also showed an intense distribution of MC-LR in the liver of MC-treated medaka fish. MC was reported to mostly localize in the cytoplasm of hepatocytes where they disturb a sequence of phosphorylation/dephosphorylation-dependent biochemical reactions, directly contributing to hepatocyte dysfunction. Additionally, MCs induced cytoskeleton disruption is likely responsible for the deformation of hepatocytes, and cellular oxidative stress is associated with cell lysis (Campos and Vasconcelos, 2010). In a few acute studies developing exposures with a high concentration of toxin, immunolabeling of MCs was also observed in the hepatic nucleus (Fischer et al., 2000; Guzman and Solter, 2002; Yoshida et al., 1998). It was even reported that the intensity of immunostaining in the nucleus of hepatocytes appeared to be remarkably higher than that in the cytoplasm in the mice exposed to 57.6 μ g.kg⁻¹ of MC-LR (24h, i.p. injection) (Yoshida et al., 1998), which is consistent with the observation provided in the present study. Protein phosphatases PP1 and PP2A, one of the main molecular "target" of MCs, localize in both nucleus and cytoplasm, and the nuclear activity of PP1 is higher than the average extra-nuclear activity (Shenolikar, 1994). Therefore, the nuclear accumulation of MCs could be the consequence of the MC specific antibodies binding to the MC-LR-PP1/PP2A adducts localized in the nucleus. The intranuclear distribution of MCs may directly affect cell cycle regulation and DNA repair, contributing to cell apoptosis and death.

In the present study, immunolabeling of MC-LR was also observed in the gonad of MC-treated medaka fish. More specifically, MC-LR was mostly localized in the connective tissue of ovary and testis, which is consistent with a previous observation in the ovary of common bream collected from MC-contaminated lakes (Trinchet et al., 2013). However, in another chronic study, MCs were not detected in the ovary or testis of medaka fish following a balneation exposure to 5 µg.L⁻¹ of MC-LR for 30 days by this immunolocalization method (Trinchet et al., 2011). There are at least three causes that may account for this discrepancy. Toxin concentration and exposure time are crucial to the results obtained. Ten µg.g⁻¹ bw of MC-LR used in the present study is a quite high concentration (the highest LD_{50} value of MCs by i.p. injection is about 1.5 $\mu g.g^{-1}$ bw in fish) (Malbrouck and Kestemont, 2006), and the short time of exposure (1 h) largely reduced the possible toxin excretion process through liver detoxification, together leading to a sufficient quantity of toxin that could access to gonad through blood stream and be detected by the immunohistological method. Besides, it was worth to mention that the process of section preparation also affected the result, since a fraction of MC-LR (mainly free MC-LR) was removed during the dehydrating process of the section preparation. The immunostaining for MC-LR indeed detected MC-LR-PP1/PP2A adducts mostly, due to a strong affinity of MC-LR to protein phosphatases PP1/PP2A distributed in both nucleus and cytoplasm (Yoshida et al., 2001). D-MeAsp and D-Glu residues of MC-LR play important roles in forming the MC-LR-PP1/PP2A adducts (Campos and Vasconcelos, 2010), and arginine at position 4 of MC-LR is crucial to the binding by Antibody MC10E7 (Zeck et al., 2001).

The immunolocalization of MC in both spermatozoids and oocytes in the genital glands of snails exposed for 5 weeks to MC-LR (Lance et al., 2010), and the localization of MC-LR in spermatogonia and Sertoli cells of rats injected with 300 μ g.kg⁻¹ bw of MC-LR for 6 days were previously reported (L. Wang et al., 2013). It is still unknown how MCs get into the gonad, even enter reproductive cells (immature and mature). It can be assumed that MCs may be transported into oocytes as protein-bound forms along with protein import during oogenesis, since the exchange of a big amount of protein and other materials are occurring, or even simply through the passive diffusion of a small quantity. Our present immunolocalization of MC-LR reveals that MCs do accumulate in the gonadal tissue of fish, being particularly intense in the connective tissue, and our result also exhibits an apparent distribution of MC in the early stage of oocytes with *in vivo* study

for the first time. The possibility of MC transferring from adults into offspring via toxin accumulation in the reproductive cell is worthy to be further investigated.

The accumulation of MCs in the connective tissue of gonads is largely associated with the potential reproductive toxicity induced by the toxin. The connective tissue of the gonad consists of massive fibers and various types of gonadal somatic cells, such as Leydig cells and Sertoli cells in the testis, and theca cells and granulosa cells in the ovary. These gonadal somatic cells are important functional cells in the gonad, being responsible for synthesizing and secreting androgens and estrogens that are essential for the development and maturation of reproductive cells, or serving as channels for the transport of nutrients into the growing reproductive cells. Therefore, MC-induced injury of gonadal somatic cells could indirectly disturb normal oogenesis and spermatogenesis process, resulting in a reproductive impairment. Furthermore, MC-induced tissue damages in the gonad, such as reduction of vitellus storage, disruption of the connection between the follicular cells and oocytes, disturbance of spermatogenesis, have been reported previously in medaka fish following a chronic exposure (Trinchet et al., 2011). In the present study, no significant histopathological modification in the gonad was observed, which could be due to the short exposure time (1h).

Both liver and gonad play important roles in the reproductive process of oviparous vertebrates. The present study exhibits a distinct localization of MC-LR in the liver and the gonad, and shows a severe tissue injury in the liver of adult medaka fish upon acute exposure to pure MC-LR. This result contributes to a better understanding of the potential reproductive toxicity of MC-LR at the histopathological level, favoring the characterization of underlying mechanisms.

Chapter 4 Hepatic dysfunction is responsible for reproduction impairment in medaka fish upon chronic exposure to MC-LR and the extract of *Microcystis aeruginosa* PCC7820

4.1 Introduction

MCs have been studied extensively by the research community and many conclusions on fish were generated based on short time and high doses of toxin exposure (Djediat et al., 2010; Hou et al., 2014; Li et al., 2005; Malbrouck and Kestemont, 2006). However, in the natural environment, fish and other aquatic organisms are usually subject to long-term exposure to a relatively low concentration of toxins. The potential deleterious effects induced by a weak quantity of MCs are potentially more realistic than those caused by acute intoxication, as noticeable and relatively low concentrations of cyanotoxins are frequently observed in even more environments worldwide. The monitoring of MC concentrations in 26 recreational lakes in Brittany (France) between 2004 and 2011 showed that MC concentration was mostly between 0.1 and 10 μ g.L⁻¹ (Pitois et al., 2016). Another large nation-wide survey of cyanotoxins (1161 lakes) in the United States, conducted in 2007, reported a mean MC concentration of 3.0 μ g.L⁻¹ (Loftin et al., 2016). In addition, the aquatic organisms exposed to cyanobacterial blooms are not only threatened by MCs, as a lot of other secondary metabolites are also produced by cyanobacteria. It has been reported that the toxic effects caused by cyanobacterial extracts could be different from those of individual MC due to the complexity of the composition of the extracts (Marie et al., 2012; Rogers et al., 2011).

Classical toxicology or ecotoxicology approaches are not able to globally characterize the toxicity of emergent pollutants such as a mixture of compounds produced by cyanobacteria. Nowadays, with the advances in high-throughput analytical methods, such as transcriptomics and proteomics, the interactive effects of such complex mixture can be further described without *a priori* hypothesis. In this sense, an integrated analysis of omic data is needed to provide useful information that may not be depicted by a single method. To the best of our knowledge, no systematic study consisting of integrated transcriptomics and proteomics analysis to delineate the

overall hepatic alteration at the cellular and molecular level in fish upon exposure to environmentally relevant concentrations of cyanotoxins has been performed before.

In the present study, a 28-day balneation exposure was conducted in adult medaka fish, a model aquatic vertebrate for toxicological and ecotoxicological study. The goal was to obtain a comprehensive understanding of the hepatic alterations at histological, proteome and transcriptome levels of fish chronically exposed to environmentally relevant concentrations of MC-LR (1 and 5 μ g.L⁻¹) and to a *Microcystis* complex extract (5 μ g.L⁻¹ of equivalent MC-LR). It has been proposed that liver dysfunctions could be responsible for indirect reproductive disorders, since it performs key functions in the regulation of different reproductive processes (Li et al., 2013). Moreover, the structural integrity and germ cell development have been evaluated by histological observation and transcriptional level evaluation of key proteins and enzymes in the gonad. Therefore, the present study seeks to better understand the potential reproductive toxicity of the cyanotoxins in aquatic organisms, contributing to advance our current knowledge about the protection of aquatic organism populations, as well as human reproductive health.

4.2 Material and methods

4.2.1 Toxin and Microcystis aeruginosa PCC 7820 extract preparation

MC-LR, purchased from Novakit® (Nantes, France), was selected as the model MC for the present experiment.

The monoclonal *Microcystis aeruginosa* strain PCC 7820, producing various MCs, among other secondary metabolites (Table 4.1), was maintained in the Paris' Museum Collection (PMC) of cyanobacteria and cultured in Z8 medium (Kotai, 1972) (25 °C, 16 h:8 h light:dark photoperiod at 16 μ mol of photons.m⁻².s⁻¹). This strain has already been used as a representative strain of bloomforming cyanobacteria in previous studies (Liu et al., 2005). Dense cultures of PCC 7820 been at the exponential growing phase (above 1,000 μ g.L⁻¹ chl a) was freshly incubated from a 50 mL inoculum in 700 mL of Z8 during two weeks (25 °C, 16 h:8 h light:dark photoperiod at -50 °C for 48 hours, weighted and stored at -20 °C prior to metabolite extraction according to previously described for this strain (Le Manach et al., 2016). The lyophilized cells were sonicated in 80% methanol on ice for 30 min (Sonics VibracelITM, amplitude 100%, 1 pulse of 30 s per 3 min). The homogenates were evaporated as previously described to get a solid extract (Djediat et al., 2011),

that was dissolved in 50% ethanol (Vol/Vol) and partially evaporated until completely removing the ethanol with a Speedvac. MCs concentration in the extract was quantified using a commercial Adda-specific AD4G2 ELISA test (Abraxis).

Metabolite class	Name	Mass (Da)	Count area	Annotation criteria
Acutiphycin		480.4	4.2	MS
Aerucyclamide	С	516.2	6.1	MS/MS
Aeruginosin	620	620.2	6.7	MS/MS
Aeruginosin		622.2	4	MS
Anabaenopeptin	Oscillamide C	956.6	11.9	MS/MS
Cyanopeptolin		938.5	45.3	MS/MS
Cyanopeptolin		924.5	4.6	MS/MS
Cyanopeptolin	972	972.5	1.5	MS
Cyanopeptolin	Micropeptin 90	962.5	0.8	-
Cyclamide	Aerucyclamide A	534.2	32.3	MS/MS
Cyclamide	Dendroamide C	536.2	12.2	MS
Cyclamide	Ulongamide D	671.4	2.8	MS
Microcyclamide	MZ602	602.1	3	MS
Microcystin	[Dmet-Asp3]MC-LR	980.5	27	MS/MS
Microcystin	MC-LR	994.6	17.5	MS/MS
Microcystin	[Dmet]MC-LR-Phe?	1127.6	2.3	MS
Microcystin		1083.6	2.1	MSMS
Microcystin	MC-LR-Phe?	1141.6	1.8	MS
Microcystin	[MDha3]MC-LR	948.5	1.8	MS
Microcystin	[D-Asp3, Dha7]MC-LR	966.5	0.9	-
Microcystin	MC-AR	952.6	0.9	-
Microginin	FR5	686.3	3.6	MS
Microginin	FR5	725.4	1.5	MS
NONAeruginosin	NON298-A	604.2	2.4	MS
Pseudo-aeruginosin	KT554	554.2	12.6	MS
unknown		552.2	63.5	-
unknown		323.2	17.9	MS
unknown		303.2	8.5	-
unknown		355.3	7.5	-
unknown		331.3	5.5	-
unknown		518.2	5.4	-
unknown		684.3	4.5	-
unknown		910.5	3.4	-
unknown		531.3	2.2	-
unknown		534.8	2	-
unknown		352.3	1.5	-
unknown		570.2	1.4	-
unknown		262.1	0.9	-
unknown		311.2	0.6	-
unknown		243.2	0.2	

Table 4. 1 Metabolite contents of Microcystis aeruginosa PCC 7820 determined by LC-ESI-MS/MS

4.2.2 Fish maintenance, exposure and sampling

The experimental procedures were conducted in accordance with the European Union regulations concerning the protection of experimental animals and the validation of experimental procedure by MNHN's ethical committees (N°68-040 for 2013-18). Medaka fish (*Oryzias latipes*) is a model aquatic vertebrate for toxicological and ecotoxicological study. Medaka fish of the inbred Cab strain maintained in the lab was used for this experiment. Five-month-old adult fish were fed in 20 L glass aquaria filled with a mixture of tap water and reverse osmosis filtered water (1:2) in a flow-through system for aeration and filtration, in a temperature controlled room (25 \pm 1 °C), with a 15 h:9 h light:dark cycle (reproductive cycle). Fish were fed three times a day with commercial dry bait for juvenile salmons.

Sixty females and sixty males were randomly selected and dispatched into 4 experimental groups, namely "Cont" with control solvent, "MC1" with 1 µg.L⁻¹ MC-LR solvent, "MC5" with 5 µg.L⁻¹ MC-LR solvent, and "Ext5" with PCC 7820 Microcystis aeruginosa strain extract containing 5 µg.L⁻¹ equivalent MC-LR. Concerning MC content of the PCC 7820, the extract predominantly contains MC-LR and [Dmet-Asp³] MC-LR variants, along with traces of other variants, in accordance with previous investigations (Le Manach et al., 2016). The MC content was globally quantified using Adda specific ELISA test and was expressed according to MC-LR equivalent. Each group comprised three glass aquaria with a flow-through system for aeration and filtration. Five females and five males were randomly transferred into each aquarium containing 11.5 L water (15 females and 15 females per group) two weeks prior to the beginning of the experiment, representing a fish loading of about 0.4 g fresh weight of fish.L⁻¹. The room temperature, light and feeding parameters were the same as described above. The balneation exposure lasted for 28 days and the respective water conditions were maintained by renewing 4.5 L stale water with fresh water containing the respective toxin contents of each condition, every 2 days. Water parameters including temperature, pH, conductivity and total dissolved solids were measured every day and nitrite and nitrate concentrations were measured once per week. MCs concentrations in aquaria were determined every 6 days by using the AD4G2 ELISA kit (Table 4.2). Fish were inspected three times a day, and no abnormal behavior nor significant mortality was observed (only 1 female under Ext5 group died on the 6th day of exposure and 1 male fish died in the control group on the 21st day). Eggs were collected every 2 days. By the end of the exposure, fish were anesthetized in buffered 0.1% MS-222, the blood (around 10 µL per individual) was collected by cutting the tail using glass capillary tubes that were rinsed with heparin, and then stored in heparinized microtubes.

Then fish were sacrificed, and the liver and the gonad were collected on ice, then prepared and stored for the different analyses. The blood of 3 fish was pooled to get 1 blood sample that was centrifuged at 1000 g for 20 min at 4 °C in order to separate the plasma. The collected plasma was stored at -20 °C for sex hormone examination.

Table 4. 2 MC concentration in different MC-containing conditions determined by using Adda-specific AD4G2 enzyme-linked immunosorbent assay (ELISA) kit every 6 days (mean ± SD, n=3, water samples were taken just after renewal stale water).

Exposure time(day)	Concentration of MCs (µg.L ⁻¹)				
	Cont	MC1	MC5	Ext5	
1	below detectable limit	0.53	5.26	3.52	
6		0.49	1.07	1.95	
11		0.19	2.06	1.55	
16		0.15	1.55	1.48	
21		0.28	1.66	1.24	
28		0.42	1.86	1.14	
Mean		0.34	2.24	1.81	
SD		0.16	1.51	0.88	

4.2.3 Reproductive parameters

The eggs attached to the abdomen of females were harvested manually at 1 PM every two days. The eggs from the same aquarium and of the same date were pooled and placed in Yamamoto medium. Only the fertilized eggs (transparent) were further kept and placed in a specific incubator maintaining a photoperiod of 12 h:12 h light:dark at 25 °C. The Yamamoto medium was renewed daily to prevent bacterial contamination. The dead eggs, which turned blue due to penetration by the Coomassie blue of the Yamamoto medium were counted and removed from the Petri dishes. When the embryos hatched, the date and the number of hatched eggs were recorded and an inspection was conducted until the last egg hatching. The reproductive performance was evaluated by the total number of fertilized eggs per female and the percentage of hatched eggs. Results are presented as the mean \pm SD and the levels of significance were evaluated by one-way ANOVA with post hoc Tukey HSD test for *p*<0.05 or *p*<0.01 using R software (version 3.2.5, package ggplot2).

4.2.4 Histological observation and immunolocalization of MCs in liver and gonad

The half liver of each individual and the whole gonad (5 fish per condition) were fixed in Davidson solution (Annex 3.1) immediately after dissection, kept at 4 °C for 24 h, then transferred into a graded series of ethanol (from 70% to 95%) for dehydration, and then embedded in paraffin.

Blocks were cut into 3 µm-thick sections, and slides were stained with HES or PAS/Alcian blue according to the respective observation purposes, or used to perform immunolocalization of MCs.

The liver lysis was determined through the full view observation of 2 sections per individual. The glycogen content detection (estimated as a surface percentage of the purple-red pixels, 5 randomly selected views per individual) was performed by ImageJ software (version 1.51d).

The HES sections of gonad were used to check the structural integrity and the status of gonad development. Different stages of germ cells were identified and classified into 4 stages (perinucleolar oocyte, cortical alveolar oocyte, vitellogenic oocyte and mature oocyte) in the ovary and 3 stages (primary spermatocyte, secondary spermatocyte and spermatid) in the testis according to their classical characteristics (Dietrich and Krieger, 2009b). The percentage of different oocytes on the full surface of 2 sections per individual, and the cell density of different stages of spermatocytes in one seminiferous duct (full view observation, 5 randomly selected views per section, 2 sections per individual) were calculated.

Statistical analysis of all the parameters in the liver and the gonad (n=5 individuals per condition for each gender) was conducted by one-way ANOVA with post hoc Tukey HSD test for p<0.05 or p<0.01 using R software.

Immunolocalization of MCs was performed under the same process used in the acute exposure (described in Section 3.2.4). The only difference is that the monoclonal antibody to MCs (AD4G2, Alexis) that recognizes all MCs by Adda residue (dilution 1:4000) was used here, in order to detect all variants of MCs from the extract condition. The secondary antibody control sections were prepared by skipping the first antibody MC10E7 incubation step. One MC-contaminated medaka liver sample from an acute high dose exposure in the lab previously was included as the technically positive control.

4.2.5 Sex hormone measurement in the plasma

Plasma sex steroid hormones, E2 and 11-ketotestosterone (11-KT), were measured using commercially fish specific ELISA kit in accordance with the manufacturer's instruction. All samples (5 samples per condition for each gender) and standards were run in duplicates. The sex hormone level was expressed by the ratio of E2 concentration to 11-KT concentration. Statistical

analysis of E2/11-KT values (n=5, mean±SD) was conducted by one-way ANOVA with post hoc Tukey HSD test for p<0.05 or p<0.01 using R software.

4.2.6 Proteomic analysis of the liver

Protein sample preparation and mass spectrometry analysis. The half liver of 4 or 5 individuals (adjusting according to the size of tissue) were pooled to get 1 pooling sample per condition (males and females, respectively). The pooled livers were homogenized and prepared as described in the method of Section 2.2.4 to get protein samples. Fifty µg of proteins of each liver were used for digestion with 3 µg of proteomic-grade trypsin (Sigma-Aldrich, USA), prior to coupling with iTRAQ 4-plex tags following the manufacturer's protocol (Sciex®). For both males and females, the pooled liver sample of control animals and the ones exposed to MC1, MC5 and Ext5 were labeled with the 114, 115, 116 and 117 tags, respectively. Protein digests were analyzed in triplicates using both a nano-LC (Ultimate 3000, Dionex) coupled to an ESI-LTQ-Orbitrap (LTQ Orbitrap XL, Thermo Scientific) mass spectrometer and a nano-LC system (ACQUITYTMUPLC® M-Class, Waters Corp.) coupled to a SYNAPT[®] G2-*Si* HDMSTM (Waters Corp.) mass spectrometer, as described below.

Mass spectrometry analysis using LTQ Orbitrap XL (Thermo Fisher). The iTRAQ-based quantitative proteomic analysis was performed using nano-LC (Ultimate 3000, Dionex) coupled with an ESI-LTQ-Orbitrap (LTQ Orbitrap XL, Thermo Scientific) mass spectrometer. Six μ g of iTRAQ-tagged liver protein digests solubilized in 10% ACN with 0.1% formic acid were injected in triplicate by the autosampler and were concentrated on a trapping column (Pepmap, C₁₈, 300 μ m x 50 mm, 3 μ m 100 Å, Dionex) with water containing 10% ACN with 0.1% formic acid (solvent A). After 5 min, the peptides were eluted onto a separation column (Pepmap, C₁₈, 75 μ m x 500 mm, 2 μ m 100 Å, Dionex) equilibrated with solvent A. The peptides were separated with a 2 h-linear gradient, increasing from 10% to 80% ACN + 0.1% formic acid (solvent B) at a flow rate of 200 nL.min⁻¹. Spectra were measured at a mass scan range of *m*/*z* 300-2000 and at a resolution of 30,000 in the profile mode followed by data dependent CID and/or HCD fragmentation of the ten most intense ions, with a dynamic exclusion window of 60 s. MS/MS data were exported in mgf format using Excalibur software.

Mass spectrometry analysis using SYNAPT G2-*Si* HDMS (Waters Corp.). The iTRAQ-based quantitative proteomic analysis was also performed using nano-LC (ACQUITYTM UPLC® M-Class, Waters Corp.) coupled with a SYNAPT® G2-*Si* HDMSTM mass spectrometer (Waters Corp.) in

HD-DDA mode. One µg of iTRAQ-tagged liver protein digests solubilized in 3% ACN with 0.1% formic acid were injected in triplicate. Samples were desalted and concentrated were concentrated on a trapping column (ACQUITYTM UPLC® M-Class Trap V/M Symmetry®, C₁₈5 μm, 180 μm x 20 mm, Waters Corp.) at 10 µL.min⁻¹ for 2 min with water containing 0.1% formic acid (solvent A). The peptides were then separated onto an analytical column at 35°C (ACQUITYTM UPLC® M-Class HSS T3 1.8 µm, 175 µm x 20 mm, Waters Corp.) with a 120 min-gradient, increasing from 1% to 35% ACN + 0.1% formic acid (solvent B) at a flow rate of 300 nL.min⁻¹, followed by a cleaning step at 85% B over 5 min. Additionally, a Lockmass reference (100 fmol.µL⁻¹ Glu-1-fibrinopeptide B) was delivered to the NanoLockSpray of the mass spectrometer at 0.5 µL.min⁻¹ and was sampled at 60 s intervals. For all measurements, the mass spectrometer was operated in data-dependant positive ESI mode at a resolution of 20,000 FWHM at 400 m/z. MS spectra were measured for 0.2 s at a mass range of m/z 350-5000. The 20 most intense ions were selected for CID fragmentation. MS/MS spectra were measured for 0.1 s at a mass range of m/z 50-2000 and the "TIC stop" parameter was set to 100,000 intensity.s⁻¹ allowing a maximum accumulation time of 250 ms (i.e. up to three tandem MS spectra of the same precursor). A dynamic exclusion of 30 s (\pm 500 mDa) was set. An ion mobility configuration was used to increase sensitivity on the fragment ions. Briefly, wave velocity was ramped from 2500 m.s⁻¹ to 400 m.s⁻¹ (start to end) and the ramp was applied over the full IMS cycle. A manual release time of 500 µs was set for the mobility trapping and a trap height of 15 V with an extract height of 0 V. The IMS wave delay was set to 1000 µs for the mobility separation after trap release. HD-DDA data were processed into ProteinLynx Global Server 3.0.2 (Waters Corp) and exported in mgf format.

Data treatment. The results obtained from all runs from both instruments were exported as mgf files and merged into a single file for each sex for further identification and quantification data treatment. All MS/MS-analyzed samples were analyzed using Mascot 2.4.1 (Matrix Science, UK) with Scaffold software (version 4.4.8; Proteome Software, USA) against Ensembl databases for a few species of fish (restricted to *Oryzias latipes, Danio rerio* and *Tetraodon nigroviridis* sequences, of the Ensembl database V78). The ion mass tolerance and the parent ion tolerance were set to 0.05 Da. The methyl methanethiosulfonate of cysteine was specified as fixed modifications. Oxidation of methionine and iTRAQ 4-plex of tyrosine for iTRAQ-derivatized samples were specified as variable modifications. Scaffold Q+ was used to quantify the isobaric tag peptide and protein identifications. Peptide identifications were accepted if they could be established with more than 95.0% probability. Protein identifications were accepted if they could be established with more than

99.0% probability and contained at least 2 identified peptides. Only identified proteins (with Ensembl ID) were considered for quantification and further analysis. Peptides were quantified using the centroid reporter ion peak intensity. The minimum quantitative value for each spectrum was calculated as 0.025% of the highest peak. Quantitative ratios were Log_2 normalized for final quantitative testing, using control value set up as a reference sample in both genders. The quantities of proteins were considered significantly changed when $|Log_2FC| > 0.5$.

4.2.7 RNA-seq analysis of the liver

Library construction and sequencing process. The half liver of female fish (2 or 3 individuals) and male fish (1 or 2 individuals) (adjusting according to the size of tissue) were pooled respectively, to get 4 pooled female samples and 3 pooled male samples per condition. Then the samples were homogenized using a bead beater. Total RNA was isolated and purified using RNeasy Plus Mini Kit with gDNA eliminator spin (Qiagen). RNA quantity and quality were evaluated using Qubit RNA Assay Kit in Qubit®2.0 Fluorometer (Life Technologies, USA) and an Agilent Bioanalyzer 2100 eukaryote total RNA Pico series II chip (Agilent Technologies Inc., USA), respectively. Twenty-eight transcriptome libraries (16 females and 12 males) were prepared from the total RNA samples with RIN value over 7.7 using Illumina TruSeq Stranded mRNA Sample Preparation kits (Illumina Inc., USA) following manufacturer's instruction. The libraries were multiplexed in lane 3-5 for females and in lane 6-7 for males. Sequencing was performed on an Illumina Hi-Seq1000 instrument using the TruSeq SBS kit V3-HS 50-cycles (Illumina Inc., San Diego, CA, USA) and 30-40 millions of 51bp single-end reads per library were generated. CASAVA-1.8.2 software was used for demultiplexing.

Data analysis. Raw reads were first cleaned by removing adaptors using Cutadapt-1.3 and then only 51 bp-long reads were kept. The overall quality of the reads was checked using the FastQC 0.10.1 and good Phred scores (>30) were obtained in all the libraries. However, the analysis of the unicity ratio shows a high level of duplications in all the libraries. This high duplication issue was also observed in another transcriptomics investigation of medaka liver published recently (Murata et al., 2015). Therefore, for our data, a step of duplicated reads removal was conducted using a python script (Annex 4.1) that analyses the quality of reads and keeps the one with the best quality score. The information of all the libraries following a serie of reads filtration was presented in Table 4.3.
Name	Treatments	Initial reads	51 bp- length reads	Unique 51 bp-length reads	Mapped reads without multiple hits
			female	2	
lib2_Ctrl_rep1	Control	38,575,606	38,342,834	5,476,326	4,187,204
lib3_Ctrl_rep2	Control	35,336,283	35,174,521	5,000,671	3,879,188
lib4_Ctrl_rep3	Control	38,425,987	38,251,845	5,532,370	4,295,146
lib5_Ctrl_rep4	Control	35,444,305	35,281,840	5,594,890	4,392,620
lib7_MC1_rep1	MC1	42,064,479	41,824,381	5,694,547	4,400,553
lib8_MC1_rep2	MC1	35,326,111	35,172,695	5,644,506	4,425,599
lib9_MC1_rep3	MC1	36,365,411	36,197,230	7,007,894	5,689,071
lib10_MC1_rep4	MC1	33,423,848	33,277,608	5,298,437	4,153,622
lib12_MC5_rep1	MC5	33,984,744	33,838,197	5,932,572	4,765,481
lib13_MC5_rep2	MC5	40,001,615	39,774,485	5,682,842	4,359,182
lib14_MC5_rep3	MC5	34,686,577	34,531,959	5,102,773	4,011,124
lib15_MC5_rep4	MC5	32,465,693	32,318,704	4,102,321	3,139,462
lib17_Ext5_rep1	Extract5	34,537,072	34,344,848	4,708,864	3,589,680
lib18_Ext5_rep2	Extract5	40,447,119	40,259,621	5,583,425	4,338,836
lib19_Ext5_rep3	Extract5	33,032,256	32,887,264	4,855,119	3,772,063
lib20_Ext5_rep4	Extract5	35,761,932	35,592,256	6,308,148	5,038,474
			male		
lib22_Ctrl_rep1	Control	36,611,265	36,417,081	6,327,852	5,152,242
lib23_Ctrl_rep2	Control	35,322,543	35,132,110	6,515,553	5,324,603
lib24_Ctrl_rep3	Control	39,302,923	39,087,605	6,854,286	5,564,618
lib26_MC1_rep1	MC1	35,550,873	35,349,581	6,405,948	5,218,647
lib28_MC1_rep2	MC1	36,899,002	36,698,809	7,104,542	5,831,664
lib29_MC1_rep3	MC1	39,815,171	39,598,761	7,802,274	6,413,366
lib31_MC5_rep1	MC5	40,987,651	40,767,086	7,066,514	5,748,889
lib33_MC5_rep2	MC5	36,790,125	36,598,766	6,147,867	5,006,770
lib35_MC5_rep3	MC5	37,884,146	37,679,205	6,429,975	5,179,368
lib36_Ext5_rep1	Extract5	35,978,425	35,785,164	6,812,324	5,562,480
lib38_Ext5_rep2	Extract5	35,856,566	35,659,033	6,616,672	5,423,793
lib39_Ext5_rep3	Extract5	34,190,517	34,005,412	6,235,008	5,084,076

Table 4. 3 Number of sequences in all libraries along the data filtration

Tophat2 (v2.0.10) (Kim et al., 2013) was used to map the clean unique reads against the medaka genome (release 81) downloaded from Ensembl. Multiple hits were removed by samtools (v0.1.18) and read counting on gene exons was accomplished by HTSeq-count (v0.6.1p1) (Anders et al., 2015) in union mode against the annotation of medaka genomes downloaded from Ensembl. DESeq2 (v1.8.1) (Love et al., 2014) was used to do differential expressed gene analysis. The script and command of these softwares were shown in Annex 4.1. Genes were considered differentially expressed when the FDR (false discovery rate) was below 0.05, using the control group as reference.

qPCR validation. For each treatment, the same RNA samples used for RNA-seq, together with

one additional replicate were used as input to conduct sequencing results validation by quantitative real-time PCR (gRT-PCR). Eleven genes (1-sf, dec1b, prmt7, asb16, cvp4v8, rab44, hsf2, rorcb, arntlla, ppp1r1b and one gene encoding un uncharacterized protein) were randomly selected from RNA-seq significantly differential expressed genes of MC5 and Ext5 conditions within the expression intensity gradient. 18s RNA and rpl7 (ribosomal protein l7) were selected as the reference genes after the relative stability measurement by geNorm (Vandesompele et al., 2002). The gene-specific primer pairs (Table 4.4) were designed by using Primer 5.0. One µg of total RNA from each sample (5 replicates for females and 4 replicates for males) was used to synthesize the first strand cDNA using iScript Advanced cDNA Synthesis Kit (Bio-rad) according to the instructions of the manufacturer. Quantitative real-time PCR analysis was performed using LightCycler FastStart DNA Master Plus SYBR GreenI(Roche) on Light Cycler 1.5 instrument (Roche). The following three-step reaction was performed: pre-denaturation at 95 °C for 10 s, 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 5 s and elongation at 72 °C for 8 s. Melt curve analysis was performed at the end of the reaction to demonstrate the reaction specificity. Each plate contained non-template control with water instead of the template. All PCR reactions were performed in duplicate. The relative quantifications of target genes were presented as Log₂FC relative to the expression level of the control group using the $^{\Delta\Delta}$ CT method (Pfaffl, 2001). All quantitative data (mean value) were compared with corresponding RNA-seq results and the linear correlation rate was calculated.

Gene ID	Gene	Forward primer	Reverse primer
ENSORLG00000010134	1-sf	TGGCACTTCAACACTTAGT	TTCTTCAATGGGGATAGG
ENSORLG00000014397	dec1b	GAGAAGAAAAGGCGTGAC	CTCCAGATGGCCCAGA
DQ118296.2(gene bank)	rpl7	TCGGGAACTATTATGTGC	GACCTTGGGGGCTGACT
AB105163.1(gene bank)	18sRNA	CCTGCGGCTTAATTTGACTC	GACAAATCGCTCCACCAACT
ENSORLG00000015041	prmt7	CCGTCTGTCTGAGCGTC	CACCTCCCGTTATGGC
ENSORLG00000015822	Uncharacterized	TTGGTTCATCCTGGTCTC	TCCGCCTGCTATCACTA
ENSORLG0000002620	asb16	ACCAAAACCAGCATCC	GCTGGGGTAGGAGTTCA
ENSORLG0000008162	cyp4v8	GTGATGAACGAGCAAGC	CTTTGTCTTCGGCTGATA
ENSORLG00000020036	rab44	GCCTTTCTCCTGATGTATG	TTCTCCCTGTTGGCTTTT
ENSORLG00000015498	hsf2	TTCAAAGCAACCGCATCA	CGTTCAGACCGTTGACAGG
ENSORLG00000014886	rorcb	CTGTGAAGGCTGTAAGGG	GCAGCGGTTTCGGTTA
ENSORLG00000019370	arntl1a	AAGACGCTCAGAGGTGC	CAGCCCACGACAAACA
ENSORLG0000003438	ppp1r1b	AACCAACCTGGACCCC	CCCACAACCCACTGATG

Table 4. 4 Gene-specific primers of RT-qPCR for RNA-seq validation

4.2.8 Ingenuity pathway analysis (IPA) of transcriptomics and proteomics data

Medaka dysregulated genes (FDR<0.05 and $|Log_2FC|>0.4$) and proteins ($|Log_2FC|>0.5$) were converted to the corresponding Human orthologous according to Ensembl online platform. IPA software (V01-07; Qiagen) was used to perform canonical pathway analysis and molecular and cellular function determination based on the specific Ingenuity Knowledge Database (using default parameters for all tissues and cell lines, with relaxed filters), which constitutes a repository of biological interactions and functional annotations.

4.2.9 Transcriptional regulation of selected genes in the gonad determined using RT-qPCR

The ovary of 2 female fish and the testis of 2 male fish were pooled, respectively, to get 5 pooled samples per gender in each condition. Total RNA from the gonad samples was isolated and purified following the same process as liver RNA extraction. RNA quantity and quality were evaluated using Qubit®2.0 Fluorometer (Life Technologies, USA) and gel electrophoresis test, respectively. All the RNA samples (20 females and 20 males) passed the quality check. One hundred ng of total RNA was used for reverse transcription, and the quantitative real-time PCR analysis was performed following the process described in Section 4.2.7. The gene-specific primer pairs of eight selected genes (*fshr, cyp19a, star, cyp17a, 3bhsd, sod, cat, bcl2*) were presented in Table 4.5. *18s RNA and rpl7* were used as the reference genes. The relative quantifications of target genes were presented as Log₂FC relative to the expression level of the control group using the $^{\Delta\Delta}$ CT method (Pfaffl, 2001). Statistical analysis of the normalized relative expression level (n=5 individuals per condition for each gender) was conducted by one-way ANOVA with post hoc Tukey HSD test for *p*<0.05 or *p*<0.01 using R software.

 Table 4. 5 Gene-specific primers of RT-qPCR for the evaluation of transcriptional changes in the selected genes in the gonad.

Gene	Description	Forward primer	Reverse primer
fshr	Follicle-stimulating hormone receptor	CCTTCGTGGGTTCCAGTGA	TTCAGGCCACTGATGATGT
		GT	TATCG
cyp19a	Cytochrome P450 19A	GGGTGTTCCTGTTGACGAG	CTGCTTGCTCCATTTCCT
		А	
star	Steroidogenic acute regulatory protein	GAAGGGCATCATTAGAGC	AACTTGGTCTTGTTTGGGT
			G
cyp17a	Cytochrome P450 17A	GAGACGGCAAAGACATCG	GCCTCAGACAGGGTGGA
3bhsd	3 beta-hydroxysteroid dehydrogenase	GCGGGACGAAACTCAG	CGTTCTCGTGGAGGCAC
sod	Superoxide dismutase	CCTCACTTCAACCCATACA	ACCACAGTTCTGCCAACA
	-	A	
cat	Catalase	GCGGTACAACAGCGCAGA	GGATGGACGGCCTTCAAG
		TGAAG	TTC
bcl2	Apoptosis regulator B-cell lymphoma	GGGATGGGATGCCTTTGT	CTTGATGGACGGCCAGTA
	2		G

4.3 Results

4.3.1 Reproductive impairment

During the exposure period, the fecundity (Figure 4.1A) and the hatchability (Figure 4.1B) significantly decrease under the three MC-containing treatments compared with the control at multiple time points (p<0.05). These results indicate that reproductive performance of medaka fish is adversely affected by MC-containing treatments.



Figure 4. 1 Reproductive performance monitored during the 28-day exposure.

A: fertilized eggs per fish (mean \pm SD, n=15); B: hatchability (mean \pm SD, n=3). * and ** indicate statistically significant differences at *p*<0.05 and *p*<0.01.

4.3.2 Histological observation and immunolocalization of MCs in the liver and the gonad

In the liver of the control fish, the hepatocytes present compact and characteristic cord-like parenchymal organization (Figure 4.2 A and E). In contrast, noticeable hepatic lesions characterized by cellular disjunctions and various lytic areas are observed in all toxic treatments, being particularly obvious in MC5 and Ext5 treatments. Some hepatocytes lose their characteristic shape and become rounded (Figure 4.2 B-D and F–H). The proportion of lytic area exhibits an increasing trend in all toxic treatments compared with the control (Figure 4.4 A).

Intense glycoprotein and/or glycogen reserves stained in purple-red by PAS are distributed on all slides of the control fish (Figure 4.3 A and E). In contrast, a severe decrease in glycoprotein and/or glycogen reserves is observed in the liver of toxin-treated females (Figure 4.3 B-D), and the numerical values are plotted in Figure 4.4 B, showing a statistically significant decrease in MC5 and Ext5 treated females.





Figure 4. 2 Representative histological observations of liver sections stained with HES. Females (A: Cont, B: MC1, C: MC5, D: Ext5) and males (E: Cont, F: MC1, G: MC5, H: Ext5), respectively.





Figure 4. 3 Representative histological observations of liver sections stained with PAS. Females (A: Cont, B: MC1, C: MC5, D: Ext5) and males (E: Cont, F: MC1, G: MC5, H: Ext5), respectively.



Figure 4. 4 Histological observation of the liver.

A: percentage of lytic area determined through the light microscopy observation of HES-stained sections (mean \pm SD, n=5); B: percentage of glycogen content determined through the light microscopy observation of PAS-stained sections (mean \pm SD, n=5). * and ** indicate significant differences at *p*<0.05 and *p*<0.01, respectively.

In the gonad of both gender of toxin-treated fish, no noticeable tissue lesion is found, no significant change in the percentage of different stages of oocytes (Figure 4.5 A), nor in the density of different stages of spermatocytes is detected (Figure 4.5 B).



Figure 4. 5 Histological observation of the gonad.

A: percentage of different stages of oocytes determined through the light microscopy observation of HES-stained sections (mean \pm SD, n=5); B: cell density of different stages of spermatocytes in one seminiferous duct determined through the light microscopy observation of HES-stained sections (mean \pm SD, n=5).

Through light microscopy, all the liver, ovary and testis of the three MC-containing treatments (MC1, MC5 and Ext5) do not exhibit any positive signal of MCs specific antibodies (Figure 4.6 A, -D) whereas the liver of technical positive control display a strong signal distributed in the hepatocytes (Figure 4.6 E), which validates our immunostaining process technically.



Figure 4. 6 Representative photos of immunolocalization of MCs in the liver and gonad of adult medaka fish.

The liver under MC5 treatment (A), the liver under Ext5 treatment (B), the testis under MC5 treatment (C), the the ovary under MC5 treatment (D), The technically positive control: MC-contaminated medaka liver sample from an acute high dose exposure in the lab previously (E), stained with both toluidine blue and MC-LR immunolabeling (AD4G2), revealed with peroxidase specific reaction of DAB.

4.3.3 Plasma sex steroid hormones

No significant change in the plasma sex steroid hormone level (E2/11-KT) is observed in any toxin treatment (Figure 4.7).



Figure 4. 7 Plasma sex steroid hormone determination using ELISA (mean \pm SD, n=5).

4.3.4 Proteomic analysis of the liver

The iTRAQ-based quantitative proteomic approach provides identification for a total of 305 and 453 proteins in females and males, respectively. Among these identified proteins, 298 and 448 of them present reliable quantifications according to relative tag intensity normalized according to the control group. 150 and 153 proteins were considered as differentially regulated proteins in females and males, respectively ($|log_2FC|>0.5$, under at least one of the three MC-containing conditions, Annex 4.2).

The up- or down-dysregulated proteins are individually assigned to functional groups according to their respective gene ontology classification (Figure 4.8). Overall, they are related to various material and energy metabolisms, oxidation-reduction processes, detoxification, translation and nuclear receptor signaling.

A complementary canonical pathway analysis performed by IPA with the human orthologous sequences of the differentially regulated proteins highlight that various pathways mainly concerning cellular stress and growth, nuclear receptor signaling, energy, amino acids and hormones metabolisms were affected in response to all the three toxic treatments (p<0.01, Figure 4.9).



Figure 4. 8 Heatmap representation of the overall alteration of hepatic proteomes with gene ontology classification. 225 dysregulated proteins ($|Log_2FC|>0.5$, under at least one toxic condition) for both genders in total are represented by Log_2FC value using Gene-E freeware (http://www.broadinstitute.org/cancer/software/GENE-E/). Up-regulated proteins are indicated in red, down-regulated proteins in green, and missing values in gray.

	Category	Canonical Pathway		P value	
		,, ,	MC1	MC5	Ext5
	cellular stress and injury	NRF2-mediated Oxidative Stress Response	1.89E-05	3.86E-04	4.06E-02
	cellular growth, proliferation	EIF2 Signaling	2.54E-04	1.31E-06	4.22E-03
	and development				
	hormones degradation	Serotonin Degradation	1.15E-03	7.34E-03	1.00E+00
	disease specific pathway	Mitochondrial Dysfunction	1.90E-03	4.26E-02	3.44E-03
	metabolic regulations biosythesis	Creatine-phosphate Biosynthesis	1.53E-02	9.56E-03	8.85E-03
	nuclear receptor signaling	LPS/IL-1 Mediated Inhibition of RXR Function	r 4.76E-03	8.34E-04	5.85E-02
		Aryl Hydrocarbon Receptor Signaling	9.17E-03	2.44E-03	2.21E-01
Female	amines and polyamines degradation	Choline Degradation I	6.13E-03	3.84E-03	1.00E+00
1 onnaio		4-aminobutyrate Degradation I	9.19E-03	5.75E-03	5.32E-03
	amino acids metabolism	Threonine Degradation II	6.13E-03	3.84E-03	3.55E-03
		Alanine Degradation III	6.13E-03	1.00E+00	3.55E-03
		Alanine Biosynthesis II	6.13E-03	1.00E+00	3.55E-03
		Glutamate Degradation II	9.19E-03	5.75E-03	5.32E-03
		Aspartate Biosynthesis	9.19E-03	5.75E-03	5.32E-03
		L-cysteine Degradation I	1.22E-02	7.66E-03	7.08E-03
		Proline Biosynthesis I	1.22E-02	7.66E-03	7.08E-03
		Glutamate Degradation III	1.53E-02	9.56E-03	8.85E-03
	cellular growth proliferation	EIF2 Signaling	2.3E-08	3.0E-21	1.9E-13
	and development	Regulation of eIF4 and p70S6K Signaling	3.8E-05	3.3E-06	2.3E-07
		mTOR Signaling	8.7E-06	1.6E-04	1.2E-06
	sugars biosynthesis	Gluconeogenesis I	1.9E-03	5.2E-03	7.1E-02
	hormone biosynthesis	Thyroid Hormone Biosynthesis	7.8E-03	1.3E-02	8.8E-03
	generation of precursor metabolites	TCA Cycle II (Eukaryotic)	2.8E-05	4.4E-03	6.5E-02
	and energy	Pentose Phosphate Pathway	2.9E-04	8.1E-04	3.7E-04
	metabolic clusters	tRNA Charging	4.6E-03	6.4E-04	5.8E-03
	cofactors, prothetic groups	S-adenosyl-L-methionine Biosynthesis	7.8E-03	1.3E-02	8.8E-03
	and electron carriers biosythesis				
Male	C1 compounds utilization	Formaldehyde Oxidation II	1.0E+00	8.6E-03	1.0E+00
	and assimilation				
	fatty acid biosynthesis	Fatty Acid Biosynthesis Initiation II	1.0E+00	8.6E-03	5.8E-03
		Palmitate Biosynthesis I (Animals)	1.0E+00	8.6E-03	5.8E-03
	amino acids metabolism	Superpathway of Methionine Degradation	7.7E-05	3.5E-04	4.0E-03
		Aspartate Degradation II	1.4E-04	3.8E-04	1.0E+00
		Cysteine Biosynthesis III (mammalia)	4.8E-02	3.0E-03	1.4E-03
		Phenylalanine Degradation I (Aerobic)	1.0E+00	1.1E-04	1.0E+00
		L-cysteine Degradation II	1.0E+00	4.3E-03	2.9E-03
		Cysteine Biosynthesis	1.0E+00	8.6E-03	5.8E-03
		Glutamate Degradation X	1.0E+00	8.6E-03	5.8E-03
		Glutamate Biosynthesis II	1.0E+00	8.6E-03	5.8E-03

Figure 4. 9 Significantly affected pathways determined with hepatic proteome data through canonical pathway analysis of IPA (p<0.01 is indicated in red).

4.3.5 Transcriptomic analysis of the liver

The high-throughput sequencing of cDNA results in the identification of transcripts corresponding to 16,262 and 15,633 different genes (read count \geq 2) for females and males, respectively. For females, 6, 168 and 418 genes are significantly differentially expressed (SDE) under MC1, MC5 and Ext5 treatments. For males, 17, 126 and 104 genes present differential expression under MC1, MC5 and Ext5 conditions ($|Log_2FC|\geq0.4$, FDR<0.05, Annex 4.3). The apparent increase in the number of SDE genes in MC5 condition compared with that in MC1 condition suggests that the dysregulation of gene expression follows a dose-dependent response.

The high correlation between the RNA-seq and RT-qPCR results provides a clear validation of the overall SDE genes obtained by the RNA-Seq analysis. A good linear correlation between these two approaches was indeed supported by R² correlation coefficient values of up to 0.96 and 0.86 in females and males, respectively (Figure 4.10 A). The global variability of the gene expression in different replicates is investigated by principal component analysis (PCA), showing an apparent distinction between replicates from control and MC5 groups, as well as between control and Ext5 groups, but not between control and MC1 in both genders (Figure 4.10 B). Therefore, the specificities of the SDE genes (representing a limited number) in response to MC1 treatment will not be further considered below in terms of function enrichment and pathway analysis. Venn diagram representations are depicted to compare the dysregulated genes between the different conditions. Only 97 (half) and 32 (one-fourth) SDE genes detected in MC5 condition are present in the SDE genes of Ext5 treatment for females and males, respectively (Figure 4.10 C).

Canonical pathway analysis indicates that the SDE genes significantly contribute to various pathways (p<0.01), including circadian rhythm signaling, cellular growth and injury, amino acid, nucleotide, fatty acid and lipid metabolisms and so on (Figure 4.11 A). The respective Log₂FC values of all these genes involved in those canonical pathways are shown in a heatmap (Figure 4.11 B).



Figure 4. 10 Analysis of hepatic transcriptome through RNA-seq.

A: RT-qPCR validation of the SDE genes determined through RNA-seq. B: PCA illustration of the global variability of individuals under different conditions in females and males, respectively. C: Venn diagram representations to compare the SDE genes between different MC-containing conditions by directly comparing the gene entries.

Α

	Category	Canonical Pathway	P value	
			MC5	Ext5
	nourotransmitters and other naryous system	Circadian Phythm Signaling	1 65 00	1 25 06
	ineurotransmitters and other hervous system		1.0E-09	1.3E-00
	signaling	Cholecystokinin/Gastrin-mediated Signaling	4.9E-01	8.4E-03
		Axonal Guidance Signaling	5.4E-01	9.2E-03
	cellular growth, proliferation and development	Sertoli Cell-Sertoli Cell Junction Signaling	1.0E+00	4.0E-03
		B Cell Activating Factor Signaling	1.0E+00	5.0E-03
	transcriptional regulation	Assembly of RNA Polymerase I Complex	57E-02	1 0E-02
			5.72-02	1.02-02
	nuclear receptor signaling		5.6E-01	3.6E-04
		LXR/RXR Activation	1.9E-01	2.7E-04
		PXR/RXR Activation	1.0E+00	1.8E-05
		TR/RXR Activation	1.1E-01	1.7E-05
		PPARa/RXRa Activation	3 3E-01	1.5E-05
		Estragon Recentor Signaling	5 7E 01	7 45 02
			3.7E-01	7.1E-03
	intracellular and second messenger signaling	Gaq Signaling	2.5E-01	4.2E-03
		Insulin Receptor Signaling	5.8E-01	2.2E-03
		Adipogenesis pathway	2.5E-04	9.0E-03
		Glucocorticoid Receptor Signaling	1.0E+00	8.9E-03
		RhoA Signaling	4 6E-02	1 3E-03
		Protoin Kingan A Signaling	2.55 01	1.50 00
			2.5E-01	1.5E-04
	disease-specific pathways	Pancreatic Adenocarcinoma Signaling	1.5E-01	2.5E-03
	cellular stress and injury	Antioxidant Action of Vitamin C	1.4E-01	3.3E-04
	cancer	ERK/MAPK Signaling	1.2E-01	5.5E-03
Female		Sonic Hedgehog Signaling	1.0E+00	1.7E-03
	organismal growth and development	Wnt/Ca+ pathway	3.1E-01	2.8E-03
	anontosis	April Mediated Signaling	1.05+00	4 15 02
			1.0E+00	4.1E-03
	cellular immune response	Interferon Signaling	1.7E-03	4.7E-01
	amino acids metabolism	Citrulline Biosynthesis	1.5E-05	7.8E-03
		Superpathway of Citrulline Metabolism	9.5E-05	2.4E-02
		Taurine Biosynthesis	1.0E+00	3.0E-04
		Phenylalanine Degradation IV (Mammalian via Side Chain)	3 7E-03	1 0E+00
	fathy acide and linide motabolism	Choline Biosynthesis III	3 25-03	1 35.03
	fatty acids and lipids metabolism	Obstantast Biografia III (cia Decreation)	5.2E-05	1.3E-03
		Cholesterol Blosynthesis III (Via Desmosterol)	3.2E-03	2.0E-01
		Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	3.2E-03	2.0E-01
		Cholesterol Biosynthesis I	3.2E-03	2.0E-01
		Superpathway of Cholesterol Biosynthesis	3.2E-05	3.9E-01
		v-linolenate Biosynthesis II (Animals)	1 1E-01	2.9E-03
		Fatty Acid Activation	8 25 02	1 25 02
		Place Activation	0.22-02	1.3E-03
		Phospholipases	5.5E-02	5.0E-04
		Triacylglycerol Degradation	1.0E+00	8.0E-03
		Mitochondrial L-carnitine Shuttle Pathway	1.1E-01	2.9E-03
	cofactors, prosthetic groups and	Superpathway of Geranylgeranyldiphosphate Biosynthesis	5.4E-03	1.0E+00
	electron carriers biosynthesis	Folate Transformations I	1.5E-03	1.0E+00
	hormones biosynthesis	1 25-dihydroxyvitamin D3 Biosynthesis	1.0E+00	5 2E-06
		A set d Co A Discustionical III (from Citysta)	1.02.00	1.75.00
	generation of precursor metabolites and energy	Acetyl-CoA Biosynthesis III (from Citrate)	6.5E-03	1.7E-02
	neurotransmitters and other nervous system	Circadian Rhythm Signaling	1.8E-05	2.0E-07
	signaling			
	growth factor signaling	Neuregulin Signaling	8.8E-03	1.0E+00
	humoral immune response	Complement System	1 0E+00	9.7E-03
		EXP/PXP Activation	2 25 02	4 OF 01
		PARINAR Activation	3.2E-03	4.0E-01
	amino acids metabolism	Cysteine Biosynthesis/Homocysteine Degradation	1.0E+00	8.0E-03
		Arginine Degradation VI (Arginase 2 Pathway)	1.0E+00	2.4E-04
		Superpathway of Methionine Degradation	1.0E+00	7.3E-03
		Arginine Degradation I (Arginase Pathway)	1.0E+00	9.5E-05
		Citrulline Degradation	4 8E-03	1 0E+00
		Citrulline Biosynthesis	3.8E-02	4 45 04
		Current and Citalling Matchelian	0.55.02	4.45.00
		Superpartiway of Citrulline Metabolism	0.5E-02	1.4E-03
Male		l aurine Biosynthesis	9.6E-03	1.0E+00
	fatty acids and lipids biosynthesis	Superpathway of Cholesterol Biosynthesis	9.4E-06	1.0E+00
	a 2011 22	Mevalonate Pathway I	3.0E-05	1.0E+00
	primidine nucleotide biosynthesis	Pyrimidine Ribonucleotides Interconversion	1.3E-01	6.1E-03
		Pyrimidine Ribonucleotides De Novo Ricevethesis	9 7E-02	2 65-04
		Lidias El abastista Discuttore	0.0E 00	2.02-04
		Undine-o-phosphate Biosynthesis	9.6E-03	8.0E-03
	nucleosides and nucleotides biosynthesis	Guanine and Guanosine Salvage I	1.0E+00	8.0E-03
	amines and polyamines biosynthesis	Spermine Biosynthesis	1.0E+00	8.0E-03
	n manana ya ya sa	Spermidine Biosynthesis I	1.0E+00	8.0E-03
	nitrogen compounds metabolism	Urea Cycle	2.8E-02	2.4E-04
	cofactors prosthetic groups and	Supernathway of Geranylgeranyldinhosphate Biosynthesis	S OF OF	1.0E+00
	alastron sarriera biosunthesia	ouperpartiway of Geranyigeranyiuiphosphate biosynthesis	0.32-05	1.02+00
	election carriers biosynthesis	Katagangaia	4 05 00	105.00
	generation of precursor metabolites and energy	Netogenesis	1.0E-03	1.UE+00

B							
	0		\$			log ₂ FC	
	MC1 MC5 +	Ext5	MC5 C	Ext5	-1.5	0	1.5
Circadian rhythm signaling					aryl hydrocarbon recepto clock circadian regulator basic helix-loop-helix e44 period circadian clock 3 cryptochrome circadian d basic helix-loop-helix e4 period circadian clock 1 cryptochrome circadian d	or nuclear translocator lik 0 clock 2 1 clock 1	e
Cellular growth, proliferation and development					nuclear factor of activate claudin 2 tight junction protein 2 spectrin beta, non-erythr TNF receptor associated glycogen synthase kinas v-rel avian reticuloendott junctional adhesion mole protein kinase, cAMP-de breast cancer anti-estrog	d T-cells 5 ocytic 2 factor 3 e 3 beta neliosis viral oncogene h icule 3 gen dent, alpha gen resistance 1	omolog A
Growth factor signaling					heat shock protein 90kD decorin	a alpha, A1	
Transcriptional regulation					polymerase (RNA) I A	leceptor	
Nuclear receptor signaling					clusterin phospholipase C beta 3 acyl-CoA oxidase 1, palr phospholipase C epsilon glycerol-3-phosphate del sterol regulatory element nuclear receptor 0B2 ATP binding cassette B1 solute carrier 4, 2 DEAD-box helicase 5 alpha-1-microglobulin/bit kruppel-like factor 9 forkhead box O3 retinoid X receptor gamn phosphoenolpyruvate ca nuclear receptor coactive cytochrome P450 27, A1 glucose-6-phosphatase, cytochrome P450 21, A1 phosphoenolpyruvate ca solute carrier 2 (facilitate complement component solute carrier 5 1 alpha apolipoprotein B ATP binding cassette B1	nitoyl 1 hydrogenase 1 t binding transcription fac kunin precursor na rboxykinase 2 ator 4 catalytic subunit rboxykinase 1 d glucose transporter), 1 4B	tor 2
Gαq Signaling					phospholipase D2 heme oxygenase 1		
Insulin receptor signaling					ATP citrate lyase growth factor receptor bo	ound protein 10	
Adipogenesis pathway					frizzled class receptor 9 lipin 1 histone deacetylase 4 nuclear receptor 1 D2		
Glucocorticoid receptor signaling					ELK1, member of ETS o protein kinase, AMP-acti nuclear receptor corepre SMAD 2 nuclear receptor coactiva	ncogene vated, gamma 2 ssor 1 ator 2	
RhoA signaling					septin 8 Rho GTPase activating p pleckstrin homology and CDC42 effector protein 4 Rap guanine nucleotide ezrin	protein 35 RhoGEF domain G5 4 exchange factor 2	
Protein kinase A signaling					Rho-associated, coiled- protein tyrosine phospha cell division cycle 14B adducin 3 A-kinase anchoring prote protein phosphatase 1, g phosphorylase kinase, g protein tyrosine phospha protein tyrosine phosphatase 1 ref	oil containing protein kin tase, receptor type K ain 13 jamma isozyme amma 2 (testis) tase, receptor type B egulatory inhibitor subuni	ase 2 t 1B
Complement system					complement factor H complement component	5	



Figure 4. 11 Significantly affected pathways determined with hepatic transcriptome data through canonical pathway analysis of IPA.

A: Significantly affected pathways determined with the SDE genes (p < 0.01 is indicated in red). B: Heatmap representation of the actual Log₂FC values of all the genes involved in the significantly affected pathway. Upregulated genes are indicated in red, down-regulated genes in green and missing values in gray.

4.3.6 Comparison of proteomics and transcriptomics results through disease and biological function analysis

Disease and biological function analysis detect 49 and 34 significantly affected molecular and cellular functions with transcriptomics and proteomics data, respectively (p < 0.05 and |z-score|>1.4, Figure 4.12). Specifically, these disturbed functions are related to various cellular processes including cell death, lipid metabolism and gene expression that appear to be globally activated. However, the treatments appear to globally inhibit various other molecular and cellular functions, such as functions concerning cell survival, three specific lipid metabolism processes, carbohydrate metabolism, small molecule biochemistry, cellular growth, proliferation and movement, DNA binding and RNA repression.

The comparison of the total dysregulated gene (Annex 4.3) and protein (Annex 4.2) entries displays a limited overlap between these two analyses (only 13 out of total 225 proteins or 654 genes are in common, Figure 4.13). However, a noticeable compatibility has been observed between the transcriptome analysis and the proteome investigation at this molecular and cellular function level (Figure 4.12). The activation of cell death and lipid metabolisms is especially consistent between our transcriptomics and proteomic results, characterized by an apparent induction of processes related to cell death, necrosis and apoptosis, and an inhibition of proteinaceous mechanisms involved in lipid uptake, synthesis, conversion and oxidation. Furthermore, transcriptome data even shows a clearer and more notable cellular response than that depicted by proteomes, which is highlighted by a clear up and down-regulation between some opposing function aspects, such as cell death and cell survival, respectively.



Figure 4. 12 Heatmap representation of significantly affected cellular and molecular functions determined with hepatic transcriptome and proteome data through IPA.

Disease and biological function analysis model of IPA is conducted with all dysregulated genes and proteins (|z-score|>1.4 and p<0.05). The result is presented by *z-score*, which indicates the activation (in red) or inhibition (in blue) effect. The uncertain effect was indicated in white (*z-score* is not available, but p<0.05) and the gray presented that the related genes/proteins were not in involved in the function category.



Figure 4. 13 Venn diagram representation to compare the dysregulated protein and gene entries directly.

4.3.7 Transcriptional regulation of selected genes in the ovary and the testis

Transcriptional regulation of eight selected genes (*fshr*, *cyp19a*, *star*, *cyp17a*, *3bhsd*, *sod*, *cat*, *bcl2*) related to oogenesis/spermatogenesis process, oxidative stress and apoptosis are evaluated in the gonad using RT-qPCR. In the ovary, transcriptional level of cytochrome P450 hydroxylase/lyase A (*cyp17a*), one important steroidogenic enzyme, significantly decreases in the MC5 and Ext5 treatments compared with the control level, while the transcriptional level of catalase (*cat*) significantly increases in the MC5 treatment (Figure 4.14 A). In the testis, the transcriptional level of superoxide dismutase (*sod*) exhibits a decreasing trend in all toxic treatments, being statistically significant in MC5-treated male fish compared with the control (Figure 4.14 B).



Figure 4. 14 Transcriptional level of selected genes related to oogenesis/spermatogenesis process, oxidative stress and apoptosis in the gonad evaluated using RT-qPCR.

A: Normalized relatively transcriptional level of genes (*fshr*, *cyp19a*, *star*, *cyp17a*, *3bhsd*, *sod*, *cat*, *bcl2*) in the ovary (mean \pm SD, n=5); B: Normalized relatively transcriptional level of genes (*fshr*, *star*, *cyp17a*, *sod*, *bcl2*) in the testis (mean \pm SD, n=5). * and ** indicate significant differences at *p*<0.05 and *p*<0.01, respectively.

4.4 Discussion and conclusion

In the present study, using C_{18} LC-MS analysis the methanol extract of *Microcystis* PCC 7820 exhibited 40 different secondary metabolites, mostly cyanopeptides, including 8 variants of MC (the predominant being the MC-LR and the [Dmet-Asp3]MC-LR variants), 4 cyanopeptolins, 3 cyclamides and some other molecules (Table 4.1). The overall composition remains broadly consistent with the previous investigation (Le Manach et al., 2016; Ríos et al., 2014), delineating MC-LR and D-Asp3-MCLR as main metabolites/cyanopeptides along with a large diversity of cyanobacterial metabolites produced by the representative strain of *Microcystis aeruginosa*. One

and five μ g.L⁻¹ MC-LR and the *Microcystis* extract containing five μ g.L⁻¹ of equivalent MC-LR were chosen as environmentally relevant concentrations, corresponding to the concentration of MCs utilized in a few previous studies and often measured in water bodies during the summer season (Loftin et al., 2016; Pitois et al., 2016; Qiao et al., 2013b; Zhao et al., 2015). Although the measured MC concentrations in the aquaria (mean values are 0.34, 2.24 and 1.81 μ g.L⁻¹ for MC1, MC5 and Ext5, respectively, Table 4.2) were lower than the designed ones due to several possible consumption mechanisms, such as photoisomerization, photodegradation and adsorption on the aquarium glass, the actual exposure concentrations still caused considerable toxic effects on the fish.

4.4.1 Reproductive impairment

The decreased fecundity and the hatchability of toxin-treated fish indicate that reproductive performance of medaka fish is adversely affected by certain MC-containing treatments at certain exposure time. MCs have been reported to exert adverse effects on physiological and behavioral reproductive parameters of mammals, fish, amphibians and birds (L. Chen et al., 2016). Especially in fish, it has been reported that the spawning behavior decreased when zebrafish were exposed to 50 µg.L⁻¹ of MC-LR for 6 days (Baganz et al., 1998). Tissue damages were observed in the gonad of fish exposed to MCs either via balneation (Qiao et al., 2013b) or intraperitoneal injection (Hou et al., 2014) exposures. In addition, few studies have focused on hypothalamo-pituitary-gonadal (HPG) axis disturbances induced by MC exposure, which disrupts the reproductive process by affecting hormone levels (Liu et al., 2016; Zhao et al., 2015). It can be concluded that MCs impact the reproductive function of fish in multiple aspects, including reproductive behavior interference, gonad damage, and HPG axis disturbance. Besides, for vertebrates and some other animals, liver is a vital organ that performs a wide range of functions, including protein synthesis, lipid metabolism, energy generation and drug detoxification. Liver function particularly ties to reproduction regulation for oviparous fish on account of hepatic VTG synthesis (Arukwe and Goksøyr, 2003). Therefore, the decrease in reproductive output of fish upon exposure to MCs might also have another principal cause and could mainly be related to an overall liver dysfunction, which can induce indirect but important deleterious consequences referred to reproductive functions.

4.4.2 Immunolocalization of MCs in the liver and gonad

Liver is the organ that presents the highest tropism for MCs due to a few liver-specific OATP members possessing high affinities and transport capacities to MCs. Therefore, a noticeable accumulation of MCs in the liver of organisms following an acute exposure has been often reported

in fish previously (Djediat et al., 2010; Marie et al., 2012), which is also found in the other experiment of this thesis, as well as in the gonad of fish acutely exposed to MC-LR (Chapter 3). However, in the chronic exposure, MCs were not distributed in any liver or gonad of toxin-treated fish. This could be due to the difference in the exposure route, toxin concentration and exposure time between the two experiments here.

In the acute exposure, although only a part of toxin could be transported across the intestinal barrier into the bloodstream due to a limited expression of MC-transporter at the intestinal cells (Zeller et al., 2011), there is still a relatively high quantity of toxin that could access to the liver through the bloodstream since fish were gavaged with a high dose of MC-LR (10 μ g.g⁻¹ bw, Chapter 3). It is believed that less amount of toxin can actually be taken up by the fish through the breath by gills (no certain evidence of MC-transporter in fish gill) in the low-dose balneation exposure compared with the acute one. Besides, during a long period of exposure, a part of MC-LR could be excreted as MC-LR-GSH and MC-LR-Cys through liver detoxification function (Gehringer et al., 2004; Wang et al., 2006; D. Zhang et al., 2012), which led to a small amount of toxins left in the blood stream that was transported through the body and being hardly detected through the immunostaining technique in liver or other organ or tissue including gonad.

Liver and gonad play fundamental roles in the reproductive process of oviparous fish. Although no detectable MCs can be observed in either liver or gonad in this chronic exposure, the declined reproductive output seems to still imply possible function disturbances in liver and gonad, but more results from different aspects including histology, molecular and cellular function are needed.

4.4.3 Gonad status regarding germ cells development, oxidative stress and apoptosis

The reduced reproductive output may reflect a defect in oogenesis/spermatogenesis and a possible structural disruption in the gonad. This phenomenon has been often observed in mice (X. Wang et al., 2013; Wu et al., 2014), rat (Li et al., 2008) and zebrafish (Liu et al., 2016; Su et al., 2016; Zhao et al., 2012, 2015) that were acutely or chronically exposed to MC-LR. However, no noticeable tissue lesion was found in the gonad in our histological observation, implying that the observed reduction of reproductive output might not be due to an apparent gonad lesion. Transcriptional expression level of oxidative stress-related genes (*sod* and *cat*) and anti-apoptosis gene (*bcl-2*) was not significantly dysregulated in most toxin-treated group, with only a slight modification of gene *cat* and *sod* expression level being observed in MC5 treated female fish

(around 30% of up-regulation) and Ext5 treated male fish (30% of down-regulation), respectively. Oxidative stress induced by MCs is considered to be one of the important mechanisms of toxicity of MCs. In the organism, the antioxidant defense system is usually affected by oxidative stress induced by exogenous toxicants, exhibiting an inhibition or induction of various antioxidant enzyme (e.g. sod and cat) activities or corresponding gene expression level (Hou et al., 2014). For the combined histological observation and the observed dysregulation of *sod* and *cat* expression, the present MC-containing treatments (MC5 and Ext5) might cause a mild oxidative stress, but not a remarkable oxidative damage in the gonad.

The ovarian and testicular development is under the regulation of a serie of factors, enzymes and hormones. The gene transcriptional level of a few selected important regulators, including follicle-stimulating hormone receptor (fshr), cytochrome P450 19A (cyp19a), steroidogenic acute regulatory protein (star), cytochrome P450 17A (cyp17a) and 3 beta-hydroxysteroid dehydrogenase (3bhsd), were determined in the gonad of the medaka fish through RT-qPCR. The result exhibited a weak response in both genders of toxin-treated fish. Only gene cyp17a was transcriptionally downregulated in the ovary of MC5 and Ext5 conditions. On the one hand, cyp17a plays a key role in the conversion of 17-hydroxyprogesterone to androstenedione, which is one forward step of conversion of androgen to estrogen in fish gonads (Fernandes et al., 2011). The observed down-regulation of *cvp17a* suggests that the oogenesis in the toxin-treated female fish might be affected due to a possible synthesis obstruction of estrogen. On the other hand, aromatase enzyme (cyp19a) catalyzes the final step of estrogen synthesis (Fenske and Segner, 2004). The unaltered transcriptional level of *cyp19a* implies that the estrogen synthesis might be unaffected or only impacted to a small extent in the toxin-treated ovary. Moreover, our histological observation showed that the ratio of different stages of oocytes and density of different stages of spermatocytes ovary and testis were not significantly affected by all MC-containing treatments. Considering the results from both gene expression level and histological observation, it is believed that the current MC-containing treatments do not significantly affect the oogenesis and spermatogenesis in the ovary of medaka fish. Therefore, the observed decrease in reproductive output might be, to a large extent, caused by another mechanism indirectly affected by MC, rather than a direct disturbance of gonadal structure and function.

4.4.4 Liver lesion

Our histological observation indicates noticeable hepatic lesions in all toxic treatments. Although there was no statistically significant difference of the proportion of lytic area due to the large individual variation (for instance, some toxin treated fish display a mild or even no response to the cyanotoxins from histological perspective), integrated transcriptome and proteome analysis of liver highlighted some responses of genes and proteins involved in cellular damage processes in the liver of fish exposed to all MC-containing treatments. A genuine activation of cell death process, including necrosis and apoptosis, accompanied by inhibitions of cell survival, cellular growth and proliferation is highlighted by IPA in the molecular and cellular function category (Figure 4.12). This finding is directly corroborated and supported by the increase in hepatocyte lytic area observed by histology, since lysis could be a major consequence of the diffuse necrosis and apoptosis (Boorman et al., 1997).

The occurrence of liver lesions, including cellular necrotic, lipidosis or apoptotic events induced by various MCs or extracts of MC-producing cyanobacteria, has been reported previously in several fish species (Atencio et al., 2008; Deng et al., 2010; Fischer et al., 2000). It is known that the inhibition of serine/threonine PPI and PP2A, the typical toxicity mechanism of MC, subsequently leads to the disruption of the hepatic cytoskeleton, and consequently results in damage to liver structure (Fischer and Dietrich, 2000). In the present study, histological observation together with omics data attests to a noticeable liver damage induced by the cyanotoxin treatments. The cellular damage might generally disrupt various hepatocyte metabolism regulations and indirectly affect liver physiological activities, including their implication in reproductive processes.

4.4.5 Hepatic glycogen store depletion

In the present study, a severe decrease in hepatic glycogen content was observed in female fish under MC5 and Ext5 concentrations, implying that a modification in liver glycogen synthesis or consumption processes might happen in the female fish exposed to cyanotoxins. Regulation of glycogen synthesis and breakdown is mainly controlled by two key enzymes: glycogen synthase and glycogen phosphorylase, respectively (García-Rocha et al., 2001). Our proteomics analysis showed an up-regulation of glycogen phosphorylase under MC1 and MC5 treatments for females and males, respectively (encoded by gene *pygl*, Annex 4.2). This implies an up-regulation of glycogen breakdown process, which is consistent with the histological observation. On the one hand, the possible inhibition of phosphatase activity induced by MCs could affect the rate of glycogen

metabolism by modifying the activity of key enzymes (Malbrouck et al., 2004), such as glucose 6phosphate that is the rate-limiting enzyme in the synthesis of the polysaccharide through the modification of their respective phosphorylation state (Bollen et al., 1998). On the other hand, the depletion of hepatic glycogen is a common response of the fish liver to stressors such as toxins, and it may reflect a prior increased energy requirement for the organism homeostasis, via tissue repair and molecular detoxification processes (Macirella et al., 2016). Some previous studies have reported that the hepatic glycogen content of fish decreased under MC exposure, and it was suggested that the increasing energy requirement contributes to that (Malbrouck et al., 2004; Marie et al., 2012).

The dysregulation of gluconeogenesis was especially detected in the Ext5 treated fish through functional analysis on integrated transcriptomics and proteomics data (Figure 4.12), implying that the glycogen-associated energy metabolism is impacted. Furthermore, the IPA canonical pathway analysis also indicates that other energy metabolism-associated pathways, such as acetyl-CoA biosynthesis 3 and ketogenesis at the transcriptional level (Figure 4.11 A), TCA cycle and pentose phosphate at the protein regulation level (Figure 4.9), are significantly affected in the fish exposed to MC1 and MC5 conditions. Taken together, these results reveal an occurrence of a disorder of energy metabolism or shortage of energy supply in the medaka fish exposed to both pure MC and complex *Microcystis* extract.

Sufficient energy supply is essential for maintaining normal physiological functions, being especially important to support the reproductive effort and guarantee its efficiency. Particularly for female fish, the depleted glycogen content associated with disturbed energy metabolism might have contributed, at least in part, to the decrease in reproductive output.

4.4.6 Oviparous-specific reproduction proteins expression dysregulation

For oviparous organisms, such as fish, large amounts of VTG and CHG are synthesized in the liver of mature female fish. These proteins, which constitute the main components of the oocyte yolk and the chorion, respectively, are secreted from the hepatocytes and then transported by the bloodstream to the ovary, where they contribute to the oocyte maturation. An appropriate production of vtg is crucial for reproductive successes, and the decrease in hepatic vtg or chg contents of female fish could directly affect the maturation of oocyte and alter their fecundability

properties. As it has been shown in the present proteomics investigation, the quantities of vtg1, vtg2, vtg3 and L-SF precursor proteins are largely down-regulated under MC1 and MC5 conditions, and to a lesser extent under Ext5 (Figure 4.8), which might be responsible for the decrease in reproductive output of female fish. The similar down-regulation of hepatic *vtg1* gene expression has been reported in female zebrafish chronically exposed to MC-LR (Qiao et al., 2013b). The expression of *vtg* genes in the liver of female fish is positively regulated by 17β-estradiol (E2). Another chronic studies in zebrafish upon exposure to MC-LR observed a decrease of E2 level, and a subsequent defect in oogenesis induced by certain concentrations of MC-LR (Liu et al., 2016; Zhao et al., 2015). However, the plasma E2/T levels measured in the present study exhibited no variation (Figure 4.7), suggesting that the observed down-regulation of vtg protein quantity might result from an overall alteration of the liver, rather than specific endocrine perturbation. It has been reported that stress induced by toxic substances, such as ammonia, could impair the liver of fish and similarly result in unspecific impaired vitellogenesis (Garric et al., 1996).

Various methods have been used to evaluate the possibility of estrogenic activity of MC or mixture compounds released by cyanobacteria (Marie et al., 2012; Oziol and Bouaïcha, 2010; Qiao et al., 2013b; Rogers et al., 2011). One *in vitro* study demonstrated a weak estrogenic potency induced by MC through a cell-based transactivation assay (Oziol and Bouaïcha, 2010). On the contrary, another *in vivo* study testified that a strong up-regulation of *vtg* gene expression was induced by the lyophilized *Microcystis*, but not by MC-LR in larval zebrafish (Rogers et al., 2011). Other *in vivo* studies also suggested that cyanobacterial extract could potentially induce estrogenic responses (Marie et al., 2012). Indeed, the *vtg* expression induction is classically considered as an evidence of a response to estrogens in experimentally tested oviparous vertebrates, particularly in males. According to our present transcriptomics and proteomics data, there is no activation of *vtg* gene expression nor over-representation of vtg protein in any male fish, demonstrating that the concentrations used here for either MC or the *Microcystis* extract do not cause any effective estrogenic response in adult medaka fish.

4.4.7 Global proteome dysregulation in the liver

The overall proteome heatmap generated with all the dysregulated protein displays a highly similar dysregulation pattern among the three toxic treatments in both genders. It seems that the molecular effects induced by MC-containing cyanobacterial extract globally correspond to those of pure MC-LR exposure. The MC-LR released by the cyanobacteria appears to have a dominant

hepatotoxic effect over the other components in the extract. Moreover, the hepatic proteome of the fish under MC1 treatment was also altered in the present study. One μ g.L⁻¹ of MC-LR has been released as a guideline value in drinking water by the world health organization (WHO , 2011). But our result suggests that the environmental exposure to MC guideline concentration value may potentially induce a health risk for aquatic organisms.

In consideration of the gene ontology classifications of these dysregulated proteins, translation regulation is one of the most represented categories in both genders, illustrated by the downregulation of numerous ribosomal proteins and eukaryotic translation factors. Ribosomal proteins are structural constituents of the ribosome, macromolecular machines responsible for protein synthesis in cells. It has been reported that dysregulated ribosomal proteins induced by Microcystis altered protein synthesis of Daphnia pulex (Asselman et al., 2012). In the study, a large set of ribosomal proteins and various translation factors are down-regulated in response to the cyanotoxin exposure, suggesting that the overall protein synthesis in the liver might be inhibited by MC-LR and Microcystis extract due to translational down-regulation. Additionally, enrichment of canonical pathways also indicates that EIF2 signaling, an important molecular signaling for protein translation controlling, is significantly affected in both genders of fish exposed to all MC-containing treatments (Figure 4.9). As it has been described above, the reproduction-specific proteins (vtg and chg) are down-regulated at the proteome level in female liver, which is in consistency with the overall protein synthesis inhibition. However, there is no corresponding observation of these reproductionspecific proteins at the transcriptome level (Annex 4.3). It seems that the cyanotoxin exposures adversely affect the synthesis of vtg and chg by down-regulating their translation processes without modifying their relative transcription levels.

Quantitative ITRAQ proteomics analysis has been applied previously to investigate the toxic effect of cyanotoxin on medaka fish in our lab (Marie et al., 2012). Le Manach and co-workers performed proteomics analysis of the liver in adult medaka fish exposed to environmentally relevant concentrations of pure MC-LR, complex extracts of MC-producing or nonproducing cyanobacterial biomasses, and of a Microcystis aeruginosa natural bloom for 28 days. (Le Manach et al., 2016). The comparison of the dysregulated proteins between the previous and the present studies under the same MC-containing treatments (MC5 and Ext5) reveals a similar hepatic alteration pattern (Table 4.6). The proteins associated with amino acid metabolism, detoxification, nuclear receptor signaling, translation and transport are generally up-regulated in both genders. Particularly, the up-regulations of glutathione S-transferase A and complement component C3-1

suggest that the cell redox homeostasis/detoxification are affected by the MC containing treatments. On the other hand, the proteins involved in lipids and nucleotides metabolisms, protein modification and oviparous specific proteins are generally down-regulated. It is noteworthy that 3 oviparous specific proteins are commonly down-regulated in the both studies and this confirms our aforementioned observation that both MC5 and Ext5 conditions impact the oviparous specific protein biosynthesis in liver and consequently resulted in impaired vitellogenesis.

Table 4. 6 Comparison of proteomic analysis between the present study and one previous in-house investigation. Data were presented as Log_2FC . Up-regulated proteins are indicated in red, down-regulated proteins in green and missing values in white.

	Freembl		Present study				Previous in-house study				
Function	ID (ENSO	Name	Fema	le	Male		Female		Male		
	RLP000)		MC	Ext	MC	Ext	MC	Ext	MC	Ext	
			5	5	5	5	5	5	5	5	
	00003019	betaine-homocysteine	0.4	-0.3	0.4	0.5	0.3	-0.1	-0.7		
,		S-methyltransferase 1-									
amino acids	00020426	glutathione S-			0.9	04	0.2	0.5			
metabolism	00020420	transferase zeta 1			0.7	0.4	0.2	0.5			
	00000114	cathepsin D			1.1	0.9	0.2	0.5			
	00024221	Glutathione S-	0.7	0.8	0.3	0.5	0.2	-0.4			
homeostasis/detoxific		transferase A-like									
ation	00014130	complement	0.6	0.6	0.0	0.3	0.4	1.1			
	00011000	component C3-1	0.0	0.5	0.1	0.1			0.1	0.1	
nuclear receptor signaling	00011903	aldehyde	0.9	0.7	0.1	0.1			0.1	0.1	
	00020672	denydrogenase 1, L1	0.5	0.6	0.1	0.1	0.2	0.3			
	00020072	transferrin-a	0.0	0.0	0.1	0.1	0.2	0.5			
	00022033	B1	0.2	0.2	0.3	0.0	0.6	0.1			
	00004115	betaine-homocysteine	0.1	0.5	0.3	0.2	0.6	-0.1			
translation		methyltransferase									
	00020346	ribosomal protein SA	0.5	0.2	0.4	0.5			0.2	0.7	
	00006568	hemoglobin		0.4	1.1	0.3			0.1	0.5	
		embryonic, alpha		0.4	1.0	0.1				0.5	
transport	00003802	hemoglobin, alpha-1- like	-0.2	0.4	1.0	0.1			0.2	0.5	
	00003772	hemoglobin, beta-1-	0.4	0.8	1.4	0.4	-0.5	-0.6	0.3	0.5	
		like									
oxidation-reduction	00017096	dihydropyrimidine	0.0	0.3	0.4	0.3	0	0.7			
process	ENICOMO	dehydrogenase b	0.0	0.4	0.2	0.4	0.1	0.1			
	ENSGMO		0.2	0.4	0.3	0.4	0.4	-0.1			
	P0000002 0886	fatty acid binding									
fatter agida and linida	0000	protein 10a, liver basic									
metabolism	00000760	C // 1 1	0.0	0.1	0.1	0.4			0.7	0.7	
metabolisiii	00020768	fatty acid amide hydrolase	0.2	0.1	-0.1	-0.4			0.7	0.7	
	00016901	fatty acid binding	-0.2	-0.1	-3.3	-3.8			-0.4	-0.6	
		protein 7, brain, a									

	00000620	acyl-CoA oxidase 3, pristanoyl			1.0	0.5			0.5	0.1
protein modification	00022552	calreticulin 3b	-0.5	-0.4	-0.3	0.0	-0.6	0.1		
	00014882	ribosome binding protein 1	-0.6	-0.2	-0.6	-0.4		-	-0.2	-0.6
nucleotides metabolism	00020213	cytidine deaminase	-0.6	-0.4	0.2	0.2	0.6	0.6		
	00008867	nucleolin	-0.2	0.0	-0.8	-0.4			-0.2	-0.6
	00012711	L-SF precursor	-0.8	-0.4			-0.6	-0.2		
oviparous specific	00007656	vitellogenin 1	-0.1	-0.1	-1.9	-2.7	-0.6	-0.5		
proteins		precursor								
r ····	00007793	vitellogenin II	-0.7	-0.5			-0.3	-0.3		
		precursor								
extracellular region	00013645	Zona pellucida	-0.4	-0.3			-0.6	-0.2		
		glycoprotein 2, like 2								

4.4.8 Global transcriptome dysregulation in the liver

The global hepatic transcription profile reveals a different effect induced by MC5 and Ext5 treatments for both genders of fish (Figure 4.10). MCs are not the only noticeable toxic compounds present in the cyanobacterial extract. Other compounds have received increasing attention for their potential bioactivity that may lead to synergistic, antagonistic or additive effects with MCs, but the information about those metabolites still remains limited. In the present study, Ext5 exposure leads to 418 SDE genes in female medaka fish being two times more numerous than the SDE genes induced by MC5, implying that the toxicity of complex cyanotoxin mixtures, including MCs, could be wider or greater than that of the pure MC. This phenomenon has also been observed previously in adult medaka and larval zebrafish exposed to cyanobacterial extract and pure MC (Marie et al., 2012; Rogers et al., 2011).

In terms of impacted pathways, more biological pathways are affected by the Ext5 treatment compared with pure MC treatments in both genders, but particularly in females. Those female supplementary pathways mostly concern signaling pathway categories, comprising a large set of nuclear receptor signals and various second messenger signals, such as estrogen receptor, PPAR α /RXR α , TR/RXR, FXR/RXR, PXR/RXR and LXR/RXR (Figure 4.11). Similar molecular effects have been reported previously, for instance, the nuclear receptor gene family *nr1d2b* was significantly down-regulated in zebrafish larvae by *Microcystis* extract exposure (Rogers et al., 2011). One purified cyanopeptide (cyanopeptolin 1020) produced by different *Microcystis* and *Planktothrix* strains, led to the expression dysregulation of the nuclear receptors related gene in zebrafish embryos (Faltermann et al., 2014). In fact, different cyanopeptolin variants are also present in the *Microcystis* extract used in the present study (Table 4.1). Considering the importance

of liver functions regulated by nuclear receptors in fatty acid oxidation and uptake, thyroid hormone metabolism, bile acid homeostasis, gluconeogenesis and reproductive hormone metabolism, *Microcystis* extract seems to have a greater potential to disturb the reproductive function of female medaka fish.

Integrated studies based on transcriptome and proteome analysis often present a limited correlation between mRNA expression and protein abundance due to different synthesis regulation time-scales, biological modifications (post-transcription machinery) or technical variations (Ghazalpour et al., 2011). In the present study, a limited overlap between these two analysis methods has been illustrated by directly comparing the dysregulated genes and proteins entries (Figure 4.13). This big variance between the gene expression level and the protein content might be, to some extent, resulted by the difference in the number of individual in each pooling sample. In this case, interpreting the omic data at the overall function level seems more appropriate than directly looking at the individual genes/proteins. The overlap between these two sets of omic data at biological function level presents a high compatibility in the cell death process, lipid and carbohydrate metabolisms (Figure 4.12), attesting to a potent hepatic cytotoxicity and consequent liver metabolic disorders induced by both MC-LR and the *Microcystis* extract.

4.4.9 Fatty acids and lipids metabolism disorder

Pathway analysis of the transcriptome data reveals that MC5 and Ext5 treatments significantly affect hepatic fatty acids and lipids metabolism in female fish, but to a much lesser extent in males (Figure 4.11). In particular, choline, cholesterol, and linolenate biosynthesis, phospholipases and triacylglycerol degradation are concerned. Whereas, male fish may also present a remarkable response concerning lipid metabolism to MC exposure, as it has been shown in quart male medaka in which a high lesion score for lipidosis in the liver was observed when fish were fed the diet containing $3.93 \ \mu g.g^{-1}$ of MC-LR (Deng et al., 2010). In the present study, the gender differences in lipid metabolism are very likely related to the massive material needs for the reproductive process in females, since the liver is responsible for a large amount of lipid and fatty acid deposits to the oocyte too.

Choline is an essential nutrient, playing an important role in fat metabolism by hastening removal or decreasing deposition of fat in the liver. The potential choline biosynthesis disturbance could be responsible for the described liver histological damages since choline deficiency has been shown to be consistently associated with liver damage in rat upon exposure to MC-LR (He et al.,

2012). Meanwhile, four cholesterol biosynthesis pathways are significantly impacted in the female fish treated with MC5. As acetyl-CoA is an important precursor molecule of cholesterol synthesis, acetyl-CoA biosynthesis perturbation, as it has also been observed here, could highly contribute to the modification of cholesterol synthesis. It has been described earlier that the phosphatase inhibition action of MCs could dysregulate glucose metabolism process through the modification of various key enzymes, which consequently affects the content of some metabolic products, such as acetyl-CoA. The perturbation of cholesterol biosynthesis was also observed in zebrafish exposed to MCs, and acetyl-CoA was suggested to be one important cause for interfering in the balance between glucose and fat metabolism (Pavagadhi et al., 2013). Furthermore, cholesterol biosynthesis perturbation may have consequences on steroid hormone and bile acid biosynthesis, since cholesterol is essential for the synthesis of steroid hormones and bile acids. Steroid hormones and bile acids are endocrine signaling molecules, which have important roles in maintaining important physiological function in fish, such as in the reproductive process. The reproductive hormone levels are highly dynamic due to complex feedback regulation mechanisms in organisms. In the present study, the reproductive hormone (E2 and 11-KT) levels in plasma are not significantly modified which might be associated with the potential bile acid level disturbance. Taken together, our results described above suggest that both MC5 and Ext5 exposure induce a disruption of major lipid metabolic pathways in medaka liver, particularly in females. MC-induced alterations in liver lipid components or lipid metabolism have been often reported in fish (Malécot et al., 2011; Marie et al., 2012; Pavagadhi et al., 2013) or rodents (Sedan et al., 2010). In the present study, this lipid metabolism disruption is another important aspect, besides energy shortage and protein biosynthesis inhibition, which could significantly contribute to the observed reproductive impairments.

4.4.10 Circadian rhythm signaling perturbation

In various organisms, circadian rhythm regulation plays an important role in a wide variety of physiological functions including sleeping, feeding, reproduction and cellular metabolism. Circadian clocks are present not only in the central nervous system but also in peripheral organs and tissue, such as the liver (Kaneko et al., 2006). The molecular mechanism of circadian rhythms has been described in *Drosophila* and in mice, functioning through oscillation feedback loops, generally consisting of four core proteins. Clock (circadian locomotor output cycles protein kaput) and bmall (brain and muscle ARNT-like 1) proteins transcriptionally regulate clock-controlled genes, and meanwhile, activate the transcription of per (period) and cry (cryptochrome) proteins, which in turn produce a negative feedback loop by suppressing clock:bmal1-mediated transcription (Dunlap,

1999). For fish, the circadian system appears to have a similar scheme, and the genes encoding these four proteins have been shown to be rhythmically expressed in peripheral tissues of zebrafish (Cahill, 2002).

In the present study, the pathway analysis of hepatic transcriptomes reveals that the circadian rhythm signaling is the most affected pathway in the liver of the two genders of fish exposed to MC5 and Ext5, evidenced by a significant down-regulation of period and cry genes accompanied by up-regulated *bmal* (Figure 4.11). This result constitutes the first report of the expression dysregulation of circadian rhythm-related genes induced by MC exposure. The liver plays important roles in glucose and lipid metabolisms. On one hand, it has been proved that glucose and lipid metabolisms are highly regulated by hepatic circadian rhythm (Gnocchi et al., 2015; Johnston, 2014). On the other hand, the alteration in metabolism processes could also affect the circadian rhythm-relevant genes in return (Johnston, 2014). In the present study, the various modified hepatic metabolic processes could be the consequence of circadian rhythm perturbation in response to MC or alternatively constitute a possible cause of hepatic circadian disturbance in fish chronically exposed to the cyanotoxins. The observed nuclear receptor signaling perturbation could partially contribute to the second hypothesis, since nuclear receptors play important roles in regulating the circadian rhythm. The underlying mechanism of the present phenomenon remains unclear, and to date, there is only one investigation that reported a circadian rhythm perturbation induced by cyanopeptolin 1020 extracted from Microcystis aeruginosa in zebrafish embryos (Faltermann et al., 2014).

Reproductive traits in medaka fish are regulated by the photoperiod. Light variations are integrated by complex feedback loops of the core clock genes located in the central suprachiasmatic nucleus (CSN) (Harmer et al., 2001). The circadian activity generated in the central SCN affects multiple physiological rhythms, including reproductive periodicity, and it also directly sets the peripheral circadian machinery (Gnocchi et al., 2015). Based on the present observation of the hepatic circadian disturbance induced by MC and *Microcystis* extract, one can hypothesize that central circadian clocks might have been influenced by the exposure to cyanotoxins as well, consequently disturbing the reproductive function. Another conjecture is that central circadian machinery, although so far there was no clear evidence showing the feedback effect of peripheral circadian on the central SCN.

Hepatic circadian rhythm regulation is associated with energy and various materials metabolism that are essential for reproductive process. In addition, it might potentially interfere with the light-period control of fish reproduction activation as it is the case in medaka. Therefore, the observed hepatic circadian perturbation could be, at least partially, responsible for the reproductive disturbance observed in the present study.

4.4.11 Conclusion

Our results indicate that both MC-LR and the *Microcystis* extract induce potential reproductive toxicity in adult medaka fish. The main cause of the observed reproduction impairment is believed to be the hepatotoxic effect induced by MC-containing treatments, rather than the directly deleterious effect on gonad. The observed hepatic alterations contribute to reproduction impairments by affecting energy supply, lipid and fatty acid metabolism, oocyte-specific protein synthesis and circadian regulation-related gene perturbation. Two interesting findings have been revealed by the two omic analyses independently. The first one is that the proteomics analysis suggests that the MC-containing treatments might inhibit overall liver protein synthesis by down-regulating ribosomal proteins and various translation factors. For the second finding, the transcriptomics investigation shows that the hepatic circadian rhythm regulation system has been impacted by the present toxin treatments.

The present study also shows that a wider range of biological pathways are disturbed by the complex mixture compounds produced by cyanobacteria compared with pure MC-LR. In the aquatic environment, low concentrations of cyanotoxins and other bioactive compounds produced by cyanobacteria widely spread. However, the information about a big number of compounds produced by cyanobacteria is still limited. More efforts on the long-term study of these diverse toxic compounds released by cyanobacteria are urgently needed to advance our current knowledge on the protection of aquatic organism populations as well as human health from chronic cyanotoxin issues.

Chapter 5 Conclusion

5.1 Summary and general discussion

Cyanobacterial blooms threaten human health as well as other living organisms of the aquatic environment, particularly due to the production of natural toxic components, so called the cyanotoxins. So far, one of the most studied cyanotoxins is the MC, which is mainly known for its potent hepatotoxicity properties. In addition to the hepatotoxicity, more attention has been recently drawn to MC due to its potential reproductive toxicity. However, little is still known regarding the direct effects of MC on the gonad and the implication of liver dysfunction in reproductive impairment, especially for oviparous vertebrates.

The liver is a key organ that plays fundamental roles in various physiological processes, being particularly important in the reproductive process for oviparous vertebrates. Moreover, the liver is also the first target organ for MCs and some other cyanotoxins, such as nodularin or cylindrospermopsin. Therefore, this thesis firstly investigated the metabolic specificities of the liver at the cellular and molecular levels in both female and male medaka fish by using histological, metabolomics, proteomics and transcriptomics approach. The results basically attested to a strong sexual dimorphism that concerned various cellular and molecular processes of hepatocytes comprising protein synthesis, amino acid, lipid and polysaccharide metabolism, steroidogenesis and detoxification. In particular, in female medaka fish, large cellular and molecular modifications were associated with massive synthesis of oocyte protein precursors, which highlights that the metabolic adjustment of the liver is important for maintaining the reproductive competency in adult medaka fish, being critically important in females.

In order to obtain a clear and noticeable view of histological changes in the liver and gonad, an acute experiment has been conducted in adult medaka fish exposed to $10 \ \mu g.g^{-1}$ bw of pure MC-LR for 1 hour. The histological and immunohistochemical examinations revealed that both liver and gonad were significantly affected by MC-LR exposure. An intense distribution of MC-LR within hepatocytes along with a severe liver lesion attested to the potent hepatotoxicity of MC. An immunogold electron microscopy analysis showed that, besides being accumulated in the
hepatocytes, MC-LR was also found in the connective tissue of the gonads and also to some extent in oocytes. This finding constitutes the first observation of the presence of MC in the reproductive cell with *in vivo* study. Both livers and gonads play important roles in the reproductive process of oviparous vertebrates. Our results of this acute exposure, which provide intracellular localization of MC-LR in the liver and gonad, advance the understanding of the potential reproductive toxicity of MC-LR in medaka fish.

However, in the natural environment, fish and other aquatic organisms are usually subject to long-term exposure to a relatively low concentration of cyanotoxins. In order to investigate the accurate environmental toxic effects of MCs and other secondary metabolites produced by cyanobacteria according on a more realistic condition, a chronic exposure has been performed in adult medaka fish that were exposed to 1 and 5 µg.L⁻¹ MC-LR and to an extract of MC-producing *Microcystis aeruginosa* PCC 7820 (5 µg.L⁻¹ of equivalent MC-LR) by balneation for 28 days. The results showed that both MC-LR and *Microcystis* extract adversely affect reproductive parameters, including fecundity and egg hatchability. In particular, the reproductive impairment appears most likely to be caused by an overall hepatotoxic effect at histological, proteome and transcriptome levels. Our result also revealed that a wider range of biological pathways were disturbed by the complex mixture compounds produced by *Microcystis* compared to the effect induced by pure MC-LR, implying that the deleterious effects induced by mixture compounds of cyanobacteria are potentially more worrying than those caused by pure toxin alone.

Overall, the thesis evaluated the reproductive toxicity of MC-LR and *Microcystis* extract by investigating the toxic effects on the liver and gonad in adult medaka fish with one acute and one chronic exposure. The specific results regarding a few specific objective questions are presented below.

- Does sexual dimorphism should be taken into consideration when interpreting the toxic effects of hepatotoxins in adult oviparous vertebrate?

Our integrated analysis of medaka liver attested to a strong sexual dimorphism that concerned various cellular and molecular processes of hepatocytes concerning energetic metabolism, detoxification and reproduction. In the present chronic experiment with MC-containing treatments, female fish liver presented to be more susceptible to hepatotoxic stressors, which could be linked with hepatic sexual dimorphism in mature oviparous vertebrates. The massive production of oocyte yolk stocks and chorion precursors in female liver subsequently impacts the global liver activities

concerning amino acid, saccharide, fatty acid and lipid metabolism, as well as liver detoxification capabilities which are implicated in the hepatic response to MCs. In some other ecotoxicological investigations on small fish, various hepatotoxic stressors such as cyanotoxins (Deng et al., 2010), pesticides (Werner et al., 2002) or aromatic hydrocarbons (Oh et al., 2012) induced a higher toxicological response in female livers, suggesting that females would be more sensitive to the effects of those molecules than males (X. Zhang et al., 2012). However, some examples also attested to a higher susceptibility of male livers according to certain specific exposure conditions to toxic chemicals (Yu et al., 2013). Therefore, a careful investigation of the dimorphic detoxification capabilities should aim at being performed for each specific hepatotoxin. To advance our systematical understanding of the hepatic stressor in environmental toxicology evaluations, hepatic sexual dimorphism of oviparous vertebrates should be taken into account.

- Does MC accumulate in the gonad of fish or enter reproductive cells?

MC has been documented to distribute and accumulate in various fish organs, including liver, intestine, kidney, gill and gonad by several field and laboratory studies (Cazenave et al., 2005; Chen et al., 2005; Djediat et al., 2010; Mezhoud et al., 2008; Papadimitriou et al., 2012; Poste et al., 2011; Schmidt et al., 2013). Specifically, the accumulation of MCs in the gonad has been previously reported through different methods, such as ELISA, LC-ESI-MC and radiotracing. However, little is known about the localization of MCs within the gonad tissue, or about the intra-tissular/-cellular distribution of MCs. The present acute study was conducted to investigate the precise localization of MC-LR in the gonad of medaka fish gavaged with 10 µg.g⁻¹ bw of MC-LR through immunohistochemical method under light microscopy and immunogold electron microscopy techniques. Under light microscopy, the immunolabeling of MC-LR was clearly observed in the connective tissue and some unidentified round cells within the ovary, whereas only some slight labeling was found in the connective tissue of the testis. A clearer observation was realized under electron microscopy, which revealed that MC-LR was indeed present in the connective tissue of ovary and testis, as well as in some oocytes. The presence of MC in the connective tissue has been reported previously in common bream collected from MC contaminated lakes (Trinchet et al., 2013). It seems that MCs are transported through bloodstream into gonads, in which they mostly accumulate in the connective tissue. Various gonadal somatic cells are distributed in the connective tissue. These gonadal somatic cells, such as Leydig cells in the testis and Sertoli cells in the ovary, are essential for the development and maturation of reproductive cells. The presence of MC in connective tissue or specific types of gonadal somatic cells could potentially affect normal

oogenesis and spermatogenesis processes, resulting in a decrease in reproductive output. Moreover, our result also exhibits an apparent distribution of MC in the reproductive cells for the first time. A few liver-specific OATPs are the known membranal transporters of MCs. Furthermore, some unidentified OATPs possessing MC-transport capability might be expressed in the gonad at a relatively low level. The expression level of 5 Oatps in the spermatogonia of rat was affected by exposure to different concentration of MC-LR and MC-LR was detected to immigrate into the isolated spermatogonia, implying that these Oatps may involve in MC-transport into the reproductive cells. (Zhou et al., 2012), but more information of other MC-transporting OATPs and their expression level in reproductive cells is still required.

- Does MC or the complex mixture of compounds produced by MC-producing *Microcystis* could adversely affect the reproductive function of medaka fish? If so, then through the disruption of liver function or the disturbance of gonad function?

Our chronic study demonstrates that both MC-LR and the extract of MC-producing *Microcystis aeruginosa* strain adversely affect reproductive function in medaka fish under environmental relevant concentrations.

The reproductive impairment appears most likely to be caused by an overall hepatic alteration instead of an adverse impact on gonad directly. Indeed, in the liver of the fish treated with such toxins, the dysregulation of the energy metabolism and the inhibition of overall protein synthesis, being particularly for vtg and chg, were directly accompanying with the decreased reproductive output, as collateral effects. This is in accordance with current knowledge, since sufficient energy and protein supplies are essential for supporting the reproductive effort and guaranteeing its efficiency. In particular, the remarkable decrease of hepatic vtg and chg quantities could be the source of adverse effects in the development of oocytes in female fish. Moreover, both MC-LR and the extract exposures significantly affected hepatic fatty acid and lipid metabolism in toxin-treated fish, particularly in females. Especially, choline, cholesterol, and linolenate biosynthesis, phospholipases and triacylglycerol degradation were concerned. These fatty acids and lipids play important roles in signal transduction, fat and glucose metabolism. The disturbance of fatty acid and lipid metabolism in the liver not only impacted the lipid deposits in the oocyte, but also affected the overall energy and material metabolism, subsequently influencing the reproductive function. In addition, the pathway analysis of hepatic transcriptomes revealed that the circadian rhythm signaling was the most affected pathway in the liver of the toxin treated-fish. It has been reported

previously that the circadian rhythm perturbation was induced by cyanopeptolin 1020 extracted from *Microcystis aeruginosa* in zebrafish embryos (Faltermann et al., 2014), the underlying mechanism being still unknown. One can conclude that hepatic circadian rhythm regulation is associated with energy and various materials metabolism, and it might, therefore, affect reproductive process indirectly too. Overall, the observed reproduction impairment of cyanotoxin-treated medaka fish is considered to be caused by an overall liver dysfunction comprising the perturbation of energy supply, lipid and fatty acid metabolism, the inhibition of oocyte-specific protein synthesis, along with the dysregulation of the hepatic circadian-related gene.

- Does MC or the complex mixture of compounds produced by MC-producing *Microcystis* have estrogenic potency *in vivo*?

It has been hypothesized that some of the compounds produced by cyanobacteria might possess endocrine disrupting activity, specifically the estrogenic activity by binding to the estrogen receptors and mimicking the action of endogenous estrogen, which consequently disturbs the normal functioning of the endocrine system. Various methods have been used to evaluate the possibility of estrogenic activity of MC (Marie et al., 2012; Oziol and Bouaïcha, 2010; Qiao et al., 2013b; Rogers et al., 2011) since MC may potentially act on estrogen receptors through the indirect interactions with phosphorylation signaling pathways. To date, a limited number of studies have reported the estrogenic potency of MC, whereas more investigations support the idea that the extracts or exudates of certain strains of cyanobacteria could induce estrogenic effects, implying that the presence of some still undetermined compounds produced by cyanobacteria, other than MCs, might be the cause of estrogenic response in organisms. The vtg expression induction is classically considered as an evidence of a response to estrogens in experimentally tested oviparous vertebrates, particularly in males. Our hepatic transcriptomics and proteomics analysis and plasma E2/11-KT determination revealed that under the investigated experimental conditions there is no activation of vtg gene expression/protein over-representation, nor induction of E2 level in any male fish, demonstrating that the concentrations used here for either MC or the extract of Microcystis PCC 7820 does not cause any effective estrogenic response in adult medaka fish. However, answering the question concerning the possible estrogenic potency of a complex mixture of compounds produced by Microcystis or other bloom-forming cyanobacteria is not so simple. The composition of a complex mixture of compounds differs among cyanobacteria species, or even among strains of the same species. It has been demonstrated that one strain of Aphanizomenon gracile elicited significant estrogenicity with EEQ reaching 280 ng.g⁻¹ d.w. of E₂ using *in vitro*

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trans-activation assays, while another strain from the same species did not have this estrogenicity (Sychrová et al., 2012). The study of Sychrová also revealed that exudates and aqueous extracts of the same strain of cyanobacteria could cause a different estrogenic response. So from the present study, what we can conclude is only relevant to this strain (bloom-forming cyanobacteria) with the concentration used, further work of comparing the effects of several species/strain of cyanobacteria commonly present in the water bodies is needed.

- Are the effects induced by pure MC-LR and the extract of MC-producing *Microcystis* different?

The global hepatic transcription profile of medaka fish following the present chronic exposure reveals a different effect induced by MC5 and Ext5 treatments for both genders of fish. For example, Ext5 exposure leads to 418 SDE genes in female medaka fish being two times more numerous than the SDE genes induced by MC5, implying that the effects of complex cyanotoxin mixtures including MCs, could be wider or greater than that of the pure MC. Those female supplementary SDE genes involve in biological pathway concerning a large set of nuclear receptor signals and various second messenger signals. Since these nuclear receptors play crucial roles in fatty acid oxidation and uptake, gluconeogenesis and reproductive hormone metabolism, *Microcystis* extract seems to have a greater potential to disturb the reproductive function of medaka fish.

MCs are not the only noticeable toxic or bioactive compounds present in the cyanobacterial extract. In the present extract, we detected various variants of cyanopeptolin, aeruginosins and cyclamides. It has been reported that Cyanopeptolin 1020 produced by different *Microcystis* and *Planktothrix* strains, led to the expression dysregulation of genes related to various important biological and physiological pathways (Faltermann et al., 2014). Aeruginosins are potential inhibitors of trypsin, chymotrypsin, and other serine proteases (Ersmark et al., 2008). Various compounds belonging to cyclamide class present noticeable cytotoxic properties (Voloshko et al., 2008). Other compounds have received increasing attention for their potential bioactivity that may lead to synergistic, antagonistic or additive effects with MCs, but the information about those metabolites still remains limited.

5.2 Improvements and future perspectives

This thesis involved a long-term study of medaka fish exposed to different MC-containing conditions and analyzed the toxicological effects from histological, proteomic and transcriptomic

perspectives. Due to practical limitations, some point of views could not be explored to its full extension, and some interesting research directions could have been evaluated more thoroughly. Some improvements and future perspectives regarding the experimental technique and strategy are presented below.

- The number of individuals in each pooling sample for omic investigation should be homogenized.

In the chronic study, the half liver of 4 or 5 individuals (adjusting according to the size of tissue) were pooled to get 1 pooled sample for proteomics analysis, while the half liver of 2 or 3 females and of 1 or 2 males were pooled, respectively, for transcriptomics study. Due to practical difficulties during the tissue collection and the sample preparation, we pooled different numbers of individuals for these omic analyses. This might affect the individual analysis of omic data on account of the interference between week positive and weak negative response from each individual. This pooling strategy might also, to some extent, cause disagreement between the transcriptomic and proteomic results.

A homogenized strategy of sampling and individual pooling should be applied in the future toxicological and ecotoxicological studies using such omic approaches in order to obtain more comparable information between transcriptome and proteome level. Furthermore, working at the individual level without pooling would let us more accurately investigate the individual response of the organisms that can especially be heterogenous with low dose/chronic exposure conditions. Work at the individual level with the various omic approach (in the same organisms) would also give us the possibility to performed more powerful correlation between the different molecular levels.

- More reproductive parameters should be taken into account to fully assess the potential reproductive toxicity of the target toxin.

In the chronic study, the fecundity, the fertility, the hatching rate and the embryonic health state were applied to assess the reproductive performance, and the proportion of different stages of oocytes and spermatocytes were determined through histological observation to evaluate the status of gonad development. Our histological study of ovary and testis showed no significant change in the ratio of different stages of reproductive cells. However, according to the histological observation, it seems that the total number of present reproductive cells in the ovary decreased in the toxin-treated fish, which might be the direct cause of the decreased fecundity. But this observation is not convincing enough since the cutting position impact the size of the section surface (although we always tried to cut the biggest surface of the block).

The gonad body index (the ratio of ovary weight and body weight), an important endpoint to assess the status of gonad development, should be included in a future chronic study regarding the reproductive toxic effects in fish. Considering the size of gonad in small fish, such as medaka, the ovary body index is particularly more informative than that of the testis. Combined with histological analysis of ovary, the ovary body index could provide an overall view of the ovary development, favoring the interpretation of the possible reproductive impairment, and the further study of the underlying mechanism at molecular level.

- Chronic exposure with multiple sampling time points could largely favor the evaluation of the possible endocrine disruption effects.

In the present chronic study, we collected all the samples once at the end of 28 day-exposure. The E2 and 11-KT levels in plasma, vtg gene expression level and protein quantity in livers, and the expression level of steroidogenic enzymes in gonads were used to evaluate the possible endocrine disruption effects of MC-containing treatments. These test endpoints and biomarkers have been often documented to be highly dynamic under the regulation of the hypothalamic–pituitary–gonadal and liver axis (HPG-liver axis) in fish (Milsk et al., 2016). At one specific exposure point, a compensatory response could be stronger than a direct effect induced by the target toxin. Therefore, the unanticipated dynamics of the biological system during the chronic exposure should be taken into account for data interpretation. If possible, a multi-sampling strategy should be applied in the future chronic study regarding potential reproductive toxicity or endocrine disruption effects, which can provide more informative data and permit us to observe the temporal effects induced by the target toxin.

- A systematic method for a long-term evaluation of reproductive toxicity of specific species of fish needs to be established.

In the present study, we did not follow strictly the OECD guideline 229: Fish short-term reproduction assay due to some actual constraints, also due to the consideration for a long term exposure. Future efforts need to be put on the establishment or development of a systematic method for the evaluation of reproductive toxicity of specific species of fish, since the response in different species of fish may vary. Considering to extend the exposure period a little bit may favor us to get a

clearer picture of the fish response to environmental toxins. The most important is that a systematic method allows us to investigate different types of cyanotoxins and bioactive compounds of cyanobacteria under a unified pipeline.

5.3 Conclusion

This thesis provides compelling findings on the potentially reproductive toxicity of MC in adult medaka fish, and also gives a further insight on the toxic effects of other secondary metabolites produced by *Microcystis*, as shown in Figure 5.1. It demonstrates that MC might directly impact gonadal function by inducing cytotoxicity in gonadal somatic cells and reproductive cells, and it could also impact the reproductive function indirectly by disturbing the general liver function. Chronic exposure to an environmentally relevant concentration of MCs, and even to the WHO guideline value of 1 μ g.L⁻¹ are shown to induce a significant reproductive impairment, suggesting that the widely spread MC brings a common and serious health risk for the aquatic organisms of natural environment contaminated by MCs. Moreover, in the real environment, the aquatic organisms exposed to cyanobacterial blooms are not only threatened by MCs, but also by a lot of other secondary metabolites produced by cyanobacteria (together with other contaminants, such as HAPs, pesticides or metals, for example) that might lead to synergistic effects with MCs, as it has been shown in the present chronic exposure.

Overall, the results of this thesis improve our understanding of the potential reproductive toxicity of cyanotoxins in model fish. This could advance our current knowledge on the protection of aquatic organism populations as well as human health from cyanotoxin issues.



Figure 5. 1 Systematic view of the results obtained from the acute and chronic experiments.

Annexes

Annex 2. 1 Complete list of metabolites that	are significantly sex-enriched in female and male
livers, and their respective metabolic pathwa	y enrichment.

Metabolites	HMDB	PubCh em	KEGG	Fold Change	P value	Up-regulated
Maltotetraose	HMDB01296	439639	C02052	384.98	0.000001	Females
Taurine	HMDB00251	1123	C00245	327.23	0.000001	Females
Rhamnose	HMDB00849	25310	C00507	210.52	0.000001	Females
cis-Aconitic acid	HMDB00072	643757	C00417	178.57	0.000001	Females
1,3-Dimethyluric acid	HMDB40177	70278	C06728	113.04	0.000001	Females
Glucose 6-phosphate	HMDB01401	5958	C00092	107.02	0.000001	Females
D-Glucose	HMDB00122	5793	C00031	44.58	0.00042	Females
Argininosuccinic acid	HMDB00052	16950	C03406	41.8	0.00002	Females
Glycerol	HMDB00131	753	C00116	41.64	0.000001	Females
Pantothenic acid	HMDB00210	988	C00864	41.24	0.000001	Females
Myoinositol	HMDB00211	NA	C00137	17.01	0.000001	Females
1,5-Anhydrosorbitol	HMDB03911	64956	C05145	16.43	0.00003	Females
Alpha-Lactose	HMDB00186	84571	C00243	15.09	0.000001	Females
Sucrose	HMDB00258	5988	C00089	14.84	0.00001	Females
L-Arabitol	HMDB01851	439255	C00532	12.91	0.000001	Females
D-Galactose	HMDB00143	439357	C00984	12.26	0.00002	Females
Glucosamine 6-sulfate	HMDB00592	72361	C04132	10.9	0.000001	Females
Allocystathionine	HMDB00455	101049 53	C00542	10.68	0.000001	Females
L-Cystathionine	HMDB00099	439258	C02291	9.92	0.000001	Females
Acetylcholine	HMDB00895	187	C01996	9.8	0.00011	Females
Guaiacol	HMDB01398	460	C01502	9.36	0.00185	Females
L-Homoserine	HMDB00719	12647	C00263	8.63	0.00005	Females
L-Cystine	HMDB00192	67678	C00491	8.55	0.000001	Females
7-Methylxanthine	HMDB01991	68374	C16353	8.26	0.00005	Females
L-Serine	HMDB00187	5951	C00065	7.93	0.00003	Females
D-Xylose	HMDB00098	135191	C00181	7.92	0.00003	Females
L-Leucine	HMDB00687	6106	C00123	7.75	0.0169	Females
Menthol	HMDB03352	16666	C00400	7.69	0.000001	Females
Dihydrothymine	HMDB00079	93556	C00906	7.67	0.00006	Females
Deoxycorticosterone	HMDB00016	6166	C03205	7.39	0.00055	Females
Alpha-Hydroxyisobutyric acid	HMDB00729	11671	NA	7.26	0.00001	Females
Cysteine-S-sulfate	HMDB00731	115015	C05824	6.41	0.04622	Females
Guanidoacetic acid	HMDB00128	763	C00581	6.11	0.00001	Females
Ureidopropionic acid	HMDB00026	111	C02642	5.96	0.00023	Females

Putrescine	HMDB01414	1045	C00134	5.95	0.0129	Females
N-Acetylgalactosamine	HMDB00212	84265	C01074	5.86	0.000001	Females
D-Alanine	HMDB01310	71080	C00133	5.69	0.000001	Females
2-Hydroxy-2-methylbutyric acid	HMDB01987	95433	NA	5.54	0.00145	Females
Sorbitol	HMDB00247	5780	C00794	5.31	0.00112	Females
Amylose	HMDB03403	534777 71	C00718	5.24	0.00038	Females
5-Methoxysalicylic acid	HMDB01868	75787	NA	4.89	0.00003	Females
Capryloylglycine	HMDB00832	84290	NA	4.61	0.00536	Females
Gamma-Caprolactone	HMDB03843	12756	NA	4.39	0.0536	Females
L-Lactic acid	HMDB00190	107689	C00186	4.37	0.0006	Females
Maltitol	HMDB02928	493591	NA	4.36	0.00004	Females
Betaine	HMDB00043	247	C00719	4	0.00024	Females
Thymidine 5'-triphosphate	HMDB01342	64968	C00459	3.94	0.02569	Females
1,3,7-Trimethyluric acid	HMDB07022	534779 57	NA	3.77	0.00089	Females
Glycolic acid	HMDB00115	757	C00160	3.65	0.00021	Females
1-Methylhistidine	HMDB00001	92105	C01152	3.64	0.0001	Females
2-Amino-3-		3857	C05672	3 51	0.0005	Famalas
phosphonopropionic acid	TIMDB00370	054222	003072	5.54	0.0005	remates
5a-Androstane-3a,17a-diol	HMDB04047	954 <i>333</i> 3	C14143	3.52	0.00002	Females
3-Hydroxybutyric acid	HMDB00357	441	C01089	3.46	0.01172	Females
Creatine	HMDB00064	586	C00300	2.87	0.00021	Females
Glycerol 3-phosphate	HMDB00126	439162	C00093	2.53	0.00024	Females
Gluconic acid	HMDB00625	10690	C00257	2.38	0.00463	Females
3-Hydroxyphenylacetic acid	HMDB00440	12122	C05593	2.26	0.00066	Females
Phosphocreatine	HMDB01511	587	C02305	2.24	0.00264	Females
Phenylpropanolamine	HMDB01942	26934	C07911	2.02	0.01086	Females
Hypotaurine	HMDB00965	107812	C00519	-50.02	0.000001	Males
Methylamine	HMDB00164	6329	C00218	-33.17	0.000001	Males
Carnosine	HMDB00033	439224	C00386	-31.58	0.000001	Males
Cortisone	HMDB02802	222786	C00762	-30.31	0.000001	Males
Tetrahydrocortisone	HMDB00903	124446 17	NA	-29.81	0.000001	Males
Mevalonic acid	HMDB00227	449	C00418	-28.59	0.000001	Males
S-Adenosylhomocysteine	HMDB00939	439155	C00021	-23.11	0.000001	Males
Scyllitol	HMDB06088	NA	C06153	-22.5	0.000001	Males
Gamma-Butyrolactone	HMDB00549	7302	C01770	-20.41	0.000001	Males
Uridine 5'-diphosphate	HMDB00295	6031	C00015	-18.85	0.000001	Males
3,7-Dimethyluric acid	HMDB07021	534779 56	NA	-16.93	0.000001	Males
Nicotinuric acid	HMDB03269	68499	C05380	-16.4	0.000001	Males
9-Methyluric acid	HMDB01973	108714	NA	-13.33	0.000001	Males
Ouinone	HMDB03364	4650	C00472	-13.32	0.000001	Males
Hydrocinnamic acid	HMDB00764	107	C05629	-13.08	0.000001	Males
== 0 ===========		- V I	200027	12.00	2.00001	

Retinal	HMDB01358	638015	C00376	-12.78	0.000001	Males
Ethanolamine	HMDB00149	700	C00189	-12.17	0.000001	Males
2-Hydroxyestrone	HMDB00343	440623	C05298	-11.59	0.000001	Males
Guanine	HMDB00132	764	C00242	-11.47	0.000001	Males
Tiglylglycine	HMDB00959	644156 7	NA	-11.31	0.000001	Males
Succinic acid	HMDB00254	1110	C00042	-11.23	0.000001	Males
Estrone	HMDB00145	5870	C00468	-11.1	0.000001	Males
L-Aspartyl-L-phenylalanine	HMDB00706	93078	NA	-10.91	0.000001	Males
Malonic acid	HMDB00691	867	C00383	-10.87	0.000001	Males
D-Lactic acid	HMDB01311	61503	C00256	-10.57	0.000001	Males
L-Octanoylcarnitine	HMDB00791	119538 14	C02838	-10.33	0.000001	Males
Guanidinosuccinic acid	HMDB03157	439918	C03139	-10.25	0.000001	Males
Dehydroascorbic acid	HMDB01264	210328	C00425	-9.91	0.000001	Males
Theophylline	HMDB01889	2153	C07130	-9.79	0.000001	Males
Salbutamol	HMDB01937	123600	C11770	-9.52	0.000001	Males
D-threo-Isocitric acid	HMDB01874	531853 2	C00451	-9.33	0.000001	Males
Glycylproline	HMDB00721	79101	NA	-8.87	0.000001	Males
Estriol	HMDB00153	5756	C05141	-8.55	0.000001	Males
N,N-Dimethylformamide	HMDB01888	6228	C03134	-8.54	0.000001	Males
Estradiol	HMDB00151	5757	C00951	-8.3	0.000001	Males
Levulinic acid	HMDB00720	11579	NA	-8.2	0.000001	Males
L-Tryptophan	HMDB00929	6305	C00078	-8.1	0.000001	Males
Coumarin	HMDB01218	323	C05851	-7.93	0.000001	Males
Cortisol	HMDB00063	657311	C00735	-7.81	0.000001	Males
Threonic acid	HMDB00943	151152	C01620	-7.65	0.000001	Males
3a,6b,7b-Trihydroxy-5b- cholanoic acid	HMDB12252	528344 6	NA	-7.63	0.000001	Males
Creatinine	HMDB00562	588	C00791	-7.26	0.000001	Males
Dimethylsulfide	HMDB02303	1068	C00580	-7.26	0.000001	Males
L-Histidine	HMDB00177	6274	C00135	-7.14	0.000001	Males
Lactulose	HMDB00740	11333	C07064	-7.09	0.000001	Males
3a,6a,7b-Trihydroxy-5b- cholanoic acid	HMDB05096	528338 9	NA	-6.79	0.000001	Males
3-Hydroxymandelic acid	HMDB00750	86957	NA	-6.48	0.000001	Males
L-Thyronine	HMDB00667	546110 3	NA	-6.46	0.000001	Males
Beta-Alanine	HMDB00056	239	C00099	-6.02	0.000001	Males
Dehydroepiandrosterone	HMDB00077	986074 4	C01227	-5.77	0.000001	Males
Isopropyl alcohol	HMDB00863	3776	C01845	-5.54	0.000001	Males
Ibuprofen	HMDB01925	3672	C01588	-5.45	0.000001	Males
Alpha-Aspartyl-lysine	HMDB04987	642700 3	NA	-5.27	0.000001	Males
Deoxycholic acid glycine conjugate	HMDB00631	228335 39	C05464	-5.21	0.000001	Males

N-Acetylputrescine	HMDB02064	122356	C02714	-5.18	0.000001	Males
Methylmalonic acid	HMDB00202	487	C02170	-5.07	0.000001	Males
Corticosterone	HMDB01547	5753	C02140	-4.95	0.000001	Males
Methionine sulfoxide	HMDB02005	847	NA	-4.85	0.000001	Males
2-Aminobenzoic acid	HMDB01123	227	C00108	-4.83	0.000001	Males
Taurodeoxycholic acid	HMDB00896	273376 8	C05463	-4.68	0.000001	Males
Sarcosine	HMDB00271	1088	C00213	-4.4	0.000001	Males
Glutathione	HMDB00125	124886	C00051	-4.12	0.000001	Males
Hexanoylcarnitine	HMDB00705	642685 3	NA	-4.09	0.000001	Males
D-Mannose	HMDB00169	18950	C00159	-4.02	0.000001	Males
4,5-Dihydroorotic acid	HMDB00528	648	C00337	-4.01	0.000001	Males
Epiandrosterone	HMDB00365	110553 99	C07635	-3.86	0.000001	Males
2,2-Dimethylsuccinic acid	HMDB00729	11671	NA	-3.85	0.000001	Males
Orotidine	HMDB00788	92751	C01103	-3.82	0.000001	Males
Deoxycholic acid	HMDB00626	222528	C04483	-3.7	0.000001	Males
Bilirubin	HMDB00054	528035 2	C00486	-3.42	0.000001	Males
L-Valine	HMDB00883	6287	C00183	-3.42	0.000001	Males
2-Ketobutyric acid	HMDB00005	58	C00109	-3.4	0.000001	Males
L-Alanine	HMDB00161	5950	C00041	-3.15	0.00001	Males
Quinic acid	HMDB03072	NA	C06746	-3.13	0.00001	Males
2-Methoxyestradiol	HMDB00405	66414	C05302	-3.11	0.00001	Males
Androsterone	HMDB00031	123067 65	C00523	-3.01	0.00001	Males
Ethylmalonic acid	HMDB00622	11756	NA	-2.99	0.00001	Males
Phenyllactic acid	HMDB00779	3848	C01479	-2.8	0.00001	Males
3-Phosphoglyceric acid	HMDB00807	724	C00597	-2.79	0.00002	Males
O-Phosphoethanolamine	HMDB00224	1015	C00346	-2.79	0.00002	Males
N-Acetyl-L-alanine	HMDB00766	88064	C00624	-2.73	0.00002	Males
Pipecolic acid	HMDB00070	849	C00408	-2.67	0.00002	Males
(R)-lipoic acid	HMDB01451	6112	C00725	-2.63	0.00003	Males
Pyruvatoxime	HMDB02455	641942 7	NA	-2.54	0.00003	Males
Homo-L-arginine	HMDB00670	9085	C01924	-2.53	0.00003	Males
Aldosterone	HMDB00037	247584 25	C01780	-2.5	0.00004	Males
Oxoglutaric acid	HMDB00208	51	C00026	-2.49	0.00005	Males
L-Alpha-aminobutyric acid	HMDB00452	80283	C02356	-2.47	0.00006	Males
Propyl alcohol	HMDB00820	1031	C05979	-2.46	0.00007	Males
Quercetin	HMDB05794	528034 3	C00389	-2.34	0.00007	Males
Selenomethionine	HMDB03966	15103	C05335	-2.33	0.00008	Males
Homocarnosine	HMDB00745	102433 61	C00884	-2.32	0.00008	Males
Hippuric acid	HMDB00714	464	C01586	-2.3	0.00009	Males

Androstenedione	HMDB00053	6128	C00280	-2.24	0.00009	Males
Testosterone	HMDB00234	6013	C00535	-2.22	0.00011	Males
Glycine	HMDB00123	750	C00037	-2.22	0.00012	Males
N-Acetylglutamic acid	HMDB01138	185	C00624	-2.2	0.00012	Males
p-Aminobenzoic acid	HMDB01392	978	C00568	-2.2	0.00017	Males
Choline	HMDB00097	305	C00114	-2.16	0.00035	Males
Oxalacetic acid	HMDB00223	970	C00036	-2.16	0.00038	Males
2-Hydroxybutyric acid	HMDB00008	11266	C05984	-2.06	0.00066	Males
Sphingosine	HMDB00252	535395 5	C00319	-2	0.00084	Males

MetaboAnalyst 3.0 Pathways (female)	Total	Expected	Hits	Raw p	Impact
Galactose metabolism	26	1.0	6	0.0003615 2	0.08757
Glycine, serine and threonine metabolism	31	1.2	5	0.0062254	0.26298
Arginine and proline metabolism	43	1.7	5	0.024582	0.1665
Starch and sucrose metabolism	22	0.9	3	0.052984	0.05445
Pyrimidine metabolism	41	1.6	4	0.075	0.04087
Cysteine and methionine metabolism	29	1.1	3	0.1037	0.1671
Pantothenate and CoA biosynthesis	15	0.6	2	0.11622	0.03571
Pentose and glucuronate interconversions	15	0.6	2	0.11622	0.08333
Glyoxylate and dicarboxylate metabolism	18	0.7	2	0.15724	0.18519
Glycerolipid metabolism	18	0.7	2	0.15724	0.30658
Synthesis and degradation of ketone bodies	5	0.2	1	0.1831	0

MetaboAnalyst 3.0 Pathways (male)	Total	Expected	Hits	Raw p	Impact
Steroid hormone biosynthesis	56	3.6385	12	0.0001396 2	0.46154
Propanoate metabolism	20	1.2995	4	0.03614	0
Alanine, aspartate and glutamate metabolism	24	1.5594	4	0.065065	0.15823
Nitrogen metabolism	9	0.58476	2	0.11155	0
Citrate cycle (TCA cycle)	20	1.2995	3	0.13581	0.20776

MetaboAnalyst 3.0 Pathways (common)	Total	Expected	Hits	Raw p	Impact
Amino sugar and nucleotide sugar metabolism	37	1.6856	5	0.02352	0.2314
Pentose and glucuronate interconversions	15	0.68335	3	0.027678	0
Starch and sucrose metabolism	22	1.0022	3	0.074781	0.3589
Aminoacyl-tRNA biosynthesis	67	3.0523	6	0.079277	0
Phenylalanine metabolism	11	0.50112	2	0.086191	0
Galactose metabolism	26	1.1845	3	0.11117	0.08786
Arginine and proline metabolism	43	1.9589	4	0.12802	0.1777
Histidine metabolism	14	0.63779	2	0.13079	0.16667

D-Arginine and D-ornithine metabolism	4	0.18223	1	0.17032	0

Annex 2. 2 Molecular pathways of the medaka liver according the whole metabolite list determined by the Metaboanalyst 3.0 online search engine.

Metabolite pathways (MetaboANALYST 3.0)	Total Cmpd	Hits	P value	FDR
Glutathione metabolism	26	5	7.51E-18	4.13E-16
Tryptophan metabolism	39	3	6.91E-17	1.90E-15
Riboflavin metabolism	11	1	1.66E-16	3.05E-15
Arginine and proline metabolism	43	11	3.80E-14	5.23E-13
Taurine and hypotaurine metabolism	7	2	6.02E-14	6.10E-13
Purine metabolism	66	2	6.65E-14	6.10E-13
Pantothenate and CoA biosynthesis	15	4	2.35E-13	1.85E-12
Glyoxylate and dicarboxylate metabolism	18	3	7.86E-13	5.40E-12
Citrate cycle (TCA cycle)	20	4	1.39E-12	8.52E-12
Propanoate metabolism	20	4	5.65E-12	3.11E-11
Pyruvate metabolism	22	3	3.48E-11	1.65E-10
Butanoate metabolism	22	3	3.89E-11	1.65E-10
Retinol metabolism	16	1	3.90E-11	1.65E-10
beta-Alanine metabolism	16	2	9.92E-11	3.90E-10
Steroid hormone biosynthesis	56	17	1.60E-10	5.81E-10
Pyrimidine metabolism	41	9	1.69E-10	5.81E-10
Terpenoid backbone biosynthesis	15	1	2.35E-10	7.61E-10
Sphingolipid metabolism	21	3	1.17E-08	3.58E-08
Valine, leucine and isoleucine degradation	38	3	4.00E-07	1.16E-06
Inositol phosphate metabolism	27	1	1.09E-06	2.85E-06
Ascorbate and aldarate metabolism	6	1	1.09E-06	2.85E-06
Fructose and mannose metabolism	21	3	1.25E-06	3.13E-06
Valine, leucine and isoleucine biosynthesis	13	4	3.02E-06	7.21E-06
Cysteine and methionine metabolism	29	5	5.19E-06	1.19E-05
Glycolysis or Gluconeogenesis	26	4	6.94E-06	1.53E-05
Caffeine metabolism	11	1	9.77E-06	2.07E-05
Selenoamino acid metabolism	17	2	3.39E-05	6.90E-05
Amino sugar and nucleotide sugar metabolism	37	6	0.0001	0.0003
Glycerophospholipid metabolism	28	6	0.0001	0.0003
Histidine metabolism	14	3	0.0002	0.0003
Pentose and glucuronate interconversions	15	5	0.0002	0.0004
Porphyrin and chlorophyll metabolism	27	2	0.0002	0.0004
Methane metabolism	9	2	0.0002	0.0004
Cyanoamino acid metabolism	6	2	0.0002	0.0004
Synthesis and degradation of ketone bodies	5	1	0.0005	0.0008
Galactose metabolism	26	10	0.0006	0.0008
Glycine, serine and threonine metabolism	31	10	0.0008	0.0013
Nitrogen metabolism	9	4	0.0014	0.0020

Glycerolipid metabolism	18	2	0.0028	0.0040
D-Glutamine and D-glutamate metabolism	5	2	0.0061	0.0084
Pentose phosphate pathway	19	1	0.0067	0.0089
Starch and sucrose metabolism	22	6	0.0108	0.0139
Fatty acid biosynthesis	38	1	0.0114	0.0139
Fatty acid elongation in mitochondria	27	1	0.0114	0.0139
Fatty acid metabolism	38	2	0.0114	0.0139
Biosynthesis of unsaturated fatty acids	42	2	0.0123	0.0147

Annex 2. 3 Complete list of proteins that are significantly sex-over-represented in female and male livers, and their respective metabolic pathway enrichment.

SNP	Ensembl Protein ID		$\log(FC)$	10910	Un-
		Associated Gene Name	10g2(1 C)	Pvalue	regulated
H2LNM8_ORYLA	ENSORLP0000007656	vitellogenin 1 precursor	9.5325	3.5723	Females
H2LP13_ORYLA	ENSORLP0000007793	vitellogenin II precursor	8.432	3.7752	Females
H2M2R4_ORYLA	ENSORLP00000012711	choriogenin L	7.9643	4.3092	Females
H2ME97_ORYLA	ENSORLP00000016901	fatty acid binding	7.7661	3.0451	Females
H2LQ37_ORYLA	ENSORLP0000008173	protein 7, brain, a vitellogenin 3, phosyitinless	7.5641	4.6265	Females
Q8JHT8_ORYLA	ENSORLP00000013645	zona pellucida glycoprotein 2, like 2	7.425	6	Females
Q8JI21_ORYLA	ENSORLP00000012647	zona pellucida glycoprotein 2, like 2	6.9405	4.4959	Females
H2L7I1_ORYLA	ENSORLP0000001793	creatine kinase, mitochondrial 1	5.8161	3.6548	Females
H2M9S6_ORYLA	ENSORLP00000015268	pyrroline-5-carboxylate reductase 1b	5.782	2.4282	Females
H2LU70_ORYLA	ENSORLP00000009644	pyridine nucleotide- disulphide	5.0981	4.7671	Females
H2MTM5_ORYLA	ENSORLP00000022129	transmembrane protein 214	5.035	4.32	Females
H2RZC5_TAKRU	ENSTRUP0000005496	threonyl-tRNA svnthetase	4.3502	3.2972	Females
H2LQA6_ORYLA	ENSORLP0000008246	endoplasmic reticulum- golgi intermediate 1	4.3225	3.6801	Females
H2MTR0_ORYLA	ENSORLP00000022167	DnaJ (Hsp40) homolog, subfamily C, member 3a	4.2925	4.4617	Females
H2MPJ8_ORYLA	ENSORLP00000020660	eukaryotic translation initiation factor 4Bb	4.247	2.1789	Females
H2MLI1_ORYLA	ENSORLP00000019559	translocon-associated protein subunit alpha- like	4.178	2.8058	Females
H2MAK2_ORYLA	ENSORLP00000015557	SEC11 homolog A, signal peptidase complex	4.127	3.0431	Females
H2L7T6_ORYLA	ENSORLP0000001903	FK506 binding protein 11	4.1229	2.6087	Females
H2LK51_ORYLA	ENSORLP0000006401	ribosomal protein L5	4.0698	4.4415	Females
H2MT95_ORYLA	ENSORLP00000021993	Cytochrome c, somatic b	3.961	2.5807	Females

H2L8H1_ORYLA	ENSORLP0000002143	DDRGK domain containing 1	3.8855	3.2326	Females
H2MPK9_ORYLA	ENSORLP00000020671	ribosomal protein L35	3.8842	4.5876	Females
H2MDR0_ORYLA	ENSORLP00000016711		3.8188	3.3354	Females
H2MJP0_ORYLA	ENSORLP00000018889	FK506 binding protein 3	3.8175	3.452	Females
H2UJW9_TAKRU	ENSTRUP00000037240	calumenin b	3.8161	3.5602	Females
H2LCF5_ORYLA	ENSORLP0000003592	abcel	3.7645	2.7144	Females
H2MLA2_ORYLA	ENSORLP00000019476	arfgap3	3.749	3.0066	Females
H2M450_ORYLA	ENSORLP00000013211	scfd1	3.7448	2.2013	Females
H2LLW5_ORYLA	ENSORLP0000007032	rps15	3.6656	4.0956	Females
H2LDD7_ORYLA	ENSORLP0000003940	nop58	3.5806	2.0727	Females
H2LBS7_ORYLA	ENSORLP0000003356	rpl36	3.4305	2.1897	Females
H2MJJ9_ORYLA	ENSORLP00000018848	serbp1a	3.4247	2.2863	Females
H2L816_ORYLA	ENSORLP00000001984	•	3.421	3.2106	Females
H2MCT7_ORYLA	ENSORLP00000016375	vars	3.3934	2.1667	Females
H2L902_ORYLA	ENSORLP0000002335	sec31a	3.375	4.043	Females
H2LIZ3_ORYLA	ENSORLP00000005976	iars	3.2401	2.3965	Females
I1SRS9_ORYME	ENSORLP00000007779	mrpl32	3.2375	3.7691	Females
H2M8M2_ORYLA	ENSORLP00000014842	clqbp	3.2331	3.5577	Females
H2LBC1_ORYLA	ENSORLP0000003192	acaa2	3.1747	2.5215	Females
H2LCK3_ORYLA	ENSORLP0000003642	rpl2211	3.1106	3.7274	Females
Q2PHF0_ORYLA	ENSORLP00000013510	fabp7	3.0907	2.1573	Females
H2LSN9_ORYLA	ENSORLP0000009104	rps10	3.0897	2.0688	Females
H2M2W5_ORYLA	ENSORLP00000012763	npm1a	3.0102	2.4352	Females
H2T3A4_TAKRU	ENSTRUP00000019142	aimp1	3.0024	2.0087	Females
H2L3N8_ORYLA	ENSORLP0000000364	ndufv1	2.9349	3.3707	Females
H2LLR3_ORYLA	ENSORLP0000006978	TMEM38B	2.9276	2.3715	Females
H2M6R6_ORYLA	ENSORLP00000014164	gfpt1	2.9001	2.6483	Females
H2L720_ORYLA	ENSORLP0000001628	erolb	2.8854	3.1127	Females
H2LEW3_ORYLA	ENSORLP00000004490	txndc12	2.8825	3.681	Females
H2L5G3_ORYLA	ENSORLP00000001035	rpl36a	2.8456	2.1843	Females
H2LTY3_ORYLA	ENSORLP0000009556	kars	2.832	2.1388	Females
H2LKH6_ORYLA	ENSORLP0000006527		2.8229	3.4635	Females
H2MSS6_ORYLA	ENSORLP00000021819	gale	2.8018	2.2628	Females
H2L7V1_ORYLA	ENSORLP00000001918	sars	2.7911	4.5045	Females
H2L6U0_ORYLA	ENSORLP0000001547	SEC61A1 (1 to many)	2.7491	2.2367	Females
H2MWK8_ORYLA	ENSORLP00000023257	gcn1 (1 to many)	2.7383	2.4528	Females
H2LR39_ORYLA	ENSORLP0000008536	copb1	2.7116	2.9384	Females
H2L8B1_ORYLA	ENSORLP0000002081	si:ch211-127115.5	2.6991	3.2771	Females
H2UAE0_TAKRU	ENSTRUP00000033907	rpl14	2.6896	3.278	Females
H2MG03_ORYLA	ENSORLP00000017531	yars	2.6752	2.0596	Females
H2LY75_ORYLA	ENSORLP00000011089	nop56	2.6186	2.5942	Females
H2L7Q5_ORYLA	ENSORLP0000001872	rps17	2.6156	2.7567	Females
H2MJS3_ORYLA	ENSORLP00000018922	arf6b	2.5951	6	Females
H2MCC8_ORYLA	ENSORLP00000016213	prep	2.5897	2.3365	Females

H2LVL8_ORYLA	ENSORLP00000010151	CYP2W1 (1 to many)	2.5818	2.6305	Females
H2M4U8_ORYLA	ENSORLP00000013469	qars	2.5578	2.5759	Females
H2L910_ORYLA	ENSORLP0000002344	dpm1	2.5335	2.8293	Females
H2N2R1_ORYLA	ENSORLP00000025750	spcs2	2.5172	2.3955	Females
H2MGU0_ORYLA	ENSORLP00000017833	eif3s10	2.4981	2.0596	Females
MT_ORYLA	ENSORLP00000019508	mt2	2.477	2.472	Females
H2M705_ORYLA	ENSORLP00000014255	taf15	2.4674	2.8426	Females
H2LGP8_ORYLA	ENSORLP00000005146	sec22ba	2.4453	2.4971	Females
H2MPF0_ORYLA	ENSORLP00000020609	rps12	2.4438	2.92	Females
H2L6Q5_ORYLA	ENSORLP0000001506	eiflb	2.4262	2.5068	Females
H2LV85_ORYLA	ENSORLP00000010015	aplgl	2.4262	2.5068	Females
H2LP66_ORYLA	ENSORLP0000007846	eif3jb	2.4228	2.6144	Females
H2LE67_ORYLA	ENSORLP0000004234	fbl	2.3562	2.6947	Females
H2LXT9_ORYLA	ENSORLP00000010951	sec61b	2.3291	4.2112	Females
H2LRE6_ORYLA	ENSORLP0000008645	rpl4	2.3164	3.5655	Females
H2MHG1_ORYLA	ENSORLP00000018068	zgc:114188	2.308	3.1044	Females
H2MSY7_ORYLA	ENSORLP00000021880	tmed10	2.3038	2.7528	Females
H2LAW8_ORYLA	ENSORLP0000003034	eiflaxb	2.2312	2.3127	Females
A0A0D5ZZW0_ORYL	ENSORLP00000013668	aamtl	2.2312	2.3127	Females
A		asinn			
H2MSB3_ORYLA	ENSORLP00000021650	rcn3	2.2111	2.4923	Females
H2MCW3_ORYLA	ENSORLP00000016402	rps25	2.2092	2.4863	Females
H2L9Z6_ORYLA	ENSORLP0000002697	rps5	2.1974	2.8182	Females
I6L4R8_ORYLA	ENSORLP00000015610	cygb1	2.1944	2.1656	Females
H2LPD8_ORYLA	ENSORLP00000007920	rps6	2.1664	2.7383	Females
H2MJS0_ORYLA	ENSORLP00000018919	prpf39	2.1	3.0165	Females
H2MYJ5_ORYLA	ENSORLP00000024083	hyoul	2.0921	2.5537	Females
H2N1B0_ORYLA	ENSORLP00000025209	rpl17	2.0862	2.1601	Females
H2M5W8_ORYLA	ENSORLP00000013856	spcs3	2.0854	2.4265	Females
H2MWK1_ORYLA	ENSORLP00000023250	canx	2.0734	2.4609	Females
H2N1K0_ORYLA	ENSORLP00000025305	srp72	2.073	2.8989	Females
Q8AXS2_ORYLA	ENSORLP0000005291	ybx1	2.0517	2.2164	Females
H2LR36_ORYLA	ENSORLP0000008533		2.0516	3.2681	Females
H2M0E5_ORYLA	ENSORLP00000011869	gars	2.0334	3.3439	Females
H2N0P8_ORYLA	ENSORLP00000024964	rpl18a	2.0326	2.2191	Females
H2M8R1_ORYLA	ENSORLP00000014882	zgc:171356	2.023	2.659	Females
H2MWK7_ORYLA	ENSORLP00000023256	zc3h15	2.0101	6	Females
H2ML65_ORYLA	ENSORLP00000019438	cnpy2	2.0005	2.2013	Females
H2M002_ORYLA	ENSORLP00000011725	mvda	1.9982	2.6265	Females
H2M8F3_ORYLA	ENSORLP00000014769	tfg	1.9981	2.6327	Females
H2LCR6_ORYLA	ENSORLP0000003706	zgc:152830	1.9715	3.3933	Females
H2LKU4_ORYLA	ENSORLP0000006651	gnb2l1	1.9478	3.6784	Females
H2LLI7_ORYLA	ENSORLP0000006901	eif3ha	1.9405	2.1571	Females
H2LCN1_ORYLA	ENSORLP0000003671	lasp1	1.9349	2.3904	Females
H2LLN8_ORYLA	ENSORLP0000006953	rpl23a	1.9295	4.3635	Females

H2MYG8_ORYLA ENSORLP00000024050 ddost 1.8803 3.4451 Females H2LCW3_ORYLA ENSORLP0000003755 rp119 (1 to many) 1.8521 3.635 Females H2LKH7_ORYLA ENSORLP0000001372 rps18 1.8188 3.828 Females H2MYT0_ORYLA ENSORLP00000014497 fabp3 1.7432 2.273 Females H2LSQ4_ORYLA ENSORLP00000014497 fabp3 1.7432 2.374 Females H2LSQ4_ORYLA ENSORLP0000002827 ssr4 1.6973 2.7474 Females H2LPD1_ORYLA ENSORLP0000002823 sec63 1.6906 2.0134 Females H2LPD1_ORYLA ENSORLP0000002823 sec63 1.625 2.0094 Females H2L6A5_ORYLA ENSORLP0000001582 rps13 1.625 2.004 Females H2LM3_ORYLA ENSORLP0000001526 rps3a 1.5577 4.3436 Females H2LM15_ORYLA ENSORLP0000001536 rps1a 1.5543 2.568 Females H2MYB_1YAKU ENSORLP00						
H2LCW3_ORYLA ENSORLP0000000375 rp119 (1 to many) 1.8521 3.6335 Females H2MKH7_ORYLA ENSORLP00000019191 arcn lb 1.8342 2.6472 Females H2L6C6_ORYLA ENSORLP0000001372 rps18 1.8188 3.8828 Females H2MYT0_ORYLA ENSORLP0000001447 fabp3 1.7432 2.5218 Females H2LSQ4_ORYLA ENSORLP00000009179 pdia4 1.7075 2.7474 Females H2LSQ6_ORYLA ENSORLP0000000877 srs4 1.6975 2.5361 Females H2LDQ_ORYLA ENSORLP0000007913 mat2ab 1.6265 2.9464 Females H2L6X5_ORYLA ENSORLP0000001528 rps13 1.6224 2.2865 Females H2L6X5_ORYLA ENSORLP0000001529 rps3a 1.5577 4.346 Females H2LGS8_ORYLA ENSORLP0000001534 rp110 1.5389 2.032 Females H2LGS9_ORYLA ENSORLP0000001549 rpt10 1.5385 2.4635 Females H2LGS9_ORYLA	H2MYG8_ORYLA	ENSORLP00000024050	ddost	1.8803	3.4451	Females
H2MKH7_ORVLA ENSORLP00000019191 arcn1b 1.8342 2.6472 Females H2L6C6_ORVLA ENSORLP0000001372 rps18 1.8188 3.8828 Females H2MYN0_ORYLA ENSORLP00000014187 fabp3 1.7432 2.5218 Females H2LSQ4_ORYLA ENSORLP0000008437 srs4 1.6973 2.7474 Females H2LSIQ_ORYLA ENSORLP0000002323 sec63 1.6906 2.0094 Females H2LPD1_ORYLA ENSORLP0000001582 rps13 1.6265 2.0094 Females H2L6X5_ORYLA ENSORLP0000001582 rps13 1.6255 2.4635 Females H216X5_ORYLA ENSORLP0000001582 rps3a 1.5577 4.3436 Females H21G9_ORYLA ENSORLP0000001536 rms3a 1.5577 4.3436 Females H21G9_ORYLA ENSORLP0000001536 rps3a 1.5572 2.4635 Females H21G9_ORYLA ENSORLP0000001364 rpl10 1.5389 2.032 Females H2MT5_ORYLA ENSORLP000001349	H2LCW3_ORYLA	ENSORLP0000003755	rpl19 (1 to many)	1.8521	3.6335	Females
H2L6C6_ORYLAENSORLP0000001372rps181.81883.8828FemalesH2MYT0_ORYLAENSORLP00000024188sod3b1.79332.0273FemalesH2LSQ4_ORYLAENSORLP00000014497fabp31.74322.5218FemalesH2LSQ4_ORYLAENSORLP0000000919pdia41.70755.6843FemalesH2LSQ1_ORYLAENSORLP0000000827ssr41.69732.7474FemalesH2LO1_ORYLAENSORLP0000007913mat2ab1.67052.5361FemalesH2LSA_TAKRUENSORLP0000001582rps131.62252.0094FemalesH2LOSA_TAKRUENSORLP0000001582rps131.62552.4635FemalesH2NJO_ORYLAENSORLP0000001582rps3a1.55432.668FemalesH2MT1_ORYLAENSORLP0000001453tmed91.55432.668FemalesH2LF08_ORYLAENSORLP0000001453tmed91.55432.062FemalesH2LF08_ORYLAENSORLP0000001453tmed91.53892.3027FemalesH2LF08_ORYLAENSORLP0000001280hpx-2.65343.8174MalesH2LF08_ORYLAENSORLP0000001280hpx-2.65343.8174MalesH2LF08_ORYLAENSORLP0000001284gcthf-2.56552.3351MalesH2LF08_ORYLAENSORLP0000002914gcthf-2.26643.2071MalesH2LW32_ORYLAENSORLP0000001294gcthf-2.26652.3331MalesH2LW42_ORYLAENSORLP000000294agtt1 (1 to many)	H2MKH7_ORYLA	ENSORLP00000019191	arcn1b	1.8342	2.6472	Females
H2MYT0_ORYLA ENSORLP0000024188 sod3b 1.7933 2.0273 Females H2M7N9_ORYLA ENSORLP0000001497 fabp3 1.7432 5.5843 Females H2LSQ4_ORYLA ENSORLP0000009119 pdia4 1.7075 5.6843 Females H2LSI8_ORYLA ENSORLP0000000887 ssr4 1.6973 2.7474 Females H2LPD_ORYLA ENSORLP00000039831 pdia3 1.6264 2.2865 Females H2LOS_ORYLA ENSORLP0000001528 mar2ab 1.6705 2.5461 Females H2L6XS_ORYLA ENSORLP0000001528 mar2ab 1.6224 2.2865 Females H2LGSQ_ORYLA ENSORLP00000014539 tmed9 1.5543 2.6435 Females H2LGYS_ORYLA ENSORLP00000018586 cyp27a7 -5.3282 2.2062 Males H2LFUS_ORYLA ENSORLP0000012850 hpx -2.634 3.8174 Males H2LWB_ORYLA ENSORLP0000023234 gchfr -2.6365 2.3351 Males H2DMYL_ORYLA ENSORLP000002324	H2L6C6_ORYLA	ENSORLP0000001372	rps18	1.8188	3.8828	Females
H2M7N9_ORYLA ENSORLP00000014497 fabp3 1.7432 2.5218 Females H2LSQ4_ORYLA ENSORLP0000009817 ssr4 1.6973 5.6843 Females H2LS18_ORYLA ENSORLP00000020823 sec63 1.6906 2.0134 Females H2LPD1_ORYLA ENSORLP00000020823 sec63 1.6265 2.0094 Females H2L6X5_ORYLA ENSORLP0000002582 rps13 1.6224 2.2865 Females H2L6X5_ORYLA ENSORLP0000001582 rps13 1.6274 2.8361 Females H2X071_ORYLA ENSORLP00000014539 tmed9 1.5543 2.568 Females H2X0FS_ORYLA ENSORLP00000014539 tmed9 1.5455 2.4635 Females H2X0FS_ORYLA ENSORLP00000018568 cyp27a7 -5.3282 2.0367 Males H2LFUS_ORYLA ENSORLP00000012334 gst1a (1 to many) -2.634 3.8174 Males H2LFUS_ORYLA ENSORLP00000012364 pch7 -2.5265 2.3351 Males H2LFUS_ORYLA <t< td=""><td>H2MYT0_ORYLA</td><td>ENSORLP00000024188</td><td>sod3b</td><td>1.7933</td><td>2.0273</td><td>Females</td></t<>	H2MYT0_ORYLA	ENSORLP00000024188	sod3b	1.7933	2.0273	Females
H2LSQ4_ORYLA ENSORLP0000009119 pdia4 1.7075 5.6843 Females H2LSI8_ORYLA ENSORLP0000008877 ssr4 1.6973 2.7474 Females H2LPD1_ORYLA ENSORLP0000007913 mat2ab 1.6906 2.5361 Females H2LPD1_ORYLA ENSORLP0000007913 mat2ab 1.6255 2.0094 Females H2LM3_ORYLA ENSORLP0000009623 rps13 1.6224 2.2865 Females RS3A_ORYLA ENSORLP0000009633 rps3a 1.5543 2.568 Females H2MT1_ORYLA ENSORLP0000014539 imced9 1.5455 2.4635 Females H2MT1_ORYLA ENSORLP0000014539 imced9 1.5455 2.4635 Females H2MT9_ORYLA ENSORLP0000012860 rp110 1.5389 2.032 Females H2MT9_ORYLA ENSORLP0000012850 rp18 -2.6534 3.817 Males H2MW1_ORYLA ENSORLP000001249 rgtfr -2.6565 2.3351 Males H2MM2_ORYLA ENSORLP000001249	H2M7N9_ORYLA	ENSORLP00000014497	fabp3	1.7432	2.5218	Females
H2LS18_ORYLA ENSORLP0000008877 ssr4 1.6973 2.7474 Females H2MQ10_ORYLA ENSORLP0000020823 sec63 1.6906 2.0134 Females H2LPD1_ORYLA ENSORLP00000039831 pdia3 1.6265 2.0094 Females H2LSAS_ORYLA ENSORLP0000001582 rps13 1.6224 2.2865 Females H2LM3_ORYLA ENSORLP0000000423 rps3a 1.5577 4.3436 Females H2MT1_ORYLA ENSORLP0000001539 tmed9 1.5343 2.568 Females H2MT9_ORYLA ENSORLP0000001534 rps10 1.5389 2.032 Females H2MT9_ORYLA ENSORLP0000001534 rp110 1.5380 2.032 Females H2MT9_ORYLA ENSORLP00000018568 cyp27a7 5.3282 2.062 Males H2M49_ORYLA ENSORLP0000001280 pdia1 1.5380 2.3567 Males H2M49_ORYLA ENSORLP0000001294 gsc112265 -2.496 3.2071 Males H2LAK3_ORYLA ENSORLP0000001294	H2LSQ4_ORYLA	ENSORLP0000009119	pdia4	1.7075	5.6843	Females
H2MQ10_ORYLA ENSORLP0000020823 see63 1.6906 2.0134 Females H2LPD1_ORYLA ENSORLP0000007913 mat2ab 1.6705 2.361 Females H2USA8_TAKRU ENSORLP0000001822 rps13 1.6224 2.2865 Females H2L6X5_ORYLA ENSORLP0000002508 mapre1b 1.5844 2.861 Females RS3A_ORYLA ENSORLP00000014339 tmed9 1.5577 4.3436 Females H2MT1_ORYLA ENSORLP0000001343 tmed9 1.5543 2.663 Females H2MT5_ORYLA ENSORLP0000001344 rpl10 1.5389 2.032 Females H2MT5_ORYLA ENSORLP0000001834 cp27a7 -5.3282 2.2062 Males H2MT5_ORYLA ENSORLP0000001850 hpx -2.634 3.8174 Males H2MW1_ORYLA ENSORLP00000012344 ggt1a (1 to many) -2.645 2.3351 Males H2MW1_ORYLA ENSORLP0000001244 rgb1 -2.2066 2.3311 Males H2LMS0_ORYLA ENSORLP000000124	H2LS18_ORYLA	ENSORLP0000008877	ssr4	1.6973	2.7474	Females
H2LPD1_ORYLA ENSORLP0000007913 mat2ab 1.6705 2.5361 Females H2USA8_TAKRU ENSTRUP00000039831 pdia3 1.6265 2.0094 Females H2L6X5_ORYLA ENSORLP0000001582 rps13 1.6224 2.2865 Females H2N103_ORYLA ENSORLP0000000586 mapre1b 1.5543 2.568 Females H2MTT1_ORYLA ENSORLP00000014539 tmcd9 1.5573 2.4635 Females H2MTS_ORYLA ENSORLP0000001354 rp10 1.5389 2.032 Females H2MTS_ORYLA ENSORLP00000018568 cyp27a7 -5.3282 2.062 Males H2LFW_ORYLA ENSORLP0000001856 cyp27a7 -5.3282 2.3967 Males H2MMJ_ORYLA ENSORLP0000001850 hpx -2.6534 3.8174 Males H2MWJ_ORYLA ENSORLP00000012850 hpx -2.6534 3.8174 Males H2MWJ_ORYLA ENSORLP0000012854 gst11a (1 to many) -2.634 3.89 Males H2MWL_ORYLA ENSORLP00000	H2MQ10_ORYLA	ENSORLP00000020823	sec63	1.6906	2.0134	Females
H2USA8_TAKRU ENSTRUP00000039831 pdia3 1.6265 2.0094 Females H2L6X5_ORYLA ENSORLP0000001582 rps13 1.6224 2.2865 Females H2N103_ORYLA ENSORLP00000025086 mapre1b 1.5844 2.8361 Females RS3A_ORYLA ENSORLP0000004239 rps3a 1.5577 4.3436 Females H2L07B_ORYLA ENSORLP0000004919 alg5 1.5453 2.568 Females H2L07B_ORYLA ENSORLP00000018568 cyp27a7 -5.3282 2.062 Males H2MT5_ORYLA ENSORLP0000001856 cyp27a7 -5.3208 2.3967 Males H2M49_ORYLA ENSORLP0000002324 gst1a (1 to many) -2.634 3.8174 Males H2M42_ORYLA ENSORLP000000214 gcthf -2.3333 2.3845 Males H2LX82_ORYLA ENSORLP0000001882 uqcrc2a -2.3333 2.3845 Males H2LMK3_ORYLA ENSORLP0000001882 uqcrc2a -2.3333 2.3845 Males H2LXM1_ORYLA ENSO	H2LPD1_ORYLA	ENSORLP0000007913	mat2ab	1.6705	2.5361	Females
H2L6X5_ORYLAENSORLP0000001582 Eps13 $rps13$ 1.62242.2865FemalesH2N103_ORYLAENSORLP0000009623 ENSORLP0000009623 $rps3a$ 1.55774.3436FemalesRS3A_ORYLAENSORLP0000009623 ENSORLP00000014539 $rps3a$ 1.55774.3436FemalesH2MTT1_ORYLAENSORLP00000014539 ENSORLP0000001343 $rps3a$ 1.55452.668FemalesH2LG98_ORYLAENSORLP0000001343 PUI0 $rp110$ 1.53892.032FemalesH2RYB3_TAKRUENSORLP0000018568 ENSORLP0000012324 $cyp27a7$ 5.32822.2062MalesH2LFU8_ORYLAENSORLP0000012324 ENSORLP0000023234 gst1a (1 to many)-2.6343.89MalesH2MR20_ORYLAENSORLP000000214 ENSORLP000000214 gchfr-2.56652.3351MalesH2LW82_ORYLAENSORLP0000012169 esc112265-2.49063.2071MalesH2LW82_ORYLAENSORLP000001249 esc112265-2.49063.2071MalesH2LW81_ORYLAENSORLP000001249 rab18b-2.21662.3351MalesH2LW81_ORYLAENSORLP0000003710 eso-2.26962.5231MalesH2LM81_ORYLAENSORLP0000003708-2.01442.4791MalesH2LM81_ORYLAENSORLP00000023708-2.01442.4791MalesH2LM81_ORYLAENSORLP0000003708-2.26952.8981MalesH2LM81_ORYLAENSORLP0000003708-2.26952.8171MalesH2LM81_ORYLAENSORLP0000003708-2.01442.4791Males<	H2USA8_TAKRU	ENSTRUP00000039831	pdia3	1.6265	2.0094	Females
H2N103_ORYLA ENSORLP0000025086 mapre1b 1.5844 2.8361 Females RS3A_ORYLA ENSORLP0000009623 rps3a 1.5577 4.3436 Females H2LG98_ORYLA ENSORLP00000014539 tmed9 1.5543 2.568 Females H2RG98_ORYLA ENSORLP00000005134 rp110 1.5389 2.032 Females H2RYB3_TAKRU ENSORLP00000018568 cyp27a7 -5.3282 2.2062 Males H2LFU8_ORYLA ENSORLP00000018850 hpx -2.6334 3.8174 Males H2MM17_ORYLA ENSORLP00000012850 hpx -2.634 3.89 Males H2MR20_ORYLA ENSORLP0000002144 gchfr -2.5665 2.3351 Males H2LW82_ORYLA ENSORLP0000002144 zgc:112265 -2.496 3.2071 Males H2LW82_ORYLA ENSORLP0000010369 abcc2 -2.3333 2.3845 Males H2LW81_ORYLA ENSORLP00000010892 uqcrc2a -2.3333 2.3845 Males H2LW82_ORYLA ENSORLP00000103701 c9 -2.2695 2.8981 Males H2LM	H2L6X5_ORYLA	ENSORLP00000001582	rps13	1.6224	2.2865	Females
RS3A_ORYLA ENSORLP0000009623 rp3a 1.5577 4.3436 Females H2M7T1_ORYLA ENSORLP0000014539 tmed9 1.5543 2.568 Females H2LG98_ORYLA ENSORLP0000001343 rp110 1.5389 2.032 Females H2RYB3_TAKRU ENSORLP00000005134 rp110 1.5389 2.032 Females H2MIT5_ORYLA ENSORLP000000018568 cyp27a7 -5.3282 2.2062 Males H2LM9_ORYLA ENSORLP00000012850 hpx -2.6534 3.8174 Males H2MM17_ORYLA ENSORLP0000002134 gstl1a (1 to many) -2.654 3.89 Males H2LM82_ORYLA ENSORLP0000002147 zgc:112265 -2.496 3.2071 Males H2LW82_ORYLA ENSORLP0000001682 uqcrc2a -2.3333 2.3845 Males H2LW1_ORYLA ENSORLP00000015937 dera -2.2696 2.5231 Males H2LK1_ORYLA ENSORLP0000002708 -2.0144 2.4791 Males H2LM1_ORYLA ENSORLP0000002708 <t< td=""><td>H2N103_ORYLA</td><td>ENSORLP00000025086</td><td>mapre1b</td><td>1.5844</td><td>2.8361</td><td>Females</td></t<>	H2N103_ORYLA	ENSORLP00000025086	mapre1b	1.5844	2.8361	Females
H2M7T1_ORYLA ENSORLP0000014539 tmed9 1.5543 2.568 Females H2LG98_ORYLA ENSORLP0000004991 alg5 1.5455 2.4635 Females H2RYB3_TAKRU ENSORLP00000018568 cyp27a7 -5.3282 2.2062 Males H2LFU8_ORYLA ENSORLP00000018568 cyp27a7 -5.3282 2.3967 Males H2LFU8_ORYLA ENSORLP00000012850 hpx -2.6534 3.8174 Males H2MW17_ORYLA ENSORLP00000023234 gsttl a (1 to many) -2.634 3.89 Males H2LAK3_ORYLA ENSORLP0000002114 gchfr -2.6565 2.3351 Males H2LW82_ORYLA ENSORLP00000010842 uqcrc2a -2.4901 2.1675 Males H2LW82_ORYLA ENSORLP00000010842 uqcrc2a -2.3333 2.3845 Males H2LW82_ORYLA ENSORLP0000001842 uqcrc2a -2.3333 2.3845 Males H2LW82_ORYLA ENSORLP0000001794 rab18b -2.2162 2.5231 Males H2LW82_ORYLA <t< td=""><td>RS3A_ORYLA</td><td>ENSORLP0000009623</td><td>rps3a</td><td>1.5577</td><td>4.3436</td><td>Females</td></t<>	RS3A_ORYLA	ENSORLP0000009623	rps3a	1.5577	4.3436	Females
$\begin{array}{llllllllllllllllllllllllllllllllllll$	H2M7T1_ORYLA	ENSORLP00000014539	tmed9	1.5543	2.568	Females
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H2MIT5_ORYLAENSORLP0000018568 $cyp27a7$ -5.3282 2.2062 MalesH2LFU8_ORYLAENSORLP0000004839dio1 -5.3208 2.3967 MalesH2M349_ORYLAENSORLP0000012850hpx -2.6534 3.8174 MalesH2MWI7_ORYLAENSORLP0000023234gstt1a (1 to many) -2.634 3.89 MalesH2MR20_ORYLAENSORLP00000021194gchfr -2.5665 2.3351 MalesH2LAK3_ORYLAENSORLP0000002914 $zgc:112265$ -2.496 3.2071 MalesH2LW82_ORYLAENSORLP0000001369 $abcc2$ -2.4901 2.1675 MalesH2LW1_ORYLAENSORLP0000001882uqcrc2a -2.3333 2.3845 MalesH2MN2_ORYLAENSORLP00000101892rab18b -2.2916 2.3762 MalesH2MN2_ORYLAENSORLP0000001799rab18b -2.2069 2.5231 MalesH2MN5_ORYLAENSORLP00000023708 -2.0144 2.4791 MalesH2MN7_ORYLAENSORLP0000002679bckdha -1.9537 2.6139 MalesH2LS08_ORYLAENSORLP00000019645HTRA2 (1 to many) -1.9289 2.2628 MalesH2LM3_ORYLAENSORLP0000001212gsta.2 -1.6778 2.704 MalesH2LS08_ORYLAENSORLP0000003799vdac3 -1.6815 3.0846 MalesH2LM3_ORYLAENSORLP000000399vdac3 -1.6815 3.0846 MalesH2LM3_ORYLAENSORLP000000399vdac3 -1.6778 2.704 Males <td>H2RYB3_TAKRU</td> <td>ENSTRUP0000005134</td> <td>rpl10</td> <td>1.5389</td> <td>2.032</td> <td>Females</td>	H2RYB3_TAKRU	ENSTRUP0000005134	rpl10	1.5389	2.032	Females
H2LFU8_ORYLA ENSORLP0000004839 diol -5.3208 2.3967 Males H2M349_ORYLA ENSORLP0000012850 hpx -2.6534 3.8174 Males H2MW17_ORYLA ENSORLP0000023234 gstt1a (1 to many) -2.634 3.89 Males H2MR20_ORYLA ENSORLP0000021194 gchfr -2.5665 2.3351 Males H2LAK3_ORYLA ENSORLP0000002914 zgc:112265 -2.496 3.2071 Males H2LW82_ORYLA ENSORLP0000001369 abcc2 -2.4901 2.1675 Males H2LW82_ORYLA ENSORLP00000101882 uqcrc2a -2.3333 2.3845 Males H2LW82_ORYLA ENSORLP00000112049 rab18b -2.2916 2.3762 Males H2MN1_ORYLA ENSORLP00000015937 dera -2.2696 2.5231 Males H2LCR1_ORYLA ENSORLP00000023708 -2.0144 2.4791 Males H2LMN5_ORYLA ENSORLP0000002679 bckdha -1.9923 2.2225 Males H2LMS_ORYLA ENSORLP0000002679 bckdha -1.9537 2.6139 Males H2LMS_ORYLA <td>H2MIT5 ORYLA</td> <td>ENSORLP00000018568</td> <td>cvp27a7</td> <td>-5.3282</td> <td>2.2062</td> <td>Males</td>	H2MIT5 ORYLA	ENSORLP00000018568	cvp27a7	-5.3282	2.2062	Males
H2M349_ORYLA ENSORLP0000012850 hpx -2.6534 3.8174 Males H2MWI7_ORYLA ENSORLP0000023234 gstt1a (1 to many) -2.634 3.89 Males H2MR20_ORYLA ENSORLP0000021194 gchfr -2.5665 2.3351 Males H2LAK3_ORYLA ENSORLP0000002914 zgc:112265 -2.496 3.2071 Males H2LW82_ORYLA ENSORLP00000010369 abcc2 -2.4901 2.1675 Males H2LW82_ORYLA ENSORLP00000012049 rab18b -2.2916 2.3762 Males H2MN5_ORYLA ENSORLP00000015937 dera -2.2695 2.8981 Males H2MN5_ORYLA ENSORLP00000023708 -2.0144 2.4791 Males H2MNT_ORYLA ENSORLP00000023708 -2.0144 2.4791 Males H2MNT_ORYLA ENSORLP0000002679 bckdha -1.9923 2.2225 Males H2MNT_ORYLA ENSORLP0000002679 bckdha -1.9537 2.6139 Males H2LS08_ORYLA ENSORLP0000001369 ydac3 -1.6815 3.0846 Males H2LS1_ORYLA ENSORLP00000	H2LFU8 ORYLA	ENSORLP0000004839	dio1	-5.3208	2.3967	Males
H2MWI7_ORYLAENSORLP0000023234 ENSORLP00000021194gth1 (1 to many) gchfr-2.6343.89MalesH2MR20_ORYLAENSORLP00000021194 gchfrgchfr-2.56652.3351MalesH2LAK3_ORYLAENSORLP0000002914 ENSORLP00000010369abcc2-2.49012.1675MalesH2LW82_ORYLAENSORLP00000010882 Uqerc2auqerc2a-2.33332.3845MalesH2LXM1_ORYLAENSORLP0000001249 ENSORLP0000015937rab18b-2.29162.3762MalesH2MRJ_ORYLAENSORLP00000015937 ENSORLP00000012378dera-2.26962.5231MalesH2MWT_ORYLAENSORLP00000023708-2.01442.4791MalesH2MWT_ORYLAENSORLP00000023708-2.01442.4791MalesH2MW7_ORYLAENSORLP0000002679bckdha-1.95372.6139MalesH2LS08_ORYLAENSORLP00000012132 gsta.2gsta.2-1.70933.134MalesH2LB31_ORYLAENSORLP00000012132 ENSORLP0000003799vdac3-1.68153.0846MalesH2LQ36_ORYLAENSORLP0000008866 ENSORLP0000008172 amacr-1.67782.7204MalesH2LU31_ORYLAENSORLP0000008172 ENSORLP0000008844 Alacsamacr-1.67782.5015MalesH2LW31_ORYLAENSORLP0000008172 ENSORLP0000008844 Alacs-1.59592.1054MalesH2LA13_ORYLAENSORLP0000008844 ENSORLP0000008844 Alacs-1.56012.5383MalesH2LA13_ORYLAENSORLP0000008844 ENSORLP0000008844 Alacs <t< td=""><td>H2M349 ORYLA</td><td>ENSORLP00000012850</td><td>hpx</td><td>-2.6534</td><td>3.8174</td><td>Males</td></t<>	H2M349 ORYLA	ENSORLP00000012850	hpx	-2.6534	3.8174	Males
H2MR20_ORYLA ENSORLP00000021194 gchfr -2.5665 2.3351 Males H2LAK3_ORYLA ENSORLP0000002914 zgc:112265 -2.496 3.2071 Males H2LW82_ORYLA ENSORLP0000010369 abcc2 -2.4901 2.1675 Males H2LW81_ORYLA ENSORLP0000010882 uqcrc2a -2.3333 2.3845 Males H2LXM1_ORYLA ENSORLP0000012049 rab18b -2.2916 2.3762 Males H2M0X2_ORYLA ENSORLP0000003701 c9 -2.2695 2.8981 Males H2LCR1_ORYLA ENSORLP00000023708 -2.0144 2.4791 Males H2MW7_ORYLA ENSORLP0000002679 bckdha -1.9923 2.2225 Males H2LNR3_ORYLA ENSORLP0000002679 bckdha -1.9537 2.6139 Males H2LNR3_ORYLA ENSORLP0000002679 bckdha -1.9289 2.2628 Males H2LS08_ORYLA ENSORLP00000012132 gsta.2 -1.7093 3.134 Males H2LS08_ORYLA ENSORLP00000012132 gsta.2 -1.6778 2.704 Males H2L43_ORYLA	H2MWI7_ORYLA	ENSORLP00000023234	gstt1a (1 to many)	-2.634	3.89	Males
H2LAK3_ORYLA ENSORLP0000002914 zgc:112265 -2.496 3.2071 Males H2LW82_ORYLA ENSORLP0000010369 abcc2 -2.4901 2.1675 Males H2LW82_ORYLA ENSORLP0000010882 uqcrc2a -2.3333 2.3845 Males H2LXM1_ORYLA ENSORLP0000012049 rab18b -2.2916 2.3762 Males H2M0X2_ORYLA ENSORLP00000015937 dera -2.2696 2.5231 Males H2LCR1_ORYLA ENSORLP00000023708 -2.0144 2.4791 Males H2MNY5_ORYLA ENSORLP00000023708 -2.0144 2.4791 Males H2LMV7_ORYLA ENSORLP0000002679 bckdha -1.9923 2.2225 Males H2LNR3_ORYLA ENSORLP0000002679 bckdha -1.9537 2.6139 Males H2LS08_ORYLA ENSORLP0000002679 bckdha -1.9537 2.6139 Males H2LS08_ORYLA ENSORLP00000012132 gsta.2 -1.7093 3.134 Males H2L31_ORYLA ENSORLP0000003099 vdac3 -1.6815 3.0846 Males H2L43_ORYLA ENSORLP0000000	H2MR20 ORYLA	ENSORLP00000021194	gchfr	-2.5665	2.3351	Males
H2LW82_ORYLA ENSORLP00000010369 abcc2 -2.4901 2.1675 Males H2LXM1_ORYLA ENSORLP00000010882 uqcrc2a -2.3333 2.3845 Males H2M0X2_ORYLA ENSORLP0000012049 rab18b -2.2916 2.3762 Males H2M0X2_ORYLA ENSORLP00000015937 dera -2.2696 2.5231 Males H2LCR1_ORYLA ENSORLP0000003701 c9 -2.2695 2.8981 Males H2MXN5_ORYLA ENSORLP00000023708 -2.0144 2.4791 Males H2MMU7_ORYLA ENSORLP0000002679 bckdha -1.9923 2.2225 Males H2LS08_ORYLA ENSORLP0000002679 bckdha -1.9537 2.6139 Males H2LS08_ORYLA ENSORLP0000001665 HTRA2 (1 to many) -1.9289 2.2628 Males H2LS08_ORYLA ENSORLP00000012132 gsta.2 -1.7093 3.134 Males H2LB31_ORYLA ENSORLP0000008866 dao.2 -1.6815 3.0846 Males H2LB31_ORYLA ENSORLP0000008172 amacr -1.6778 2.7204 Males H2L413_ORYLA </td <td>H2LAK3 ORYLA</td> <td>ENSORLP0000002914</td> <td>zgc:112265</td> <td>-2.496</td> <td>3.2071</td> <td>Males</td>	H2LAK3 ORYLA	ENSORLP0000002914	zgc:112265	-2.496	3.2071	Males
H2LXMI_ORYLA ENSORLP00000010882 uqcrc2a -2.3333 2.3845 Males H2M0X2_ORYLA ENSORLP00000012049 rab18b -2.2916 2.3762 Males H2MBL3_ORYLA ENSORLP00000015937 dera -2.2696 2.5231 Males H2LCR1_ORYLA ENSORLP0000003701 c9 -2.6055 2.8981 Males H2MXN5_ORYLA ENSORLP00000023708 -2.0144 2.4791 Males H2MMU7_ORYLA ENSORLP0000002679 bckdha -1.9923 2.2225 Males H2LS08_ORYLA ENSORLP0000002679 bckdha -1.9537 2.6139 Males H2LS08_ORYLA ENSORLP00000019645 HTRA2 (1 to many) -1.9289 2.2628 Males H2LS08_ORYLA ENSORLP00000012132 gsta.2 -1.7093 3.134 Males H2LB31_ORYLA ENSORLP00000003099 vdac3 -1.6815 3.0846 Males H2LS06_ORYLA ENSORLP00000008172 amacr -1.6778 2.7204 Males H2LQ36_ORYLA ENSORLP0000000676 zgc:56235 -1.5967 2.5015 Males H2L413_	H2LW82 ORYLA	ENSORLP00000010369	abcc2	-2.4901	2.1675	Males
H2M0X2_ORYLA ENSORLP00000012049 rab18b -2.2916 2.3762 Males H2MBL3_ORYLA ENSORLP00000015937 dera -2.2696 2.5231 Males H2LCR1_ORYLA ENSORLP0000003701 c9 -2.2695 2.8981 Males H2MXN5_ORYLA ENSORLP00000023708 -2.0144 2.4791 Males H2MMU7_ORYLA ENSORLP0000002679 bckdha -1.9923 2.2225 Males H2LQS9_ORYLA ENSORLP0000002679 bckdha -1.9537 2.6139 Males H2LS08_ORYLA ENSORLP00000012132 gsta.2 -1.8171 2.0155 Males H2LB31_ORYLA ENSORLP0000003099 vdac3 -1.6815 3.0846 Males H2LB31_ORYLA ENSORLP0000003099 vdac3 -1.6815 3.0846 Males H2LQ36_ORYLA ENSORLP0000008172 amacr -1.6778 2.7204 Males H2LQ36_ORYLA ENSORLP00000008172 amacr -1.6778 2.5015 Males H2LQ36_ORYLA ENSORLP00000008172 amacr -1.66778 2.5015 Males H2LQ36_ORYLA <	H2LXM1 ORYLA	ENSORLP00000010882	ugere2a	-2.3333	2.3845	Males
H2MBL3_ORYLA ENSORLP00000015937 dera -2.2696 2.5231 Males H2LCR1_ORYLA ENSORLP0000003701 c9 -2.2695 2.8981 Males H2MXN5_ORYLA ENSORLP00000023708 -2.0144 2.4791 Males H2MMU7_ORYLA ENSORLP0000002679 bckdha -1.9923 2.2225 Males H2MS_ORYLA ENSORLP0000002679 bckdha -1.9537 2.6139 Males H2MLR3_ORYLA ENSORLP00000019645 HTRA2 (1 to many) -1.9289 2.2628 Males H2LS08_ORYLA ENSORLP00000012132 gsta.2 -1.7093 3.134 Males H2LB31_ORYLA ENSORLP0000008172 amacr -1.6778 2.7204 Males H2LQ36_ORYLA ENSORLP00000008172 amacr -1.6778 2.7204 Males H2L413_ORYLA ENSORLP0000000676 zgc:56235 -1.5967 2.5015 Males H2LQ36_ORYLA ENSORLP00000003894 amt -1.5601 2.5383 Males H2LQ36_ORYLA ENSORLP00000003894 amt -1.5601 2.5383 Males H2LA92_ORYLA	H2M0X2 ORYLA	ENSORLP00000012049	rab18b	-2.2916	2.3762	Males
H2LCR1_ORYLA ENSORLP0000003701 c9 -2.2695 2.8981 Males H2MXN5_ORYLA ENSORLP0000023708 -2.0144 2.4791 Males H2MMU7_ORYLA ENSORLP0000020043 agmat -1.9923 2.2225 Males H2L9X9_ORYLA ENSORLP0000002679 bckdha -1.9537 2.6139 Males H2MLR3_ORYLA ENSORLP00000019645 HTRA2 (1 to many) -1.9289 2.2628 Males H2LS08_ORYLA ENSORLP00000012132 gsta.2 -1.7093 3.134 Males H2LB31_ORYLA ENSORLP0000003099 vdac3 -1.6815 3.0846 Males H2LQ36_ORYLA ENSORLP00000008172 amacr -1.6778 2.7204 Males H2L413_ORYLA ENSORLP0000000676 zgc:56235 -1.5967 2.5015 Males H2L43_ORYLA ENSORLP0000000676 zgc:56235 -1.5959 2.1054 Males H2L43_ORYLA ENSORLP00000003894 amt -1.5601 2.5383 Males H2L43_ORYLA ENSORLP00000003894 amt -1.5601 2.5383 Males GNAI2_ORYLA	H2MBL3 ORYLA	ENSORLP00000015937	dera	-2.2696	2.5231	Males
H2MXN5_ORYLA ENSORLP0000023708 -2.0144 2.4791 Males H2MMU7_ORYLA ENSORLP0000020043 agmat -1.9923 2.2225 Males H2L9X9_ORYLA ENSORLP0000002679 bckdha -1.9537 2.6139 Males H2MLR3_ORYLA ENSORLP00000019645 HTRA2 (1 to many) -1.9289 2.2628 Males H2LS08_ORYLA ENSORLP00000012132 gsta.2 -1.8171 2.0155 Males H2LB31_ORYLA ENSORLP00000003099 vdac3 -1.6815 3.0846 Males H2LQ36_ORYLA ENSORLP00000008172 amacr -1.6778 2.7204 Males H2L413_ORYLA ENSORLP0000000676 zgc:56235 -1.5967 2.5015 Males H2LQ36_ORYLA ENSORLP00000008172 amacr -1.6778 2.7204 Males H2L413_ORYLA ENSORLP00000008166 zgc:56235 -1.5967 2.5015 Males H2L413_ORYLA ENSORLP00000003894 amt -1.5601 2.5383 Males H2L405_ORYLA ENSORLP00000003894 amt -1.5601 2.5383 Males H2LAP2	H2LCR1 ORYLA	ENSORLP0000003701	c9	-2.2695	2.8981	Males
H2MMU7_ORYLAENSORLP0000020043 ENSORLP0000002679agmat-1.99232.2225MalesH2L9X9_ORYLAENSORLP0000002679bckdha-1.95372.6139MalesH2MLR3_ORYLAENSORLP0000019645HTRA2 (1 to many)-1.92892.2628MalesH2LS08_ORYLAENSORLP00000012132gsta.2-1.81712.0155MalesH2M151_ORYLAENSORLP00000012132gsta.2-1.70933.134MalesH2LB31_ORYLAENSORLP0000003099vdac3-1.68153.0846MalesH2LQ36_ORYLAENSORLP0000008172amacr-1.67782.7204MalesH2L413_ORYLAENSORLP0000000676zgc:56235-1.59672.5015MalesGNA12_ORYLAENSORLP00000003894amt-1.56012.5383MalesH2LD95_ORYLAENSORLP0000002954AKR1D1 (1 to many)-1.5462.1104Males	H2MXN5 ORYLA	ENSORLP00000023708		-2.0144	2.4791	Males
H2L9X9_ORYLAENSORLP0000002679bckdha-1.95372.6139MalesH2MLR3_ORYLAENSORLP00000019645HTRA2 (1 to many)-1.92892.2628MalesH2LS08_ORYLAENSORLP0000008866dao.2-1.81712.0155MalesH2M151_ORYLAENSORLP00000012132gsta.2-1.70933.134MalesH2LB31_ORYLAENSORLP0000003099vdac3-1.68153.0846MalesH2LQ36_ORYLAENSORLP0000008172amacr-1.67782.7204MalesH2L413_ORYLAENSORLP000000676zgc:56235-1.59672.5015MalesGNAI2_ORYLAENSORLP00000003894amt-1.56012.5383MalesH2LAP2_ORYLAENSORLP0000002954AKR1D1 (1 to many)-1.5462.1104Males	H2MMU7_ORYLA	ENSORLP00000020043	agmat	-1.9923	2.2225	Males
H2MLR3_ORYLAENSORLP00000019645HTRA2 (1 to many)-1.92892.2628MalesH2LS08_ORYLAENSORLP0000008866dao.2-1.81712.0155MalesH2M151_ORYLAENSORLP00000012132gsta.2-1.70933.134MalesH2LB31_ORYLAENSORLP0000003099vdac3-1.68153.0846MalesH2LQ36_ORYLAENSORLP0000008172amacr-1.67782.7204MalesH2L4I3_ORYLAENSORLP000000676zgc:56235-1.59672.5015MalesGNAI2_ORYLAENSORLP00000003894amt-1.56012.5383MalesH2LAP2_ORYLAENSORLP00000002954AKR1D1 (1 to many)-1.5462.1104Males	H2L9X9_ORYLA	ENSORLP0000002679	bckdha	-1.9537	2.6139	Males
H2LS08_ORYLA ENSORLP0000008866 dao.2 -1.8171 2.0155 Males H2M151_ORYLA ENSORLP00000012132 gsta.2 -1.7093 3.134 Males H2LB31_ORYLA ENSORLP0000003099 vdac3 -1.6815 3.0846 Males H2LQ36_ORYLA ENSORLP00000008172 amacr -1.6778 2.7204 Males H2L4I3_ORYLA ENSORLP0000000676 zgc:56235 -1.5967 2.5015 Males GNAI2_ORYLA ENSORLP00000000114 ctsd -1.5959 2.1054 Males H2LD95_ORYLA ENSORLP00000003894 amt -1.5601 2.5383 Males H2LAP2_ORYLA ENSORLP00000002954 AKR1D1 (1 to many) -1.546 2.1104 Males	H2MLR3_ORYLA	ENSORLP00000019645	HTRA2 (1 to many)	-1.9289	2.2628	Males
H2M151_ORYLA ENSORLP00000012132 gsta.2 -1.7093 3.134 Males H2LB31_ORYLA ENSORLP0000003099 vdac3 -1.6815 3.0846 Males H2LQ36_ORYLA ENSORLP0000008172 amacr -1.6778 2.7204 Males H2L4I3_ORYLA ENSORLP000000676 zgc:56235 -1.5967 2.5015 Males GNAI2_ORYLA ENSORLP0000000114 ctsd -1.5959 2.1054 Males H2LD95_ORYLA ENSORLP00000003894 amt -1.5601 2.5383 Males H2LAP2_ORYLA ENSORLP00000002954 AKR1D1 (1 to many) -1.546 2.1104 Males	H2LS08_ORYLA	ENSORLP0000008866	dao.2	-1.8171	2.0155	Males
H2LB31_ORYLA ENSORLP0000003099 vdac3 -1.6815 3.0846 Males H2LQ36_ORYLA ENSORLP0000008172 amacr -1.6778 2.7204 Males H2L4I3_ORYLA ENSORLP000000676 zgc:56235 -1.5967 2.5015 Males GNAI2_ORYLA ENSORLP0000000114 ctsd -1.5959 2.1054 Males H2LD95_ORYLA ENSORLP00000003894 amt -1.5601 2.5383 Males H2LAP2_ORYLA ENSORLP00000002954 AKR1D1 (1 to many) -1.546 2.1104 Males	H2M151_ORYLA	ENSORLP00000012132	gsta.2	-1.7093	3.134	Males
H2LQ36_ORYLA ENSORLP0000008172 amacr -1.6778 2.7204 Males H2L4I3_ORYLA ENSORLP0000000676 zgc:56235 -1.5967 2.5015 Males GNAI2_ORYLA ENSORLP0000000114 ctsd -1.5959 2.1054 Males H2LD95_ORYLA ENSORLP00000003894 amt -1.5601 2.5383 Males H2LAP2_ORYLA ENSORLP0000002954 AKR1D1 (1 to many) -1.546 2.1104 Males	H2LB31_ORYLA	ENSORLP0000003099	vdac3	-1.6815	3.0846	Males
H2L4I3_ORYLA ENSORLP0000000676 zgc:56235 -1.5967 2.5015 Males GNAI2_ORYLA ENSORLP0000000114 ctsd -1.5959 2.1054 Males H2LD95_ORYLA ENSORLP00000003894 amt -1.5601 2.5383 Males H2LAP2_ORYLA ENSORLP0000002954 AKR1D1 (1 to many) -1.546 2.1104 Males	H2LQ36_ORYLA	ENSORLP0000008172	amacr	-1.6778	2.7204	Males
GNAI2_ORYLA ENSORLP0000000114 ctsd -1.5959 2.1054 Males H2LD95_ORYLA ENSORLP00000003894 amt -1.5601 2.5383 Males H2LAP2_ORYLA ENSORLP00000002954 AKR1D1 (1 to many) -1.546 2.1104 Males	H2L4I3_ORYLA	ENSORLP0000000676	zgc:56235	-1.5967	2.5015	Males
H2LD95_ORYLA ENSORLP0000003894 amt -1.5601 2.5383 Males H2LAP2_ORYLA ENSORLP0000002954 AKR1D1 (1 to many) -1.546 2.1104 Males	GNAI2_ORYLA	ENSORLP0000000114	ctsd	-1.5959	2.1054	Males
H2LAP2_ORYLA ENSORLP0000002954 AKR1D1 (1 to many) -1.546 2.1104 Males	H2LD95_ORYLA	ENSORLP0000003894	amt	-1.5601	2.5383	Males
	H2LAP2_ORYLA	ENSORLP0000002954	AKR1D1 (1 to many)	-1.546	2.1104	Males
H2LPA9_ORYLA ENSORLP00000007889 pck2 -1.513 3.2149 Males	H2LPA9_ORYLA	ENSORLP0000007889	pck2	-1.513	3.2149	Males
H2M6J4_ORYLA ENSORLP00000014089 nme4 -1.5052 2.8779 Males	H2M6J4_ORYLA	ENSORLP00000014089	nme4	-1.5052	2.8779	Males

Pathway (female)	Total	Expected	Hits	P Value
Aminoacyl-tRNA biosynthesis	41	0.50252	7	1.8214E-07
N-Glycan biosynthesis	43	0.52704	2	0.095159
Pathway (male)	Total	Expected	Hits	P.Value
Primary bile acid biosynthesis	17	0.13482	3	0.0002379
D-Arginine and D-ornithine metabolism	1	0.0079308	1	0.0079308
Glycine, serine and threonine metabolism	37	0.29344	2	0.032738
Arginine and proline metabolism	57	0.45205	2	0.071938

Annex 2. 4 Complete list of transcripts that are significantly sex-over-represented in female and male livers, and their respective metabolic pathway enrichment.

Encoded protein ID	Gene name	log ₂ FC (F Vs M)	P Value	FDR	Up- regulated in
ENSORLP0000007656	ol-vit1	19.04901259	1.9998E-129	6.8189E-126	Females
ENSORLP0000008173	vtg3	17.15791086	3.0635E-257	6.2675E-253	Females
ENSORLP00000007793	vit-6	16.68117866	1.0491E-232	1.0732E-228	Females
ENSORLP0000007667	Uncharacterized	14.74461592	1.31E-143	6.7002E-140	Females
ENSORLP0000006088	wfdc2 (2 of 2)	14.25368922	2.2689E-128	6.6312E-125	Females
ENSORLP0000006074	wfdc2 (1 of 2)	13.37623589	4.8437E-110	1.1011E-106	Females
ENSORLP00000016715	Uncharacterized	12.02096482	1.06609E-86	1.45407E-83	Females
ENSORLP0000002332	FAM46A (1 of 2)	11.7460469	5.37154E-34	3.66321E-31	Females
ENSORLP00000023360	Uncharacterized	9.961630534	2.47455E-20	7.55625E-18	Females
ENSORLP00000010151	CYP2W1 (1 of 3)	9.780346847	4.259E-145	2.9045E-141	Females
ENSORLP0000000173	Uncharacterized	9.73735334	3.234E-37	2.28153E-34	Females
ENSORLP00000016711	Uncharacterized	9.508210699	7.3385E-120	1.8767E-116	Females
ENSORLP0000007553	hebp3	9.459499633	1.1845E-106	2.4233E-103	Females
ENSORLP00000012870	BEND7	9.347158175	1.12336E-32	6.56651E-30	Females
ENSORLP00000020046	UNC79	9.293794405	4.16693E-31	2.24345E-28	Females
ENSORLP00000013184	tspan13b	9.246012785	2.62068E-32	1.48935E-29	Females
ENSORLP00000023336	ANO9 (2 of 2)	9.16300104	1.31236E-07	6.83195E-06	Females
ENSORLP00000023667	gabra6b	8.943498947	1.94382E-11	2.23419E-09	Females
ENSORLP0000008208	rxfp3	8.821282095	2.39684E-12	3.08408E-10	Females
ENSORLP0000009654	TMEM235	8.527287882	9.92174E-19	2.81929E-16	Females
ENSORLP00000017075	slc6a2	8.488384962	2.1705E-09	1.66941E-07	Females
ENSORLP0000006121	rtn4rl1b	8.418573558	1.86124E-14	3.30398E-12	Females
ENSORLP00000013216	baiap212b	8.134276403	4.52512E-14	7.65119E-12	Females
ENSORLP00000013188	fam20cl	8.110112947	1.6409E-142	6.7142E-139	Females
ENSORLP0000007978	adora1	8.101543368	1.10832E-12	1.52182E-10	Females
ENSORLP00000013645	Uncharacterized	8.082511552	5.83853E-97	9.18849E-94	Females
ENSORLP0000005013	Uncharacterized	7.795099383	1.82705E-08	1.13962E-06	Females

ENSORLP0000006963	Uncharacterized	7.640521091	0.000645474	0.008295075	Females
ENSORLP0000005276	Uncharacterized	7.442170515	2.14117E-05	0.000535528	Females
ENSORLP00000017229	si:dkey-113d16.9	7.411756504	2.69609E-05	0.000645138	Females
ENSORLP00000014713	SLC41A3 (2 of 2)	7.403928621	2.78202E-76	3.16207E-73	Females
ENSORLP00000016901	fabp7a	7.381590606	4.61854E-79	5.55828E-76	Females
ENSORLP00000013368	ela31	7.107732051	1.21651E-06	4.7955E-05	Females
ENSORLP0000005046	myof	7.058316333	1.78235E-53	1.82325E-50	Females
ENSORLP00000012820	cpt1b	7.051353268	1.10532E-30	5.65344E-28	Females
ENSORLP0000005945	gpnmb	7.048749361	2.79031E-37	2.03882E-34	Females
ENSORLP00000010295	col6a3	7.04787136	0.000199544	0.003261406	Females
ENSORLP00000013721	si:ch211-120k19.1	6.978857464	1.80464E-08	1.12909E-06	Females
ENSORLP0000005679	slc35a3b	6.852768017	3.71784E-31	2.05577E-28	Females
ENSORLP0000005193	fabp2	6.777317558	4.50369E-51	4.38767E-48	Females
ENSORLP00000025183	PRIMA1	6.714849749	1.17375E-08	7.89924E-07	Females
ENSORLP0000000509	slc12a3	6.583342339	2.62861E-07	1.27438E-05	Females
ENSORLP00000012711	1-sf	6.55199034	1.5208E-99	2.59286E-96	Females
ENSORLP00000010079	chrne	6.530395525	8.11247E-55	8.73542E-52	Females
ENSORLP0000006831	cdh16	6.50388335	0.000112518	0.002076112	Females
ENSORLP0000006836	dachc	6.487802727	3.13921E-17	7.92902E-15	Females
ENSORLP0000007975	kcnn4	6.423794632	4.72736E-25	1.97382E-22	Females
ENSORLP00000012647	Uncharacterized	6.351254274	6.7013E-102	1.24639E-98	Females
ENSORLP00000014639	scn12aa	6.267686151	0.000442183	0.006166754	Females
ENSORLP00000020573	CEL (3 of 3)	6.228409605	6.40147E-06	0.000194603	Females
ENSORLP00000018928	ocstamp	6.222041145	4.86815E-05	0.001052827	Females
ENSORLP00000015751	caprin2	6.186353055	2.11916E-05	0.000530673	Females
ENSORLP00000014396	LAMA4	6.165712957	1.41693E-06	5.50074E-05	Females
ENSORLP00000020494	slc48a1b	6.086185147	5.42522E-16	1.16836E-13	Females
ENSORLP00000020755	dmbx1b	6.064472132	4.13182E-06	0.000134821	Females
ENSORLP00000018192	esr1	6.055461964	1.90024E-91	2.77692E-88	Females
ENSORLP00000011848	fam20cb (2 of 2)	6.039141653	2.60334E-33	1.61399E-30	Females
ENSORLP00000021777	BEST3	6.037999423	0.000100827	0.001903204	Females
ENSORLP00000024038	Uncharacterized	6.029242672	0.000378606	0.005451024	Females
ENSORLP0000002027	scamp21	6.026206666	0.000150983	0.002624442	Females
ENSORLP0000007867	fam134b	6.017327667	6.64038E-83	8.49097E-80	Females
ENSORLP00000018950	cxcl12	5.86199777	0.000742892	0.009267582	Females
ENSORLP00000012583	mapk8ip2	5.799238827	1.5004E-05	0.000399214	Females
ENSORLP00000019253	Uncharacterized	5.727034439	1.30049E-05	0.000352408	Females
ENSORLP00000018089	CEL (1 of 3)	5.651567591	6.33318E-05	0.00131664	Females
ENSORLP0000007279	vav3a	5.634446022	9.78514E-25	4.00388E-22	Females
ENSORLP00000025558	Uncharacterized	5.623509765	0.000100829	0.001903204	Females
ENSORLP00000007104	ca6	5.617307316	0.000678262	0.008645477	Females
ENSORLP0000007858	Uncharacterized	5.612923154	0.000220799	0.003523654	Females
ENSORLP00000018833	il12rb2	5.588912846	1.10444E-09	9.26058E-08	Females

ENSORLP00000010749	pdia2	5.580591347	2.08125E-05	0.0005231	Females
ENSORLP00000024615	CPA1 (2 of 2)	5.559690876	0.000635396	0.008191277	Females
ENSORLP00000011217	mapk11	5.558025502	0.000255026	0.003940762	Females
ENSORLP0000000293	c6ast3	5.557156145	0.000163098	0.002791255	Females
ENSORLP00000018128	CEL (2 of 3)	5.500643325	0.000211859	0.003431844	Females
ENSORLP00000016600	Uncharacterized	5.489644209	0.000489614	0.006699097	Females
ENSORLP00000015574	ccr9b	5.46989674	3.42823E-17	8.55344E-15	Females
ENSORLP0000000360	si:ch211-255i20.3	5.449351442	0.000753426	0.009359041	Females
ENSORLP00000011348	cxcl12a	5.430611056	2.17329E-05	0.000540258	Females
ENSORLP00000023742	Uncharacterized	5.426520074	0.000229779	0.003647046	Females
ENSORLP0000009717	CNNM1 (2 of 2)	5.40995524	0.000272574	0.00415166	Females
ENSORLP00000014348	cldn11a	5.406473195	0.000238308	0.003741782	Females
ENSORLP00000001177	cpa2	5.40246916	1.01575E-05	0.00028585	Females
ENSORLP00000021751	rgs9b	5.382792653	5.84024E-44	4.77942E-41	Females
ENSORLP00000012585	cpb1	5.350927191	0.000117448	0.002143506	Females
ENSORLP00000011160	gem	5.266951299	0.000778985	0.009623942	Females
ENSORLP00000016216	fbln2	5.249843385	1.77475E-12	2.40461E-10	Females
ENSORLP0000005766	ela21	5.221388901	0.000101103	0.001905077	Females
ENSORLP00000010338	Uncharacterized	5.213302655	0.000112639	0.002076112	Females
ENSORLP00000021509	phgdh	5.206676674	6.17087E-05	0.00128695	Females
ENSORLP00000010182	SLCO5A1 (3 of 3)	5.184139067	0.000997845	0.011659002	Females
ENSORLP00000024264	ASTL (10 of 10)	5.162883265	0.000634925	0.008190368	Females
ENSORLP00000016815	ca8	5.126917919	0.000351797	0.00512271	Females
ENSORLP0000006061	slc7a1	5.125136664	1.8531E-09	1.45817E-07	Females
ENSORLP00000015007	FBXO2	5.121141471	0.000297572	0.004463361	Females
ENSORLP00000024837	zgc:92137	5.101018624	8.73837E-05	0.001710798	Females
ENSORLP0000002399	itga2.2	5.071565537	0.000936056	0.011130212	Females
ENSORLP00000010919	Uncharacterized	5.041166872	6.47245E-13	9.26013E-11	Females
ENSORLP00000022987	pla2g1b	5.021747327	0.000105673	0.001968996	Females
ENSORLP00000024892	endou	4.971424424	3.48942E-06	0.000117225	Females
ENSORLP0000009833	excl19	4.968538385	5.14841E-07	2.27007E-05	Females
ENSORLP00000013922	bach2a	4.962600292	0.000203341	0.003312228	Females
ENSORLP0000005703	ela2	4.948154353	0.000314433	0.004678528	Females
ENSORLP0000004007	SLC7A1 (1 of 2)	4.85755463	7.27705E-34	4.80262E-31	Females
ENSORLP0000008068	ctrl	4.841873869	0.000260099	0.004004034	Females
ENSORLP0000009657	esyt1a	4.712288297	6.20246E-12	7.64435E-10	Females
ENSORLP00000010623	mctp2a	4.69683809	0.000309175	0.004610354	Females
ENSORLP00000021501	Uncharacterized	4.651206352	0.000180627	0.003029064	Females
ENSORLP0000008474	adgrl1a	4.615448153	0.000190116	0.003139289	Females
ENSORLP0000005443	KIF5B (2 of 2)	4.608621376	9.64221E-08	5.17601E-06	Females
ENSORLP0000009786	rbms2b	4.572592734	2.34309E-20	7.26322E-18	Females
ENSORLP0000002178	rims1a	4.571383984	0.000515758	0.006955769	Females
ENSORLP0000003068	si:ch73-22a13.3	4.48918266	8.91871E-20	2.64446E-17	Females

ENSORLP0000004687	adgrb1a	4.459543511	9.9808E-05	0.001894223	Females
ENSORLP00000025406	abca1b	4.459344897	2.89573E-08	1.71721E-06	Females
ENSORLP00000007899	Uncharacterized	4.379462376	0.000892657	0.010723945	Females
ENSORLP0000001793	ckmt1	4.372750717	6.65298E-31	3.49008E-28	Females
ENSORLP00000025295	Uncharacterized	4.322028723	0.000181995	0.003044506	Females
ENSORLP0000008141	cd151	4.313042074	0.000258576	0.003989433	Females
ENSORLP00000004112	CNTN1 (1 of 2)	4.302645483	6.46102E-05	0.001332519	Females
ENSORLP00000022633	egln3	4.264073863	1.9971E-08	1.23068E-06	Females
ENSORLP00000018909	si:dkey-109a10.2	4.23828629	8.29022E-05	0.001643504	Females
ENSORLP00000020884	dnase1	4.232977237	0.000560053	0.007444971	Females
ENSORLP00000020887	selp	4.213405968	2.18764E-06	7.84687E-05	Females
ENSORLP00000023324	matn4	4.206282769	0.00017983	0.003020635	Females
ENSORLP00000012409	gorasp1	4.202327016	1.23762E-08	8.19644E-07	Females
ENSORLP00000019466	CKAP4	4.180952494	1.47675E-08	9.56101E-07	Females
ENSORLP00000015282	mfsd4a	4.156995704	1.09762E-32	6.56651E-30	Females
ENSORLP00000016290	evala	4.15161816	3.784E-05	0.00085638	Females
ENSORLP0000008618	wscd2	4.120013847	4.1911E-10	3.71193E-08	Females
ENSORLP00000005129	ROBO2 (1 of 3)	4.106327666	2.10323E-08	1.28832E-06	Females
ENSORLP00000018347	ZBTB16 (3 of 3)	4.101325378	2.54784E-08	1.52788E-06	Females
ENSORLP00000023352	pim2	4.061711218	6.30208E-14	1.03147E-11	Females
ENSORLP00000020875	GLUL (2 of 2)	4.059934864	4.20589E-30	2.04877E-27	Females
ENSORLP0000006110	csflra	4.052923551	1.36682E-11	1.6164E-09	Females
ENSORLP00000011736	arhgef10lb	4.042577081	0.000160736	0.002765765	Females
ENSORLP0000009610	ccr9a	4.031761381	4.8913E-13	7.19937E-11	Females
ENSORLP00000013378	HELZ2	4.02238488	2.29643E-07	1.12668E-05	Females
ENSORLP00000019967	slc12a8	3.999332218	0.000230964	0.003660185	Females
ENSORLP00000017065	tryp	3.998900386	0.000162164	0.002781953	Females
ENSORLP0000001374	ACSL6	3.994565096	2.55406E-08	1.52788E-06	Females
ENSORLP0000009140	Uncharacterized	3.990746909	0.000183112	0.003055706	Females
ENSORLP0000005540	klb	3.987581903	0.000266014	0.004073642	Females
ENSORLP00000010020	rdh10a	3.95595527	8.66467E-05	0.001704524	Females
ENSORLP0000006894	myo5c	3.949300512	2.63435E-24	1.05679E-21	Females
ENSORLP0000008826	dennd4a	3.924420179	4.24705E-08	2.46148E-06	Females
ENSORLP00000015990	Uncharacterized	3.921472095	3.0887E-06	0.000105319	Females
ENSORLP00000010087	Uncharacterized	3.895858438	5.14804E-26	2.19424E-23	Females
ENSORLP00000005140	Uncharacterized	3.894986105	1.20165E-08	8.00803E-07	Females
ENSORLP00000013835	sytl1	3.892605929	1.89183E-09	1.48295E-07	Females
ENSORLP00000015813	mapk15	3.887393652	0.000941086	0.011148621	Females
ENSORLP00000010028	sox8	3.870947955	3.13953E-05	0.000736602	Females
ENSORLP00000020475	pglyrp2 (2 of 2)	3.86038307	1.00855E-07	5.38744E-06	Females
ENSORLP00000018345	ZBTB16 (2 of 3)	3.768668377	6.01555E-06	0.000185131	Females
ENSORLP00000025702	try	3.76831731	0.000269848	0.004116944	Females
ENSORLP0000006878	klhl5	3.758590208	4.29831E-06	0.000140031	Females
	ENSORLP0000004687ENSORLP0000007899ENSORLP0000007891ENSORLP00000025295ENSORLP00000025295ENSORLP00000026331ENSORLP0000020884ENSORLP0000020384ENSORLP0000020384ENSORLP0000023324ENSORLP0000012409ENSORLP0000015282ENSORLP0000015282ENSORLP0000016290ENSORLP0000016290ENSORLP0000016290ENSORLP0000016290ENSORLP00000016290ENSORLP00000013372ENSORLP00000013372ENSORLP00000013374ENSORLP00000013378ENSORLP00000013378ENSORLP00000013378ENSORLP00000013378ENSORLP00000013378ENSORLP00000013378ENSORLP00000013378ENSORLP00000013378ENSORLP00000013378ENSORLP00000013378ENSORLP00000013378ENSORLP00000013378ENSORLP00000013378ENSORLP00000013378ENSORLP00000013836ENSORLP00000013835ENSORLP0000013835ENSORLP0000013835ENSORLP0000013835ENSORLP0000013835ENSORLP0000013835ENSORLP0000013835ENSORLP0000013835ENSORLP00000013835ENSORLP00000013835ENSORLP00000013835ENSORLP00000013835ENSORLP00000013835ENSORLP00000013835ENSORLP00000018345ENSORLP00000018345ENSORLP00000018345ENSORLP00000018345ENSORLP00000018345ENSORLP00000018345ENSORLP000000035402 <t< th=""><th>ENSORLP0000004687adgrb1aENSORLP0000025406abca1bENSORLP0000007399UncharacterizedENSORLP00000025295UncharacterizedENSORLP00000025295UncharacterizedENSORLP0000001120CNTN1 (1 of 2)ENSORLP00000026333egln3ENSORLP0000002884dnase1ENSORLP0000002884dnase1ENSORLP0000002884gorasp1ENSORLP0000012409gorasp1ENSORLP0000012409gorasp1ENSORLP0000012409eva1aENSORLP0000012409eva1aENSORLP0000012528mfsd4aENSORLP0000012528mfsd4aENSORLP0000012529ROBO2 (1 of 3)ENSORLP000001232jmi2ENSORLP0000001334ZBTB16 (3 of 3)ENSORLP000001335jmi2ENSORLP0000011736aftgef10lbENSORLP0000011736aftgef10lbENSORLP0000011736aftgef10lbENSORLP0000013378HELZ2ENSORLP0000013378HELZ2ENSORLP0000013378HELZ2ENSORLP0000013374ACSL6ENSORLP000001374ACSL6ENSORLP0000001374ACSL6ENSORLP0000001374JUncharacterizedENSORLP0000001374JUncharacterizedENSORLP000001374JUncharacterizedENSORLP000001374JUncharacterizedENSORLP000001374JUncharacterizedENSORLP000001374JUncharacterizedENSORLP000001374JUncharacterizedENSORLP000001374JUncharacterizedENSORLP000001374JUncharacterized</th><th>ENSORLP0000004687adgrb1a4.459543511ENSORLP0000007590Uncharacterized4.379462376ENSORLP0000007595Uncharacterized4.322028723ENSORLP00000025255Uncharacterized4.322028723ENSORLP0000008141ed1514.313042074ENSORLP0000002633egln34.264073863ENSORLP0000002884dnase14.232977237ENSORLP0000002887selp4.213405968ENSORLP0000002887selp4.213405968ENSORLP0000002324matn44.206282769ENSORLP0000012409gorasp14.20232716ENSORLP0000012409gorasp14.20232716ENSORLP0000012409gorasp14.20327016ENSORLP0000012409gorasp14.15695704ENSORLP000001252mfsd4a4.15695704ENSORLP0000001290eva1a4.1616316ENSORLP0000001290eva1a4.10132378ENSORLP0000001292ROBO2 (1 of 3)4.1032378ENSORLP0000001334ZBTB16 (3 of 3)4.10325378ENSORLP0000001335gin24.051711218ENSORLP0000001336ers1a4.052923551ENSORLP0000001337HELZ24.0232848ENSORLP0000013374ACSL63.994565096ENSORLP00000013374ACSL63.994565096ENSORLP0000001344ACSL63.994765096ENSORLP0000001345klb3.987581903ENSORLP0000001345klb3.987581903ENSORLP0000001345klb3.98486105ENSORLP0000001345yrl13.892605929<th>ENSORLP0000004687 adgrb1a 4.459543511 9.9808E-05 ENSORLP00000025406 abca1b 4.459344897 2.89573E-08 ENSORLP00000007393 Uncharacterized 4.37245376 0.000892657 ENSORLP00000001239 ckm1 4.3124273717 6.65298E-31 ENSORLP00000001213 CUncharacterized 4.3202473 0.000258576 ENSORLP00000001214 CNTN1 (1 of 2) 4.302645483 6.46102E-05 ENSORLP0000002884 dase1 4.232977237 0.00056053 ENSORLP0000002884 dnase1 4.2023277016 1.23762E-08 ENSORLP0000002884 dnase1 4.202327016 1.23762E-08 ENSORLP0000002832 matn4 4.206282769 0.00017983 ENSORLP00000012409 gorasp1 4.2031847 4.1911E-10 ENSORLP0000001529 eva1a 4.151618 3.784E-05 ENSORLP00000005129 eva1a 4.160327666 2.10323E-08 ENSORLP00000005129 eva1a 4.151618 3.948E-05 ENSORLP0000001332 pim2 4.061711218 6.30208E-14</th><th>ENSORI.P0000004687 adgrb la 4.459543511 9.9808F-05 0.001894231 ENSORI.P00000025400 abcalb 4.459344897 2.89573E-08 1.71721F-06 ENSORI.P0000001793 ckmtl 4.372462376 0.00089267 0.00734450 ENSORI.P00000025295 Uncharacterized 4.322028723 0.000181995 0.003044506 ENSORI.P00000025395 Uncharacterized 4.322028723 0.000181905 0.003345106 ENSORI.P00000022633 egln3 4.26407863 1.9971E-08 1.23068E-06 ENSORI.P0000002384 dnasel 4.232277127 0.00056053 0.007144971 ENSORI.P0000002334 man4 4.206282769 0.00017983 0.003020635 ENSORI.P00000012409 goraspl 4.213405968 1.3762E-08 8.16641E-07 ENSORI.P0000001240 goraspl 4.20628769 0.00017983 0.0085638 ENSORI.P0000001240 goraspl 4.20628766 1.0076E-32 6.56651E-30 ENSORI.P0000001240 goraspl 4.15161816 3.784E-05 0.00085638 ENSORI.P0000001530</th></th></t<>	ENSORLP0000004687adgrb1aENSORLP0000025406abca1bENSORLP0000007399UncharacterizedENSORLP00000025295UncharacterizedENSORLP00000025295UncharacterizedENSORLP0000001120CNTN1 (1 of 2)ENSORLP00000026333egln3ENSORLP0000002884dnase1ENSORLP0000002884dnase1ENSORLP0000002884gorasp1ENSORLP0000012409gorasp1ENSORLP0000012409gorasp1ENSORLP0000012409eva1aENSORLP0000012409eva1aENSORLP0000012528mfsd4aENSORLP0000012528mfsd4aENSORLP0000012529ROBO2 (1 of 3)ENSORLP000001232jmi2ENSORLP0000001334ZBTB16 (3 of 3)ENSORLP000001335jmi2ENSORLP0000011736aftgef10lbENSORLP0000011736aftgef10lbENSORLP0000011736aftgef10lbENSORLP0000013378HELZ2ENSORLP0000013378HELZ2ENSORLP0000013378HELZ2ENSORLP0000013374ACSL6ENSORLP000001374ACSL6ENSORLP0000001374ACSL6ENSORLP0000001374JUncharacterizedENSORLP0000001374JUncharacterizedENSORLP000001374JUncharacterizedENSORLP000001374JUncharacterizedENSORLP000001374JUncharacterizedENSORLP000001374JUncharacterizedENSORLP000001374JUncharacterizedENSORLP000001374JUncharacterizedENSORLP000001374JUncharacterized	ENSORLP0000004687adgrb1a4.459543511ENSORLP0000007590Uncharacterized4.379462376ENSORLP0000007595Uncharacterized4.322028723ENSORLP00000025255Uncharacterized4.322028723ENSORLP0000008141ed1514.313042074ENSORLP0000002633egln34.264073863ENSORLP0000002884dnase14.232977237ENSORLP0000002887selp4.213405968ENSORLP0000002887selp4.213405968ENSORLP0000002324matn44.206282769ENSORLP0000012409gorasp14.20232716ENSORLP0000012409gorasp14.20232716ENSORLP0000012409gorasp14.20327016ENSORLP0000012409gorasp14.15695704ENSORLP000001252mfsd4a4.15695704ENSORLP0000001290eva1a4.1616316ENSORLP0000001290eva1a4.10132378ENSORLP0000001292ROBO2 (1 of 3)4.1032378ENSORLP0000001334ZBTB16 (3 of 3)4.10325378ENSORLP0000001335gin24.051711218ENSORLP0000001336ers1a4.052923551ENSORLP0000001337HELZ24.0232848ENSORLP0000013374ACSL63.994565096ENSORLP00000013374ACSL63.994565096ENSORLP0000001344ACSL63.994765096ENSORLP0000001345klb3.987581903ENSORLP0000001345klb3.987581903ENSORLP0000001345klb3.98486105ENSORLP0000001345yrl13.892605929 <th>ENSORLP0000004687 adgrb1a 4.459543511 9.9808E-05 ENSORLP00000025406 abca1b 4.459344897 2.89573E-08 ENSORLP00000007393 Uncharacterized 4.37245376 0.000892657 ENSORLP00000001239 ckm1 4.3124273717 6.65298E-31 ENSORLP00000001213 CUncharacterized 4.3202473 0.000258576 ENSORLP00000001214 CNTN1 (1 of 2) 4.302645483 6.46102E-05 ENSORLP0000002884 dase1 4.232977237 0.00056053 ENSORLP0000002884 dnase1 4.2023277016 1.23762E-08 ENSORLP0000002884 dnase1 4.202327016 1.23762E-08 ENSORLP0000002832 matn4 4.206282769 0.00017983 ENSORLP00000012409 gorasp1 4.2031847 4.1911E-10 ENSORLP0000001529 eva1a 4.151618 3.784E-05 ENSORLP00000005129 eva1a 4.160327666 2.10323E-08 ENSORLP00000005129 eva1a 4.151618 3.948E-05 ENSORLP0000001332 pim2 4.061711218 6.30208E-14</th> <th>ENSORI.P0000004687 adgrb la 4.459543511 9.9808F-05 0.001894231 ENSORI.P00000025400 abcalb 4.459344897 2.89573E-08 1.71721F-06 ENSORI.P0000001793 ckmtl 4.372462376 0.00089267 0.00734450 ENSORI.P00000025295 Uncharacterized 4.322028723 0.000181995 0.003044506 ENSORI.P00000025395 Uncharacterized 4.322028723 0.000181905 0.003345106 ENSORI.P00000022633 egln3 4.26407863 1.9971E-08 1.23068E-06 ENSORI.P0000002384 dnasel 4.232277127 0.00056053 0.007144971 ENSORI.P0000002334 man4 4.206282769 0.00017983 0.003020635 ENSORI.P00000012409 goraspl 4.213405968 1.3762E-08 8.16641E-07 ENSORI.P0000001240 goraspl 4.20628769 0.00017983 0.0085638 ENSORI.P0000001240 goraspl 4.20628766 1.0076E-32 6.56651E-30 ENSORI.P0000001240 goraspl 4.15161816 3.784E-05 0.00085638 ENSORI.P0000001530</th>	ENSORLP0000004687 adgrb1a 4.459543511 9.9808E-05 ENSORLP00000025406 abca1b 4.459344897 2.89573E-08 ENSORLP00000007393 Uncharacterized 4.37245376 0.000892657 ENSORLP00000001239 ckm1 4.3124273717 6.65298E-31 ENSORLP00000001213 CUncharacterized 4.3202473 0.000258576 ENSORLP00000001214 CNTN1 (1 of 2) 4.302645483 6.46102E-05 ENSORLP0000002884 dase1 4.232977237 0.00056053 ENSORLP0000002884 dnase1 4.2023277016 1.23762E-08 ENSORLP0000002884 dnase1 4.202327016 1.23762E-08 ENSORLP0000002832 matn4 4.206282769 0.00017983 ENSORLP00000012409 gorasp1 4.2031847 4.1911E-10 ENSORLP0000001529 eva1a 4.151618 3.784E-05 ENSORLP00000005129 eva1a 4.160327666 2.10323E-08 ENSORLP00000005129 eva1a 4.151618 3.948E-05 ENSORLP0000001332 pim2 4.061711218 6.30208E-14	ENSORI.P0000004687 adgrb la 4.459543511 9.9808F-05 0.001894231 ENSORI.P00000025400 abcalb 4.459344897 2.89573E-08 1.71721F-06 ENSORI.P0000001793 ckmtl 4.372462376 0.00089267 0.00734450 ENSORI.P00000025295 Uncharacterized 4.322028723 0.000181995 0.003044506 ENSORI.P00000025395 Uncharacterized 4.322028723 0.000181905 0.003345106 ENSORI.P00000022633 egln3 4.26407863 1.9971E-08 1.23068E-06 ENSORI.P0000002384 dnasel 4.232277127 0.00056053 0.007144971 ENSORI.P0000002334 man4 4.206282769 0.00017983 0.003020635 ENSORI.P00000012409 goraspl 4.213405968 1.3762E-08 8.16641E-07 ENSORI.P0000001240 goraspl 4.20628769 0.00017983 0.0085638 ENSORI.P0000001240 goraspl 4.20628766 1.0076E-32 6.56651E-30 ENSORI.P0000001240 goraspl 4.15161816 3.784E-05 0.00085638 ENSORI.P0000001530

ENSORLP00000018639	gria4b	3.73778783	2.46535E-07	1.20092E-05	Females
ENSORLP00000010769	PDILT	3.723478655	0.000833924	0.010137409	Females
ENSORLP0000006070	mapk4	3.718024245	0.000130507	0.002338034	Females
ENSORLP00000010543	Uncharacterized	3.71747897	1.62064E-12	2.21045E-10	Females
ENSORLP0000000712	wu:fd14a06 (1 of 2)	3.709468233	1.81469E-16	4.26744E-14	Females
ENSORLP00000021579	si:ch211-240119.5	3.699080239	0.000516114	0.006955974	Females
ENSORLP00000012484	COL21A1	3.684190213	9.75845E-05	0.00186239	Females
ENSORLP0000007351	mfsd2ab	3.651557754	1.07835E-06	4.30898E-05	Females
ENSORLP0000001338	LGALS4	3.635425838	1.19105E-08	7.9633E-07	Females
ENSORLP00000009148	ablim1b	3.634854535	3.50853E-06	0.000117674	Females
ENSORLP00000011429	ppala	3.629541519	0.000144736	0.00254084	Females
ENSORLP00000015268	pycr1b	3.574911984	3.81288E-18	1.02642E-15	Females
ENSORLP00000010810	Uncharacterized	3.570749991	5.64587E-06	0.000175813	Females
ENSORLP0000008411	tle3a	3.566112851	3.2661E-10	2.98309E-08	Females
ENSORLP0000004312	arfip2a	3.554748844	8.81209E-10	7.54337E-08	Females
ENSORLP00000001981	arhgap4b	3.551209308	8.54063E-06	0.000247848	Females
ENSORLP00000011704	cdk16 (2 of 2)	3.547133598	4.85257E-05	0.001050974	Females
ENSORLP00000011580	mat2aa	3.546518824	2.23797E-18	6.18739E-16	Females
ENSORLP00000010093	Uncharacterized	3.543258664	6.48557E-16	1.36792E-13	Females
ENSORLP00000013616	ppp2r3b	3.538111785	1.46699E-09	1.18629E-07	Females
ENSORLP00000025163	irbp	3.531227346	1.51782E-06	5.81519E-05	Females
ENSORLP0000008394	cyp24a1	3.523987908	0.000384397	0.005526613	Females
ENSORLP00000004477	col5a1	3.482506478	1.13687E-06	4.52513E-05	Females
ENSORLP0000008956	WNK3	3.460261456	0.00024048	0.003772985	Females
ENSORLP00000009115	hdac9b	3.450120332	1.31114E-28	6.09652E-26	Females
ENSORLP0000005370	ezh1	3.434765903	1.14874E-47	9.79253E-45	Females
ENSORLP0000008434	ADGRE5 (1 of 2)	3.4345609	5.79522E-07	2.52803E-05	Females
ENSORLP0000000516	Uncharacterized	3.431849828	1.75886E-22	6.42581E-20	Females
ENSORLP00000012985	Uncharacterized	3.410500121	1.51782E-05	0.000400424	Females
ENSORLP00000022269	ak7a	3.377239015	1.46624E-09	1.18629E-07	Females
ENSORLP00000013817	efcab1	3.367739777	0.000115518	0.002117727	Females
ENSORLP00000020179	aspg	3.340684867	3.50963E-18	9.5738E-16	Females
ENSORLP0000002468	Uncharacterized	3.305332305	0.000572337	0.007564246	Females
ENSORLP0000009237	TCP11	3.302554407	3.42164E-06	0.0001159	Females
ENSORLP00000015440	nav3	3.265323675	2.04257E-05	0.000515277	Females
ENSORLP0000002502	efemp2a	3.237150319	3.90175E-07	1.79788E-05	Females
ENSORLP00000015970	Uncharacterized	3.185130365	2.789E-05	0.00066349	Females
ENSORLP00000010770	IFI44L	3.175164782	0.000560765	0.007444971	Females
ENSORLP00000014918	specc1	3.162758579	5.20117E-07	2.28349E-05	Females
ENSORLP00000020950	CCDC148	3.160778222	2.70089E-07	1.30324E-05	Females
ENSORLP0000004208	waif2	3.160032689	0.000563581	0.00746781	Females
ENSORLP00000013609	cax2	3.159640591	2.69028E-09	2.01613E-07	Females
ENSORLP00000018612	c6	3.150445698	2.62646E-05	0.000631848	Females

ENSORLP0000004641	PRSS23	3.142425843	0.0009819	0.011526736	Females
ENSORLP0000007986	smox	3.129941123	6.07813E-24	2.34627E-21	Females
ENSORLP0000007071	trpc6a	3.127455318	2.37049E-15	4.70853E-13	Females
ENSORLP00000014508	creld2	3.11270233	1.77565E-30	8.8605E-28	Females
ENSORLP0000001023	cdc42ep3	3.109238956	0.000496303	0.006773761	Females
ENSORLP0000009265	zgc:154058	3.108509768	1.6817E-26	7.32042E-24	Females
ENSORLP0000004298	ctnnal1	3.100731502	8.10923E-21	2.59229E-18	Females
ENSORLP0000007954	CCDC86 (1 of 2)	3.098941884	0.00029315	0.004413207	Females
ENSORLP0000003891	pik3r5	3.09512042	0.000321139	0.004757557	Females
ENSORLP00000017990	ptrfb	3.090443498	2.51608E-06	8.85999E-05	Females
ENSORLP0000009644	pyroxd2	3.074326874	1.57856E-28	7.17685E-26	Females
ENSORLP00000021856	pabpc4	3.069822079	1.50004E-09	1.20824E-07	Females
ENSORLP00000018061	cpz	3.063199233	2.99269E-11	3.32758E-09	Females
ENSORLP00000025166	Uncharacterized	3.055797556	3.04552E-06	0.000104195	Females
ENSORLP00000020144	KIF21B	3.055760401	0.000216293	0.003470701	Females
ENSORLP0000008198	slc45a2	3.046127519	1.80684E-07	9.03818E-06	Females
ENSORLP00000021903	runx3	3.042195332	6.95544E-05	0.001410321	Females
ENSORLP0000003185	ccdc88b	3.021642909	5.30687E-06	0.000167293	Females
ENSORLP0000006505	lgi1b	3.011032128	1.55943E-16	3.75346E-14	Females
ENSORLP00000012926	LPAR4	2.983902221	0.000101125	0.001905077	Females
ENSORLP00000013400	klf9	2.982749862	5.7244E-12	7.09791E-10	Females
ENSORLP0000002582	Uncharacterized	2.981511152	0.000384357	0.005526613	Females
ENSORLP00000019610	dram1	2.964960152	0.000148833	0.002595376	Females
ENSORLP00000011769	VAV1	2.949343124	4.17483E-10	3.71193E-08	Females
ENSORLP00000011190	rad54b	2.936313042	9.93742E-05	0.001889495	Females
ENSORLP00000024820	slc6a15	2.93604553	0.000497463	0.006785063	Females
ENSORLP00000016268	efhc1	2.931746876	0.000204247	0.003323519	Females
ENSORLP00000010030	pleca	2.913385626	6.48223E-09	4.58893E-07	Females
ENSORLP00000022639	faima	2.893661168	1.2676E-05	0.000344407	Females
ENSORLP0000005319	arhgef16	2.885550848	6.71586E-05	0.001373998	Females
ENSORLP00000019171	den	2.870895076	0.000163718	0.002798253	Females
ENSORLP00000020777	Uncharacterized	2.867579484	5.05877E-07	2.24994E-05	Females
ENSORLP0000009503	myo1ha	2.855348844	1.90993E-05	0.000485407	Females
ENSORLP00000012779	ADAP2 (2 of 2)	2.846816546	2.32384E-05	0.00057075	Females
ENSORLP00000013110	tfec	2.846762735	6.94536E-05	0.001409673	Females
ENSORLP0000005334	or129-1	2.846019994	5.87606E-07	2.55241E-05	Females
ENSORLP00000013946	numa1	2.816991805	0.000166313	0.002837863	Females
ENSORLP00000014511	TACC2	2.812899062	6.86836E-07	2.90931E-05	Females
ENSORLP0000001311	mctp1b	2.812602042	6.1206E-05	0.001279075	Females
ENSORLP00000014315	Uncharacterized	2.81204006	4.05609E-10	3.63963E-08	Females
ENSORLP00000012075	angpt2a	2.806423807	4.56294E-05	0.00100057	Females
ENSORLP00000015983	RBBP6 (2 of 2)	2.79676716	3.8881E-06	0.000128095	Females
ENSORLP00000019363	Uncharacterized	2.789401701	1.80175E-05	0.000464256	Females

ENSORLP00000011123	pdp1	2.779855859	1.20158E-16	2.92655E-14	Females
ENSORLP0000007945	si:dkey-79d12.5	2.773664199	5.77283E-14	9.52471E-12	Females
ENSORLP00000018112	mmp13b	2.755375579	5.54018E-06	0.000173048	Females
ENSORLP0000000230	DOC2A (1 of 2)	2.751872858	0.000681608	0.008656128	Females
ENSORLP00000012738	chkb	2.742880976	7.27582E-12	8.75624E-10	Females
ENSORLP00000025070	STX11 (2 of 2)	2.73963305	0.000127586	0.002291739	Females
ENSORLP0000007491	aadac	2.736442376	0.000796675	0.009796346	Females
ENSORLP0000007515	p2ry12	2.72331995	4.08928E-10	3.65339E-08	Females
ENSORLP00000017861	olgc9	2.723277196	4.22683E-07	1.93028E-05	Females
ENSORLP0000007511	spock3 (1 of 2)	2.721327357	0.000727301	0.009123152	Females
ENSORLP00000022967	grk6	2.715420236	5.44646E-06	0.000170642	Females
ENSORLP00000021787	matk	2.710384545	1.08652E-05	0.000304509	Females
ENSORLP0000000992	prex1	2.703776872	5.79023E-09	4.14204E-07	Females
ENSORLP00000017312	adgrd1	2.701936908	1.31728E-09	1.0867E-07	Females
ENSORLP00000013240	si:ch211-236c15.2	2.660746522	0.000291774	0.004396502	Females
ENSORLP00000014516	fzd9b	2.653944112	5.71884E-11	6.2235E-09	Females
ENSORLP0000008275	Uncharacterized	2.649112892	1.24119E-07	6.49452E-06	Females
ENSORLP00000018358	fam118b	2.633197871	2.72538E-07	1.31197E-05	Females
ENSORLP00000013542	Uncharacterized	2.63211292	0.000573786	0.007574201	Females
ENSORLP00000010561	pprc1	2.612261849	2.38504E-12	3.08408E-10	Females
ENSORLP00000016474	dph6	2.609649414	1.7846E-06	6.57859E-05	Females
ENSORLP00000019880	kif15	2.581867663	0.000288362	0.00435716	Females
ENSORLP00000020301	TLR5 (1 of 2)	2.579732969	2.18423E-10	2.12796E-08	Females
ENSORLP00000012031	VILL	2.558998581	0.000829531	0.010110563	Females
ENSORLP00000010549	Uncharacterized	2.557694823	2.99497E-07	1.41511E-05	Females
ENSORLP0000000888	cacna2d4b	2.555393837	1.52467E-13	2.43698E-11	Females
ENSORLP0000006699	bicc1a	2.553554462	0.000877184	0.010550444	Females
ENSORLP00000015190	tagapb	2.54791008	1.07493E-06	4.3037E-05	Females
ENSORLP0000008599	ankrd33ba	2.537035442	2.65732E-05	0.000637353	Females
ENSORLP0000001690	mprip	2.521534432	1.40354E-05	0.000376839	Females
ENSORLP0000006486	dffa	2.517616274	0.000254124	0.003929798	Females
ENSORLP00000021330	PLD4	2.512742294	0.000107744	0.002003934	Females
ENSORLP00000010875	arhgap15	2.509180692	1.64016E-05	0.000428556	Females
ENSORLP00000017178	zgc:92287	2.498395897	2.91383E-07	1.38316E-05	Females
ENSORLP00000018214	sh3d21	2.497233845	0.000975073	0.011464948	Females
ENSORLP00000024908	fkbp5	2.484802665	1.95889E-09	1.52384E-07	Females
ENSORLP00000018200	syk	2.468967315	0.000121089	0.002195094	Females
ENSORLP00000022276	tlr8a	2.468295243	0.00015308	0.002651877	Females
ENSORLP00000019268	hmha1b	2.463476633	2.16413E-08	1.32167E-06	Females
ENSORLP0000009402	slc7a8b	2.461534293	0.000212032	0.003431935	Females
ENSORLP00000010515	Uncharacterized	2.453688113	3.5961E-16	7.82687E-14	Females
ENSORLP00000012436	Uncharacterized	2.452905273	1.23496E-05	0.000336881	Females
ENSORLP00000024981	mylkb	2.433700096	1.33507E-06	5.23262E-05	Females

ENSORLPO	0000013452	ches1	2.433231368	1.30329E-10	1.32657E-08	Females
ENSORLPO	0000007229	slit2	2.432440541	8.96995E-05	0.001747773	Females
ENSORLPO	0000020092	ADORA1 (2 of 2)	2.428867728	0.000896059	0.010745875	Females
ENSORLPO	0000009467	OTUD7A	2.426374178	0.000455404	0.006291099	Females
ENSORLPO	0000015781	IDH2 (2 of 2)	2.426170207	5.04636E-18	1.34082E-15	Females
ENSORLPO	0000009068	Uncharacterized	2.409300152	2.81878E-06	9.74146E-05	Females
ENSORLPO	0000009049	il10ra	2.40822557	2.09596E-05	0.00052615	Females
ENSORLPO	0000008201	B3GNT3 (1 of 4)	2.395597506	7.59018E-06	0.000225054	Females
ENSORLPO	0000019021	stat4	2.389879211	2.80223E-10	2.62986E-08	Females
ENSORLPO	0000012607	FAM124A	2.386532348	0.000628526	0.008123186	Females
ENSORLPO	0000017517	Uncharacterized	2.384808694	0.00035586	0.005170843	Females
ENSORLPO	0000022933	Uncharacterized	2.378922137	0.000737194	0.009208833	Females
ENSORLPO	0000014030	gpr183a	2.377519541	0.000440418	0.006154722	Females
ENSORLPO	0000010109	slc2a11b (2 of 2)	2.374152258	0.000454218	0.006283195	Females
ENSORLPO	0000011525	myo1f	2.373643279	1.2184E-05	0.000333697	Females
ENSORLPO	0000017550	crlfla	2.372548115	6.31467E-08	3.52021E-06	Females
ENSORLPO	0000019520	bcl6a	2.362753518	1.79801E-10	1.80321E-08	Females
ENSORLPO	0000021665	CACNA1E (1 of 2)	2.359808226	6.41665E-05	0.001325943	Females
ENSORLPO	0000017172	mmp2	2.359175897	0.000437188	0.006125911	Females
ENSORLPO	0000010930	prkeba	2.338002864	8.62988E-06	0.000250083	Females
ENSORLPO	0000014218	il13ra1	2.331871739	0.000415256	0.005880193	Females
ENSORLPO	0000013640	Uncharacterized	2.319141939	0.000113785	0.002091579	Females
ENSORLPO	0000006441	col2a1b	2.318082789	3.97335E-05	0.000892687	Females
ENSORLPO	0000002300	slc7a3a	2.317778035	8.55163E-07	3.5282E-05	Females
ENSORLPO	0000004802	TRIM69	2.314322629	5.68829E-06	0.000176596	Females
ENSORLPO	0000007700	ahcyl1 (2 of 2)	2.299146197	0.000897054	0.010751506	Females
ENSORLPO	0000006100	mpeg1.2	2.297368698	4.20857E-08	2.44611E-06	Females
ENSORLPO	0000006326	slc43a2b	2.295800194	1.67737E-06	6.24823E-05	Females
ENSORLPO	0000000818	blnk	2.290726407	8.71268E-06	0.000252125	Females
ENSORLPO	0000004346	adap2	2.286912531	0.000190597	0.003142167	Females
ENSORLPO	0000016040	csflrb	2.284789201	6.04991E-05	0.00126689	Females
ENSORLPO	0000001826	isg20	2.279987513	2.68542E-09	2.01613E-07	Females
ENSORLPO	0000015994	Uncharacterized	2.279121124	0.000287497	0.004351666	Females
ENSORLPO	0000015793	ZNF710 (2 of 2)	2.273263219	0.000424092	0.005975548	Females
ENSORLPO	0000010772	plekhh1	2.26929512	0.000816489	0.009984785	Females
ENSORLPO	0000017587	ENDOD1 (5 of 5)	2.268847789	4.25429E-05	0.000943364	Females
ENSORLPO	0000019457	Uncharacterized	2.268561162	2.05669E-13	3.21205E-11	Females
ENSORLPO	0000008577	zgc:194312	2.238907727	0.000366859	0.005308039	Females
ENSORLPO	0000006725	Uncharacterized	2.236681673	0.000186567	0.00310179	Females
ENSORLPO	0000019546	CLSTN2	2.23240186	0.000150617	0.002622536	Females
ENSORLPO	0000018136	csf3r	2.231736042	2.1856E-07	1.07586E-05	Females
ENSORLPO	0000018346	CCDC134	2.228253237	2.78598E-10	2.62665E-08	Females
ENSORLPO	0000008608	Uncharacterized	2.224345494	0.000573831	0.007574201	Females

ENSORLP0000008528	PDLIM7	2.22174831	9.58181E-10	8.10059E-08	Females
ENSORLP0000009618	snx10b	2.220782354	0.000452739	0.006279725	Females
ENSORLP00000021498	Uncharacterized	2.214922854	0.000279993	0.004252686	Females
ENSORLP0000009050	Uncharacterized	2.211924189	0.000133166	0.002372313	Females
ENSORLP0000003850	ahnak	2.20282943	2.6896E-13	4.13734E-11	Females
ENSORLP00000013816	ttyh2	2.196389705	1.50193E-05	0.000399214	Females
ENSORLP0000004288	Uncharacterized	2.189982379	5.37719E-05	0.001147153	Females
ENSORLP00000013657	tmem263	2.187012239	9.18989E-13	1.27038E-10	Females
ENSORLP0000008273	jak3	2.185915986	0.000112313	0.002076112	Females
ENSORLP0000002048	ITGAL	2.181510441	2.17033E-06	7.80367E-05	Females
ENSORLP0000004247	MEDAG	2.174085838	6.28449E-06	0.000191902	Females
ENSORLP00000001508	col7a1	2.172481852	1.83762E-05	0.000469947	Females
ENSORLP00000022682	CEP250	2.166485307	0.00013997	0.002475065	Females
ENSORLP0000009215	dock10	2.159289261	0.000464962	0.006405831	Females
ENSORLP00000025392	cxcr4b	2.157727822	0.000222627	0.003547301	Females
ENSORLP00000012678	Uncharacterized	2.15381638	6.37299E-05	0.001321391	Females
ENSORLP00000019871	tfeb	2.152323645	7.5206E-15	1.4116E-12	Females
ENSORLP00000004891	rbms1a	2.137951763	0.000615616	0.008001839	Females
ENSORLP00000014360	arhgap27	2.128988642	0.000438338	0.006134032	Females
ENSORLP00000015151	slc41a1	2.120562709	2.00253E-22	7.18769E-20	Females
ENSORLP0000006742	entpd3	2.115094096	5.65691E-06	0.000175889	Females
ENSORLP0000005685	PHOSPHO1 (2 of 2)	2.113516669	4.4115E-08	2.54957E-06	Females
ENSORLP00000015173	ezrb	2.113318821	2.03875E-10	1.99573E-08	Females
ENSORLP00000011554	fes	2.110348296	0.000110136	0.002042866	Females
ENSORLP0000008643	ptprh	2.105567575	0.000676748	0.008645477	Females
ENSORLP0000002049	cybb	2.099555177	0.000982796	0.011526736	Females
ENSORLP0000001307	ampd3b	2.091214658	5.77998E-06	0.000179171	Females
ENSORLP00000011076	asns	2.088437354	2.96084E-09	2.20276E-07	Females
ENSORLP00000010202	prickle2b	2.086179148	7.19022E-11	7.70182E-09	Females
ENSORLP00000013149	ercla	2.086093326	1.86269E-12	2.4746E-10	Females
ENSORLP00000011941	ar	2.075936905	1.09733E-15	2.29085E-13	Females
ENSORLP0000008861	vwa9	2.069735284	1.26445E-10	1.29347E-08	Females
ENSORLP00000001595	sytl4	2.069107847	5.817E-15	1.11224E-12	Females
ENSORLP0000000496	piezo1	2.06232896	1.74224E-06	6.44566E-05	Females
ENSORLP0000007406	ptk2ba	2.059370626	9.45631E-05	0.00182172	Females
ENSORLP00000015669	Uncharacterized	2.054540769	2.1958E-06	7.85205E-05	Females
ENSORLP00000020977	PKP4	2.051816237	8.36256E-15	1.55536E-12	Females
ENSORLP0000009417	b4galnt1a	2.048379612	0.000734987	0.009191379	Females
ENSORLP0000005077	DOCK2	2.047568429	2.10626E-05	0.000528088	Females
ENSORLP00000020699	NCKAP5L	2.045270399	2.93084E-05	0.00069481	Females
ENSORLP0000003023	si:ch1073-139a18.1	2.041557292	0.000324458	0.004799769	Females
ENSORLP00000017936	SLC25A13 (1 of 2)	2.039683821	1.41818E-08	9.2403E-07	Females
ENSORLP00000013287	dock4b	2.039150875	1.87647E-06	6.8924E-05	Females

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ENSORLP0000005921	hnrpkl	2.022035263	2.14242E-09	1.65403E-07	Females
ENSORLP00000012587	eaf2	2.015569695	1.91462E-12	2.52717E-10	Females
ENSORLP00000012465	ptprea	2.015336473	0.000193781	0.003184396	Females
ENSORLP00000020216	adam28	2.015311498	6.11642E-06	0.00018761	Females
ENSORLP00000016026	pmepal	-2.006838987	1.44745E-05	0.000387102	Males
ENSORLP0000004502	epo	-2.009912123	2.61407E-06	9.15774E-05	Males
ENSORLP00000018665	rorcb	-2.012163779	7.5991E-08	4.14586E-06	Males
ENSORLP00000010275	tlr3	-2.013983388	1.60836E-06	6.05992E-05	Males
ENSORLP00000012824	slmapb	-2.017248895	1.8809E-08	1.16964E-06	Males
ENSORLP00000025125	Uncharacterized	-2.019889527	1.89046E-05	0.000481916	Males
ENSORLP00000011471	miox	-2.030569196	2.77696E-15	5.46286E-13	Males
ENSORLP00000023782	pir	-2.049118967	3.71411E-06	0.000123757	Males
ENSORLP00000014096	eps811	-2.054775972	7.7915E-09	5.36722E-07	Males
ENSORLP00000013097	tspan15	-2.07601978	4.78199E-05	0.00103969	Males
ENSORLP00000021160	cds1	-2.076642745	1.79229E-21	6.11139E-19	Males
ENSORLP00000010795	Uncharacterized	-2.089351516	3.25529E-07	1.53456E-05	Males
ENSORLP0000002153	si:ch211-14c7.2	-2.089421674	0.000178206	0.002998282	Males
ENSORLP0000001162	Uncharacterized	-2.113308819	9.10013E-15	1.67729E-12	Males
ENSORLP00000023156	si:dkey-266f7.9	-2.119336273	8.94404E-08	4.8409E-06	Males
ENSORLP00000017102	Uncharacterized	-2.120483425	5.74851E-09	4.14204E-07	Males
ENSORLP0000005597	TIMP4	-2.123951213	0.000232487	0.003675768	Males
ENSORLP00000010781	rnf34b	-2.125743767	0.000246979	0.003852025	Males
ENSORLP0000009840	SEPT4 (2 of 2)	-2.142771522	5.99714E-09	4.27511E-07	Males
ENSORLP00000013722	TMCC1 (2 of 2)	-2.151134775	6.77617E-20	2.03873E-17	Males
ENSORLP0000003238	krt222 (1 of 2)	-2.153272152	3.58439E-05	0.000819364	Males
ENSORLP0000003137	pvrl1b	-2.181719456	1.36588E-09	1.12227E-07	Males
ENSORLP0000008355	npnt	-2.194727586	1.30787E-07	6.82597E-06	Males
ENSORLP00000017291	CCIN	-2.211182351	1.65579E-06	6.20436E-05	Males
ENSORLP0000004936	Uncharacterized	-2.215559915	3.3457E-21	1.12212E-18	Males
ENSORLP00000019992	cux2b	-2.233010217	0.000831399	0.010112715	Males
ENSORLP0000008888	ass1	-2.238632054	4.00148E-06	0.000131493	Males
ENSORLP00000020975	SCN1B (2 of 3)	-2.240694576	7.86141E-05	0.001578377	Males
ENSORLP00000021699	zgc:101846 (6 of 6)	-2.24277439	9.74199E-06	0.000276055	Males
ENSORLP0000008754	RNH1 (11 of 14)	-2.264466536	0.000386489	0.005548892	Males
ENSORLP00000017103	Uncharacterized	-2.278373534	3.17618E-06	0.000107943	Males
ENSORLP00000016624	Uncharacterized	-2.281706041	0.000839519	0.010199358	Males
ENSORLP0000006731	ddc	-2.286799906	2.18423E-16	5.07808E-14	Males
ENSORLP00000013437	ESYT2 (2 of 2)	-2.288811555	2.28156E-05	0.000561714	Males
ENSORLP00000021034	zgc:194209	-2.295514928	1.12343E-05	0.000311861	Males
ENSORLP0000005409	HCN2	-2.311318371	1.80597E-05	0.000464424	Males
ENSORLP00000017509	EFNA2	-2.31314574	3.41013E-06	0.000115701	Males
ENSORLP00000005901	ptprdb	-2.351857889	1.62029E-05	0.000425804	Males
ENSORLP00000023724	ccdc135	-2.352096699	2.92979E-08	1.73239E-06	Males

ENSORLP00000020892	agmo	-2.375842064	1.87332E-14	3.30398E-12	Males
ENSORLP00000022475	CD200 (2 of 2)	-2.404314779	0.000341135	0.005006661	Males
ENSORLP00000011566	RERG	-2.428249027	0.000289081	0.004364801	Males
ENSORLP00000011013	slc5a2	-2.449228385	3.35654E-05	0.000778588	Males
ENSORLP00000020833	LPIN3	-2.485014265	1.56379E-15	3.17658E-13	Males
ENSORLP0000009759	TLN2 (1 of 2)	-2.506603932	1.53817E-06	5.87115E-05	Males
ENSORLP00000013019	hprt11	-2.510473307	6.16143E-06	0.000188708	Males
ENSORLP00000024806	hhla2a.1	-2.511575665	1.43665E-06	5.54573E-05	Males
ENSORLP00000020425	nr2f5	-2.55551011	1.70086E-13	2.69751E-11	Males
ENSORLP00000013504	ZNHIT6	-2.594572193	4.98337E-07	2.22519E-05	Males
ENSORLP0000001852	fgflb	-2.614355998	5.7184E-11	6.2235E-09	Males
ENSORLP0000008000	aldoa	-2.617309079	9.54999E-12	1.14259E-09	Males
ENSORLP00000023406	cishb	-2.618490696	2.64497E-14	4.62508E-12	Males
ENSORLP00000020398	CCDC176	-2.620033788	7.2115E-09	5.0355E-07	Males
ENSORLP0000004839	dio1	-2.631052489	1.70188E-33	1.08809E-30	Males
ENSORLP00000012458	nuf2	-2.645497819	3.05584E-06	0.000104373	Males
ENSORLP0000005698	pcdh17	-2.654442492	1.35344E-08	8.85555E-07	Males
ENSORLP00000010168	sgsm1b	-2.657874446	4.03123E-05	0.000903339	Males
ENSORLP00000024395	MFAP4 (3 of 5)	-2.704427655	0.000504214	0.006861264	Males
ENSORLP0000007029	trim71	-2.737488816	0.000505582	0.006863777	Males
ENSORLP0000000032	zgc:113142	-2.749145105	0.000539099	0.007230205	Males
ENSORLP0000008002	Uncharacterized	-2.752587049	6.64086E-05	0.001362417	Males
ENSORLP00000016949	fasn	-2.847191018	6.72287E-07	2.85953E-05	Males
ENSORLP00000021003	scn1ba	-2.849086781	5.32599E-09	3.87774E-07	Males
ENSORLP0000007580	ndst3	-2.855337848	2.12831E-06	7.69313E-05	Males
ENSORLP00000018374	acp5a	-2.882574611	5.48316E-13	7.92195E-11	Males
ENSORLP0000003050	kcnj9	-2.9189413	9.39296E-05	0.001811221	Males
ENSORLP00000016822	barx2	-2.929834916	6.37476E-05	0.001321391	Males
ENSORLP0000000620	acox3 (1 of 3)	-2.933193247	3.19509E-24	1.25708E-21	Males
ENSORLP0000008012	si:dkey-238d18.4	-2.963093169	2.42881E-11	2.76061E-09	Males
ENSORLP0000003801	tmem117	-2.974633056	8.19854E-07	3.43014E-05	Males
ENSORLP00000001594	MAPKAPK3 (1 of 2)	-2.997513268	5.09177E-19	1.48818E-16	Males
ENSORLP00000017197	wnt5b	-3.013795944	1.59498E-06	6.02061E-05	Males
ENSORLP00000014350	BPIFC (2 of 2)	-3.034098978	0.000897699	0.010752941	Males
ENSORLP00000025345	pcdh18b	-3.037826952	1.26075E-06	4.95082E-05	Males
ENSORLP00000001271	mgat4c (1 of 2)	-3.039796558	0.000995745	0.011647764	Males
ENSORLP00000018637	myom3	-3.06943305	2.42198E-12	3.09695E-10	Males
ENSORLP00000022724	Uncharacterized	-3.146236332	6.81405E-09	4.79068E-07	Males
ENSORLP0000002465	agpat91	-3.162291124	1.00734E-14	1.8401E-12	Males
ENSORLP00000004697	loxhd1b	-3.165188525	0.000122243	0.002213243	Males
ENSORLP00000020462	REPS2	-3.168587539	7.44007E-15	1.40941E-12	Males
ENSORLP0000004675	HMOX1 (1 of 2)	-3.183794536	7.46964E-23	2.77857E-20	Males
ENSORLP00000012009	pvalb5	-3.18971389	2.82751E-07	1.35171E-05	Males

ENSORLP0000008458	cyp2r1	-3.217370522	1.40893E-14	2.5321E-12	Males
ENSORLP00000011397	CIT (1 of 2)	-3.22470304	0.000398221	0.005677495	Males
ENSORLP00000020911	Uncharacterized	-3.261832196	2.28129E-08	1.38907E-06	Males
ENSORLP0000006591	gb	-3.29331855	9.78915E-07	3.98164E-05	Males
ENSORLP00000022096	abhd3	-3.297171382	9.58323E-05	0.001840971	Males
ENSORLP0000002349	card14	-3.310999967	2.90412E-07	1.38175E-05	Males
ENSORLP00000016285	hpdb	-3.332813777	0.000295646	0.004444254	Males
ENSORLP00000004422	oxgr1b	-3.368324077	1.18719E-16	2.92635E-14	Males
ENSORLP00000020686	FLVCR2 (2 of 2)	-3.384883357	3.87792E-10	3.49509E-08	Males
ENSORLP00000020096	Uncharacterized	-3.432812729	0.000235826	0.003711356	Males
ENSORLP0000007544	lpar3	-3.445416445	7.45833E-07	3.14619E-05	Males
ENSORLP0000000225	ociad2	-3.459581792	9.73928E-06	0.000276055	Males
ENSORLP0000001788	aacs	-3.498611857	1.0057E-39	7.9137E-37	Males
ENSORLP00000022535	nmrk2	-3.537065715	0.000161696	0.00277761	Males
ENSORLP00000014702	ankha	-3.537599596	7.42449E-23	2.77857E-20	Males
ENSORLP00000019292	DEPDC4	-3.543126512	3.50783E-05	0.000806238	Males
ENSORLP00000013243	lrp8	-3.547404714	0.000583926	0.007692563	Males
ENSORLP00000010352	sema4ba	-3.582598062	2.01228E-05	0.000508263	Males
ENSORLP00000023389	syce2	-3.588719812	0.000456828	0.006306514	Males
ENSORLP0000002773	igfbp2a	-3.619293949	7.7022E-09	5.34167E-07	Males
ENSORLP00000010869	gria3a	-3.66708935	3.05412E-05	0.000719036	Males
ENSORLP0000003916	ctss	-3.78947201	3.4687E-07	1.62394E-05	Males
ENSORLP0000002352	eno1b	-3.857301808	1.18441E-21	4.10708E-19	Males
ENSORLP00000001063	Uncharacterized	-3.895847067	0.000415887	0.005880193	Males
ENSORLP00000021189	cyp1c1	-3.928750342	2.72169E-08	1.61869E-06	Males
ENSORLP00000022526	slc4a11	-3.993835096	5.70777E-11	6.2235E-09	Males
ENSORLP0000002601	foxg1b	-4.0027021	6.49052E-05	0.001337255	Males
ENSORLP00000014408	cacng8b	-4.010871993	6.65138E-21	2.16001E-18	Males
ENSORLP00000016421	astn1	-4.057603453	1.32132E-07	6.84374E-06	Males
ENSORLP00000015592	slc1a8b	-4.122670875	9.59819E-06	0.000273115	Males
ENSORLP00000019009	CYP27B1	-4.129737709	7.66326E-18	1.95978E-15	Males
ENSORLP0000004289	rxfp2b	-4.155270328	9.46661E-19	2.72785E-16	Males
ENSORLP0000003656	KCNIP3 (1 of 2)	-4.172365405	2.78211E-05	0.000662623	Males
ENSORLP0000009880	Uncharacterized	-4.297099279	1.89148E-05	0.000481916	Males
ENSORLP00000011888	rorc	-4.307545949	1.25857E-15	2.60091E-13	Males
ENSORLP0000004433	rbp7b	-4.324299081	0.000100671	0.001903204	Males
ENSORLP0000005859	rab3c	-4.448817116	1.5811E-06	5.97923E-05	Males
ENSORLP00000012735	dact1	-4.513955393	3.96029E-28	1.76138E-25	Males
ENSORLP00000018527	prkacab	-4.531734572	4.19065E-21	1.38285E-18	Males
ENSORLP00000021362	Uncharacterized	-4.554328515	2.77108E-06	9.59281E-05	Males
ENSORLP0000000379	EMB	-4.675487701	9.98519E-39	7.56618E-36	Males
ENSORLP00000011833	pdc	-4.737274107	6.13249E-05	0.00128025	Males
ENSORLP0000005925	grk7	-4.764901737	3.8273E-12	4.8335E-10	Males

ENSORLP00000017280	CDH18	-4.769584363	3.03678E-16	6.75321E-14	Males
ENSORLP0000000296	si:ch211-106h11.3	-4.855304532	5.33969E-08	3.01781E-06	Males
ENSORLP00000011335	homer1b	-4.883346587	0.000720267	0.009055068	Males
ENSORLP00000020348	afp4	-4.906841012	4.17227E-07	1.90963E-05	Males
ENSORLP0000008675	tnxb	-5.245267422	1.61168E-50	1.49879E-47	Males
ENSORLP00000013984	pgam2	-5.436243473	9.21416E-48	8.1962E-45	Males
ENSORLP00000018307	NTRK3 (1 of 2)	-5.549230736	5.30169E-30	2.5225E-27	Males
ENSORLP00000025649	Uncharacterized	-5.572434381	6.58461E-18	1.70525E-15	Males
ENSORLP00000019301	hecw2a	-5.607199145	3.64405E-05	0.000832071	Males
ENSORLP00000018419	iqsec3a	-5.689477217	0.000309018	0.004610354	Males
ENSORLP00000015392	MGAM	-5.923992858	0.000218515	0.003495388	Males
ENSORLP00000025336	SLC7A11	-6.200580875	5.4984E-13	7.92195E-11	Males
ENSORLP00000015278	esl	-6.279356319	2.10603E-06	7.62606E-05	Males
ENSORLP00000025276	asah2	-6.471218783	2.92213E-05	0.000693548	Males
ENSORLP00000019146	mag	-6.963211271	1.61663E-16	3.84589E-14	Males
ENSORLP0000004810	enpp2 (2 of 2)	-6.976555795	1.45133E-11	1.69673E-09	Males
ENSORLP00000018473	GABRR1 (1 of 2)	-7.099185474	0.000547488	0.007317905	Males
ENSORLP00000020188	sall4	-7.581417085	1.4732E-07	7.5729E-06	Males
ENSORLP0000006491	irx2a	-7.681834939	4.56161E-05	0.00100057	Males
ENSORLP00000013332	bco2l	-7.835132376	6.99777E-09	4.90299E-07	Males
ENSORLP00000011357	otc	-8.190420751	1.08537E-18	3.04186E-16	Males
ENSORLP0000005353	hsd17b3	-8.230979135	1.11417E-10	1.15125E-08	Males

Metaboanalyst 3.0 Pathways (female)	Total	Expected	Hits	P Value
Steroid biosynthesis	18	0.27253	2	0.028878
Arginine and proline metabolism	57	0.86301	3	0.051748
Alanine, aspartate and glutamate metabolism	32	0.4845	2	0.082477
Cysteine and methionine metabolism	35	0.52992	2	0.096305

Metaboanalyst 3.0 pathways (male)	Total	Expected	Hits	P Value
Ubiquinone and other terpenoid-quinone biosynthesis	2	0.033165	1	0.032902
Steroid biosynthesis	18	0.29849	2	0.034262
Phenylalanine metabolism	18	0.29849	2	0.034262
Fatty acid biosynthesis	6	0.099495	1	0.095626

Annex 2. 5 Complete list of IPA molecular pathways that are significantly different between male and female medaka livers, according to transcriptome and metabolome data.

Category	<i>p</i> -value, corrected threshold
Lipid Metabolism	2,65E-14-1,13E-03
Molecular Transport	2,65E-14-1,13E-03
Small Molecule Biochemistry	2,65E-14-1,13E-03
Inflammatory Response	1,19E-13-1,11E-03
Organismal Development	3,53E-12-1,13E-03
Vitamin and Mineral Metabolism	5,06E-12-3,66E-04
Free Radical Scavenging	8,76E-12-1,24E-05
Cell Death and Survival	4,32E-10-9,2E-04
Cardiovascular System Development and Function	2,03E-09-1,14E-03
Metabolic Disease	2,4E-09-1,07E-03
Neurological Disease	2,4E-09-9,65E-04
Psychological Disorders	2,4E-09-9,65E-04
Carbohydrate Metabolism	3,62E-09-9,43E-04
Cardiovascular Disease	4,31E-09-9,76E-04
Cancer	9,25E-09-1,14E-03
Organismal Injury and Abnormalities	9,25E-09-1,14E-03
Hematological Disease	4,18E-08-9,72E-04
Energy Production	4,46E-08-3,79E-04
Endocrine System Disorders	1,08E-07-3,65E-04
Gastrointestinal Disease	1,08E-07-1,06E-03
Renal and Urological Disease	1,08E-07-7,67E-04
Amino Acid Metabolism	1,54E-07-8,86E-04
Cellular Movement	9,32E-07-1,11E-03
Respiratory Disease	1,03E-06-2,69E-04
Hepatic System Disease	1,56E-06-1,06E-03
Hematological System Development and Function	1,69E-06-1,14E-03
Tissue Morphology	1,69E-06-1,14E-03
Cellular Development	2,92E-06-8,62E-04
Digestive System Development and Function	3,09E-06-4,31E-04
Organismal Survival	3,4E-06-6,4E-04
Connective Tissue Development and Function	4,1E-06-7,85E-04
Immunological Disease	5,79E-06-7,85E-04
Inflammatory Disease	5,79E-06-1,04E-03
Organ Morphology	6,89E-06-1,05E-03
Embryonic Development	7,12E-06-5,58E-04
Dermatological Diseases and Conditions	8,19E-06-5,31E-04
Immune Cell Trafficking	9,41E-06-1,11E-03
Renal and Urological System Development and Function	1,03E-05-7,04E-04
Cell-To-Cell Signaling and Interaction	1,04E-05-8,12E-04
Cellular Function and Maintenance	1,1E-05-1,11E-03
Nucleic Acid Metabolism	1,18E-05-6,86E-04
Cellular Assembly and Organization	1,18E-05-7,62E-04

Reproductive System Development and Function	1,56E-05-1E-03
Connective Tissue Disorders	1,76E-05-1,04E-03
Skeletal and Muscular Disorders	1,76E-05-1,04E-03
Cellular Growth and Proliferation	1,89E-05-7,95E-04
Tissue Development	2,87E-05-9,76E-04
Developmental Disorder	4,23E-05-6,38E-04
Hepatic System Development and Function	5,32E-05-4,31E-04
Humoral Immune Response	7,82E-05-7,82E-05
Endocrine System Development and Function	9,25E-05-8,77E-04
Lymphoid Tissue Structure and Development	9,3E-05-5,25E-04
Hereditary Disorder	9,47E-05-1,06E-03
Cell Morphology	1,27E-04-1,11E-03
Nutritional Disease	1,32E-04-6,88E-04
Reproductive System Disease	1,36E-04-1,14E-03
Tumor Morphology	1,38E-04-9,2E-04
Protein Synthesis	1,6E-04-1,14E-03
Cellular Compromise	1,6E-04-9,76E-04
Organ Development	2,87E-04-1,14E-03
Post-Translational Modification	2,93E-04-1,14E-03
Organismal Functions	3,24E-04-5,15E-04
Nervous System Development and Function	3,66E-04-4,41E-04
Skeletal and Muscular System Development and Function	5,58E-04-8,62E-04
Ophthalmic Disease	6,63E-04-6,63E-04
Drug Metabolism	7,1E-04-7,1E-04
Cell Cycle	7,62E-04-7,62E-04
Hematopoiesis	1,11E-03-1,11E-03
Respiratory System Development and Function	1,14E-03-1,14E-03

Annex 3. 1 Fixing solution for paraffin sections (100 mL)

Formaldehyde (37-40%)	20 mL
Glycerol	10 mL
Glacial acetic acid	10 mL
Absolute ethanol	30 mL
Distilled water	30 mL

Annex 3. 2 Fixing solution for resin sections (100 mL)

Paraformaldehyde (16%)	25 mL
Glutaraldehyde (50%)	0.4 mL

Picric acid	0.1 mL
0.1M pH 7.4 sorensen buffer	74.5 mL

Annex 3. 3 HES staining protocol

Dewax the sections in toluene, 2 baths, 15 min per each + 1 bath, 10 min.

Hydrate

- Ethanol 100%, 3min
- Ethanol 95%, 2min
- Rinse in distilled water, 4 times

Hematoxylin, 2 min

Rinse in tap water, 4 times

Rinse in distilled water, 2 times, 1 min per each

HCL solution. The sections turn pink

Rinse in distilled water, 1 min 30 s

Lithium carbonate solution, 3 min

Rinse in tap water

Eosine, 1 min

Rinse in tap water

Rinse in distilled water, 1 min 30 s

Ethanol 100% , 30 s

Saffron, 7 min

Dehydrate, ethanol 100%, 2 baths

Toluene, 2 baths

The eosin solution: dissolve 4 g erythrosine, 4 g eosin, and 4 g thymol in 1 L distilled water.

The saffron solution: add 10 g saffron to 1 L absolute ethanol and heat at 40 °C for 1 month.

HCL solution: 7 drops HCL in 400 mL distilled water.

Lithium carbonate solution: add lithium carbonate to distilled water until saturation.

Annex 3. 4 PAS staining protocol

Dewax and hydrate the sections. Put the slides into the periodic acid solution (0.5%-0.8%) for 10 min. Rinse in running tap water, then in distilled water. Place in Schiff reagent for 20 min, sections become light pink. Rinse in sulfurous water for 2 times. Rinse in running tap water (2-5 min) Counterstain in light green solution for 3 min Dehydrate and put the coverslip on the sections

The sulfurous water: 10 mL sodium metabisulfite (10%) +10 mL HCL (0.1 N) +180 mL distilled water.

Light green solution: 0.15 g light green + 100 Ml distilled water + 0.2 mL glacial acetic acid.

Annex 4. 1 Main script and command in the data treatment of RNA-seq.

Removing duplicates from the beginning fastq file by python:

#!/usr/bin/python
-*- coding: utf-8 -*-

usage = "

remove_dup permits to remove reads duplications in fastq files. -f = fastq input file -o = fastq ouput file

...

def read_file(infile):
 "" linearization of the fastQ format""

print("\tREAD FILE")
```
fl = open(infile, "r")
     cpt = 0
     cpt line = 0
     data = \{\}
     while True:
          cpt = cpt + 1
          line = f1.readline()
          #if (cpt line%500000 == 0):
          #
                print("\tmanagement of "+str(cpt line) + " sequences")
          if (line == ""):
               break
          if (cpt == 1):
               ID = line.strip().replace(" ", " ")
          if (cpt == 2):
               seq = line.strip()
          if (cpt == 4):
               cpt = 0
               cpt line = cpt line + 1
               if (cpt line%500000 == 0):
                    print("\tmanagement of "+str(cpt_line) + " sequences")
               quality = line.strip()
               if (data.get(seq)):
                    old ID = data[seq][0]
                    old quality = data[seq][1]
                    best ID, best quality = get bestQuality(ID, quality, old ID, old quality)
                    data[seq] = (best ID, best quality)
               else:
                    data[seq] = (ID, quality)
     fl.close()
     return(data)
def get bestQuality(ID, quality, old ID, old quality):
     if (get_quality(quality) >= get_quality(old_quality)):
          return (ID, quality)
     else:
          return(old_ID, old_quality)
def get_quality(qual):
     global qual = 0
     for i in qual:
          global qual = global qual + (ord(i) - 33)
     return(global qual)
def write data(data, outfile):
```

```
153
```

print("\tWRITE FILE")

```
sequences = list(data.keys())
f2 = open(outfile, 'w')
for seq in sequences:
    data_seq = data[seq]
    f2.write(data_seq[0] + "\n")
    f2.write(seq + "\n")
    f2.write("+\n")
    f2.write(data_seq[1] + "\n")
```

```
f2.close()
```

```
def main(infile, outfile):
data = read_file(infile)
write_data(data, outfile)
```

if name == " main ":

#getting of parameters
args = sys.argv[1:]

try:

```
optlist, args = getopt.getopt(args, "hf:o:", ["help", "file=", "output="])
```

```
except getopt.GetoptError, err:
    # print help information and exit:
    print ("option not recognized")
    sys.stderr.write(usage)
    sys.exit(2)
```

```
for opt, arg in optlist:
    if opt in ("-f", "--file"):
        infile = str(arg)
    elif opt in ("-o", "--output"):
        outfile = str(arg)
    elif opt in ("-h","--help") :
        print (usage)
        sys.exit()
    else :
        assert False, "unhandled option"
        print (usage)
        sys.exit()
```

```
main(infile, outfile)
```

use bash file to launch the pyton script
#!/bin/bash

```
## DIRECTORIES
```

DATA_FASTQ=/rddm/BM020315/DATA_INIT OUT_DIR=/rddm/BM020315/ANALYSES/WITH_RMDUP

PROGRAM

RMDUP="python remove_duplicates_fastq_v2.py"

####

removing of replicates before the mapping
###-----

for fqfile in \$DATA_FASTQ/*.fastq;

do

NAME=`basename \$fqfile`; NAME CUT=`echo \$NAME | cut -d \. -f 1`;

echo "Management of \$NAME ...";

OUTFILE=\$OUT_DIR/\$NAME_CUT"_rmdup.fastq"; OUTFILE_LOG=\$OUT_DIR/\$NAME_CUT"_rmdup.log";

launch bash file
./bot_remove_dup_pre_mapping.sh 1>2 2> bash.log &

Mapping the reads on the medaka genome by using a bash file to launch Tophat2 (v2.0.10):

#!/bin/bash

DIRECTORIES DATA_FASTQ=/rddm/BM020315/DATA_INIT OUT_DIR=/rddm/BM020315/ANALYSES INDEXES=/rddm/BM020315/medaka ensembl/medaka release78

PROGRAM
TOPHAT2=/rddm/duvernois/SOFTS/tophat-2.0.10.Linux_x86_64/tophat2
#####
mapping with tophat2
####------

for fqfile in \$DATA_FASTQ/*.fastq ;

do

NAME=`basename \$fqfile`; NAME_CUT=`echo \$NAME | cut -d \. -f 1`;

echo "Management of \$NAME ...";

OUTFILE_FOLDER=\$OUT_DIR/\$NAME_CUT"_tophat2_default"; OUTFILE_LOG=\$OUT_DIR/\$NAME_CUT"_tophat2_defaultlog";

\$TOPHAT2 -o \$OUTFILE_FOLDER -p 6 \$INDEXES \$fqfile > \$OUTFILE_LOG

done

Removing the multiple hints of mapping by samtools (v0.1.18), and counting reads on gene exons by HTSeqcount (v0.6.1p1). Using a bash file to launch the two softwares:

!/bin/bash ## DIRECTORIES DATA FOLDER=/rddm/BM020315/ANALYSES OUT DIR=/rddm/BM020315/ANALYSES/RemMH OUT COUNT=/rddm/BM020315/ANALYSES/READ COUNT MEDAKA=/rddm/BM020315/medaka ensembl/Oryzias latipes.MEDAKA1.78.gff3 **#PROGRAMS** RMMH="samtools view -bq 4" HTSEQ="htseq-count -f bam -s no" #### # mapping with tophat2 ####-----for folder in \$DATA FOLDER/* default/; do FOLDER CUT=`basename \$folder`; echo "Management of \$FOLDER CUT"; for bam in \$folder*.bam; do NAME=`basename \$bam`; if [["\$NAME" =~ accepted hits\.bam\$]]; then #\$RMMH \$bam > \$OUT DIR"/"\$FOLDER CUT" remMH.bam" #echo "\$HTSEQ \$OUT DIR/\$FOLDER CUT_remMH.bam \$MEDAKA > \$OUT COUNT/\$FOLDER CUT remMH.count" \$HTSEQ \$OUT DIR"/"\$FOLDER CUT" remMH.bam" \$MEDAKA > \$OUT COUNT"/"\$FOLDER CUT" remMH.count" fi done done Principal component analysis of the count file by "R": ### Import library(DESeq) library(FactoMineR) library(RColorBrewer) library(gplots) ### COLOR FOR CLUSTERING hmcol = colorRampPalette(brewer.pal(9, "GnBu"))(100) ### Principal Analysis Component counttable = read.table("BM020315.count", header= T, row.names=1) conditions = c("Ctrl", "Ctrl", "Ctrl", "T1", "T1", "T1", "T2", "T2", "T2", "T3", "T3", "T3") conditions legend = c("Ctrl", "T1", "T2", "T3") colors_legend=c("red","chartreuse4", "darkcyan", "darkorchid4") colors pca= c("red", "red", "red", "chartreuse4", "chartreuse4", "chartreuse4", "darkcyan", "darkcyan",

"darkcyan", "darkorchid4", "dar korchid4", "darkorchid4")

```
#### normalization, SVT and PCA
cds = newCountDataSet(counttable, conditions)
cds=estimateDispersions(cds, method="blind")
vsd=varianceStabilizingTransformation(cds)
data_vsd = getVarianceStabilizedData(cds)
transpose=t(data_vsd)
res.pca = PCA(transpose, scale.unit=FALSE, ncp=7, graph=F)
pdf("PCA_normVSD_noRMDUP_BM020315.pdf", onefile = TRUE)
for (i in 1:6){
    shift = i+1
    for (j in shift:7){
        plot.PCA(res.pca, choix="ind", habillage="ind", axes=c(i,j), cex=1, col.hab=colors_pca,
title=paste("PCA dim",i, "/dim", j, sep=""), new.plot=FALSE)
        legend("bottomleft", legend=conditions_legend, col=colors_legend, pch=15, cex =1, border="white")
```

}
}
dev.off()

Ensembl ID	Gene	Description	Log ₂ (fold			Log ₂ (fold change) Male		
	паше		MC1	MC5	Ext5	MC1	MC5	Ext5
(ENSORLP)0 0000001793	ckmt1	creatine kinase, mitochondrial 1	-1.65	-0.95	-1.08	-1.57	-2.06	-2.14
0000007793	vit-6	vitellogenin II precursor	-0.93	-0.69	-0.49			
00000010034	suclg2	succinate-CoA ligase, GDP-forming, beta	-0.92	-0.54	-0.54	-0.35	-0.20	-0.10
0000007624	lman1	lectin, mannose-binding, 1	-0.83	-0.15	-0.23	-0.48	-0.54	-0.40
0000008877	ssr4	signal sequence receptor, delta	-0.80	-0.07	-0.09	-0.42	-0.75	-0.66
00000012711	1-sf	L-SF precursor	-0.76	-0.83	-0.42			
00000015268	pycr1b	pyrroline-5-carboxylate reductase 1b	-0.76	-0.61	-0.84			
00000025081	etfdh	electron-transferring-flavoprotein dehydrogenase	-0.72	-0.79	-0.73	-0.56	-0.54	-0.22
0000008173	vtg3	vitellogenin 3, phosvitinless	-0.70	-0.20	-0.28			
00000013215	gcat	glycine C-acetyltransferase	-0.68	-0.57	-0.87	-0.55	-0.21	-0.39
00000022167	dnajc3a	Hsp40 homolog C, 3a	-0.66	-0.50	-0.34			
00000011869	gars	glycyl-tRNA synthetase	-0.66	0.06	-0.45	-0.85	-0.74	-0.94
0000002774	hdlbpa	high density lipoprotein binding protein a	-0.64	-0.35	-0.54	-0.48	-0.68	-0.56
0000006524	rpl31	ribosomal protein L31	-0.63	-0.39	-0.52			
00000016160	hmgcs1	3-hydroxy-3-methylglutaryl-CoA synthase 1	-0.63	-0.78	-0.70	-0.41	-0.17	-0.06
0000002123	ppib	peptidylprolyl isomerase B	-0.58	-1.01	-0.18	-0.68	-0.60	-0.49
0000006626	rps28	ribosomal protein S28	-0.58	-0.28	-0.25	-0.43	-0.66	-0.46
00000013645	\	uncharacterized protein	-0.57	-0.45	-0.31			
00000018349	mlec	malectin	-0.57	-0.11	-0.65			
ENSAMXP00 000013947	golga4	golgin A4	-0.56	-0.32	-0.15			

Annex 4. 2 Complete list of dysregulated proteins

	00000009170	pfn2l	profilin 2 like	-0.56	-0.55	-0.35	0.01	-0.48	-0.21
	00000007499	hspa4b	heat shock protein 4b	-0.56	-0.34	-0.42			
	00000009295	ndufb4	NADH dehydrogenase 1 beta subcomplex 4	-0.55	-0.62	-0.71	0.01	-0.13	-0.16
	0000006153	rpl34	ribosomal protein L34	-0.54	0.00	-0.05	0.21	0.12	-0.17
	00000009644	pyroxd2	pyridine nucleotide-disulphide oxidoreductase domain 2	-0.54	-0.51	-0.08	0.16	-1.08	-0.57
	00000020213	cda	cytidine deaminase	-0.53	-0.60	-0.39	-0.06	0.16	0.17
	00000009590	rpl13	ribosomal protein L13	-0.53	-0.40	-0.37	-0.44	-0.53	-0.40
	ENSONIP000 00004475	mat2aa	methionine adenosyltransferase II, alpha a	-0.53	-0.25	-0.48			
	00000010951	sec61b	Sec61 beta	-0.52	-0.84	-0.93			
	0000003206	rps27a	ribosomal protein S27a	-0.51	-0.16	-0.32	0.11	-0.05	-0.02
	00000016805	aars	alanyl-tRNA synthetase	-0.51	-0.31	-0.58	-0.58	-0.97	-0.87
	0000007555	ndufs1	NADH dehydrogenase Fe-S protein 1	-0.50	-0.59	-0.80	-0.32	0.27	-0.38
	00000018263	copg2	coatomer protein complex, gamma 2	-0.50	-0.72	-0.67			
	00000012527	rps27.1	ribosomal protein S27, 1	-0.49	-0.62	-0.19	-0.52	-0.50	-0.33
	00000019508	mt2	metallothionein 2	-0.48	0.08	0.90			
	00000011569	pls3	plastin 3	-0.47	0.02	-0.78			
	00000014882	rrbp1	ribosome binding protein 1	-0.47	-0.57	-0.16	-0.55	-0.60	-0.37
	00000019321	cirbpb	cold inducible RNA binding protein b	-0.46	-0.76	-0.27	0.17	-0.33	-0.45
	00000007656	ol-vit1	vitellogenin 1 precursor	-0.46	-0.14	-0.08	-2.12	-1.88	-2.71
	00000009119	pdia4	protein disulfide isomerase A, 4	-0.43	-0.10	-0.16	-0.77	-1.03	-0.79
	00000023883	cndp2	CNDP dipeptidase 2	-0.41	-0.32	-0.52	0.18	0.19	0.17
	ENSONIP000 00022082	aimp1	aminoacyl tRNA synthetase complex- interacting protein 1	-0.41	-0.30	-0.32	-0.45	-0.79	-0.91
	00000023179	eef1b2	eukaryotic translation elongation factor 1 beta 2	-0.40	-0.21	-0.21	-0.08	-0.58	0.01
	00000022552	calr3b	calreticulin 3b	-0.39	-0.51	-0.36	-0.10	-0.28	-0.04
	00000001910	rps26	ribosomal protein S26	-0.39	-0.50	-0.52	-0.44	-0.30	-0.56
	00000016162	rps14	ribosomal protein S14	-0.39	-0.43	-0.35	-0.27	-0.34	-0.50
	00000023880	rpl23	ribosomal protein L23	-0.35	-0.43	-0.18	-0.37	-0.57	-0.43
	00000014382	eIF-5A	eukaryotic translation initiation factor 5A	-0.35	-0.16	-0.02	-0.31	-0.80	-0.56
	0000008283	rpl10a	ribosomal protein L10a	-0.34	-0.34	-0.33	-0.32	-0.59	-0.32
	00000015979	rpl24	ribosomal protein L24	-0.34	-0.17	0.26	-0.56	-0.66	-0.48
	0000001825	pdia3	protein disulfide isomerase A, 3	-0.32	-0.21	-0.23	0.01	-0.52	-0.17
	00000013387	rpl8	ribosomal protein L8	-0.31	-0.21	-0.21	-0.51	-0.58	-0.69
	00000004423	h6pd	hexose-6-phosphate dehydrogenase	-0.28	-0.05	0.49	0.01	0.67	1.37
	ENSAMXP00	rp110	ribosomal protein L 10	0.27	0.17	0.00	0.35	0.41	0.55
	000013824	Ipiio	noosoniai protein L10	-0.27	0.17	0.09	-0.35	-0.41	-0.55
	00000019400	tpt1	translationally-controlled tumor protein	-0.27	-0.51	-0.63	-1.52	-1.38	-1.63
	0000009353	myl6b	myosin light chain 6B	-0.25	-0.14	-0.57			
	0000003949	acadm	acyl-CoA dehydrogenase, C-4 to C-12 straight chain	-0.22	-0.25	-0.55	-0.42	-0.14	-0.26
	0000007226	mdh2	malate dehydrogenase 2, NAD	-0.22	0.25	-0.07	0.63	0.66	0.45
	00000016901	fabp7a	fatty acid binding protein 7, brain, a	-0.20	-0.19	-0.08	-1.94	-3.26	-3.77
	00000013731	rpl18	ribosomal protein L18	-0.19	-0.17	-0.36	-0.37	-0.61	-0.81
ļ	00000012015	rps4x	ribosomal protein S4, X-linked	-0.19	-0.16	-0.01	-0.31	-0.60	-0.32
	0000005051	btd	biotinidase	-0.18	-0.27	-0.04	0.41	-0.02	0.52
	0000009623	rps3a	V-Fos transformation effector	-0.16	-0.03	-0.01	-0.60	-0.58	-0.47
	00000006901	eif3ha	eukaryotic translation initiation factor 3, Ha	-0.13	-0.68	-0.47			

0000007410	rplp21	ribosomal protein, large P2, like	-0.12	-0.45	-0.30	-0.43	-0.80	-0.39
00000025589	acol	aconitase 1, soluble	-0.10	0.04	-0.26	-0.57	0.01	-0.31
ENSTNIP000 00014271	ak2	adenylate kinase 2	-0.09	-0.68	-0.52			
00000016402	rps25	ribosomal protein S25	-0.09	-0.17	-0.06	-0.36	-0.61	-0.24
00000023060	stoml2	stomatin-like 2	-0.08	-0.22	-0.94			
0000005291	ybx1	Y-box binding protein	-0.08	-0.75	-0.18			
0000000748	rps3	ribosomal protein S3	-0.07	0.25	0.04	-0.30	-0.08	-0.55
00000014497	fabp3	fatty acid binding protein 3, muscle and heart	-0.05	-0.29	-0.22	1.18	1.38	-0.40
00000017642	rps19	ribosomal protein S19	0.00	-0.08	-0.10	-0.50	-0.52	-0.56
00000005146	sec22bb	SEC22 vesicle trafficking protein homolog B, b	0.00	-0.23	0.11	-0.01	-0.77	-0.33
0000001372	rps18	ribosomal protein S18	0.05	0.22	0.02	-0.38	-0.20	-0.51
0000006749	cct8	chaperonin containing TCP1, 8	0.08	0.02	-0.36	-0.13	-0.78	-0.60
0000003802	gb	hemoglobin, alpha-1-like	0.11	-0.15	0.35	0.22	1.02	0.15
00000004230	pvalb4	parvalbumin 4	0.17	0.92	-0.13			
00000009992	rpl7	ribosomal protein L7	0.17	0.53	0.18	-0.09	-0.11	-0.17
0000008867	ncl	nucleolin	0.19	-0.19	-0.02	-0.64	-0.83	-0.42
00000024964	rpl18a	ribosomal protein L18a	0.19	0.68	0.31			
00000020346	rpsa	ribosomal protein SA	0.21	0.51	0.23	0.77	0.38	0.47
00000020299	myh9b	myosin, heavy chain 9b, non-muscle	0.23	-0.14	-0.09	0.07	-0.60	-0.25
0000007030	rpl9	ribosomal protein L9	0.24	-0.02	0.03	-0.49	-0.66	-0.36
0000003571	\	uncharacterized protein	0.25	0.18	-0.06	-0.59	-0.72	-0.25
0000006568	Gb-alpha1	hemoglobin embryonic, alpha	0.26	0.00	0.39	0.18	1.12	0.26
ENSAMXP00 000019028	npepps	aminopeptidase puromycin sensitive	0.29	0.57	0.13	0.56	0.12	0.38
0000002988	dmgdh	dimethylglycine dehydrogenase	0.34	0.08	0.22	0.22	0.80	0.38
00000017546	apoa2	apolipoprotein A-II	0.35	-0.20	-0.01	-0.42	-0.68	-0.48
00000020703	aldh8a1	aldehyde dehydrogenase 8, A1	0.36	-0.02	0.38	0.38	0.50	0.48
ENSAMXP00 000018192	slc25a3b	solute carrier family 25	0.36	-0.04	0.04	-0.68	-0.19	-0.10
0000006843	agla	amylo-alpha-1, 6-glucosidase	0.37	-0.75	-0.33	-0.18	0.28	0.28
00000014655	dbnlb	drebrin-like b	0.40	0.42	-0.05	-0.43	-0.82	0.05
00000017582	pcbd1	dimerization cofactor of hepatocyte nuclear factor 1 alpha	0.40	0.24	0.02	0.18	0.54	0.27
00000015542	g6pd	glucose-6-phosphate dehydrogenase	0.41	0.22	-0.10	0.91	0.09	0.43
ENSONIP000	glud1b	aglutamate dehydrogenase 1b	0.44	0.19	0.20	0.40	0.55	0.50
00019996	8		0.44	0.04	0.74	0.00	0.00	0.40
00000003671	laspl	LIM and SH3 protein I	0.44	0.24	0.74	0.69	0.09	0.48
0000001833	krt18	keratin 18	0.45	0.22	0.16	0.63	0.14	0.19
00000019384	1Vd	isovaleryl-CoA dehydrogenase	0.45	0.51	0.50	-0.32	0.22	0.11
00000025316	ywnag1	tyrosine 3-monooxygenase gamma	0.4/	0.10	0.10	-0.44	-0.34	-0.73
00000014297		uncharacterized protein	0.48	0.61	0.45	-0.11	-0.02	-0.05
00000017772	aldh9a1a.2	aldehyde dehydrogenase 9, Ala	0.48	0.01	0.06	0.61	0.42	0.27
00000007503	\	uncharacterized protein	0.49	0.64	0.21	0.00	1.00	0.25
00000003772	gb	hemoglobin, beta-1-like	0.49	0.38	0.75	0.28	1.36	0.35
00000019715	apoala	apolipoprotein A-la	0.50	0.10	0.17	-0.32	-0.43	-0.46
0000011903	0000011903 aldh111 aldehyde dehydrogenase 1, L1		0.51	0.93	0.72	0.10	0.10	0.06
00000015097	000015097 gstk1 glutathione S-transferase kappa 1 ISCMOROO fatty and him find metating to a line to a		0.51	0.26	0.35	-0.28	-0.23	-0.11
ENSGMOP00 000020886	fabp10a	basic	0.52	0.25	0.40	0.57	0.35	0.43
00000011267	got2b	glutamic-oxaloacetic transaminase 2b	0.52	0.60	0.58	0.18	0.89	0.26

ENSTRUP000	histone h4	histone cluster 1, H4h	0.54	0.30	0.14	0.14	0.26	-0.04
000039718	abhadh	anovil CoA hydrotosa	0.55	0.43	0.05	0.07	0.27	0.03
00000020034	eimaun aun%h1	entophroma D450 8 D1	0.55	0.43	-0.05	0.07	0.27	-0.03
50000022033 FNSTNIP000	cypoor	cytoenrome 1450 8, B1	0.55	0.23	0.16	0.00	0.34	0.05
00009392	\	uncharacterized protein	0.55	0.68	0.51			
00000014130	c3-1	complement component C3-1	0.56	0.55	0.59	0.20	0.00	0.27
00000004115	bhmt	betaine-homocysteine methyltransferase	0.57	0.12	0.46	0.33	0.30	0.17
0000007243	aldh7a1	aldehyde dehydrogenase 7, A1	0.57	0.62	0.38	0.16	0.24	0.34
ENSXMAP00 000008629	pcxb	pyruvate carboxylase b	0.57	0.11	0.08	-0.14	0.19	0.25
00000024800	mvp	major vault protein	0.57	0.16	0.31	0.18	0.26	0.25
00000021207	fh	fumarate hydratase	0.57	0.37	0.47	-0.44	-0.16	-0.27
00000020768	faah	fatty acid amide hydrolase	0.57	0.17	0.10	-0.87	-0.12	-0.40
0000006519	grhprb	glyoxylate reductase b	0.57	0.18	0.24	0.13	0.47	0.47
0000004329	dhrs9	dehydrogenase/reductase 9	0.58	0.40	0.13	1.46	0.50	0.08
00000011229	pygl	phosphorylase, glycogen, liver	0.59	0.02	0.31	0.08	0.62	0.11
00000017960	akr1a1b	aldo-keto reductase 1, A1b	0.59	0.30	0.36			
0000009104	rps10	ribosomal protein S10	0.61	0.86	0.64	-0.40	-0.98	-1.21
0000000559	cbr1	carbonyl reductase 1	0.64	0.83	0.55	-0.04	0.18	-0.19
00000015780	tnilb	triosephosphate isomerase 1b	0.64	0.22	0.40	0.17	0.13	0.11
0000023909	haol	hydroxyacid oxidase 1	0.66	0.23	0.04	0.06	0.33	0.20
00000011061	anxa4	annexin max1	0.67	0.48	0.30	0.13	0.14	-0.19
00000000835	ech1	enovl CoA hydratase 1 peroxisomal	0.67	0.59	0.36	0.13	0.60	0.15
00000012961	akr1b1	aldo-keto reductase 1 B1	0.67	0.35	0.55	-0.04	0.18	0.18
00000012901	dnydb	dihydronyrimidine dehydrogenase h	0.00	0.04	0.35	-0.13	0.10	0.10
00000017090	ldhha	lactate debydrogenase Ba	0.08	0.04	0.29	-0.15	1.06	0.27
00000012333	luali	lusozuma a lika 1	0.09	0.21	0.55	0.50	1.00	0.42
00000000382	lygII	Tysozymie g-mee i	0.70	2.94	-0.11			
00000000914	\	uncharacterized protein	0.71	0.42	0.50	0.45	0.09	0.45
0000018709	1	4 hudroughonylpurguate diouygonogo	0.71	0.50	0.32	0.45	0.08	0.45
00000013971	hpda	a	0.72	0.31	0.28	-0.03	0.38	0.17
00000022717	allc	allantoicase	0.72	0.14	0.30	0.25	0.57	0.42
0000005364	park7	parkinson protein 7	0.75	0.28	0.51	-0.16	-0.30	-0.22
0000003701	c9	complement component 9	0.78	0.36	0.25	0.34	0.10	0.19
00000009160	gpt	glutamic-pyruvate transaminase	0.79	0.22	0.57	-0.05	0.27	-0.03
00000020672	tfa	transferrin-a	0.81	0.51	0.60	0.26	0.12	0.10
00000013136	pbld2	phenazine biosynthesis-like protein domain 2	0.85	0.26	0.65	0.37	0.68	0.56
00000007910	uox	urate oxidase	1.14	0.13	0.45	-0.09	0.08	0.00
00000023717	\	uncharacterized protein	1.14	0.92	0.55	0.13	-0.44	0.29
00000024221	\	uncharacterized protein	1.16	0.68	0.85	0.47	0.26	0.49
0000003019	\	uncharacterized protein	1.24	0.38	-0.31	0.68	0.43	0.46
00000010655	steap4	STEAP family 4	1.49	1.03	1.15	0.68	0.04	0.66
00000015461	epdl1	ependymin-like 1	1.85	0.67	0.81			
00000010644	vcp	valosin containing protein	2.33	2.99	3.07	-0.27	-0.21	-0.40
ENSAMXP00 000020715	eno3	enolase 3				0.82	0.48	0.50
ENSDARP000 00006445	psma8	proteasome subunit, alpha type, 8				-0.03	-0.45	-0.89
ENSDARP000 00012693	pdha1b	pyruvate dehydrogenase alpha 1b				-0.37	-0.63	-0.58
ENSDARP000	fbl	fibrillarin				-0.96	-0.49	-0.66

00070509					
ENSGMOP00	nrd. 5	norovirodovin 5	0.69	0.40	0.40
000014625	pruxs	peroxitedoxili 5	0.08	0.49	0.49
ENSONIP000 00008514	hpgd	hydroxyprostaglandin dehydrogenase 15-(NAD)	0.45	0.48	0.53
ENSONIP000 00018247	hsd17b4	hydroxysteroid dehydrogenase 4	-0.02	0.94	0.03
ENSONIP000	\	uncharacterized protein	-0.05	1.04	-0.06
00018900	ated	oothensin D	0.73	1 1 2	0.02
00000000114	cisu	callepsill D	0.75	1.12	0.92
00000000020	acox5	acyl-COA oxidase 5, pristanoyi	0.28	1.03	0.30
00000000977	\ 	uncharacterized protein	0.10	0.87	0.22
0000001035	rp130a	ribosomai protein L30A	-0.49	-0.55	-0.60
00000001773	taluol	three and the DNA completence like 2	0.75	0.35	0.72
0000001984	tars12	Classin A shifts	-0.88	-1.11	-0.81
00000002469	fina	filamin A, alpha	-0.19	-0.48	-0.52
00000003314	dexr	dicarbonyl/L-xylulose reductase	0.41	0.79	0.34
00000003356	rp136	ribosomal protein L36	-0.62	-0.89	-0.76
00000003722	\	uncharacterized protein	-0.71	-0.69	-0.65
00000003856	\	uncharacterized protein	0.26	0.58	-0.34
00000004493	uqere2b	ubiquinol-cytochrome c reductase core protein IIb	-0.57	0.00	-0.62
00000005223	oxct1b	3-oxoacid CoA transferase 1b	-0.89	-0.10	-0.05
00000005433	sdr16c5a	short chain dehydrogenase 5a	0.43	0.57	0.41
00000005718	cmpk	cytidylate kinase	-1.05	-0.60	-0.64
0000006224	pkmb	pyruvate kinase, muscle, b	-0.04	-0.35	-0.75
0000006341	plin2	perilipin 2	0.23	-0.05	-0.62
0000006439	rpl28	ribosomal protein L28	-0.43	-0.64	-0.41
0000006596	gb	hemoglobin subunit beta-like	0.34	1.95	1.13
0000006739	aldocb	aldolase C, fructose-bisphosphate, b	-0.34	-0.78	-0.46
0000007296	bf/c2	complement factor B	0.74	0.14	0.77
0000007254		ubiquinol-cytochrome c reductase,	0.22	0.60	0.10
00000007354	uqerq	complex III subunit VII	0.23	0.60	-0.10
00000007754	sdha	succinate dehydrogenase complex,flavoprotein	-0.59	-0.31	-0.52
00000007913	mat2ab	methionine adenosyltransferase II,	-0.60	-0.82	-0.93
00000008568	acadvl	acyl-CoA dehydrogenase, very long	-0.94	-0.91	-0.75
	1 14	chain	0.5	0.71	0.70
00000009206	hacll	2-hydroxyacyl-CoA lyase 1	0.06	0.74	0.21
00000009330	hgd	homogentisate 1,2-dioxygenase	0.36	0.68	0.19
00000009413	decr2	2,4-dienoyl CoA reductase 2, peroxisomal	0.41	0.89	0.32
00000009518	pgamla	phosphoglycerate mutase 1a	0.42	0.51	0.54
00000009694	got1	glutamic-oxaloacetic transaminase 1, soluble	-0.52	-0.36	0.38
00000009744	cthl	cystathionase, like	0.35	1.02	0.88
00000011110	\	uncharacterized protein	0.99	1.06	0.82
00000011702	prss16	protease, serine, 16	-0.67	-0.22	-0.48
0000012245	ddw20a	DEAD (Asp-Glu-Ala-Asp) box	0.69	0.40	0.00
0000015245	uux398	polypeptide 39A	-0.08	-0.40	-0.68
00000013519	cuta	cutA divalent cation tolerance	0.67	0.09	0.26
00000013581	fabri 1a	fatty acid hinding protein 11a	-0.45	_1 13	_0 71
00000013301	gush	glucuronidase heta	-0.42	-0.67	-0.06
55555001 (120	0400	Bravaroniaube, ova	J. 14	0.07	0.00

00000014641	mccc1	methylcrotonoyl-CoA carboxylase 1 alpha	-0.01	0.59	0.10
00000014839	eif2s1a	eukaryotic translation initiation factor 2	-0.48	-0.56	-0.78
00000015497	anxa1	annexin max3	0.19	-0.33	-0.52
00000015597	eefldb	eukaryotic translation elongation factor 1 delta b	-0.54	-0.56	-0.64
00000015852	apobb.1	apolipoprotein Bb, tandem duplicate 1	-0.14	-0.20	-0.79
00000015937	dera	deoxyribose-phosphate aldolase	-0.75	-0.49	-1.02
00000015985	naaa	N-acylethanolamine acid amidase	0.34	0.81	0.36
00000016949	fasn	fatty acid synthase	0.21	0.78	-0.64
00000017065	tryp	trypsinogen	1.95	0.76	0.71
00000017507	esd	esterase D/formylglutathione hydrolase	0.44	0.69	0.32
00000018568	cyp27a7	cytochrome P450 27, A7	-0.05	0.35	0.66
00000018701	cyp2ad2	cytochrome P450 2, AD2	1.18	1.10	1.09
00000019361	cs	citrate synthase	-0.20	0.59	-0.16
00000020098	\	uncharacterized protein	0.17	-0.64	-0.67
00000020426	gstz1	glutathione S-transferase zeta 1	0.50	0.85	0.37
00000020543	farsa	phenylalanyl-tRNA synthetase, alpha	-0.44	-0.60	-0.39
00000020660	eif4bb	eukaryotic translation initiation factor 4Bb	-0.68	-0.47	-0.72
00000020671	rpl35	ribosomal protein L35	-0.40	-0.62	-0.51
00000020791	amdhd1	amidohydrolase domain containing 1	0.53	0.91	0.53
00000021981	fubp3	far upstream element binding protein 3	-0.59	0.40	-0.42
00000022083	adh5	alcohol dehydrogenase Class VI	1.28	0.78	0.63
00000022170	gpt2l	glutamic pyruvate transaminase 2, like	0.14	0.58	0.46
00000022695	rps7	ribosomal protein S7	-0.64	0.02	-0.47
00000023077	psmc2	proteasome 26S, ATPase 2	-0.31	-0.83	-0.20
00000023234	gstt1a	glutathione S-transferase theta 1a	-0.24	0.64	0.78
00000024298	pdhb	pyruvate dehydrogenase beta	0.26	0.05	0.50
00000024957	qdpra	quinoid dihydropteridine reductase a	0.36	0.65	0.30
00000024982	\	uncharacterized protein	-0.52	-0.23	-0.81
00000025197	cpne1	copine I	0.32	0.66	-0.06
00000025818	selenbp1	selenium binding protein 1	-0.52	-0.31	-0.40

Annex 4. 3 Complete list of significantly differential expressed genes

Femlae	Cont vs MC1		Base		
id (ENSORLG)	gene name	Description	mean	log ₂ FC	padj
0000001205	f10	coagulation factor X	2043.70	-0.36	0.03
0000004305	bsg	basigin	762.30	-0.39	0.03
0000007438	cideb	cell death-inducing DFFA-like effector b	679.93	-0.54	0.03
00000016451	\	Uncharacterized protein	1118.62	-0.41	0.03
00000019185	\	Uncharacterized protein	1212.94	-0.40	0.02
00000020801	ctsl	Oryzias latipes cathepsin L (ctsl), mRNA.	1333.69	-0.40	0.03

Femlae	Cont vs MC5	Descrip	tion					Base		
id (ENSORLG)	gene name							mean	log ₂ FC	padj
00000015399	nr1d4b	nuclear	receptor	subfamily	1,	group	D,	49.30	-2.33	0.00

		member 4b			
00000012462	cry5	cryptochrome 5	45.88	-1.87	0.00
0000002650	ciarta	circadian associated repressor of transcription	74.72	-1.50	0.00
00000015822	si:dkey- 246g23.4	a si:dkey-246g23.4	195.63	-1.39	0.00
00000009768	cry-dash	cryptochrome DASH	81.13	-1.35	0.00
00000017118	ehd3	EH-domain containing 3	182.88	-1.35	0.00
0000009329	si:ch73-25i22.2	si:ch73-25i22.2	37.86	-1.32	0.00
0000006929	per1b	period circadian clock 1b	397.05	-1.23	0.00
00000014144	tefb	thyrotrophic embryonic factor b	82.17	-1.23	0.00
00000016382	cry1b	cryptochrome circadian clock 1b	60.30	-1.19	0.00
00000015498	hsf2	heat shock factor 2	117.41	-1.18	0.00
00000014803	usp2b	ubiquitin specific peptidase 2b	189.59	-1.16	0.00
0000007662	lox14	lysyl oxidase-like 4	39.87	-1.15	0.00
00000011483	slc25a48	solute carrier family 25, member 48	45.26	-1.14	0.01
00000011028	\	Uncharacterized protein	44.24	-1.13	0.00
00000010962	aspa	aspartoacylase	99.11	-1.12	0.00
00000014640	ZBTB16	zinc finger and BTB domain containing 16	55.40	-1.12	0.01
00000016390	mterf2	mitochondrial transcription termination factor 2	27.35	-1.10	0.00
00000007735	lonrfl	LON peptidase N-terminal domain and ring finger 1	93.29	-1.09	0.00
00000015238	\	Uncharacterized protein	206.31	-1.06	0.00
00000011681	lpin1	lipin 1	667.00	-1.04	0.00
0000005656	slmo2	slowmo homolog 2 (Drosophila)	119.27	-1.02	0.00
0000007741	got1	glutamic-oxaloacetic transaminase 1, soluble	580.98	-1.00	0.00
00000018068	egln3	egl-9 family hypoxia-inducible factor 3	40.31	-1.00	0.03
00000008119	si:ch73- 209e20.3	si:ch73-209e20.3	326.19	-1.00	0.01
00000012977	si:ch73-7i4.2	si:ch73-7i4.2	198.91	-0.98	0.00
00000009215	acsl3a	acyl-CoA synthetase long-chain family member 3a	66.27	-0.96	0.01
00000012573	Clorf50	chromosome 1 open reading frame 50	156.91	-0.94	0.03
00000010295	CIART	circadian associated repressor of transcription	30.97	-0.93	0.01
00000016431	nr1d2a	nuclear receptor subfamily 1, group D, member 2a	461.47	-0.93	0.00
00000016767	PKP4	plakophilin 4	113.84	-0.91	0.00
00000011412	dbpb	D site albumin promoter binding protein b	179.96	-0.90	0.01
00000016287	atraid	all-trans retinoic acid-induced differentiation factor	66.44	-0.89	0.00
00000016102	gpatch4	G patch domain containing 4	56.65	-0.87	0.03
0000007513	padi2	peptidyl arginine deiminase, type II	128.17	-0.86	0.00
00000016123	aspg	asparaginase homolog (S. cerevisiae)	64.54	-0.84	0.03
00000011492	TRIM14	tripartite motif containing 14	235.00	-0.80	0.03
00000010503	usp1	ubiquitin specific peptidase 1	106.46	-0.80	0.00
0000006356	smox	spermine oxidase	543.53	-0.79	0.02
00000018755	gch2	GTP cyclohydrolase 2	204.07	-0.79	0.03
00000014701	cry3	cryptochrome 3	63.16	-0.75	0.01
00000020036	rab44	RAB44, member RAS oncogene family	211.98	-0.75	0.02
00000020377	wdr74	WD repeat domain 74	52.18	-0.72	0.03
00000016195	znf395b	zinc finger protein 395b	177.12	-0.72	0.04
0000006457	ipo4	importin 4	150.08	-0.72	0.00

00000009088	FLVCR2	feline leukemia virus subgroup C cellular receptor family, member 2	1067.2 7	-0.72	0.05
00000017385	ptprb	protein tyrosine phosphatase, receptor type, b	463.03	-0.69	0.00
00000015456	PER2	period circadian clock 2	326.51	-0.69	0.00
0000004239	errfi1	ERBB receptor feedback inhibitor 1	462.43	-0.69	0.03
00000010201	dhrs12	dehydrogenase/reductase (SDR family) member 12	78.75	-0.68	0.04
00000009998	acaca	acetyl-CoA carboxylase alpha	288.96	-0.68	0.04
0000002582	tefa	thyrotrophic embryonic factor a	517.87	-0.67	0.04
00000011586	fzd9b	frizzled class receptor 9b	766.65	-0.66	0.04
00000014616	AKAP13	A kinase (PRKA) anchor protein 13	184.75	-0.65	0.02
00000011011	PPFIBP1	PTPRF interacting protein, binding protein 1 (liprin beta 1)	352.22	-0.65	0.00
00000010698	tm7sf2	transmembrane 7 superfamily member 2	482.68	-0.60	0.04
0000002287	abhd2a	abhydrolase domain containing 2a	293.04	-0.59	0.00
00000016920	GRB14	growth factor receptor-bound protein 14	226.11	-0.59	0.03
00000009917	hprt1	hypoxanthine phosphoribosyltransferase 1	396.32	-0.59	0.00
00000012173	pdk2a	pyruvate dehydrogenase kinase, isozyme 2a	858.09	-0.59	0.01
0000003438	ppp1r1b	protein phosphatase 1, regulatory (inhibitor) subunit 1B	273.53	-0.59	0.02
00000018344	fam65a	family with sequence similarity 65, member A	334.96	-0.59	0.03
0000008263	CTPS1	CTP synthase 1	207.97	-0.59	0.03
00000013059	ddx27	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27	378.39	-0.57	0.03
00000013053	znf598	zinc finger protein 598	172.93	-0.57	0.04
0000003269	ncoa4	nuclear receptor coactivator 4	823.92	-0.57	0.00
0000003342	zzz3	zinc finger, ZZ-type containing 3	196.30	-0.55	0.00
00000015098	ddx5	DEAD (Asp-Glu-Ala-Asp) box helicase 5	877.81	-0.55	0.00
00000020419	si:ch211- 235e9.8	si:ch211-235e9.8	514.50	-0.54	0.00
0000003836	chchd3b	coiled-coil-helix-coiled-coil-helix domain containing 3b	192.93	-0.51	0.03
00000013091	khk	ketohexokinase	707.66	-0.50	0.04
00000013422	argl	arginase 1	1490.4 9	-0.49	0.00
00000005932	lonrf11	LON peptidase N-terminal domain and ring finger 1, like	464.01	-0.48	0.01
0000001724	EXT1	exostosin glycosyltransferase 1	269.17	-0.48	0.05
0000000890	polr1b	polymerase (RNA) I polypeptide B	247.67	-0.48	0.02
00000012493	gls2a	glutaminase 2a (liver, mitochondrial)	739.80	-0.43	0.02
00000001082	\	Guanine nucleotide-binding protein G(i) subunit alpha-2	393.36	0.46	0.04
00000020656	acol	aconitase 1, soluble	856.91	0.54	0.02
00000018951	mthfr	methylenetetrahydrofolate reductase (NAD(P)H)	236.51	0.55	0.05
00000015433	cirbpb	cold inducible RNA binding protein b	662.53	0.55	0.00
0000005368	GOLM1	golgi membrane protein 1	166.51	0.56	0.03
0000003244	tom112	target of myb1-like 2 (chicken)	156.25	0.58	0.04
0000000785	acox1	acyl-CoA oxidase 1, palmitoyl	542.07	0.59	0.01
0000004715	cpn1	carboxypeptidase N, polypeptide 1	211.64	0.59	0.01
0000003686	si:dkey-222f8.3	si:dkey-222f8.3	204.47	0.65	0.00
0000004545	PLEKHA5	pleckstrin homology domain containing, family A member 5	177.55	0.68	0.01
00000015424	cab39	calcium binding protein 39	157.71	0.69	0.00
00000015078	nars	asparaginyl-tRNA synthetase	525.99	0.70	0.01

00000017844	chmp5a	charged multivesicular body protein 5a	41.71	0.71	0.05
0000008721	\	Uncharacterized protein	267.64	0.71	0.00
0000008654	abhd10b	abhydrolase domain containing 10b	74.21	0.73	0.02
0000007922	lztr1	leucine-zipper-like transcription regulator 1	142.84	0.73	0.00
00000012643	klhdc4	kelch domain containing 4	74.88	0.73	0.01
0000006927	arhgap35a	Rho GTPase activating protein 35a	265.08	0.74	0.00
00000013063	vars	valyl-tRNA synthetase	479.55	0.75	0.01
0000005864	esyt2a	extended synaptotagmin-like protein 2a	395.46	0.77	0.00
0000004751	ptk2aa	protein tyrosine kinase 2aa	63.39	0.78	0.03
00000012745	ca2	carbonic anhydrase II	347.18	0.79	0.00
00000016265	KIF13B	kinesin family member 13B	61.56	0.80	0.04
00000010119	SLC6A6	solute carrier family 6 (neurotransmitter transporter), member 6	274.40	0.82	0.00
00000006229	mthfd2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	138.45	0.83	0.01
0000001509	crbn	cereblon	53.82	0.85	0.01
00000010041	fdps	farnesyl diphosphate synthase (farnesyl	271.95	0.86	0.01
		pyrophosphate synthetase, dimethylallyltranstransferase, geranyltranstransferase)			
00000016635	myo10l3	myosin X-like 3	23.20	0.87	0.04
00000020273	slc1a4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	84.69	0.88	0.04
00000013832	wnk1b	WNK lysine deficient protein kinase 1b	203.34	0.89	0.00
00000016750	\	Uncharacterized protein	230.70	0.89	0.00
00000017773	clu	clusterin	198.48	0.90	0.03
0000002409	lancl1	LanC antibiotic synthetase component C-like 1 (bacterial)	97.50	0.91	0.01
0000003753	HMOX1	heme oxygenase 1	191.34	0.91	0.03
00000018133	hivep2a	human immunodeficiency virus type I enhancer binding protein 2a	56.25	0.91	0.02
00000014572	ccl34b.3	chemokine (C-C motif) ligand 34b, duplicate 3	20.35	0.92	0.03
00000015374	klf8	Kruppel-like factor 8	68.17	0.92	0.00
00000014663	SYNE1	spectrin repeat containing, nuclear envelope 1	115.93	0.94	0.00
00000011746	spsb3a	splA/ryanodine receptor domain and SOCS box containing 3a	28.43	0.94	0.02
00000004495	clocka	clock circadian regulator a	93.34	0.95	0.00
00000015345	si:ch211- 244b2.4	si:ch211-244b2.4	32.56	0.95	0.04
00000016725	selp	selectin P	17.55	0.96	0.04
00000015663	cd83	si:ch211-149p10.2	21.72	0.96	0.04
0000000313	lygl1	lysozyme g-like 1	174.43	0.96	0.03
0000006140	herc7	hect domain and RLD 7	48.33	0.96	0.01
0000005096	\	Uncharacterized protein	141.53	0.96	0.01
0000001444	ttc7a	tetratricopeptide repeat domain 7A	21.56	0.97	0.03
00000016158	tap 1	transporter 1, ATP-binding cassette, sub- family B (MDR/TAP)	147.66	0.97	0.04
0000002786	pvrl4	poliovirus receptor-related 4	26.30	0.98	0.01
0000004691	cd82b	CD82 molecule b	57.91	0.98	0.00
00000013204	sypl1	synaptophysin-like 1	14.72	0.99	0.03
0000003196	sqlea	squalene epoxidase a	376.54	0.99	0.00
0000008715	socs1b	suppressor of cytokine signaling 1b	44.38	1.00	0.03
00000011909	etv4	ets variant 4	34.05	1.00	0.00

00000012898	hmgcs1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	391.30	1.00	0.01
00000004882	rbpja	recombination signal binding protein for immunoglobulin kappa I region a	34.77	1.00	0.00
00000013720	ERC2	ELKS/RAB6-interacting/CAST family member 2	61.71	1.00	0.00
0000006506	GTPBP2	GTP binding protein 2	74.91	1.01	0.00
00000013878	aclyb	ATP citrate lyase b	142.58	1.02	0.00
0000001447	aacs	acetoacetyl-CoA synthetase	61.53	1.02	0.01
0000005073	\	Uncharacterized protein	25.29	1.03	0.02
0000004218	uck1	uridine-cytidine kinase 1	24.01	1.03	0.00
0000010346	tgif1	TGFB-induced factor homeobox 1	28.15	1.05	0.01
0000009764	\	Uncharacterized protein	47.51	1.06	0.01
00000006618	abcb3	Oryzias latipes antigen peptide transporter 2- like (abcb3), mRNA.	102.03	1.08	0.01
00000011239	nme4	NME/NM23 nucleoside diphosphate kinase 4	252.68	1.08	0.01
0000007786	TLN2	talin 2	38.82	1.09	0.01
0000001248	TRIM14	tripartite motif containing 14	28.00	1.09	0.01
0000002792	pld1b	phospholipase D1b	37.39	1.11	0.00
0000003293	rwdd2b	RWD domain containing 2B	15.20	1.13	0.00
00000014397	dec1b	Oryzias latipes bHLH protein DEC1b (dec1b), mRNA.	373.76	1.14	0.00
0000007252	im:7160594	im:7160594	23.44	1.16	0.00
0000000447	clip3	CAP-GLY domain containing linker protein 3	16.40	1.16	0.01
0000001079	\	Uncharacterized protein	26.34	1.20	0.00
0000003644	\	Uncharacterized protein	17.98	1.20	0.00
00000011245	eps811	eps8-like1	33.29	1.22	0.00
00000009245	LONRF3	LON peptidase N-terminal domain and ring finger 3	40.76	1.23	0.00
00000013771	erc1b	ELKS/RAB6-interacting/CAST family member 1b	270.64	1.24	0.00
00000012335	nav3	neuron navigator 3	28.81	1.28	0.00
00000010486	adgrv1	adhesion G protein-coupled receptor V1	74.04	1.32	0.00
00000014730	nfil3-5	nuclear factor, interleukin 3 regulated, member 5	16.92	1.33	0.00
0000009825	\	Uncharacterized protein	48.18	1.34	0.00
00000011116	sesn2	sestrin 2	26.97	1.34	0.00
00000016718	\	Uncharacterized protein	18.76	1.38	0.00
0000009426	npas2	neuronal PAS domain protein 2	64.25	1.44	0.00
00000001978	agpat91	1-acylglycerol-3-phosphate O-acyltransferase 9, like	29.66	1.45	0.00
00000019370	arntlla	aryl hydrocarbon receptor nuclear translocator- like 1a	80.38	1.57	0.00
0000006377	si:dkey- 238d18.4	si:dkey-238d18.4	18.25	1.60	0.00
00000016569	mknk1	MAP kinase interacting serine/threonine kinase 1	80.95	1.61	0.00
0000001131	irf1b	interferon regulatory factor 1b	184.86	1.75	0.00
00000010946	ARNTL2	aryl hydrocarbon receptor nuclear translocator- like 2	20.45	1.90	0.00
00000014886	rorcb	RAR-related orphan receptor C b	88.21	2.28	0.00

Femlae	Cont vs Ext5		Base		
Id (ENSORLG)	Gene name	Description	mean	log ₂ FC	padj
0000000234	fryl	turry homolog, like	/28./1	0.34	0.01
0000000325	trim33	tripartite motif containing 33	179.98	-0.65	0.03
00000000592	kdr	kinase insert domain receptor (a type III receptor tyrosine kinase)	89.17	-0.62	0.01
0000000785	acox1	acyl-CoA oxidase 1, palmitoyl	575.46	0.70	0.00
0000000890	polr1b	polymerase (RNA) I polypeptide B	250.97	-0.49	0.04
00000001152	/	Uncharacterized protein	43.14	-0.72	0.02
00000001254	ACSBG1	acyl-CoA synthetase bubblegum family member 1	364.66	-0.69	0.00
00000001278	plekha7a	pleckstrin homology domain containing, family A member 7a	258.35	0.39	0.03
0000001284	NAALADL1	N-acetylated alpha-linked acidic dipeptidase- like 1	474.69	0.66	0.00
0000001298	sytl4	synaptotagmin-like 4	161.79	-1.05	0.00
0000001313	sept8a	septin 8a	182.83	0.49	0.03
0000001348	mid1ip11	MID1 interacting protein 1, like	558.61	-0.88	0.00
0000001414	impact	impact RWD domain protein	162.93	-0.54	0.02
0000001477	isg20	interferon stimulated exonuclease gene	43.41	-1.26	0.00
0000001498	abhd2b	abhydrolase domain containing 2b	80.18	0.56	0.05
0000001573	bcat2	branched chain amino-acid transaminase 2, mitochondrial	162.21	-0.92	0.00
0000001581	klc3	kinesin light chain 3	62.66	0.63	0.02
0000001642	xpola	exportin 1 (CRM1 homolog, yeast) a	135.21	0.73	0.04
0000001693	oaz2a	ornithine decarboxylase antizyme 2a	330.86	-0.72	0.00
0000001913	dlg3	discs, large homolog 3 (Drosophila)	344.80	0.49	0.00
00000001940	kdrl	kinase insert domain receptor like	123.73	-0.62	0.01
0000001978	agpat91	1-acylglycerol-3-phosphate O-acyltransferase 9, like	22.46	1.07	0.00
0000001981	SLC25A5	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	400.37	-0.41	0.04
0000002004	hif1al	hypoxia-inducible factor 1, alpha subunit, like	778.05	-1.35	0.00
0000002005	nrp1b	neuropilin 1b	237.93	0.74	0.00
0000002021	skiv2l	superkiller viralicidic activity 2-like (S. cerevisiae)	100.74	0.49	0.04
0000002070	/	Uncharacterized protein	42.65	0.94	0.00
0000002078	ptdss2	phosphatidylserine synthase 2	148.21	1.37	0.00
0000002146	ppm1h	protein phosphatase, Mg2+/Mn2+ dependent, 1H	109.94	0.72	0.00
0000002216	dnajc4	DnaJ (Hsp40) homolog, subfamily C, member 4	133.62	-0.70	0.00
0000002221	\	Uncharacterized protein	48.75	0.64	0.05
0000002325	\	Clathrin heavy chain	25.70	0.75	0.05
0000002446	cdon	cell adhesion associated, oncogene regulated	113.08	0.67	0.02
0000002456	lonp1	lon peptidase 1, mitochondrial	278.39	-0.42	0.03
0000002582	tefa	thyrotrophic embryonic factor a	534.82	-0.67	0.00
0000002650	ciarta	circadian associated repressor of transcription a	83.79	-1.22	0.00
0000002786	pvrl4	poliovirus receptor-related 4	30.40	1.15	0.00
0000002792	pld1b	phospholipase D1b	32.97	0.93	0.01
0000002877	rsl1d1	ribosomal L1 domain containing 1	146.75	-0.51	0.02
0000002886	dmxl2	Dmx-like 2	211.85	0.68	0.00
0000003075	TEP1	telomerase-associated protein 1	64.28	0.67	0.02
0000003101	si:ch211- 170n20.3	si:ch211-170n20.3	626.56	-0.47	0.04

0	0000003103	si:ch211- 170n20.3	si:ch211-170n20.3	1032.20	-0.48	0.03
0	0000003162	nop58	NOP58 ribonucleoprotein homolog (yeast)	183.43	-0.55	0.01
0	0000003234	\	Uncharacterized protein	407.69	0.69	0.00
0	0000003244	tom112	target of myb1-like 2 (chicken)	190.80	0.96	0.00
0	0000003269	ncoa4	nuclear receptor coactivator 4	898.35	-0.36	0.00
0	0000003342	zzz3	zinc finger, ZZ-type containing 3	202.77	-0.51	0.01
0	0000003390	cdo1	cysteine dioxygenase, type I	682.05	-0.58	0.02
0	0000003438	ppp1r1b	protein phosphatase 1, regulatory (inhibitor) subunit 1B	295.43	-0.43	0.01
0	0000003572	TMEM102	transmembrane protein 102	43.61	0.81	0.03
0	0000003577	EGFR	epidermal growth factor receptor	921.99	-0.64	0.02
0	0000003706	zgc:136858	zgc:136858	116.22	0.63	0.00
0	0000003724	cnp	2',3'-cyclic nucleotide 3' phosphodiesterase	358.74	0.57	0.01
0	0000003734	NFIX	nuclear factor I/X (CCAAT-binding transcription factor)	148.68	0.58	0.01
0	0000003753	HMOX1	heme oxygenase 1	214.81	1.18	0.00
0	0000003765	rorca	RAR-related orphan receptor C a	156.29	0.72	0.03
0	000003836	chchd3b	coiled-coil-helix-coiled-coil-helix domain containing 3b	194.86	-0.54	0.02
0	000003838	pank1a	pantothenate kinase 1a	260.74	-0.58	0.00
0	0000003931	gpr137c	G protein-coupled receptor 137c	81.24	0.69	0.02
0	0000003939	cdc14b	cell division cycle 14B	221.02	0.80	0.00
0	0000003980	surf6	surfeit 6	145.54	-0.60	0.01
0	0000004003	ankrd12	ankyrin repeat domain 12	183.06	0.45	0.04
0	0000004109	ubr4	ubiquitin protein ligase E3 component n-recognin 4	893.53	0.51	0.02
0	0000004368	tcea3	transcription elongation factor A (SII), 3	154.11	0.70	0.02
0	0000004442	nr0b2a	nuclear receptor subfamily 0, group B, member 2a	332.53	0.63	0.04
0	0000004461	slc12a7b	solute carrier family 12 (potassium/chloride transporter), member 7b	581.42	-0.64	0.01
0	0000004495	clocka	clock circadian regulator a	86.49	0.79	0.01
0	0000004514	SRCIN1	SRC kinase signaling inhibitor 1	249.26	0.86	0.00
0	0000004545	PLEKHA5	pleckstrin homology domain containing, family A member 5	195.38	0.82	0.00
0	0000004563	bcl6ab	B-cell CLL/lymphoma 6a, genome duplicate b	35.14	-0.78	0.04
0	0000004591	ndfip2	Nedd4 family interacting protein 2	485.11	0.30	0.04
0	0000004610	creb3l3a	cAMP responsive element binding protein 3-like 3a	462.82	0.49	0.01
0	0000004691	cd82b	CD82 molecule b	51.19	0.78	0.00
0	0000004715	cpn1	carboxypeptidase N, polypeptide 1	245.49	0.84	0.00
0	0000004734	stoml3b	stomatin (EPB72)-like 3b	80.31	-0.72	0.01
0	0000004762	dusp7	dual specificity phosphatase 7	117.08	0.84	0.00
0	0000004810	me2	malic enzyme 2, NAD(+)-dependent, mitochondrial	32.29	-0.88	0.01
0	0000004926	tsr1	TSR1, 20S rRNA accumulation, homolog (S. cerevisiae)	321.11	-0.66	0.00
0	0000004930	mal2	mal, T-cell differentiation protein 2 (gene/pseudogene)	42.25	0.71	0.03
0	0000005102	col2a1b	collagen, type II, alpha 1b	48.03	1.14	0.00
0	0000005192	jam3a	junctional adhesion molecule 3a	123.47	-0.65	0.03
0	0000005200	plce1	phospholipase C, epsilon 1	203.82	0.73	0.00
0	0000005224	igsf8	immunoglobulin superfamily, member 8	122.15	0.70	0.02

0000005226	soat2	sterol O-acyltransferase 2	409.59	0.47	0.02
0000005250	raph1b	Ras association (RalGDS/AF-6) and pleckstrin	268.84	0.63	0.01
		homology domains 1b			
0000005289	cobl	cordon-bleu WH2 repeat protein	281.49	0.42	0.04
0000005315	grb10b	growth factor receptor-bound protein 10b	68.98	-0.74	0.01
0000005327	hnrnpaba	heterogeneous nuclear ribonucleoprotein A/Ba	380.77	-0.74	0.00
00000005471	plcb3	phospholipase C, beta 3 (phosphatidylinositol-	147.70	0.79	0.00
		specific)			
00000005521	MAST3	microtubule associated serine/threonine kinase 3	121.13	0.46	0.04
00000005522	PRRC2B	proline-rich coiled-coil 2B	382.82	0.40	0.03
0000005634	ppan	peter pan homolog (Drosophila)	194.72	-0.71	0.00
0000005656	slmo2	slowmo homolog 2 (Drosophila)	129.16	-0.82	0.00
0000005757	slc25a33	solute carrier family 25 (pyrimidine nucleotide	654.39	-0.44	0.05
		carrier), member 33			
0000005783	NCOA2	nuclear receptor coactivator 2	271.06	0.62	0.01
00000005796	timm17a	translocase of inner mitochondrial membrane 17	103.38	-0.59	0.02
0000005040	1 1 10	homolog A (yeast)	07 (2	0.50	0.02
0000005840	mpnosph10	m-phase phosphoprotein 10 (03 small nucleolar	87.63	-0.59	0.03
0000005864	esyt2a	extended synaptotagmin-like protein 2a	397 30	0.78	0.00
00000005804	csyt2a	sulfotransferase family 1 cytosolic	185.82	0.70	0.00
00000003993	Sullisto	sulfotransferase 6	405.02	0.55	0.05
0000006127	rab3il1	RAB3A interacting protein (rabin3)-like 1	31.51	-0.95	0.01
0000006140	herc7	hect domain and RLD 7	46.61	0.91	0.00
0000006208	PITPNM2	phosphatidylinositol transfer protein.	22.39	0.79	0.03
		membrane-associated 2			
0000006215	ctbs	chitobiase, di-N-acetyl-	231.80	0.73	0.02
0000006259	minppla	multiple inositol-polyphosphate phosphatase 1a	887.52	-0.52	0.04
0000006274	PNPLA2	patatin-like phospholipase domain containing 2	204.70	-0.78	0.01
0000006279	nmd3	NMD3 ribosome export adaptor	778.39	-0.42	0.03
0000006281	pck2	phosphoenolpyruvate carboxykinase 2	2369.99	-0.35	0.04
	-	(mitochondrial)			
0000006330	stx1a	syntaxin 1A (brain)	41.82	0.75	0.05
0000006475	SLC19A1	solute carrier family 19 (folate transporter),	804.29	-0.31	0.03
000000000000	1 0 5 0 0	member 1	1.4.4.02	0.50	0.01
0000006483	slc25a22	solute carrier family 25 (mitochondrial carrier:	144.93	0.73	0.01
0000006506	GTDDD)	GTP hinding protein 2	66 15	0.73	0.01
00000000500	ologra	CLVA according corrigo/orgining rich protein	00.45	0.75	0.01
000000000000000000000000000000000000000	clasip	cLK4-associating serine/arginine fich protein	03.30	0.75	0.01
0000000098	sicz/ala	member 1a	270.31	0.39	0.02
0000006705	sox6	SRY (sex determining region Y)-box 6	71 38	0.62	0.05
00000006720	ADGRE5	adhesion G protein-coupled receptor E5	25.69	-0.88	0.03
0000006737	cvp2r1	cytochrome P450 family 2 subfamily R	147 64	1.07	0.00
0000000757	cyp211	polypeptide 1	117.01	1.07	0.00
0000006747	phldb2b	pleckstrin homology-like domain, family B,	336.24	0.63	0.03
	-	member 2b			
0000006844	ankrd33ba	ankyrin repeat domain 33ba	121.58	0.88	0.00
0000006847	gpd1b	glycerol-3-phosphate dehydrogenase 1b	149.92	0.50	0.03
0000006916	st5	suppression of tumorigenicity 5	170.00	0.64	0.02
0000006927	arhgap35a	Rho GTPase activating protein 35a	301.93	0.99	0.00
0000006929	per1b	period circadian clock 1b	457.70	-0.78	0.00
0000006939	si:dkey-8k3.2	si:dkey-8k3.2	3672.38	-0.46	0.03
0000007028	prkacaa	protein kinase, cAMP-dependent, catalytic,	253.69	-0.56	0.03
		alpha, genome duplicate a			

0000007055	ncl	nucleolin	1289.77	-0.34	0.00
00000007092	mrpl12	mitochondrial ribosomal protein L12	133.11	-0.54	0.01
0000007252	im:7160594	im:7160594	26.05	1.40	0.00
0000007311	lzts2a	leucine zipper, putative tumor suppressor 2a	163.78	0.90	0.00
0000007357	sptbn2	spectrin, beta, non-erythrocytic 2	735.33	0.57	0.00
0000007366	CIART	circadian associated repressor of transcription	259.72	-0.77	0.00
0000007373	cdc42ep4a	CDC42 effector protein (Rho GTPase binding)	78.52	0.78	0.00
0000007473	slc2a1b	solute carrier family 2 (facilitated glucose transporter) member 1b	71.00	-0.96	0.01
0000007479	march8	membrane-associated ring finger (C3HC4) 8	99.77	0.60	0.02
0000007483	rab11fip3	RAB11 family interacting protein 3 (class II)	199.51	0.55	0.02
0000007543		Uncharacterized protein	283.26	0.52	0.03
00000007662	lox14	lysyl oxidase-like 4	45.62	-0.75	0.02
0000007664	lrba	LPS-responsive vesicle trafficking beach and	74 67	0.68	0.02
0000007001	nou	anchor containing	/ 1.0 /	0.00	0.02
00000007689	timp-2b	tissue inhibitor of metalloproteinase-2b	215.02	-0.84	0.02
0000007735	lonrfl	LON peptidase N-terminal domain and ring finger 1	108.48	-0.58	0.03
0000007761	usp36	ubiquitin specific peptidase 36	312.24	-0.76	0.00
0000007786	TLN2	talin 2	52.89	1.22	0.00
0000007794	phkg2	phosphorylase kinase, gamma 2 (testis)	72.24	-0.77	0.00
0000007888	fgd5a	FYVE, RhoGEF and PH domain containing 5a	72.12	-0.63	0.04
0000007922	lztr1	leucine-zipper-like transcription regulator 1	142.51	0.69	0.00
00000007930	mast3b	microtubule associated serine/threonine kinase	125.06	0.78	0.03
0000008013	zgc:153372	zgc:153372	71.44	0.58	0.04
0000008021	pld2	phospholipase D2	35.47	0.75	0.02
0000008044	gltpd2	glycolipid transfer protein domain containing 2	236.20	-0.50	0.02
0000008058	csad	cysteine sulfinic acid decarboxylase	71.24	0.85	0.02
00000008111	slc46a1	solute carrier family 46 (folate transporter).	63.28	0.75	0.01
0000000111	510.0001	member 1	00.20	0.70	0.01
00000008119	si:ch73- 209e20.3	si:ch73-209e20.3	309.01	-1.59	0.00
0000008138	fatla	FAT atypical cadherin 1a	516.97	0.76	0.00
0000008236	abhd15a	abhydrolase domain containing 15a	226.90	-1.18	0.00
0000008263	CTPS1	CTP synthase 1	215.87	-0.54	0.02
0000008275	glis2b	GLIS family zinc finger 2b	25.19	0.87	0.02
0000008402	dgcr2	DiGeorge syndrome critical region gene 2	311.19	0.44	0.05
0000008403	chd2	chromodomain helicase DNA binding protein 2	254.81	0.67	0.00
0000008453	upfl	upfl regulator of nonsense transcripts homolog (veast)	531.40	0.37	0.03
00000008457	SGK3	serum/glucocorticoid regulated kinase family, member 3	38.79	-0.84	0.01
0000008489	mef2aa	myocyte enhancer factor 2aa	139.63	-0.71	0.00
0000008624	epg5	ectopic P-granules autophagy protein 5 homolog (C. elegans)	206.10	0.57	0.01
0000008661	riok2	RIO kinase 2 (yeast)	197.12	-0.54	0.01
0000008698	spata13	spermatogenesis associated 13	455.83	-0.43	0.02
0000008721	\	Uncharacterized protein	287.45	0.82	0.00
0000008753	polrla	polymerase (RNA) I polypeptide A	252.98	-0.61	0.00
0000008796	ubap2a	ubiquitin associated protein 2a	284.12	0.65	0.00
0000008833	nop56	NOP56 ribonucleoprotein homolog	917.63	-0.46	0.00
1	-				

0000008838	eif4a?	eukarvotic translation initiation factor 4A	707 97	-0.38	0.02
0000000000000	011 142	isoform 2	101.91	0.50	0.02
00000009023	foxk1	Oryzias latipes forkhead box K1 (LOC100301617) mRNA	253.28	-0.68	0.00
00000009024	mknk2a	MAP kinase interacting serine/threonine kinase	143.66	-0.81	0.03
00000009088	FLVCR2	feline leukemia virus subgroup C cellular	1095.42	-0.77	0.00
00000009141	miox	myo-inositol oxygenase	1176 23	0.95	0.00
00000009156	linea	linase hormone-sensitive a	94 99	-1 43	0.00
00000009165	ananc16	anaphase promoting complex subunit 16	62 39	-0.84	0.00
00000009105	acel3a	acyl-CoA synthetase long-chain family member	68.05	-1.00	0.00
000000000000000000000000000000000000000	ues15u	3a	00.05	1.00	0.00
00000009245	LONRF3	LON peptidase N-terminal domain and ring finger 3	55.52	1.65	0.00
00000009269	abcb4	ATP-binding cassette, sub-family B (MDR/TAP), member 4	67.64	0.65	0.03
0000009329	si:ch73- 25i22.2	si:ch73-25i22.2	36.66	-1.49	0.00
0000009421	\	Uncharacterized protein	118.18	0.60	0.01
0000009426	npas2	neuronal PAS domain protein 2	59.65	1.41	0.00
0000009432	EIF4G1	eukaryotic translation initiation factor 4 gamma,	207.07	0.59	0.00
		1			
0000009480	adka	adenosine kinase a	703.43	-0.71	0.00
0000009542	gse1	Gse1 coiled-coil protein	151.52	0.56	0.03
0000009573	lmtk2	lemur tyrosine kinase 2	671.44	0.55	0.01
0000009601	hgfb	hepatocyte growth factor b	135.84	-1.05	0.00
0000009627	nt5c2l1	5'-nucleotidase, cytosolic II, like 1	71.86	-0.84	0.00
0000009673	uckl1b	uridine-cytidine kinase 1-like 1b	59.00	-0.66	0.03
0000009690	ksr1a	kinase suppressor of ras 1a	119.27	0.82	0.02
00000009715	slc43a2a	solute carrier family 43 (amino acid system L	74.07	-1.08	0.00
0000000768	any dash	cruptochrome DASH	85 73	1 / 2	0.00
0000009708	cry-uasii	dyptoenione DASH	05.75	-1.45	0.00
0000009814	usp2a	ubiquitin specific pentidese 2a	204.70	1.30	0.00
00000009912	usp2a hprt1	humoventhing phosphorihogultransferaça 1	417.70	-1.50	0.00
00000009917	tan	translin	417.70	-0.49	0.00
00000010049	tSII nlumh 1 h	uansiin nlovin h1h	91.09 206.10	-0.55	0.05
00000010071		plexil 010	200.19	0.01	0.00
0000010119	SLCOAO	transporter) member 6	251.05	0.00	0.01
0000010256	LAMB3	laminin beta 3	309 30	-1 25	0.00
00000010295	CIART	circadian associated repressor of transcription	31.52	-0.91	0.01
00000010299	ambn	alpha-1-microglobulin/bikunin precursor	256 79	-0.62	0.01
00000010320	nfat5a	nuclear factor of activated T-cells 5 tonicity-	144.09	0.02	0.01
00000010343	inat5a	responsive a	1 (0, 02	0.71	0.00
00000010363	tnpo2	transportin 2 (importin 3, karyopherin beta 2b)	168.03	-0.67	0.02
00000010486	adgrvl	adhesion G protein-coupled receptor V1	71.49	1.30	0.00
00000010503	uspl	ubiquitin specific peptidase 1	101.80	-0.97	0.00
0000010690	klt9	Kruppel-like factor 9	196.97	-1.15	0.00
00000010729	ches1	checkpoint suppressor 1	78.71	-1.83	0.00
00000010812	eef2k	eukaryotic elongation factor 2 kinase	507.81	-1.29	0.00
00000010819	UBE2H	ubiquitin-conjugating enzyme E2H	152.17	-0.88	0.00
00000010829	MAF1	MAF1 homolog (S. cerevisiae)	176.58	0.58	0.01
00000010895	asmtl	acetylserotonin O-methyltransferase-like	273.24	0.43	0.03

00000010915	urb2	URB2 ribosome biogenesis 2 homolog (S. cerevisiae)	173.08	-0.55	0.02
00000010943	GPSM2	G-protein signaling modulator 2	162.21	-0.83	0.01
00000010960	galnt2	UDP-N-acetyl-alpha-D-	76.70	0.68	0.00
		galactosamine:polypeptide N-			
00000100/0		acetylgalactosaminyltransferase 2	114.00	0.51	0.00
00000010962	aspa	aspartoacylase	114.23	-0.71	0.02
00000011011	PPFIBP1	PTPRF interacting protein, binding protein 1 (liprin beta 1)	345.34	-0.80	0.00
00000011028	\	Uncharacterized protein	47.63	-0.93	0.00
00000011065	SLC29A2	solute carrier family 29 (equilibrative	765.50	-0.68	0.00
		nucleoside transporter), member 2			
00000011067	adpgk2	ADP-dependent glucokinase 2	167.46	0.48	0.03
00000011077	si:ch211- 105f12.2	si:ch211-105f12.2	81.14	0.59	0.02
00000011100	\	Serine/threonine-protein phosphatase	420.06	-0.41	0.02
00000011104	rela	v-rel avian reticuloendotheliosis viral oncogene	186.32	-0.65	0.03
		homolog A			
00000011116	sesn2	sestrin 2	24.14	1.15	0.00
00000011156	bmpla	bone morphogenetic protein 1a	257.44	-0.53	0.00
00000011157	\	Uncharacterized protein	170.06	0.83	0.00
00000011238	MTMR4	myotubularin related protein 4	198.24	0.97	0.00
00000011239	nme4	NME/NM23 nucleoside diphosphate kinase 4	215.63	1.03	0.00
00000011245	eps811	eps8-like1	32.24	1.70	0.00
00000011255	ptprk	protein tyrosine phosphatase, receptor type, K	113.69	0.71	0.00
00000011263	tfb2m	transcription factor B2, mitochondrial	159.21	-0.56	0.01
00000011274	gusb	glucuronidase, beta	356.18	-0.43	0.05
00000011313	eea1	early endosome antigen 1	250.75	0.60	0.00
00000011321	AKR1D1	aldo-keto reductase family 1, member D1	61.90	0.83	0.01
00000011323	plekhg7	pleckstrin homology domain containing, family G (with RhoGef domain) member 7	270.31	-0.84	0.00
00000011327	\	Uncharacterized protein	52.78	-0.90	0.00
00000011398	porb	P450 (cytochrome) oxidoreductase b	1380.58	-0.35	0.02
00000011412	dbpb	D site albumin promoter binding protein b	177.43	-1.19	0.00
00000011464	gdpd1	glycerophosphodiester phosphodiesterase	61.08	-0.89	0.00
		domain containing 1			
00000011491	mycbp2	MYC binding protein 2	298.97	0.53	0.00
00000011518	sema4gb	sema domain, immunoglobulin domain (Ig),	129.26	-1.16	0.00
		transmembrane domain (TM) and short			
00000011550	a a dh 1 h	cytoplasmic domain, (semaphorin) 4Gb	241 40	0.65	0.01
00000011559		protocadierin 10	541.40 77.95	0.03	0.01
0000001150/	SIIX I 8D	Softing nextri 180	77.85	-0.70	0.03
00000011081	ipini nldla	IIPIII I nhaanhalinaaa Dla	/0/.80	-0.04	0.01
00000011699		phospholipase DTa	101.30	0.92	0.00
00000011706	ubp1	upstream binding protein 1 (LBP-1a)	344.66	-0.70	0.00
0000011/46	sps03a	containing 3a	25.90	0.80	0.03
00000011807	\	Uncharacterized protein	195.86	-0.54	0.03
00000011834	\	Mothers against decapentaplegic homolog	64.22	0.71	0.03
00000011909	etv4	ets variant 4	30.15	0.76	0.03
00000011915	ypela	Oryzias latipes yippee-like a (ypela), mRNA.	139.72	0.64	0.02
00000011992	ncor1	nuclear receptor corepressor 1	972.88	0.87	0.00
00000012037	fnbp11	formin binding protein 1-like	187.93	-0.50	0.01
00000012082	musk	muscle, skeletal, receptor tyrosine kinase	166.91	0.65	0.00

00000012106	slc41a1	solute carrier family 41 (magnesium transporter), member 1	850.70	-0.75	0.00
00000012128	ezrb	ezrin b	215.31	-1.15	0.00
00000012164	slc4a2b	solute carrier family 4 (anion exchanger), member 2b	335.02	0.44	0.03
00000012237	si:ch211- 117k10.3	si:ch211-117k10.3	45.24	-0.88	0.00
00000012247	slc30a1a	solute carrier family 30 (zinc transporter), member 1a	70.47	-0.67	0.04
00000012307	zfand5a	zinc finger, AN1-type domain 5a	330.00	-0.91	0.01
00000012335	nav3	neuron navigator 3	26.46	1.08	0.00
00000012410	sec1411	SEC14-like 1 (S. cerevisiae)	535.77	-0.68	0.00
00000012462	cry5	cryptochrome 5	53.48	-1.34	0.00
00000012468	cygb1	cytoglobin 1	135.77	0.75	0.00
00000012541	lcat	lecithin-cholesterol acyltransferase	670.25	-0.60	0.00
00000012552	qkia	QKI, KH domain containing, RNA binding a	86.35	0.68	0.01
00000012573	Clorf50	chromosome 1 open reading frame 50	157.06	-1.12	0.00
00000012594	ca5a	carbonic anhydrase Va	785.14	-0.84	0.00
00000012652	ELL3	elongation factor RNA polymerase II-like 3	148.02	-0.69	0.01
00000012686	egln1a	egl-9 family hypoxia-inducible factor 1a	127.16	-0.86	0.00
00000012723	DYNC1H1	dynein, cytoplasmic 1, heavy chain 1	1230.51	0.49	0.02
00000012731	rock2b	rho-associated, coiled-coil containing protein kinase 2b	308.15	0.55	0.02
00000012739	decla	Oryzias latipes bHLH protein DEC1a (dec1a), mRNA.	111.41	-0.97	0.00
00000012745	ca2	carbonic anhydrase II	408.58	1.04	0.00
00000012746	TRPM7	transient receptor potential cation channel, subfamily M, member 7	429.68	0.58	0.02
00000012799	tmem64	transmembrane protein 64	232.86	-0.73	0.00
00000012805	pck1	phosphoenolpyruvate carboxykinase 1 (soluble)	1476.10	-1.01	0.00
00000012977	si:ch73-7i4.2	si:ch73-7i4.2	205.99	-0.84	0.01
00000013059	ddx27	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27	363.56	-0.80	0.00
00000013382	tox	thymocyte selection-associated high mobility group box	28.68	-1.33	0.00
00000013415	slco2b1	solute carrier organic anion transporter family, member 2B1	104.67	0.63	0.02
00000013422	argl	arginase 1	1576.64	-0.37	0.00
00000013450	foxo3b	forkhead box O3b	477.29	-0.53	0.02
00000013474	tjp2a	tight junction protein 2a (zona occludens 2)	414.76	0.50	0.03
00000013489	pwp1	PWP1 homolog (S. cerevisiae)	243.27	-0.43	0.03
00000013621	pl10	pl10	1149.46	-0.47	0.01
00000013626	dpydb	dihydropyrimidine dehydrogenase b	3850.33	-0.48	0.00
00000013720	ERC2	ELKS/RAB6-interacting/CAST family member 2	61.22	0.96	0.00
00000013771	erc1b	ELKS/RAB6-interacting/CAST family member 1b	249.29	1.15	0.00
00000013802	phf20a	PHD finger protein 20, a	194.36	-0.51	0.01
00000013821	ndrg3a	ndrg family member 3a	84.70	-0.75	0.03
00000013832	wnk1b	WNK lysine deficient protein kinase 1b	254.16	1.26	0.00
00000013878	aclyb	ATP citrate lyase b	134.11	0.94	0.00
00000013965	ret	ret proto-oncogene receptor tyrosine kinase	39.53	0.90	0.02
00000013995	zfpm1	zinc finger protein, FOG family member 1	114.26	0.60	0.01
00000013999	apoa2	apolipoprotein A-II	10112.34	-0.38	0.00
00000014083	chadla	chondroadherin-like a	64.78	-0.62	0.03

00000014144	tefb	thyrotrophic embryonic factor b	79.87	-1.46	0.00
00000014181	PMM1	phosphomannomutase 1	166.01	-0.96	0.00
00000014292	gdpd5b	glycerophosphodiester phosphodiesterase domain containing 5b	76.62	-0.71	0.04
00000014324	tspan33	tetraspanin 33	57.81	0.89	0.00
00000014373	pi4k2b	phosphatidylinositol 4-kinase type 2 beta	205.09	-0.52	0.03
00000014397	dec1b	Oryzias latipes bHLH protein DEC1b (dec1b),	439.26	1.41	0.00
		mRNA.			
00000014443	dock5	dedicator of cytokinesis 5	114.89	0.84	0.00
00000014522	evalba	eva-1 homolog Ba (C. elegans)	268.09	-0.73	0.00
00000014554	JMJD1C	jumonji domain containing 1C	195.32	-0.79	0.00
00000014603	ralgps2	Ral GEF with PH domain and SH3 binding motif 2	276.11	-0.43	0.03
00000014616	AKAP13	A kinase (PRKA) anchor protein 13	188.71	-0.67	0.00
00000014640	ZBTB16	zinc finger and BTB domain containing 16	54.74	-1.49	0.00
00000014663	SYNE1	spectrin repeat containing, nuclear envelope 1	143.45	1.16	0.00
00000014672	srebf2	sterol regulatory element binding transcription factor 2	394.61	0.52	0.03
00000014701	cry3	cryptochrome 3	68.44	-0.57	0.03
00000014792	tbx2b	T-box 2b	283.93	0.94	0.00
00000014803	usp2b	ubiquitin specific peptidase 2b	187.74	-1.31	0.00
00000014886	rorcb	RAR-related orphan receptor C b	99.03	2.49	0.00
00000014893	cirh1a	cirrhosis, autosomal recessive 1A (cirhin)	271.56	-0.53	0.05
00000014935	im:7148292	im:7148292	127.82	-0.58	0.02
00000014987	ulk2	unc-51 like autophagy activating kinase 2	640.89	-0.79	0.01
00000015077	dnaja2	DnaJ (Hsp40) homolog, subfamily A, member 2	480.54	-0.40	0.04
00000015098	ddx5	DEAD (Asp-Glu-Ala-Asp) box helicase 5	894.22	-0.55	0.00
00000015152	add3a	adducin 3 (gamma) a	329.61	0.51	0.01
00000015153	ccng2	cyclin G2	178.01	0.55	0.03
00000015170	CYP27B1	cytochrome P450, family 27, subfamily B, polypeptide 1	151.84	0.91	0.02
00000015224	impdh1b	IMP (inosine 5'-monophosphate) dehydrogenase 1b	153.07	0.63	0.00
00000015227	bptf	bromodomain PHD finger transcription factor	189.25	0.53	0.01
00000015238	\	Uncharacterized protein	234.91	-0.63	0.01
00000015354	cldn2	claudin 2	164.81	0.50	0.01
00000015374	klf8	Kruppel-like factor 8	73.14	0.99	0.00
00000015399	nr1d4b	nuclear receptor subfamily 1, group D, member 4b	52.37	-2.13	0.00
00000015424	cab39	calcium binding protein 39	155.41	0.64	0.00
00000015433	cirbpb	cold inducible RNA binding protein b	634.19	0.40	0.01
00000015446	COQ10A	coenzyme Q10 homolog A (S. cerevisiae)	50.27	-1.12	0.00
00000015456	PER2	period circadian clock 2	350.81	-0.49	0.04
00000015458	als2a	amyotrophic lateral sclerosis 2a (juvenile)	148.55	0.46	0.03
00000015498	hsf2	heat shock factor 2	129.69	-1.07	0.00
00000015657	rchyl	ring finger and CHY zinc finger domain containing 1	157.52	-0.82	0.00
00000015753	mcl1b	myeloid cell leukemia 1b	655.90	-0.42	0.01
00000015822	si:dkey- 246g23.4	sı:dkey-246g23.4	240.42	-0.70	0.02
00000015856	llph	LLP homolog, long-term synaptic facilitation (Aplysia)	240.88	-0.38	0.04
00000015858	mbnl1	muscleblind-like splicing regulator 1	141.13	0.62	0.02
00000015921	ube2g1b	ubiquitin-conjugating enzyme E2G 1b (UBC7	115.75	-0.57	0.02

		homolog, yeast)			
00000015948	ppp1cc	protein phosphatase 1, catalytic subunit, gamma isozyme	282.04	-0.59	0.01
00000016102	gpatch4	G patch domain containing 4	60.97	-0.73	0.03
00000016152	emilin1a	elastin microfibril interfacer 1a	248.42	0.92	0.00
0000016237	mtss1la	metastasis suppressor 1-like a	272.22	-1.15	0.00
00000016238	slc25a38a	solute carrier family 25, member 38a	330.50	-1.24	0.00
00000016287	atraid	all-trans retinoic acid-induced differentiation factor	64.45	-1.11	0.00
00000016311	snx17	sorting nexin 17	386.87	-0.91	0.00
00000016315	nr2f5	nuclear receptor subfamily 2, group F, member 5	27.85	0.88	0.02
00000016382	cry1b	cryptochrome circadian clock 1b	64.66	-0.98	0.00
00000016390	mterf2	mitochondrial transcription termination factor 2	28.40	-1.09	0.00
00000016431	nr1d2a	nuclear receptor subfamily 1, group D, member 2a	537.05	-0.47	0.05
00000016511	slc2a12	solute carrier family 2 (facilitated glucose transporter), member 12	156.87	-0.62	0.03
00000016527	C14orf1	chromosome 14 open reading frame 1	107.05	-0.77	0.00
00000016569	mknk1	MAP kinase interacting serine/threonine kinase 1	59.93	1.10	0.00
00000016623	dlst	dihydrolipoamide S-succinyltransferase	909.64	-0.30	0.03
00000016641	CASKIN1	CASK interacting protein 1	347.07	0.66	0.00
00000016643	LPIN3	lipin 3	31.21	0.78	0.03
00000016690	rxrgb	retinoid X receptor, gamma b	684.52	-0.37	0.04
0000016737	upp2	uridine phosphorylase 2	2706.07	-0.65	0.00
00000016750	\	Uncharacterized protein	239.42	0.98	0.00
0000016767	PKP4	plakophilin 4	119.18	-0.78	0.00
00000016772	kmt2cb	lysine (K)-specific methyltransferase 2Cb	312.44	0.56	0.03
00000016783	prkag2b	protein kinase, AMP-activated, gamma 2 non- catalytic subunit b	64.19	0.79	0.01
00000016809	loxl2a	lysyl oxidase-like 2a	38.28	-1.49	0.00
00000016848	MEGF9	multiple EGF-like-domains 9	43.67	0.76	0.01
00000016882	wdfy3	WD repeat and FYVE domain containing 3	158.18	0.60	0.00
00000016912	cds1	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 1	107.09	0.67	0.00
00000016920	GRB14	growth factor receptor-bound protein 14	225.63	-0.65	0.01
0000016927	\	Uncharacterized protein	395.48	-0.46	0.05
00000016930	COBLL1	cordon-bleu WH2 repeat protein-like 1	98.67	-0.60	0.02
00000016950	elk1	ELK1, member of ETS oncogene family	24.89	0.86	0.01
0000016997	lpl	lipoprotein lipase	1225.64	-0.68	0.00
00000017001	pex12	peroxisomal biogenesis factor 12	114.60	-0.50	0.03
00000017007	EIF4G3	eukaryotic translation initiation factor 4 gamma, 3	91.76	0.58	0.01
00000017105	dnttip2	deoxynucleotidyltransferase, terminal, interacting protein 2	115.29	-0.63	0.01
00000017118	ehd3	EH-domain containing 3	184.06	-1.55	0.00
00000017177	cebpz	CCAAT/enhancer binding protein (C/EBP), zeta	259.67	-0.46	0.04
00000017338	aldh3b1	aldehyde dehydrogenase 3 family, member B1	277.77	-0.49	0.01
00000017385	ptprb	protein tyrosine phosphatase, receptor type, b	524.57	-0.35	0.04
00000017428	TYMP	thymidine phosphorylase	677.99	-0.60	0.00
00000017473	ncl1	nicalin	417.17	-0.42	0.02
00000017606	cx28.1	connexin 28.1	570.14	-0.67	0.00
00000017608	cx32.3	connexin 32.3	810.16	-0.77	0.00

00000017754	fam102aa	family with sequence similarity 102, member Aa	94.90	-0.54	0.03
00000017767	nol10	nucleolar protein 10	208.54	-0.42	0.05
00000017773	clu	clusterin	162.80	0.65	0.04
00000017776	rasgef1ba	RasGEF domain family, member 1Ba	35.59	-1.19	0.00
00000017950	gsk3b	glycogen synthase kinase 3 beta	184.06	-0.55	0.04
00000017989	rpia	ribose 5-phosphate isomerase A (ribose 5- phosphate epimerase)	305.13	-0.43	0.03
00000018068	egln3	egl-9 family hypoxia-inducible factor 3	36.94	-1.55	0.00
00000018092	hdac4	histone deacetylase 4	230.43	-0.53	0.02
00000018170	NAT8	N-acetyltransferase 8 (GCN5-related, putative)	21.72	0.89	0.02
0000018196	ahr2	aryl hydrocarbon receptor 2	206.70	-0.78	0.00
00000018236	wdr43	WD repeat domain 43	343.27	-0.53	0.01
00000018531	mibp	muscle-specific beta 1 integrin binding protein	93.65	-1.48	0.00
00000018585	bcar1	breast cancer anti-estrogen resistance 1	271.23	-0.83	0.00
00000018731	g6pca.1	glucose-6-phosphatase a, catalytic subunit, tandem duplicate 1	529.48	-0.81	0.01
0000018737	cishb	cytokine inducible SH2-containing protein b	83.10	1.75	0.00
00000018748	dkc1	dyskeratosis congenita 1, dyskerin	377.78	-0.33	0.04
00000018785	stk35	serine/threonine kinase 35	52.97	-1.01	0.01
00000018862	nob1	NIN1/RPN12 binding protein 1 homolog (S. cerevisiae)	141.35	-0.48	0.03
00000019036	dip2bb	DIP2 disco-interacting protein 2 homolog Bb (Drosophila)	28.10	0.77	0.04
00000019075	pdlim4	PDZ and LIM domain 4	81.48	0.82	0.01
00000019097	cry2a	cryptochrome 2a	382.48	0.74	0.00
00000019281	PLEKHG5	pleckstrin homology domain containing, family G (with RhoGef domain) member 5	149.63	0.55	0.01
00000019370	arntl1a	aryl hydrocarbon receptor nuclear translocator- like 1a	80.53	1.52	0.00
00000019493	traf3	TNF receptor-associated factor 3	49.40	-0.74	0.04
00000019720	enpep	glutamyl aminopeptidase	78.09	-0.87	0.01
00000019952	vps13d	vacuolar protein sorting 13 homolog D (S. cerevisiae)	545.14	0.55	0.00
00000020002	cyp51	cytochrome P450, family 51	1652.41	-0.55	0.01
00000020011	trub2	TruB pseudouridine (psi) synthase family member 2	58.46	-1.06	0.00
0000020036	rab44	RAB44, member RAS oncogene family	199.60	-0.86	0.02
00000020045	grid1b	glutamate receptor, ionotropic, delta 1b	58.27	0.66	0.03
00000020186	rapgef2	Rap guanine nucleotide exchange factor (GEF) 2	171.33	-0.86	0.00
00000020302	antxr1c	anthrax toxin receptor 1c	38.80	1.05	0.00
00000020419	si:ch211- 235e9.8	si:ch211-235e9.8	536.55	-0.47	0.00
00000020475	LRP4	low density lipoprotein receptor-related protein 4	32.39	0.77	0.04
00000020624	C4orf29	chromosome 4 open reading frame 29	607.14	-0.31	0.02
0000020656	acol	aconitase 1, soluble	876.49	0.54	0.01

Male	Cont vs MC1					Base		
id (ENSORLG)	Gene name	Description				mean	log2FC	padj
00000018932	\	Uncharacterized	l protein			1817.89	-1.09	0.00
0000003454	abcb11b	ATP-binding	cassette,	sub-family	В	2253.29	-0.76	0.02

		(MDR/TAP), member 11b			
00000013639	\	Uncharacterized protein	4646.91	-0.72	0.00
0000001418	pld3	phospholipase D family, member 3	907.36	-0.71	0.02
00000020447	cldn15lb	claudin 15-like b	914.67	-0.71	0.00
0000000637	\	Uncharacterized protein	1223.03	-0.66	0.00
0000003462	\	Uncharacterized protein	1618.55	-0.64	0.00
0000003862	fn1b	fibronectin 1b	9040.86	-0.59	0.01
00000018413	slc26a5	solute carrier family 26 (anion exchanger),	794.14	-0.57	0.04
		member 5			
0000002039	osmr	oncostatin M receptor	753.47	-0.57	0.01
0000001777	mat2al	methionine adenosyltransferase II, alpha-like	1009.62	-0.52	0.01
0000001080	xdh	xanthine dehydrogenase	2313.80	-0.49	0.04
0000007561	slc51a	solute carrier family 51, alpha subunit	1035.27	-0.49	0.04
00000011826	ncehla	neutral cholesterol ester hydrolase 1a	1321.37	-0.44	0.04
00000015672	\	Uncharacterized protein	5285.00	-0.40	0.03
00000013140	habp2	hyaluronan binding protein 2	13292.81	-0.40	0.02
00000012898	hmgcs1	3-hydroxy-3-methylglutaryl-CoA synthase 1	938.38	1.00	0.00
		(soluble)			

Male	Cont vs MC5		Base		
id (ENSORLG)	Gene name	Description	mean	log ₂ FC	padj
0000001085	LGALS4	lectin, galactoside-binding, soluble, 4	36.83	1.39	0.01
00000001491	si:ch211- 133j6.3	si:ch211-133j6.3	17.45	1.49	0.00
0000001556	GLDC	glycine dehydrogenase (decarboxylating)	760.50	-0.85	0.03
0000001693	oaz2a	ornithine decarboxylase antizyme 2a	330.97	-0.66	0.05
0000001828	slc20a2	solute carrier family 20 (phosphate transporter), member 2	186.75	-0.91	0.05
00000001916	TEX2	testis expressed 2	644.37	-0.66	0.03
0000002146	ppm1h	protein phosphatase, Mg2+/Mn2+ dependent, 1H	251.56	-0.87	0.01
0000002287	abhd2a	abhydrolase domain containing 2a	444.58	-0.93	0.00
0000002369	bnip1b	BCL2/adenovirus E1B interacting protein 1b	326.71	-0.97	0.00
0000002409	lancl1	LanC antibiotic synthetase component C-like 1 (bacterial)	159.46	0.85	0.03
0000002620	asb16	ankyrin repeat and SOCS box containing 16	146.96	1.45	0.00
0000002650	ciarta	circadian associated repressor of transcription a	82.23	-1.28	0.00
0000003084	apol1	apolipoprotein L, 1	18.66	-2.18	0.00
0000003454	abcb11b	ATP-binding cassette, sub-family B (MDR/TAP), member 11b	1821.24	-1.35	0.00
0000003577	EGFR	epidermal growth factor receptor	508.47	-0.97	0.00
0000003862	fn1b	fibronectin 1b	7892.80	-0.76	0.03
0000003931	gpr137c	G protein-coupled receptor 137c	64.18	-1.33	0.01
00000004461	slc12a7b	solute carrier family 12 (potassium/chloride transporter), member 7b	453.46	-0.98	0.00
00000004640	hmg20b	high mobility group 20B	73.79	1.03	0.04
00000004913	cd74a	CD74 molecule, major histocompatibility complex, class II invariant chain a	886.28	0.75	0.01
0000005783	NCOA2	nuclear receptor coactivator 2	208.90	-1.10	0.01
0000005983	mroh1	maestro heat-like repeat family member 1	274.21	-0.85	0.03
0000006177	si:ch211- 153b23.5	si:ch211-153b23.5	145.46	1.36	0.01
0000006357	fads2	fatty acid desaturase 2	138.73	-1.71	0.00

0000006929	per1b	period circadian clock 1b	365.71	-1.46	0.00
0000007031	dennd5a	DENN/MADD domain containing 5A	499.19	-0.86	0.00
0000007145	isg15	ISG15 ubiquitin-like modifier	21.09	1.31	0.02
0000007151	\	Uncharacterized protein	40.04	1.21	0.03
0000007210	tmprss13b	transmembrane protease, serine 13b	33.72	1.25	0.03
0000007561	slc51a	solute carrier family 51, alpha subunit	911.56	-0.64	0.03
0000007735	lonrfl	LON peptidase N-terminal domain and ring finger 1	135.87	-0.90	0.04
0000007737	\	Uncharacterized protein	54.53	-1.00	0.04
0000008058	csad	cysteine sulfinic acid decarboxylase	112.24	1.01	0.03
00000008162	0.00 / 1.0	cytochrome P450, family 4, subfamily V,	1725.04	0.85	0.01
0000008102	Сурчув	polypeptide 8	1/33.74	0.85	0.01
0000008373	\	Uncharacterized protein	301.50	0.98	0.00
0000008377	DNAAF5	dynein, axonemal, assembly factor 5	30.91	1.23	0.02
0000008489	mef2aa	myocyte enhancer factor 2aa	115.06	-1.02	0.02
0000008604	KIF21A	kinesin family member 21A	821.81	-0.84	0.01
0000008635	ABCD2	ATP-binding cassette, sub-family D (ALD), member 2	347.03	-0.98	0.01
0000008703	cad	carbamoyl-phosphate synthetase 2, aspartate	21.08	1.24	0.03
0000008769	slc5a2	solute carrier family 5 (sodium/glucose cotransporter) member 2	50.75	-1.21	0.04
00000009056	ote	ornithine carbamovltransferase	22.15	-1 45	0.01
00000009133	furina	furin (paired basic amino acid cleaving enzyme) a	2350 71	-0.71	0.01
000000000000000000000000000000000000000	si:ch73-		20000.71	1.04	0.01
0000009329	25i22.2	s1:ch/3-25122.2	27.89	-1.24	0.02
0000009351	mvda	mevalonate (diphospho) decarboxylase a	122.11	1.25	0.02
0000009768	cry-dash	cryptochrome DASH	90.65	-1.39	0.00
0000009998	acaca	acetyl-CoA carboxylase alpha	636.64	-0.89	0.01
00000010071	plxnb1b	plexin b1b	396.53	-1.15	0.00
00000010101	zgc:101785	zgc:101785	25.39	1.37	0.01
00000010228	slmapb	sarcolemma associated protein b	53.11	-1.15	0.04
00000010256	LAMB3	laminin, beta 3	356.09	-1.39	0.00
00000010258	hkdc1	hexokinase domain containing 1	15.47	1.27	0.01
00000010295	CIART	circadian associated repressor of transcription	37.30	-1.31	0.02
00000010486	adgrv1	adhesion G protein-coupled receptor V1	157.82	0.96	0.03
00000010533	prdx1	peroxiredoxin 1	419.61	0.80	0.03
00000010698	tm7sf2	transmembrane 7 superfamily member 2	938.36	-0.86	0.02
00000011120	numal	nuclear mitotic apparatus protein 1	30.24	1.62	0.00
00000011239	nme4	NME/NM23 nucleoside diphosphate kinase 4	567.28	0.97	0.04
00000011401	bhlhe41	basic helix-loop-helix family, member e41	176.21	-1.12	0.02
00000011412	dbpb	D site albumin promoter binding protein b	206.17	-1.10	0.02
00000011642	dusp7	dual specificity phosphatase 7	29.01	1.25	0.02
00000011759	\	Uncharacterized protein	567.78	-0.74	0.02
00000011805	scube1	signal peptide, CUB domain, EGF-like 1	317.53	-1.39	0.00
00000012115	\	Uncharacterized protein	55.65	-0.99	0.04
00000012175	jag1b	jagged 1b	229.84	-0.85	0.04
00000012454	slc1a8b	solute carrier family 1 (glutamate transporter), member 8b	23.74	-1.26	0.02
00000012462	cry5	cryptochrome 5	39.93	-1.84	0.00
00000012653	apobb.1	apolipoprotein Bb, tandem duplicate 1	19949.34	-0.77	0.03
00000012898	hmgcs1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	992.67	1.11	0.03

00000013226	acat2	acetyl-CoA acetyltransferase 2	532.64	-0.72	0.03
00000013444	adamts13	ADAM metallopeptidase with thrombospondin type 1 motif, 13	616.92	-0.68	0.02
00000013626	dpydb	dihydropyrimidine dehydrogenase b	6880.76	-0.68	0.02
00000013678	adipor2	adiponectin receptor 2	483.65	-0.70	0.02
00000013684	dhrs13b	dehydrogenase/reductase (SDR family) member 13b	28.85	1.43	0.01
00000014024	rbp2b	retinol binding protein 2b, cellular	450.53	0.78	0.04
00000014144	tefb	thyrotrophic embryonic factor b	55.60	-1.17	0.03
00000014181	PMM1	phosphomannomutase 1	223.87	-0.83	0.05
00000014422	col18a1	collagen type XVIII, alpha 1	18.75	1.74	0.00
00000014586	si:dkey- 224e22.2	si:dkey-224e22.2	51.31	1.14	0.03
00000014660	acp5a	acid phosphatase 5a, tartrate resistant	123.58	-1.11	0.04
00000014730	nfil3-5	nuclear factor, interleukin 3 regulated, member 5	36.56	1.62	0.00
00000014811	cyp27a7	cytochrome P450, family 27, subfamily A, polypeptide 7	1087.98	-0.68	0.02
00000014877	\	Uncharacterized protein	356.12	-0.69	0.04
00000014886	rorcb	RAR-related orphan receptor C b	174.82	0.98	0.05
00000014900	usp32	ubiquitin specific peptidase 32	377.13	-0.96	0.00
00000015041	prmt7	protein arginine methyltransferase 7	106.86	1.01	0.01
00000015238	\	Uncharacterized protein	229.69	-1.07	0.00
00000015308	den	decorin	31.78	1.10	0.05
00000015348	TP53INP2	tumor protein p53 inducible nuclear protein 2	96.70	-1.01	0.03
00000015399	nr1d4b	nuclear receptor subfamily 1, group D, member 4b	65.61	-1.78	0.00
00000015456	PER2	period circadian clock 2	367.72	-1.37	0.00
00000015498	hsf2	heat shock factor 2	154.38	-1.28	0.00
00000015554	MYO7B	myosin VIIB	24.88	1.27	0.02
00000015598	nup93	nucleoporin 93	120.25	0.97	0.03
00000015714	tagln	transgelin	155.07	0.89	0.05
00000015735	cbln11	cerebellin 11	4393.81	1.16	0.02
00000015810	klhl24a	kelch-like family member 24a	154.97	-1.08	0.03
00000015822	si:dkey- 246g23.4	si:dkey-246g23.4	394.50	-0.98	0.01
00000015952	per3	period circadian clock 3	633.14	-0.87	0.01
00000015979	sort1a	sortilin 1a	456.56	-0.85	0.03
00000016006	mtmr3	myotubularin related protein 3	175.36	-1.00	0.02
00000016343	REPS2	RALBP1 associated Eps domain containing 2	64.66	-1.47	0.00
00000016390	mterf2	mitochondrial transcription termination factor 2	24.47	-1.56	0.00
00000016522	FLVCR2	feline leukemia virus subgroup C cellular receptor family, member 2	33.10	-1.28	0.02
00000016528	PTGR1	prostaglandin reductase 1	18.29	1.34	0.01
00000016551	zgc:158846	zgc:158846	42.79	1.47	0.00
00000016561	krt4	keratin 4	175.45	1.43	0.00
00000016886	LAMA3	laminin, alpha 3	409.29	-0.83	0.03
00000017533	hsp90aa1.2	heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2	72.78	1.23	0.02
00000017721	esr2	estrogen receptor beta	114.44	-1.02	0.02
00000017889	slc5a3b	solute carrier family 5 (sodium/myo-inositol cotransporter), member 3b	26.60	-1.21	0.04
00000017987	slc4a11	solute carrier family 4, sodium borate transporter, member 11	35.04	-1.44	0.01
00000017996	atrn	attractin	433.09	-0.85	0.03

00000018344	fam65a	family with sequence similarity 65, member A	497.47	-0.87	0.03
00000018805	rfc2	replication factor C (activator 1) 2	81.70	1.03	0.02
00000018887	\	Uncharacterized protein	177.71	0.88	0.03
00000019017	ccdc135	coiled-coil domain containing 135	47.95	-1.44	0.00
00000019097	cry2a	cryptochrome 2a	442.97	0.64	0.05
00000019738	APOB	apolipoprotein B	112.41	-1.38	0.00
00000019740	APOB	apolipoprotein B	85.02	-1.52	0.00
00000019785	hgh1	HGH1 homolog (S. cerevisiae)	64.25	1.01	0.03
00000020036	rab44	RAB44, member RAS oncogene family	312.59	-1.50	0.00
00000020367	igf2r	insulin-like growth factor 2 receptor	168.55	-1.14	0.01
00000020660	ugt5b4	UDP glucuronosyltransferase 5 family, polypeptide B4	742.12	-0.65	0.05
00000020682	ADARB1	adenosine deaminase, RNA-specific, B1	13.46	1.38	0.01
00000020839	anp32e	acidic (leucine-rich) nuclear phosphoprotein 32 family, member E	79.71	1.11	0.01

Male id (ENSORLG)	Cont vs Ext5 Gene name	Description	Base mean	log ₂ FC	padj
0000000233	hsp70.3	heat shock cognate 70-kd protein, tandem duplicate 3	64.87	0.97	0.04
0000000979	OGDH	oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	982.08	-1.01	0.00
00000001120	kcnq1	potassium voltage-gated channel, KQT-like subfamily, member 1	92.44	-0.84	0.04
0000001252	zgc:113516	zgc:113516	689.08	-0.54	0.03
0000001274	ccni2	cyclin I family, member 2	42.04	1.30	0.00
0000001343	tspan7	tetraspanin 7	558.48	-0.74	0.00
0000001693	oaz2a	ornithine decarboxylase antizyme 2a	326.76	-0.79	0.00
0000002409	lancl1	LanC antibiotic synthetase component C-like 1 (bacterial)	181.64	1.00	0.00
0000002582	tefa	thyrotrophic embryonic factor a	724.73	-0.64	0.02
0000002620	asb16	ankyrin repeat and SOCS box containing 16	71.00	1.11	0.00
0000002650	ciarta	circadian associated repressor of transcription a	83.08	-1.17	0.00
0000003438	ppp1r1b	protein phosphatase 1, regulatory (inhibitor) subunit 1B	524.62	-0.84	0.00
0000003454	abcb11b	ATP-binding cassette, sub-family B (MDR/TAP), member 11b	2155.97	-0.73	0.02
0000003869	fam20a	family with sequence similarity 20, member A	519.38	-0.76	0.01
0000004059	ugt5d1	UDP glucuronosyltransferase 5 family, polypeptide D1	879.64	-0.65	0.02
00000004146	fabp2	fatty acid binding protein 2, intestinal	40.87	1.62	0.00
0000004334	C21orf59	chromosome 21 open reading frame 59	25.91	0.95	0.05
00000004419	cyp27c1	cytochrome P450, family 27, subfamily C, polypeptide 1	77.97	-1.47	0.00
0000005093	DMPK	dystrophia myotonica-protein kinase	12.59	1.03	0.01
00000005432	agla	amylo-alpha-1, 6-glucosidase, 4-alpha- glucanotransferase a	1581.02	-0.61	0.04
0000006140	herc7	hect domain and RLD 7	58.64	1.11	0.00
0000006194	cbsb	cystathionine-beta-synthase b	347.34	-0.66	0.02
0000006265	selm	selenoprotein M	146.20	-0.83	0.02
0000006357	fads2	fatty acid desaturase 2	154.72	-1.15	0.00
0000006475	SLC19A1	solute carrier family 19 (folate transporter), member 1	1791.36	-0.60	0.01

0000006494	lxn	latexin	282.01	-0.89	0.00
0000006793	\	Uncharacterized protein	480.55	0.76	0.01
0000006876	chst3a	carbohydrate (chondroitin 6) sulfotransferase 3a	99.11	0.94	0.03
0000006929	per1b	period circadian clock 1b	388.67	-1.07	0.00
00000007510	CLEC3A	C-type lectin domain family 3, member A	67.85	1.12	0.01
00000007542	klf13	Kruppel-like factor 13	66.34	0.89	0.05
00000007592	pgamla	phosphoglycerate mutase 1a	563.13	-0.62	0.05
00000007735	lonrfl	LON peptidase N-terminal domain and ring finger	142.77	-0.76	0.05
00000007777	cant1a	calcium activated nucleotidase 1a	992.78	-0.55	0.02
00000007912	scaper	S-phase cyclin A-associated protein in the ER	76.07	0.80	0.05
00000008162	cyp4v8	cytochrome P450, family 4, subfamily V, polypeptide 8	1683.99	0.73	0.00
00000008604	KIF21A	kinesin family member 21A	879.07	-0.68	0.01
00000008648	ift172	intraflagellar transport 172	70.17	0.99	0.01
	1	carbamovl-phosphate synthetase 2. aspartate		0.55	0.01
00000008703	cad	transcarbamylase, and dihydroorotase	25.67	1.23	0.00
0000008866	pdp1	subunit 1	62.53	1.00	0.02
00000009329	si:ch73- 25i22.2	si:ch73-25i22.2	26.86	-1.26	0.00
00000009768	cry-dash	cryptochrome DASH	88.91	-1.48	0.00
00000009917	hprt1	hypoxanthine phosphoribosyltransferase 1	453.80	-0.92	0.00
00000009933	arg2	arginase 2	29.54	-1.03	0.02
00000009998	acaca	acetyl-CoA carboxylase alpha	656.17	-0.89	0.00
00000010071	plxnb1b	plexin b1b	438.26	-0.78	0.03
00000010295	CIART	circadian associated repressor of transcription	35.96	-1.51	0.00
00000011031	clmn	calmin	231.49	-0.76	0.04
00000011120	numal	nuclear mitotic apparatus protein 1	27.39	1.32	0.00
00000011239	nme4	NME/NM23 nucleoside diphosphate kinase 4	555.80	0.93	0.00
00000011349	mmp28	matrix metallopeptidase 28	125.88	0.88	0.01
00000011398	porb	P450 (cytochrome) oxidoreductase b	2192.81	-0.53	0.05
00000011401	bhlhe41	basic helix-loop-helix family, member e41	172.01	-1.01	0.02
00000011536	pim3	Pim-3 proto-oncogene, serine/threonine kinase	155.90	-0.80	0.03
00000011711	\	Uncharacterized protein	79.45	0.92	0.05
00000012126	klhdc8a	kelch domain containing 8A	147.78	0.71	0.04
00000012175	jag1b	jagged 1b	238.85	-0.77	0.03
00000012383	rgs4	regulator of G-protein signaling 4	84.95	-1.03	0.02
00000012462	crv5	cryptochrome 5	42.36	-1.37	0.00
00000012573	Clorf50	chromosome 1 open reading frame 50	124.54	-1.13	0.00
00000012601	IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial	180.37	0.94	0.01
00000012739	dec1a	Oryzias latipes bHLH protein DEC1a (dec1a), mRNA	180.39	-1.27	0.00
00000012935	fbln2	fibulin 2	25 36	1 16	0.00
00000013091	khk	ketohexokinase	563.44	-1.21	0.00
00000013422	argl	arginase 1	3589 68	-0.71	0.00
00000013476	CRAT	carnitine O-acetyltransferase	195 29	0.93	0.00
00000013626	dpvdb	dihydropyrimidine dehydrogenase b	6742.82	-0.85	0.00
00000013749	1100t1	LIDP-glucose glyconrotein glucosyltransferase 1	570.45	-0.89	0.00
00000013767		Uncharacterized protein	95 93	0.83	0.04
00000013826	\ \	Uncharacterized protein	69 72	0.87	0.07
00000013993	kean1a	kelch-like ECH-associated protein 1a	299.82	-0.78	0.02
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00000014243	tmem237a	transmembrane protein 237a	257 20	0.96	0.00
00000014245	nlbd1	phospholinase B domain containing 1	616 51	0.50	0.00
0000014200	piour	5-methyltetrahydrofolate-homocysteine	010.01	0.50	0.05
00000014271	MTR	methyltransferase	2163.44	-0.48	0.05
00000014730	nfil3-5	nuclear factor, interleukin 3 regulated, member 5	36.65	1.22	0.00
00000014886	rorcb	RAR-related orphan receptor C b	183.13	0.88	0.05
00000015164	cacna2d1a	calcium channel, voltage-dependent, alpha 2/delta subunit 1a	102.38	-1.63	0.00
00000015204	amd1	adenosylmethionine decarboxylase 1	732.06	-0.57	0.02
00000015224	impdh1b	IMP (inosine 5'-monophosphate) dehydrogenase 1b	208.41	0.78	0.03
00000015238	\	Uncharacterized protein	238.05	-0.99	0.00
00000015297	\	Uncharacterized protein	1035.29	0.69	0.01
00000015399	nr1d4b	nuclear receptor subfamily 1, group D, member 4b	63.92	-1.89	0.00
00000015425	tcp1112	t-complex 11, testis-specific-like 2	181.61	-0.77	0.01
00000015456	PER2	period circadian clock 2	388.44	-1.21	0.00
00000015498	hsf2	heat shock factor 2	152.51	-1.33	0.00
00000015696	atp1b1a	ATPase, Na+/K+ transporting, beta 1a polypeptide	330.59	-0.66	0.02
00000015699	\	Uncharacterized protein	68.94	-0.98	0.02
00000015822	si:dkey- 246g23.4	si:dkey-246g23.4	364.71	-1.47	0.00
00000015919	nipa1	non imprinted in Prader-Willi/Angelman syndrome 1	151.08	-0.70	0.04
00000016390	mterf2	mitochondrial transcription termination factor 2	25.39	-1.23	0.00
00000016809	loxl2a	lysyl oxidase-like 2a	30.27	-0.98	0.04
00000017149	igfbp3	insulin-like growth factor binding protein 3	28.77	1.50	0.00
00000017161	igfbp1b	insulin-like growth factor binding protein 1b	250.03	1.13	0.00
00000017314	ABHD1	abhydrolase domain containing 1	288.41	-0.75	0.01
00000017460	cers1	ceramide synthase 1	187.66	-0.82	0.03
00000017552	c5	complement component 5	23449.00	-0.54	0.01
00000017636	eif4eb	eukaryotic translation initiation factor 4eb	268.00	-0.68	0.04
00000018153	\	Uncharacterized protein	617.44	-0.62	0.05
00000018263	ppdpfb	pancreatic progenitor cell differentiation and proliferation factor b	844.15	0.61	0.03
00000018673	ANO9	anoctamin 9	123.13	4.15	0.00
00000019097	cry2a	cryptochrome 2a	466.96	0.69	0.00
00000019370	arntlla	aryl hydrocarbon receptor nuclear translocator-like la	147.02	0.78	0.03
0000020036	rab44	RAB44, member RAS oncogene family	328.24	-1.49	0.00
00000020385	\	Uncharacterized protein	76.74	-1.02	0.02

Annex 5. 1 Article 1 An integrated omic analysis of hepatic alteration in medaka fish chronically exposed to cyanotoxins with possible mechanisms of reproductive toxicity



An integrated omic analysis of hepatic alteration in medaka fish chronically exposed to cyanotoxins with possible mechanisms of reproductive toxicity*



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ABSTRACT

Cyanobacterial blooms threaten human health as well as the population of other living organisms in the aquatic environment, particularly due to the production of natural toxic components, the cyanotoxin. So far, the most studied cyanotoxins are microcystins (MCs). In this study, the hepatic alterations at histological, proteome and transcriptome levels were evaluated in female and male medaka fish chronically exposed to 1 and 5 μ g L⁻¹ microcystin-LR (MC-LR) and to the extract of MC-producing *Microcystis aer*uginosa PCC 7820 (5 µg L⁻¹ of equivalent MC-LR) by balneation for 28 days, aiming at enhancing our understanding of the potential reproductive toxicity of cyanotoxins in aquatic vertebrate models. Indeed, both MC and Microcystis extract adversely affect reproductive parameters including fecundity and egg hatchability. The liver of toxin treated female fish present glycogen storage loss and cellular damages. The quantitative proteomics analysis revealed that the quantities of 225 hepatic proteins are dysregulated. In particular, a notable decrease in protein quantities of vitellogenin and choriogenin was observed, which could explain the decrease in reproductive output. Liver transcriptome analysis through Illumina RNAseq reveals that over 100–400 genes are differentially expressed under 5 $\mu g \ L^{-1}$ MC-LR and Microcystis extract treatments, respectively. Ingenuity pathway analysis of the omic data attests that various metabolic pathways, such as energy production, protein biosynthesis and lipid metabolism, are disturbed by both MC-LR and the Microcystis extract, which could provoke the observed reproductive impairment, The transcriptomics analysis also constitutes the first report of the impairment of circadian rhythmrelated gene induced by MCs. This study contributes to a better understanding of the potential consequences of chronic exposure of fish to environmental concentrations of cyanotoxins, suggesting that Microcystis extract could impact a wider range of biological pathways, compared with pure MC-LR, and even 1 µg L⁻¹ MC-LR potentially induces a health risk for aquatic organisms.

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Emerging environmental pollution due to the expansion of anthropogenic activities has profoundly altered the quality and availability of water resources. The release of phosphates and

1. Introduction

nitrogen from fertilizer runoff, fossil fuel, animal waste, and industrial discharge has been largely responsible for the eutrophication in the fresh waters worldwide, resulting in recurrent and massive cyanobacterial bloom developments. Harmful cyanobacterial blooms threaten human health, as well as the population of any living organism in the water, particularly due to the production of natural toxic components, so-called cyanotoxins (Pavagadhi and Balasubramanian, 2013).

Microcystins (MCs) are the most prevalent cyanobacterial hepatotoxins with more than 100 structural variants, being produced by at least six genera of cyanobacteria (Puddick et al., 2014). Among all these known variants, microcystin-LR (MC-LR) is considered to be the most common and potently toxic (Puddick et al., 2014). MCs are transported through cell membranes by organic anion transporting polypeptides (OATP) and they specifically inhibit eukaryotic serine/threonine protein phosphatases 1 and 2A, which causes the disruption of numerous cellular signals and processes (Fischer et al., 2005; MacKintosh et al., 1990). OATPs are detected in various tissues of the organism and they are mostly present in the hepatocyte membranes (Fischer et al., 2005). Therefore, MCs could be distributed and accumulate in various fish organs including liver, kidney, gill, gonad and, to a lesser extent, in other tissues or organs (Mezhoud et al., 2008).

Although MCs have been studied extensively by the research community (Marie et al., 2012; Mezhoud et al., 2008), many conclusions were generated based on short time and high doses of toxin exposure. However, in the natural environment, fish and other aquatic organisms are usually subject to long-term exposure to a relatively low concentration of toxins. The potential deleterious effects induced by a weak quantity of MCs are potentially more worrying than those caused by acute intoxication, as noticeable and relatively low concentrations of cyanotoxins are frequently observed in even more environments worldwide. The monitoring of MC concentrations in 26 recreational lakes in Brittany (France) between 2004 and 2011 showed that MC concentration was mostly between 0.1 and 10 μ g L⁻¹ (Pitois et al., 2016). Another large nationwide survey of cyanotoxins (1161 lakes) in the United States, conducted in 2007, reported a mean MC concentration of 3.0 μ g L⁻¹ (Loftin et al., 2016). In addition, the aquatic organisms exposed to cyanobacterial blooms are not only threatened by MCs, as a lot of other secondary metabolites are also produced by cyanobacteria. It has been reported that the toxic effects caused by cyanobacterial extracts could be different from those of individual MCs due to the complexity of the composition of the extracts (Marie et al., 2012; Rogers et al., 2011).

Classical toxicology or ecotoxicology approaches are not able to globally characterize the toxicity of emergent pollutants such as a mixture of compounds produced by cyanobacteria. Nowadays, with the advances in high-throughput analytical methods, such as transcriptomics and proteomics, the interactive effects of such complex mixture can be further described without *a priori* hypothesis. In this sense, an integrated analysis of the transcriptomic and proteomic data is needed to provide useful information that may not be depicted by a single method. To the best of our knowledge, no systematic study consisting of integrated transcriptomics and proteomics analysis to delineate the overall hepatic alteration at cellular and molecular level in fish upon exposure to environmentally relevant concentration of cyanotoxins has been performed before.

In the present study, a 28-day balneation exposure was conducted in adult medaka fish, a model aquatic vertebrate for toxicological and ecotoxicological study. The goal was to obtain a comprehensive understanding of the hepatic alterations at histological, proteome and transcriptome levels of fish chronically exposed to environmentally relevant concentrations of MC-LR (1 and 5 μ g L⁻¹) and to a *Microcystis* complex extract (5 μ g L⁻¹ of equivalent MC-LR). It has been proposed that liver dysfunction could be responsible for indirect reproductive disorders, since it performs key functions in the regulation of different reproductive processes (Li et al., 2013). Therefore, the present study also seeks to better understand the potential reproductive toxicity of the cyanotoxins in aquatic organisms, contributing to advance our current knowledge about the protection of aquatic organism populations, as well as human reproductive health.

2. Materials and methods

2.1. Toxin and Microcystis aeruginosa PCC 7820 extract preparation

MC-LR, purchased from Novakit[®] (Nantes, France), was selected as the model MC for the present experiment.

The monoclonal Microcystis aeruginosa strain PCC 7820, producing various MCs, among other secondary metabolites (Table S1), was maintained in the Paris' Museum Collection (PMC) of cyanobacteria and cultured in Z8 medium (Kotai, 1972) (25 °C, 16 h:8 h light:dark photoperiod at 16 μ mol of photons m⁻² s⁻¹). This strain has already been used as a representative strain of bloom-forming cyanobacteria in previous studies (Liu et al., 2005). Dense cultures of PCC 7820 been at the exponential growing phase (above 1000 μ g L⁻¹ chl a) was freshly incubated from a 50 mL inoculum in 700 mL of Z8 during two weeks (25 °C, 16 h:8 h light:dark photoperiod at 30 μmol of photons $m^{-2}~s^{-1}$). The cyanobacterial cells were then centrifuged at 4000 g, freeze-dried at -50 °C for 48 h, weighted and stored at -20 °C prior to metabolite extraction according to previously described for this strain (Le Manach et al., 2016). The lyophilized cells were sonicated in 80% methanol on ice for 30 min (Sonics Vibracell™, amplitude 100%, 1 pulse of 30 s per 3 min). The homogenates were evapored as previously described to get a solid extract (Djediat et al., 2011), that was dissolved in 50% ethanol (Vol/Vol) and partially evaporated until completely removing the ethanol with a Speedvac. MCs concentration in the extract was quantified using a commercial Addaspecific AD4G2 ELISA test (Abraxis).

2.2. Fish maintenance, exposure and sampling

The experimental procedures were conducted in accordance with the European Union regulations concerning the protection of experimental animals and the validation of experimental procedure by MNHN's ethical committees (N°68-040 for 2013-18). Medaka fish (*Oryzias latipes*) is a model aquatic vertebrate for toxicological and ecotoxicological study. Medaka fish of the inbred Cab strain maintained in the lab was used for this experiment. Five month-old adult fish were fed in 20 L glass aquaria filled with a mixture of tap water and reverse osmosis filtered water (1:2) in a flow-through system for aeration and filtration, in a temperature controlled room (25 \pm 1 °C), with a 15 h:9 h light:dark cycle (reproductive cycle). Fish were fed three times a day with commercial dry bait for juvenile salmons.

Sixty females and sixty males were randomly selected and dispatched into 4 experimental groups, namely "Cont" with control solvent, "MC1" with 1 µg L^{-1} MC-LR solvent, "MC5" with 5 µg L^{-1} MC-LR solvent, and "Ext5" with PCC 7820 *Microcystis aeruginosa* strain extract containing 5 µg L^{-1} equivalent MC-LR. Concerning the PCC 7820 MC content, the extract predominantly contains MC-LR and [Dmet-Asp3]MC-LR variants, along with traces of other variants, in accordance with previous investigations (Le Manach et al., 2016). The MC content was globally quantified using Adda specific ELISA test and was expressed according to MC-LR equivalent. Each group comprised three glass aquaria with a flow-

through system for aeration and filtration, containing 5 females and 5 males that were randomly transferred into each aquarium (containing 11.5 L water) two weeks prior to the beginning of the experiment, representing a fish loading of about 0.4 g fresh weight of fish L^{-1} . The temperature, light and feeding parameters were the fish same as described above. The balneation exposure lasted for 28 viri days and the respective water conditions were maintained by renewing 4.5 L stale water with fresh water containing the respective toxin contents of each condition, every 2 days. MCs concentrations in aquaria were determined every 6 days by using

concentrations in aquaria were determined every 2 days. Mcs concentrations in aquaria were determined every 6 days by using the AD4G2 ELISA kit (Table S2). Fish were inspected three times a day, and no abnormal behavior nor significant mortality was observed (only 1 female and 1 male died). Eggs were collected every 2 days. By the end of the exposure, fish were anesthetized in buffered 0.1% MS-222, sacrificed, and the livers were collected on ice, then prepared and stored for the different analyses.

2.3. Reproductive parameters

The eggs attached to the abdomen of females were harvested manually at 1 p.m. every two days. The eggs from the same aquarium and of the same date were pooled and placed in Yamamoto medium. Only the fertilized eggs (transparent) were further kept and placed in a specific incubator maintaining a photoperiod of 12 h:12 h light:dark at 25 °C. The Yamamoto medium was renewed daily to prevent bacterial contamination. The dead eggs, which turned blue due to penetration by the Coomassie blue of the Yamamoto medium were counted and removed from the Petri dishes. When the embryos hatched, the date and the number of hatched eggs were recorded and an inspection was conducted until the last egg hatching. The reproductive performance was evaluated by the total number of fertilized eggs per female and the percentage of hatched eggs. Results are presented as mean ± standard deviation (SD) and the levels of significance were evaluated by one-way ANOVA with post hoc Tukey HSD test for p < 0.05 or p < 0.01 using R software (version 3.2.5, package ggplot2).

2.4. Histological observation of liver

The half liver of each individual were fixed in Davidson solution immediately after dissection, kept at 4 °C for 24 h, then transferred into a graded series of ethanol (from 70% to 95%) for dehydration, and then embedded in paraffin. Blocks were cut into 3 μ m thick sections, and slides were stained with hematoxylin-eosin-saffron (HES) or periodic acid-Schiff (PAS)/Alcian blue according to the respective observation purposes.

The liver lysis was determined through the full view observation of 2 sections per individual. The glycogen content detection (estimated as a surface percentage of the purple-red pixels, 5 randomly selected views per individual) was performed by ImageJ software (version 1.51d). Statistical analysis of both parameters (n = 5 individuals per condition for each gender) was conducted by one-way ANOVA with post hoc Tukey HSD test for p < 0.05 or p < 0.01 using R software.

2.5. Proteomics

2.5.1. Protein sample preparation and mass spectrometry analysis

The half liver of 4 or 5 individuals (adjusting according to the size of tissue) were pooled to get 1 pooling sample per condition (males and females, respectively), as shown in Table S3. The pooled samples were prepared for the proteomics analysis through the isobaric tags for relative and absolute quantitation (iTRAQ)-based quantitative proteomics analysis following the protocol described in Supplementary Method.

2.5.2. Data treatment

All MS/MS-analyzed samples were analyzed using Mascot 2.4.1 (Matrix Science, UK) with Scaffold software (version 4.4.8; Proteome Software, USA) against Ensembl databases for a few species of fish (restricted to Oryzias latipes, Danio rerio and Tetraodon nigroviridis sequences, of the Ensembl database V78). The ion mass tolerance and the parent ion tolerance were set to 0.05 Da. The methyl methanethiosulfonate of cysteine was specified as fixed modifications. Oxidation of methionine and iTRAQ 4-plex of tyrosine for iTRAQ-derivatized samples were specified as variable modifications. Scaffold Q+ was used to quantify the isobaric tag peptide and protein identifications. Peptide identifications were accepted if they could be established with more than 95.0% probability. Protein identifications were accepted if they could be established with more than 99.0% probability and contained at least 2 identified peptides. Only identified proteins (with Ensembl ID) were considered for quantification and further analysis. Peptides were quantified using the centroid reporter ion peak intensity. The minimum quantitative value for each spectrum was calculated as 0.025% of the highest peak. Quantitative ratios were Log2 normalized for final quantitative testing, using control value set up as a reference sample in both genders. The quantities of proteins was considered significantly changed when $|Log_2FC| > 0.5$.

2.6. RNA-seq

2.6.1. Libraries construction and sequencing process

The half liver of female fish (2 or 3 individuals) and male fish (1 or 2 individuals) (adjusting according to the size of tissue) were pooled respectively, to get 4 pooled female samples and 3 pooled male samples per condition, as shown in Table S3. Then the samples were homogenized using a bead beater. Total RNA was isolated and purified using RNeasy Plus Mini Kit with gDNA eliminator spin (Qiagen). RNA quantity and quality were evaluated using Qubit RNA Assay Kit in Qubit[®]2.0 Fluorometer (Life Technologies, USA) and an Agilent Bioanalyzer 2100 eukarvote total RNA Pico series II chip (Agilent Technologies Inc., USA), respectively. Twenty-eight transcriptome libraries (16 females and 12 males) were prepared from the total RNA samples with RIN value over 7.7 using Illumina TruSeq Stranded mRNA Sample Preparation kits (Illumina Inc., USA) following manufacturer's instruction. The libraries were multiplexed in lane 3-5 for females and in lane 6-7 for males. Sequencing was performed on an Illumina Hi-Seq1000 instrument using the TruSeq SBS kit V3-HS 50-cycles (Illumina Inc., San Diego, CA, USA) and 30-40 millions of 51bp single-end reads per library were generated. CASAVA-1.8.2 software was used for demultiplexing.

2.6.2. Data analysis

Raw reads were first cleaned by removing adaptors using Cutadapt-1.3 and then only 51 bp-long reads were kept. The overall quality of the reads was checked using the FastQC 0.10.1 and good Phred scores (>30) were obtained in all the libraries. However, the analysis of the unicity ratio shows a high level of duplications in all the libraries. This high duplication issue was also observed in another transcriptomics investigation of medaka liver published recently (Murata et al., 2015). Therefore, for our data, a step of duplicated reads removal was conducted using a python script that analyzes the quality of reads and keeps the one with the best quality score. The information of all the libraries following a serie of reads filtration was presented in Table S4.

Tophat2 (v2.0.10) (Kim et al., 2013) was used to map the clean unique reads against the medaka genome (release 81) downloaded from Ensembl. Multiple hits were removed by samtools (v0.1.18) and read counting on gene exons was accomplished by HTSeqcount (v0.6.1p1) (Anders et al., 2015) in union mode against the annotation of medaka genomes downloaded from Ensembl. DESeq2 (v1.8.1) (Love et al., 2014) was used to do differential expressed gene analysis. Genes were considered differentially expressed when the FDR (false discovery rate) was below 0.05, using the control group as reference.

2.6.3. qPCR validation

For each treatment, the same RNA samples used for RNA-seq, together with one additional replicate were used as input to conduct sequencing results validation by quantitative real-time PCR (qRT-PCR). Eleven genes (1-sf, dec1b, prmt7, asb16, cyp4v8, rab44, hsf2, rorcb, arnt11a, ppp1r1b and one gene encoding un uncharacterized protein) were randomly selected from RNA-seq SDE genes of MC5 and Ext5 conditions within the expression intensity gradient (Table S5). 18s RNA and ef1 (elongation factor 1, alpha) were selected as the reference genes after the relative stability measurement by geNorm (Vandesompele et al., 2002). The process was described in Supplementary Method. The quantitative results (mean value) were compared with corresponding RNA-seq results and the linear correlation rate was calculated.

2.7. Ingenuity pathway analysis (IPA) of transcriptomics and proteomics data

Medaka dysregulated genes (FDR < 0.05 and $|Log_2FC| > 0.4$) and proteins ($|Log_2FC > 0.5|$) were converted to the corresponding Human orthologous according to Ensembl online platform. IPA software (V01-07; Qiagen) was used to perform canonical pathway analysis and molecular and cellular function determination based on the specific Ingenuity Knowledge Database (using default parameters for all tissues and cell lines, with relaxed filters), which constitutes a repository of biological interactions and functional annotations.

3. Results and discussion

For vertebrates and some other animals, the liver is a vital organ that performs a wide range of functions, including protein synthesis, lipid metabolism, energy generation and drug detoxification. Besides, liver function is particularly tied to reproduction regulation for oviparous fish on account of hepatic vitellogenin (vtg) synthesis (Arukwe and Goksøyr, 2003). Therefore, this integrated investigation with detailed information of altered liver proteomes and transcriptomes could provide insights into the potential reproductive toxicity of these cyanotoxins in vertebrate models.

In the present study, using C18 LC-MS analysis the methanol extract of Microcystis PCC 7820 exhibits 40 different secondary metabolites, mostly cyanopeptides, including 8 variants of MC (the predominant being the MC-LR and the [Dmet-Asp3]MC-LR variants), 4 cyanopeptolins, 3 cyclamides and some other molecules (Table S1). The overall composition remains broadly consistent with previous investigation (Le Manach et al., 2016; Ríos et al., 2014), delineating MC-LR and D-Asp3-MCLR as main metabolites/ cyanopeptides along with a large diversity of cyanobacterial metabolites produced by the representative strain of Microcystis aeruginosa. One and five $\mu g L^{-1}$ MC-LR and the Microcystis extract containing five µg L⁻¹ of equivalent MC-LR are chosen as environmentally relevant concentrations, corresponding to the concentration of MCs utilized in a few previous studies and often measured in water bodies during the summer season (Loftin et al., 2016; Pitois et al., 2016; Qiao et al., 2013; Zhao et al., 2015). Although the measured MC concentrations in the aquaria (mean values are 0.34, 2.24 and 1.81 $\mu g \; L^{-1}$ for MC1, MC5 and Ext5, respectively, Table S2) are lower than the designed ones due to several possible consumption mechanisms, such as photoisomerization, photodegradation and adsorption on the aquarium glass, the actual exposure concentrations still causes considerable toxic effects on the fish.

3.1. Reproductive impairment

During the exposure period, the fecundity (Fig. 1A) and the hatchability (Fig. 1B) significantly decrease under all MC-containing treatments compared with the control at multiple time points of the four weeks (p < 0.05). These results indicate that reproductive performance of medaka fish is adversely affected by all cyanotoxin treatments.

MCs have been reported to exert adverse effects on physiologically and behaviorally reproductive parameters of mammals, fishes, amphibians and birds (Chen et al., 2016). Especially in fish, it has been reported that the spawning behavior decreased when zebrafish were exposed to 50 μ g L⁻¹ of MC-LR for 6 days (Baganz et al., 1998). Ultrastructural tissue damages were also observed in the ovary and testis of medaka fish exposed to 5 $\mu g \, L^{-1}$ of MC-LR for 30 days (Trinchet et al., 2011). Such histological lesions in the gonad of fish exposed to MCs have been reported in other studies either via balneation (Qiao et al., 2013) or intraperitoneal injection (Hou et al., 2014) exposures. In addition, few studies have focused on hypothalamo-pituitary-gonadal (HPG) axis disturbances induced by MC exposure, which disrupts the reproductive process by affecting hormone levels (Liu et al., 2016; Zhao et al., 2015). It can be concluded that MCs impact the reproductive function of fish in multiple aspects, including reproductive behavior interference, gonad damage, and HPG axis disturbance. Besides, the decrease in reproductive output of fish upon exposure to MCs might also have another principal cause and could mainly be related to an overall liver dysfunction, which can induce indirect but important deleterious consequences referred to reproductive functions.

3.2. Liver lesion

In the liver of the control fish, the hepatocytes present compact and characteristic cord-like parenchymal organization (Fig. S1, A and E). In contrast, noticeable hepatic lesions characterized by cellular disjunctions and various lytic areas are observed in all toxic treatments, being particularly obvious in MC5 and Ext5 treatments. Some hepatocytes lose their characteristic shape and become rounded (Fig. S1, B-D and F-H). The proportion of lytic area exhibits an increasing trend in all toxic treatments compared with the control (Fig. 2A). Although there is no statistically significant difference due to the large individual variation (for instance, some toxin treated fish display a mild or even no response to the cyanotoxins from the histological perspective), integrated transcriptome and proteome analysis of liver highlights some responses of genes and proteins involved in cellular damage processes in the liver of fish exposed to all MC-containing treatments. A genuine activation of cell death process, including necrosis and apoptosis, accompanied by inhibitions of cell survival, cellular growth and proliferation is highlighted by IPA in the molecular and cellular function category (Fig. 7). This finding is directly corroborated and supported by the increase in hepatocyte lytic area observed by histology, since lysis could be a major consequence of the diffuse necrosis and apoptosis (Boorman et al., 1997).

The occurrence of liver lesions, including cellular necrotic or apoptotic events induced by various MCs or extracts of MCproducing cyanobacteria, has been reported previously in several fish species (Atencio et al., 2008; Fischer and Dietrich, 2000). It is known that the inhibition of serine/threonine PPI and PP2A, the typical toxicity mechanism of MC, subsequently leads to the destruction of the hepatic cytoskeleton, and consequently results in

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Fig. 1. Reproductive performance monitoring during the 28-day exposure. A: fertilized eggs per fish (mean \pm SD, n = 15); B: hatchability (mean \pm SD, n = 3). * and ** indicate statistically significant differences at p < 0.05 and p < 0.01, respectively.



Fig. 2. Histological observation of the liver. A: percentage of lytic area determined through the light microscopy observation of HES-stained sections (mean \pm SD, n = 5); B: percentage of glycogen content determined through the light microscopy observation of PAS-stained sections (mean \pm SD, n = 5). * and ** indicate significant differences at p < 0.05 and p < 0.01, respectively.

damages to liver structure (Fischer and Dietrich, 2000). In the present study, histological observation together with omic data attests to noticeable liver damages induced by the cyanotoxin treatments. This cellular damage might generally disrupt various hepatocyte metabolism regulations and indirectly affect liver physiological activities, including their implication in reproductive processes.

3.3. Hepatic glycogen store depletion

In the present study, intense glycoprotein and/or glycogen reserves stained in purple-red by PAS are distributed on all slides of the control fish (Fig. S2, A and E). In contrast, a severe decrease in glycoprotein and/or glycogen reserves is observed in the liver of toxin-treated females (Fig. S2, B-D), and the numerical values are plotted in Fig. 2B, showing a statistically significant decrease in MC5 and Ext5 treated females.

These results imply that a modification in liver glycogen synthesis or consumption processes might happen in the female fish exposed to cyanotoxins. Regulation of glycogen synthesis and breakdown is mainly controlled by two key enzymes: glycogen synthase and glycogen phosphorylase, respectively (García-Rocha et al., 2001). Our proteomics analysis shows an up-regulation of glycogen phosphorylase under MC1 and MC5 treatments for females and males, respectively (encoded by gene *pygl*, Table S6). This implies an up-regulation of glycogen breakdown process, which is consistent with the histological observation. On the one hand, the possible inhibition of phosphatase activity induced by MCs could affect the rate of glycogen metabolism by modifying the activity of key enzymes, through the modification of their respective phosphorylation states (Malbrouck et al., 2004). On the other hand, the depletion of hepatic glycogen is a common response of the fish liver to stressors such as toxins, and it may reflect a prior increased energy requirement for the organism homeostasis, via tissue repair and molecular detoxification processes (Macirella et al., 2016). Some previous studies have reported that the hepatic glycogen content of fish decreases under MC exposure, and it has been suggested that the increasing energy requirement contributes to that (Malbrouck et al., 2004; Marie et al., 2012).

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The dysregulation of gluconeogenesis is especially detected in the Ext5 treated fish through functional analysis on integrated transcriptomics and proteomics data (Fig. 7), implying that the glycogen-associated energy metabolism is impacted. Furthermore, the IPA canonical pathway analysis also indicates that other energy metabolism-associated pathways, such as acetyl-CoA biosynthesis 3 and ketogenesis at the transcriptional level (Fig. 6A), TCA cycle and pentose phosphate at the protein regulation level (Fig. 4), are significantly affected in the fish exposed to MC1 and MC5 conditions. Taken together, these results reveal an occurrence of a disorder of energy metabolism or shortage of energy supply in the medaka fish exposed to both pure MC and complex *Microcystis* extract. Sufficient energy supply is essential for maintaining normal physiological functions, being especially important to support the reproductive effort and guarantee its efficiency. Particularly for female fish, the depleted glycogen content associated with disturbed energy metabolism might have contributed, at least in part, to the decrease in reproductive output.

3.4. Oviparous-specific reproduction proteins expression dysregulation

For oviparous organisms, such as fish, large amounts of vitellogenin (vtg) and choriogenin (chg) are synthesized in the liver of mature female fish. These proteins, which constitute the main components of the oocyte yolk and the chorion, respectively, are secreted from the hepatocytes and then transported by the bloodstream to the ovary, where they contribute to the oocyte maturation. An appropriate production of vtg is crucial for reproductive successes, and the decrease in hepatic vtg or chg contents of female fish could directly affect the maturation of oocyte and alter their fecundability properties. As it has been shown in the present proteomics investigation, the quantities of vtg1, vtg2, vtg3 and L-SF precursor proteins are largely down-regulated under MC1 and MC5 conditions, and to a lesser extent under Ext5 (Fig. 3), which might be responsible for the decrease in reproductive output of female fish. The similar down-regulation of hepatic vtg1 gene expression has been reported in female zebrafish chronically exposed to MC-LR (Qiao et al., 2013). As the expression of vtg genes in the liver of female fish is positively regulated by 17β-estradiol (E2), the plasma E2/T levels were measured, but no significant variation was observed (Fig. S3), suggesting that the observed down-regulation of vtg protein quantity might result from an overall alteration of the liver, rather than specific endocrine perturbation. It has been reported that stress induced by toxic substances, such as ammonia, could impair the liver of fish and similarly result in unspecific impaired vitellogenesis (Garric et al., 1996).

Various methods have been used to evaluate the possibility of estrogenic activity of MC or mixture compounds released by cyanobacteria (Marie et al., 2012; Oziol and Bouaïcha, 2010; Qiao et al., 2013; Rogers et al., 2011). One in vitro study demonstrated a weak estrogenic potency induced by MC through a cell-based transactivation assay (Oziol and Bouaïcha, 2010). On the contrary, another in vivo study testified that a strong up-regulation of vtg gene expression was induced by the lyophilized Microcystis, but not by MC-LR in larval zebrafish (Rogers et al., 2011). Other in vivo studies also suggested that cyanobacterial extract could potentially induce estrogenic responses (Marie et al., 2012). Indeed, the vtg expression induction is classically considered as an evidence of a response to estrogens in experimentally tested oviparous vertebrates, particularly in males. According to our present transcriptomics and proteomics data, there is no activation of vtg gene expression nor over-representation of vtg protein in any male fish, demonstrating that the concentrations used here for either MC or the Microcystis extract does not cause any effective estrogenic response in adult medaka fish.

3.5. Global proteome dysregulation

The iTRAQ-based quantitative proteomic approach provides identification for a total of 305 and 453 proteins in females and males, respectively. Among these identified proteins, 298 and 448 of them present reliable quantifications according to the relative tag intensity normalized according to the control group. 150 and 153 proteins are considered as differentially regulated proteins in females and males, respectively (|Log₂FC|>0.5, under at least one of

the three MC-containing conditions, Table S6). The fold change of these dysregulated proteins is illustrated in an overall heatmap representation (Fig. S4), displaying a highly similar dysregulation pattern among the three toxic treatments in both genders. It seems that the molecular effects induced by MC-containing cyanobacterial extract globally correspond to those of pure MC-LR exposure. The MC-LR released by the cyanobacteria appears to have a dominant hepatotoxic effect over the other components in the extract. Moreover, the hepatic proteome of the fish under MC1 treatment was also altered in the present study. One μ g L⁻¹ of MC-LR has been released as a guideline value in drinking water by the world health organization (WHO, 2011). But our result suggests that the chronic exposure to MC guideline concentration value may potentially induce a health risk for aquatic organisms.

The up- or down- dysregulated proteins are individually assigned to functional groups according to their respective gene ontology classification (Fig. 3). Overall, they are related to various material and energy metabolisms, oxidation-reduction processes, detoxification, translation and nuclear receptor signaling. Among them, translation regulation is one of the most represented categories in both genders, illustrated by the down-regulation of numerous ribosomal proteins and eukaryotic translation factors. Ribosomal proteins are structural constituents of the ribosome, macromolecular machines responsible for protein synthesis in cells. It has been reported that dysregulated ribosomal proteins induced by Microcystis altered protein synthesis of Daphnia pulex (Asselman et al., 2012). In the study, a large set of ribosomal proteins and various translation factors are down-regulated in response to the cyanotoxin exposure, suggesting that the overall protein synthesis in the liver might be inhibited by MC-LR and Microcystis extract due to translational down-regulation. Additionally, enrichment of canonical pathways also indicates that EIF2 signaling, an important molecular signaling for protein translation controlling, is significantly affected in both genders of fish exposed to all MC-containing treatments (Fig. 4). As it has been described above, the reproduction-specific proteins (vtg and chg) are downregulated at the proteome level in female liver, which is in consistency with the overall protein synthesis inhibition. However, there is no corresponding observation of these reproductionspecific proteins at the transcriptome level (Table S7). It seems that the cyanotoxin exposures adversely affect the synthesis of vtg and chg by down-regulating their translation processes without modifying their relative transcription levels.

3.6. Global transcriptome dysregulation

The high-throughput sequencing of cDNA results in the identification of transcripts corresponding to 16,262 and 15,633 different genes (read count \geq 2) for females and males, respectively. For females, 6, 168 and 418 genes are significantly differentially expressed (SDE) under MC1, MC5 and Ext5 treatments. For males, 17, 126 and 104 genes present differential expression under MC1, MC5 and Ext5 conditions ($|Log_2FC| \ge 0.4$, FDR < 0.05, Table S7). The apparent increase in the number of SDE genes in MC5 condition compared with that in MC1 condition suggests that the dysregulation of gene expression follows a dose-dependent response. The high correlation between the RNA-seq and RT-qPCR results provides a clear validation of the overall SDE genes obtained by the RNA-Seq analysis. A good linear correlation between these two approaches was indeed supported by R² correlation coefficient values of up to 0.96 and 0.86 in females and males, respectively (Fig. 5A).

The global variability of the gene expression in different replicates is investigated by principal component analysis (PCA), showing an apparent distinction between replicates from control

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Fig. 3. Heatmap representation of the overall alteration of hepatic proteomes with gene ontology classification. 225 dysregulated proteins (|Log₂FC| > 0.5, under at least one toxic condition) for both genders in total are represented by Log₂FC value using Gene-E freeware (http://www.broadinstitute.org/cancer/software/GENE-E/). Up-regulated proteins are indicated in red, down-regulated proteins in green, and missing values in gray. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and MC5 groups, as well as between control and Ext5 groups, but not between control and MC1 in both genders (Fig. 5B). Therefore, the specificities of the SDE genes (representing a limited number) in response to MC1 treatment will not be further considered below in terms of function enrichment and pathway analysis.

Venn diagram representations are depicted to compare the dysregulated genes between the different conditions. Only 97 (half) and 32 (one-fourth) SDE genes detected in MC5 condition are

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_	Category	Canonical Pathway	P value			
			MC1	Ext5		
	cellular stress and injury	NRF2-mediated Oxidative Stress Response	1.89E-05	3.86E-04	4.06E-02	
	cellular growth, proliferation	EIF2 Signaling	2.54E-04	1.31E-06	4.22E-03	
	and development					
	hormones degradation	Serotonin Degradation	1.15E-03	7.34E-03	1.00E+00	
	disease specific pathway	Mitochondrial Dysfunction	1.90E-03	4.26E-02	3.44E-03	
	metabolic regulations biosythesis	Creatine-phosphate Biosynthesis	1.53E-02	9.56E-03	8.85E-03	
	nuclear receptor signaling	LPS/IL-1 Mediated Inhibition of RXR Function	4.76E-03	8.34E-04	5.85E-02	
Female		Aryl Hydrocarbon Receptor Signaling	9.17E-03	2.44E-03	2.21E-01	
	amines and polyamines degradation	Choline Degradation I	6.13E-03	3.84E-03	1.00E+00	
		4-aminobutyrate Degradation I	9.19E-03	5.75E-03	5.32E-03	
	amino acids metabolism	Threonine Degradation II	6.13E-03	3.84E-03	3.55E-03	
		Alanine Degradation III	6.13E-03	1.00E+00	3.55E-03	
		Alanine Biosynthesis II	6.13E-03	1.00E+00	3.55E-03	
		Glutamate Degradation II	9.19E-03	5.75E-03	5.32E-03	
		Aspartate Biosynthesis	9.19E-03	5.75E-03	5.32E-03	
		L-cysteine Degradation I	1.22E-02	7.66E-03	7.08E-03	
		Proline Biosynthesis I	1.22E-02	7.66E-03	7.08E-03	
		Glutamate Degradation III	1.53E-02	9.56E-03	8.85E-03	
	cellular growth proliferation	EIF2 Signaling	2.3E-08	3.0E-21	1.9E-13	
	and development	Regulation of eIF4 and p70S6K Signaling	3.8E-05	3.3E-06	2.3E-07	
		mTOR Signaling	8.7E-06	1.6E-04	1.2E-06	
	sugars biosynthesis	Gluconeogenesis I	1.9E-03	5.2E-03	7.1E-02	
	hormone biosynthesis	Thyroid Hormone Biosynthesis	7.8E-03	1.3E-02	8.8E-03	
	generation of precursor metabolites	TCA Cycle II (Eukaryotic)	2.8E-05	4.4E-03	6.5E-02	
	and energy	Pentose Phosphate Pathway	2.9E-04	8.1E-04	3.7E-04	
	metabolic clusters	tRNA Charging	4.6E-03	6.4E-04	5.8E-03	
	cofactors, prothetic groups	S-adenosyl-L-methionine Biosynthesis	7.8E-03	1.3E-02	8.8E-03	
	and electron carriers biosythesis					
Male	C1 compounds utilization	Formaldehyde Oxidation II	1.0E+00	8.6E-03	1.0E+00	
	and assimilation					
	fatty acid biosynthesis	Fatty Acid Biosynthesis Initiation II	1.0E+00	8.6E-03	5.8E-03	
		Palmitate Biosynthesis I (Animals)	1.0E+00	8.6E-03	5.8E-03	
	amino acids metabolism	Superpathway of Methionine Degradation	7.7E-05	3.5E-04	4.0E-03	
		Aspartate Degradation II	1.4E-04	3.8E-04	1.0E+00	
		Cysteine Biosynthesis III (mammalia)	4.8E-02	3.0E-03	1.4E-03	
		Phenylalanine Degradation I (Aerobic)	1.0E+00	1.1E-04	1.0E+00	
		L-cysteine Degradation II	1.0E+00	4.3E-03	2.9E-03	
		Cysteine Biosynthesis	1.0E+00	8.6E-03	5.8E-03	
		Glutamate Degradation X	1.0E+00	8.6E-03	5.8E-03	
		Glutamate Biosynthesis II	1.0E+00	8.6E-03	5.8E-03	

Fig. 4. Significantly affected pathways determined with hepatic proteome data through canonical pathway analysis of IPA (*p* < 0.01 is indicated in red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

present in the SDE genes of Ext5 treatment for females and males, respectively (Fig. 5C). MCs are not the only noticeable toxic compounds present in the cyanobacterial extract. Other compounds have received increasing attention for their potential bioactivity that may lead to synergistic or antagonistic effects together with MCs, but the information about those metabolites still remains limited. In the present study, Ext5 exposure leads to 418 SDE genes in female medaka fish being two times more numerous than the SDE genes induced by MC5, implying that the toxicity of complex cyanotoxin mixtures, including MCs, could be wider or greater than that of the pure MC. This phenomenon has also been observed previously in adult medaka and larval zebrafish exposed to cyanobacterial extract and pure MC (Marie et al., 2012; Rogers et al., 2011).

Canonical pathway analysis indicates that the SDE genes significantly contribute to various pathways (p < 0.01), including circadian rhythm signaling, cellular growth and injury, amino acid, nucleotide, fatty acid and lipid metabolisms and so on (Fig. 6A). The respective Log₂FC values of all these genes involved in those canonical pathways are shown in a heatmap (Fig. 6B). In terms of impacted pathways, more biological pathways are affected by the Ext5 treatment compared with pure MC treatments in both genders, but particularly in females. Those female supplementary pathways mostly concern signaling pathway categories, comprising a large set of nuclear receptor signals and various second

messenger signals, such as estrogen receptor, PPARa/RXRa, TR/RXR, FXR/RXR, PXR/RXR and LXR/RXR. Similar molecular effects have been reported previously, for instance, the nuclear receptor gene family nr1d2b was significantly down-regulated in zebrafish larvae by Microcystis extract exposure (Rogers et al., 2011). One purified cyanopeptide (cyanopeptolin 1020) produced by different Microcystis and Planktothrix strains, led to the expression dysregulation of nuclear receptors related gene in zebrafish embryos (Faltermann et al., 2014). In fact, different cyanopeptolin variants are also present in the Microcystis extract used in the present study (Table S1). Considering the importance of liver functions regulated by nuclear receptors in fatty acid oxidation and uptake, thyroid hormone metabolism, bile acid homeostasis, gluconeogenesis and reproductive hormone metabolism, Microcystis extract seems to have a greater potential to disturb the reproductive function of female medaka fish.

Disease and biological function analysis detects 49 significantly affected molecular and cellular functions (p < 0.05 and |z-score| > 1.4, Fig. 7). Specifically, these disturbed functions are related to various cellular processes including cell death, lipid metabolism and gene expression that appear to be globally activated. However, the treatments appear to globally inhibit various other molecular and cellular functions, such as functions concerning cell survival, three specific lipid metabolism processes, carbohydrate metabolism, small molecule biochemistry, cellular growth,



Fig. 5. Analysis of hepatic transcriptome through RNA-seq. A: RT-qPCR validation of the SDE genes determined through RNA-seq. B: PCA illustration of the global variability of individuals under different conditions in females and males, respectively. C: Venn diagram representations to compare the SDE genes between different MC-containing conditions by directly comparing the gene entries.

proliferation and movement, DNA binding and RNA repression. Integrated studies based on transcriptome and proteome analysis often present a limited correlation between mRNA expression and protein abundance due to different synthesis regulation timescales, biological modifications (post-transcription machinery) or technical variations (Ghazalpour et al., 2011). In the present study, the comparison of the dysregulated gene and protein entries display a limited overlap between these two analyses (only 13 out of total 225 proteins or 654 genes are in common, Fig. S5). This big variance between the gene expression level and the protein content might be, to some extent, resulted by the difference in the number of individual in each pooling sample. However, a noticeable compatibility has been observed between the transcriptome analysis and the proteome investigation at this molecular and cellular function level (Fig. 7). The activation of cell death and lipid metabolisms is especially consistent between our transcriptomics and proteomic results, characterized by an apparent induction of processes related to cell death, necrosis and apoptosis, and an inhibition of proteinaceous mechanisms involved in lipid uptake, synthesis, conversion and oxidation. Furthermore, transcriptome data even shows a clearer and more notable cellular response than that depicted by proteomes, which is highlighted by a clear up and down-regulation between some opposing function aspects, such as cell death and cell survival, respectively.

3.7. Fatty acids and lipids metabolism disorder

Pathway analysis of the transcriptome data reveals that MC5 and Ext5 treatments significantly affect hepatic fatty acids and lipids metabolism in female fish, but to a much lesser extent in males (Fig. 6A). In particular, choline, cholesterol, and linolenate biosynthesis, phospholipases and triacylglycerol degradation are concerned. The gender differences in lipid metabolism are very likely related to the massive material needs for reproductive 128

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	Category	Canonical Pathway	P value MC5	Ext5	
-	neurotransmitters and other nervous system	Circadian Rhythm Signaling	1.6E-09	1.3E-06	
	signaling	Cholecystokinin/Gastrin-mediated Signaling	4.9E-01	8.4E-03	
		Axonal Guidance Signaling	5.4E-01	9.2E-03	
	cellular growth, proliferation and development	Sertoli Cell-Sertoli Cell Junction Signaling	1.0E+00	4.0E-03	
	termentational resultation	B Cell Activating Factor Signaling	1.0E+00	5.0E-03	
	transcriptional regulation	EXP/RXP Activation	5.7E-02	1.0E-02	
	nuclear receptor signaling	LXR/RXR Activation	1.9E-01	2 7E-04	Circadian rhythm si
		PXR/RXR Activation	1.0E+00	1.8E-05	
		TR/RXR Activation	1.1E-01	1.7E-05	
		PPARa/RXRa Activation	3.3E-01	1.5E-05	
		Estrogen Receptor Signaling	5.7E-01	7.1E-03	
	intracellular and second messenger signaling	Goq Signaling	2.5E-01	4.2E-03	
		Insulin Receptor Signaling	5.8E-01	2.2E-03	Cellular growth, prolit and develo
		Adipogenesis pathway	2.5E-04	9.0E-03	
		Glucocorticoid Receptor Signaling	1.0E+00	8.9E-03	
		RhoA Signaling	4.6E-02	1.3E-03	
		Protein Kinase A Signaling	2.5E-01	1.5E-04	
	disease-specific pathways	Pancreatic Adenocarcinoma Signaling	1.5E-01	2.5E-03	Growth factor si
	centular stress and injury	ERK/MARK Signaling	1.95-01	5.3E-04	Transcriptional reg
nale	cancer	Sonic Hedgehon Signaling	1.0E+00	1.7E-03	1000 March 8 (1000) 10 -
mane	organismal growth and development	Wnt/Ca+ oathway	3 1E-01	2.8E-03	
	apontosis	April Mediated Signaling	1.0E+00	4.1E-03	
	cellular immune response	Interferon Signaling	1.7E-03	4.7E-01	
	amino acids metabolism	Citrulline Biosynthesis	1.5E-05	7.8E-03	
		Superpathway of Citrulline Metabolism	9.6E-05	2.4E-02	
		Taurine Biosynthesis	1.0E+00	3.0E-04	
		Phenylalanine Degradation IV (Mammalian, via Side Chain	3.7E-03	1.0E+00	Nuclear receptor si
	fatty acids and lipids metabolism	Choline Biosynthesis III	3.2E-03	1.3E-03	
		Cholesterol Biosynthesis III (via Desmosterol)	3.2E-03	2.0E-01	
		Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	3.2E-03	2.0E-01	
		Cholesterol Biosynthesis I	3.2E-03	2.0E-01	
		Superpathway of Cholesterol Biosynthesis	3.2E-05	3.9E-01	
		y-linolenate Biosynthesis II (Animals)	1.1E-01	2.9E-03	
		Fatty Acid Activation	8.2E-02	1.3E-03	
		Phospholipases	5.5E-02	5.0E-04	
		I nacylglycerol Degradation	1.0E+00	8.0E-03	Gaq Sig
	asfastes exectivity groups and	Mitochondrial L-carnitine Shuttle Pathway	1.1E-01	2.9E-03	Insulin recentor di
	coractors, prostnetic groups and	Superpatriway of Geranyigeranyidiphosphate Biosynthesis	5.4E-03	1.0E+00	Insulin receptor si
	electron carriers biosynthesis	Folate Transformations I	1.6E-03	1.0E+00	
	normones biosynthesis	Acatul-CoA Biosunthesis III (from Citrate)	C. EE-02	1.7E-02	Adipogenesis pa
-	generation or precursor metabolites and energy	Acety-CoA biosynthesis in (nom citrate)	0.0E-03	1.76-02	
	neurotransmitters and other nervous system	Circadian Rhythm Signaling	1.8E-05	2.0E-07	Glucocorticoid receptor sig
	growth factor signaling	Neuregulin Signaling	8.8E-03	1.0E+00	
	humoral immune response	Complement System	1.0E+00	9.7E-03	
	nuclear receptor signaling	FXR/RXR Activation	3.2E-03	4.0E-01	
	amino acids metabolism	Cysteine Biosynthesis/Homocysteine Degradation	1.0E+00	8.0E-03	RhoA si
		Arginine Degradation VI (Arginase 2 Pathway)	1.0E+00	2.4E-04	
		Superpathway of Methionine Degradation	1.0E+00	7.3E-03	
		Arginine Degradation I (Arginase Pathway)	1.0E+00	9.5E-05	
		Citrulline Degradation	4.8E-03	1.0E+00	
		Citrulline Biosynthesis	3.8E-02	4.4E-04	Protein kinase A si
		Superpathway of Citrulline Metabolism	6.5E-02	1.4E-03	
Male		Taurine Biosynthesis	9.6E-03	1.0E+00	
	fatty acids and lipids biosynthesis	Superpathway of Cholesterol Biosynthesis	9.4E-06	1.0E+00	
		Mevalonate Pathway I	3.0E-05	1.0E+00	Complement
	primidine nucleotide biosynthesis	Pyrimidine Ribonucleotides Interconversion	1.3E-01	6.1E-03	
		Pyrimidine Ribonucleotides De Novo Biosynthesis	9.7E-03	2.6E-04	Interferon si
		Undine-b-phosphate Biosynthesis	9.6E-03	8.0E-03	
	nucleosides and nucleotides biosynthesis	Guanine and Guanosine Salvage I	1.0E+00	8.0E-03	ERK/MADY -
	amines and polyamines biosynthesis	Spermine Biosynthesis	1.0E+00	8.0E-03	Entry MAPK SI
	a line many de anatalis e lleve	Spermane closynthesis I	1.02+00	8.0E-03	
	nitrogen compounds metabolism	Supermethyse of Geranulgeranulginhoenhate Discusterais	2.8E-02	2.4E-04	Axonal guidance si
	electron carriers biosynthesis	Superparate Blosynthesis	0.92-05	1.00+00	and the second second second
	The second				



Fig. 6. Significantly affected pathways determined with hepatic transcriptome data through canonical pathway analysis of IPA. A: Significantly affected pathways determined with the SDE genes (*p* < 0.01 is indicated in red). B: Heatmap representation of the actual Log₂FC values of all the genes involved in the significantly affected pathway. Up-regulated genes are indicated in red, down-regulated genes in green and missing values in gray. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

process in females, since the liver is responsible for a large amount of lipid and fatty acid deposits to the oocyte too.

Choline is an essential nutrient, playing an important role in fat metabolism by hastening removal or decreasing deposition of fat in the liver. The potential choline biosynthesis disturbance could be responsible for the described liver histological damages since choline deficiency has been shown to be consistently associated with liver damage in rat upon exposure to MC-LR (He et al., 2012). Meanwhile, four cholesterol biosynthesis pathways are significantly impacted in the female fish treated with MC5. As acetyl-CoA Q. Qiao et al. / Environmental Pollution 219 (2016) 119-131

z score transcript level protein level \$ 8 \$ б molecular and cellular functions cell death of epithelial cell cell death cell death of carcinoma cell lines cell death necrosis of epithelial tissue necrosis of brain cancer cell lines necrosis apoptosis internalization by tumor cell lines reorganization of filaments oxidation of lipid uptake of lipid conversion of lipid lipid metabolism oxidation of fatty acid concentration of lipid synthesis of lipid transcription of DNA gene expression activation of DNA endogenous promoter quantity of amino acids metabolism of protein accumulation of triacyglycerol accumulation of lipid molecular transport secretion of molecule metabolism of nucleic acid component or derivative metabolism of terpencid lipid metabolism concentration of acylglycerol steroid metabolism quantity of carbohydrate carbohydrate metabolism gluconeogenesis concentration of triacylglycerol fatty acid metabolism small molecule biochemistry synthesis of purine nucleotide secretion of steroid concentration of D-glucose quantity of hdl cholesterol in blood transport of molecule cell viability of breast cancer cell lines cell viability of brain cancer cell lines cell survival cell viability of tumor cell lines cell surviva proliferation of cells cell proliferation of squamous cell carcinoma cell lines cell proliferation of tumor cell lines cellular growth and proliferation cvtostasis of tumor cel lines cytostasis migration of cells invasion of cells invasion of tumor cell lines cell movement cellular movement migration of tumor cell lines cell movement of prostate cancer cell lines migration of prostate cancer cell lines migration of tumor cells cell proliferation of ovarian cancer cell lines binding of cells metabolism of carbohydrate binding of DNA gene expression sion of RNA repres conversion of amino acids

Fig. 7. Heatmap representation of significantly affected cellular and molecular functions determined with transcriptome and proteome data through IPA. Disease and biological function analysis model of IPA is conducted with all dysregulated genes and proteins (*|z-score|* > 1.4 and *p* < 0.05). The result is presented by *z-score*, which indicates the activation (in red) or inhibition (in blue) effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

is an important precursor molecule of cholesterol synthesis, acetyl-CoA biosynthesis perturbation, as it has also been observed here, could highly contribute to the modification of cholesterol synthesis. It has been described earlier that the phosphatase inhibition action of MCs could dysregulate glucose metabolism process through the modification of various key enzymes, which consequently affects the content of some metabolic products, such as acetyl-CoA. The perturbation of cholesterol biosynthesis was also observed in zebrafish exposed to MCs, and acetyl-CoA was suggested to be one important cause for interfering in the balance between glucose and fat metabolism (Pavagadhi et al., 2013). Furthermore, cholesterol biosynthesis perturbation may have consequences on steroid hormone and bile acid biosynthesis, since cholesterol is essential for the synthesis of steroid hormones and bile acids. Steroid hormones and bile acids are endocrine signaling molecules, which have important roles in maintaining important physiological function in fish, such as in the reproductive process. The reproductive hormone levels are highly dynamic due to complex feedback regulation mechanisms in organisms. In the present study, the reproductive hormone (E2 and 11-KT) levels in plasma are not significantly

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modified (Fig. S3), however, a potential disturbance of steroid hormone synthesis is still suggested through the disturbed cholesterol synthesis. Other associated substances, such as bile acids, not only facilitate the absorption and digestion of lipidsoluble nutrients but also act as endocrine signaling molecules. They can activate multiple nuclear and membrane receptormediated signaling pathways. In fact, the perturbation of nuclear receptor and intracellular signaling is another highly impacted aspect evidenced by pathway analysis of the hepatic transcriptomes of the toxin-treated female fish (Fig. 6A), which might be associated with the potential bile acid level disturbance. Taken together, our results described above suggest that both MC5 and Ext5 exposure induce a disruption of major lipid metabolic pathways in medaka liver, particularly in females. Besides energy shortage and protein biosynthesis inhibition, this lipid metabolism disruption is another important aspect that could significantly contribute to the observed reproductive impairments.

3.8. Circadian rhythm signaling perturbation

In various organisms, circadian rhythm regulation plays an important role in a wide variety of physiological functions including sleeping, feeding, reproduction and cellular metabolism. Circadian clocks are present not only in the central nervous system but also in peripheral organs and tissue, such as the liver (Kaneko et al., 2006). The molecular mechanism of circadian rhythms has been described in Drosophila and in mice, functioning through oscillation feedback loops, generally consisting of four core proteins. Clock (circadian locomotor output cycles protein kaput) and bmal1 (brain and muscle ARNT-like 1) proteins transcriptionally regulate clock-controlled genes, and meanwhile, activate the transcription of per (period) and cry (cryptochrome) proteins, which in turn produce a negative feedback loop by suppressing clock:bmal1-mediated transcription (Dunlap, 1999). For fish, the circadian system appears to have a similar scheme, and the genes encoding these four proteins have been shown to be rhythmically expressed in peripheral tissues of zebrafish (Cahill, 2002).

In the present study, the pathway analysis of hepatic transcriptomes reveals that the circadian rhythm signaling is the most affected pathway in the liver of the two genders of fish exposed to MC5 and Ext5, evidenced by a significant down-regulation of period and crv genes accompanied by up-regulated bmal (Fig. 6). This result constitutes the first report of the expression dysregulation of circadian rhythm related genes induced by MC exposure. The liver plays important roles in glucose and lipid metabolisms. On one hand, it has been proved that glucose and lipid metabolisms are highly regulated by hepatic circadian rhythm (Gnocchi et al., 2015; Johnston, 2014). On the other hand, the alteration in metabolism processes could also affect the circadian rhythm-relevant genes in return (Johnston, 2014). In the present study, the various modified hepatic metabolic processes could be the consequence of circadian rhythm perturbation in response to MC or alternatively constitute a possible cause of hepatic circadian disturbance in fish chronically exposed to the cyanotoxins. The observed nuclear receptor signaling perturbation could partially contribute to the second hypothesis, since nuclear receptors play important roles in regulating the circadian rhythm. The underlying mechanism of the present phenomenon remains unclear, and to date, there is only one investigation that reported a circadian rhythm perturbation induced by cyanopeptolin 1020 extracted from Microcystis aeruginosa in zebrafish embryos (Faltermann et al., 2014).

Reproductive traits in medaka fish are regulated by the photoperiod. Light variations are integrated by complex feedback loops of the core clock genes located in the central suprachiasmatic nucleus (SCN) (Harmer et al., 2001). The circadian activity generated in the central SCN affects multiple physiological rhythms, including reproductive periodicity, and it also directly sets the peripheral circadian machinery (Gnocchi et al., 2015). Based on the present observation of the hepatic circadian disturbance induced by MC and *Microcystis* extract, one can hypothesize that central circadian clocks might have been influenced by the exposure to cyanotoxins as well, consequently disturbing the reproductive function. Another conjecture is that central circadian clocks are impacted through the interplay between the central and hepatic circadian machinery, although so far there was no clear evidence showing the feedback effect of peripheral circadian on the central SCN.

Hepatic circadian rhythm regulation is associated with energy and various materials metabolism that are essential for reproductive process. In addition, it might potentially interfere with the light-period control of fish reproduction activation as it is the case in medaka. Therefore, the observed hepatic circadian perturbation could be, at least partially, responsible for the reproductive disturbance observed in the present study.

4. Conclusion

Our results indicate that both MC-LR and the Microcystis extract exert hepatotoxic effects at different levels, comprising structural disruption and function disorder. The observed hepatic alterations contribute to reproduction impairments by affecting energy supply, lipid and fatty acid metabolism, oocyte-specific protein synthesis and circadian regulation-related gene perturbation. It is worth mentioning that the integrated proteomics analysis and the transcriptomics investigation highlight cell death process, lipid and carbohydrate metabolisms coherently, attesting to a potent hepatic cytotoxicity and consequent liver metabolic disorders induced by both MC-LR and the Microcystis extract. Furthermore, two interesting findings have been revealed by the two omic analyses independently. The first one is that the proteomics analysis suggests that the MC-containing treatments might inhibit overall liver protein synthesis by down-regulating ribosomal proteins and various translation factors. For the second finding, the transcriptomics investigation shows that the hepatic circadian rhythm regulation system has been impacted by the present toxin treatments.

The present study also shows that a wider range of biological pathways are disturbed by the complex mixture compounds produced by cyanobacteria compared with pure MC-LR. In the aquatic environment, low concentrations of cyanotoxins and other bioactive compounds produced by cyanobacteria widely spread. However, the information about a big number of compounds produced by cyanobacteria is still limited. More efforts on long-term study of these diverse toxic compounds released by cyanobacteria are urgently needed to advance our current knowledge on the protection of aquatic organism populations as well as human health from chronic cyanotoxin issues.

Author contribution

Q.Q., S.L.M., H.H., M.E. and B.M. conceived the experiments, Q.Q., S.L.M., H.H, C.D., B.S. and B.M. conducted the experiments, Q.Q., S.L.M., H.H, E.D.B., S.C., L.P., A.M, L.M., S.L., G.B., C.D. and B.M. analyzed the results. All authors reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http:// dx.doi.org/10.1016/j.envpol.2016.10.029.

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Annex 5.2 Article 2 Deep sexual dimorphism in adult medaka fish liver highlighted by multi-omic approach

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OPEN Deep sexual dimorphism in adult

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Sexual dimorphism describes the features that discriminate between the two sexes at various biological levels. Especially, during the reproductive phase, the liver is one of the most sexually dimorphic organs, because of different metabolic demands between the two sexes. The liver is a key organ that plays fundamental roles in various physiological processes, including digestion, energetic metabolism, xenobiotic detoxification, biosynthesis of serum proteins, and also in endocrine or immune response. The sex-dimorphism of the liver is particularly obvious in oviparous animals, as the female liver is the main organ for the synthesis of occyte constituents. In this work, we are interested in identifying molecular sexual dimorphism in the liver of adult medaka fish and their sex-variation in response to hepatotoxic exposures. By developing an integrative approach combining histology and different high-throughput omic investigations (metabolomics, proteomics and transcriptomics), we were able to globally depict the strong sexual dimorphism that concerns various cellular and molecular processes of hepatocytes comprising protein synthesis, amino acid, lipid and polysaccharide metabolism, along with steroidogenesis and detoxification. The results of this work imply noticeable repercussions on the biology of oviparous organisms environmentally exposed to chemical or toxin issues.

Sexual dimorphism terminology is widely used for organisms that perform sexual reproduction to describe physiological differences between two sexes at various biological levels. Although sexual dimorphism is generally considered at the anatomical or the behavioural level, it can also be extended to differences in the physiology of functions not directly involved in reproductive processes. According to different strategies for survival fitness of the two sexes and the consideration of their respective sexual specificities, exogenous perturbations could induce noticeable dissimilarities of endogenous response of individuals of the two sexes. Recent research observations have shown that a large number of genes exhibited deep sexual differences at the transcriptomic level in various tissues, suggesting that, in fact, sex-dependent genetic and hormonal regulations could also affect non-gonadal organs such as brain or liver¹⁻³. This assumption is supported by numerous reports that have addressed that males and females may differ in their susceptibility to environmental or biological stresses, as well as in the differential responsiveness of the liver to various xenobiotics⁴.

The liver is a key organ that plays fundamental roles in various physiological processes, including digestion, energetic metabolism, xenobiotic detoxification, biosynthesis of serum proteins, and also in endocrine or immune responses. Because of the different metabolic needs between sexes, especially during the reproductive phase, the liver is one of the most sexually dimorphic organs in terms of gene expression⁵. The first evidence of a sex-related

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difference in the rat hepatic steroid metabolism was published in 1953⁶. Based on these initial observations, five decades of research have since then established the existence of a gonadal-hypothalamo-pituitary-liver axis that determines the differences between male and female liver. Moreover, the importance of hormone secretion patterns has been revealed and the understanding of hepatic gene regulation at the molecular level has advanced in mammals⁷. For example, various studies have shown that many hepatic genes associated with xenobiotic metabolisms, such as cytochrome P450, are expressed in a sex-dependent manner during the detoxification process⁴. Particularly, the sex-dimorphism of the liver is obvious in oviparous animals, as the female liver is the main organ for the synthesis of oocyte constituents, such as the yolk protein precursors (vitellogenins) and the zona pellucida proteins (choriogenins)⁸.

As continental aquatic environments are threatened by a large spectrum of xenobiotics and pollutants, freshwater oviparous organisms such as fish are especially impacted by these potential toxicants, and their liver detoxification capabilities constitute essential defences for the fitness of these organisms. In this context, one can suppose that the various sexual dimorphisms of oviparous organisms, concerning energetic metabolism, detoxification and reproduction processes may drastically influence the hepatic responses of different sexes.

In this work, we were interested in identifying the molecular sexual dimorphism in the liver of adult medaka fish and illustrating its implication in response to hepatotoxic exposures. Small fish such as the Japanese medaka (*Oryzias latipes*) have emerged as useful vertebrate model organisms, suitable for studying various physiological processes⁹, toxicological mechanisms¹⁰ and also ecotoxicological effects¹¹. Medaka fish presents the advantages of small size, established models produced from inbred lines, rapid development and growth, high fecundity, omnivorousness, and also shows sugar and lipid metabolic profiles similar to those of mammals⁹. By developing an integrative approach comprising histology and different high-throughput omic investigations (*i.e.* metabolomics, proteomics and transcriptomics), we are now able to globally describe the sex-dimorphism in the medaka liver. To our knowledge, this constitutes the first systematic investigation of the liver sex-dimorphism in this model organism. Furthermore, under hepatotoxin perturbed conditions, sex-specific variation in molecular responses was investigated using quantitative proteomic analyses, implying potential different repercussions on the biology of fish environmentally exposed to chemical issues.

Results

Histology. The liver of organisms under undisturbed lab condition presents in both sexes a characteristic architectural organization with polyhedral hepatocytes organized around the capillary sinusoids and the bile canaliculi, appearing in characteristic cord-like parenchymal structures. As shown in Fig. 1, medaka fish liver presents a sexual dimorphism at the cellular level based on histological observations of the hepatocytes. Indeed, male and female hepatocytes present obvious differences in their global cytoplasm appearance with a distinct distribution of vesicles that are revealed using hematoxylin-eosin-saffron (HES), periodic acid-Schiff/alcian blue (PAS) or toluidin blue staining. Whereas female hepatocytes present large isolated reserve vesicles (mostly one per hepatocyte) with dense contents of glycoprotein and/or glycogen (Fig. 1C,D,G,H), male hepatocytes exhibit more diffuse small vesicles (Fig. 1A,B,E,F).

NMR metabolomics. The hydrophilic fraction of the liver of medaka bred under undisturbed lab condition was investigated on 18 males and 18 females by ¹H NMR analysis as shown in Fig. 2. Up to 237 different potential metabolites have been detected and relatively quantified according to the Batman R package analysis. The global analysis of the molecular pathway involved in liver metabolism reveals that the medaka liver metabolome



Figure 2. Metabolomics of male and female medaka livers by ¹H NMR. Frincipal component analysis (PCA) performed with the quantification values of the 237 metabolites detected by Batman' R package algorithm (Supplementary Table S2) from the 18 males and 18 female individual NMR spectra (A). Volcano plot representation of the 237 metabolites according to female/male fold change average and significance of the differences ($|log_2 FC| > 1$ corresponding to |FC| > 2 and log_{10} Pvalue < 1.3 corresponding to Pvalue < 0.05) (B). Female and male over-represented metabolites are determined with positive and negative significant FC (F/M) values and are shown in red and blue, respectively, and are represented with darker colours when metabolite presenting VIP values are superior to 1, according to PLS-DA analysis. Top-25 lists of the putative annotations of male- and female-representative metabolites (C and D, respectively).

presents a significant enrichment in a very wide diversity of processes, comprising principally glutathione, taurine, amino acid, carbohydrate, lipid, steroid hormone and tricarboxylic acid (TCA) cycle metabolisms (supplementary Table S1). Although our metabolomic analysis was performed on the hydrophilic fraction of the liver, we were able to observe various mostly hydrophobic metabolites, such as steroidal compounds, that testify of the intense lipid metabolism, especially in males. Principal component analysis (PCA) clearly discriminates specific liver metabolome between males and females according to its component 1 that comprises 76% of the total sample variation (Fig. 2A).

Significantly over-represented metabolites in both sexes (|FC| > 2 and Pvalue < 0.05) were highlighted in a volcano plot (Fig. 2B). Whereas 59 molecules are over-represented in females, 103 appear to be over-represented in male metabolome (supplementary Table S2). According to their putative annotation provided by the Batman algorithm, a pathway enrichment analysis indicates that female-enriched metabolomes would rather be implicated in some saccharide and amino acid metabolic processes, whereas male-enriched metabolomes seem to exhibit signatures of steroid hormone biosynthesis and energy (TCA, nitrogen) processes, when the common metabolite set is more relevant to other amino acid and saccharide metabolism (Fig. 2C,D). Another interesting noticeable specificity of the medaka fish metabolome concerns the relative quantity of taurine and hypotaurine that are strongly over-represented in females and males, with 327 and 50 |FC|, respectively.

Proteomics. The cytosolic fraction of the liver proteome of medaka bred under undisturbed lab condition was investigated by high-throughput method of bottom-up proteomics. The trypsin-cleaved peptides were separated by nano-LC and analysed by high-resolution mass spectrometry, then the proteins were identified thanks to a large genomic dataset available for the Japanese medaka¹². Among the 820 proteins identified in the three male pools, 64 appear to be potentially male-biased, because they were detected in both 3 male pools, and not in any of the three female pools. For females, 178 seem to be similarly biased of the 934 total proteins identified in the three is also illustrated with semi-quantification of the proteins according to both MS and MS/MS data, represented in a volcano plot (Fig. 2B). Among 1241 identified proteins, 25 and 125 are significantly over-represented in males



Figure 3. Proteomics of male and female medaka livers. Unscaled Venn's diagram of the protein identified with at least 95% protein identification certainty in all of the 3 different 3-individual pools of male and/or female medaka livers (**A**). Volcano plot representation of the 1241 proteins according to female/male fold change average and significance of the differences ($|log_2 \ FC| > 1.5$ corresponding to |FC| > 3 and log_{10} Pvalue < 2 corresponding to Pvalue < 0.01) determined according to Scaffold 4.5.1 semi-quantitative values based on both MS and MS/MS data (**B**). Female and male over-represented proteins are determined with positive and negative significant FC (F/M) values and are shown in red and blue, respectively. Top-25 lists of the male- and female-superabundant proteins (**C** and **D**, respectively).

and females, respectively (|FC| > 3 and p < 0.01) (Supplementary Table S3). A global analysis of these sex-biased proteins according to the described functions of their human orthologs suggests that female-biased proteins are significantly related to tRNA and nucleotide sugar metabolism, that might be related with intense gene expression and synthesis processes, whereas male-biased proteins are related to bile and amino acid metabolisms, and common proteins to other TCA, sugar, lipid and amino acid metabolisms, characteristic of classical liver metabolic pathways (Supplementary Table S3).

Among the top highly-abundant proteins in female livers dominate various isoforms of vitellogenins and choriogenins, together with fatty acid-biding, cytochrome P450 and various isoforms of ribosomal and translation-related proteins, whereas male-enriched liver proteins interestingly present other cytochrome P450 (CYP) isoforms, complement proteins, glutathione S-transferases (GSTs) and various TCA metabolism-related proteins, together with wap65 - a protein of unknown function whose transcript over-expression appears characteristic of the male Gulf pipe *Syngnathus scovelli*¹³ - highlighting important singularities of the liver proteome in both sexes (Fig. 3C,D).

Transcriptomics. PCA clearly discriminates between all male and female liver transcriptomes of medaka bred under undisturbed lab condition investigated by RNA-Seq analysis (Fig. 4A), and the volcano plot representation indicates a colossal over-expression of some genes in females compared with males (Fig. 4B). Indeed, some genes such as *vitellogenin 1*, 3 or 6 reach above 15 to 19 |log₂ FC| variations (namely up to 500,000 |FC|) (Supplementary Table S4), representing a large portion of the total female liver transcriptome. In contrast, in male livers the most over-expressed gene, *hydroxysteroid dehydrogenase* 3, exhibits only up to 8 |log₂ FC| (≈250 |FC|) variation in comparison with females. Additional pathway analyses, performed with the 375 and 147 significantly enriched transcripts in females and males, considering their human orthologs (Supplementary Table S4), indicate global enrichments (p < 0.1) of steroid and amino acid processes in both sexes when males also exhibit terpenoid-quinone and fatty acid biosynthesis over-representations.

The list of the over-expressed genes in females comprises, along with some genes whose expressions are well known to be female-specific, such as *vitellogenins, choriogenins* and *chorionic protease inhibitors*, various forms of *FAM20C* genes, belonging to the serine threonine kinase 20c-like family. Various isoforms of the FAM20C



Figure 4. Transcriptomics of male and female medaka livers investigated by RNA-seq approach. Principal component analysis (PCA) performed according to the transcript count for the 16,523 genes encoding medaka liver proteins (Supplementary Table S4) from the 3 male and 4 female pooled cDNA sequenced by Hiseq 1000 comprising at least 30 million reads per libraries (A). Volcano plot representation of the gene expression according to female/male fold change average and significance of the differences ($|log_2 FC| > 2$ corresponds to |FC| > 4 and log_{10} Pvalue < 3 corresponds to Pvalue < 0.001) (B). Female and male over-expressed genes are determined with positive and negative significant FC (F/M) values and are shown in red and blue, respectively. Top-25 lists of the male- and female-over-expressed genes (C and D, respectively).

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protein family were similarly over-expressed in the female liver of the Gulf pipefish¹³. Although the biological function of the proteins of this family remains poorly documented, they are interestingly annotated in GO library as "cellular response to estrogen stimulus" genes, and may constitute female-specific markers in fish livers. With some agreement with our proteomic observation, other genes of interest belong to the *cytochrome-P450 (CYP)* family. Some member such as, for example, *cytochrome-P450 27b1* and *cytochrome-P450 2w1* appear clearly over-expressed in male and female medaka livers, respectively (Fig. 4C,D).

Integrated pathway analysis. To investigate and visualize the biological connectivity of the sex-enriched metabolites and transcripts, the network-generating algorithm of ingenuity pathway analysis (IPA) was used to maximize the interconnectedness of molecules based on all known connectivity in the database developed from Human molecular knowledge in the liver. The results of the IPA biological function analysis (Supplementary Table S6), represented as a bar chart and a heatmap, are shown in Fig. 5A,B, respectively. Lipid metabolism, molecular transport, small molecule biochemistry, inflammatory response, organismal development, vitamin and mineral metabolism, and free radical scavenging appear to be the most significantly represented functional categories according to the combined liver transcriptome and metabolome dataset. The sex-specificity of the molecules involved in these processes is indicated in the heatmap representation (Fig. 5B) that clearly shows a global up-regulation of molecules involved in small molecule biochemistry, lipid metabolism, lissue development, vitamin and mineral metabolism, energy production and carbohydrate metabolism in males, whereas in females most of the molecules involved in cellular movement, haematological system, inflammatory response, or immune cell trafficking appear largely up-regulated.

The IPA network search shows that 2 of the top networks consisted predominantly of only female- and male-enriched molecules are related respectively to RNA post-transcriptional modification and lipid metabolism processes, as shown in Fig. 5C,D. These molecular network representations clearly illustrate the selected massive induction of some genes and metabolites related to RNA post-translational modification in females (Fig. 5C), and specific lipid metabolism processes connected with cholesterol metabolism and steroidogenesis, in males (Fig. 5D).

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Specificities of the toxicological molecular responses. The quantitative proteomic analysis of liver of the medaka exposed to various hepatotoxic treatments was performed on nanoLC-ESI-MS/MS, leading to the identification of 1114 proteins and to the differential quantification of 177 and 185 proteins in males and females, respectively (Fig. 6A). Only above a quarter of them (49 proteins) appears to be common between the two sexes. Protein quantification in each group of adult medaka fish, chronically exposed to the various cyanobacterial hepatotoxic treatments (CHT1-3) was reported, according to the relative intensity normalized with the controls set at 0, and the reliable quantifications in male and female livers were represented in a heatmap with hierarchical cluster analysis. This analysis clearly revealed a distinguishable sex-dependent response of medaka fish to the various cyanobacterial hepatotoxic treatments according to the group distribution given by the clustering analysis, which is based on the pattern of relative abundance of up- and down-regulated proteins (Fig. 6B). These cyanobacterial hepatotoxic treatments dysregulate various molecular function categories comprising lipid, amino acid, carbohydrate and TCA metabolisms and detoxification processes (Supplementary Table S5). The sex-specificity of the liver molecular response suggests that identical hepatic stress could impact these various molecular processes differently, potentially inducing dissimilar biological repercussions in the organisms of two sexes. In our example, male-enriched protein dysregulations concern rather TCA, steroid, fatty acid, amino acid and vitamin B6-7 metabolism pathway categories, whereas female-enriched protein dysregulations are rather related to tRNA biosynthesis, amino acid, glutathione, xenobiotic and drug metabolism pathways.

Discussions

The liver is a key organ in vertebrates performing a large diversity of vital functions, including processing and storage of nutrients, maintenance of serum composition, bile production, and xenobiotic detoxification. It is



primarily an exocrine gland, secreting bile into the intestine, but it is also an endocrine organ and a blood filter. The liver is a metabolic factory, which synthesizes and breaks down a variety of substances, comprising the production of bile salt anions, the synthesis of urea and many plasma proteins, the metabolism of glycogen, cholesterol and fatty acids, the detoxification of many drugs, and the processing of steroid hormones and vitamin D. Studies carried out in rodents have established that sex-based differences in liver function also characterize many drug-metabolizing enzymes⁴. The sexual dimorphism of liver gene expression is not confined to DMEs, and it concerns more than 1000 genes in these organisms², including plasma lipoproteins, pheromone binding proteins, regulators of fatty acid homeostasis, nuclear receptors, and other transcription factors³.

Similar to the mammalian liver, the teleost liver plays an important role in the metabolic homeostasis of the whole organism, in addition to that, oviparous vertebrate-specific processes related to the synthesis of various oocyte protein precursors (*i.e.* mainly vitellogenins and choriogenins, together with other minor vitamin-binding proteins) are synthesized in females under the direct control of estrogens, which bind to estrogen receptor complex and activate the translation of messenger RNA via *cis*-regulation mechanisms^{8,14}. The massive rate of synthesis of vitellogenin in the egg-laying animal causes considerable ultrastructural changes in liver cells, which are characterized by extensive proliferation of the rough endoplasmic reticulum and the Golgi apparatus¹⁵. In mature female medaka, a remarkable part of the liver metabolism might be dedicated to these reproduction-related processes, as each female can spawn above 30 mature oocytes daily¹⁶. This massive synthesis is known to induce large cellular and molecular modifications, as it can also increase the lipid synthesis of hepatocytes⁵. This metabolic adjustment to maintain the reproductive competency of the female constitutes one of the physiological bases for the extended sexual dimorphism in fish livers.

Our histological observations of male and female medaka livers clearly showed differences of reserve vesicle within hepatocytes, which is consistent with ultrastructural modifications in the liver cells between males and females. Interestingly, previous observation of mature medaka liver under transmission electron microscope shows that, in the perinuclear region, granular endoplasmic reticulum, mitochondria, and peroxisomes appear largely increased in number in female hepatocytes¹⁵. These hepatocyte sexual differences in cellular organization and content might be related to the intense activity of protein synthesis and consequently the high energy requirement of female hepatocytes. Indeed, in sexually mature fish, as in other oviparous vertebrates, livers globally present morphological, molecular and functional sexual-dimorphisms^{15,17,18}. The liver of female performs an important function in the synthesis of a large set of proteins involved in the oocyte envelop and vitellogen reserves, whereas male liver hepatocytes do not exhibit such activity.

Although the precise function of taurine and hypotaurine, which were highly abundant in female and male livers, respectively, and the balance between the two remains poorly documented in fish liver¹⁹, one previous study has reported the influence of taurine on egg maturation²⁰. To date, only one investigation performed in adult zebrafish has attempted to compare male and female metabolomes, according to various analytical approaches, including GC-MS, LC-MS and NMR, and has observed a significant up-regulation in various fatty acids, together with valine, acetate, glutamate, glutamine, creatinine and betaine in female liver²¹.

The sex-biased pattern depicted in our transcriptomic analyses appears acutely congruent and even more contrasted than our global proteome investigation. These strong sex-biases testify of the intensity of the female liver efforts for the gene expression and the synthesis of the oocyte precursor proteins^{8,14}, and the involvement of the male liver in steroid hormone and metabolism processes²², such as urea and energy cycles⁹, respectively.

Previous investigation of medaka fish has observed that liver transcriptome globally exhibits significant enrichment in the expression of genes related to macromolecule, RNA, and nitrogen compound metabolic processes with regard to the gene expression in other tissues, but it has not considered the differences between sexes^{23,24}. Previous works performed on zebrafish and Gulf pipefish have highlighted large sets of genes whose expression appears to be driven by sex-dependent processes^{16,25}. On one side, zebrafish transcriptomic approach reveals that the female-over-expressed gene list included *vitellogenins* and *zona pellucida glycoproteins*, many ribosomal proteins, and *estrogen receptor 1*, in contrast, the list of male-over-expressed genes contains *fatty acid-binding protein2*, *apolipoprotein 4*, and also genes that are supposed to be involved in anti-inflammatory processes, such as *complement factors 9b* and 3b, together with several *chitinases*²⁵. On the other side, transcriptomic investigation performed on Gulf pipefish shows a quite similarly high over-expression in characteristic genes of females, such as *vitellogenin b* and *c*, *choriogenin h*, and *zpc* 4-like, in addition to *estrogen receptor 1* and various *fam20c* isoforms, whereas males exhibit less intense over-expression of specific genes, comprising various metabolism related genes such as *hint3*, *cll*, *nsun3* and *wap65*¹³. Moreover, primary investigation of the medaka fish liver transcriptome by microarray analysis indicates that the female-specific transcript is comprises some previously characterized female-specific genes such as *vitellogenins*, *choriogenins*, *ZP* family genes, *cyclins B* and *42S nucleoprotein*, whereas most of the male-specific transcripts have not yet been assigned or characterized²⁶.

Similarly, previous proteinaceous investigations have highlighted that female liver proteome moreover contains massive amounts of oocyte precursor proteins (*i.e.* vitellogenins, choriogenins and fatty acid-binding proteins) that are being secreted by the hepatocytes¹⁷, together with variations between sexes in drug metabolism capabilities⁴. CYPs constitute a diverse group of enzymes that are potentially involved in key reactions of oxidation of organic substances, such as drug detoxification¹⁷ and steroid hormone metabolism^{4,7,13}. The sexual polymorphism in the expression of these enzymes may have fundamental repercussion on liver physiology such as drug-metabolism processes.

In addition to our transcriptomic data that gives a congruent view with previously published observations, our systematic investigation constitutes an unprecedented opportunity to globally depict the medaka liver sexual dimorphism at different molecular and cellular levels. By developing an integrative approach combining high output proteomic, RNA-Seq transcriptomic and non-targeted metabolomics outputs, together with histological examinations, we are able to appreciate the wideness and the deepness of the sexual dimorphism, in terms of both number and intensity of the sex-dependent dysregulations. At the mRNA level, twice more sex-over-expressed transcripts appear in females, comprising some genes involved in ovogenesis (i.e. vitellogins and choriogenins), and reach very high fold change values (up to 50,000 and 250 FC in females and males, respectively). Proteomic investigations also show more proteins that appear to be specific of female proteomes, with higher fold change too (up to 750 and 50 FC in females and males, respectively). The quantitative metabolome analysis performed by NMR indicates that the difference of fold changes between female- and male-enriched metabolites appear to be much higher in female (up to 350 and 50 FC in females and males, respectively). The observations of globally more intensive and numerous molecular up-regulations in female livers are in agreement with the conception of an oviparous female liver that is in charge of extra metabolic activity, according to their massive production of oocyte yolk stocks and chorion precursors, which have substantial impacts on both amino acid, saccharide and fatty acid metabolism of the global liver activities8.

As the mature female liver is considered to be more physiologically and energetically solicited than male's, we assume that female liver could be consequently more susceptible to hepatotoxic stressors. In our experiments and according to other investigations on small fish ecotoxicology, various hepatotoxic stressors such as cyanotoxins¹¹, pesticides^{27,28} or aromatic hydrocarbons²⁰ induce a higher toxicological response in female livers, suggesting that females would be more sensitive to the effects of those molecules than males^{15,17,18}. However, some examples also attest to a higher susceptibility of male livers according to certain specific exposure conditions to toxic chemicals³⁰, and a careful investigation of the dimorphic detoxification capabilities should aim at being performed for each specific chemical or hepatic stressor evaluation. Indeed, as the liver, being the principal detoxification organ, presents noticeable sex-differences in its drug metabolism (*e.g.* CYP P450 isoforms) and homeostasis capabilities⁴, it is likely that for some hepatic stressors the detoxification performance, which could be clearly distinguishable between the two sexes, should be systematically considered in environmental toxicology evaluations.

Overall, in addition to providing first insight into the molecular mechanism underlying the sex-specificity of the livers of oviparous organisms, and concerning important liver processes, such as energetic metabolism, detoxification and reproduction, our molecular integrated research demonstrates also that, freshwater oviparous organisms such as medaka fish present a net sex-dimorphism in the molecular response specifically induced by chronic hepatotoxin exposures.

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Furthermore, numerous reports show that fish populations are adversely affected by environmental estrogens, which can potentially present ecological adverse effects, via the induction of the synthesis of oogenesis proteins. These xenoestrogens can also impact various liver processes that also exhibit sex-dimorphisms, such as metabolism and biotransformation enzymes that directly influence the stress resistance capabilities of the organism. Additional studies are still needed to validate these findings at higher levels of biological organization, and to fully estimate their consequences for different populations of the global ecosystem.

Methods

Medaka fish. Medaka fish (*Oryzias latipes*) of the inbred Cab strain were used for this experiment. The animals were handled and experiments were performed in accordance with European Union regulations concerning the protection of experimental animals and the experimental procedures were approved (N°68-040 for 2013-18) by the "Cuvier's ethical committee" of the Muséum National d'Histoire Naturelle (French national number C2EA - 68). All fish used in this study (during summer 2014) originate from the same broodstock (F0 from genitors provided in November 2013 by the Amagen CNRS/INRA platform - Gif-sur-Yvette, France).

All histopathology, metabolomics, proteomic and transcriptomic analyses, except for iTRAQ proteomic with chronically exposed fish, were performed on the untreated fish breed and kept under control conditions, described as follow. Six month-old adult fish (around 0.55 ± 0.08 and 0.59 ± 0.10 g, for males and females, respectively; n = 36), mature and sexually active (with secondary sexual character well developed; sex determination was confirmed by further histology of the gonads for 36 individuals), were maintained at 25 ± 1 °C, with 15h:9h light/dark cycle (in reproductive cycle). Fish were raised in 20 L glass aquaria (in triplicate tanks, containing above 10 male and 10 female per tank) filled with a continuously-aerated mixture of tap water and reverse osmosis filtered water (1/3-2/3, respectively), which was renewed once a week. Fish were fed three times a day with commercial food for juvenile salmon, supplemented once a day with fresh artemia, and were inspected three times daily, and no abnormal behaviour, nor mortality was observed. Fish were randomly selected, briefly anesthetized in buffered 0.1% MS-222, sacrificed. The liver samples were collected and prepared for further analysis, as described below.

Mature and sexually active fish, bred in the same conditions of light, temperature and nourishing, were used for chronic exposure to hepatotoxin then for iTRAQ quantitative proteomic analysis, as further described in the following paragraph. Five month-old fish were transferred to experimental tanks (15-L glass tanks, containing 5 males and 5 females each) two weeks prior to the beginning of the experimentation.

Hepatotoxic mixture exposure. Fish were exposed during 21 days to environmental concentrations of 3 different cyanobacterial hepatotoxic treatments³¹ (CHT), called CHT1-3, as well as to solvent control conditions (Control). The experiment was performed in triplicate tanks for each treatment (comprising 30 fish, 15 males and 15 females per treatment) and the exposure conditions were maintained by the renewal of two-thirds of the total aquaria volume (10 L) containing hepatotoxic mixture every 2 days. Fish were inspected three times daily, and no abnormal behaviour, nor mortality was observed during all the experimentation. At the end of the experiment, fish were anesthetised in 0.1% MS-222, euthanized and then livers were sampled for further analyses using iTRAQ quantitative proteomics.

Histopathology. Liver samples from at least 9 males and 9 females bred under unstressed control conditions were fixed in cold 10% buffered formalin (4 °C, 48 h), then transferred into 70% ethanol, dehydrated in successive baths of ethanol (from 70 to 95%), and then embedded in paraffin. Blocks were cut in $3-5\mu$ m-thick sections, and slides were stained with hematoxylin-eosin-saffron (HES) or periodic acid-Schiff/alcian blue (PAS), according to the standard histological procedure. Alternatively, liver samples were fixed with a mixture of paraformaldehyde (2%), glutaraldehyde (0.5%), pictic acid (0.5%) and sucrose (0.18 M) in 0.1 M pH 7.4 Sørensen buffer prior to post-fixation in osmium tetroxide (1%). Samples were then dehydrated in ethanol, embedded in the epoxy mixture (Spur's resin), and cut in semi-thin 0.5 μ m-thick sections and stained with toluidine blue (TB).

Metabolome ¹**H-NMR spectra.** Liver extraction was carried out using the methanol/chloroform/water method (ratio 2/2/1.8). Fresh frozen livers of 18 individuals for each sex (6 individuals from each triplicate tank) bred under unstressed control conditions were weighted and then homogenized in the ice cold methanol (8 mL.g⁻¹ of tissue; AnalaR Normapur, min. 99.8%) and ice cold milliQ water (2.5 mL.g^{-1}) and then vortexed for 1 min. Subsequently, ice cold chloroform (4 mL.g⁻¹; Normapur, 99.3%) and milliQ water (4 mL.g^{-1}) were added. Then, the mixture was vortexed for 1 min and incubated on ice for 10 min to partition. The supernatant was then centrifuged at 4°C for 10 min at 2,000 g. The upper polar fraction was then transferred to 2 mL Eppendorf tubes, dried under Speed-vac device and then kept at -80 °C until NMR analysis. The extracts were dissolved in 550 µL of 0.1 M sodium phosphate buffer prepared in D₂O (10% v/v) containing 0.25 mM sodium -3-tri-methylsilylpropionate (TMSP) as an internal standard, then were transferred to a 5-mm NMR tube (Norell, France) and analyzed immediately by ¹H-NMR.

All NMR data were recorded at 298 K on a 600 MHz Bruker AVANCE III HD spectrometer equipped with a 5 mm TCI CryoProbe (¹H-¹³C-¹⁵N) with Z-gradient. One-dimensional ¹H NMR spectra were acquired using a standard Bruker noesygppr1d pulse sequence to suppress water resonance. Each spectrum consisted of 512 scans of 32,768 data points with a spectra width of 7.2 kHz, a relaxation delay of 3 s and an acquisition time of 2.3 s. A Quality Check (QC) sample was injected every 6 samples, in order to verify that no significant drift of the analysis occurs, according to expected reference. Spectra were then processed with Topspin software (Bruker) for alignment and noise reduction, and analyzed for bucketing, annotation and quantification with the Batman R package³². Individual metabolite intensities were compared according to sex groups using Metaboanalyst 3.0 online tool³³ for PCA, PLS-DA, volcano plot reconstruction and metabolite pathway enrichment analyses. **Proteomic analysis.** Nine livers from adult male and female medaka fish bred under unstressed control conditions were randomly pooled (one fish liver from each triplicate tank in each triplicate pool) and homogenized on ice with a Dounce homogenizer in 500μ L of a solution of 6 M guanidine hydrochloride, 500 mM triethylammonium bicarbonate buffer (TEAB, pH 8.3), 0.1% Triton X-100 and 10µg of protease inhibitor mixture (Roche, Switzerland). The homogenates were centrifuged at 4 °C (12,000 g; 10 min), and then the supernatants were collected. Proteins were precipitated with cold acetone (-20 °C; overnight), centrifuged at 4 °C (2,000 g; 4 °C), and then resuspended in 500 mM TEAB with 6 M urea and 0.1% SDS. The protein concentration was measured using a micro-BCA kit (Sigma-Aldrich, USA), with BSA as a protein standard.

100 µg of each liver protein pool was used for digestion with 5µg of proteomic-grade trypsin (Sigma-Aldrich, USA), reduced with 2 mM tris-(2-carboxyethyl)phosphine (TCEP) with and cysteine-blocked with 10 mM methyl methane-thiosulfonate (MMTS), prior to analyses with a Q ExactiveTM Hybrid Quadrupole-OrbitrapTM mass spectrometer (Thermofisher Scientific). Liver protein digests were concentrated on C18 stages tips, recovered in 40 µL 2% aqueous TFA, 2% ACN before injection in triplicates (6µL injected). NanoLC was performed on an Ultimate 3000 RSLCnano System (Thermofisher Scientific): digests were desalted on a trap column (Pepmap, C₁₈ 300 µm x 5 mm, 5 µm 100 Å, Dionex) with water containing 2% ACN with 0.1% formic acid (solvent A) for 6 min, and the peptides were finally eluted from a separation column (Pepmap, C18 75 µm, x 500 mm, 3 µm 100 Å, Dionex). The separation gradient as optimized for the samples and is divided into 3 successive slopes: 2–20% in 120 min, 20–35% in 45 min and 35–80% ACN +0.1% formic acid (solvent B) at a flow rate of 300 nL.min⁻¹. Each MS spectrum acquisition (*m/z* 400–2000, 70,000 Res.) was followed by up to ten data dependent HCD MS/MS spectra (first fixed mass *m/z* 90, 17,500 Res., 30 normalized collision energy) with an isolation window *m/z* 2 and a dynamic exclusion window of 30 s.

All MS/MS-analyzed samples were analyzed using Mascot 2.4.1 (Matrix Science, UK) and X!Tandem with Scaffold software (version 4.5.1; Proteome Software, USA) to search Uniprot databases of Teleostei (downloaded in December 2015). The ion mass tolerance and the parent ion tolerance were set to 20 mDa and 10 ppm, respectively. The methyl methanethiosulfonate of cysteine was specified as fixed modifications. Oxidation of methionine and deamination of N and Q were specified as variable modifications. The Scaffold was used to probabilistically validate the protein identifications derived from MS/MS sequencing results. Normalized semi-quantifications and identification probability of male and female identified proteins were estimated using Scaffold+ default parameters from MS and MS/MS data for proteins presenting at least two peptides.

iTRAQ based quantitative proteomic was performed in triplicates for each experimental group on 3-pooled livers from nine livers from adult male and female medaka fish for each condition (CHT1, CHT2, CHT3 and Control) that were randomly pooled (one fish liver from each triplicate tank in each triplicate pool) as previously described³⁴. Nano-LC-MS/MS analysis of the 8-plex tagged peptide digests was performed using the same top-10 strategy. A Quality Check (QC) sample was injected every 5 samples, in order to confirm that no significant drift of the analysis occurs, according to expected minimal identification number. Protein quantification was performed with Scaffold Q+ (4.5.1) using the isobaric tag peptide and protein identifications. Protein identifications were accepted if they could be established with more than 99.0% probability, and contained at least 2 identified peptides that were quantified using the centroid reporter ion peak intensity. Protein quantitative ratios were calculated as the median of all peptide ratios of the three consecutive runs. Quantitative ratios were log₂ normalized for final quantitative testing, using Gene-E freeware (http://www.broadinstitute.org/cancer/software/ GENE-E/) using Spearman correlation's value for sample and protein clustering analyses.

RNA-Seq. Two or three pooled livers from untreated adult males and females randomly selected from the three replicate tanks were homogenized using a bead beater. Total RNA of pooled female and male livers (6 biological replicates, for each sex) was isolated and purified using RNeasy Plus Mini Kit with gDNA eliminator spin (Qiagen). RNA quantity and quality were evaluated using Qubit RNA Assay Kit in Qubit[®]2.0 Fluorometer (Life Technologies, USA) and an Agilent Bioanalyzer 2100 eukaryote total RNA Pico series II chip (Agilent Technologies Inc., USA), respectively. The RIN values of all samples (3 pool samples for males and 4 pool samples for females) further selected for RNA-seq analysis were over 7.7. The transcriptome libraries were prepared from total RNA using Illumina TruSeq Stranded mRNA Sample Preparation kits (Illumina Inc., USA) following the manufacturer's handbook. Briefly, the mRNA was purified using poly-T oligo-attached magnetic beads and then fragmented into small pieces that were then used for synthesizing the first- and second-strand cDNA. After end repair, single nucleotide A (adenine) addition and adaptor ligation, the fragments were amplified with a 10-cycles PCR program. The libraries were sequenced on the Illumina Hi-Seq1000 instrument using the 51 bp single-end sequencing strategy with the TruSeq SBS kit V3-HS 50-cycles (Illumina Inc., USA).

Raw reads were first cleaned by removing adaptors using Cutadapt-1.3 and only 51 bp-long reads were kept. The global quality of the reads was checked using the FastQC 0.10.1 and good global Phred scores (>30) were obtained in all the libraries. However, the analysis of the unicity of reads shows a high level of sequence duplications, so a step of duplicated reads removal was conducted using a python script that analyses the quality of reads and keeps the one with the best global quality score. Tophat2 (v2.0.10)³⁵ was used to map the clean unique reads against the medaka genome (release 81) downloaded from Ensembl (ftp://ftp.ensembl.org/pub/release-81/fast4) oryzias_latipes/dna/). Multiple hits were removed by samtools (v0.1.18) and read counting on gene exons was accomplished by HTSeq-count (v0.6.1p1)³⁶ in union mode against the annotation of medaka genomes downloaded from Ensembl (ftp://ftp.ensembl.org/pub/release-81/gtst4). DESeq2 v1.8.1³⁷ was used to do differential expressed gene analysis on the raw count data. Genes were considered differently expressed when the *p* value below 0.001, using the control group as reference. Furthermore, we only included genes with expression level of at least 4 [FC] in order to capture more physiologically relevant genes. Then, we identified 375 female- and 147 male-enriched transcripts (Supplementary Table S4). Messenger RNA expression levels were secondarily

confirmed by RT-qPCR on 8 genes randomly selected within the expression intensity gradient that presented correlation coefficient greater than 0.95 between these two techniques in both sexes.

Molecular network analysis. Molecular pathway was determined for our merged transcriptome and metabolome data using the Ingenuity Pathway analysis software (V01-04; Qiagen) with the Human orthologous of medaka proteins available from Ensembl online platform (http://www.ensembl.org), according to specific Ingenuity Knowledge Database (Genes and Endogenous Chemicals), which is a repository of biological interactions and functional annotations. The fold change values (females vs males) and p values calculated according to the quantifications of all replicates for 2214 gene expressions (FDR < 0.05) and 245 metabolites (HMDB numbers) were imported into IPA, then "Core Analysis" was performed, with default setting on liver tissue and relaxed filters, including both direct and indirect relationship between our dataset and the reference annotations, in order to interpret data in the context of biological pathways, molecular functions and networks.

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Author Contributions

O.Q., S.L.M., B.S., G.B., A.M., H.H., C.B., M.E. and B.M. conceived the experiments, Q.Q., S.L.M., B.S., C.D. and B.M. conducted the experiments, Q.Q., S.L.M., B.S., B.M., E.D.-B., A.P., L.P., A.M., A.B., L.M., J.V., C.D. and B.M. performed the analyses and analysed the results. All authors reviewed the manuscript.

Additional Information

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Annex 5. 3 Article 3 Gender-specific toxicological effects of chronic exposure to pure microcytin-LR or complex *Microcystis aeruginosa* extracts on adult medaka fish.

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Gender-Specific Toxicological Effects of Chronic Exposure to Pure Microcystin-LR or Complex *Microcystis aeruginosa* Extracts on Adult Medaka Fish

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S Supporting Information

ABSTRACT: Cyanobacterial blooms often occur in freshwater lakes and constitute a potential health risk to human populations, as well as to other organisms. However, their overall and specific implications for the health of aquatic organisms that are chronically and environmentally exposed to cyanobacteria producing hepatotoxins, such as microcystins (MCs), together with other bioactive compounds have still not been clearly established and remain difficult to assess. The medaka fish was chosen as the experimental aquatic model for studying the cellular and molecular toxicological effects on the liver after chronic exposures (28 days) to environmentally relevant concentrations of pure MC-LR, complex extracts of MC producing or nonproducing cyanobacterial biomasses, and of a *Microcystis aeruginosa* natural bloom. Our results showed a higher susceptibility of females to the different treatments compared to males at both the cellular and the molecular levels. Although hepatocyte lysis increased with MC-containing treatments, lysis



always appeared more severe in the liver of females compare to males, and the glycogen cellular reserves also appeared to decrease more in the liver of females compared to those in the males. Proteomic investigations reveal divergent responses between males and females exposed to all treatments, especially for proteins involved in metabolic and homeostasis processes. Our observations also highlighted the dysregulation of proteins involved in oogenesis in female livers. These results suggest that fish populations exposed to cyanobacteria blooms may potentially face several ecotoxicological issues.

INTRODUCTION

Cyanobacteria play important roles in aquatic ecosystems because they make a significant contribution to primary production and, for some species, to the fixation of atmospheric nitrogen.¹ However, local cyanobacteria proliferations can also disrupt the functioning of aquatic ecosystems and constitute a major cause of concern for both public health and ecology as a result of the ability of several species and genera to proliferate and to produce harmful toxins, so-called cyanotoxins.² The most common and well-studied cyanotoxin-producing cyanobacterium, *Microcystis*, is one of the most widespread and important toxin-producing cyanobacteria genera in worldwide lakes, in terms of both abundance and distribution.³⁻⁶

The concerns about the toxicological potential of these freshwater cyanobacteria have mainly focused on their production of microcystins (MCs), a diverse family of cyclic heptapeptide hepatotoxins that are considered the most common toxins of cyanobacteria. Among MC structural diversity variants, the MC-LR variant, the most frequently detected MC variant in the environment, is also the variant that exhibits the highest concentrations in lakes⁷ and has the greatest potential for toxicity to aquatic organisms.⁸

Cyanotoxins, such as MCs, mostly enter an organism using the food pathway, cross the intestinal wall, and move through the portal venous system to reach the liver, in which they accumulate: the liver exhibits very high tropism for various drugs and/or chemicals, such as cyanotoxins, and notably, MCs.^{9,10} MCs are known to enter a hepatocyte liver cell thanks to their high content in transmembranal anionic biliary-acid transporters, and to induce molecular defects though a cascade of reactions following the inhibition of phosphatase PP1 and PP2 activity due to MC-specific fixation on those proteins.^{7,8,11} The mechanisms

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of MC toxicity and detoxification in fish are believed to be similar to those reported in mammals. $^{\rm 12-15}$

However, for aquatic organisms such as fish, most investigations on MC toxicity remain based on gavage experiments, one-time force-feeding experiments, ¹⁶ or short-term dietary exposure bioassays¹⁷ that determined the acute effects at cellular and molecular levels.^{18–21} The main toxicological pathways that resulted in the acute effects of MC on hepatocytes are the production of reactive oxygen species production, the occurrence of oxidative stress, and cytoskeletal dysregulation, together with the induction of apoptosis.^{18–22}

To date, only limited information is available on the fine chronic effects of an aqueous MC exposure on fish under balneation, $^{20,23-26}$ which might potentially be the major and natural route of MC toxicity to fish in their environment. 9,27,28 A small number of these studies were dedicated to the investigation of chronic effects of MC exposure at the cellular or molecular levels, and all of them were focused on pure MC-LR or -RR effects only. $^{4,20,26,29-32}$ Only a restricted number of ecotoxicological studies investigated the potential chronic effects of complex cyanobacterial cells and lysates that contain other compounds, $^{24,25,33-35}$ many of which are only now being identified, $^{36-40}$ and their potential toxicological effects are being revealed. $^{33,41-45}$ In addition to the classically described cyanotoxins (microcystins, cylindrospermopsins, anatoxins, or saxitoxins), cyanobacteria can also produce numerous other secondary metabolites such as microviridins, 46 microginins, 37 oscillapeptins, 47 cyanopeptolins, or aeruginosins, 40 through nonribosomal peptide synthase/polyketide synthase (NRPS/ PKS) pathways; 37 these secondary metabolites may also have concrete deleterious biological effects on fish.

Although various studies have already been performed to determine pure cyanotoxin effects, it remains a key issue to elucidate the underlying molecular mechanisms of the toxicological response of aquatic organisms, such as fish, that are chronically exposed to a range of cyanobacterial metabolites for both environmental and toxicological purposes. To learn more about the chronic effects of the Microcystis aeruginosa secondary metabolites, comprising MCs, we investigated their cellular and molecular effects on a fish model, the medaka Oryzias latipes,¹⁸ exposed to various cyanobacterial extracts. To investigate the ecotoxicological effect of various Microcystis biomass containing or not MCs, we performed complementary pathological and molecular approaches on the liver of chronically exposed adult medaka fish. The fish liver represents the most suitable organs for this study because it both constitutes the primary target of hepatotoxins and is the principal detoxification organ, integrating the whole organism responsiveness to xenobiotics or toxins.^{11,17,32} Through the systematic analyses of the cellular and molecular alterations induced in adult medaka after chronic exposures to various cyanobacterial extracts, containing or not MCs, we contribute to generate new information on the environmental hazard and risk assessment of cyanobacteria to aquatic organisms.

EXPERIMENTAL SECTION

Preparation of Exposure Extracts. *Microcystis Strain Cultures.* The monoclonal *Microcystis aeruginosa* strains PCC 7820 and PMC 570.08, high-producer and nonproducer of MCs, respectively, along with other secondary metabolites (Supporting Information Figure S1A,B). The strains were maintained in the Paris Museum Collection (PMC) of cyanobacteria and cultured in Z8 medium⁴⁸ (25 °C, 16 h:8 h light/dark photoperiod at 16 μ mol of photon·m⁻²·s⁻¹) under nonaxenic conditions for large biomass production, prior to methanol extraction.

Microcystis Bloom Sampling. The recreational lake located near the city of Champs-sur-Marne (48°51′47″ N, 02°35′53″ E, France) has a surface area of 0.1 km² with an average depth of 2.5 m, and since 2006, it has experienced several episodes of cyanobacterial blooms.⁴⁹ During the summer of 2011, raw water was sampled weekly, the chlorophyll *a* (Chl *a*) concentration was measured and the cyanobacterial genera or species (>20 μ m) were determined as described previously.⁵⁰ The MC concentration was determined using AD4G2 ELISA tests (Abraxis). During one of the main *Microcystis aeruginosa* bloom (98% of the total phytoplankton biomass) events with high production of MCs that occurred on 09/19/2011, a large cyanobacteria biomass was concentrated with a specific net for phytoplankton (200 μ m) and was collected in a 2 L-bottle for the secondary metabolite extraction (Figure S2).

Microcystis Secondary Metabolite Extraction. The *Microcystis aeruginosa* biomasses from the 2 cultured strains and from the bloom described above were filtered and freeze-dried. The lyophilized cells were then sonicated in 80% methanol, centrifuged at 4 °C (4000g; 10 min) and filtered (GF/C 1.2 μ m); then, the supernatant was evaporated as described previously.⁵¹ The dried extract was dissolved in 50% ethanol (Vol/Vol) and then partially evaporated to remove the ethanol prior to the experimentation. The metabolite compositions of the 3 extracts were then investigated using LC-MS/MS performed on ESI-qTOF/TOF, and the MCs were quantified using AD4G2 ELISA tests (Abraxis).

Metabolite Analysis by Mass Spectrometry. High performance liquid chromatography (HPLC) was performed on 5 μ L of each of the metabolite extracts using a capillary 1 mmdiameter C₁₈ column (Discovery Bio wide pore 5 μ m, Sigma) at a 50 μ L.min⁻¹ flow rate with a gradient of acetonitrile in 0.1% formic acid (10 to 80% in 60 min). The metabolite contents were analyzed at least three times using an electrospray ionization hybrid quadrupole time-of-flight (ESI-QqTOF) hybrid mass spectrometer (QStar Pulsar i, Applied Biosystems, France) on positive mode with information dependent acquisition (IDA), which allowed for switching between MS and MS/MS experiments, as previously described.⁴⁴ The data were acquired and analyzed with the Analyst QS software (Version1.1). Peak lists were generated from MS/MS spectra between 10 and 55 min, with a filtering noise threshold at 2% maximal intensity and combining various charge states and related isotopic forms. Metabolite annotation was attempted according to the precise mass of the molecules and their respective MS/MS fragmentation patterns with regards to an in-house database of above 600 cyanobacteria metabolites that were previously described in reference publications.

Medaka Chronic Exposure. Experimental Design. Medaka fish (*Oryzias latipes*) belonging to the inbred Cab strain were reared, and experiments were performed in accordance with European Union regulations concerning the protection of experimental animals and the validation of experimental procedures by the ethical committee of the "museum national d'histoire naturelle" –MNHN ($N^{\circ}68$ –040 for 2013–18). One month prior to the chronic experiments, 5 month-old adult fish were maintained at 25 ± 1 °C with a 15 h:9 h light/dark cycle (reproductive cycle) to induce their reproductive activity. Fish were randomly assigned to one of the 5 experimental groups, namely "control" for no toxin balneation with control solvent

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Figure 1. Metabolite detection in the various *Microcystis aerugonisa* extracts using LC-ESI-MS/MS. Mass spectra of the various molecules detected in the methanol-extracts of (A) the PMC 570.08 non MC-producing strain (MicA–), (B) the PCC 7820 MC-producing strain (MicA+) and (C) the natural bloom that contained MCs, which was collected from Champs-sur-Marne' water body containing MC (Bloom+). Metabolite annotation was attempted according to precise mass and respective MS/MS spectra with regards to an in-house database of above 600 known cyanobacteria metabolites and are represented in a Venn' diagram (D). MCs are indicated in red and other metabolites are indicated in green.

conditions; "MC-LR" for the exposure to pure MC-LR, "MicA+" for the Microcystis aeruginosa MC-producing strain; "MicA-" for the Microcystis aeruginosa non- producing MC strain; and "Bloom +" for Microcystis aeruginosa bloom producing MC. Each treatment comprised 15 males and 15 females maintained in a 30-L aquarium. The exposure dose of MCs was fixed to the frequently observed environmental concentration of 5 μ g equivalent MC-LR L⁻¹ for MC-LR, MicA+ and Bloom+ groups, whereas the quantity of MicA- extract was adjusted to be equivalent to the biomass used for the MicA+ concentration, and no toxin or extract was added to the control tank. The experiment was performed for 28 days, and the exposure condition maintained by renewal of a third of the total aquarium volume (10 L) every 2 or 3 days. Fish were inspected three times daily, and no abnormal behavior, nor mortality was observed throughout the experiment. After 28 days of exposure, the fish were briefly anesthetized in buffered 0.1% MS-222 and sacrificed, and the liver samples were collected for analysis. Circulating estradiol E2 levels were measured in 3 plasma pools of 3 males and 3 females using a commercial ELISA test (Biosense laboratories) following protein quantification using a bicinchoninic acid (BCA) test, which was performed with a bovine serum albumin (BSA) protein standard.

Liver Sample Analyses. *Histopathology.* Liver samples were fixed in cold 10% buffered formalin ($4 \,^{\circ}$ C, 48 h), transferred into 70% ethanol, dehydrated in successive baths of ethanol (from 70 to 95%), and then embedded in paraffin. Blocks were cut into 3- to 5- μ m thick sections, and slides were stained with

hematoxylin-eosin-saffron (HES) or periodic acid-Schiff (PAS), according to standard histological procedure. For each individual, the hepatocyte lysis surface was determined from a blind assessment of 3-5 liver sections of the HES-stained slides, and the glycolysis index (scores = 0-3) was visually determined on 3 subsampled areas from one section of PAS-stained livers by two different researchers. Significant differences among the various experimental groups (n = 6-9 individual for each sex) were investigated with nonparametric tests using Kruskal–Wallis or Mann and Whitney-Wilcoxon methods, which are suitable for small data sets.

Quantitative Proteomic Analysis. Liver tissues from 3 fish per treatment were pooled, and the content protein was extracted and quantified as previously described.⁴⁴ One hundred μ g of each liver protein pool, prepared as described above, was used for the digestion with 5 μ g of proteomic-grade trypsin (Sigma-Aldrich, U.S.A.) and the sample was labeled, following the manufacturer's protocol for the 8-plex iTRAQ kit (Applied Biosystems, France).

Mass Spectrometry Analysis. iTRAQ-based quantitative proteomic analysis was performed using nano-LC (Ultimate 3000, Dionex) coupled with an ESI-LTQ-Orbitrap (LTQ Orbitrap XL, Thermo Scientific) mass spectrometer. Six μ g of iTRAQ-tagged liver protein digests solubilized in 10% ACN with 0.1% formic acid were injected in triplicate by the autosampler and were concentrated on a trapping column (Pepmap, C₁₈, 300 μ m × 50 mm, 3 μ m 100 Å, Dionex) with water containing 10% ACN with 0.1% formic acid (solvent A). After 5 min, the peptides were eluted onto a separation column (Pepmap, C₁₈, 75 μ m x

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Figure 2. Liver histopathological investigations. Representative histological observations of adult medaka fish liver stained with HES under a light microscope (x200 and x1000, A and C, B and C, respectively) for females (A,B) and males (C,D) exposed to control conditions. Scale bares represent 20 μ m. n, nucleus; m, membrane; c, cytosol; and v, vesicle. Histopathological effects of the various *Microcystis aeruginosa* extract exposures on the medaka fish liver (E,F). Percentage of hepatocyte lyses (E) measured on HES stained liver thick sections (n = 3-5 sections per fish) in both males and females (n = 6-9 fishes per condition). Intrahepatocyte glycogen reserve indexes (F) estimated according to the intensity of purple staining on PAS stained liver thick sections (n = 3 sections per fish) in both males and females (n = 6 fishes per condition). Significant differences from the control and from MC-LR treatment are indicated by * and #, respectively; one or two symbols indicate that the p values are less than 0.05 or 0.01, respectively.

500 mm, 2 μ m 100 Å, Dionex) equilibrated with solvent A. The peptides were separated with a 2 h-linear gradient, increasing from 10% to 80% ACN + 0.1% formic acid (solvent B) at a flow rate of 200 nL.min⁻¹. Spectra were measured at a mass scan range of m/z 300–2000 at a resolution of 30 000 in the profile mode followed by data dependent CID and/or HCD fragmentation of the ten most intense ions, with a dynamic exclusion window of 60 s.

Proteomic Data Treatment. All data were processed using Mascot 2.4.1 (Matrix Science, U.K.) and using X!Tandem with Scaffold software (version 3.0; Proteome Software, U.S.A.) compared against Ensembl databases for fishes (restricted to Oryzias latipes, Danio rerio and Tetraodon nigroviridis sequences in the Ensembl database V68). The ion mass tolerance and the parent ion tolerance were set to 0.50 Da. The methylmethanethiosulfonate of cysteine was specified as a fixed modification. The oxidation of methionine and the iTRAQ 8-plex of tyrosine for iTRAQ-derivatized samples were specified as variable modifications. Scaffold was used to probabilistically validate the protein identifications derived from the MS/MS sequencing results using the X!Tandem algorithms.

Data Analyses. Scaffold Q+ was used to quantify the isobaric tag peptide and protein identifications as previously described.⁴⁴ Quantitative ratios were \log_2 normalized for final quantitative testing, and the control value was used as the reference sample in both sexes. The heatmap protein quantification was represented using Gene-E freeware (http://www.broadinstitute.org/cancer/software/GENE-E/) with Spearman correlation's value for hierarchical clustering analysis for both samples and proteins. The statistical significance of the differential expression of the proteins was investigated using Kruskal–Wallis tests with a 0.5 \log_2 fold change (FC) threshold. The molecular pathway was determined using the Ingenuity Pathway Analysis software (V01–04; Qiagen) with the Human orthologous of medaka

proteins available from the Ensembl online platform (http:// www.ensembl.org), according to the specific Ingenuity Knowledge Database (using default parameters for all tissues and cell lines, with relaxed filters), which constitutes a repository of biological interactions and functional annotations.

RESULTS AND DISCUSSION

Metabolite Compositions of Microcystis Culture and Bloom Extracts. The secondary metabolite compositions of the two monoclonal Microcystis strains and the Microcystis dominated bloom were determined using liquid chromatography coupled with mass spectrometry (ESI-MS/MS) as represented in Figure 1(A-C). Both extracts exhibited a large diversity of molecules, and their molecular annotations were performed according to their respective precise mass and, if available, with their corresponding MS/MS fragmentation patterns, containing a global matching pattern or several signature ions that are specific to some metabolite families³⁷ (Table S1). We observed 40, 25, and 28 metabolites for the MicA+, MicA-, and bloom+ extracts, respectively, and a global composition comparison showed that only a very limited number of metabolites were common between the different extracts (Figure 1D). In MicA+ extract, 6 MCs variants could be detected and annotated: MC-LR, (DAsp³)-MC-LR, (DMha³)-MC-LR, (DAsp³-DMha⁷)-MC-LR, MC-AR, and 1 other putative uncharacterized variant along with these MC variants, together with microginin FR5, 5 cyclamides, 4 cyanopeptolins, and other uncharacterized molecules were also detected in the MicA+ extract. The Microcystis bloom+ extracts contained cyanopeptides, comprising higher peak counts for MC-LR, MC-YR, MC-RR, and (DAsp³)-MC-LR (in decreasing order), along with 3 other putative uncharacterized MC variants, 6 potential cyanopeptolins, 2 potential aeruginosins, 1 aeruginosamide 560, and other uncharacterized molecules. In



Figure 3. Representative view of male and female medaka hepatocytes observed on thick HES-stained liver sections exhibiting different examples of lysis area.

contrast, the MicA- extract, which lack any MCs, contained different microginin variants (FR3 and FR4, plus potentially Tyr-Tyr deleted fragments), along with cyanopeptolin 974, anabaenopeptin F, and various other components of unknown structures.

We confirm here that *Microcystis aeruginosa* produced a wide diversity of secondary metabolites, including microcystins, microginins, aeruginosins, cyanopeptolins, cyclamides, or anabaenopeptides, as was suggested through recent genome mining approaches performed in this species. These observations also highlight that blooms might produce a wider metabolite diversity as blooms comprise a codominance of various clones, producing different metabolite sets.^{3,5,6} These combined observations illustrate the complexity, and the global dissimilarities of the studied *Microcystis* extracts. Therefore, the chronic deleterious effects were further investigated on adult medaka fish, with regard to similar environmental concentrations of MCs.

Effect on Liver Hepatocyte Lysis and Glycogen Contents. The liver is an important organ that performs various vital functions, which include the process and the storage of nutrients, maintenance of serum composition, bile production, and xenobiotic detoxification. Liver from both sexes of medaka exposed to control conditions presented a typical architectural organization with polyhedral hepatocytes organized around the capillary sinusoids and the bile canaliculi, appearing in typical cord-like parenchymal structures. As shown in Figure 2A–D, the liver of medaka fish exposed to control conditions presented a noteworthy sexual dimorphism: this is illustrated at the cellular level from the histological observations of the hepatocytes. Hepatocytes of female fish presented large reserve vesicles (very

likely containing glycoprotein and/or glycogen, stained in purple with PAS⁴⁴), which appears isolated from the rest of the cytoplasm contents, whereas hepatocytes of male fish exhibited a more diffuse cytoplasm that contained small inclusions. Indeed, in mature fish, as in other oviparous vertebrates, the liver of the female plays an important function in the production of the oocyte envelope and vitellogenin reserves, whereas the male liver hepatocytes do not exhibit such activity. The liver globally presents sexual morphologic, molecular and functional dimorphisms. $^{\rm S2-S6}$

Here, we determined that significant cellular impacts were detected by histology observation of liver sections in both hepatocyte lysis and glycogen content levels with exposures to MC-LR, MicA+ and bloom+ treatments in both sexes (Figure 2E,F and Figure 3). Those increases in the hepatocyte lysis area, concomitant with a clear decrease in intrahepatocyte glycogen reserves, represent genuine evidence of the cellular hepatotoxicity of the various treatments that contain at least 5 μ g·L⁻¹ MC-LR or equivalent MC content. The increase in the hepatocyte lysis area may be the result of diffuse cellular necrotic or apoptotic events induced by hepatotoxic treatments,⁵⁷ and its association with the decrease of intracellular glycogen contents might reveal the induction of a true chronic hepatic stress induction. Interestingly, previous investigations performed on whitefish chronically exposed to MC-producing Planktothrix rufescens34 have shown gastrointestinal histological alterations characterized by "granulated cytosol, reduced glycogen stores, disintegration of the parenchymal liver architecture, cell dissociation,...", the severity of these effects being dependent on the quantity of toxic cells. Taken together, these observations

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Figure 4. Global proteomic effects of the various *Microcystis aeruginosa* extract exposures on medaka fish liver. A heatmap representation of proteinaceous dysregulation observed using an iTRAQ quantitative proteomic approach for both males and females. All samples were normalized to the male and female controls and the ratios are represented on a log_2 scale. Down-regulated proteins are indicated in red, up-regulated proteins in green, and missing values in gray. Clustering was performed with Pearson's correlation coefficient values.

2		20	Part of the second s	R. m		F MC-LR	F MicA+	F MicA-	F Bloom+
WC		1	Carlos Carlos	0	METABOLISM	0.0	4.7	4 7	4.4
0				• /	Histidine ammonia-iyase	-0.9	-1.7	-1.7	-1.7
					Alle synthase subunit alpha	-0.8	-0.5	-1.8	-1.6
					Aldehyde dehydrogenase 4A1	-0.3	-1.3	-0.7	-0.4
					Phosphoethanolamine methyltransferase	-0.5	-0.6	-0.4	-0.4
					ODP glucuronosyltransferase 1	0.1	0.2	-1./	-0.1
15 (२ २	4			Fatty acid binding protein 100	0.4	-0.7	0.1	0.4
		- /			Phytanoyl-CoA 2-hydroxylase*	0.3	0	0	0.6
					Fatty acid binding protein 10a	0.4	-0.1	0.4	0.9
					UDP-glucose pyrophosphorylase 2a	0.1	0.1	0.2	0.7
					Sulturtransferase	0.2	0.6	0.3	0.3
					Betaine-homocysteine methyltransferase 1*	0.3	-0.1	0	0.8
					Betaine-homocysteine methyltransferase 2	0.6	-0.1	0.2	0.8
					4-aminobutyrate aminotransferase	0.7	0	0.4	0.5
					Dihydropyrimidine dehydrogenase b	0	0.7	0	1.2
					Alanine-glyoxylate aminotransferase a	0.6	0	0.2	1
					Acetyl-CoA acetyltransferase 2	1.5	2.2	1.7	1.1
					REPRODUCTION PROCESS	1000	1000	00000	
					Vitellogenin 1	-0.6	-0.5	-0.5	-1
	IVI IVIC-LK	WI WIICA+	IVI IVIICA-	IVI BIOOM+	Vitellogenin II precursor	-0.3	-0.3	-0.2	-0.7
METABOLISM	- 10				Choriogenin H precursor	-0.6	-0.2	0.1	-0.9
Glycosyltransferase	0	-0.6	-0.5	-0.1	Choriogenin L	-0.6	-0.2	-0.2	-0.9
Acyl-CoA binding protein	-0.4	-1.1	-0.4	-0.6	HOMEOSTASIS PROCESS				
Betaine-homocysteine methyltransferase 1*	-0.7	0	0	-0.8	Glutaredoxin	0.4	-0.7	0.2	0.2
Fatty acid-binding protein	-0.4	-0.6	2.5	-0.7	Transferrin-a	0.2	0.3	0.1	0.6
Saccharopine dehydrogenase a	-0.4	-1	-0.3	-0.4	Uricase	0.4	-0.1	0.1	0.7
Aldehyde dehydrogenase 1	0.1	0.1	0.8	0	Catalase	0.3	-0.1	0	0.7
Acyl-Coenzyme A oxidase 3	0.5	0.1	0.6	0.3	Cytochrome P450 4B*	0.6	-0.3	-0.3	0.6
Acetyl-Coenzyme A acyltransferase 1	0.6	0.3	0.3	0.5	Cytochrome P450 3A	0.7	0.1	0.1	0.7
Fatty acid amide hydrolase	0.7	0.7	0.7	0.4	Cytochrome P450 8B	0.6	0.1	-0.1	0.9
Phytanoyl-CoA 2-hydroxylase*	0.7	0.5	0.4	0.6	Cytochrome P450 2A	0.7	0.1	0.3	0.4
Phenylalanine hydroxylase	0.6	0.7	0.6	1.1	Glutathione S-transferase A-like	0.2	-0.4	-0.1	1
HOMEOSTASIS PROCESS			2000		Glutathione S-transferase zeta 1	0.2	0.5	0.1	0.8
Alpha-type globin	0.1	0.5	0.6	0	Superoxide dismutase 1	0.5	0.5	0.5	0.6
Alpha-type globin	0.2	0.5	0.7	0.3	OTHER FUNCTIONS				
Beta-type globin	0.3	0.5	0.8	0.2	Calreticulin like	-0.6	0.1	-0.2	-0.2
Cytochrome P450 4B*	0.6	0.9	0.4	0.6	Ribosomal protein S17	-0.7	-0.1	-0.2	-0.1
TRANSLATION					Uncharacterized protein	0.4	0.2	0.1	0.8
Ribosome binding protein 1	-0.2	-0.6	-0.5	-0.1	Cathepsin D	0.2	0.5	0.3	0.8
Nucleolin	-0.2	-0.6	-0.1	0	Complement component C3-1	0.4	1.1	0.1	0.8
Ribosomal protein SA	0.2	0.7	0.2	0.1	Cytidine deaminase	0.6	0.6	0.4	0.5

Figure 5. Lists of the differentially regulated proteins (>0.5 $\log_2 FC$) in male and female livers. Proteinaceous quantification according to iTRAQ analyses was normalized according to male (M) and female (F) control quantification values and is represented with a \log_2 fold change scale. Down-regulated proteins are indicated in red and up-regulated proteins in green (threshold of 0.5 $\log_2 FC$, darker colors indicate when dysregulation is greater than 1 $\log_2 FC$). *Indicates the three proteins highly dysregulated in both sexes.

support the idea that noticeable cellular liver damages are induced in a dose-dependent manner by an environmentally relevant amount cyanobacterial toxic compounds, such as the MCs.

We observed also that in all of the experiment treatments that the fish were exposed to in this study, the female medaka fish livers exhibited higher lysis areas associated with lower glycogen content than the male livers (p < 0.01); this suggests that female and male medaka fish might present variable responsiveness to hepatic stressor, as indeed control females (low/no stress condition) already present primal traces of hepatic impairments. These differences of those two hepatic stress markers between

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sexes might be related to additional energetic and metabolic involvement of the female fish liver in reproductive processes, such as the synthesis of the oocyte contents. Furthermore, liver enzymes such as cytochrome P450, GST, aldehyde dehydrogenase, and hydroxysteroid dehydrogenase, were differentially expressed between the two sexes, underlying the sexual differences in the xenobiotic detoxification capabilities.^{58–60}

Effects on Global Protein Contents and Sexual Dimorphism of the Liver Responses. The analysis of protein digests of liver tagged with iTRAQ 8-plex on the nanoLC-ESI-MS/MS XL Orbitrap led to the identification of 321 and 304 proteins in males and females, respectively (Figure S3), using Mascot and X!tandem (with 2 unique peptides, with 99% global protein confidence score). Protein quantification in each treatment group was reported for relative tag intensity normalized according to the controls, and reliable quantifications for 219 proteins identified in male and female livers were represented in a heatmap with hierarchical cluster analysis (Figure 4). This analysis clearly revealed a distinguishable sexdependent response of medaka fish to the various hepatotoxic Microcystis treatments according to the group distribution, which was based on the pattern of relative abundance of up-regulated (green) and down-regulated (red) proteins. Previous studies on changes in zebrafish livers have observed dimorphic gene expression patterns that would influence their respective response to xenobiotics or pollutants.^{54,58,61} Interestingly, MC-LR and bloom+ treated fish livers presented the most similar protein dysregulation pattern, as they appeared regrouped by the clustering analysis in both males and females.

Previous molecular investigations of acute exposure to various model fish species observed specific liver responses to MCs (mostly to MC-LR), which were characterized mostly by lipid and carbohydrate metabolism, translation and protein synthesis, or detoxification;^{18,44,45} however, the dysregulation of proteins induced by our present chronic exposures revealed that among 321 proteins identified in male livers, 18 (6%) appeared to be differentially regulated by the treatments (log₂ |FC| > 0.5 and *P* < 0.05), while 37 proteins (12%) were dysregulated among 304 identified proteins in female medaka livers. These observations suggest that, globally, the liver of females seemed to be more responsive to the different treatments, as they exhibited more numerous molecular dysregulations than the liver of males.

Among these 18 and 37 most differentially quantified proteins in males and females, respectively, only 3 of them, namely phytanoyl-CoA 2-hydrolase, betaine-homocysteine methyltransferase 1, and cytochrome P450 4b, were common between the 2 sexes (Figure 5). All the differentially regulated proteins were sorted into 4 main categories according to their respective gene ontology (GO) classification terms, including metabolism, homeostasis, translation and reproduction process (Table S2a,b). Metabolism and homeostasis appeared to be the two main protein categories in both male and female medaka livers. Primarily, these observations support the idea that a chronic exposure to MC induced a different molecular response in medaka than the response induced by acute treatments.¹ Globally, although the male and female responses appearred primarily dimorphic, the four MC and Microcystis extract treatments (MC-LR, MicA+, MicA-, and bloom+) induced comparable protein responses within each sex, with more dysregulation pattern similarities shared between MC-LR and bloom+ than shared with MicA+.

This observation suggests that the chronic treatments of those various hepatotoxic mixtures may induce comparable molecular

effects on hepatocytes. Whereas males globally presented a net up-regulation of various α and β -type globin proteins together with up or down-regulation of various tricarboxylic acid (TCA) metabolism related proteins, the females exhibited a large downregulation of vitellogenins and choriogenins, and a clear upregulation of various homeostasis related proteins, such as various cytochrome P450, glutathione S-transferase (GST) or superoxide dismutase (SOD). Females also presented a dysregulation of various metabolism-related proteins, comprising a down-regulation of histidine ammonia-lyase and ATP synthase and a strong up-regulation of acetyl-CoA acetyltransferase 2. These results are further detailed and discussed below.

Metabolism Proteins. Various important liver functions are related to the capabilities of the liver to intensively metabolize and/or catabolize different type of molecules, such as biliary acids, carbohydrates, lipids, or amino acids. The differentially regulated proteins in male and female livers were mainly involved in metabolic processes (Figure 5). Indeed, in females, there was a higher level of up-regulation of acetyl-CoA acetyltransferase 2 after exposure to all treatment compared to the control level (above 2 log₂ FC). This protein, which catalyzes the acetyl-CoA condensation in acetoacetyl-CoA, is potentially involved in various metabolic processes, such as lipid or various amino acid metabolisms. Various other proteins related to the amino acid metabolism, such as alanine-glyoxylate aminotransferase a, 4aminobutyrate aminotransferase, dihydropyrimidine dehydrogenase b, and 2 betaine-homocysteine methyltransferases presented noticeable up-regulation under both MC-LR and bloom+ treatments. However, different amino acid metabolismrelated proteins, such as histidine ammonia-lyase and aldehyde dehydrogenase 4A1, presented a net down-regulation pattern, attesting to a global rearrangement in proteins related to amino acid metabolism.

Alternatively, in males various acetyl-CoA related proteins associated with energy metabolism processes, such as acyl-CoA binding protein, acyl-CoA oxidase 3, acetyl-CoA acyltransferase 1 and phytanoyl-CoA 2-hydroxylase presented important upand down-regulations. Fatty acid-binding protein and fatty acid amide hydrolase were also differentially regulated in males, which indicated modulations in specific lipid processes.

Homeostasis and Detoxification Proteins. One other important function of the vertebrate liver is that it has on its high detoxification capabilities related to its high content in drugmetabolizing enzymes and to its high tropism for xenobiotics. It also presents noticeable sex differences in its drug metabolism and homeostasis capabilities.⁶⁰ Our proteomic analyses (Figure 5) described a large distinction in liver homeostasis and detoxification processes between male and female medaka.

In females, in which all treatments induced an up-regulation of SOD, 1 catalase (CAT) presented important increase after bloom+ exposure, suggesting that these treatments, and especially the bloom+, could induce an increase in the recruitment of the enzymatic oxidative defenses in the liver. This increase in enzymatic defenses might aim at coping an increase of the intracellular production of reactive oxygen species (ROS) that can be induced by cyanotoxins, such as MCs, as it has been previously proposed, avoiding the appearance of cellular oxidative damage.¹⁰ In parallel, the highest levels of various cytochrome P450 (Cyt P450 2A, 3A, 4B, and 8B) were detected in the MC-LR and bloom+ treatments, which suggests an important deployment of phase 1 detoxification enzymes, very likely aim at detoxifying potentially toxic component such as MC or other cyanotoxins and secondary metabolites.^{10,22} Further-

more, bloom+ treated female fish exhibited an additional induction of detoxification enzymes of phase 2, such as the 2 GST isoforms observed here. This observation is in accordance with previous studies showing an increase in GST expression in order to promote the conjugation MCs with glutathione in the detoxification process.^{11,14,15} Interestingly, MicA+ exposed fish exhibited a quite distinct pattern, which comprised a down-regulation of glutaredoxin and an up-regulation of GST zeta1. This suggests that the redox effects and detoxification processes induced by this complex *Microcystis* extract might somehow differ from other hepatotoxic conditions.

In males, all treatments induced an up-regulation of Cyt P450 4B, one of the major xenobiotic and toxin metabolizing enzymes that are potentially involved in phase 1 detoxification processes. Additionally, the significant up-regulation of 3 hemoglobinconstituting proteins (2α -type and 1 β -type globin subunits) in both MicA+ and MicA- suggest that a recruitment of erythrocytes or alternatively a slight intrahepatic hemorrhage may occur in the liver following these treatments (although this has not been confirmed by histology).

Oviparous-Specific Reproductive Proteins. Beyond the clear sexual dimorphism in the response of the proteins involved in metabolism and homeostasis functions, we also observed that some proteins, specific to the reproductive process of oviparous vertebrates, were strongly impacted by the exposure to the various treatments and present obvious sex-biased responses (Figure 5). In mature female medaka fish, a large portion of the liver metabolism is dedicated to the synthesis of choriogenins and vitellogenins, the main oocyte constituents, as each female can spawn above 30 mature oocytes daily.⁶² This massive protein synthesis is known to induce large cellular and molecular modifications, as it can also increase the lipid synthesis of hepatocytes.⁶³

All the present chronic exposures to the various hepatotoxic contents, principally in exposures to bloom+, MC-LR, and MicA +, and to a lesser extent, to MicA-, induced a clear decrease in vitellogenins 1 and H and in choriogenins L and H precursors in female medaka fish. This observation strongly suggests that exposed female livers synthesize fewer amount of these proteins, which is very likely due to a global dysregulation of the hepatic functions induced by the MC and/or Microcystis mixtures. The decrease in oocyte content synthesis by the liver could directly adversely affect the maturation of oocytes and alter the reproductive success of the fish. Although both vitellogenin and choriogenin syntheses are under the control of estrogen, we do not believe that the observed decreases in the synthesis of these proteins in females is related to endocrine perturbation, such as a modification of circulating hormone estradiol levels (E2), as no significant changes of this hormone were measured here in the medaka plasma (Figure S4). In addition, male medaka fish did not present any significant evidence of estrogenic effect such as circulating estradiol induction, as it has been observed for true endocrine-disrupting components.⁶⁴ In the present case, we assume that the decreases in vitellogenins and choriogenins in females instead result from a global alteration of the liver, as was observed in our histological analysis, with indirect consequences on the liver capability to synthesize oocyte protein precursors. A similar effect scenario recently was proposed for zebrafish exposed to pure MC-LR,^{26,30} and supports our assumption. However, previous studies report that some Microcystis strains produce potential estrogenic compounds,^{45,65} but the molecules involved in the monitored bioactivity are still not identified. In addition, other studies suggested that some other strain would

lack such bioactive components,⁴³ and these assertions still need to be substantially documented.

Molecular Pathway Networks (IPA). Ingenuity Pathway Analysis (IPA) was performed with a subset of 189 proteins presenting Human orthologs. The top molecular pathways impacted by the various treatments concern gene expression; protein synthesis; lipid, small molecules, and amino acid metabolisms; and organism injury and cellular diseases (Figures S5 and S6). The IPA interaction network for the various proteins involved in the lipid metabolism pathway in females (Figure S7) illustrated the higher global similarities of protein dysregulations induced by MC-LR and bloom+, and MicA+ treatments, compared to the MicA- treatment that exhibited a more different protein dysregulation pattern. Similar distinctions between the molecular effects induced by the treatments were also observed for protein networks related to protein synthesis, carbohydrate metabolism, and post-translational modification processes, as illustrated in Figures S8-10 for females and males.

Effects of MC-LR and of Microcystis Metabolite Cocktails. Our results show deep differences between male and female adult medaka⁴ in their molecular and toxic responses that have not been observed before in acute exposure experiments.^{18,44} Previously, a first attempt to characterize the liver molecular response of a chronic exposure of zebrafish to MC-LR²⁰ did not distinguish between male and female organisms. This 2-D electrophoresis proteomic investigation reported a dysregulation of 22 proteins involved in cytoskeleton assembly, macromolecule metabolism, oxidative stress, and signal transduction. Interestingly, in our analysis, only one differentially deregulated protein, the SOD, appears to be similarly upregulated by MC-LR exposure in the female livers. However, this global discrepancy might result from the significant technical differences between the experimental and/or the proteomic approaches developed in both studies.

According to toxicological mode of action of MC deduced from acute exposure experiments, MC inhibits the PP1 and PP2 hepatocyte activity, with direct cascade effect on the regulation, via their different phosphorylation states, of various proteins involved in oxidative stress, protein maturation, apoptosis or cellular proliferation. Our proteomic analyses do not highlight protein phosphatase dysregulation (actually protein phosphatase were not quantifiable in the present iTRAQ experiment), and it seems that dedicated proteomic analysis would be required in order to be able to further investigate potential alterations of PP1 and PP2 by MCs and their consequence on protein phosphorylation.

Notably, the exposure to the bloom+ extract induced in fish more cellular changes and more intense protein dysregulation compared to those induced after the exposures to MC-LR alone or to MicA+ extract (Figures 2E,F, 3, 4, and 5). These wider toxic effects could potentially be additive or synergistic effects of the MC alone or of the MC together with the other metabolites or various exogenous chemical toxins that compose the bloom+ extract mixture.^{66,67} However, previous in vivo and in vitro bioassays showed that pure MC-LR displayed a greater dosedependent toxicological effect than the other MC isoforms, such as the demethyl-MC, which is present in various cyanobacterial crude extracts.^{68,69} Alternatively, mixture effects of MCs with other cyanobacteria metabolites have not been investigated to date. However, some studies also previously suggested that the toxicity of complex cyanotoxin mixtures could be greater than that of the purified cyanotoxin alone.^{70,7}

Interestingly, although the MicA-treatment showed a limited effect at the hepatocyte cellular level, it induced noticeable effects at the proteinaceous level, comprising in females a large upregulation of a fatty acid-binding protein not observed in males, and an obvious down-regulation of the UDP glucuronosyl-transferase 1 involved in metabolism process (Figure 5). Among the list of MicA-extract metabolites, we can only speculate which ones are responsible for the observed molecular effects, and further investigation concerning the potential bioactivity of various cyanobacteria metabolite families, such as microginins, anabaenopeptins, or aerucyclamides^{38,40,72} are still needed. Interestingly, recent work on purified cyanopeptides, such as cyanopeptolin 1020, highlighted the potential deleterious effects on original molecular pathways such as DNA repair or circadian cycle regulation.⁴²

Hepatotoxic effects were observed in these studies after chronic exposures of medaka to an environmentally relevant concentration of MCs and other cyanobacterial metabolites, which are the concentrations often observed during *Microcystis* blooms that can occur and persist numerous weeks, especially in summer.^{8,27,73} These observations suggest that fish from natural environments in which MC-producing cyanobacteria blooms occur similarly could be deleteriously affected. However, this assumption and its ecotoxicological consequences on fish guilds and populations from natural environments still need to be more deeply investigated⁷⁴ and are still under debate: to date, only very scarce data linking environmental issues directly to cyanotoxin production have been reported.^{75–77}

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b01903.

Figure S1. Microcystis aeruginosa strains and bloom used in this study. Figure S2. Weekly monitoring of the phytoplanktonic and cyanobacteria dominance. Figure S3. A Venn diagram of the identified proteins within male and female medaka livers through proteomics. Figure S4. Quantification of circulating estradiol-2 (E2). Figure S5. Ingenuity pathway categories. Figure S6. Example of the heatmap of IPA categories. Figure S7. IPA network of proteins related to lipid metabolism, small molecule biochemistry, and acid nucleic metabolism. Figure S8. IPA network of proteins related to protein synthesis. Figure S9. IPA network of proteins related to carbohydrate metabolism. Figure S10. IPA network of proteins (PDF) Table S1. List of metabolites observed in the methanol extract. Table S2a,b. Extended lists of significantly disregulated proteins in males and females, respectively (XLSX)

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Notes

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